Journal of Pharmacy and Pharmacology

Published by
The Pharmaceutical Society
of Great Britain

British Pharmaceutical Conference
One Hundred and First Meeting
Edinburgh 1964

Volume 16: Supplement
December 1964

British Pharmaceutical Conference

Edinburgh 1964

CHAIRMAN

W. Mitchell

Journal of Pharmacy and Pharmacology
17 Bloomsbury Square, London, W.C.1

Journal of Pharmacy and Pharmacology

British Pharmaceutical Conference

Supplement

Editor: D. W. Mathieson, B.Sc., Ph.D., F.R.I.C.
Press Editor: J. R. Fowler, B.Pharm., F.P.S.

December 1964 Vol. 16

CONTENTS

CONTENTS

Science Papers—(*continued*)

CONTENTS

Science Papers—(*continued*)

Journal of Pharmacy and Pharmacology Supplement, 1963, **16**, 1*T*–8*T*

British Pharmaceutical Conference

One hundred and First Annual Meeting, Edinburgh, 1964

Report of Proceedings

OFFICERS:

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

Chairman:
W. MITCHELL, B.Sc., Ph.D., F.R.I.C., London.

Past-Chairmen (Members of the Executive):
H. TREVES BROWN, B.Sc., F.P.S., London.
W. H. LINNELL, D.Sc., Ph.D., F.P.S., F.R.I.C., London.
D. C. GARRATT, D.Sc., Ph.D., F.R.I.C., Nottingham.
J. C. HANBURY, M.A., B.Pharm., F.P.S., F.R.I.C., Ware.
H. G. ROLFE, B.Sc., F.P.S., F.R.I.C., London.

Honorary Treasurer:
J. M. ROWSON, M.Sc., Ph.D., F.P.S., F.L.S., Bradford.

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

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

*A. ALDINGTON, M.P.S., London.
A. H. BECKETT, D.Sc., Ph.D., F.P.S., F.R.I.C., London.
*J. C. BLOOMFIELD, M.P.S., Portsmouth.
G. BRYAN, M.P.S., London.
*Miss M. A. BURR, M.P.S., Nottingham.
S. DURHAM, M.P.S., Sheffield.
D. W. HUDSON, M.P.S., Hove.
C. A. JOHNSON, B.Pharm., B.Sc., F.P.S., F.R.I.C., London.
J. J. LEWIS, M.Sc., F.P.S., Glasgow.
H. D. C. RAPSON, B.Sc., Ph.D., D.I.C., A.R.I.C., London.
E. SHOTTON, B.Sc., Ph.D., F.P.S., F.R.I.C., London.
G. SYKES, M.Sc., F.R.I.C., Nottingham.

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

REPORT OF PROCEEDINGS

PROCEEDINGS OF CONFERENCE

EDINBURGH 1964

THE OPENING SESSION

The opening session of the Conference was held in the Leith Town Hall on Monday, September 14, with Mr. C. W. Maplethorpe, President of the Conference (President of the Pharmaceutical Society), in the Chair. On the platform were the Chairman of the Conference (Dr. W. Mitchell), Bailie T. Morgan, the Chairman (Mr. E. Knott) and Secretary (Mr. A. W. Paterson) of the Local Committee, the Conference Treasurer and the Honorary General Secretaries, together with members of the Conference Executive.

The President introduced Bailie Morgan, who welcomed the Conference on behalf of the citizens of Edinburgh. On the motion of the President a vote of thanks to Bailie Morgan was carried by acclamation.

The President then handed over further conduct of the Conference to the Chairman (Dr. W. Mitchell), who delivered his address entitled "On a Sense of Proportion in Science", which is printed in the *Pharmaceutical Journal*, 1964, **193**, 247–251.

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

CIVIC RECEPTION

On the evening of Monday, September 14, the Conference members were the guests of the Lord Provost, Magistrates and Council of the City of Edinburgh at a reception and dance in the Assembly Rooms. In the absence of the Lord Provost, guests were received by Bailie N. McQueen and Mrs. McQueen.

THE SCIENCE SESSIONS

Meetings were held on Monday, Wednesday and Friday, September 14, 16 and 18, at the Heriot-Watt College, the Chairman and immediate past Chairman presiding. During the sessions the following 32 papers were presented.

Thin-layer chromatography of corticosteroids. By A. Hall, B.Sc.

Some aspects of the use of thin-layer chromatography in a limit test for related foreign steroids. By C. J. Clifford, B.Sc., A.R.I.C., J. V. Wilkinson, B.Sc. and J. S. Wragg, B.Sc., F.R.I.C.

The detection and identification of other 17,21-di-hydroxy-20-oxosteroids in corticosteroids. By C. A. Johnson, B.Pharm., B.Sc., F.P.S., F.R.I.C. and Sylvia Fowler, F.P.S.

Polarographic determination of microgramme quantities of chlorpromazine. By G. S. Porter, F.P.S.

A specific method for the determination of amphetamine in urine. By A. H. Beckett, D.Sc., Ph.D., F.P.S., F.R.I.C. and M. Rowland, B.Pharm., M.P.S.

Determination of methyl salicylate in pharmaceutical preparations. By S. G. E. Stevens, B.Sc., F.R.I.C. and B. Warren, L.R.I.C.

The influence of the method of evaluation on the recovery of a phage after phenol treatment. By W. R. L. Brown, B.Pharm., Ph.D., F.P.S., A. M. Cook, B.Pharm., Ph.D., Dip.Bact., F.P.S., F.R.I.C. and J. Oduro-Yeboah, B.Pharm.

The effect of polysorbate (Tween) 80 on the growth rate of *Pseudomonas aeruginosa*. By M. R. W. Brown, M.Sc., Ph.D., M.P.S., M.I.Biol. and R. M. E. Richards, B.Pharm., F.P.S.

The unsuitability of the B.P. tests for sterility to detect fungi. By N. M. Chauhan, B. Pharm., Ph.D., M.P.S. and V. Walters, B.Pharm., Ph.D., F.P.S.

Effect of polysorbate (Tween) 80 on the resistance of *Pseudomonas aeruginosa* to chemical inactivation. By M. R. W. Brown, M.Sc., Ph.D., M.P.S., M.I.Biol. and R. M. E. Richards, B.Pharm., F.P.S.

The effect of chlorhexidine on the permeability and succinoxidase activity of *Micrococcus lysodeikticus*. By D. Wiseman, B.Sc., M.P.S.

Influence of oil : water ratio on the activity of some bactericides against *Escherichia coli* in liquid paraffin and water dispersions. By H. S. Bean, B.Pharm., Ph.D., F.P.S. and S. M. Heman-Ackah, B.Pharm., F.P.S.

REPORT OF PROCEEDINGS

The relationship between analgesic activity, acute toxicity and chemical structure in esters of 14-hydroxycodeinone. By W. R. Buckett, B.Pharm., M.P.S., M.I.Biol.

An isolated parasympathetically-innervated oesophagus preparation from the chick. By W. C. Bowman, B.Pharm., Ph.D., M.P.S. and Sally D. Everett, B.Pharm.

Metabolism of some dimethylaminoazobenzene derivatives. By P. J. Robinson, M.Sc., A. J. Ryan, M.Sc., Ph.D. and S. E. Wright, D.Sc., Ph.D., Dip.Pharm.

Antihistamine protection against histamine-induced gastric ulceration in guinea-pigs. By J. Watt, M.B., Ch.B., M.D. and C. B. Eagleton, B.Sc., M.D.

A simple method for the evaluation of local anaesthetic activity using earthworms. By B. P. Block, B.Pharm., Ph.D., M.P.S., D. J. Potts, B.Pharm., M.P.S. and R. S. H. Finney, B.Sc., M.Sc.

Neuromuscular blocking agents: alkyl and heterocyclic analogues of simple linear-trisonium compounds. By Fiona Macleod Carey, B.Sc., C. I. Furst, B.Sc., Ph.D., M.P.S., J. J. Lewis, M.Sc., F.P.S. and J. B. Stenlake, D.Sc., Ph.D., F.P.S., F.R.I.C., F.R.S.E.

The influence of vehicles on skin penetration. By C. W. Barrett, B.Pharm., M.P.S., J. W. Hadgraft, F.P.S., F.R.I.C. and I. Sarkany, M.R.C.P.

A physical indicator for sterilisation procedures. By D. E. Simpkins, B.Pharm. and G. R. Wilkinson, F.P.S.

Some observations on the effect of lubrication on the crushing strength of tablets. By E. Shotton, B.Sc., Ph.D., F.P.S., F.R.I.C. and C. J. Lewis, B.Pharm., Ph.D.

Tensile strength of sterilised surgical catgut. By J. Owen Dawson, B.Sc., F.P.S., T. W. Roylance, B.Sc. and T. Smith.

Bactericidal effect upon *Pseudomonas aeruginosa* of chemical agents for use in ophthalmic solutions. By W. B. Hugo, B.Pharm., Ph.D., F.P.S. and J. H. S. Foster, M.Pharm., M.P.S.

Some properties of bronopol, a new antimicrobial agent active against *Pseudomonas aeruginosa*. By Betty Croshaw, M.Sc., M.I.Biol., M. J. Groves, M.Pharm., M.P.S. and B. Lessel, B.Pharm., Ph.D.

Umbelliferous fruit identification by thin layer chromatography. By T. J. Betts, B.Pharm., Ph.D., A.R.I.C.

The assay of nux vomica and its preparations. By H. M. Perry, M.Sc., F.R.I.C. and M. L. Sheppard, A.R.I.C.

Preparation and biological activity of some complexes of trypanocidal phenanthridinium compounds. By M. J. Groves, M.Pharm., M.P.S. and E. C. Wilmshurst, B.Pharm., B.Sc.

A temperature dependent micellar change. By J. E. Adderson, M.Pharm., F.P.S., L.R.I.C. and H. Taylor, B.Sc., Ph.D., F.P.S., F.R.I.C.

Reproducibility of extinctions measured on the slopes of absorption curves. By M. Ismail, B.Pharm., B.Sc. and A. L. Glenn, B.Pharm., B.Sc., Ph.D., F.P.S.

Mathematical treatment of oral sustained release drug formulation. By M. Rowland, B.Pharm., M.P.S. and A. H. Beckett, D.Sc., Ph.D., F.P.S., F.R.I.C.

Alkaloids of the leaves of *Rauwolfia vomitoria* Afz. By M. Patel, Ph.D., M.P.S., J. Poisson, Pharmacien, Docteur-ès-Sciences, J. L. Pousset, Pharmacien, Licencée-ès-Sciences and J. M. Rowson, Ph.D., M.Sc., F.P.S.

Reaction of ephedrine with chloroform. By H. Williams, M.Sc., A.R.I.C.

THE CONFERENCE LECTURE

A lecture on "Transplantation of Tissues and Organs," was given on Tuesday, September 15, by Professor M. F. A. Woodruff. The Chairman presided. The lecture is printed in the *Pharmaceutical Journal*, 1964, **193**, 252–255.

THE SYMPOSIUM SESSION

A symposium on "The General Aspects of Aerosols" was held on Thursday, September 17. The Chairman presided. The introductory papers were by Messrs. J. Pickthall, R. Marsden, G. F. Phillips and K. Dixon. The meeting is reported in the *Pharmaceutical Journal*, 1964, **193**, 391–410.

REPORT OF PROCEEDINGS

PROFESSIONAL SESSIONS

With the President of the Conference, Mr. C. W. Maplethorpe, in the Chair, professional sessions were held on the mornings of Wednesday, September 16, when Dr. G. R. Boyes read a paper on "Pharmaceutical Problems of Self Medication", and Friday, September 18, when Mr. F. W. Adams introduced the report on "The Membership Survey and Future Needs for Pharmaceutical Manpower". This was commented upon by Messrs. H. G. Moss, H. S. Grainger, J. C. Hanbury and Professor A. H. Beckett. Full reports of the papers and discussions were published in the *Pharmaceutical Journal*, 1964, **193**, 275–284; 313–323.

THE CLOSING SESSION

The closing session of the Conference was held on Friday, September 18, in the Heriot-Watt College, the Chairman presiding.

Vote of Thanks to Local Committee

The Chairman called upon Dr. W. R. L. Brown to propose a vote of thanks to the Local Committee. This was seconded by Mr. A. C. McWhinney. Mr. E. Knott (Chairman of the Local Committee) replied to the vote of thanks. The Chairman then presented to the Edinburgh and South-Eastern Scottish Branch an inscribed gavel provided by the Bell & Hills Fund. Mr. W. S. Tait (Chairman of the Branch) accepted and acknowledged the gift on behalf of the Branch.

Annual Report

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

Your Executive has pleasure in presenting the one-hundred-and-first Annual Report.

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

Reports on 1963 Meeting.—The report of the meeting of the Conference in London in 1963 together with the science papers, discussions, and the Conference Lecture were published as a supplement to the 15th Volume of *The Journal of Pharmacy and Pharmacology*. The papers and discussions at the Professional Sessions were published in *The Pharmaceutical Journal* Series IV, Volume 137.

Conference Papers, 1964.—The Executive agreed as an experiment for the Edinburgh meeting to invite an additional type of contribution in the form of Discussion Topics, and six of these were submitted. However, it was considered that none of the topics was such as to lead to a general and informed discussion and while thanking the authors for these contributions the Executive decided to omit Discussion Topics from this meeting. Forty-six research papers were submitted, and of these twenty-five full papers and seven short communications were presented. The Executive thanks the authors of these papers and also the authors of the papers presented to the Symposium and the Professional Sessions for their contributions. The Executive is grateful to the Editor of the *Journal of Pharmacy and Pharmacology* and to the Editor of *The Pharmaceutical Journal* for making galley proofs of the papers available before this meeting.

Conference Lecture.—The 1964 Conference Lecture was delivered by Professor M. F. A. Woodruff, Department of Surgical Science, Edinburgh University, on the subject of Transplantation of Tissues and Organs, and the Executive wish to record their thanks to him for his contribution to the Conference.

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

Future Meetings.—An invitation will be presented at this meeting for the Conference to meet in Cardiff during the week commencing September 6, 1965, and your Executive has provisionally accepted an invitation to hold the Conference in Manchester during the week commencing September 5, 1966. Other

branches of the Society have made preliminary enquiries regarding the possibility of entertaining the Conference in future years and the Executive is grateful for these offers of hospitality.

OFFICERS AND EXECUTIVE OF THE CONFERENCE.—Your Executive has nominated the following Officers for 1964–65.

Chairman: T. C. Denston; *Honorary Treasurer:* J. M. Rowson.

It is with regret that the Executive has to report that Dr. D. Train was unable to accept renomination as one of the Honorary General Secretaries owing to pressure of business commitments and in consequence E. F. Hersant and K. A. Lees have been nominated.

In accordance with Rule 6, the Executive has nominated for election by this meeting, the following three persons to serve on the Executive for the period 1964–67: A. M. Cook, T. O. Martin and S. G. E. Stevens.

The remaining elected members of the Executive are G. Bryan, S. Durham, C. A. Johnson, J. J. Lewis, H. D. C. Rapson and G. Sykes.

The following five past-Chairmen will serve on the Executive:—W. H. Linnell, D. C. Garratt, J. C. Hanbury, H. G. Rolfe and W. Mitchell.

The above persons together with the President of the Conference (the President of the Pharmaceutical Society of Great Britain), the three persons nominated by the Council of the Pharmaceutical Society of Great Britain, 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 1964–65.

ACKNOWLEDGEMENTS.—The Executive wish to record thanks to the Chairman, Officers and Members of the Edinburgh 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. In this connection the Executive wish to place on record their grateful thanks for the willing and efficient services over a period of many years of Miss Whiteside, who will no longer be associated with the work of the Conference.

Miss C. Mozley-Stark proposed the acceptance of the report and the election of officers of the Conference for the ensuing year. Mr. E. J. Fitchett seconded.

Mr. M. T. C. Denston thanked the Conference on behalf of the newly-elected officers.

A motion by Mr. M. I. Barnett that a vote of thanks be recorded to Dr. D. Train for his services was carried with acclamation.

TREASURERS' REPORT

During the financial year ended December 31, 1963, the £500 3% Exchequer Stock 1962–63 was redeemed and the proceeds were used to meet the expenses of the Centenary celebrations of the Conference.

The Local Committee Fund loan of £250 has been made to the Edinburgh Local Committee.

Changes in the Constitution have no effect on the accounts for 1963 but will result in an income from subscriptions during the present year.

The subscriptions of the elected members, including a composition fee from the Pharmaceutical Society of Northern Ireland, amounted to £206 and were credited to the account of the *Journal of Pharmacy and Pharmacology*.

J. M. ROWSON,
Honorary Treasurer.

PLACE OF MEETING FOR 1965

Mr. J. Edwards on behalf of the Branch of the Society extended an invitation to hold the Conference in Cardiff in 1965. Mr J. D. Mackenzie proposed that the invitation be accepted, and the President seconded. The vote was put to the meeting and unanimously carried.

VOTE OF THANKS TO CHAIRMAN

Miss S. M. Rivers proposed a vote of thanks to the Chairman.

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

Dr. Mitchell briefly responded.

REPORT OF PROCEEDINGS

BRITISH PHARMACEUTICAL CONFERENCE

Inaugural Meeting held at Newcastle upon Tyne in 1863*

Years	*Places of Meeting*	*Chairmen*	*Local Secretaries*
1923	LONDON ..	F. W. GAMBLE	W. J. U. WOOLCOCK, C.B.E.
1924	BATH	E. WHITE, B.Sc., F.I.C.	P. J. THOMPSON W. H. HALLETT
1925	GLASGOW ..	E. WHITE, B.Sc., F.I.C.	P. M. DUFF
1926	LEICESTER ..	D. LLOYD HOWARD, J.P.	J. BARKER
1927	BRIGHTON ..	D. LLOYD HOWARD, J.P.	F. W. BURGESS
1928	CHELTENHAM ..	R. R. BENNETT, B.Sc., F.R.I.C.	P. JAMES
1929	DUBLIN ..	R. R. BENNETT, B.Sc., F.R.I.C.	V. E. HANNA
1930	CARDIFF ..	J. T. HUMPHREY	J. MURRAY
1931	MANCHESTER ..	J. H. FRANKLIN	R. G. EDWARDS
1932	ABERDEEN ..	H. SKINNER	H. M. DUGAN
1933	LONDON ..	C. H. HAMPSHIRE, C.M.G., M.B., B.S., B.Sc., F.R.I.C.	H. N. LINSTEAD
1934	LEEDS	C. H. HAMPSHIRE, C.M.G., M.B., B.S., B.Sc., F.R.I.C.	G. C. CRUMMACK J. F. SIMON
1935	BELFAST ..	F. W. CROSSLEY-HOLLAND, L.M.S.S.A.	D. L. KIRKPATRICK
1936	BOURNEMOUTH	H. DEANE, B.Sc., F.R.I.C.	V. J. SCAMPTON
1937	LIVERPOOL ..	T. E. LESCHER, O.B.E.	W. E. HUMPHREYS
1938	EDINBURGH ..	J. RUTHERFORD HILL, O.B.E.	C. G. DRUMMOND
1939	BIRMINGHAM ..	J. RUTHERFORD HILL, O.B.E.	D. J. 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 ..	N. EVERS, B.Sc., Ph.D., F.R.I.C.	A. WILSON
1949	BLACKPOOL ..	N. EVERS, B.Sc., 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., 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.	D. F. SMITH
1960	NEWCASTLE UPON TYNE	W. H. LINNELL, D.Sc., Ph.D., F.R.I.C.	A. McGUCKIN
1961	PORTSMOUTH ..	D. C. GARRATT, D.Sc., Ph.D., F.R.I.C.	N. L. BANKS
1962	LIVERPOOL ..	J. C. HANBURY, M.A., B.Pharm., F.R.I.C.	D. L. REES
1963	LONDON ..	H. G. ROLFE, B.Sc., F.R.I.C.	K. R. CAPPER
1964	EDINBURGH ..	W. MITCHELL, B.Sc., Ph.D., F.R.I.C.	A. W. PATTERSON

Honorary Treasurers (One)

1912 to 1927, R. R. BENNETT, B.Sc., F.R.I.C.
1927 to 1934, F. W. CROSSLEY-HOLLAND, L.M.S.S.A.
1934 to 1936, T. E. LESCHER, O.B.E.
1936 to 1940, A. R. MELHUISH
1940 to 1947, T. MARNES
1947 to 1952, G. R. BOYES, L.M.S.S.A., B.Sc., F.R.I.C.
1952 to 1953, H. DAVIS, C.B.E., B.Sc., Ph.D., F.R.I.C.
1953 to 1958, H. TREVES BROWN, B.Sc.
1958 to 1962, H. G. ROLFE, B.Sc., F.R.I.C.
1962 to , J. M. ROWSON, M.Sc., Ph.D., F.L.S.

Honorary General Secretary (Two)

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 1964, D. TRAIN, M.C., B.Pharm., B.Sc., Ph.D., F.R.I.C., A.M.I.Chem.E.
1964 to , K. A. LEES, F.P.S.

* For details before 1923 see Report of Proceedings for 1962.

REPORT OF PROCEEDINGS

BRITISH PHARMACEUTICAL CONFERENCE

INCOME AND EXPENDITURE ACCOUNT, 1963

Expenditure	£	*s.*	*d.*	*Income*	£	*s.*	*d.*
Gavel—memento to Host Branch ..	–	–	–	Interest on 2½% Consols	40	5	0
Replica of Chairman's Badge, engraving, etc.	7	16	6	Interest on 3% Savings Bonds ..	6	0	0
Engraving Sports Trophies		3	6	Interest on 3% Exchequer Stock ..	7	10	0
Secretaries' Expenses	23	9	6	Interest on Bank Deposit Account ..	7	2	5
Treasurer's Expenses	13	17	10	Donation from Pharmaceutical Society of Northern Ireland ..	25	0	0
Expenses of Speakers	–	–	–	Donation from Pharmaceutical Society of Ireland	50	0	0
Honorarium to Conference Lecturer	26	5	0	Liverpool Local Committee Donation	–	–	–
Income Tax	22	7	7	Deficit carried to Accumulated Fund	380	9	8
Cheque Book		5	0				
Expenses of Young Authors of Conference Papers	38	14	9				
Executive Centenary Dinner ..	383	7	5				
	£516	7	1		£516	7	1

BALANCE SHEET AT DECEMBER 31, 1963

Liabilities	£	*s.*	*d.*	*Assets*	£	*s.*	*d.*
Accumulated Fund as at 31.12.62 ..	2,032	8	11	Investments at cost:			
Add: Surplus on redemption of Exchequer Stock	26	15	2	£1,610 2½% Consols	1,250	0	0
	2,059	4	1	£200 3% Savings Bonds 1960–70..	200	0	0
Less: Deficit 1963	380	9	8	£500 3% Exchequer Stock 1963–63 (Redeemed 1963)	–	–	–
	1,678	14	5		1,450	0	0
Creditor	–	–	–	(Total market value at December 31, 1963: £879; 1962: £1,401)			
Local Committee Fund	250	0	0	Stock of replicas (2) of Chairman's Badge	14	14	0
				Loan to Local Committee	250	0	0
				Cash at Bank—			
				Deposit Account 210 4 4			
				Current Account 3 16 1			
					214	0	5
	£1.928	14	5		£1,928	14	5

Audited and found correct

T. HESELTINE
T. C. DENSTON

May 6, 1964.

BRITISH PHARMACEUTICAL CONFERENCE

REVISED CONSTITUTION AND RULES

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

2. The Conference shall consist of:

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

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

(*c*) elected members.

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

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

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

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

5. The Executive Committee of the Conference shall consist of the Officers of the Conference, five past-Chairmen who have most recently filled the office of Chairman, together with three persons nominated by the Council of the Pharmaceutical Society of Great Britain and nine other members of the Conference. The Chairman of the Executive of the Scottish Department of the Pharmaceutical Society of Great Britain, the President of the Pharmaceutical Society of Ireland, the President of the Pharmaceutical Society of Northern Ireland, the Editor of the *Journal of Pharmacy and Pharmacology*, the Chairman of the Local Committee and the Honorary Local Secretary shall be *ex officio* members of the Executive Committee. Student members may not be members of the Executive Committee.

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

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

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

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

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

J. Pharm. Pharmacol., 1964, **16**, *Suppl.*, 9T–10T Received April 29, 1964

Science Papers

SHORT COMMUNICATION

Thin-layer chromatography of corticosteroids

A. HALL

PAPER chromatography used routinely for examining corticosteroids is time consuming and relatively insensitive. Thin-layer chromatography is simple and useful for handling a number of samples rapidly. In conjunction with 2,5-diphenyl-3(4-styrylphenyl)tetrazolium (Brooks & others, 1958) as a spray reagent, it is capable of detecting less than 1 μg of corticosteroid (0·25 μg of cortisone acetate).

Two solvent systems are described which allow the separation of closely related substances. Two analyses using these systems suffice to identify and separate the corticoids in accordance with the results listed in Table 1.

APPARATUS

Spread thin-layer chromatography plates 20 × 20 cm with a 0·25 mμ layer of Kieselgel G (Macherey Nagel) and dry for 1 hr at 110°.

Line two tanks with Whatman No. 4 filter paper, one containing approximately 1½ cm of solvent system 1 and the other of solvent system 2. 1 μl disposable pipettes.

REAGENTS

2,5-Diphenyl-3(4-styrylphenyl)tetrazolium chloride 0·5% w/v in ethanol. Dilute 5 ml of the above solution to 50 ml with 2N sodium hydroxide immediately before using as spray reagent.

Solvent system 1. Shake together dichloroethane (100 ml), methyl acetate (50 ml) and water (50 ml). Allow to separate and run the lower layer through a filter paper into one tank.

Solvent system 2. Shake together methylene chloride (100 ml), dioxan (50 ml) and water (50 ml). Allow to separate and run the lower layer through a filter paper into the second tank.

Saturation of the solvent systems with water reduces the size of the spot.

METHOD

Dissolve 50 mg ± 1 mg of the sample in 10 ml of chloroform : methanol mixture 1 : 1. Select 2 plates and mark off at 1·5 cm intervals, 3 cm from

From Glaxo Laboratories Limited, Montrose, Angus.

the end. Apply 1 μl of the solution of the substance under test to the first mark on each plate. To subsequent marks apply as standards 1 μl of a 0·5% solution of the authentic specimen, 1 μl of a 0·005% and 1 μl of a 0·01% solution of both hydrocortisone and cortisone acetate (equivalent to 1% and 2% impurity). Where impurities other than hydrocortisone and cortisone acetate are possible, these may be used instead provided one substance of low Rf value and one substance of high Rf are used.

Place one plate in each tank with the spots at the lower end and allow the solvent system to rise to 3 cm from the top of the plates.

Allow the plates to dry at room temperature for 5 min, heat at 105° for 5 min, then spray with alkaline 2,5-diphenyl-3(4-styrylphenyl)tetrazolium solution. The sample should give a spot on each plate of the same intensity and in the same position as the authentic specimen. By comparison with the standards the amount of any impurities shown as subsidiary spots can be estimated and may possibly be identified.

RESULTS

These are summarised in Table 1. One point deserves mention. Under the conditions used, dexamethasone and betamethasone could not be separated and the separation of dexamethasone acetate from betamethasone acetate was marginal. However, on plates spread with alumina (Fluka), separation of the latter pair of substances was possible using solvent system 1.

TABLE 1. Rf VALUES OF CORTICOSTEROIDS IN SOLVENT SYSTEMS 1 AND 2

Sample	Rf value of sample: Rf of cortisone acetate in system 1	Sample	Rf value of sample: Rf of hydrocortisone alcohol in system 2
Hydrocortisone	0·19	Hydrocortisone hydrogen succinate	0·18
6-Methylhydrocortisone	0·19	Triamcinolone	0·59
Betamethasone	0·26	Prednisolone	0·83
Triamcinolone acetonide	0·50	Methylprednisolone	0·89
Fluocinolone acetonide	0·53	Dexamethasone	1·0
Prednisolone acetate	0·53	Betamethasone	1·0
Hydrocortisone acetate	0·66	6-Methylhydrocortisone	1·05
Betamethasone 17-valerate	0·71	Prednisone	1·21
Betamethasone acetate	0·76	Cortisone 21-alcohol	1·37
Dexamethasone acetate	0·79	Triamcinolone acetonide	1·39
Prednisone acetate	0·79	Fluocinolone acetonide	1·40
Fludrocortisone acetate	0·87	Cortisone acetate	1·88
Prednisolone trimethyl acetate	1·10		
Betamethasone 21-valerate	1·34		
Deoxycortone acetate	1·92		
Deoxycortisone trimethylacetate	2·22		

Reference

Brooks, S. G., Evans, R. M., Green, G. F. H., Hunt, J. S., Long, A. G., Mooney, B. & Wyman, L. J. (1958). *J. chem. Soc.*, 4614.

The paper was presented by THE AUTHOR.

J. Pharm. Pharmacol., 1964, **16**, *Suppl.*, 11*T*–16*T* Received April 30, 1964

Some aspects of the use of thin-layer chromatography in a limit test for related foreign steroids

C. J. CLIFFORD, J. V. WILKINSON AND J. S. WRAGG

To attain the maximum separation of the steroid impurities in pharmaceutically important synthetic corticosteroids the running of two or more chromatograms using different solvent systems is recommended. Using adsorption chromatography, solvent systems of the type 1,2-dichloroethane : methanol : water, 95 : 5 : 0·2, give the most satisfactory general separations. Some steroids can best be separated by partition chromatography, using formamide as the stationary phase and chloroform : ether : water, 80 : 20 : 0·5, or cyclohexane : tetrachloroethane : water, 50 : 50 : 0·1, as the mobile phase. A spray reagent of 0·05% tetrazolium blue and 8% sodium hydroxide in methanol is recommended. The amounts of foreign related steroids can be limited by running standard amounts of impurities alongside the steroid under test and comparing the colour intensities of the impurities in the steroid with those of the standards.

CORTISONE acetate and a number of related synthetic steroids are assayed in the British Pharmacopoeia 1963 by measuring the colour of the formazan produced by the reducing action of the steroids on triphenyltetrazolium. In addition, to limit the presence of impurities that are tetrazolium-reactive, a paper chromatographic procedure is included in which the impurities are separated and then reacted with a triphenyltetrazolium reagent.

The well-known advantages of thin-layer chromatography over paper chromatography have led us to investigate this newer technique as an alternative means of testing for tetrazolium-reacting impurities.

Experimental and results

MATERIALS AND APPARATUS

Adsorbents. Kieselgel G and Kieselgel GF 254 (Merck). With Kieselgel GF 254 the chromatograms were examined in ultra-violet light at 254 mμ.

The steroid solutions were applied to the thin layers by micro-capillary (Micro-cap) pipettes. The reproducibility of the delivery of ten 5μl pipettes was checked by weighing the amount of water delivered. For any given pipette, no delivery deviated by more than 2% from the mean and the means ranged from 4·7–5·0 μl.

The steroids examined are those listed in Table 1.

Analytical grade solvents were used, purified where necessary (Bush, 1961). 2-Methoxyethyl acetate was purified by the procedure 19·054 of the Official Methods of Analysis of the A.O.A.C. (1960).

GENERAL PROCEDURE

Shake the adsorbent (25 g) for 1 min with water (50 ml) and spread the slurry at a thickness of 0·25 mm over twenty 5 × 20-cm or five

From the Analytical Development Group, Standards Department, Boots Pure Drug Company Ltd., Station Street, Nottingham.

TABLE 1. CHROMATOGRAPHY IN VARIOUS SOLVENT SYSTEMS

Steroid	Number of hydroxyl groups	Number of carbonyl groups	Running distances (hydrocortisone acetate = 1·00) Solvent system A	Solvent system B	Solvent system C	Running distances (hydrocortisone = 1·00) Solvent system D	Running distances (hydrocortisone acetate = 1·00) Solvent system E
Deoxycortone acetate	0	2	2·44	2·51	1·42	3·0	2·0
Reichstein's Compound S 21-acetate	1	2	1·62	1·90	1·36	3·0	2·0
Deoxycortone	1	2	1·76	1·96	0·98	3·0	2·0
Cortisone acetate	1	3	1·25	1·45	1·02	2·9	1·72
Prednisone acetate	1	3	1·16	1·24	0·89	2·8	1·54
Dexamethasone acetate	2	2	1·11	1·14	1·18	2·7	1·05
Fludrocortisone acetate	2	2	1·03	1·14	1·16	2·6	0·62
6-α-Methylhydrocortisone 21-acetate	2	2	1·00	1·10	1·08	2·8	1·42
Hydrocortisone acetate	2	2	1·00	1·00	1·00	2·7	1·00
Methylprednisolone acetate	2	2	0·92	0·90	1·00	2·7	1·19
Reichstein's Compound S	2	2	0·84	1·02	0·86	2·7	1·27
Presnisolone acetate	2	2	0·78	0·82	0·92	2·5	0·72
Triamcinolone acetonide	2	2	0·59	0·73	0·74	2·6	0·99
Cortisone	2	3	0·51	0·63	0·53	1·92	0·24
Prednisone	2	3	0·43	0·51	0·44	1·70	0·16
6-α-Methylhydrocortisone	3	2	0·27	0·31	0·50	1·55	0·13
Dexamethasone	3	2	0·27	0·35	0·68	0·91	0·04
Hydrocortisone	3	2	0·24	0·29	0·45	1·00	0·06
Fludrocortisone	3	2	0·24	0·33	0·63	0·70	0·01
Betamethasone	3	2	0·22	0·35	0·69	0·91	0·04
Methylprednisolone	3	2	0·22	0·24	0·45	1·24	0·09
Presnisolone	3	2	0·19	0·22	0·39	0·72	0·04
16-α-Hydroxyfludrocortisone	4	2	0·14	0·18	0·31	0·19	0·01
Triamcinolone	4	2	0·08	0·14	0·27	0·04	0

On chromatograms run 15 cm, the approximate distances in cm travelled by hydrocortsone acetate in solvent systems A, B, C and E are 3·7, 5·1, 6·2 and 6·8 and by hydrocortisone in solvent system D is 4·7.
Solvent systems: A—1,2-Dichloroethane : methanol : water—95 : 5 : 0·2.
B—1,2-Dichloroethane : 2-methoxyethyl acetate : water—80 : 20 : 1.
C—Cyclohexane : ethyl acetate : water—25 : 75 : 1.
D—Stationary phase: Chromatoplate run in 20% v/v solution of formamide in acetone. Mobile phase: Chloroform : ether : water—80 : 20 : 0·5.
E—Stationary phase: Chromatoplate run in 25% v/v solution of formamide in acetone. Mobile phase: Cyclohexane : tetrachloroethane : water—50 : 50 : 0·1.

20 × 20-cm glass plates. Activate the resulting thin layers at 105–110° for 1 hr after drying at room temperature for 30 min. Remove a thin border of silica gel from the sides of the chromatoplate to reduce "edge-effects." Store the chromatoplates, unless used immediately, in desiccators over anhydrous silica gel, for a maximum of four days.

Introduce the solvent mixture into the tank, line the sides of the tanks with Whatman No. 1 filter-paper, saturated with solvent and allow to equilibrate for 1–2 hr.

Spot a suitable quantity of the steroid, usually 5–10 μg in 2–5 μl of chloroform, 2 cm from the lower edge of the chromatoplate. Allow the running solvent to ascend the chromatogram until 15 cm past the spotting line. Remove the solvent by drying for 10 min in a stream of cold air from a hair drier.

SOLVENT SYSTEMS

(a) *Adsorption chromatography.* The running distances of the steroids, relative to the movement of hydrocortisone acetate, in three useful solvent systems on Kieselgel GF 254 are listed in Table 1 (systems A, B and C). System A is similar to those used by Bennett & Heftmann (1962). They were selected from 1500 systems examined.

(*a*) *Partition chromatography.* An examination of 400 partition systems was made using formamide on silica gel as the stationary phase. The formamide was applied by running the activated chromatoplate in a solution of formamide in acetone. The steroid solutions were spotted and the chromatograms run in the mobile phase. The running distances of the steroids, relative to that of hydrocortisone or of hydrocortisone acetate, in two partition systems are listed in Table 1 (systems D and E).

TETRAZOLIUM BLUE REAGENT

A spray reagent of 0·05% tetrazolium blue and 8% sodium hydroxide in methanol reacts with the steroids on the chromatogram to give blue spots on a white background. The reagent is in two parts. Part A, 0·1% w/v tetrazolium blue in methanol. Part B, 16% w/v sodium hydroxide in methanol. Equal parts of A and B are mixed immediately before use. Over-spraying with the reagent must be avoided, otherwise the chromatogram will disintegrate. 1–1·5 ml is suitable for a 5 × 20 cm chromatogram.

The tetrazolium blue reagents suggested by Matis, Adamec & Galvánek (1962) and Nishikaze & Staudinger (1962) and the 2,5-diphenyl-3-(4-styryl-phenyl)tetrazolium reagent suggested by Stevens (1964) were examined but they were found to be appreciably less sensitive than the proposed reagent. Other tetrazolium salts were examined but were also less sensitive.

Cortisone, hydrocortisone, prednisolone, prednisone, prednisolone acetate, hydrocortisone acetate, cortisone acetate, prednisone acetate, dexamethasone and betamethasone all gave approximately the same response to the tetrazolium blue reagent. The approximate limit of detection of each was about 0·03 μg when each was run 3 cm in the same solvent system.

FACTORS AFFECTING THE RESPONSE TO THE TETRAZOLIUM BLUE REAGENT

(*a*) *Removal of excess solvent from the chromatogram.* The use of heat to remove excess solvent from the chromatogram results in a loss of sensitivity when the steroids are subsequently sprayed with the reagent. The limit of detection of prednisolone after chromatography with 1,2-dichloroethane: acetone (2:1), was 0·02 μg when the chromatogram was dried under a stream of cold air from a hair-drier for 10 min but 0·03 μg when dried at 100° for 10 min.

(*b*) *Application of steroid solution to the chromatoplate.* The area occupied by the steroid when applied to the chromatoplate initially, determines the area it occupies after chromatography and hence its apparent response to the reagent. This area is dependent upon the solvent used to dissolve the steroid, the way in which the solution is applied and the concentration of the solution.

5 μg of prednisolone, dissolved in chloroform, chloroform:methanol (97·5 + 2·5) and dioxane and spotted in one 5 μl application gave spots at the point of application of 2·9, 3·5 and 9·0 mm diameter, respectively.

5 μg of prednisolone dissolved in chloroform, when applied in two 2·5 μl amounts and in one 5 μl amount, gave spots of 2·6 and 2·9 mm diameter, respectively. 5-μl applications of 2·5 and 10 μg amounts of prednisolone dissolved in chloroform gave spots of 2·5 and 3·0 mm diameter, respectively.

(*c*) *Running distance.* The distance moved by a steroid on the chromatogram affects its response to the reagent. The approximate limit of detection of hydrocortisone acetate, run 1, 2, 3 and 5 cm, was 0·01, 0·02, 0·03 and 0·04 μg, respectively.

(*d*) *Amount of steroid and concentration of impurity.* Various amounts of cortisone acetate, prednisone acetate and prednisolone acetate were added to solutions of "pure" hydrocortisone acetate and 5-μl applications were chromatographed in the solvent system A. The response of the added impurities is indicated in Table 2.

TABLE 2. DETECTION OF IMPURITIES AT DIFFERENT CONCENTRATIONS

Amount of hydrocortisone acetate applied in one 5-μl application μg	Added steroid	Response of added steroids at concentrations of					Running distance of the added steroid cm
		0·5%	1·0%	2·0%	3·0%	4·0%	
5	Cortisone acetate	–	?	+	++	+++	4·8
5	Prednisone acetate	–	?	+	++	+++	4·2
5	Prednisolone acetate	?	+	+	++	+++	2·7
10	Cortisone acetate	?	+	++	+++	+++	5·1
10	Prednisone acetate	?	+	++	+++	+++	4·5
10	Prednisolone acetate	+	++	+++	+++	+++	2·8
15	Cortisone acetate	+	++	+++	+++	+++	5·1
15	Prednisone acetate	+	++	+++	+++	+++	4·5
15	Prednisolone acetate	++	++	+++	+++	+++	2·7

Symbols: – Not detectable. ? Very faint spot. + Faint spot. ++ Easily detectable. +++ Very easily detectable.

Discussion

Using adsorption chromatography, solvent systems such as ethylene dichloride (or chloroform)/methanol (or dioxane or 2-methoxyethyl acetate) separate the steroids according to the number of hydroxyl and carbonyl groups each possesses (Table 1, solvent systems A and B). Steroids that differ only in their less polar groups are less easily separated by this type of solvent system. Prednisolone and hydrocortisone are just separated from each other, but fludrocortisone and dexamethasone are not separated from hydrocortisone nor is methylprednisolone separated from prednisolone.

The solvent system of cyclohexane: ethyl acetate, though giving a less effective separation of the steroids according to their polarity, does separate fludrocortisone and dexamethasone from hydrocortisone.

Up to 2% water added to the solvent system often results in a considerable reduction of the tailing to which the more polar steroids are prone. An optimum separation of steroids within a certain range of polarity

is obtained by adjusting the proportions of the solvents. Thus 1,2-dichloroethane : methanol : water, 95 : 5 : 0·2 is suitable for separating the acetates listed in Table 1, while the alcohols are better separated when the solvents are in the proportions 92 : 8 : 0·5.

Compared with adsorption chromatography, the disadvantages of partition chromatography are that the spot sizes tend to be larger, especially those of the less polar steroids, and that it is a more time-consuming procedure. The advantages of partition chromatography are that the spread of the steroids over the chromatogram is improved and that some separations are achieved that are not possible by adsorption chromatography. Thus a better separation is obtained between hydrocortisone and prednisolone and a good separation is obtained between prednisolone and methylprednisolone.

The apparent response of a steroid on a chromatogram to the reagent depends on the nature and volume of the solvent in which the steroid is applied to the chromatoplate, the amount of steroid applied and the distance moved by the steroid. In developing a test it is necessary to standardise these conditions or to compensate for any that cannot be readily controlled.

A test in which a sample is deemed satisfactory when the impurities are not visible suffers from two disadvantages: (1) It is often difficult to decide whether or not an impurity is just visible. (2) Any variations in test conditions that affect the distance the impurities travel on the chromatogram will affect their limits of detection.

These disadvantages can be overcome by applying standard impurities to each chromatoplate and comparing the intensities of the sample and standard impurity spots. The standard impurities should be easily visible, but not too intensely coloured or comparison becomes difficult.

Assuming a limit of not more than 3% of any tetrazolium-reactive impurity, hydrocortisone for example, could be examined using the following adsorption systems and standards:

(i) Apply 5 μl of a 0·10% chloroform solution of the sample and 5 μl of a chloroform solution containing 0·10% of pure hydrocortisone and 0·003% each of prednisolone, prednisone and prednisolone acetate. Run the chromatogram with the solvent system 1,2-dichloroethane : methanol : water, 92 : 8 : 0·5.

(ii) Apply 5 μl of a 0·10% chloroform solution of the sample and 5 μl of a chloroform solution containing 0·10% of pure hydrocortisone and 0·003% each of cortisone, hydrocortisone acetate and cortisone acetate. Run the chromatogram with the solvent system 1,2-dichloroethane : methanol : water, 95 : 5 : 0·2.

(iii) Apply 5 μl of a 0·10% chloroform solution of the sample and 5 μl of a chloroform solution containing 0·10% of pure hydrocortisone and 0·003% of fludrocortisone. Run the chromatogram with the solvent system cyclohexane : ethyl acetate : water, 25 : 75 : 1.

After the spraying with the tetrazolium blue reagent, for chromatograms (i) and (ii) compare the intensity of any impurity from the sample

lying within the region of the chromatogram between the highest and lowest standard impurity spots with that of the nearest standard impurity spot; for chromatogram (iii) compare with the standard impurity spots the intensity of any impurity from the sample at a similar running distance.

References

Bennett, R. D. & Heftmann, E. (1962). *J. Chromatog.*, **9**, 348–352.
British Pharmacopoeia 1963, pp. 1074–1076.
Bush, I. E. (1961). *The Chromatography of Steroids*, pp. 347–357, London: Pergamon Press.
Official Methods of Analysis of the Association of Official Agricultural Chemists, 9th Edition, 1960, p. 254.
Matis, J., Adamec, O & Galvánek, M. (1962). *Nature, Lond.*, **194**, 477.
Nishikaze, O. & Staudinger, H. J. (1962). *Klin. Wschr.*, **40**, 1014.
Stevens, P. J. (1964). *J. Chromatog.*, **14**, 269–273.

The paper was presented by MR. WRAGG.

J. Pharm. Pharmacol., 1964, **16**, *Suppl.*, 17*T*-23*T* Received April 28, 1964

The detection and identification of other 17,21-dihydroxy-20- oxosteroids in corticosteroids

C. A. JOHNSON AND SYLVIA FOWLER

A method is described for the separation of corticosteroids by paper chromatography. It is more sensitive than the present official test and will control the presence of related foreign steroids to a uniform degree and in a reproducible manner. Details of the application of this test to many pharmaceutically important steroids are given and the relationship of molecular structure to mobility in the solvent systems used is discussed.

THE paper chromatography of steroids has been extensively reviewed by Bush (1961). Much of this work has been concerned with the isolation, identification and estimation of steroids as an aid to clinical studies. Our interest, on the other hand, was to define a test to limit the proportion of related foreign steroids in corticosteroids of pharmaceutical significance. With many such substances, now available, each capable of manufacture by a variety of syntheses, the problem of devising a test of general applicability is complex. To be effective as an official criterion of purity, such a test should control all possible related compounds to approximately the same extent, and it should be uniformly applicable in all laboratories. Tests designed to fulfil this need, and based on the solvent systems used by Zaffaroni, Burton & Keutmann (1950) and Burton, Zaffaroni & Keutmann (1951a, b) have been described (British Pharmacopoeia 1963; United States Pharmacopeia XVI). These systems have proved particularly effective because they have a sufficiently high capacity to allow an adequate sample to be chromatographed so that minor constituents may be recognised and estimated; no doubt, however, other systems might also be applicable. Considerable work in conjunction with the establishment of a collection of authentic specimens of various corticosteroids has shown that the published tests are not uniformly sensitive for all 17,21-dihydroxy-20-oxosteroids since diffusion of a substance as it moves down the paper necessarily results in a decrease of sensitivity. The extent to which this diffusion can affect the conclusions to be drawn from the test has been determined and a standardised procedure has been developed that will enable impurities to be equally detected, irrespective of their Rf values.

Experimental and results

The sensitivity of various means of detection, in relation to distance moved, was studied as follows: Papers (Whatman No. 1) were dipped in a 40% v/v solution of formamide in methanol, and blotted. Chromatograms were developed as described in the British Pharmacopoeia 1963 page 1075 for Related Foreign Steroids, various loadings of cortisone acetate were used and the chromatograms were run (with mobile phase B)

From the Laboratory of the British Pharmacopoeia Commission, General Medical Council Office, 44 Hallam Street, London, W.1.

for different lengths of time. After removal from the tank, the papers were dried in a current of air for 5 min then heated at 105° for 40 min.

The minimum quantities of cortisone acetate detectable under different conditions are given in Table 1.

TABLE 1. DETECTABILITY OF CORTISONE ACETATE WITH VARIOUS REAGENTS IN RELATION TO DISTANCE MOVED

Distance of spot from starting line (cm)	Method of detection used				
	254 mμ U.V. light	TPTZ	TPTZ fluorescence after heat*	BT	DPST
	Amount visible (μg)				
4	0·25	4 (?)	0·5	0·25	0·25
6·5	0·5	8	1	0·25	0·25
8	0·5	8	0·5	0·5	0·25
13	1	8	0·5	0·5	0·5
15	2	8	1	1	0·5
22	2	12	2	1	0·5
30	2	12	1	2	1

TPTZ. Alkaline triphenyltetrazolium chloride solution. B.P. 1963, page 1076.

BT. Blue tetrazolium solution. A 0·1% w/v solution of blue tetrazolium in 2N sodium hydroxide.

DPST. A 0·1% solution of diphenylstyrylphenyltetrazolium chloride prepared as described in the recommended procedure.

* No fluorescence is obtained with $\Delta^{3,4}$ corticosteroids.

A second series of experiments explored the sensitivity of diphenylstyrylphenyltetrazolium chloride (Brooks & others, 1958); this is the preferred means of detection because at the concentration specified, it has good sensitivity and gives a violet spot on a pale yellow background.

Solutions were prepared containing equal amounts of six steroids having different Rf values. These solutions were then chromatographed by the official method (using mobile phase B), but using diphenylstyrylphenyltetrazolium chloride (DPST) as the detecting reagent. Loadings equivalent to 0·125, 0·25, 0·5, 1, 2 and 4 μg of each steroid were used: the distance moved and the minimum quantity detected were then recorded for each steroid (Table 2).

TABLE 2. DETECTABILITY OF CORTICOSTEROIDS* WITH DIPHENYLSTYRYLPHENYLTETRAZOLIUM CHLORIDE IN RELATION TO DISTANCE MOVED

	Distance moved (cm)	Minimum quantity detected (μg)
Solution 1		
Dexamethasone	2·3	0·125
Methylprednisolone	3·6	0·125
Prednisone	5·9	0·25
Fludrocortisone acetate	17	0·5
Dexamethasone acetate	22·5	0·5
Cortisone acetate	30·2	1·0
Solution 2		
Prednisolone	2·1	0·125
Hydrocortisone	3·3	0·125
Prednisolone acetate	18·5	0·5
Hydrocortisone acetate	22·1	0·5
Prednisone acetate	28·0	0·5
Prednisolone trimethylacetate	33·4	1·0

* When each of these steroids is spotted on prepared Whatman No. 1 paper, the minimum quantity detectable with DPST reagent is the same for all, viz. 0·08 μg.

The results in Tables 1 and 2 show that a 4- to 8-fold difference in detectability is to be expected according to the Rf value of the steroid, and that triphenyltetrazolium chloride is insufficiently sensitive for use in a limit test. The precise loss in sensitivity depends on the degree to which the spots diffuse during chromatography, but we believe that the results quoted are typical for the standardised procedure detailed below.

The tables also indicate the minimum amount of a corticosteroid that can be detected at any given distance from the starting line.

The test described below has been designed to limit related foreign steroids to about 3%, a figure at present judged to be an acceptable practical limit. By increasing the loading or decreasing the distance that the solvent front moves, however, it is possible to make the test more stringent. In the standardised procedure, strict adherence to the details is necessary if inter-laboratory agreement is to be achieved. For example, there are many valid ways in which paper may be impregnated with formamide, each giving rise to different amounts of stationary phase. Such differences can lead to considerable variation in the time required for the mobile phase to move a given distance: in our experience this affects the degree of diffusion of the spot.

Apparatus. A rectangular glass tank sufficiently tall to enable a descending chromatogram about 45 cm in length to be prepared.

Reagents. Mobile phase A. A saturated solution of formamide in chloroform.

Mobile phase B. A saturated solution of formamide in a mixture of equal volumes of benzene and chloroform.

Formamide. Reagent grade material (vacuum distillation is unnecessary).

Diphenylstyrylphenyltetrazolium chloride solution (DPST solution). Add one volume of a 0·1% w/v solution of 2,5-diphenyl-3-(4-styrylphenyl)-tetrazolium hydrochloride in ethanol (95%) to nine volumes of 2N sodium hydroxide just before use. This reagent is 66 times weaker than the reagent of Brooks & others (1958).

Other reagents were of analytical reagent quality.

METHOD

Saturate the tank by placing some of the specified mobile phase in the bottom *and also in the solvent trough*,* close the lid and allow to stand (24 hr) at 26° ± 1°. Impregnate suitable sheets of Whatman filter paper No. 1 by dipping in a 40% v/v solution of formamide in methanol and blotting twice between sheets of clean filter paper. 100 ml of the formamide solution, which should be prepared immediately before use, is sufficient to treat four papers 20 cm × 52 cm. With the minimum of delay, apply separately to the paper in 5 µl of acetone, the quantities specified in Table 3 of (*a*) the substance being examined, (*b*) an authentic

* With a tall tank this ensures that both the top and the bottom of the tank is saturated.

specimen of the substance and (*c*) cortisone acetate, limiting the spot diameters to a maximum of 4 mm. Transfer the prepared papers to the tank as soon as possible, having first emptied and dried the solvent trough. Allow the closed tank to equilibrate for an hour and a half,

TABLE 3. LOADINGS TO BE APPLIED IN THE RECOMMENDED METHOD

Mobile phase	Distance of solvent front from starting line (cm)	Authentic specimen loading (μg)	Substance under examination loading (μg)	Cortisone acetate loading (μg)
A	35±2	25	25 50	1·0
B	22±2	25	25 —	0·5

introduce the specified mobile phase into the solvent trough and let the solvent front travel the distance from the starting line specified in Table 3. Remove the paper from the tank, allow to dry at room temperature for 5 min, heat at 105° for 40 min and develop by rapid passage through a shallow layer of DPST solution. Blot between sheets of filter paper and lay on a clean sheet of filter paper for examination. Secondary spots develop more slowly than the primary spot and also fade more quickly. The chromatograms should be examined in a subdued light for 2 min immediately after development. *The cortisone acetate spot must always be visible; if not the test should be rejected.*

The substance being examined and the authentic specimen each yield, equidistant from the starting line, a violet spot. If no secondary spots are visible when using mobile phase B, the substance passes the test. With mobile phase A, if no secondary spots are visible above 22 cm from the starting line on the 25 μg loading, or below 22 cm from the starting line on the 50 μg loading, the substance passes the test.

Where the substance does not pass the test, the following procedure is adopted. Measure the distance that any secondary spot has travelled from the starting line and repeat the procedure described above, using the loadings of the substance being examined as specified in Table 4 and the same loading of cortisone acetate as in the previous test. The larger of the two loadings may show a secondary spot but the smaller loading should not. As before, the cortisone acetate spot must be visible.

TABLE 4. LOADINGS TO BE APPLIED IN THE RECOMMENDED METHOD WHEN A SECOND CHROMATOGRAM IS NECESSARY

Distance of secondary spot from starting line (cm)	Larger loading (μg)	Smaller loading (μg)
4	4	2
8	8	5
12	12	7
16	16	10
20	20	12
24	24	14
28	30	18
32	38	23
36	50	30

With mobile phase B, certain impurities may move such a short distance that it is not possible to suggest suitable loadings. Where any impurity has moved less than 4 cm, the test should be repeated using mobile phase A.

The recommended mobile phase for individual corticosteroids, together with Rf values, are listed in Table 5. With each substance, materials from as many manufacturing sources as possible were examined, together with W.H.O. Authentic Chemical Substances and U.S.P. Reference Preparations, where available.

TABLE 5. RECOMMENDED MOBILE PHASES AND RF VALUES FOR THE MORE FREQUENTLY ENCOUNTERED CORTICOSTEROIDS

Mobile phase A	Rf	Mobile phase B	Rf
Betamethasone	0·16	Betamethasone acetate	0·44
Cortisone	0·62	Betamethasone 17-valerate	0·86
Dexamethasone	0·21	Betamethasone 21-valerate	0·89
Hydrocortisone	0·26	*Cortisone acetate	0·80
6-Methylhydrocortisone	0·36	Dexamethasone acetate	0·60
Methylprednisolone	0·27	Fludrocortisone acetate	0·45
Prednisolone	0·15	Fluocinolone acetonide	0·40
Prednisone	0·55	Hydrocortisone acetate	0·59
		Prednisolone acetate	0·49
		Prednisolone trimethylacetate	0·89
		Prednisone acetate	0·74
		Triamcinolone acetonide	0·52

* As a test of reproducibility, seven samples of cortisone acetate from different sources have been examined repeatedly over a period of several months. Values recorded for the Rf ranged between 0·76 and 0·80.

Hydrocortisone sodium and hydrogen succinates, betamethasone sodium phosphate and the hydrogen and sodium phosphates of prednisolone remain on the starting line with both mobile phases; with these substances the test is only of value in detecting unesterified material. Triamcinolone remains on the starting line with both mobile phases and in this case the test may be used to detect impurities having no hydroxyl group at position 16. Deoxycortone acetate and deoxycortone trimethylacetate move with the solvent front in both mobile phases and the test can be applied to detect impurities substituted with a hydroxyl or oxo group at position 11.

The proposed test depends on the assumption that all the corticosteroids under consideration (and impurities having the 17,21-dihydroxy-20-oxo-side-chain) respond with equal sensitivity to the reagent (see footnote to Table 2). This assumption is also supported by the similar responses that many corticosteroids show to 2,3,5-triphenyltetrazolium chloride in the assays of the British Pharmacopoeia. With substances esterified with a large group at position 21 (hydrocortisone sodium succinate and prednisolone trimethylacetate for example) the response to DPST is delayed a little probably owing to the time required for hydrolysis of the ester. The inclusion of a small loading of cortisone acetate with each test ensures that the overall sensitivity of that test is satisfactory and thus that any conclusions drawn are valid.

Discussion

The test is designed to limit the presence of any one foreign steroid in a particular corticosteroid to not more than about 3%. However if two or more impurities are each present in amounts just below this limit, the sample would often pass the test despite the aggregate of impurities exceeding the desired limit. The presence of more than one impurity in

significant quantity however, is usually detected in a preliminary chromatogram. It is then often possible to gain further information by chromatographing with suitably larger loadings or for shorter distances.

Information about the nature of unknown impurities can be gained by comparing the Rf values with those of corticosteroids of known structure. Such correlations have been extensively discussed by Bush (1961). In the test described above, using mobile phase B the following relationships between molecular structure and mobility have been noted. Acetylation of the 21-hydroxyl group brings about an increase in Rf value of approximately 0·5 whilst esterification with trimethylacetic acid (pivalic acid) increases the value still more. In a 21-acetate, replacement of the 11-hydroxyl group by an 11-oxo group causes an increase of between 0·2 and 0·25; similar replacement in a free 21-alcohol produces an increase of about 0·1. In the absence of hydroxyl or oxo substituents at position 11 and of hydroxyl substitution at position 17 (as in deoxycortone acetate and deoxycortone trimethylacetate), there is a marked increase in mobility. A double bond in the 1,2 position in addition to that in the 3,4 position causes a decrease of between 0·05 and 0·1, both in the free 21-alcohols and the corresponding esters. The presence of a non-polar substituent such as a methyl group (usually in position 6 or 16) enhances mobility so that 6-methylhydrocortisone and 6-methylprednisolone have Rf values approximately 0·05 greater than the unsubstituted compounds. Polar groups, on the other hand, reduce mobility and 9-fluorohydrocortisone acetate has an Rf value about 0·14 less than that of hydrocortisone acetate. In dexamethasone acetate, which has the 9-fluoro- substituent and also a methyl group at position 16, the opposing effects of polar and non-polar groups eliminate their respective influences so that this substance is comparable to hydrocortisone acetate in mobility. Very strongly polar substitution prevents movement in mobile phase B and substances such as hydrocortisone hydrogen (or sodium) succinate, prednisolone acid phosphate and triamcinolone (which has fluoro-substitution at position 9 and hydroxyl substitution at position 16) remain at the point of application. For triamcinolone acetonide, where a 16-hydroxyl group participates in acetonide formation, considerable mobility is restored (Rf, 0·52) whilst in fluocinolone acetonide, which differs only in having a second fluoro-group at position 6, the Rf value is reduced by 0·12. This reduction is similar to that caused by the introduction of a single fluoro- group into hydrocortisone acetate.

With the slower moving corticosteroids these differences may be accentuated by running in mobile phase A, but similar relationships exist. Dexamethasone and betamethasone, which differ only in the orientation of the methyl group at position 16, can be differentiated in this system when allowed to run side by side on the same paper. The Rf value for dexamethasone (16α-methyl) is 0·21 and that for betamethasone (16β-methyl), 0·16. From these observations the substituents present in unexpected contaminants can often be deduced and related to the synthetic process used.

Acknowledgements. We thank members of the Hormones Committee of the British Pharmacopoeia for helpful comments.

References

Brooks, S. G., Evans, R. M., Green, G. F. H., Hunt, J. S., Long, A. G., Mooney, B. & Wyman, L. J. (1958). *J. chem. Soc.*, 4614–4628.
Burton, R. B., Zaffaroni, A. & Keutmann, E. H. (1951a). *J. biol. Chem.*, **188**, 763–771.
Burton, R. B., Zaffaroni, A. & Keutmann, E. H. (1951b). *Ibid.*, **193**, 749–767.
Bush, I. E. (1961). *The Chromatography of Steroids*, London: Pergamon Press.
Zaffaroni, A., Burton, R. B. & Keutmann, E. H. (1950). *Science*, **111**, 6–8.

The paper was presented by MR. JOHNSON

J. Pharm. Pharmacol., 1964, **16**, *Suppl.*, 24T–26T Received April 15, 1964

SHORT COMMUNICATION

Polarographic determination of microgramme quantities of chlorpromazine

G. S. PORTER

FEW polarographic procedures have been described for the determination of phenothiazine derivatives.

Kabasakalian & McGlotten (1959) reported anodic oxidation of about 1·5 mg quantities, while Blazek (1956) employed an amperometric method on 50 mg quantities. The method used by Chuen & Riedel (1961) is useful at the level of 350 μg.

A method sensitive at least to 2 μg of this class of compound was required, and initially chlorpromazine has been examined. Direct cathodic polarography of chlorpromazine solutions was not satisfactory, but treatment of a solution of the substance with bromine water produced a reducible solution with a well-marked polarographic wave. Bromine water was chosen as a suitable oxidant as excess bromine was readily removed by flushing with nitrogen.

Thin-layer chromatography of chlorpromazine after oxidation with bromine water showed no spot corresponding to unchanged starting material.

After examination of the effect of pH on the cathodic wave, 0·5N hydrochloric acid was chosen as a suitable electrolyte.

The determination is applicable to small quantities (0·002 to 0·1 ml) of chlorpromazine injection (25 mg/ml) and to corresponding amounts of chlorpromazine tablets and syrup.

EXPERIMENTAL AND RESULTS

Equipment. A Southern Analytical Ltd. K1000 cathode ray polarograph was used. Determinations were made at 25° using a mercury pool anode.

Calibration graph. Chlorpromazine base (50 mg) was dissolved in and made up to one litre with 0·5N hydrochloric acid. Quantities of 1, 2, 3, 4 and 5 ml of this solution were placed in each of 25 ml graduated flasks and diluted to the mark with 0·5N hydrochloric acid.

Each 25 ml of solution was treated with two drops of saturated bromine water, shaken, allowed to stand (1 min) and 1 ml transferred to a polarographic cell. After being flushed with nitrogen (3 min) the solution was polarographed using an initial potential of −0·5 V.

A clear, well formed wave resulted, with a peak potential of about −0·75 V, increasing in negativity with chlorpromazine concentration.

From the Pharmaceutical Chemistry Laboratories, Department of Pharmacy, College of Technology, New Building, Byrom Street, Liverpool.

Values of peak current and peak potential were recorded for each concentration (Table 1).

TABLE 1. PEAK CURRENTS AND PEAK POTENTIALS OF BROMINATED CHLORPROMAZINE TEST SOLUTIONS

Concentration of chlorpromazine (μg/ml)	Peak voltage (V)	Peak current (μA)
2	−0·740	0·31
4	−0·745	0·63
6	−0·750	0·96
8	−0·760	1·26
10	−0·775	1·60

The effect of pH was examined using Britton Robinson buffer solutions. An increase in pH resulted in more negative peak potentials and gave waves whose heights were difficult to read at the low concentrations employed because of the long slope imparted to the less negative side of the peak. 0·5N Hydrochloric acid, however, was satisfactory, giving a clearly defined wave at concentrations of chlorpromazine well below 0·5 μg/ml.

To assess the reproducibility of the method, 28 solutions of chlorpromazine in 0·5N hydrochloric acid were assayed as described above. The results, derived from the calibration graph, are summarised in Table 2.

TABLE 2. PEAK POTENTIALS AND ANALYSES OF BROMINATED CHLORPROMAZINE SOLUTIONS

Peak potential range V	Range of concentrations examined μg/ml	Standard deviation
−0·74 to −0·78	1 to 2	0·037
	2 to 4	0·037
	4 to 6	0·110
	6 to 8	0·085
	8 to 10	0·142

Assay of chlorpromazine injection, tablets and syrup. The sample (0·1 ml for liquid preparations, 0·2 g for tablets) was diluted or extracted with 0·5N hydrochloric acid to give 25 ml of a solution containing between 1 and 8 μg/ml of chlorpromazine. This solution was oxidised with bromine water and polarographed as described; the concentration was determined from the calibration graph. The mean of four assays on each type of preparation is shown in Table 3.

TABLE 3. RESULTS FOR POLAROGRAPHIC ASSAY OF CHLORPROMAZINE PREPARATIONS

Preparation	Concentration of chlorpromazine base in original preparation	
	Nominal	Found
Injection	22·4 mg/ml*	21·7 mg/ml*
Syrup	6·2 mg/ml	5·8 mg/ml
Tablets	11·8% w/w	11·3% w/w

* A fresh ampoule 25 mg/ml was used for each assay.

Method of standard addition applied to chlorpromazine injection. 25 ml of a solution containing about 2 μg of chlorpromazine per ml was prepared by diluting the injection with 0·5N hydrochloric acid. This was oxidised with bromine water, 1 ml pipetted out and flushed with nitrogen, polarographed and the peak current recorded.

25 ml of a solution in 0·5N hydrochloric acid, containing exactly 6 μg/ml of pure chlorpromazine was oxidised with bromine water and a few ml flushed with nitrogen. 1 ml of this standard was then added to the 1 ml of test already in the polarographic cell, nitrogen again passed to mix, and the peak current recorded.

The unknown concentration (C_1) is calculated from the formula

$$C_1 = \frac{i_1 \cdot v \cdot C_s}{i\,(V + v) + i_1 v}, \text{ where}$$

V = volume of unknown solution (ml); v = volume of standard added (ml); C_s = concentration of the standard (μg/ml); i = the increase in peak current (μA); i_1 = original peak current (μA).

DISCUSSION

The method described is rapid and simple. Compared to spectrophotometric methods it shows better sensitivity; it is also more specific. Preparation of a five point calibration graph takes about 30 min; a single determination takes about 6 min. The calibration is reproducible for any given capillary, and checks were made from time to time using two different standard solutions.

Using this method it has been possible to show directly the diminution in concentration of chlorpromazine in acid solutions on exposure to air and light. Such decomposition products as are formed have no effect on the characteristic wave.

The nature of the reaction product of bromine and chlorpromazine is unknown: it is not chlorpromazine sulphoxide.

Acknowledgements. My thanks are due to Mr. S. G. E. Stevens (Smith, Kline & French Laboratories Ltd.) for helpful advice, and to Mrs. J. Beresford for much help in the experimental work. Chlorpromazine base was kindly supplied by Messrs. May & Baker Ltd.

References

Blazek, J. (1956). *Ceskoslov. Farm.*, **4**, 210–212.
Chuen, N. & Riedel, B. E. (1961). *Canad. Pharm. J., Sci. Sect.*, **94**, No. 4, 51–53.
Kabasakalian, P. & McGlotten, J. (1959). *Analyt. Chem.*, **31**, 431–433.

The paper was presented by THE AUTHOR.

J. Pharm. Pharmacol., 1964, **16**, *Suppl.*, 27*T*–31*T* Received May 1, 1964

A specific method for the determination of amphetamine in urine

A. H. BECKETT AND M. ROWLAND

A specific and sensitive method for the quantitative determination of amphetamine in urine, by gas chromatography, is described.

A NUMBER of methods have been used for the determination of amphetamine in urine, but all are non-specific. Richter (1938) used a picric acid dye-complexing method and this was modified by Jacobsen & Gad (1940), and by Harris, Searle & Ivy (1947). The methyl orange method of Brodie & Udenfriend (1945) was modified by Keller & Ellenbogen (1952) and found to be more sensitive than previous methods. Subsequent modifications of this have been used by Axelrod (1954), Utena, Ezoe & Kato (1955), Connell (1956) and Chapman, Shenoy & Campbell (1959).

Amphetamine has also been determined by coupling the molecule with diazotised *p*-nitroaniline (Beyer & Skinner, 1940) and measuring spectrophotometrically the red colour produced under alkaline conditions. Combined with protein precipitation and steam distillation this has been used by McNally, Bergman & Polli (1947).

Alles & Wiesgarver (1961) claim that the method of McNally & others (1947) gave inconsistent results, but by suitable standardisation obtained a working procedure. The method was slightly modified by Krivulka (1962).

Primary amines are present in urine and these react in the diazotisation method. Other amines are also present in urine and all these bases will interact to some extent in the complexing method. Since the total amine content in the urine may vary for example with the diet, time of day and smoking, a more selective method for the determination of amphetamine in urine is required for biological studies.

We now describe the development of an analytical method of sufficient specificity and sensitivity to measure the urinary excretion of amphetamine in man after oral doses of as low as 5 mg of amphetamine sulphate.

Experimental

REAGENTS

Chloroform was refluxed (12 hr) with 1% amphetamine base, washed with 1 N HCl, and twice with distilled water. It was dried over $MgSO_4$ (anhyd.) distilled, and the middle 80% fraction collected: 2% v/v absolute ethanol was then added. Absence of interfering substances was checked by evaporating 6 ml, dissolving the residue in amine-acetone solvent (see below) (150 μl) and testing for the absence of a peak (with about the same t_R value as amphetamine) on the gas chromatograph. Checks were also

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

made by placing known amounts of amphetamine hydrochloride in the chloroform solvent and assaying by the procedure given below to see that there was no interaction of amphetamine with the particular batch of chloroform.

Methoxyphenamine hydrochloride. 1% w/v in distilled water was stored at 4°.

Amine-acetone solvent. Triethylamine 5% v/v, distilled water 10% v/v, acetone to 100%.

APPARATUS AND OPERATING CONDITIONS

The equipment used was a Griffin & George V.P.C. Apparatus M.K.IIB fitted with a hydrogen flame ionisation detector and an integrator (Gas Chromatography Limited type IE.165).

Stationary phase. Polyethyleneglycol 6,000 (PEG), 10% w/w and potassium hydroxide 5% w/w on Celite 545 (acid washed 100–120 mesh). The column was prepared by applying the potassium hydroxide in methanol to the Celite, removing the methanol, then applying the polyethyleneglycol in chloroform.

Working conditions. Column length, 4 ft. (copper tubing); column temperature, 150°; mobile phase, $H_2 : N_2$ (4 : 1); flow rate, 2 litres/hr.

PROCEDURE

To urine (50 ml), add 20% sodium hydroxide solution (10 ml). Using all glass distillation apparatus, steam distil until 50 ml distillate is collected in a receiver containing hydrochloric acid solution (dilute, B.P.) (2 ml). To the acid solution add methoxyphenamine hydrochloride (300 μg) and pass steam through the acid solution until 50 ml distillate is collected. Evaporate the residual acid solution to dryness over a steam-bath. Transfer the residue to a 5 ml flask, and wash the evaporating dish with 3 × 1 ml of chloroform, transferring the chloroform to the flask. Reflux for 5 min, cool, and pipette the chloroform into a wide necked short boiling tube, via a No. 2 porosity glass filter. Reflux the residue with another 2 ml of chloroform and transfer the solution as above. Wash the sintered filter with chloroform (0·5 ml) and collect this solution. Evaporate the combined chloroform extracts to dryness by passing hot air over the tubes. Dissolve the residue in chloroform (0·5 ml) and transfer to a burette equipped with a Teflon tap. Flash distil the solution in a B7 container (0·3 ml total capacity) in a bath at about 98°. The boiling tube is then washed with chloroform (0·5 ml) and this solution also concentrated in the B7 container. Dissolve the residue in amine-acetone solvent (150 μl) in the B7 container, close with a slightly silicone-greased stopper, leave for 2 hr and then chromatograph 10 μl of this solution. Obtain the ratio of the amphetamine to methoxyphenamine peak areas.

Calculate the concentration of the amphetamine in the sample by making reference to a calibration curve obtained by plotting the ratio of amphetamine to methoxyphenamine peak areas against the concentration of amphetamine using a fixed concentration of methoxyphenamine (0·2%

w/v solution of methoxyphenamine hydrochloride in the amine-acetone solvent).

Results and discussion

PREPARATION OF THE SAMPLE FOR GAS CHROMATOGRAPHY

Problems were encountered in the attempted gas chromatography of free amphetamine. For example pronounced tailing was experienced with an alkaline-treated PEG column, a PEG glass bead column, or with a silicone elastomer column (Fales & Pisano, 1962) coated on Diatoport S (a silizanised treated celite) or glass beads. This tailing was not due to lack in uniformity in coating, or to improper packing, since other materials, e.g., naphthalene always produced a symmetrical peak. By using the amphetamine-acetone derivative, a more symmetrical peak resulted (Brochmann-Hanssen & Svendsen, 1962).

In the method described, amphetamine is present as the hydrochloride throughout the concentration procedure, to prevent loss of the compound. Before gas chromatography the free base has to be generated, since protonated primary amines do not react with acetone (Bergel & Lewis, 1955). Triethylamine was most convenient for this purpose. It is a stronger base than either amphetamine or methoxyphenamine and emerges from the chromatography column with the solvent peak. In addition, 10% water was required since in anhydrous acetone no amphetamine-acetone complex was formed. This proportion of water allowed the acetone to react with the amphetamine but not with unknown interfering compounds sometimes present in urine. The hydrochloride of methoxyphenamine produced marked tailing in gas chromatography and, therefore, had to be converted to the free base (Nicholls, Makisum & Saroff, 1963).

The conversion of the free base to the acetone derivative occurs slowly, being almost complete in 2 hr. The equilibrium mixture is stable for at least two months. The small proportion of free amphetamine in the equilibrium mixture does not prejudice the assay procedure, since the calibration curve is linear.

In the steam distillation, much ammonia is collected along with the amphetamine in the hydrochloric acid solution used to trap the bases. Evaporation of this solution gave large quantities of ammonium chloride which blocked the gas chromatography column. Amphetamine hydrochloride is much more soluble in chloroform than ammonium chloride and, therefore, chloroform extraction was used to separate the two. The chloroform was specially purified because of the known interaction of impurities in commercial chloroform with many bases (Caws & Foster, 1957). Ethanol was added to purified chloroform to prevent formation of carbonyl chloride because of the latter's reaction with amphetamine and consequent interference with the assay procedure.

INSTRUMENTAL CONSIDERATIONS

The column. Tailing of the amphetamine derivative occurs if the

column is not uniformly coated. The columns described have a long working life (2,000 chromatograms over 5 months).

Choice of internal marker. Benzylamine and methoxyphenamine with suitable t_R values were chosen.* The use of benzylamine had the disadvantage that to get complete conversion to the benzylamine acetone derivative, the water content in the acetone solution could not be allowed to exceed 1%. At this concentration of water, materials in the urine gave complexes with acetone with t_R values in the region of the amphetamine-acetone derivative peak. Since methoxyphenamine does not form an acetone adduct, the water content of the acetone is unimportant to its gas chromatographic peak. A typical gas chromatograph from a urine sample to which the internal marker has been added, is shown in Fig. 1.

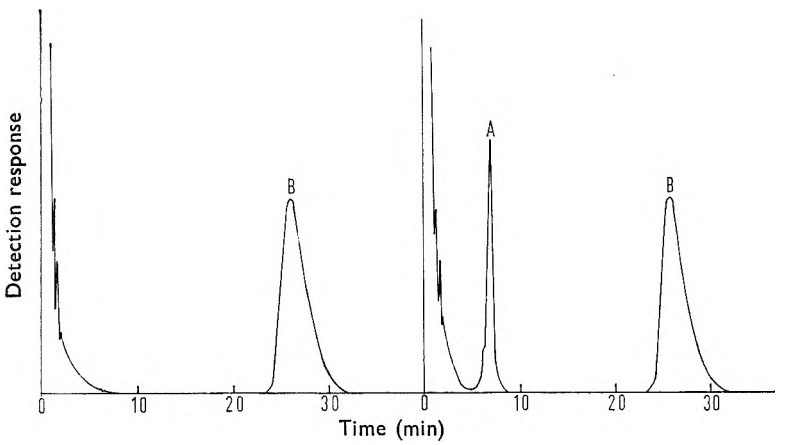


FIG. 1. Chromatograms of urine + marker and urine + marker containing 2 μg/ml amphetamine base. A. Amphetamine-acetone derivative ($t_R = 6{\cdot}5$ min). B. Methoxyphenamine (t_R 26 min).

RECOVERY, REPRODUCIBILITY AND SPECIFICITY OF THE METHOD

The corrected recovery of amphetamine from urine containing from 1–6 μg amphetamine hydrochloride per ml by the above method, was $100 \pm 5\%$. The actual amount of amphetamine recovered was about 85%. Amphetamine concentrations in the urine as low as $0{\cdot}2$ μg/ml, may be detected with ease. The amphetamine was stable in the urine, stored at 4°, for at least three days, with no concomitant rise in the urine blanks.

Specificity. The assumption is made that, in man, over 24 hr, 30% of any amount of amphetamine administered may be recovered unchanged (Beyer & Skinner, 1940). For a dose of 10 mg amphetamine sulphate and the normal urine output of 1,500 ml daily the concentration of amphetamine in the urine is $1{\cdot}5$ μg/ml base. Hence if a detailed urinary excretion

* 4-benzylpyridine; *NN*-diethylaniline; benzylmethylamine; 1-phenylpiperidine; aminodimethylmethane; anisidine; *o*-chlorobenzylamine; *p*-chlorobenzylamine; 4-methylbenzylamine; α-ethylbenzylamine; 2,4-dichlorobenzylamine were also investigated.

study is to be conducted the "blank" urine levels must be much lower than 1·5 μg/ml.

Previously published methods gave the following blank values (as μg apparent amphetamine base/ml) based upon the assumed urine output of 1,500 ml daily: with the picric acid assay, Jacobsen & Gad (1940), obtained 0–3; Alles & Wiesgarver (1961) 5–12: with the diazotisation method, Harris, Searle & Ivy (1947 found 1·76 (s.d. 1·11); Alles & Wiesgarver, (1961) gave 1·6 and Krivulka (1962) 1·2–1·7; the methyl orange procedure gave 0·9–2·0 (Utena & others 1955), 0·65 (Connell, 1958), 1·5 (s.d. 0·35) (Chapman & others, 1959).

The chromatographic method described herein gave virtually zero blank values and on no occasion did a value reach 0·1 μg amphetamine/ml of urine. This method is being used for a detailed examination of the urinary excretion pattern of amphetamine in man and other species.

Acknowledgement. One of us (M.R.) thanks the Medical Research Council for a grant held while carrying out this research.

References

Alles, G. A. & Wisegarver, B. B. (1961). *Toxicol. App. Pharmacol.*, **3**, 678–688.
Axelrod, J. (1954). *J. Pharmacol.*, **110**, 315–326.
Bergel, F. & Lewis, G. E. (1955). *Chem & Ind.*, 774–775.
Beyer, K. H. & Skinner, J. T. (1940). *J. Pharmacol.*, **68**, 419–432.
Brochmann-Hanssen, E. & Svendsen, A. B. (1962). *J. pharm. Sci.*, **51**, 938–940.
Brodie, B. B. & Udenfriend, S. (1945). *J. biol. Chem.*, **158**, 705–714.
Caws, A. C. & Foster, G. E. (1957). *J. Pharm. Pharmacol.*, **9**, 824–832.
Chapman, D. G., Shenoy, K. G. & Campbell, J. A. (1959). *Canad. med. Ass. J.*, **81**, 470–477.
Connell, P. H. (1957). *Biochem. J.*, **65**, 7P.
Connell, P. H. (1958). *Amphetamine Psychosis*, p. 92. London: Oxford University Press.
Fales, H. M. & Pisano, J. J. (1962). *Analyt. Biochem.*, **3**, 337–342.
Harris, S. C., Searle, L. M., & Ivy, A. C. (1947). *J. Pharmacol.*, **89**, 92–100.
Jacobsen, E. & Gad, I. (1940). *Arch. exp. Path. Pharmak.*, **196**, 280–289.
Keller, R. E. & Ellenbogen, W. C. (1952). *J. Pharmacol.*, **106**, 77–82.
Krivulka, R. L. (1962). M.Sc. Thesis. University of California.
McNally, W. D., Bergman, W. L. & Polli, J. F. (1947). *J. Lab. clin. Med.*, **32**, 913–916.
Nicholls, C. N., Makisumi, S. & Saroff, H. A. (1963). *J. Chromatog.*, **11**, 327–331.
Richter, D. (1938). *Biochem. J.*, **32**, 1763–1769.
Utena, H., Ezoe, T. & Kato, N. (1955). *Psychiatr. et. Neurol. Jap.*, **57**, June.

The paper was presented by MR. ROWLAND.

J. Pharm. Pharmacol., 1964, **16**, *Suppl.*, 32*T*–34*T* Received March 4, 1964

Determination of methyl salicylate in pharmaceutical preparations

S. G. E. STEVENS AND B. WARREN

A method based on gas-liquid chromatographic technique is described for the determination of methyl salicylate in pharmaceutical preparations. 10% carbowax 20M is used as a stationary phase to resolve mixtures containing amyl, ethyl and methyl salicylates, menthol and camphor. The determinations are based on the area triangulation method against a diphenyl reference standard.

ALTHOUGH many methods have been described for the estimation of methyl salicylate (Garratt, 1935; Hatfull, 1948; Haslam, Grossman, Squirrell & Loveday, 1953; Valsman & Benderskaya, 1958; Gengrinovich & Kadyrov, 1958; Benzuglyi & Dmitrieva, 1959; Bloom, P., personal communication), the use of gas chromatography appeared to offer a more elegant technique than any previously used. It promised to be more specific, accurate and time saving. The application of this technique to complex mixtures has been described by Domange & Longuevalle (1958) but no quantitative results on methyl salicylate were reported.

The work now reported covers the investigation of the conditions leading to the separation of methyl salicylate from a range of pharmaceutical formulations.

Experimental

Apparatus and running conditions. An F & M model 720 gas chromatograph incorporating a katharometer detector was used for the experiments. Stainless steel columns, measuring 2 ft. × ¼ in. O.D. were packed with 10% carbowax 20M on 60/80 mesh Celite. The carrier gas was helium flowing at 60 cc/min. Temperatures used were: oven, 150° isothermal; detector, 300°; injection port, 250°. The katharometer bridge current was 150 mA and the chart speed: 2 inches/min.

Under these conditions the methyl salicylate was well separated from those compounds with which it is commonly associated in pharmaceutical preparations.

The retention times of camphor, menthol, methyl salicylate, and amyl salicylate were 1·9, 2·7, 4·8 and 14·2 min respectively. To resolve methyl and ethyl salicylate where both were present it was necessary to increase the column length to 6 ft. and to programme the temperature from 140 to 200° at 1°/min.

The peak areas of the chromatogram were calculated by the area triangulation method: area × 2 = height × base (Birchfield & Storrs, 1962). The base of the triangle is obtained by drawing tangents at the points of inflection on the peak and measuring the distance between the points of intersection of the tangents and the base line. The height

From Smith Kline & French Laboratories Limited, Welwyn Garden City, Hertfordshire.

measurement is the vertical distance in the same units from the intersection of the tangents to the base line.

Selection of an internal standard. The compounds investigated as possible internal standards (Ray, 1954) were: diphenyl, naphthalene, diphenylmethane and diphenylethane. Diphenyl with a retention time of 10·5 min was chosen because of its complete resolution from all compounds likely to be encountered. The reagent grade material was checked to ensure it yielded a single peak under test conditions. A *standard diphenyl solution* was prepared by accurately weighing approximately 4 g into 100 ml graduated flask and making up with toluene.

Detector response to methyl salicylate and diphenyl. This was linear over a volume range of 0·02 to 3·0 μl. The minimum detectable quantity of methyl salicylate was 0·02 μl.

Calibration curve. Mixtures of methyl salicylate and diphenyl in varying proportions were prepared and the peak areas of their chromatograms measured.

The calibration plot of methyl salicylate area/diphenyl area against the percentage w/w methyl salicylate gave a straight line passing through the origin and corresponding to the expression $y = mx$ where $m = 2 \times 0{\cdot}58 = 1{\cdot}16$ since the peak area of the diphenyl internal standard was halved to assist in the plotting of the ratio of the methyl salicylate area against diphenyl area.

DETERMINATION OF METHYL SALICYLATE IN PHARMACEUTICAL PREPARATIONS

Accurately weigh an amount of ointment containing approximately 80 mg methyl salicylate into a 10 ml graduated flask. Pipette 5 ml standard diphenyl solution into the flask together with about 2 ml toluene. Shake the flask mechanically until the ointment has completely dissolved or has been uniformly dispersed. Dilute the solution to 10 ml with toluene. If the preparation yields any insoluble matter, the mixture should be centrifuged. Inject approximately 50 μl of the clear toluene extract by means of a Hamilton syringe on to the column. Calculate the peak area ratio of methyl salicylate against diphenyl and from the calibration curve determine the weight of methyl salicylate and hence its concentration in the original sample.

A solution of the sample in toluene, clarified as before but without diphenyl should be similarly examined to establish that no peaks from the sample interfere with the use of diphenyl as a reference standard.

With oil in water formulations which are immiscible with toluene, the preparation should be dissolved in absolute ethanol: the diphenyl standard solution should then be similarly prepared.

Reproducibility of method. To establish the reproducibility, a series of replicate analyses were made on a proprietary ointment for which the determinations gave 4·9% (s.d. 0·13).

Recovery experiments. A series of experiments were carried out in which the methyl salicylate concentration in a plain iodine ointment base was varied from 5 to 55% w/w. The method gave a mean recovery of 99·5% (s.d. 0·63).

Results

The method has been applied to a range of official and proprietary formulations with the results shown in Tables 1 and 2.

TABLE 1. DETERMINATION OF METHYL SALICYLATE IN OFFICIAL PREPARATIONS

	Stated content % w/w	% w/w*	
		by B.P.C. method	by G.L.C. method
1. Ointment of Methyl Salicylate B.P.C.	50	45·0 45·2	53·6 52·4
2. Ointment of Methyl Salicylate Comp. B.P.C. ..	50	46·1 46·3	50·2 51·5
3. Ointment of Iodine and Methyl Salicylate B.P.C.†	5·9	5·2 5·2	5·9 5·8
4. Liniment of Methyl Salicylate B.P.C.	≡30	31·4 31·4	31·6 31·8

Note. The results are individual analyses. However, the preparation† contained some dross so the solution of the ointment was centrifuged before injection. In these instances the methyl salicylate was between 5·8 and 5·9% w/w.

TABLE 2. DETERMINATION OF METHYL SALICYLATE IN PROPRIETARY PREPARATIONS

	Samples tested	Methyl salicylate % w/w	
		Stated contents	Found
Ointments	6	5·0	4·7–5·0
	2	2·3	2·1
	1	1·3	1·5
Liniment	2	≡3·74	3·6, 3·7
Balm	1	1·15	0·9
	2	12·8	11·7, 11·8
	2	12·39	11·7
Balsam	2	15·0	15·8, 15·9

The method provides reproducible results in a relatively short time and in its application to the analysis of ointments appears to meet a recent criticism of Garratt (1964) that no really satisfactory method for the determination of methyl salicylate in ointments has yet been described.

References

Bezuglyi, V. D. & Dmitrieva, V. M. (1959). *Analyt. Abstr.*, 4037.
Birchfield, H. P. & Storrs, E. E. (1962). *Biochemical Applications of Gas Chromatography*, p. 122. London: Academic Press.
British Pharmaceutical Codex (1963). 488, London: The Pharmaceutical Press.
Garratt, D. C. (1935). *Quart. J. Pharm. Pharmacol.*, **8**, 472–478.
Garratt, D. C. (1964). *The Quantitative Analysis of Drugs*, 3rd ed., p. 433. London: Chapman & Hall Ltd.
Gengrinovich, A. N. & Kadyrov, Yak (1958). *Analyt. Abstr.*, 1560.
Haslam, J. Grossman, S. Squirrell, D. C. N. & Loveday, S. F. (1953). *Analyst*, **78**, 92–106.
Domange, L. & Longuevalle, S. (1958). *C.R. Acad. Sci., Paris*, **247**, 209–211.
Hatful, R. S. (1948). *Analyst*, **73**, 559.
Ray, N. A. (1954). *J. appl. Chem.*, **4**, 21.
Valsman, G. A. & Benderskaya, S. N. (1958). *Analyt. Abstr.*, 1676.

The paper was presented by MR. WARREN.

J. Pharm. Pharmacol., 1964, **16**, *Suppl.*, 35*T*–40*T* Received May 1, 1964

The influence of the method of evaluation on the recovery of a phage after phenol treatment

W. R. L. BROWN, A. M. COOK AND J. ODURO-YEBOAH

The inactivation of coliphage T6r by phenol was investigated using 4 methods of estimating surviving infective phage. The regression of log % survivors upon log time was linear. A tube dilution counting method and an extinction time method, both using peptone water as recovery medium, showed a close correlation in the numbers of infective phage recovered. A high recovery was obtained with both a surface drop method of counting on peptone agar and with an agar layer method. With the latter method the increased recovery was most pronounced towards the end of the inactivation. It is suggested that these effects are due to an unidentified constituent common to 4 agars tested.

IN previous studies of the inactivation of bacteriophages, the titres of surviving infective phage particles have been estimated, most commonly, by the agar layer method of plaque counting (Gratia, 1936; Hershey, Kalmanson & Bronfenbrenner, 1943; Adams, 1959). The composition of the medium used in counting phage after exposure to an inactivating agent may affect the count obtained (Heicken & Spicher, 1956, 1959; Hofschneider, 1960). Four methods of estimating phage surviving exposure to phenol have now been compared.

Experimental

MATERIALS

Bacteriophage and bacterial host. The methods for cultivating the host (*Escherichia coli*) and the phage (coliphage T6r) have been described previously (Cook & Brown, 1963).

For plaque counts by the soft agar layer method suspensions of the host were prepared from 24 hr peptone agar slope cultures. In all other procedures 18–24 hr host cultures in peptone water, incubated without aeration, were used. The diluent used in phage counts was peptone water.

Agar media. Except where otherwise stated, the agar medium used consisted of peptone water solidified with Japanese agar, 2·5% w/v for normal peptone agar and 0·7% w/v for soft agar. In one experiment, Davis New Zealand agar at 1·5% w/v and 0·35% w/v, Oxoid Ionagar No. 2 or Oxoid agar No. 3 at 1·0% w/v and 0·3% w/v were used.

METHODS OF COUNTING INFECTIVE PHAGE PARTICLES

Soft agar layer method. The method used was based on that described by Adams (1959). It consisted of mixing 0·5 ml of suitable dilutions of phage suspension with 2·5 ml of molten soft agar at 46°, adding, from a

From the School of Pharmacy, University of London, 29–39 Brunswick Square, London, W.C.1.

standardised dropping pipette (Cook & Yousef, 1953), 2 drops (1/30 ml) of a suspension of *E. coli* in peptone water containing approximately 1×10^{10} viable cells per ml and pouring the mixture over the surface of an overdried agar plate. At least 6 replicate plates were prepared for each count, incubated at 37° for 12–16 hr and the plaques counted.

Surface drop method. This was a modification of the method described by Williams-Smith (1951, 1953) in which agar plates were overdried for 1 hr, flooded with 3 ml of a 24 hr peptone water culture of *E. coli*, drained and redried for 15 min at 37°. Four separate drops (1/60 ml) of diluted phage suspensions were placed on each of 5 seeded plates, the drops absorbed at room temperature, the plates incubated at 37° for 6 hr and the plaques counted.

Tube dilution method. This was based on the procedure described by Fisher & Yates (1949) for the estimation of the densities of micro-organisms. From a suitable initial dilution, 7 two-fold dilutions (5 ml + 5 ml) were prepared and 0·1 ml of each dilution transferred to 10 tubes containing 10 ml peptone water. Each tube was then inoculated with 0·1 ml of a 24 hr peptone water culture of *E. coli*. After incubation at 37° for 12–24 hr the presence of infective phage was shown by lysis of the host while normal growth of the host indicated the absence of phage. From the distribution of the phage-positive tubes the count of the initial dilution was calculated.

INVESTIGATIONS OF VIRICIDAL EFFECT OF PHENOL

Ten ml of aqueous phenol solution, maintained at 25 ± 0·05° were inoculated with 0·1 ml of a phage suspension in peptone water. After the required contact time, 0·1 ml samples of the reaction mixture were diluted with peptone water and the surviving infective phage particles counted. The degree of dilution used was such as to reduce the phenol concentration to not more than 0·1% w/v, a concentration previously shown to have no effect on the multiplication of the phage.

DETERMINATION OF THE MEAN SINGLE SURVIVOR TIME (M.S.S.T.)

The method used was that previously described in detail (Cook & Brown, 1963) and the results of 20 replicate extinction time determinations were subjected to the analysis of Mather (1949).

Results

COMPARISON OF METHODS OF COUNTING UNTREATED PHAGE

The titre of a single phage suspension stored at 5° was determined by each of the 3 methods of counting on each of 5 successive days, Table 1. From an analysis of variance on the results, neither the within nor the between methods variances were significantly greater than the residual variance ($F^4_8 = 1{\cdot}1393$, $F^2_8 = 1{\cdot}1356$).

The mean of the 15 counts was $7{\cdot}63 \times 10^9$ with 95% confidence limits of $\pm 0{\cdot}47 \times 10^9$.

TABLE 1. COUNTS ON A SUSPENSION OF COLIPHAGE T6r IN PEPTONE WATER STORED AT 5°

Day	Mean counts ($\times 10^9$ per ml)		
	Surface drop method	Soft agar layer method	Tube dilution method
1	7·75	7·71	8·74
2	8·77	6·87	7·51
3	7·14	7·37	6·01
4	7·17	6·40	8·74
5	7·85	7·68	7·74
Mean	7·74	7·21	7·95

REPRODUCIBILITY OF COUNTS ON PHENOL TREATED PHAGE

The inactivation of coliphage T6r by 2·5% w/v phenol was investigated using each of the 3 methods of counting. All inocula were taken from the same phage suspension stored at 5° and 3 replicate investigations were made using each method. The mean phage inoculum was 7·71 $\pm$ 1·18 $\times$ 10^7 particles per ml of reaction mixture.

The course of the inactivation of the phage is shown for one experiment with each method in Fig. 1 where the log % survivors are plotted against time. The regressions of log % survivors upon log time for the 9 experiments were linear and are summarised in Table 2.

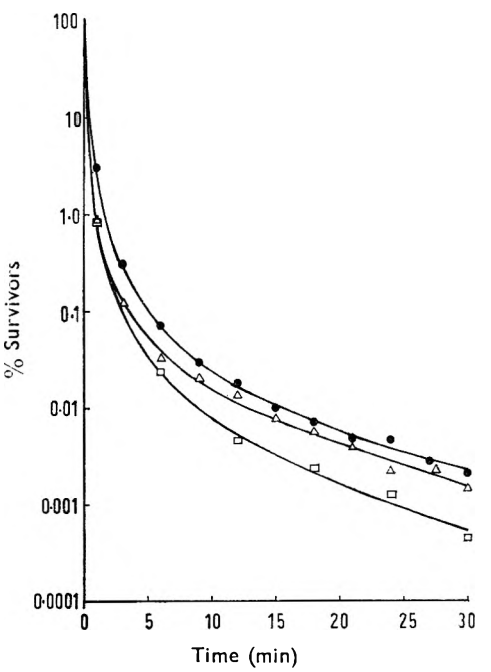


FIG. 1. The inactivation of coliphage T6r by 2·5% w/v phenol (25°) as shown by 3 methods of counting. ●——●, Surface drop method; △——△, soft agar layer method; □——□, tube dilution method.

TABLE 2. REGRESSION OF LOG % SURVIVOR UPON LOG TIME FOR THE INACTIVATION OF COLIPHAGE T6r BY 2·5% W/V PHENOL (25°) USING 3 METHODS OF COUNTING

Method of counting	Experiment	Correlation coefficient (r)	Degrees of freedom	Regression coefficient (b)	Variation about the regression line
Surface drop	1	−0·9992	9	−2·1100	0·001522
	2	−0·9953	8	−2·0629	0·009363
	3	−0·9886	9	−2·1616	0·02509
Soft agar layer	1	−0·9956	9	−1·8247	0·006811
	2	−0·9987	9	−1·8688	0·002089
	3	−0·9958	9	−1·8797	0·006944
Tube dilution	1	−0·9968	4	−2·1363	0·01095
	2	−0·9743	3	−2·0496	0·04263
	3	−0·9972	4	−2·1305	0·009925

The regressions of log % survivors upon log time from the replicate determinations with each method could not be represented by a common regression line (Yousef, 1954) but Table 3 illustrates their proximity.

TABLE 3. INACTIVATION OF COLIPHAGE T6r BY 2·5% W/V PHENOL (25°) AS DETERMINED BY 3 METHODS OF COUNTING

% surviving infective phage particles	Calculated contact times (min)											
	Surface drop method				Soft agar layer method				Tube dilution method			
	Experiment				Experiment				Experiment			
	1	2	3	Mean	1	2	3	Mean	1	2	3	Mean
1·0	1·7	0·8	2·1	1·5	0·5	0·9	1·3	0·9	1·0	0·9	1·3	1·1
0·1	5·2	2·6	6·0	4·6	3·5	3·1	4·4	3·7	2·9	2·5	3·7	3·0
0·01	15·3	7·8	17·1	13·4	12·1	10·5	14·7	12·4	8·5	7·2	10·9	8·9
0·001	45·6	23·6	48·4	39·2	42·4	35·9	49·6	42·6	24·7	20·9	31·8	25·8

To test for reproducibility within and between the methods the regressions of log % survivors upon log time were tested for parallelity (Yousef, 1954). Within each method the regression lines proved parallel but a significant deviation from parallelity existed between methods. When the slopes of one regression from each method were compared (Bailey, 1959), no significant difference existed between the slopes of the tube dilution and surface drop methods but both were significantly different from that of the agar layer method.

EFFECT OF AGAR CONTENT ON PHAGE RECOVERY

The inactivation of the phage by 2·5% w/v phenol was determined using the soft agar layer method of counting and with media containing Japanese agar, New Zealand agar, Oxoid Ionagar No. 2 or Oxoid agar No. 3. The regressions obtained are summarised in Table 4 and these may be represented by a common regression line.

TABLE 4. THE INFLUENCE OF AGAR COMPOSITION ON THE REGRESSION OF LOG % SURVIVOR UPON LOG TIME FOR THE INACTIVATION OF COLIPHAGE T6r BY 2·5% W/V PHENOL (25°), SOFT AGAR LAYER METHOD OF COUNTING

Agar	Correlation coefficient (r)	Degrees of freedom	Regression coefficient (b)	Variation about regression line
Japanese	−0·9910	7	−1·7313	0·01416
Davis New Zealand	−0·9882	7	−1·8032	0·01997
Oxoid Ionagar No. 2 ..	−0·9964	7	−1·7759	0·005800
Oxoid Agar No. 3	−0·9866	7	−1·8641	0·02421

CORRELATION BETWEEN COUNTING METHODS AND M.S.S.T. ESTIMATE

Using the same phage inoculum suspension as in the previous experiments, the M.S.S.T. with 2·5% w/v phenol was 171·14 ± 10·69 min (second calculated estimate).

Since the reaction mixture sample volume distributed in this determination was 0·1 ml. the M.S.S.T. is the time at which the reaction mixture contains 10 infective phage particles per ml. The contact time expected to produce this number of survivors was calculated for each of the regressions in Table 2 and the values are shown in Table 5.

TABLE 5. CONTACT TIMES CALCULATED TO PRODUCE % SURVIVORS EQUIVALENT TO THAT AT THE M.S.S.T. FOR 2·5% W/V PHENOL (171·14 ± 10·69 MIN)

Method of counting	Calculated contact time (min)			
	Regression			
	1	2	3	Mean
Surface drop	342·10	210·00	367·80	306·63
Soft agar layer	429·10	345·00	463·50	412·53
Tube dilution	149·60	166·10	266·10	193·93

Discussion

The reproducibility of the counts of untreated phage and their independence of the methods of counting clearly shows that the media and methods provide satisfactory conditions for the multiplication of untreated phage. The ability of phenol-treated phage to multiply was influenced by the medium and conditions of cultivation although the results with any one method were reproducible.

Deviations from the kinetics of a first order reaction have been reported for the inactivation of a staphylococcus phage by mercuric chloride (Krueger & Baldwin, 1934), for the T-even coliphages by formaldehyde (Heicken & Spicher, 1959) and for other viruses with formaldehyde (Gard, 1960). The inactivation of coliphage T6r by phenol, under the conditions used here, is not exponential with time, but the regression of log surviving fraction of phage upon log contact time proved linear. This relationship was confirmed by each of the counting methods for the inactivation of at

least 99·998% of the phage particles in the inocula. The close correlation between the M.S.S.T. estimate and the contact time giving an equivalent number of survivors (as calculated from the tube dilution counts) indicates that the relationship persists until the inactivation is virtually complete when the recovery medium for both methods was the same.

The recovery of at least the most resistant 1% of the phenol-treated phage was higher on the agar media than in the fluid medium and this suggests that the increased recovery is due to some factor associated with the agar. Since the recovery of phenol-treated phage is almost the same with all four agars the unknown constituent is common to them all. It is of interest to note that the adsorption of a phage to its host depends upon a suitable ionic environment (Hershey, Kalmanson & Bronfenbrenner, 1944; Garen & Puck, 1951; Puck, Garen & Cline, 1951) and although the agars tested differed, they all contained appreciable amounts of electrolytes (Sykes, 1956; Oxoid Manual, 1961).

With the soft agar layer method there is progressive increase of recovery of the phage particles with increase in time of contact with phenol (Table 2).

The effect may be associated with the intimate contact between medium and host cells or with the effect of the slightly elevated temperature of the soft agar with which the phage and host are mixed. An increase in the temperature at which phage particles are adsorbed to the host cells has been shown to increase the efficiency of adsorption (Garen & Puck, 1951).

References

Adams, M. H. (1959). *Bacteriophages*, pp. 27–34 & 450, New York: Interscience.

Bailey, N. T. J. (1959). *Statistical Methods in Biology*, p. 98, London: English Universities Press.

Cook, A. M. & Brown, W. R. L. (1963). *J. Pharm. Pharmacol.*, **15**, *Suppl.*, 150*T*–157*T*.

Cook, A. M. & Yousef, R. T. (1953). *J. Pharm. Pharmacol.*, **5**, 141–144.

Fisher, R. A. & Yates, F. (1949). *Statistical Tables for Biological, Agricultural & Medical Research*, 3rd ed., pp. 6–8, London: Oliver & Boyd.

Gard, S. (1960). *Ann. N.Y. Acad. Sci.*, **83**, 638–648.

Garen, A. & Puck, T. T. (1951). *J. exp. Med.*, **94**, 177–189.

Gratia, A. (1936). *Ann. Inst. Pasteur*, **57**, 652–676.

Heicken, K. & Spicher, G. (1956). *Zbl. Bakt. Abt.* 1, *Orig.*, **167**, 97–122.

Heicken, K. & Spicher, G. (1959). *Ibid.*, **175**, 11–26 & 321–332.

Hershey, A. D., Kalmanson, G. & Bronfenbrenner, J. (1943). *J. Immunol.*, **46**, 267–279.

Hershey, A. D., Kalmanson, G. & Bronfenbrenner, J. (1944). *Ibid.*, **48**, 221–239.

Hofschneider, P. H. (1960). *Nature, Lond.*, **186**, 330.

Krueger, A. P. & Baldwin, D. M. (1934). *J. gen. Physiol.*, **17**, 499–505.

Mather, K. (1949). *Biometrics*, **5**, 127–143.

Puck, T. T., Garen, A. & Cline, J. (1951). *J. exp. Med.*, **93**, 65–87.

Sykes, G. (1956). *Constituents of Bacteriological Culture Media*, p. 26–30, Cambridge: University Press.

Williams-Smith, H. (1951). *J. gen. Microbiol.*, **5**, 458–471.

Williams-Smith, H. (1953). *Ibid.*, **8**, 116–134.

Yousef, R. T. (1954). Ph.D. Thesis, University of London.

The paper was presented by DR. BROWN.

J. Pharm. Pharmacol., 1964, **16**, *Suppl.*, 41T–45T Received April 21, 1964

The effect of Polysorbate (Tween) 80 on the growth rate of *Pseudomonas aeruginosa*

M. R. W. BROWN AND R. M. E. RICHARDS*

Problems associated with growth rate measurement for *Pseudomonas aeruginosa* have been investigated. There is an apparent change in the rate of growth at an early stage when determined spectrophotometrically. This change is associated with cell clumping. The presence of polysorbate 80 in the culture medium eliminated this effect. The reproducibility of the growth rate measurement has been established.

THE resistance of *Pseudomonas aeruginosa* to chemical antibacterial agents causes serious difficulties in ophthalmology (Brown, Foster, Norton & Richards, 1964) and in the control of cross infection (Rogers, 1960). Brown & Garrett (1964) investigated the activity of chemical agents by measuring their effect on the growth rate of exponentially dividing cells of *Escherichia coli*. The advantages of this method were discussed by them.

The present work was undertaken to find out if the method is suitable for use with *Ps. aeruginosa*. There are difficulties associated with determining the growth rate of this organism because of its tendency to clump and also to form a pellicle. This paper reports results of adding polyoxyethylene sorbitan mono-oleate [polysorbate (Tween) 80] to the culture media in an attempt to overcome these difficulties.

Experimental

Ps. aeruginosa (NCTC 8203) was the test organism and Oxoid nutrient broth No. 1 was used as the basic medium. Incubation was at 37·5°. A broth culture from an isolated colony was used to inoculate replicate agar slants which were stored frozen. A fresh slant was used for each experiment.

GROWTH RATE DETERMINATIONS

Preliminary experiments showed that aeration of log phase cultures stimulated pellicle formation to the extent that rate determination was not possible. Without artificial aeration the formation of a pellicle was delayed for the minimum time necessary to establish the rate (about 40 min). Growth rates of log phase cells were measured using a Unicam 600 spectrophotometer at 420 mμ (Fig. 1 plain broth); in addition direct microscopic observation was made. The procedure of Brown & Garrett (1964) was used to reduce any lag phase to a minimum. An overnight broth culture was used to inoculate pre-warmed broth and the absorbance was then measured at intervals. At a suitable value, such that the cells were known to be dividing exponentially, a sample was further diluted with broth in an aluminium capped 250 ml conical flask maintained at

* From the School of Pharmacy, Bristol College of Science and Technology, Ashley Down, Bristol, 7.

37·5°, the final volume being 100 ml. This final culture was incubated until the cell concentration was once more sufficiently high to allow absorbance readings (about 130 min). Measurements were then made at intervals to determine the growth rate. Growth rates of the initial log phase for five replicate cultures were determined on each of three days (Table 1). The reproducibility of these measurements was determined by an analysis of variance (Table 2).

TABLE 1. GROWTH RATE CONSTANTS $\times 10^4$ FOR REPLICATE *Ps. aeruginosa* CULTURES ON DIFFERENT DAYS IN PRESENCE AND ABSENCE OF TWEEN 80

	Day 1	Day 2	Day 3
Nutrient broth	12·22	12·55	12·89
	12·31	12·50	12·37
	12·41	12·50	12·22
	12·38	12·59	12·55
	12·85	12·63	12·86
Nutrient broth + Tween 80, 0·02%	9·87	9·44	9·60
	9·52	9·60	9·44
	9·02	10·13	9·65
	9·52	9·65	10·16

TABLE 2. ANALYSIS OF VARIANCE OF GROWTH RATE CONSTANTS IN TABLE 1.

	Source of variation	Sum of squares	Degrees of freedom	Mean square	Variance ratio (F)
Nutrient broth	Between days	$5·95 \times 10^{-10}$	2	$2·97 \times 10^{-10}$	1·7
	Within days	$6·0 \times 10^{-9}$	12	$5·0 \times 10^{-10}$	
	Totals	$6·6 \times 10^{-9}$	14		
	s.d.: % of mean = 1·7				
	Tabulated value of (F) at 0·05 significance level = 19·4				
Nutrient broth + Tween 80, 0·02%	Between days	$1·4 \times 10^{-9}$	2	$7·0 \times 10^{-10}$	1·4
	Within days	$9·2 \times 10^{-9}$	9	$1·0 \times 10^{-9}$	
	Totals	$10·6 \times 10^{-9}$	11		
	s.d.: % of mean = 3·2				
	Tabulated value of (F) at 0·05 significance level = 19·3				

EFFECT OF TWEEN 80 ON GROWTH CURVE

The shortness of the initial log phase (Fig. 1 plain broth) caused some difficulty in making a sufficient number of measurements to establish the growth rates of several cultures growing simultaneously. Microscopic examination showed that the end of the initial log phase occurred at the same time as clumping of cells. *Ps. aeruginosa* produces considerable amounts of slime (Rhodes, 1959) and it seemed possible that this contributed to the clumping.

Tween 80 was added to the broth in an attempt to disperse the cells and thus prolong the initial phase. Replicate inocula were added to nutrient broth containing eight concentrations of Tween 80 graded between 0·004 and 0·5%. Fig. 1 illustrates representative growth curves obtained with and without Tween 80 in unaerated broth. Growth rates were determined for four replicate cultures on each of three days using nutrient broth containing 0·02% Tween 80 (Table 1). An analysis of variance is given in Table 2. The effect of aeration on cultures growing in the presence of 0·1% Tween 80 is illustrated in Fig. 2. Aeration was effected by shaking the flask at a rate of 120 throws/min.

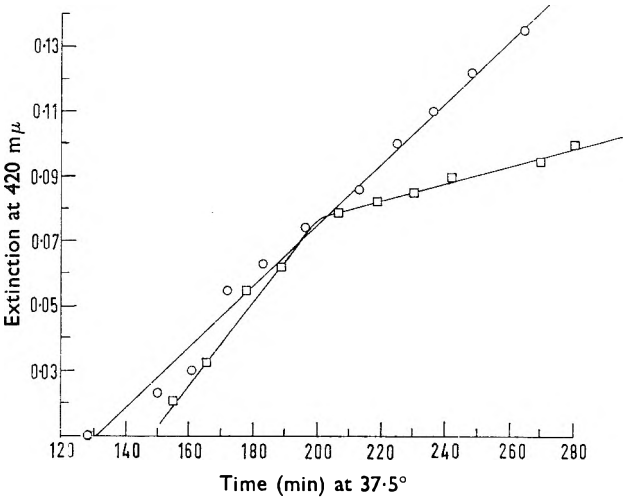


FIG. 1. Effect of Tween 80 on the growth curve of *Ps. aeruginosa* in nutrient broth. ○ Nutrient broth + Tween 80, 0·02%. □ Nutrient broth.

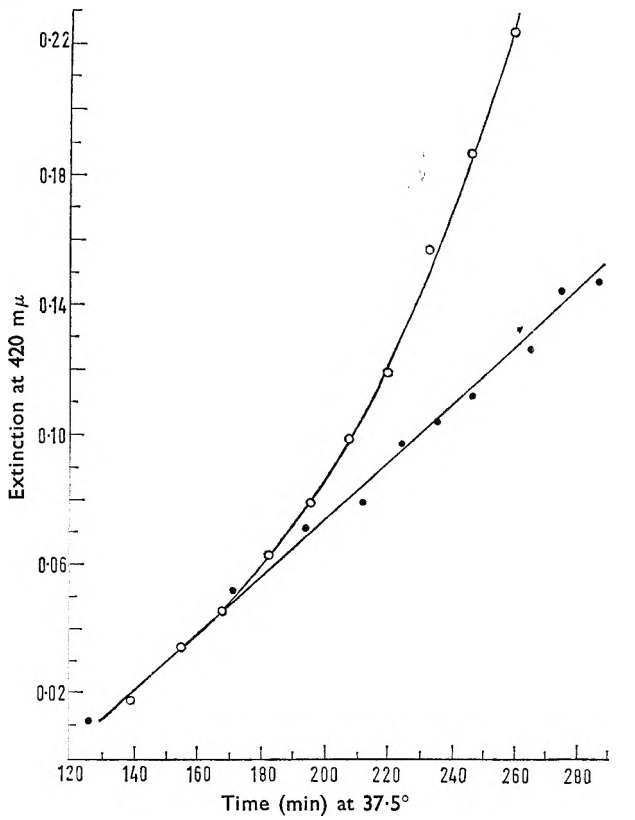


FIG. 2. Effect of aeration on the growth curve of *Ps. aeruginosa* in nutrient broth + Tween 80, 0·1%. ○ Culture shaken at 120 throws/min. ● Control, not aerated.

EFFECT OF ADDING WATER TO LOG PHASE CELLS

Initial work showed that the addition of 1 ml of distilled water to 100 ml (approx.) cultures of log phase cells had an effect greatly in excess of that expected from dilution alone. This phenomenon is important because it is intended to investigate the activity of chemical antibacterial agents by measuring alterations in growth rate after the addition of small volumes of an aqueous solution of the agent. Replicate log phase cultures were incubated in a water bath at 37·5°. Sterile distilled water was boiled and cooled to 37·5° immediately before use and added to one or more of the cultures as follows:

(*a*) 1 ml added to the culture using a 1 ml pipette and the culture afterwards briefly shaken by hand to mix.

(*b*) As in (*a*) except that the culture is briefly shaken by hand during addition of water.

These experiments were repeated using 0·5 ml water. Representative results are illustrated in Fig. 3.

Results and discussion

Fig. 1 illustrates the characteristic shoulder to the growth curve for *Ps. aeruginosa* in nutrient broth. The presence of 0·02% or more Tween 80 in the medium eliminated this shoulder. Microscopic examination made concurrently showed that clumping was absent in the presence of the Tween. This would suggest that Tween 80 has the effect of dispersing cell aggregates which contributed to the observed apparent alteration in rate. The growth rate in nutrient broth in the presence of concentrations of Tween 80 within the range 0·02–0·5% showed no difference. In cultures which were not aerated, pellicle formation occurred after 250 min in plain broth but took progressively longer in the Tween broth as the concentration of the agent increased: 0·05% Tween 80 delayed formation until after 290 min. The presence of Tween 80 in the aerated cultures also delayed but did not prevent pellicle formation. Increasing the concentration of Tween 80 to 0·1% failed to delay the pellicle formation in aerated cultures after 220 min (Fig. 2).

Tables 1 and 2 show that the reproducibility of the growth rate determinations in the presence and absence of Tween 80 is satisfactory. The presence of Tween 80 satisfactorily prolonged the log phase and made it feasible to investigate chemical antibacterial activity against *Ps. aeruginosa* by measuring the effect on growth rate (Brown & Garrett, 1964).

The addition of 0·5 ml water to cultures of log phase cells while being gently shaken by hand (for mixing without aeration) produced no measurable effect on the growth rate compared with that of a control culture (Fig. 3A). When 1 ml water was added to log phase cultures not shaken during the addition it had the effect of reducing the growth rate to zero for a significant time after which the initial rate of growth was resumed (Fig. 3B). *Ps. aeruginosa* is known to be very sensitive to osmotic effects (Bernheim, 1963) and this may account for the phenomenon.

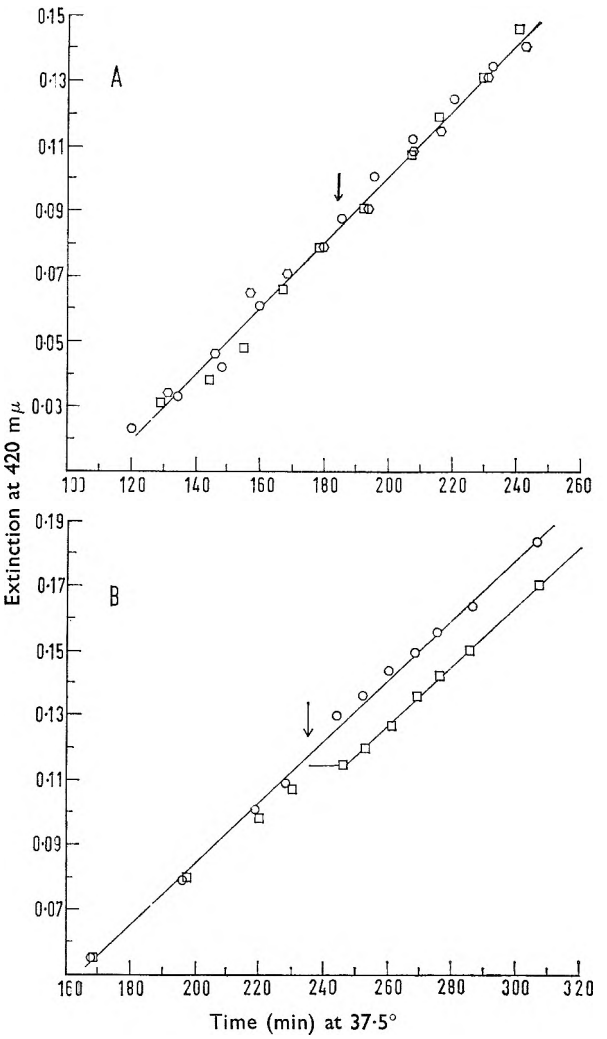


FIG. 3. Effect on growth rate of method of adding water to cultures of *Ps. aeruginosa*. A. Shaken during addition. ○ Nutrient broth + Tween 80, 0·02%. □, ⬡ Replicate cultures containing nutrient broth + Tween 80, 0·02% + 0·5 ml water added after 184 min. B. Not shaken during addition. ○ Nutrient broth + Tween 80, 0·02%. □ Nutrient broth + Tween 80, 0·02% + 1·0 ml water added after 235 min.

References

Bernheim, F. (1963). *J. gen. Microbiol.*, **30**, 53–58.
Brown, M. R. W., Foster, J. H. S., Norton, D. A. & Richards, R. M. E. (1964). *Pharm. J.*, **192**, 8.
Brown, M. R. W. & Garrett, E. R. (1964). *J. pharm. Sci.*, **53**, 179–183.
Rhodes, M. E. (1959). *J. gen. Microbiol.*, **21**, 221–263.
Rogers, K. B. (1960). *J. appl. Bact.*, **23**, 533–537.

The paper was presented by MR. RICHARDS.

J. Pharm. Pharmacol., 1964, **16**, *Suppl.*, 46*T*–50*T* Received April 28, 1964

The unsuitability of the B.P. tests for sterility to detect fungi

N. M. CHAUHAN AND V. WALTERS*

Five of sixteen fungi examined would not be detected by the B.P. Tests for Sterility. Sabouraud liquid medium containing 0·1 to 0·15% agar incubated at 24–25° has been found suitable.

THE B.P. Tests for Sterility are designed to detect small numbers of viable aerobic and anaerobic bacteria, if present, in pharmaceutical products. The U.S.P. includes also a test for fungi. This is applicable to liquids and suspensions which have not been heated in their final containers to at least 100° for 15 min, and also to crystalline or powdered solids, surgical dressings and sutures.

The inclusion of a test for fungi in the B.P. has been advocated from time to time, e.g. Sykes, 1961; we now report evidence supporting this recommendation.

Experimental

Spore suspensions. "Oxoid" Sabouraud glucose agar slopes in 6 oz emulsion bottles plugged with cotton wool, were inoculated separately, in triplicate, with cultures of the various fungi (Table 1). These were

TABLE 1. EFFECT OF MEDIUM AND INCUBATION TEMPERATURE ON THE GROWTH OF FUNGI

Fungus	Inoculum 1 ml No. of spores/ml	No. of tubes out of 10 showing growth after 7 days incubation at: 31° Aerobic broth	31° Anaerobic broth	31° Sabouraud	31° Sabouraud + 0·15% agar	24·5° Sabouraud	24·5° Sabouraud + 0·15% agar
Fusarium solani ..	50	10B++	10N++	4S 6B++	10S	9S 1B++	10S
	5	10B+	3N++ 4N+	2S 8B++	10S	6S 4B++	10S
Rhizopus arrhizus ..	50	4S 6B++	10S	10S	10S	10S	10S
	5	1S 9B+	6S 2N++	10S	10S	10S	8S
Penicillium lilacinum	50	10B+	10N+	10B+	10N−+	10B++	6S 4N++
	5	5B+	3N+	7B+	8N+	9B+	4N++ 3N+
P. chrysogenum ..	50	10B+	8N+	10B++	10N++	10B++	6S 4N++
	5	5B+	3N+	5B++ 1B+	4N+	6B++	1S 5N++

From the Department of Pharmacy, University of Ife, Ibadan Branch, Ibadan, Nigeria.

* Present address: School of Pharmacy, University of London.

TABLE I—*continued*

Fungus	Inoculum 1 ml No. of spores/ml	No. of tubes out of 10 showing growth after 7 days incubation at: 31° Aerobic broth	31° Anaerobic broth	31° Sabouraud	31° Sabouraud + 0·15% agar	24·5° Sabouraud	24·5° Sabouraud + 0·15% agar
P. cyclopium ..	50	0	0	0	0	10B++	10S
	5	0	0	0	0	10B++	7S 2N+
P. digitatum ..	50	0	0	0	0	10B++	10S
	5	0	0	0	0	9B++	4S 4N++
P. expansum ..	50	0	0	0	0	10B++	10S
	5	0	0	0	0	10B++	5S 2N++
P. notatum	50	10B+	10N+	10B+	10N++	1S 9B++	10S
	5	5B+	3N+	8B+	8N+	9B++	8S
Aspergillus flavus (brown var.) ..	50	10B++	10S	10B++	10S	10B++	10S
	5	9B++	4S 3N++	9B++	5S 3N++	9B++	5S 2N++
A. flavus (green var.)	50	5S 5B++	10S	3S 7B++	10S	10B++	10S
	5	7B++	1S 4N++ 2N+	9B++	8S	8B++	4S 3N++
A. niger	50	10B++	6S 4N++	10B++	10S	10B++	10S
	5	9B+	4N++ 4N+	10B++	10S	9B++	9S
Neurospora crassa ..	50	0	0	8B++	10S	8B++	10S
	5	0	0	1B++	5S 2N++	2B++	10S
N. sitophila ..	50	0	0	10B++	10S	10S	10S
	5	0	0	9B++	8S	9S 1B++	10S
Scopulariopsis brevicaulis ..	50	10B++	10S	10B++	10N++	10B++	10N++
	5	10B++	4S 4N++	10B++	10N++	10B++	10N++
Trichoderma viride ..	50	10B++	10N++	10B++	10S	10B++	10S
	5	9B++	2N+	8B++	9S	8B++	10S
Rhodotorula rubra ..	50	10+−	10++	10++	10++	10++	10++
	5	5+−	1+	10++	9++	3++ 6+	1++ 9+

S = dense sporulating mycelium on surface of medium; N = growth just below surface; B = growth towards the bottom of the medium; ++ = dense mycelium; + = scanty mycelium, except with *R. rubra* where ++ and + = good and poor growth respectively.

obtained from the Commonwealth Mycological Institute. One of each triplicate set was incubated at 25°, one at 28° and one at 31° for 7 to 10 days. Spore suspensions were prepared from these by the method of

Chauhan & Walters (1961) selecting in each instance that culture showing most growth of the three incubated at the different temperatures; dilutions were made to give suspensions containing 5 and 50 spores per ml.

Preliminary experiments showed that with test tubes containing 10 ml of media, there was no difference in the amount of growth produced when either cotton wool plugs or aluminium caps were used as closures; with the 50 ml containers better growth was obtained in those closed by means of cotton wool plugs, presumably because screw caps prevent gaseous diffusion.

Media. 10 ml quantities of media were sterilised in 6 in. × $\frac{3}{4}$ in. aluminium capped test tubes. The media used were: (1) "Oxoid" Nutrient Broth No. 2; (2) "Oxoid" Nutrient Broth No. 2 containing dextrose 0·5%, sodium thioglycollate 0·11%, methylene blue 0·0002%, "Davis" New Zealand agar 0·05%; (3) "Oxoid" Sabouraud liquid medium; (4) "Oxoid" Sabouraud liquid medium containing "Davis" New Zealand agar 0·15%. Ten tubes of each of these media were inoculated with 1 ml of fungal spore suspension (5 spores/ml); the tubes were then shaken and incubated for 7 days at 31°. Ten tubes each of inoculated media 3 and 4 were incubated also at 24·5°. The procedure was repeated using inocula of 50 spores/ml.

Inactivation of antifungal substances. The effectiveness of inactivation of antifungal substances was examined using Medium 4 and the 5 fungi recorded in Table 2. The procedure used was as follows. 0·5 ml amounts of the agent in solution were added to 25 × 5 oz bottles containing 50 ml of Medium 4. The bottles were plugged with cotton wool and the contents mixed. To sets of 5 bottles, 0·5 ml quantities of one of the suspensions (Table 2) containing 100 spores/ml were added and the bottles shaken.

The procedure was applied to solutions of phenol 1% and chlorocresol 0·2%, using Medium 4 in quantities of 10, 20 and 50 ml; to phenylmercuric nitrate 0·002% using 10, 20 and 50 ml medium containing 0·1% sodium thioglycollate and also to sulphacetamide sodium 20% using 20 ml amounts of medium containing 0·05% p-aminobenzoic acid. Inoculated media without the antifungal substances were prepared as controls. Also, with phenylmercuric nitrate and sulphacetamide sodium, inoculated media containing these substances, but without the neutralising agents, were used.

Results

With most of the fungi tested, daily examination of the culture tubes showed that growth occurred sooner and more profusely in Sabouraud medium with and without 0·15% agar than in either aerobic or anaerobic broth; there was no growth at all at 31° in the latter two media inoculated with the *Neurospora* spp. *Penicillium cyclopium*. *P. digitatum* and *P. expansum* did not grow in any of the media at 31° but at 24·5° abundant mycelia were produced in Sabouraud medium and dense sporulating surface mycelia in most tubes when agar was present (Table 1). Of these

three *Penicillium* spp., only *P. digitatum* did not grow in aerobic or anaerobic broth at 24·5°.

Table 2 shows that phenol, chlorocresol and sulphacetamide sodium can be inactivated by dilution or neutralisation, although chlorocresol requires dilution greater than that for bacteria. Sodium thioglycollate 0·1% much inhibited the growth of *N. crassa* and slightly reduced that of *P. notatum* and *F. solani*.

TABLE 2. EFFECT OF DILUTION AND NEUTRALISATION ON THE ACTIVITY OF SUBSTANCES INHIBITING FUNGAL GROWTH

Medium	Quantity of medium ml	Inoculum 0·5 ml of suspension containing 50 spores				
		Neurospora sitophila	*Neurospora crassa*	*Penicillium notatum*	*Rhizopus arrhizus*	*Fusarium solani*
M (control)	50 or 10	5S	5S	5S	5S	5S
M + 0·5 ml 1% phenol solution	50	5S	5S	5S	5S	3S 2N++
M + 0·5 ml 0·2% chlorocresol solution..	10	0	0	0	5N+	2N+
	20	5N++	0	5N+	5S	3S 2N++
	50	5S	5N++	5N++	5S	5S
M + 0·1% sod. thioglycollate (control)	10	5S	3N++	3S 2N++	5S	2S 2N++
M + 0·5 ml 0·002% phenylmercuric nitrate solution ..	10	0	0	0	0	0
M + 0·1% sod. thioglycollate + 0·5 ml 0·002% phenylmercuric nitrate solution	10	2N++	0	2N+	5S	0
	20	2S	0	1N+	5S	3N++
	50	3S	0	3S	5S	2S 2N++
M + 0·05% *p*-aminobenzoic acid (control)	20	5N++	5S	5S	5S	2S 3N++
M + 0·5 ml 20% sulphacetamide sodium solution	20	5N+	5N++	5N+	5N++	0
M + 0·05% paba + 0·5 ml 20% sulphacetamide sodium solution	20	5S	5S	5S	5S	3S 2N++

M = Sabouraud medium containing 0·15% agar.
In each case 5 containers of media were inoculated and the figures indicate those showing growth after 7 days incubation at 24–25°. Notation as in Table 1.

Discussion

The organisms used were a selection of common airborne saprophytes and, therefore, represented potential sources of contamination of pharmaceutical preparations. Of the sixteen fungi tested, five would not be detected by the B.P. Tests for Sterility; *N. sitophila* and *N. crassa* because of the unsuitability of the media, *P. cyclopium* and *P. expansum* because

of the unsuitable incubation temperature and *P. digitatum* for both these reasons.

It was observed in some of the earlier experiments that growth occurred nearer to the surface and was greater in anaerobic broth than in aerobic broth. This was attributed to spores remaining suspended in anaerobic broth due to its greater viscosity and density. The addition of 0·05%, 0·1% or 0·15% agar to Sabouraud medium also improved this medium since growth was more rapid and abundant than when agar was absent. Dense mycelia bearing spores were present on the surface of the medium often within two to three days of inoculation. Since sporulation occurred slightly sooner in the medium containing 0·15% agar, this medium was also used in subsequent experiments.

Pharmaceutical preparations sterilised in their final containers by a heat process may be assumed to be free from viable fungal spores since the heat resistance of fungal spores is similar to that of vegetative bacteria (Wilson & Miles, 1957; Cochrane, 1958; Sykes, 1958). It is suggested that preparations not sterilised by a heat process be tested for the presence of common airborne fungi using Sabouraud medium containing 0·1 to 0·15% agar and incubating at 24 to 25° for 7 to 14 days.

When substances possessing antifungal activity are to be tested, sufficient medium must be added to give a dilution of the substance greater than that which is fungistatic. Alternatively, a suitable inactivator must be found. With phenylmercuric nitrate further investigation is necessary to discover a suitable inactivator; not only is sodium thioglycollate 0·1% itself growth inhibitory to some fungi but also it does not entirely neutralise the activity of phenylmercuric nitrate. A suitably diluted spore suspension of one of the fungi in Table 1, or a suspension of *Candida albicans* as recommended by the U.S.P., may be used to inoculate controls.

References

Chauhan, N. M. & Walters, V. (1961). *J. Pharm. Pharmacol.*, **13**, 470–478.
Cochrane, V. W. (1958). *Physiology of Fungi*, p. 424. London: Chapman and Hall Ltd.
Sykes, G. (1958). *Disinfection and Sterilisation*, p. 93. London: Spon Ltd.
Sykes, G. (1961). Contribution to discussion in *Symposium on Sterilisation of Surgical Materials*, p. 213. London: The Pharmaceutical Press.
Wilson, G. S. & Miles, A. A. (1957). Topley and Wilson's *Principles of Bacteriology and Immunity*, 4th ed., p. 129. London: Arnold Ltd.

The paper was presented by DR. WALTERS.

J. Pharm. Pharmacol., 1964, 16, *Suppl.*, 51T–55T Received April 30, 1964

Effect of Polysorbate (Tween) 80 on the resistance of *Pseudomonas aeruginosa* to chemical inactivation

M. R. W. BROWN AND R. M. E. RICHARDS

Log phase cultures of *Pseudomonas aeruginosa* in nutrient broth containing polysorbate 80 were much less resistant to the action of benzalkonium chloride, chlorhexidine diacetate and polymyxin B sulphate than cells grown in plain broth.

THE present work was started in an attempt to investigate the resistance of *Pseudomonas aeruginosa* to chemical agents. The method used was based on the principles proposed by Brown & Garrett (1964) and modified by Brown & Richards (1964) for use with this organism.

The increase in numbers of exponentially dividing cells can be described by

$$X = X_0 e^{kt} \qquad (1)$$

where X is the number of cells per unit volume at time t, and X_0 is the concentration at time $t = t_0$: the apparent first order rate constant k describes the rate of growth and may be derived from the slope of the line described by the logarithmic transformation of equation (1)

$$\log X = {}^{kt}/2{\cdot}303 + \log X_0 \qquad (2)$$

When a chemical agent is added to a culture of log phase cells, any change in the constant k may be attributed to the action of the chemical.

Benzalkonium, chlorhexidine and polymyxin B sulphate were initially examined because *Ps. aeruginosa* is less resistant to these agents than to others. (Kohn, Gershenfeld & Barr, 1963a,b; Riegelman, Vaughan & Okumoto, 1956; Anderson & Stock, 1958).

Experimental

The test organism used was *Ps. aeruginosa* strain NCTC 8203; the basic medium was Oxoid nutrient broth No. 1 whilst the incubation and reaction temperature was 37·5°. Growth rates were followed by measuring the absorbance at 420 mμ with a Unicam 600 spectrophotometer. Antibacterial agents used were benzalkonium chloride B.P.C., chlorhexidine diacetate (I.C.I.) and polymyxin B sulphate kindly supplied by Messrs Burroughs Wellcome & Co. Details of methods and materials have been described previously (Brown & Richards, 1964).

EFFECT OF CHEMICAL AGENTS ON GROWTH RATE

Replicate inocula of log phase cells in nutrient broth were added separately to pre-warmed broth containing graded concentrations of Tween 80. Each concentration was duplicated. Every culture was allowed to grow until it was at a suitable stage in the log phase when one of each pair was inoculated with 0·5 ml of an aqueous solution of the antibacterial agent. The other one of each pair was simultaneously

From the School of Pharmacy, College of Science and Technology, Bristol.

inoculated with water. The final volumes were about 100 ml. Spectrophotometric readings were taken at intervals and representative results with benzalkonium (35 μg/ml), chlorhexidine (10 μg/ml) and polymyxin (3·3 units/ml) are shown in Figs 1–3 respectively.

EFFECT OF TWEEN 80 ON SURVIVAL TIME WITH CHLORHEXIDINE

An end-point experiment was made to determine whether the enhancing effects of Tween 80 on the action of chlorhexidine against exponentially dividing cells could also be shown with washed log phase cells.

Preparation of inoculum. An overnight culture was used to inoculate nutrient broth to give a final volume of 100 ml: this was then incubated. Replicate samples of log phase cells were then used to inoculate separately, plain broth and broth with 0·5% Tween 80. These two cultures were allowed to grow into the log phase when 8 ml of each were removed, the cells washed once by centrifugation and resuspended in 80 ml water to give two suspensions, P (from plain broth) and T (from broth with Tween).

Preparation of reaction mixtures. Two series of 15 tubes were made, each consisting of 3 replicates of 5 concentrations of chlorhexidine in water (10, 20, 50, 100 and 200 μg/ml).

One series was inoculated with 0·5 ml samples from P and the other with 0·5 ml samples from T. These were designated reaction mixtures and were maintained at 37·5°.

Determination of survival time. Samples of 0·5 ml were taken from each reaction mixture after 0·25, 0·5 and 2 hr; they were added to 5 ml recovery medium and incubated one week. The recovery medium was that of Riegelman, Vaughan & Okumoto (1956) without agar. Colony counts showed that the reaction mixtures initially contained about 4×10^3/ml viable cells. Positive controls to test the efficiency of recovery media consisted of 0·5 ml samples of P and T separately added to 9·5 ml distilled water at 37·5°. Samples of 0·5 ml were then withdrawn and added to 5 ml of recovery media containing 0·5 ml of the 200 μg/ml chlorhexidine solution. Negative controls consisted of chlorhexidine solutions of each concentration used. These were incubated at 37·5° and 0·5 ml samples withdrawn and used to inoculate 5 ml recovery media.

Results and discussion

EFFECT OF CHEMICAL AGENTS ON GROWTH RATE

There was no appreciable effect when benzalkonium was added to log phase cells in nutrient broth to produce a concentration of 35 μg/ml (Fig. 1A). The same concentration immediately reduced to about zero the growth rate in broth containing 0·02% Tween (Fig. 1B). This enhancing effect is particularly remarkable because Tween 80 is used as an antagonist of benzalkonium (Kohn, Gershenfeld & Barr, 1963a). Such antagonism is shown by Figs 1B and 1C, a comparison of which shows that a 10 fold increase in Tween 80 almost eliminated the inhibitory effect of 35 μg/ml benzalkonium. It was not possible to use concentrations of

benzalkonium greater than about 35 μg/ml in plain broth because of precipitation effects.

A similar phenomenon was observed with 10 μg/ml chlorhexidine which

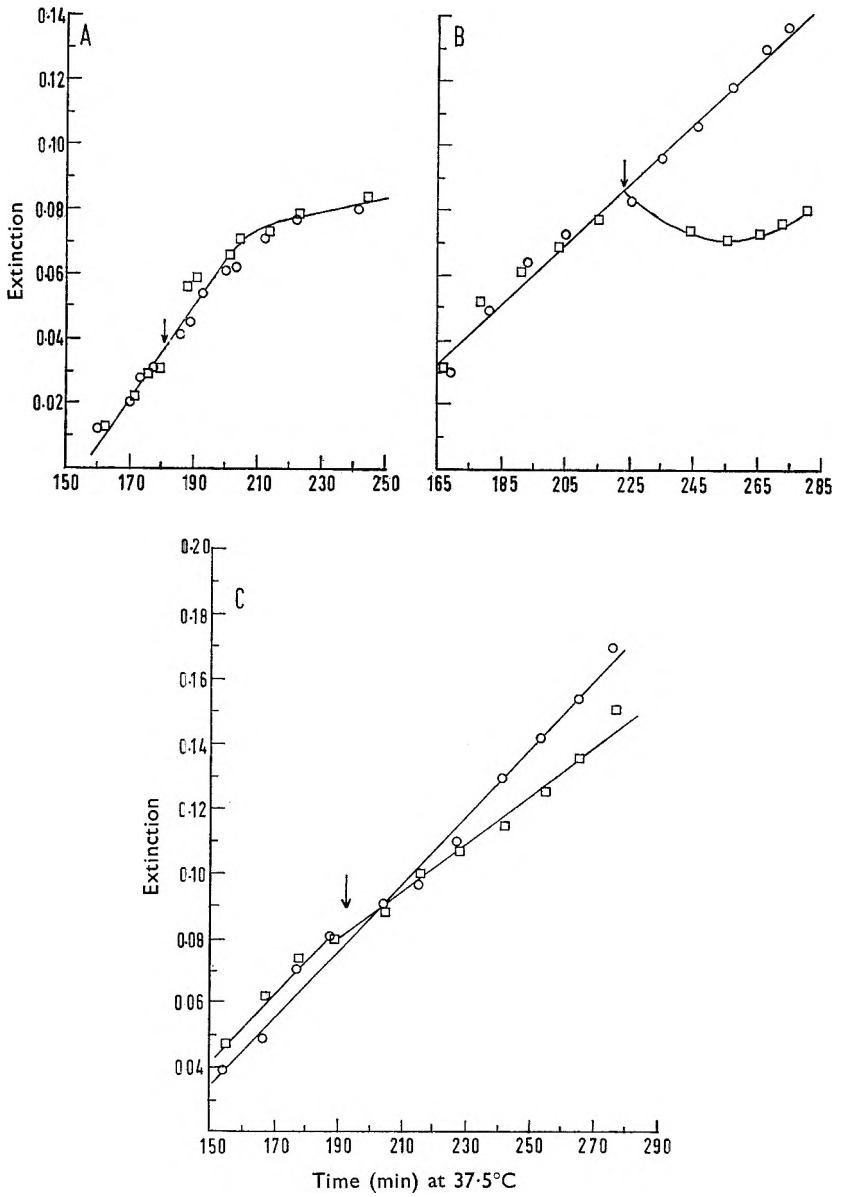


FIG. 1. Effect of Tween 80 on the action of 35 μg/ml benzalkonium chloride against log phase cultures of *Ps. aeruginosa*. ○, Control culture. □, Test culture. A, nutrient broth. Benzalkonium added after 180 min. B, nutrient broth + Tween 80, 0·02%. Benzalkonium added after 223 min. C, nutrient broth + Tween 80, 0·2%. Benzalkonium added after 193 min.

produced lysis in plain broth (Fig. 2A). The rate of lysis was increased in the presence of 0·02% Tween 80, but 0·5% eliminated any observable effect of the chlorhexidine (Fig. 2B). The enhancing of chlorhexidine activity by Tween 80 was much less marked than with benzalkonium and polymyxin.

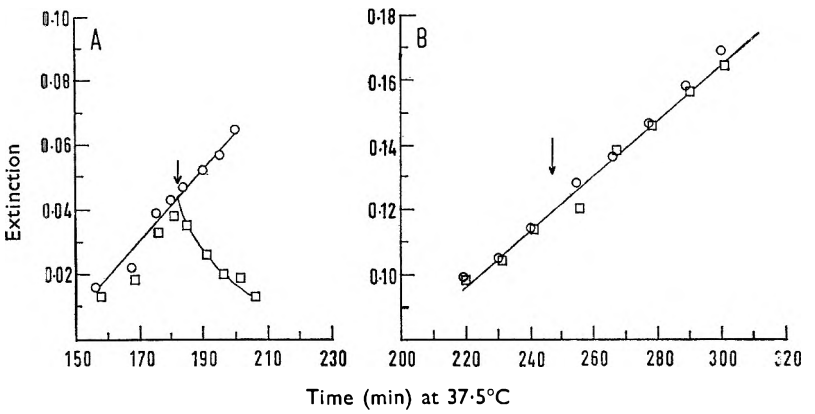


FIG. 2. Effect of Tween 80 on the action of 10 μg/ml chlorhexidine diacetate against log phase cultures of *Ps. aeruginosa*. ○, Control culture. □, Test culture. A, nutrient broth. Chlorhexidine added after 182 min. B, nutrient broth + Tween 80, 0·5%. Chlorhexidine added after 247 min.

The inhibitory effect of 3·3 units/ml polymixin B sulphate on the growth rate of *Ps. aeruginosa* was apparently enhanced in the presence of each of the concentrations (up to 0·5%) of Tween 80 tested. The effect increased with increasing concentration of Tween 80 (Fig. 3A and B).

Antibacterial agents were added at different stages in the log phase and with one exception the results were similar to those shown in Figs 1–3. When benzalkonium was added to plain broth cultures sufficiently early in the log phase it did not affect the rate of growth but did affect the onset of the final stationary phase which occurred sooner than with the control. This phenomenon did not occur in the presence of Tween 80.

These experiments were repeated using another strain of *Ps. aeruginosa* NCTC 7244, isolated from an eye infection. Tween 80 gave substantially the same effect with these chemical agents.

EFFECT OF TWEEN 80 ON SURVIVAL TIME WITH CHLORHEXIDINE

There is a correlation between the effects of Tween 80 on growth rate and lysis of *Ps. aeruginosa* in the presence of chlorhexidine observed spectrophotometrically, and its effects on survival time of washed cells. Log phase cells grown in the presence of Tween 80 and then washed showed no recovery after any of the contact periods with any concentration of chlorhexidine. Log phase cells grown in plain broth and washed were more resistant to the chlorhexidine and showed growth after 120 min contact with 20 μg/ml and up to 15 min contact with 50 μg/ml. In both

systems cells grown in the presence of Tween 80 were less resistant than cells grown in its absence.

The resultant concentration of Tween 80 in the survival time reaction mixtures, where the cells were washed with water, was much less than that previously shown to have an effect on the reactions measured optically with log phase cells. However, *Ps. aeruginosa* produces much slime

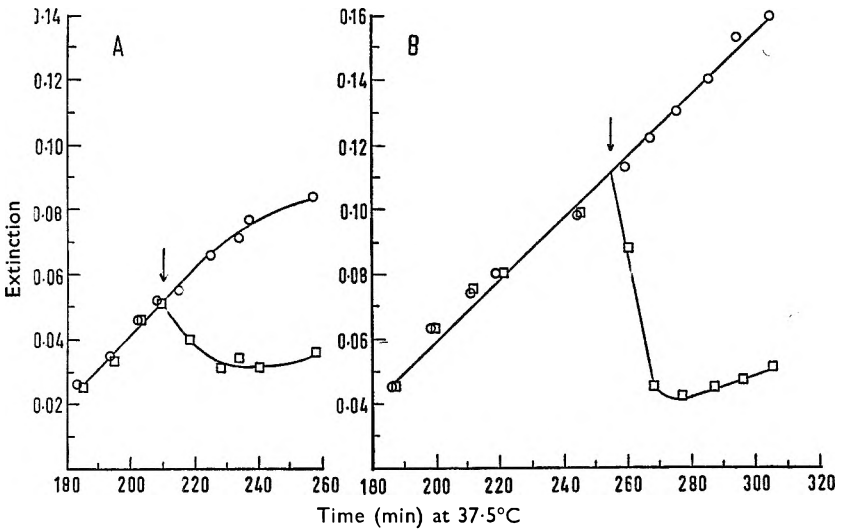


FIG. 3. Effect of Tween 80 on the action of 3·3 units/ml polymyxin B sulphate against log phase cultures of *Ps. aeruginosa*. ○, Control culture. □, Test culture. A, nutrient broth. Polymyxin added after 210 min. B, nutrient broth + Tween 80, 0·5%. Polymyxin added after 254 min.

(Rhodes, 1959) and we have verified that strains NCTC 8203 and 7244 are not exceptions. It seems likely that in all instances the presence of Tween 80 increased the rate at which the slime dispersed from the surface of the actively dividing cells and this may have rendered the organism more sensitive to chemical attack, both during growth and after washing.

Another possibility is that Tween 80 is affecting cell membrane permeability and allowing the penetration of chemicals in low concentrations which would not enter the cell in the absence of Tween. We are testing both these hypotheses.

References

Anderson, R. A. & Stock B. H. (1958). *Aust. Pharm. J.*, **39**, 1,110–1,113.
Brown, M. R. W. & Garrett, E. R. (1964). *J. pharm. Sci.*, **53**, 179–183.
Brown, M. R. W. & Richards, R. M. E. (1964). *J. Pharm. Pharmacol.*, **16**, *Suppl.* 41*T*–45*T*.
Kohn, S. R., Gershenfeld, L. & Barr, M. (1963a). *J. pharm. Sci.*, **52**, 967–974.
Kohn, S. R., Gershenfeld, L. & Barr, M. (1963b). *Ibid.*, **52**, 1,126–1,129.
Riegelman, S., Vaughan, D. G. & Okumoto, M. (1956). *J. Amer. pharm. Ass.*, Sci. Ed., **45**, 93–98.
Rhodes, M. E. (1959). *J. gen. Microbiol.*, **22**, 221–263.

The paper was presented by DR. BROWN.

J. Pharm. Pharmacol., 1964, **16**, *Suppl.*, 56*T*–57*T* Received May 1, 1964

SHORT COMMUNICATION

The effect of chlorhexidine on the permeability and succinoxidase activity of *Micrococcus lysodeikticus*

DAVID WISEMAN

CHLORHEXIDINE causes the release of phosphorus-containing compounds from *Micrococcus lysodeikticus* (Rye & Wiseman, 1964). Its effect on the release of pentose and material absorbing at 260 mμ, and on the succinoxidase activity of this organism has now been investigated.

Materials and methods were as described previously (Rye & Wiseman, 1964), except that radioactive phosphate was omitted from the medium. The supernatant fluids from treated cell suspensions were examined for pentose by Mejbaum's method (Mejbaum, 1939) and for material absorbing at 260 mμ in a Unicam S.P. 700 spectrophotometer. The extinction at 260 mμ was corrected for the presence of residual chlorhexidine by a modification of Holbrook's method (Holbrook, 1958).

The uptake of oxygen by washed cells in the presence of succinate was measured by the Warburg manometric method and the reduction of potassium ferricyanide by such cells by a modification of Weibul's method (Weibul, 1959).

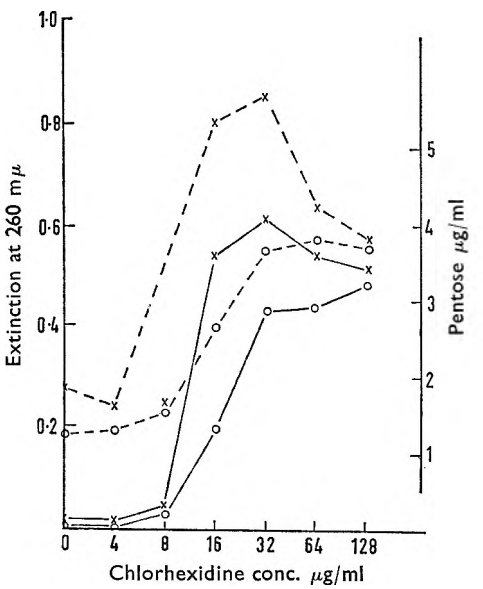


FIG. 1. The release of pentose and 260 mμ absorbing material from *Micrococcus lysodeikticus* treated with chlorhexidine. Temperature 30°; suspending medium M/15 phosphate buffer pH 7·2. ○ 2 hr; × 12 hr.; ○——○ Pentose; ○ - - - ○ 260 mμ absorbing material.

From the Department of Pharmacy, Institute of Technology, Bradford.

RESULTS AND DISCUSSION

Chlorhexidine causes the release from *Micrococcus lysodeikticus* of pentose and of material absorbing at 260 mμ (Fig 1). The pattern of release is similar to that observed for phosphorus-containing compounds (Rye & Wiseman, 1964). Salton has shown that the 260 mμ absorbing material released from cells treated with cetyltrimethylammonium bromide contains purines and pyrimidines (Salton, 1951). These compounds together with pentose and phosphate are the constituents of nucleotides and nucleic acids and this may give some clue to the nature and origin of the released materials.

Concentrations of chlorhexidine which cause the release of cell constituents inhibit the uptake of oxygen by cells in the presence of succinate but increase their rate of reduction of ferricyanide (Fig. 2). The uptake of oxygen by such cells is a measure of their succinoxidase activity. The succinoxidase system consists of succinic dehydrogenase linked with the cytochrome system and in some bacteria it has been shown to be an integral part of the cytoplasmic membrane (Weibul, 1959). Ferricyanide can replace the cytochrome system as an electron acceptor during succinate oxidation and it is reduced directly by succinic dehydrogenase (Guiditta & Singer, 1959). The above results thus show that chlorhexidine does not inhibit succinic dehydrogenase but does block the transfer of electrons along the cytochrome chain.

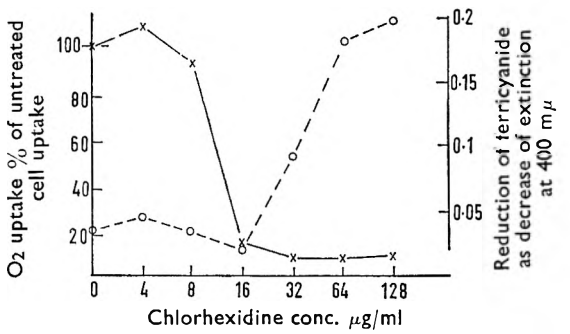


FIG. 2. The uptake of oxygen and reduction of ferricyanide by *Micrococcus lysodeikticus* in the presence of succinate and chlorhexidine. ×——× oxygen uptake; ○ - - - ○ ferricyanide reduction.

The two effects of chlorhexidine viz., (1) the disruption of the permeability barrier of the cells and (2) the blocking of electron transport in the cytochrome system, can both be attributed to chlorhexidine combining with the cytoplasmic membrane and causing an alteration or breakdown of its structure.

References

Guiditta, A. & Singer, T. P. (1959). *J. biol. Chem.*, **234**, 662–665.
Holbrook, A. (1958). *J. Pharm. Pharmacol.*, **10**, 370–374.
Mejbaum, W. (1939). *Z. Physiol. Chem.*, **258**, 117–120.
Rye, R. M. & Wiseman, D. (1964). *J. Pharm. Pharmacol.*, **16**, 516–521.
Salton, M. R. J. (1951). *J. gen. Microbiol.*, **5**, 391–404.
Weibul, C. (1959). *J. gen. Microbiol.*, **20**, 519–531.

The paper was presented by THE AUTHOR.

J. Pharm. Pharmacol., 1964, **16**, *Suppl.*, 58*T*–67*T* Received May 1, 1964

Influence of oil : water ratio on the activity of some bactericides against *Escherichia coli* in liquid paraffin and water dispersions

H. S. BEAN AND S. M. HEMAN-ACKAH†

The activity of bactericides in oil : water dispersions is dependent on their concentration in the aqueous phase and at the oil : water interface both of which are controlled by the oil : water ratio. The activity is also governed by the oil : water partition coefficient (K_w^o) which in turn is influenced by temperature. The closer K_w^o is to unity the smaller the effect on activity of a change in oil : water ratio (i.e., the smaller the phase-volume coefficient); the direction of change of activity depends on whether K_w^o is greater or less than one. In some circumstances the activity may be independent of the temperature or of the oil : water ratio. *Ad hoc* addition of preservatives to oil : water dispersions is meaningless.

PHARMACEUTICAL and cosmetic emulsion systems may undergo microbial deterioration during storage or during use. To prevent this, chemical preservatives may be incorporated. The effective concentration of a preservative is determined empirically, a procedure which is not only expensive but often leads to unexplained failures.

Preservative activity in oil : water systems is a function of the oil : water partition properties of the preservatives (Husa & Radin, 1932; Atkins, 1950; Garrett & Woods, 1953; Hibbott & Monks, 1961; Bean & Heman-Ackah, 1963). The activity of phenols in oil : water dispersions depends on the concentration in the aqueous phase and is influenced by the oil : water interface both of which are controlled by the proportion of oil (Bean, Richards & Thomas, 1962). It has also been shown that for any given overall concentration of phenol, if the oil : water partition coefficient $K_w^o < 1$ then an increase in the proportion of oil leads to an increase in the *concentration* of phenol in *both* phases: where $K_w^o > 1$ there results a decrease in both phases (Bean & others, 1962).

We now describe the activity of a specified overall concentration of a bactericide in systems of different oil : water ratio maintained at various temperatures. The simple oil : water model systems described by Bean & others (1962) have been used because in emulsified products many interacting factors influence the activity of included bactericides.

Experimental

MATERIALS

Liquid paraffin B.P.C. 1963. S.G. (0·830–0·870). Phenol (A.R. quality), *p*-chloro-*m*-cresol (Laboratory reagent), Phenylmercuric acetate (Laboratory reagent), thioglycollic acid (Laboratory reagent).

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

† Permanent address: Kwame Nkrumah University of Science and Technology, Kumasi, Ghana.

INFLUENCE OF WATER : OIL RATIO ON BACTERICIDE ACTIVITY

Nutrient broth. "Oxoid" granules (C.M.1) at a concentration of 1·3% w/v.

Indicator broth. Nutrient broth containing 1% w/v lactose and 0·0016% w/v bromocresol purple as indicator.

Nutrient agar. Nutrient broth solidified with 2% w/v powdered Kobé No. 1 agar ("Oxoid").

Organism. Escherichia coli (NCTC 5933 IMViC^{++--}, 44° +), cultivated and maintained as described by Bean & others (1962). The inoculum was a nephelometrically standardised suspension of the organism stored in sterile water at 4°. The resistance of such suspensions to the bactericides remained constant for several weeks.

The undernoted experiments determined the bactericidal activity against *E. coli* of a given overall concentration of the bactericide in oil : water dispersions of different oil : water ratios at 5, 15, 25, 35 and 45°.

PARTITION COEFFICIENT (K_w^o) BETWEEN OIL AND WATER AT DIFFERENT TEMPERATURES

Oil : water : bactericide mixtures containing a known concentration of the bactericide were allowed to equilibrate at 5, 15, 25, 35 or 45° for 24 hr with shaking at intervals. After separation the aqueous phase was assayed for bactericide content using a spectrophotometric method (phenol at 270 mμ, chlorocresol at 280 mμ, phenylmercuric acetate at 256·5 mμ). The partition coefficients were calculated from these results.

EVALUATION OF BACTERICIDAL ACTIVITY

Preparation of bactericide:oil:water reaction mixture. The bactericidal activity was determined for phenol, chlorocresol and phenylmercuric acetate in liquid paraffin : water dispersions. Pilot reactions determined suitable overall concentrations to yield reasonable extinction times in the different oil : water dispersions. These concentrations were phenol, 0·5% w/v, chlorocresol, 0·075% w/v and phenylmercuric acetate 0·002% w/v. A volume of oil containing an appropriate bactericide concentration was mixed with the bacterial suspension, followed immediately by an equal volume of aqueous bactericide solution. All solutions were temperature-equilibrated before mixing and the order of mixing ensured that the organisms were not subjected even momentarily to a concentration of bactericide greater than in the reaction mixture; the bacterial suspension contained sufficient organism to give 200×10^6 viable *E. coli* per ml of the aqueous phase.

Determination of extinction times. Within each series of experiments several sets of reaction mixtures containing different proportions of oil were prepared and the extinction time of *E. coli* in each system was determined at 5, 15, 25, 35 and 45°. The reaction mixtures were shaken at intervals of approximately 1/7th of the expected extinction time and 1 ml samples immediately transferred to 24 ml nutrient broth (0·1% w/v thioglycollic acid was incorporated in the broth as an inactivator when phenylmercuric acetate was the bactericide). 1 ml of the mixture was

further transferred to 24 ml nutrient indicator broth and incubated at 37° for 3 days. The resulting colour change enabled turbidity produced by the growing organisms to be clearly distinguished from that due to the oil droplets in suspension. Each experiment consisted of not less than five replicate determinations and the results are expressed as a mean extinction time.

Results

EFFECT OF TEMPERATURE ON THE PARTITION COEFFICIENT (K_w^o) OF THE BACTERICIDES

The oil:water partition coefficient K_w^o for chlorocresol in liquid paraffin and water is nearly doubled over the temperature range of 5–45°, whereas that of phenylmercuric acetate in liquid paraffin and water is halved over the same temperature range; in the case of phenol in liquid paraffin and water there is a $2\frac{1}{2}$-fold increase in the K_w^o (Fig. 1).

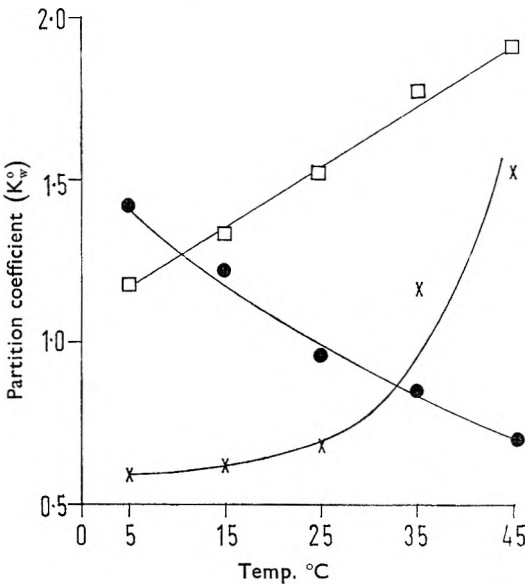


FIG. 1. Effect of temperature on the partition coefficient of the bactericides between liquid paraffin and water. ×—Phenol ($K_w^o \times 10$). □—Chlorocresol ($K_w^o \times 1$). ●—Phenyl mercuric acetate ($K_w^o \times 10$).

EFFECT OF THE PROPORTION OF OIL AND OF TEMPERATURE ON THE CONCENTRATION OF THE BACTERICIDE PARTITIONED INTO THE AQUEOUS PHASE

At constant temperature and for any given overall concentration of phenol or phenylmercuric acetate in the liquid paraffin:water mixture, the concentration of bactericide in the aqueous phase increases as the proportion of oil increases (Figs 2 and 4). With chlorocresol under the same conditions the concentration in the aqueous phase falls as the proportion of oil increases (Fig. 3).

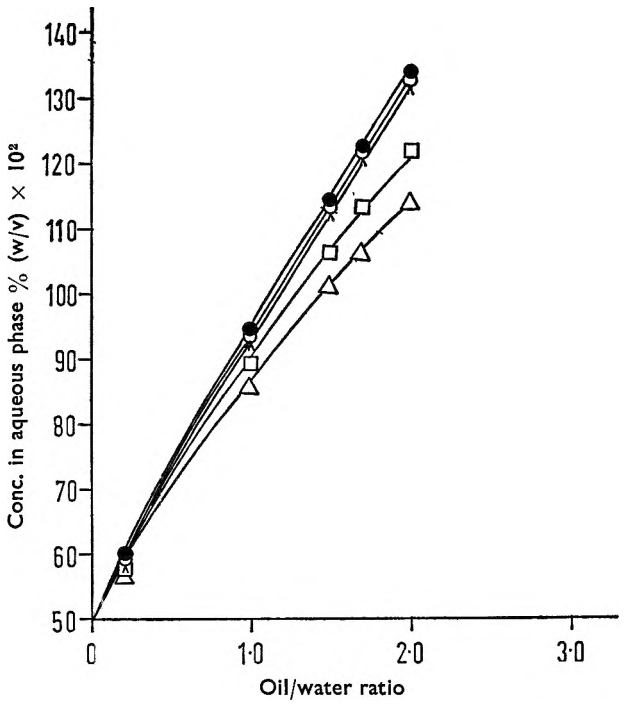


FIG. 2. Effect of oil/water ratio on the concentration of phenol partitioned to the aqueous phase. ● = 5°; ○ = 15°; × = 25°; □ = 35°; △ = 45°.

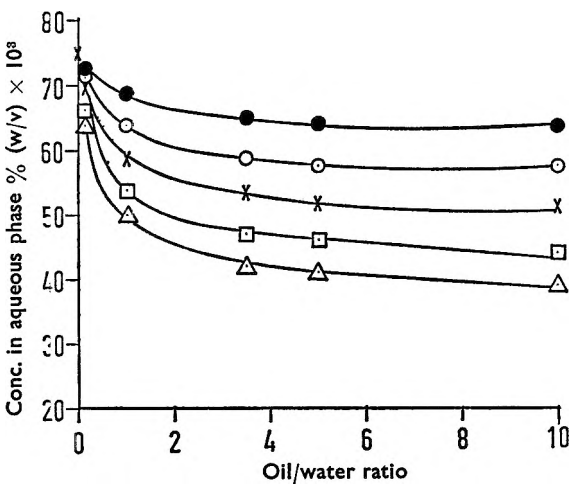


FIG. 3. Effect of oil/water ratio on the concentration of chlorocresol partitioned to the aqueous phase. ● = 5°; ○ = 15°; × = 25°; □ = 35°; △ = 45°.

ACTIVITY OF THE BACTERICIDES IN OIL : WATER DISPERSIONS

An overall concentration of phenol of 0·5% w/v in a liquid paraffin and water dispersion has a greater activity than in water alone. The activity

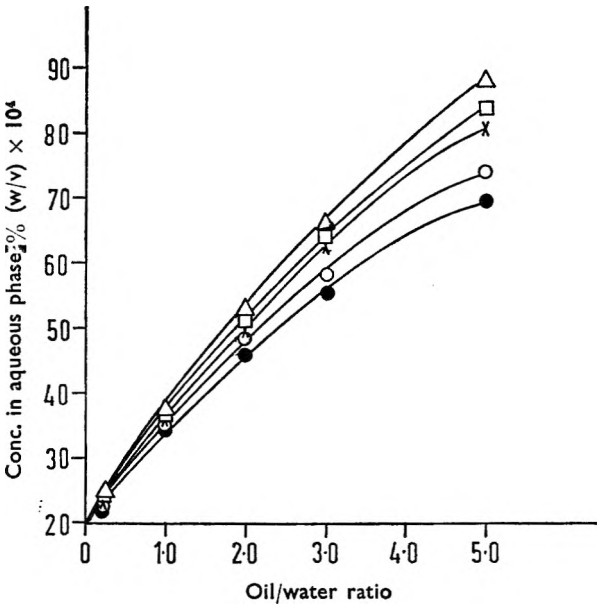


FIG. 4. Effect of oil/water ratio on the concentration of phenylmercuric acetate partitioned to the aqueous phase. ● = 5°; ○ = 15°; × = 25°; □ = 35°; △ = 45°.

increases markedly as the proportion of oil is increased, but the rate of change of activity decreases as the temperature is raised (Fig. 5).

An overall concentration of chlorocresol 0·075% w/v is, on the other hand, less active in liquid paraffin and water than in water alone. The activity changes with the proportion of oil in a rather complex way as illustrated in Fig. 6, the probable significance of this is discussed below.

Phenylmercuric acetate at an overall concentration of 0·002% w/v is more active in liquid paraffin and water dispersions than in water alone. Its activity not only increases with increase in the proportion of oil, but also the rate of change of activity increases with temperature (Fig. 7).

Discussion

EFFECT OF AQUEOUS PHASE CONCENTRATION

The partition coefficient for phenol between liquid paraffin and water is less than 1·0 and therefore a given weight of phenol produces a higher concentration in the aqueous phase of a dispersion than does the same weight dissolved in a volume of water equal to that of the dispersion

(i.e., the aqueous reference). The concentration in the aqueous phase is related to the overall concentration by the expression:

$$Cw = C\left(\frac{\phi + 1}{K_w^o\ \phi + 1}\right)$$

Where Cw = concentration in the aqueous phase % w/v
C = overall concentration % w/v
K_w^o = oil : water partition coefficient
ϕ = oil : water ratio

The activity of the dispersions is greater than that of the aqueous reference and is influenced by the oil : water interface at which there is an

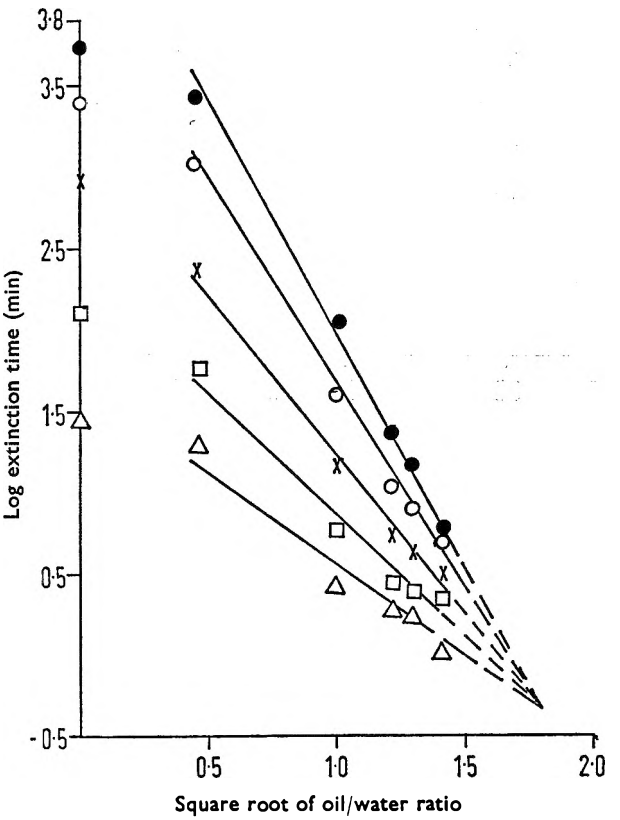


FIG. 5. Activity against *E. coli* of 0·5% w/v overall concentration phenol in liquid paraffin/water dispersions. ● = 5°; ○ = 15°; × = 25°; □ = 35°; △ = 45°.

increased concentration of phenol and, therefore, a region of enhanced activity (Bean & others, 1962). These effects are controlled by the proportion of oil, and the activity of the dispersions increases progressively with it. Thus, at 25°, a dispersion of 50% v/v liquid paraffin in

water is about 50 times more active than an aqueous solution containing the same overall concentration of phenol: a dispersion of 66·67% v/v liquid paraffin is 250 times more active. Phenylmercuric acetate in the dispersions ($K_w^o < 1$ at 5–45°) behaves similarly though the increase in activity with increase in the proportion of oil is not as great. This is due to the low concentration exponent of phenylmercuric acetate and therefore to a smaller interfacial effect on activity.

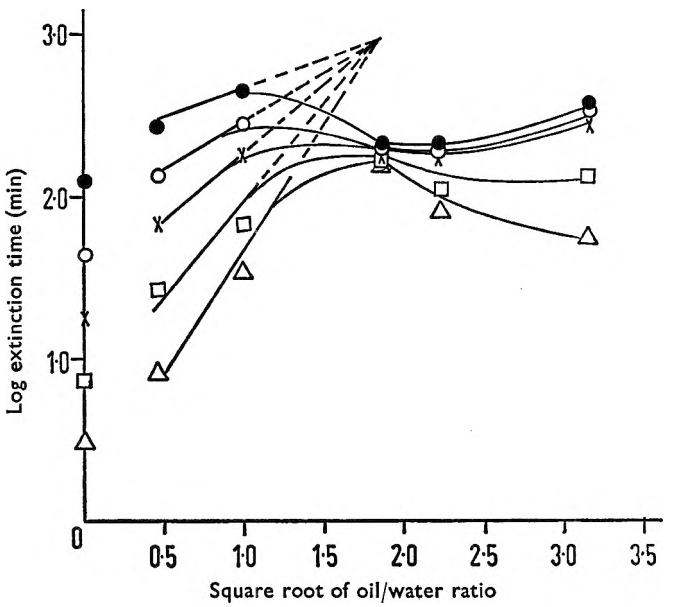


FIG. 6. Activity against *E. coli* of 0·075% overall concentration chlorocresol in liquid paraffin/water dispersions. ● = 5°; ○ = 15°; × = 25°; □ = 35°, △ = 45°.

In contrast, chlorocresol in the dispersions is partitioned in favour of the oil phase ($K_w^o > 1$ at 5–45°) and the concentration in the aqueous phase is, therefore, less than the aqueous reference of the same overall concentration. The activity of the dispersions is controlled by two opposing factors: (i) diminished activity due to reduction of chlorocresol concentration in the aqueous phase and (ii) enhanced activity due to the oil : water interface. The net result is that the dispersions of chlorocresol are less active than the aqueous reference. For low proportions of oil, the interfacial effect is very small and the activity is dependent mainly on the aqueous phase concentration. As the proportion of oil is increased, the interface, with its relatively high concentration of chlorocresol, has a greater effect on the activity which decreases less than the fall in concentration in the aqueous phase would suggest. For example, at 25° the activity of a 50% v/v liquid paraffin dispersion is 1/10th that of the aqueous reference whereas the concentration in the aqueous phase of the dispersion *per se* would indicate only 1/11th that of the aqueous reference. The activity of a 90·9% v/v dispersion, in which the interface

has an even greater effect, is 1/15th that of the aqueous reference whereas the aqueous phase concentration would indicate only 1/260th.

For each of the three bactericides, the regression of log extinction time on the oil: water ratio (ϕ) is distinctly curvilinear; and, except for chlorocresol, the log-extinction time is rectilinearly related to $\sqrt{\phi}$ (Figs 5–7). For any given overall concentration of bactericide, ϕ, determines its concentration in the aqueous phase, and the log extinction time is therefore related linearly to some function of the aqueous phase concentration, that is, to the combined effect of the aqueous phase concentration and the interface. The relationship between the concentration and activity of aqueous solution of bactericides is well established. The log

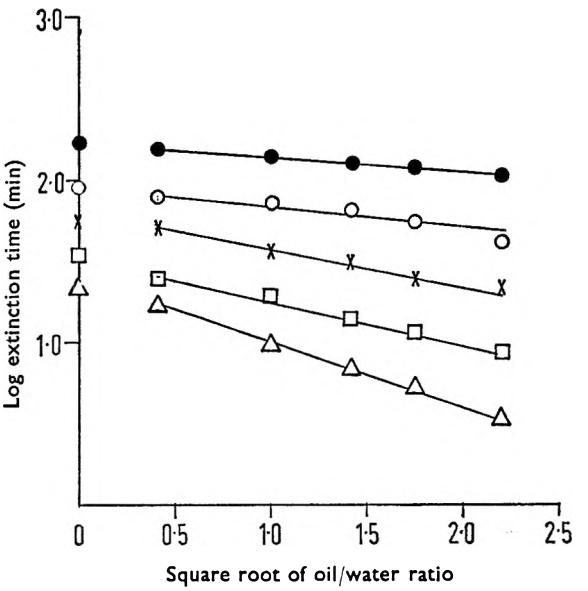


FIG. 7. Activity against *E. coli* of 0·002% w/v overall concentration of phenylmercuric acetate in liquid paraffin/water dispersions. ● = 5°; ○ = 15°; × = 25°; □ = 35°; △ = 45°.

extinction time is linearly related to the log concentration; the slope of the regression is known as the concentration exponent of the bactericide. Thus, for oil: water dispersions, the slope of the linear regression relating log extinction time to $\sqrt{\phi}$ is analogous to the concentration exponent of aqueous solutions of bactericides, and is called the *phase-volume coefficient*. The activity of the dispersions and the proportion of oil are related as follows:

$$\log t = A - p\sqrt{\phi}$$

where t = extinction time; A = constant; p = slope of the regression of log-extinction time on $\sqrt{\phi}$ (*phase-volume coefficient*); ϕ = oil: water ratio.

EFFECT OF OIL : WATER RATIO AND TEMPERATURE

In the dispersions containing phenol, the higher the temperature, the smaller is the effect of changing the proportion of oil on the aqueous phase concentration and, therefore, on the activity, i.e. the smaller the phase-volume coefficient (Table 1). The greater the proportion of oil the smaller is the effect of temperature change on activity and at an oil : water ratio of about 3 : 1, the activity is independent of temperature (Fig. 5).

TABLE 1. PHASE-VOLUME COEFFICIENTS FOR BACTERICIDES IN LIQUID PARAFFIN: WATER DISPERSIONS

Temperature °C (T)	Phase-volume coefficient (p)	Oil-water partition coefficient (K_w^o)
Phenol		
5	2·594	0·059
15	2·426	0·062
25	1·963	0·068
35	1·526	0·117
45	1·205	0·154
Phenylmercuric acetate		
5	0·077	0·141
15	0·148	0·120
25	0·234	0·095
35	0·271	0·085
45	0·371	0·070
Chlorocresol		
5	0·439	1·192
15	0·542	1·341
25	0·814	1·528
35	0·866	1·782
45	1·143	2·000

In the dispersions containing phenylmercuric acetate the reverse is the case and greater changes in activity occur as the proportion of oil and temperature are increased, i.e. the phase-volume coefficient increases with temperature (Fig. 7 and Table 1). There cannot be an oil : water ratio at which activity is independent of temperature.

The influence of the proportion of oil and temperature on the activity of chlorocresol in the dispersions is complex. An increase in the ratio of oil reduces both the aqueous phase concentration (Fig. 3) and the activity (Fig. 6), the effects increasing with temperature rise. Therefore, an increase in the proportion of oil reduces the effect of temperature change on activity: at an oil : water ratio of 3·5 : 1 the activity would be independent of temperature. The latter was confirmed experimentally but the extinction times were less than those theoretically predicted (Fig. 6) due to the interface enhancing activity. As the proportion of oil is increased above 3·5 : 1 the activity is reduced by progressive depletion of the aqueous phase although at the higher temperatures this is offset by the normal influence of temperature on activity. Thus, with chlorocresol in liquid paraffin : water dispersions, the phase-volume coefficient is only constant at low oil : water ratios. It varies with the proportion of oil as well as with temperature at the higher oil : water ratios.

The phase-volume coefficient for phenol and phenylmercuric acetate, and for chlorocresol at low oil:water ratios, is directly related to the temperature (Table 1) by the equation

$$p = kT + C$$

where p = phase-volume coefficient; T = temperature °C; k and C = constants.

From this it can be shown that at 78° the phase-volume coefficient for phenol in the dispersions is zero. The corresponding values for chlorocresol and phenylmercuric acetate are −19° and −6·5° respectively. At these temperatures the activity would be independent of oil:water ratio and the partition coefficient (K^{o}_{w}) would approach unity.

References

Atkins, F. (1950). *Mfg. Chem.*, **21**, 51–54.
Bean, H. S. & Heman-Ackah, S. M. (1963). 23rd International Congress of Pharmaceutical Sciences, Munster, W. Germany.
Bean, H. S. & Richards, J. P. & Thomas, J. (1962). *Boll. Chim. Farm.*, **101**, 339–346.
Carlson, V. & Bennett, E. O. (1960). *Lub. Eng.*, **16**, 572–574.
Hibbott, H. W. & Monks, J. (1961). *J. Soc. cosmet. Chem.*, **12**, 1–10.
Husa, W. J. & Radin, J. M. (1932). *J. Amer. pharm. Ass.*, **21**, 861–869.
Garrett, E. R. & Woods, O. R. (1953). *J. Amer. pharm. Ass., Sci. Ed.*, **42**, 736–739.

The paper was presented by MR. HEMAN-ACKAH.

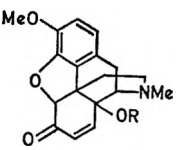
J. Pharm. Pharmacol., 1964, 16, *Suppl.*, 68*T*–71*T* Received May 1, 1964

The relationship between analgesic activity, acute toxicity and chemical structure in esters of 14-hydroxycodeinone

W. R. BUCKETT*

Acylation of 14-hydroxycodeinone with long chain unbranched fatty acids produced compounds of varying analgesic potency, maximal activity being in 14-n-heptoyloxycodeinone. In 14-phenylalkyloxy derivatives maximal analgesic potency was found in 14-cinnamoyloxycodeinone. All codeinone derivatives studied had approximately one-third the duration of morphine in mice. Intravenous and subcutaneous toxicities in mice were generally similar in compounds causing death by convulsions, but differed widely in those causing death by respiratory depression.

IT has recently been shown (Buckett, Farquharson & Haining, 1964) that hydroxylation and subsequent acylation of codeinone at the 14-position produced marked changes in analgesic activity and acute toxicity. Change of the acyl group from acetyl to valeryl gave compounds with increased analgesic potency and decreased subcutaneous acute toxicity in mice.



It seemed possible that analgesic potency could be enhanced still further in compounds with higher acyl groups at the 14-position. This paper reports on such compounds and on a limited study of the influence of introducing a double bond into the esterifying group.

Methods

Acute toxicity. The LD50 of each compound was determined using albino mice (18–22 g). Water soluble salts were dissolved in 0·9% w/v sodium chloride. Bases were dissolved in either 10% phosphoric acid or 0·1N hydrochloric acid and adjusted to pH 6·0 using 5% w/v sodium bicarbonate solution, then made up to volume with 0·9% w/v sodium chloride. Animals received a dose of 0·2 ml/20 g body weight, either by the intravenous or subcutaneous route. Intermittent observation was continued for 3 hr after injection and mortalities recorded 24 hr later. The LD50 was calculated by the method of Litchfield & Wilcoxon (1949).

Analgesia. Estimates of analgesic potency, peak activity time and duration of action in mice were made using the method of Bianchi &

From the Research Department, Edinburgh Pharmaceutical Industries Ltd., Wheatfield Road, Edinburgh, 11.

*Present address: Pharmacology Research Department, Organon Laboratories Ltd., Newhouse, Lanarkshire, Scotland.

Franceschini (1954). Albino mice (18–22 g) were tested for sensitivity to a bulldog artery clip covered with rubber tubing, which was applied to the base of the tail; only those attempting to remove the clip within 15 sec were used. Each compound was administered subcutaneously at three or four dose levels to groups of 10 animals. Animals were tested at 10 min intervals and the proportion in each group which made no attempt to remove the clip within 30 sec was determined. Testing continued until the analgesic effect had terminated, when duration curves could be plotted. The ED50 for analgesia at the time of maximum effect was determined, and the potency relative to morphine calculated by the method of Litchfield & Wilcoxon (1949).

Materials. All compounds were used in the form of bases except 14-cinnamoyloxycodeinone phosphate (R = COCH:CH·Ph) and 14-phenylpropionoxycodeinone hydrochloride (R = $COCH_2CH_2$·Ph); codeine phosphate B.P. and morphine hydrochloride B.P. were used as standards.

Results

Acute toxicity. The LD50 for each compound is given in Table 1. After intravenous administration, death usually occurred rapidly and followed either convulsions or catalepsy and respiratory depression. Death occurred later after subcutaneous injection but the toxic effects were essentially similar. Both intravenous and subcutaneous acute toxicities decreased with the change from codeinone to 14-hydroxycodeinone and through the acetoxy (Krueger, Eddy & Sumwalt, 1943) to the propionoxy compound. The reduced toxicity was particularly marked on converting the 14-hydroxy compound to its acetate (R = COMe). These four compounds produced death by convulsions and the acute toxicities differed little after either subcutaneous or intravenous administration.

Table 1 shows that intravenous toxicity (but not subcutaneous toxicity)

TABLE 1. THE ACUTE TOXICITY OF ESTERS OF 14-HYDROXYCODEINONE IN MICE

[The LD50 values are expressed in mg/kg in terms of base. Limits of error (P = 0·95) are given in parentheses]

14-substituent R	Intravenous LD50	Subcutaneous LD50	Toxic effects
None	5·0 (4·2–6·0)	11·0 (9·8–12·3)*	Violent convulsions
-H	11·8 (10·6–13·1)	28 (25–30)*	Convulsions
-COMe	105 (91–121)	127†	Convulsions
-COEt	110 (88–137)	150 (110–190)*	Convulsions
-COPr	66 (58–75)	>500	Respiratory depression
-COBu	38 (28–52)	495 (410–600)*	Respiratory depression
$-COC_5H_{11}$	9·0 (7·8–10·4)	>500	Respiratory depression
$-COC_6H_{13}$	26 (21–32)	>500	Respiratory depression
$-COC_7H_{15}$	40 (32–50)	>500	Respiratory depression
$-COC_9H_{19}$	61 (51–72)	>500	Respiratory depression
$-COC_{11}H_{23}$	120 (112–128)	>500	Respiratory depression
$-COCH_2$·Ph	71 (65–77)	300*	Respiratory depression
$-COCH_2$·CH_2·Ph	45 (39–52)	300	Convulsions
-COCH = CH·Ph	31 (24–39)	200	Respiratory depression
-COCH = CH·Me	41 (36–47)	200	Respiratory depression

* Buckett, Farquharson & Haining (1964).
† Krueger, Eddy & Sumwalt (1943).

increased in the series n-butyrate ($R = COC_3H_7$), n-valerate ($R = COC_4H_9$), and n-hexoate ($R = COC_5H_{11}$). Further increase in the length of the ester group up to n-lauroyloxy ($R = COC_{11}H_{23}$) decreased the intravenous toxicity. In all compounds with an ester group higher than propionate, death followed acute respiratory depression irrespective of the route of administration.

All the 14-phenylalkoxy derivatives tested were more toxic intravenously than subcutaneously. Death was due to acute respiratory depression except with the 14-β-phenylpropionate ($R = COCH_2 \cdot CH_2 \cdot Ph$) which produced convulsions. Replacing the single bond between the methylene groups in the side-chain with a double bond ($R = COCH : CH \cdot Ph$), increased the acute toxicity and changed the mode of death. With the crotonate ($R = CO \cdot CH : CH \cdot Me$) rather than the cinnamate ($R = CO \cdot CH : CH \cdot Ph$) the toxicity did not alter significantly and the manner of death was similar.

Analgesia. Esterification of 14-hydroxycodeinone produced derivatives with enhanced analgesic potency (Table 2). Esterification to give 14-

TABLE 2. THE ANALGESIC ACTIVITY OF ESTERS OF 14-HYDROXYCODEINONE IN MICE AFTER SUBCUTANEOUS ADMINISTRATION

[All results are expressed in terms of base. Limits of error (P = 0·95) are given in parentheses].

14-substituent R	Relative analgesic potency (morphine = 1·0)	Onset of peak activity (min)	Duration of analgesic ED50 (min)
None	Not determined due		
-H	to toxic excitation		
-COMe	4·0 (2·27–7·05)	10	30
-COEt	18·8 (12·6–28·3)	10	30
-COPr	28·7 (16·0–51·5)	10	30
-COBu	38·8 (22·1–68·4)	10	30
$-COC_5H_{11}$	47·2 (26·5–84·6)	10	30
$-COC_6H_{13}$	60·1 (39·0–92·3)	10	30
$-COC_7H_{15}$	5·1 (2·9–8·9)	10	50
$-COC_9H_{19}$	1·12 (0·56–2·21)	10	30
$-COC_{11}H_{23}$	0·034 (0·023–0·051)	10	30
$-COCH_2Ph$	52 (33–82)	10	30
$-COCH_2CH_2Ph$	115 (78–168)	10	30
$-COCH = CH \cdot Ph$	177 (101–310)	10	30
$-COCH = CH \cdot CH_3$	31 (19–48)	10	30
Standard drugs			
Morphine	1·0	20	90
Codeine	0·49 (0·35–0·7)	10–20	40

acetoxycodeinone produced a compound with four times the potency of morphine. Further increases in the length of the acylating group at position 14 gradually increased the analgesic potency. The maximum potency (sixty times that of morphine) was obtained with 14-heptoyloxycodeinone ($R = COC_6H_{13}$). Still further increases reduced the analgesic activity until with 14-lauroyloxycodeinone ($R = COC_{11}H_{23}$) a compound having only one-thirtieth the potency of morphine was obtained.

Buckett & others (1964) have previously shown that benzoylation at the 14-position reduced analgesic potency but the introduction of a single methylene group between the ester carbonyl and terminal phenyl gives a compound ($R = CO \cdot CH_2 \cdot Ph$) of high analgesic potency. A further

methylene group (R = CO·CH_2·CH_2·Ph) increased the activity still more to give a compound having over one hundred times the potency of morphine and the introduction of a double bond (R = CO·CH:CH·Ph) did not diminish this activity. With the crotonate (R = CO·CH:CH·Me) the potency was equivalent to that of the corresponding saturated straight chain compound (R = COPr). In these two pairs of compounds a double bond in the 14-substituent has no effect on analgesic potency or duration of action. Both the onset and duration of analgesia of all these compounds in mice are shorter than either morphine or codeine (Table 2).

Discussion

Beckett, Casy, Harper & Phillips (1956) postulated an analgesic receptor surface consisting of a charged anionic site separated from a flat surface by a cavity. The flat surface accommodates the aromatic position of the analgesic molecule on the basis of Van der Waals forces and the cavity allows close contact between drug and receptor in a third dimension.

The compounds in this series would appear to fit such a receptor at the anionic site, the flat surface and possibly at the cavity. Since the 14-substituent consists of a flexible acyl grouping, its position relative to the codeinone nucleus is a matter for conjecture at the present time.

Acknowledgements. I thank Dr. D. Davidson and Mr. J. Gillon for supplying all new derivatives of codeinone also Miss Doreen Wilson who assisted in the pharmacological work.

References

Beckett, A. H., Casy, A. F., Harper, N. J. & Phillips, P. M. (1956). *J. Pharm. Pharmacol.*, **8**, 860–873.

Bianchi, C. & Franceschini, J. (1954). *Brit. J. Pharmacol.*, **9**, 360–366.

Buckett, W. R., Farquharson, M. E. & Haining, C. G. (1964). *J. Pharm. Pharmacol.*, **16**, 174–182.

Krueger, H., Eddy, N. B. & Sumwalt, M. (1943). *The Pharmacology of the Opium Alkaloids*, Part 2, p. 944. Washington: U.S. Government Printing Office.

Litchfield, J. T. & Wilcoxon, F. (1949). *J. Pharmacol.*, **95**, 99–113.

The paper was presented by THE AUTHOR.

J. Pharm. Pharmacol., 1964, **16**, *Suppl.*, 72T–79T Received April 23, 1964

An isolated parasympathetically-innervated oesophagus preparation from the chick

W. C. BOWMAN AND SALLY D. EVERETT

An isolated parasympathetically-innervated preparation from the chick oesophagus is described. This preparation is essentially similar to mammalian parasympathetically-innervated smooth muscles in its responses to drugs, and because of its simplicity, robustness and cheapness it is recommended for use in student practical classes.

ISOLATED oesophagus preparations from the cat, the rabbit and the rat have been briefly described by Rabinovitch (1928), by Bain & McSwiney (1936) and by Hughes & McDowall (1954) respectively. Rand & Stafford (1964) have recently described in detail a study of the properties of isolated innervated oesophagus preparations from various species including that of the adult domestic fowl. While preparations from some species contain both striated and smooth muscle, those from the cat and the chicken contain only smooth muscle. This paper describes some pharmacological responses of the isolated innervated oesophagus of the chick which appears to possess several advantages as an experimental preparation over that taken from the adult bird.

Methods

Male chicks (Silver Link) aged between 1 and 7 days after hatching were starved overnight to empty the crop and then deeply anaesthetised or killed with ether. The upper oesophagus as far as the crop was removed together with as much as possible of one or of both parasympathetic nerve trunks. The oesophagus in the domestic fowl receives its parasympathetic innervation via branches from the vagus and glossopharyngeal nerves which run together along the course of the jugular veins on both sides (Rand & Stafford, 1964). Apart from about a 1 cm length at the free ends, the nerves were not separated from the jugular veins so that damage to the fine nerve branches passing to the oesophagus was avoided. The preparation was suspended in Krebs-Henseleit solution (NaCl 6·95, KCl 0·34, $CaCl_2$ 0·28, KH_2PO_4 0·162, $MgSO_4$ 0·294, $NaHCO_3$ 2·1, dextrose 2 g/litre) continuously gassed with 95% O_2 and 5% CO_2. The nerves were passed through stimulating electrodes of the type described by Burn & Rand (1960). Contractions of the oesophagus were recorded on smoked paper with an isotonic frontal writing lever loaded with 2 g and amplifying the contractions 6 times. The nerves were stimulated at various frequencies with rectangular pulses of 0·5 or 1 msec duration and of a strength such that the contraction for a given frequency was maximal. The body temperature of the chick is about 42° and in preliminary experiments the temperature of the bath

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

solution was varied over the range 32–42°. Similar responses to nerve stimulation occurred throughout this range but at 32° spontaneous pendular movements were less pronounced and the preparation survived much longer. In all of the experiments reported, the bath temperature was maintained at 32°.

The drugs used were: acetylcholine chloride (Roche), physostigmine sulphate (BDH), nicotine hydrogen tartrate (BDH), tetramethylammonium bromide (BDH), dimethylphenyl piperazinium iodide (Light & Co.), hexamethonium bromide (May & Baker), mecamylamine (Merck, Sharpe & Dohme), pempidine tartrate (May & Baker), atropine sulphate (BDH),

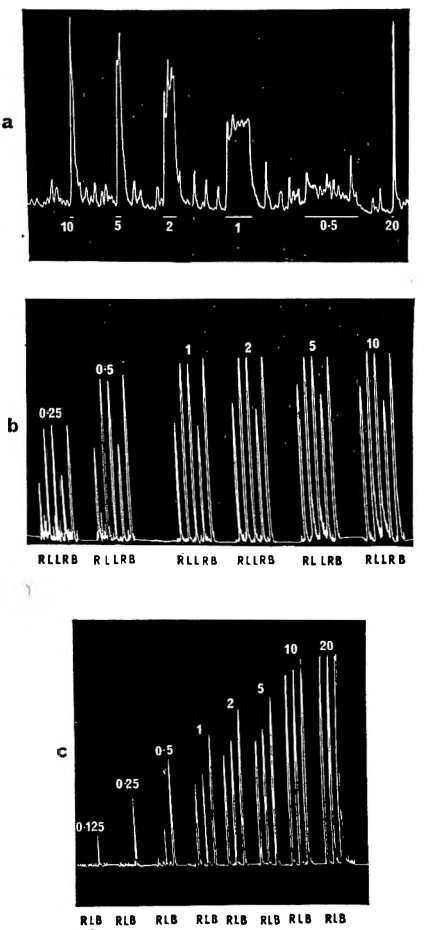


FIG. 1. a. Responses to a constant number of stimuli applied at different frequencies to the right nerve trunk. The numerals denote the frequency (stimuli/sec) applied for the periods marked by the horizontal lines. Each response is to 50 stimuli. b and c. Responses to stimulation of the right (R), the left (L) and both (B) nerve trunks for 10 sec at different frequencies denoted by the numerals above each group of responses. The responses marked B were produced by synchronous stimulation of both trunks in b and by asynchronous stimulation in c.

(−)-adrenaline (BDH), (−)-noradrenaline (Light & Co.), (−)-isopropylnoradrenaline bitartrate (Wyeth), phentolamine (Ciba), pronethalol (I.C.I.) and guanethidine sulphate (Ciba). The concentrations in the text refer to the bases.

Results

Stimulation of either nerve trunk caused contraction of the oesophagus. When the oesophagus was lying free in a petri dish, stimulation of the left nerve trunk caused it to curl to the left and stimulation of the right nerve trunk caused a similar movement in the opposite direction indicating that, as in the rabbit (Rand & Stafford, 1964), each nerve chiefly innervated the longitudinal muscle fibres on the corresponding side. The threshold frequency of stimulation in most preparations was 0·5 to 1/sec, but a few preparations responded to single shocks. The frequency required to produce maximal contractions varied in different preparations. In some it was as low as 2/sec but in most it was 10/sec. Fig. 1a illustrates responses, each to 50 stimuli applied to the right nerve trunk at different frequencies. Stimulation of the left nerve trunk produced slightly greater contractions than stimulation of the right (Fig. 1b and c). When both nerves were stimulated together and the stimuli to each nerve were synchronised (i.e. supplied by the same stimulator), the contractions produced were equal in size to those produced by stimulation of the left nerve alone (Fig. 1b). However, when both nerves were stimulated together but with the stimuli to each slightly out of phase, the contractions produced at low frequencies of stimulation were greater than those produced by stimulation of either nerve alone. For example, in the experiment illustrated in Fig. 1c, stimulation of both nerves at a frequency of 0·5/sec produced contractions similar in size to those produced by stimulation of the left nerve alone at a frequency of 2/sec. On the other hand, with a frequency of 20/sec, contractions of equal size were produced by stimulation of either nerve alone or of both together.

The right nerve trunk was easier to prepare than the left and in all the experiments in which the effects of drugs were studied, contractions of the oesophagus were elicited by stimulation of the right nerve at a frequency of 5 or 10/sec for 10 sec in every 2 min. With this pattern of stimulation comparable responses could be elicited for several hours. Regular stimulation of this type inhibited and usually completely abolished the spontaneous pendular movements of the oesophagus. On stopping the stimulation, the spontaneous activity usually slowly returned.

ACETYLCHOLINE, PHYSOSTIGMINE AND ATROPINE

Contraction of the oesophagus was produced by acetylcholine in concentrations of 0·025 μg/ml and above. The responses to acetylcholine and those to nerve stimulation were potentiated by physostigmine (0·05–0·1 μg/ml). Spasm of the oesophagus gradually developed about 10 min after the addition of physostigmine. Changing the bath fluid quickly restored the tone of the preparation to normal, but the potentiation of the responses to acetylcholine and to nerve stimulation persisted. Atropine

in a concentration of 0·01 μg/ml abolished the responses to acetylcholine and markedly depressed those to nerve stimulation. Larger concentrations of atropine (0·02 μg/ml and above) completely blocked the responses to nerve stimulation. Fig. 2 illustrates the effects of physostigmine and atropine on responses to acetylcholine and to nerve stimulation.

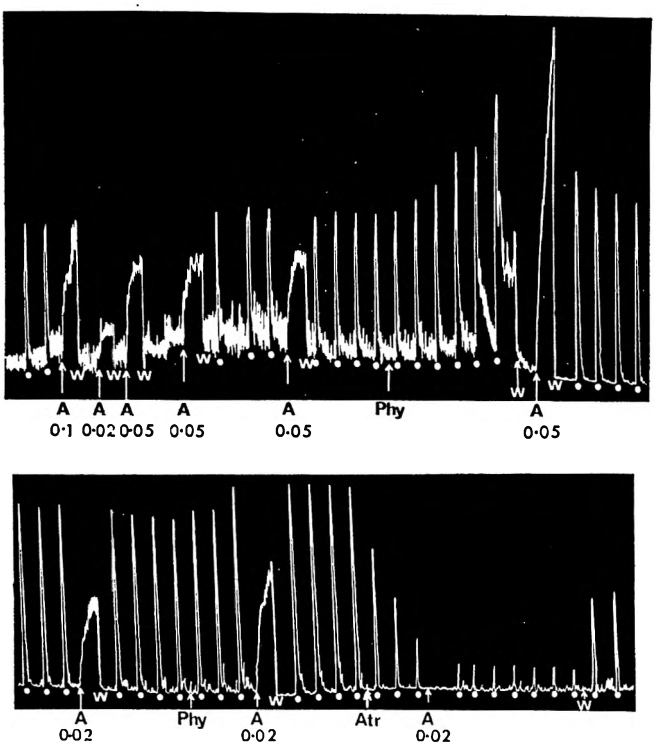


FIG. 2. Effects of acetylcholine, physostigmine and atropine. At the white dots, the right nerve trunk was stimulated at a frequency of 10/sec for 10 sec. Except when acetylcholine was added, the interval between stimulation periods was 2 min. At A, acetylcholine was added; the numerals denote the bath concentration in μg/ml. At Phy, physostigmine was added to give a bath concentration of 0·1 μg/ml in the upper record and 0·05 μg/ml in the lower. At Atr, atropine was added to give a bath concentration of 0·01 μg/ml. At W, the bath fluid was changed.

GANGLION STIMULANT AND BLOCKING DRUGS

The effects of nicotine, dimethylphenylpiperazinium (DMPP) and tetramethylammonium (TMA) were studied. In low concentrations (nicotine 2–5 μg/ml, DMPP, 0·5–1 μg/ml, TMA 2–4 μg/ml) all three enhanced or initiated rhythmic pendular movements and potentiated the responses to nerve stimulation. This effect of TMA is illustrated in Fig. 3a. Larger concentrations (nicotine 8–10 μg/ml, DMPP 2–4 μg/ml, TMA 4–6 μg/ml) produced an abrupt contraction of the oesophagus which often quickly waned before changing the bath fluid, especially with nicotine and DMPP (Fig. 3a).

The stimulant actions of nicotine, DMPP and TMA were abolished after the addition of hexamethonium (2–4 μg/ml), mecamylamine (0·5–1 μg/ml) or pempidine (0·5–1 μg/ml). The same doses of these ganglion blocking drugs produced a 50–80% depression of contractions evoked by nerve stimulation and in 6 out of 9 experiments additional amounts produced complete block. Fig. 3b illustrates the effect of pempidine on contractions

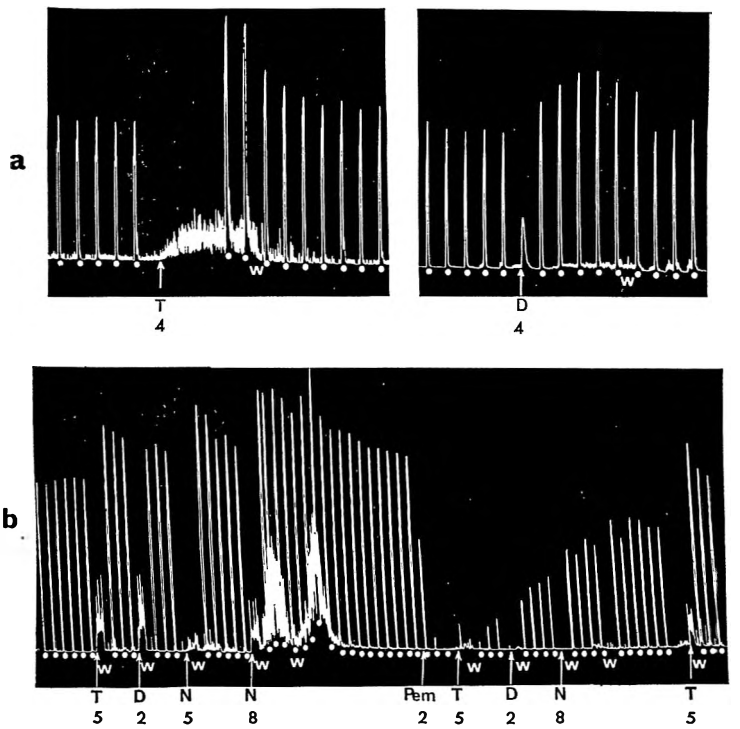


FIG. 3. Effect of ganglion stimulant and blocking drugs. At the white dots, the right nerve trunk was stimulated for 10 sec at a frequency of 5/sec in a and 10/sec in b. Except when ganglion stimulant drugs were added, the interval between stimulation periods was 2 min. At T, TMA, at D, DMPP, at N, nicotine and at Pem, pempidine was added to the bath. The numerals denote the bath concentrations in μg/ml. At W, the bath fluid was changed.

produced by nerve stimulation and by TMA, DMPP and nicotine. In two experiments with hexamethonium and in one with mecamylamine complete block of contractions evoked by nerve stimulation could not be obtained. In these 3 experiments the maximal degree of block was produced by 4 μg/ml hexamethonium and 1 μg/ml mecamylamine. Subsequent additions to a total concentration of 100 μg/ml of each drug did not produce a greater effect.

When added during partial block produced by a ganglion blocking agent, nicotine, TMA and DMPP restored the contractions evoked by nerve stimulation. The recovery from pempidine block illustrated in Fig. 3b was partly due to the addition of these ganglion stimulant drugs. In

other experiments, in which ganglion stimulant drugs were not added, recovery after pempidine was very slow despite frequent washings.

SYMPATHOMIMETIC AMINES AND ANTI-ADRENALINE DRUGS

(—)-Adrenaline (0·01–0·05 μg/ml), (—)-noradrenaline (0·02–0·1 μg/ml) and (—)-isopropylnoradrenaline (0·02–0·05 μg/ml) produced comparable inhibitions of the spontaneous pendular movements, when present, and of the contractions produced by nerve stimulation (Fig. 4, upper panels)

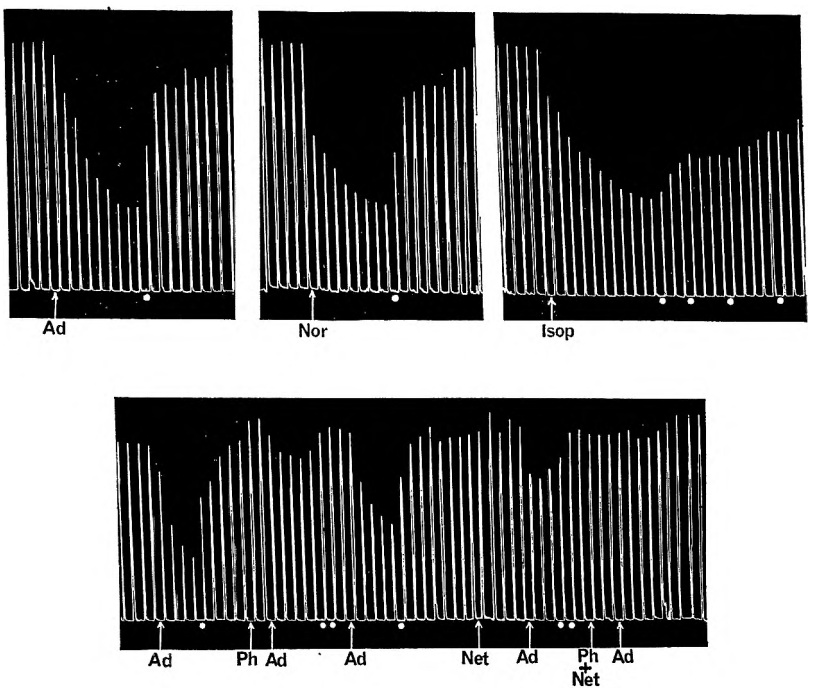


FIG. 4. Effects of sympathomimetic amines and anti-adrenaline drugs. Responses are to stimulation of the right nerve trunk (10/sec for 10 sec every 2 min). At Ad, (—)-adrenaline (0·05 μg/ml), at Nor, (—)-noradrenaline (0·1 μg/ml), at Isop, (—)-isopropylnoradrenaline (0·05 μg/ml), at Ph phentolamine (2·5 μg/ml) and at Net, pronethalol (1 μg/ml) were added to the bath. Phentolamine (2·5 μg/ml) and pronethalol (1 μg/ml) were added together before the last addition of adrenaline. At the white dots, the bath fluid was changed.

or by acetylcholine. In the same preparation, the depression of contractions produced by nerve stimulation was more marked the lower the frequency of stimulation. Contractions produced by acetylcholine and by nerve stimulation were reduced to a similar extent suggesting that the effect was due to inhibition of the smooth muscle rather than to a depression of ganglionic transmission of the type described by Marrazzi (1939). The relative potency of the three sympathomimetic amines varied in different experiments, but in most (—)-isopropylnoradrenaline and (—)-adrenaline were about equipotent and (—)-noradrenaline was about half

as active. The effects of adrenaline and noradrenaline were quickly removed by washing but that of isopropylnoradrenaline was persistent and full recovery was often not achieved despite repeated washing (Fig. 4, upper panels).

Phentolamine, in a concentration of 2·5 μg/ml. or pronethalol, in a concentration of 1 μg/ml, slightly potentiated the contractions produced by nerve stimulation and antagonised, but did not abolish the depressant effect of adrenaline added 4 to 10 min later (Fig. 4, lower panels). The addition of both anti-adrenaline drugs together in the above concentrations produced a greater antagonism of the adrenaline effect than either blocking drug added alone (Fig. 4, lower panels).

GUANETHIDINE

Guanethidine, in concentrations up to 2 μg/ml, was without effect on contractions of the oesophagus evoked by nerve stimulation. Larger amounts (up to 20 μg/ml) depressed the contractions.

Discussion

Rand & Stafford's (1964) results with the oesophagus of the adult fowl showed that it was relatively insensitive to drugs. In cases where the same drugs were used, we found concentrations 25–50 times smaller to be effective in preparations from the young chick. Rand & Stafford also found that preparations from the adult fowl responded slowly to drugs and their actions were reversed with difficulty. They attributed this to a tough connective tissue barrier which retarded the diffusion of drugs. The chick preparation, on the other hand, is relatively free of connective tissue and responds briskly to drugs which, in most instances, may be easily washed out.

The oesophagus receives its sympathetic innervation from the 1st thoracic segment. Had the point of stimulation of the nerve trunk been left *in situ*, it would have been craniad to the segment of oesophagus removed; it is unlikely therefore that the stimulated part of the nerve trunk contained any adrenergic inhibitory fibres. If such fibres had been present, however, blockade of their effects would have enhanced the responses to nerve stimulation; indeed a slight effect of this type was produced by the anti-adrenaline drugs. It is to be noted that the adrenergic neurone blocking drug, guanethidine, did not enhance the contractions, its only effect being to depress them in large doses. This result suggests that the only efferent fibres stimulated belonged to the parasympathetic division of the autonomic system. The slight enhancement of contractions by anti-adrenaline drugs may be attributed to their weak anticholinesterase action (Boyd, Chang & Rand, 1960), and the depression by large doses of guanethidine to its weak ganglion blocking action (Rand & Wilson, 1964).

The only unusual response from the preparation was the occasional inability of ganglion blocking drugs to block completely the contractions evoked by nerve stimulation. The resistant part of the contraction could

not be attributed to excitation of striated muscle fibres, since the time course of the responses, together with the fact that small concentrations of atropine always completely abolished them, demonstrated the absence of any contribution by such fibres. It is possible that in a few preparations, some fibres synapsed more centrally than the stimulating electrodes and that the stimulated nerve trunk therefore contained some post-ganglionic fibres. However, another possibility may also be considered. Martin & Pilar (1963a, b) have recently obtained evidence that at many of the synapses in the ciliary ganglion of the chick, transmission is mediated by electrical coupling between pre- and post-synaptic elements. Such ephaptic transmission is not susceptible to ganglion blocking agents and it may be that a similar transmission mechanism is occasionally present in some of the synapses of other parasympathetic ganglia in this species. Apart from this minor exception, the responses of the preparation to drugs were qualitatively similar to those expected from a parasympathetically-innervated mammalian smooth muscle preparation.

Several isolated sympathetically-innervated preparations are available for pharmacological investigations, for example, those of Finkleman 1930) and Hukovic (1961), but few tissues are easily isolated with their parasympathetic nerves. Because of its simplicity, robustness and cheapness, the isolated parasympathetically-innervated oesophagus of the chick provides a useful preparation, particularly for students' practical classes.

Acknowledgement. This work was supported by a grant from the British Egg Marketing Board.

References

Bain, W. A. & McSwiney, B. A. (1936). *J. Physiol. Lond.*, **86**, 17P.
Boyd, H., Chang, V. & Rand, M. J. (1960). *Brit. J. Pharmacol.*, **15**, 525–531.
Burn, J. H. & Rand, M. J. (1960). *J. Physiol. Lond.*, **150**, 295–305.
Finkleman, B. (1930). *Ibid.*, **70**, 145–157.
Hughes, F. B. & McDowall, R. J. S. (1954). *Ibid.*, **123**, 1P.
Hukovic, S. (1961). *Brit. J. Pharmacol.*, **16**, 188–194.
Marrazzi, A. S. (1939). *J. Pharmacol.*, **65**, 395–404.
Martin, A. R. & Pilar, G. (1963a). *J. Physiol. Lond.*, **168**, 443–463.
Martin, A. R. & Pilar, G. (1963b). *Ibid.*, **168**, 464–475.
Rabinovitch, M. (1928). *Ibid.*, **65**, XXXV.
Rand, M. J. & Stafford, A. (1964). *Brit. J. Pharmacol.* Demonstration to the January meeting of the British Pharmacological Society.
Rand, M. J. & Wilson, J. (1964). Communication to the January meeting of the British Pharmacological Society.

The paper was presented by MISS EVERETT.

J. Pharm. Pharmacol., 1964, **16**, *Suppl.*, 80T–82T Received April 27, 1964

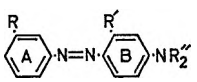
SHORT COMMUNICATION

Metabolism of some dimethylaminoazobenzene derivatives

P. J. ROBINSON, A. J. RYAN AND S. E. WRIGHT

RECENT knowledge about the carcinogenic action of 4-dimethylamino-azobenzenes on the liver of the rat has been summarised by Arcos & Arcos (1962), but the metabolism of these compounds has received little attention. Stevenson, Dobriner & Rhoads (1942) showed that the rat metabolised 4-dimethylaminoazobenzene (DAB) to *p*-aminophenol and *p*-phenylenediamine, which were then excreted as conjugates. The sequence in which the oxidation of the phenyl ring, reduction of the azo link, and demethylation of the tertiary nitrogen occurs, still remains uncertain.

No quantitative work has been done on the excretion of the dye metabolites although Berenbom & White (1951) examined the distribution of isotopic nitrogen in the tissues and excreta of rats dosed with each of the three possible ^{15}N-labelled dyes: no estimation of individual metabolites was included. We have measured from urine the amount of each aromatic ring arising from the metabolic fission of four fat soluble azo dyes, DAB (I; R=R′=H; R″=Me), 3′-methyl-DAB (I; R=R″=Me; R′=H), 2-methyl-DAB (I; R=H; R′=R″=Me) and 4-aminoazobenzene (I; R=R′=R″=H). We have also examined the excretion of *p*-phenylenediamine.



I

Of these compounds, the first two are carcinogenic to rat liver (Miller, Miller, Kline & Rusch, 1948) and the other two are non-carcinogenic (Miller, Sapp & Miller, 1949).

The first three dyes were each labelled with tritium in one ring and carbon-14 in the other; 4-aminoazobenzene was labelled with carbon-14 in both rings. Aniline and *m*-toluidine were tritiated by the Wilzbach (1957) procedure. Tritiated *NN*-dimethyl-*m*-toluidine was prepared by methylation of tritiated *m*-toluidine with trimethyl phosphate. A similar procedure gave dimethylaniline-^{14}C. The distribution of tritium in the tritiated molecules was determined in order to correct for loss due to metabolic reactions (Table 1).

The labelled dye (10 mg) and *m*-toluidine (5 mg) dissolved in arachis oil and *p*-phenylenediamine (5 mg) dissolved in water were given to rats by intraperitoneal injection. Urine was collected over 24, 48 and 72 hr. Known amounts of acetates of the expected metabolites (see Table 2)

From the Department of Pharmacy, University of Sydney, Sydney Australia.

TABLE 1. DISTRIBUTION OF ACTIVITY IN TRITIATED AMINES

Compound	% activity in ring positions			
	*o**	*m**	*p*	methyl
Aniline	8·8	26·4	29·8	—
m-Toluidine ..	—	—	25·9	0·7

* activity found for 1 position

were added to each sample and the urine was hydrolysed with acid. The hydrolysed urine was extracted with ether and the metabolites separated on a silica gel column; suitable derivatives were purified to constant specific activity. Counting was in a Packard Tricarb liquid scintillation counter, model 314.

The results in Table 2 show a distinct difference in the metabolism of each aromatic compound derived from fission of the azo link. With DAB it can be seen that there is a clear difference in the amount of *p*-phenylenediamine excreted compared to *p*-aminophenol; a similar though less

TABLE 2. EXCRETION OF DYE METABOLITES AS CUMULATIVE % OF DOSE ADMINISTERED (INDIVIDUAL RESULTS)

Dye and metabolite	Time, hr		
	24	48	72
DAB			
p-Aminophenol	35, 64, 73	71, —, 71	67, 67, 73
p-Phenylenediamine	5, 9, 13	7, 7, 10	10, 10, 11
3′-Methyl-DAB			
4-Amino-2-methylphenol	25, —, 32	23, 28, —	23, —, 32
p-Phenylenediamine	18, 11, 18	18, 19, 27	18, 19, 20
2-Methyl-DAB			
p-Aminophenol	37, 47, 47	36, 71, 75	80, 80, 83
2,5-Diaminotoluene	13, 15, 15	30, 37, 43	70, 86, —
4-Aminoazobenzene			
p-Aminophenol	76, —, 103	91, 89, 106	81, —, —
p-Phenylenediamine	28, 17, 15	18, 17, 19	20, 19, 19
p-Phenylenediamine	40, —, 58	39, 50, 59	51, 59, 61

marked difference occurs with *p*-phenylenediamine and the accompanying 4-amino-2-methylphenol derived from 3′-methyl-DAB. A second interesting point is that in both dyes the excretion of the metabolites assayed reached a maximum at about 24 hr; thereafter no significant amounts were excreted.

For the non-carcinogenic dye 2-methyl-DAB there is a significant increase in the amount of metabolite from the aromatic ring to which the basic group is attached in the original dye. However, the excretion of *p*-phenylenediamine from 4-aminoazobenzene is only about the same as that from 3′-methyl-DAB, though greater than that from DAB itself. The amount of *p*-aminophenol arising from the ring A is approximately equal to the amount excreted from the carcinogenic dyes.

p-Phenylenediamine separately administered is excreted to a much greater extent than when derived from the dyes. With the exception of 3′-methyl-DAB, the excretion of ring A from the dyes does not differ

significantly from the amounts excreted when aniline is administered (Parke, 1960). With 3'-methyl-DAB it may be that this ring is metabolised by a route other than hydroxylation.

A possible explanation for the results obtained is that the dyes are bound in the tissues, possibly by a reaction between the amino-group and cell constituents. The bound dye is then reduced and ring A rapidly metabolised and excreted. The bound ring is retained and excreted much more slowly. It may be that the bound ring is important in the carcinogenic process. We are examining this problem further.

Acknowledgements. This work forms part of a research programme supported by the National Health and Medical Research Council of Australia, the Council of the Australian Food Technologists Association and Grant EF-258 of the National Institutes of Health, U.S. Public Health Service.

References

Arcos, J. C. and Arcos, M. (1962). *Progress in Drug Research*, **4**, 409.
Berenbom, M. and White, J. (1951). *J. Nat. Cancer Inst.*, **12**, 583–590.
Miller, E. C., Miller, J. A., Kline, B. E. & Rusch, H. P. (1948). *J. Exp. Med.*, **88**, 89–98.
Miller, J. A., Sapp, R. W. & Miller, E. C. (1949). *Cancer Res.*, **9**, 652–660.
Parke, D. V. (1960). *Biochem. J.*, **77**, 493.
Stevenson, E. S., Dobriner, K. & Rhoads, C. P. (1942). *Cancer Res.*, **2**, 160–167.
Wilzbach, K. E. (1957). *J. Amer. chem. Soc.*, **79**, 1013.

The paper was presented by PROFESSOR WRIGHT.

J. Pharm. Pharmacol., 1964, **16**, *Suppl.*, 83*T*–84*T* Received April 30, 1964

SHORT COMMUNICATION

Antihistamine protection against histamine-induced gastric ulceration in the guinea-pig

J. WATT AND G. B. EAGLETON

IT is generally believed that antihistamines have no protective action against histamine-induced gastric ulceration (Ivy, Grossman & Bachrach 1952). Evidence has been based on animal experiments in which acute gastric ulcers were produced over periods ranging from 24 hr to 40 days and in which repeated large doses of histamine, usually in a beeswax and oil base, were used (Halpern & Martin, 1946; Friesen, Baronofsky & Wangensteen, 1946; Crane, Lindsay & Dailey, 1947; Winter & Mushett, 1948).

We have studied in the adult guinea-pig the protective effect of mepyramine maleate on gastric ulceration produced in less than 6 hr by a much smaller single dose of histamine in aqueous solution.

METHODS

Male albino guinea-pigs of 700 g average body weight were used. In most of the experiments, fasted animals were used. They were deprived of food for about 10 hr before the start of each experiment and wore loosely fitting perspex collars to prevent coprophagy. Non-fasted animals wore no collars. All animals received water *ad lib.*

Gastric ulceration was induced by a single injection of histamine acid phosphate (HAP) in aqueous solution (1 mg/ml) given either intramuscularly or intraperitoneally according to the method previously described (Eagleton & Watt, 1964). The dose by intramuscular injection was 1 mg/kg and by the intraperitoneal route either 1 mg or 5 mg/kg. Animals protected with mepyramine maleate were given 10 mg/kg intramuscularly 1 hr before and again 2 hr after the injection of histamine.

All animals were killed by a sharp blow on the head 6 hr after the administration of the histamine. The occurrence of ulceration was assessed after fixation of the stomach in formol-saline.

RESULTS

The incidence of histamine-induced ulceration and the protection afforded by mepyramine maleate are shown in Table 1. Protection was greatest (100%) in the animals which received 1 mg HAP/kg intraperitoneally. In the fasted and non-fasted animals which received 5 mg HAP/kg intraperitoneally, the antihistamine drug reduced the incidence of ulceration (and greatly lessened the severity of lesions). In addition, the protected animals showed no signs of abdominal discomfort as observed in control groups which received only histamine intraperitoneally.

From the Department of Pathology, University of Liverpool

TABLE 1. THE INCIDENCE OF HISTAMINE-INDUCED GASTRIC ULCERATION IN MALE ADULT GUINEA-PIGS WITH AND WITHOUT ANTIHISTAMINE PROTECTION (2 × 10 mg/kg i.m.)

Histamine acid phosphate mg/kg	Without mepyramine		With mepyramine	
	No. of animals with gastric ulcers	% with gastric ulcers	No. of animals with gastric ulcers	% with gastric ulcers
1 i.m.	8/11	73	1/6	16
1 i.p.	7/10	70	0/9	0
5 i.p.	15/15*	100	2/5*	40
	10/10†	100	1/6†	16

* Fasted animals. † Non-fasted animals.

DISCUSSION

Our results do not support the current belief that antihistamines are ineffective in preventing histamine-induced gastric ulceration. In contrast with other workers, we used smaller amounts of histamine which were nonetheless effective in producing ulcers. This permitted the effective antagonistic ratio of antihistamine drug to be increased and by restricting the period of ulcerogenesis to not more than 6 hr, this ensured a high concentration of antihistamine throughout the experiment. Provided adequate doses of antihistamine are given it seems possible that protection can be afforded against histamine ulceration produced over longer periods than were used in our experiments. Thus, in work that appears to have been overlooked, Van Meter & Oleson (1949) observed that the antihistamine chlorothen citrate (Tagathen) caused a marked reduction in the incidence and severity of gastric ulcers induced by histamine over a period of several days.

References

Crane, J. T., Lindsay, S. & Dailey, M. E. (1947) *Amer. J. Digest. Dis.*, **14**, 56–57.
Eagleton, G. B. & Watt, J. (1964). *Gastroenterology*. In the press.
Friesen, S. R., Baronofsky, G. D. & Wangensteen, O. H. (1946). *Proc. Soc. exp. Biol. N.Y.*, **63**, 23–25.
Halpern, B. N. & Martin, J. (1946). *C.R. Soc. Biol.*, **140**, 830.
Ivy, A. C., Grossman, M. I. & Bachrach, W. H. (1952). *Peptic Ulcer*, 1st ed., p. 217, London: Churchill.
Van Meter, J. C. & Oleson, J. J. (1949). *Proc. Soc. exp. Biol. N.Y.*, **71**, 163.
Winter, C. A. & Mushett, C. W. (1948). *Federation Proc.*, **7**, 136.

The paper was presented by DR. WATT.

J. Pharm. Pharmacol., 1964, **16**, *Suppl.*, 85*T*–88*T* Received April 27, 1964

A simple method for the evaluation of local anaesthetic activity using earthworms

B. P. BLOCK,* D. J. POTTS AND R. S. H. FINNEY

Earthworms are immersed in local anaesthetic solution and tested for anaesthesia at 1 min intervals by dipping their tails into weak acid. The time of onset of anaesthesia is used as a measure of anaesthetic activity. The method is simple, inexpensive and has an end-point which is unmistakable.

SEVERAL tests have been described for assessing topical anaesthesia using frogs (Munch, Pratt & de Ponce, 1933), guinea-pig cornea (Chance & Lobstein, 1944, and others), the guinea-pig sneeze reflex (Nieshultz, Hoffman & Popendiker, 1958), rabbit cornea (McIntyre & Sievers, 1937, and others) and the human larynx (Clarke, Orkin & Rovenstine, 1954).

We now describe a method for estimating topical anaesthesia using earthworms which was designed primarily for screening purposes but is equally suitable for comparing local anaesthetic potencies.

Experimental

MATERIAL

Earthworms from several genera were collected but those from the genus *Lumbricus* were used in all of the later experiments. They can be identified by the following features (Cernosvitov and Evans, 1947): (*a*) They are tanylobic; (*b*) they are thicker than other worms of similar length; (*c*) they are less opaque than worms of other genera; (*d*) they are reddish rather than brown in colour; (*e*) they have a spatulate tail.

The worms varied in weight from 0·5 to 5·0 g. They were kept in groups of about 50 at room temperature in 6½ inch flower pots filled with garden soil containing 25–30% moisture. Powdered dried cow manure was sprinkled daily on the surface of the soil to provide extra organic matter (Guild, 1957).

METHOD

This is based on a sharp withdrawal response produced when the tail is dipped into 0·0125N hydrochloric acid to a depth of about 0·25 in. The response can be abolished by total immersion in local anaesthetic solution.

Before testing, the worms are maintained in aerated worm "Ringer,"† which is also used for preparing the local anaesthetic solutions.

From the Department of Biology, College of Technology, The Newarke, Leicester.

* Present address: British Industries Biological Research Association, Carshalton, Surrey.

† NaCl, 3·67; dextrose, 1·33; $NaHCO_3$, 0·20; NaH_2PO_4, 0·67 g; KCl, 15%, 0·62 ml; $CaCl_2$, 16%, 0·50 ml; distilled water to 1 litre. Final pH 6·6. (Modified after B. I. Roots, personal communication to J. A. Edson.)

Worms, in groups of five, were first tested for a positive response to acid, washed with water and dried with absorbent paper. Five worms were now immersed in anaesthetic solution and after 1 min were removed in turn for the acid test. No individual identification of the animals was made. Thereafter, the group was tested at minute intervals. The time of response was noted and the means calculated. Separate groups were used for each concentration of anaesthetic.

FACTORS INFLUENCING THE METHOD

Concentrations of acid higher than 0·0125N caused damage to the tail, lower concentrations gave an unreliable response.

Differences in weight affected the mean time of onset of anaesthesia, large worms taking a longer time to show inhibition of the response. Consequently the animals were distributed evenly by weight amongst the groups.

Worms stored in small volumes of aerated Ringer solution showed a decreased sensitivity to the acid with length of storage time. This effect was not seen when they were stored in 250 ml of Ringer in which they retained their responsiveness for over 8 hr.

Worms from the genus *Lumbricus* showed (1), a characteristically sharper response to acid; (2), greater sensitivity to acid; (3), no acid damage which had been seen in other worms. They were, however, less sensitive to the anaesthetics than other genera.

Worms used two or three times each day showed a less sharp response to acid on the second and third occasions, thus affecting the end-point. If less than 2 hr were allowed for recovery, damage occurred and the results were unreliable. Although groups tested on consecutive days gave reproducible results, deterioration occurred if the worms were used more often than once in about 4 days.

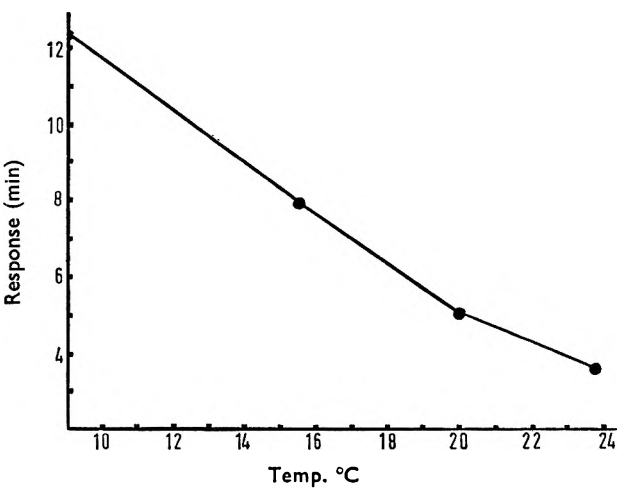


FIG. 1. The effect of temperature on the time of onset of anaesthesia.

Groups of 10 worms were tested at different temperatures with the same concentration of anaesthetic. An increase in temperature reduced the time of onset of anaesthesia at a rate of 0·6 min/°C (Fig. 1). Variation in temperature should therefore be avoided.

Results

A linear relationship between log concentration of cinchocaine hydrochloride and response was obtained (Fig. 2). This was an early experiment using worms of mixed genera. The slope of the curve ($b = -15{\cdot}5$) is significantly different from zero ($s/b = 2{\cdot}26$). The straight line portion of the graph lies between responses of about 4 and 16 min.

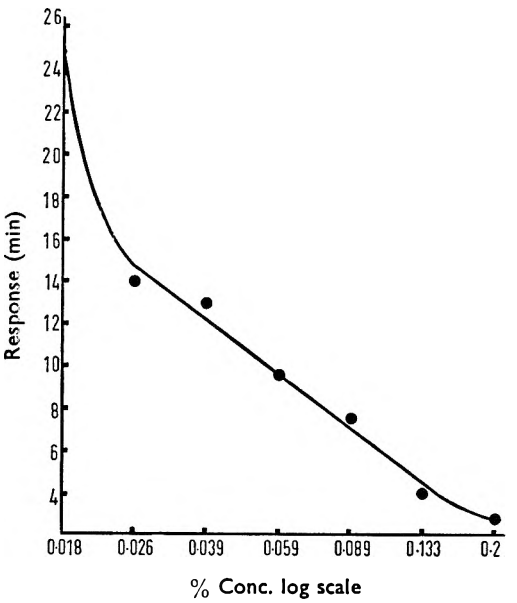


FIG. 2. Dose response relationships for cinchocaine hydrochloride. Each point is the mean of the responses of 10 worms from several genera.

Log concentration/response curves for cinchocaine, lignocaine and cocaine using *Lumbricus* are given in Fig. 3. The limits of linearity for cocaine and lignocaine are approximately 5 and 15 min.

The relative potencies were expressed as the antilog of the difference between the log concentrations producing the mean response (10 min) for each drug. Lignocaine was 7% and cocaine 13% as potent as cinchocaine. These figures agree with those recorded by Adriani (1956).

Discussion

The method of evaluating local anaesthetic activity using *Lumbricus* is simple, requires little skill and uses materials which are cheap and easily available. It eliminates the difficulty, inherent in the cornea

method, of obtaining reproducible stimulations. The end-point is unmistakable.

The sensitivity of the method is comparable with that of other methods and the ratio of potencies of the three drugs tested agreed well with those recorded in the literature.

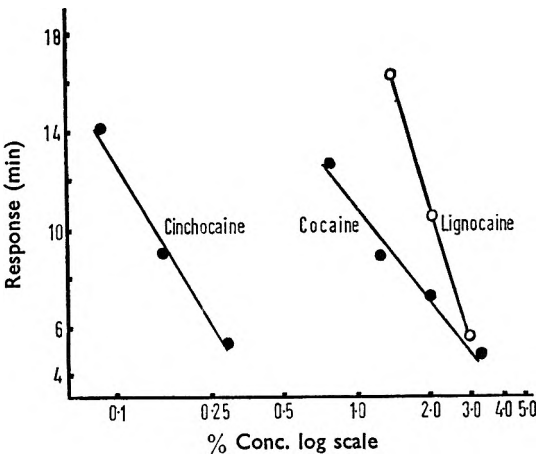


FIG. 3. Dose response curves for cinchocaine, lignocaine and cocaine hydrochlorides. Worms of the genus *Lumbricus* only were used.

Although the method measures the time of onset of anaesthesia, the duration of anaesthesia can be determined easily by removing the animals from the anaesthetic solution and replacing them in aerated Ringer.

The slopes of the dose response curves are steep. Although the use of worms of varying weights within groups is not conducive to low standard errors, the reproducibility of group means is good (± 1 min for those results in which anaesthesia occurred after 10 min immersion in anaesthetic solution).

The method is quick and suitable for rapid screening as well as for comparison work.

References

Cernosvitov, L. & Evans, A. C. (1947). *Synopsis of the British Fauna* No. 6 Lumbricidae. Linnean Society of London.

Chance, M. R. & Lobstein, H. (1944). *J. Pharmacol.*, **82**, 203–210.

Clarke, R. E., Orkin, L. R. & Rovenstine, F. A. (1954). *Anaesthesiology*, **15**, 161–173.

Guild, W. J. M. (1957). The U.F.A.W. Handbook on *The Care and Management of Laboratory Animals*, 2nd ed., pp. 875–888, London: U.F.A.W.

McIntyre, A. R. & Sievers, R. F. (1937). *J. Pharmacol.*, **61**, 107–120.

Munch, J. C., Pratt, M. J. & de Ponce, A. M. (1933). *J. Amer. pharm. Ass.*, **22**, 1078–1080.

Nieshultz, O., Hoffman, I. & Popendiker, K. (1958). *Arzneimitt. Forsch.*, **8**, 539–544.

The paper was presented by MR. POTTS.

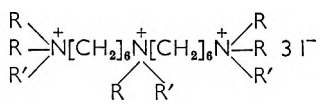
J. Pharm. Pharmacol., 1964, **16**, *Suppl.*, 89T–103T Received April 30, 1964

Neuromuscular blocking agents: alkyl and heterocyclic analogues of simple linear trisonium compounds

FIONA MACLEOD CAREY,* C. I. FURST,† J. J. LEWIS* AND J. B. STENLAKE†

A further series of linear hexamethylene-*NNN*-trisonium compounds has been synthesised and tested for neuromuscular blocking activity. In the alkyl-substituted derivatives, at least one Et is required for significant potency in mammals. Apart from the Et_2Bu^n and $EtBu^n_2$ compounds, larger alkyl substituents lower potency. High potency in mammals requires at least two Et groups on the terminal nitrogens, if the third group is not Me. Only weak activity was shown by the morpholino-deriatives but the fowl was highly sensitive towards the piperidino- and tetrahydropapaverino-compounds. The results are discussed.

THE effect of varying alkyl substitution upon the neuromuscular blocking activity in the linear hexamethylene-*NNN*-trisonium compounds (I) has been described earlier (Edwards, Lewis, Stenlake & Zoha, 1958; Carey, Edwards, Lewis & Stenlake, 1959; Edwards, Lewis, McPhail, Muir and Stenlake, 1960; Edwards, Stenlake, Lewis & Stothers, 1961). Further compounds of this type have now been synthesised with the object

(I) 

of completing the series and investigating the effect on the neuromuscular blocking potency of this basic structure produced by progressively substituting methyl, ethyl, n-propyl and n-butyl groups at R and R′.

It is well established that bis-onium compounds, in which the onium ion forms part of a heterocyclic system, are tubocurarine-like rather than decamethonium-like (Stenlake, 1963). Tris-onium compounds of structure (I) in which the terminal onium groups incorporate piperidinium, morpholinium and tetrahydropapaverinium substituents have now been prepared, and their potencies recorded.

Chemical

Two routes to the intermediate bis-6-dialkylaminohexylalkylamines (II) have been described earlier (Edwards, Lewis, Stenlake & Zoha, 1958;

$R_2N{\cdot}[CH_2]_6{\cdot}N(R'){\cdot}[CH_2]_6{\cdot}NR_2$ (II) $R_2N{\cdot}[CH_2]_6{\cdot}Br$ (III) $R_2N{\cdot}[CH_2]_6{\cdot}NHR'$ (IV)

$R_2N{\cdot}CO{\cdot}[CH_2]_2COCl$ (V) $R_2N{\cdot}CO[CH_2]_4{\cdot}CO{\cdot}N(R'){\cdot}[CH_2]_6{\cdot}NR_2$ (VI)

Carey, Edwards, Lewis & Stenlake, 1959). The method described

* From the Experimental Pharmacology Division, Institute of Physiology, University of Glasgow.
† From the Department of Pharmacy, University of Strathclyde.

in the latter publication was adopted in the present work, though an alternative route to the diamines (IV) through the corresponding *NNN'*-trialkyladipamides (VII) has been examined.

$R_2N{\cdot}CO[CH_2]_4CONHR'$ (VII) $R_2N{\cdot}CO{\cdot}[CH_2]_4{\cdot}COOH$ (VIII)

The adipamic acids (VIII) (R = Me, Bu^n, piperidyl, morpholinyl, or tetrahydropapaverinyl) were prepared from ethyl hydrogen adipate by methods similar to those previously described (Carey & others, 1959). The adipamic acids are all freely soluble in cold water and can be isolated only by keeping the volume of hydrolysate to a minimum and extracting continuously with an organic solvent. Traces of contaminating adipic acid can be removed by making use of its insolubility in benzene.

In contrast to *NN*-diethyl- and *NN*-dipropyladipamic acids, both the *NN*-dimethyl and *NN*-dibutyl compounds partly disproportionated to adipic acid and the corresponding bis-amide on vacuum distillation. This occurred when an air-leak was used to promote even boiling but with an orange stick in place of an air-leak, distillation was accomplished without disproportionation. Similar observations on the disproportionation of *NN*-diethyldiglycollamic acid have been reported by Edwards, Lewis, McPhail, Muir & Stenlake (1960) and on *N*-substituted adipamic and succinamic acids by Prelog (1930).

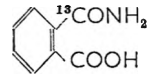
When *NN*-dibutyladipamic acid was heated (1 hr) at 250° either under nitrogen or in presence of air, both conditions led to the same amount of disproportionation. Reaction temperature also appears to be unimportant, since the distillation temperature of *NN*-dimethyladipamic acid (178°/0·05 mm) and *NN*-dibutyladipamic acid (198°/0·03 mm) do not differ markedly from those of *NN*-diethyladipamic acid (182°/0·05 mm) and *NN*-dipropyladipamic acid (198°/0·5 mm) which do not disproportionate.

NN-Dibutyladipamic acid was not formed when an equimolecular mixture of adipic acid and *NNN'N'*-tetrabutyladipamide was heated at 150° for $1\frac{1}{2}$ hr either under nitrogen, or with air being drawn through the mixture. The reaction therefore is not reversible. The mechanism is probably one of intramolecular catalysis, several analogous cases being well-established.

Leach & Lindley (1953) observed the hydrolysis of the terminal amide links in glycyl-L-asparagine (IX, R = H) and L-leucyl-L-asparagine (IX, $R = (CH_3)_2CH{\cdot}CH_2$) in aqueous solution between pH 1·2 and 3·5. Both reactions were of first order in $\overset{+}{N}H_3{\cdot}CHR{\cdot}CONH{\cdot}CH(CH_2{\cdot}CONH_2){\cdot}COOH$ and were independent of the external hydrogen ion concentration in this pH range, indicating that the undissociated carboxyl group is the reacting species. Together with the small negative entropies of activation this is consistent with an intramolecular reaction involving proton transfer from the unionised carboxyl to the terminal amide link. A six-membered hydrogen-bonded ring structure was postulated for the peptides in solution. Phthalamic acid (X) is likewise hydrolysed 10^5 times faster than benzamide

at pH 3 and the rate of reaction is independent of pH between pH 1·3 and

$NH_2{\cdot}CHR{\cdot}CONH{\cdot}CH(CH_2{\cdot}CONH_2){\cdot}COOH$ (IX)



(X)

2·6 (Bender 1957). Hydrolysis of a ^{13}C-labelled phthalamic acid (as X) with $H_2{}^{18}O$ (Bender, Chow & Chloupek, 1958) yields phthalic acid which, on decarboxylation, forms both $^{13}C^{16}O^{18}O$ and $^{12}C^{16}O^{16}O$. The carboxyl carbonyl group thus behaves as a bifunctional catalyst, simultaneously attacking the carbonyl atom of the amide and donating a proton to the departing ammonia molecule with the formation of the anhydride. A similar mechanism also appears to operate in the hydrolysis of *NN*-dimethylaminomaleamic acid (Dahlgren & Simmerman, 1963).

The infra-red spectra of all four adipamic acids in carbon tetrachloride show evidence of intramolecular hydrogen bonding (submaxima at 2,650 cm^{-1}) which would appear to favour anhydride formation by a mechanism similar to that postulated by Bender (1957) for the phthalamic acids. No satisfactory explanation of the stability of *NN*-diethyl and *NN*-dipropyl adipamic acids relative to that of the *NN*-dimethyl and *NN*-dibutyladipamic acids can be advanced on the evidence available at present.

The adipamic acids (VIII) were converted to the corresponding diamides in the usual way by reacting the acid chloride with the appropriate base. Reduction of the diamides with lithium aluminium hydride in ether or ether-tetrahydrofuran gave the required diamines (IV). 6-Dimethylaminohexylmethylamine, 6-di-n-butylaminohexyl-n-butylamine, 6-(1′-piperidino) hexylethylamine, 6-(1′-morpholino)hexylethylamine and 6-(2′ tetrahydropapaverinyl)hexylethylamine, obtained in this way, were each condensed with the corresponding adipamic acid chloride (V) and the product reduced with lithium aluminium hydride to yield bis(6-dimethylaminohexyl)methylamine, bis(6-di-n-butylaminohexyl)n-butylamine, bis-(6-morpholinohexyl)ethylamine and bis(6,2′-tetrahydropapaverinylhexyl)-ethylamine respectively. The quaternary ammonium salts (Ia–Im) shown in Table 1 were obtained in the usual way. Trichloroacetic acid which has been reported (Vilsmeier, 1958) to give highly crystalline double salts of the type $R_4N^+Cl_3C{\cdot}CO{\cdot}O^-Cl_3C{\cdot}COOH$ from hygroscopic mono- and bis-quaternary halides gave only oily products with the hygroscopic piperidinium and morpholinium halides examined.

Experimental

Ethyl NN-*dimethyladipamate.* Ethyl hydrogen adipate (40 g) was refluxed (1½ hr) at 90–100° with excess thionyl chloride (50 ml). Excess thionyl chloride was removed in the usual way. The crude acid chloride in dry ether (50 ml) was slowly added to a stirred solution of anhydrous dimethylamine (35 g) in dry ether (300 ml) at 0°. The reaction mixture was then refluxed for one hr. The precipitated dimethylamine hydrochloride was filtered off, washed with more ether and the filtrate and

washings were extracted with water, then with aqueous sodium carbonate and finally dried (Na_2SO_4). Removal of the ether and distillation gave the required product as a pale-yellow oil (36·5 g, 79%) b.p. 128°/1·5 mm, n_D^{24} 1·4560. (Andrews, Bergel & Morrison, 1953, found b.p. 102–106°/0·25 mm, n_D^{20} 1·4573). Found: N, 7·0. Calc. for $C_{10}H_{19}NO_3$: N, 7·0%.

The following amide esters were similarly prepared from ethyl hydrogen adipate, except that the acid chloride was added to the reaction vessel at room temperature and, during the extraction procedure, the ethereal solution was first washed with dilute hydrochloric acid to remove excess of the secondary amine.

Ethyl NN-*di-n-butyladipamate*, yellow oil (52 g, 93%), b.p. 146°/0·1 mm, n_D^{16} 1·4580. Johnson (1958), quotes b.p. 136–138°/0·15 mm, n_D^{16} 1·4569. Found: N, 4·9. Calc. for $C_{16}H_{31}NO_3$: N, 4·9%.

Ethyl N-*adipoylpiperidine*, yellow oil (60·5 g, 78%), b.p. 166–167°/0·2 mm. Avison (1951) gives b.p. 148–152°/0·5 mm. Found: N, 6·0. Calc. for $C_{13}H_{23}NO_3$: N, 5·8%.

Ethyl N-*adipoylmorpholine*, pale yellow oil (52 g, 71%), b.p. 177–183°/0·1 mm. Found: N, 5·8. $C_{12}H_{21}NO_4$ requires N, 5·8%.

6-*Hydroxyhexyldimethylamine*. Ethyl *NN*-dimethyladipamate (21 g) in dry ether (50 ml) was added slowly to a stirred, refluxing suspension of lithium aluminium hydride (5 g) in dry ether (120 ml) and the solution was refluxed for 5 hr. The reaction mixture was cooled in an ice-bath and brine added cautiously to decompose the complex and excess lithium aluminium hydride. Sodium hydroxide (20% solution, 100 ml) was added with stirring to produce a gel from which the ethereal supernatant was decanted. The gel was extracted with more ether (2 × 200 ml) and the combined ether extracts dried (Na_2SO_4). Evaporation of the solvent yielded a mobile liquid which was distilled to give the product as a colourless oil (10 g, 66%), b.p. 70°/5 mm, n_D^{20} 1·4485. Andrews & others, 1953, give b.p. 114–116°/12 mm, $n_D^{20\cdot5}$ 1·4482. Found: N, 9·5; titration equivalent 148·3. Calc. for $C_8H_{19}NO$: 9·7%; equiv. 145·2.

The *hydrobromide*, recrystallised from ethanol-ether in white leaflets, m.p. 85–88°. Found: N, 6·0; Br, 35·4. $C_8H_{20}BrNO$ requires N, 6·2; Br, 35·8%.

The *methiodide*. Methyl iodide (1 ml) was slowly added to the base (0·6 g) in ether (4 ml). Recrystallisation of the dense white precipitate (ethanol-ether) gave a quantitative yield of white leaflets, m.p. 126°. Found: N, 4·9; I, 44·4. $C_9H_{22}INO$ requires N, 4·9; I, 44·25%.

NN-*Dimethyladipamic acid*. Ethyl *NN*-dimethyladipamate (12·5 g) was refluxed (1 hr) with ethanolic potassium hydroxide (approx. 2/3 N; 135 ml), the solution cooled and just neutralised with dilute hydrochloric acid. Ethanol was removed under reduced pressure and benzene (2 × 50 ml) added and similarly evaporated to remove the last traces of ethanol. The residual potassium salt was acidified with hydrochloric acid (20 ml, 25%) and the precipitated potassium chloride filtered off. Continuous extraction of the filtrate with ether gave the required *acid* as a viscous grey oil which was not distilled (8·2 g, 76%). Found: N, 7·9; titration equivalent 175·6. $C_8H_{15}NO_3$ requires N, 8·1; equiv. 173·2. Subsequent

distillation of the acid gave a yellow oil (b.p. 178°/0·05 mm, equiv. 172·2) which crystallised as rosettes of thick white needles after several months.

NNN'-*Trimethyladipamide.* Thionyl chloride (8 ml) in benzene (30 ml) was added to *NN*-dimethyladipamic acid (10 g) suspended in benzene (70 ml) and the excess reagent and the solvent were removed almost immediately below 50° under reduced pressure: benzene (20 ml) was added and removed in the same way. The sparingly soluble crude *NN*-dimethyladipamoyl chloride suspended in benzene (50 ml) was stirred at 0° and dry methylamine passed in for 2 hr until the uptake was complete. The precipitated methylamine hydrochloride was filtered off, washed with benzene and the combined benzene extracts evaporated to leave a yellow oil (10·5 g, 98%) which crystallised rapidly. The *product* crystallised from dry acetone-ether in fine colourless needles, m.p. 54–56° which were collected under nitrogen. Alternatively the oil distilled, b.p. 218–220°/0·2 mm. Found: N, 14·8. $C_9H_{18}N_2O_2$ requires N, 15·0%. The diamide was extremely hygroscopic and almost insoluble in ether.

6-*Dimethylaminohexylmethylamine.* *NNN'*-trimethyladipamide (6·4 g) in dry tetrahydrofuran (30 ml) was added over 25 min to a stirred refluxing suspension of excess lithium aluminium hydride (5 g) in tetrahydrofuran (60 ml). Refluxing was continued for 5 hr and the excess reagent was decomposed with water. The supernatant tetrahydrofuran was decanted and the gel extracted with several further volumes of tetrahydrofuran (250 ml). The combined extracts were dried (Na_2SO_4), the solvent removed and the product distilled to give 6-*dimethylaminohexylmethylamine*, as a colourless, mobile oil (3·0 g, 55%), b.p. 78°/0·2 mm. Found: N, 17·4; titration equivalent 79·2. $C_9H_{22}N_2$ requires N, 17·7%; titration equivalent 79·1.

Bis(6-*dimethylaminohexyl*)*methylamine.* *NN*-Dimethyladipamic acid (2·9 g) was treated with thionyl chloride (1·8 ml) as previously described, and a suspension of the acid chloride in benzene (18 ml) was added slowly to a stirred, refluxing solution of 6-dimethylaminohexylmethylamine (5·5 g) in benzene (35 ml). After refluxing for a further 30 min, the reaction mixture was extracted with dilute hydrochloric acid (2 × 25 ml), the acid solution basified with sodium hydroxide (35 ml, 20%), and extracted with benzene (4 × 50 ml). The benzene solution was dried (Na_2SO_4) and the brown mobile oil (6·8 g) which remained after evaporation of the solvent gave, on distillation, some 6-dimethylaminohexylmethylamine (*ca.* 0·75 g), b.p. 58–60°/0·1 mm. The crude undistilled *N*-dimethylaminohexyl-*NN'N'*-trimethyladipamide in ether was reduced with lithium aluminium hydride (1·5 g) and the product extracted as described for 6-hydroxyhexyldimethylamine. Fractional distillation gave a fore-run of the diamine (1·8 g), and *bis*(6-*dimethylaminohexyl*)*methylamine*, as a pale-yellow oil (2·3 g, 48%), b.p. 130°/0·07 mm, n_D^{20} 1·4533. Found: N, 15·0; titration equivalent 95·3. $C_{17}H_{39}N_3$ requires N, 14·7%; titration equivalent 95·2.

NN-*Di-n-butyladipamic acid.* The acid was prepared by saponification of ethyl *NN*-di-n-butyladipamate (50 g), as described for the preparation of *NN*-dimethyladipamic acid. Continuous extraction of the aqueous

acid solution with ether, followed by distillation gave the required *product*, as a very viscous, yellow oil (39·2 g., 85%) b.p. 198°/0·03 mm, $n_D^{16.5}$ 1·4720. Found: N, 5·2; titration equivalent 257·8. $C_{14}H_{27}NO_3$ requires N, 5·4%; titration equivalent 257·4.

NNN'-*Tri-n-butyladipamide.* Crude *NN*-di-n-butyladipamoyl chloride was prepared by refluxing the acid (28 g) and thionyl chloride (16·5 ml) in benzene (25 ml) at 90–95° for 7 min: it was isolated as described for *NN*-dimethyladipamoyl chloride. The acid chloride in benzene (75 ml) was then added to an ice-cold solution of n-butylamine (25 ml) in benzene (100 ml) and the solution refluxed for 1 hr during which a further 5 ml of n-butylamine and 50 ml of benzene were added. The reaction mixture was filtered and washed with dilute hydrochloric acid (20 ml) and water (10 ml) and dried (Na_2SO_4). Evaporation of the solvent left a brown viscous oil which was fractionally distilled, rejecting the first fraction. The required *product* was a viscous brown oil (26·5 g, 78%), b.p. 214°/0·04 mm. Found: N, 9·0. $C_{18}H_{36}N_2O_2$ requires N, 9·0%.

6-*Di-n-butylaminohexyl-n-butylamine* was obtained as a pale-yellow oil (14·3 g, 79%), $n_D^{23.5}$ 1·4502, by the reduction of the diamide (20 g) in ether with lithium aluminium hydride (5 g) as described under the preparation of 6-hydroxyhexyldimethylamine. b.p. 132°/0·08 mm. Found: N, 9·8; titration equivalent 143·1. $C_{18}H_{40}N_2$ requires N, 9·9%; titration equivalent 142·3.

Bis(6-di-n-butylaminohexyl)n-butylamine. *NN*-Di-n-butyladipamoyl chloride in benzene (25 ml), prepared from the acid (6·9 g) as previously described, was added (15 min) to a stirred, refluxing solution of 6-di-n-butylaminohexyl-n-butylamine (15 g) in benzene (50 ml). The mixture was refluxed for 30 min. The solution gelled on cooling and was, therefore, reheated on the water-bath and extracted as described for the preparation of bis(6-dimethylaminohexyl)methylamine. On distillation, 6-di-n-butylaminohexyl-n-butylamine (5·6 g, b.p. 124°/0·03 mm) was recovered, but almost no residue was left in the distillation flask.

In a further experiment, evaporation of the reaction mixture left a viscous residue which was dried *in vacuo* and reduced with lithium aluminium hydride (3·5 g) in ether. The product was fractionally distilled and yielded the required *base* only (5·2 g), b.p. 214–218°/0·05 mm, with a fore-run (1·0 g), b.p. 130–214°/0·05 mm. (Total yield 47%.) Found: N, 8·6; titration equivalent 162·4; fore-run, equivalent 165·0. $C_{32}H_{69}N_3$ requires N, 8·5%; titration equivalent 165·3.

N-*Adipoylpiperidine.* Ethyl *N*-adipoylpiperidine (17 g) was refluxed with ethanolic potassium hydroxide (250 ml, approx. 2/3 N) for 3 hr. The solution was neutralised and the ethanol removed under reduced pressure: benzene (2 × 25 ml) was then added and likewise removed. The residue was acidified with hydrochloric acid (15 ml) and water (10 ml), filtered, and extracted with chloroform (100 ml). After drying (Na_2SO_4) and evaporation of the chloroform, a golden-yellow oil was obtained which crystallised spontaneously in rosettes of long needles. These were dissolved in a small volume of benzene and filtered to remove any adipic acid. Evaporation of the benzene and recrystallisation (charcoal) from

chloroform-ether-light petroleum (b.p. 40–60°) gave the pure *product* in colourless rosettes (14·5 g, 96%), m.p. 81–83°. Found: N, 6·7; titration equivalent 212·9. $C_{11}H_{19}NO_3$ requires N, 6·6%; titration equivalent 213·3.

N-*Adipoylmorpholine* was prepared similarly from ethyl *N*-adipoylmorpholine (20 g). Recrystallisation (chloroform-ether-light petroleum, b.p. 40–60°) gave rosettes of white needles (14 g, 79%), m.p. 63–65°. Found: N, 6·65; titration equivalent 215·9. $C_{10}H_{17}NO_4$ requires N, 6·5%; titration equivalent 215·2.

N-*Ethyladipamoylpiperidine*. *N*-Adipoylpiperidine (13 g) in benzene (50 ml) was treated with thionyl chloride (10 ml) at 70–80° for 10 min, and the solvent and excess reagent, plus two additional volumes of benzene were successively removed under reduced pressure. Anhydrous ethylamine (15 ml) in benzene (20 ml) was added with stirring to the crude acid chloride in benzene (80 ml) at 0°. The reaction mixture was allowed to stand for several hr, filtered and evaporated, leaving a viscous black oil. A solution of this oil in chloroform (100 ml) was washed with water (2 × 15 ml) and dried (Na_2CO_3). Fractional distillation, as for *NNN'*-tri-n-butyladipamide, gave N-*ethyladipamoylpiperidine* (10 g, 68%), b.p. 236–242°/0·13 mm, as a dark viscous oil which solidified very slowly on standing. Found: N, 11·1. $C_{13}H_{24}N_2O_2$ requires N, 11·7%.

N-*Ethyladipamoylmorpholine* was prepared similarly from *N*-adipoylmorpholine (20·5 g), but the acid was treated with thionyl chloride at 60–75° for 20 min. Fractional distillation gave a light-brown oil (16°8 g, 73%), b.p. 234°/0·1 mm, which crystallised on standing, m.p. 61–64° with softening at 56°. Found: N, 11·6. $C_{12}H_{22}N_2O_3$ requires N, 11·6%.

6-*Piperidinohexylethylamine*. *N*-Ethyladipamoylpiperidine (22 g) in a mixture of ether and tetrahydrofuran (100 ml, approx. 3 : 1) was slowly added to a refluxing suspension of lithium aluminium hydride in a similar mixture (100 ml) and refluxed for 5 hr. Excess reagent was decomposed by the successive addition of water (8 ml), sodium hydroxide (6 ml, 20%) and water (28 ml) giving a granular precipitate which was readily extracted with ether. Fractional distillation gave 6-*piperidinohexylethylamine* as a mobile, colourless oil (14·25 g, 73%), b.p. 112–118°/0·07 mm, n_D^{21} 1·4685. Found: N, 12·7; titration equivalent 109·3. $C_{13}H_{28}N_2$ requires N, 13·5%; titration equivalent 106·2.

Reineckate. This crystallised from aqueous acetone in fine, pink platelets which were dried *in vacuo* below 50°. The decomposition point was *ca.* 186°. Found: N, 22·3; titration equivalent 438. $C_{21}H_{44}Cr_2N_{14}O_2S_8$ requires N, 22·2%; titration equivalent 442·7.

6-*Morpholinohexylethylamine* was prepared similarly to 6-piperidinohexylethylamine from *N*-ethyladipamoylmorpholine (16·7 g). The *product* was obtained as a colourless oil (10·2 g, 69%), b.p. 110–114°/0·1 mm, $n_D^{17.5}$ 1·4680. Found: N, 13·5; titration equivalent 108·6. $C_{12}H_{26}N_2O$ requires N, 13·1%; titration equivalent 107·2.

Bis(6-*piperidinohexyl*)*ethylamine*. The acid chloride prepared from *N*-adipoylpiperidine (7 g) as previously described, was rapidly added in benzene (35 ml) to a stirred solution of 6-piperidinohexylethylamine

(13 g) in benzene (50 ml). After refluxing for 45 min, the precipitated 6-piperidinohexylethylamine hydrochloride was filtered off, and the filtrate evaporated to give a viscous dark oil. This was dissolved in chloroform (200 ml), washed with water (2 × 20 ml) and dried (Na_2SO_4). Recovered 6-piperidinohexylethylamine base weighed 4·2 g.

Evaporation of the main chloroform solution yielded crude *N*-6-piperidinohexyl-*N*-ethyladipamoylpiperidine (12·5 g) which was further dried in a vacuum desiccator before reducing with lithium aluminium hydride (3·5 g) in ether as described under 6-piperidinohexylethylamine. Fractional distillation gave a fore-run of the required product and 6-hydroxyhexyl-1′-piperidine (1 g), b.p. 129–222°/0·05 mm, and then the required *product* only (8·5 g, 68%), b.p. 226–232°/0·05 mm. Found: N, 11·0; titration equivalent 127·1. $C_{24}H_{49}N_3$ requires N, 11·1%; titration equivalent 126·6.

Bis(6-morpholinohexyl)ethylamine was prepared from *N*-adipoylmorpholine (5·5 g) and 6-morpholinohexylethylamine (10 g) by the method described for the synthesis of bis(6-piperidinohexyl)ethylamine. Fractional distillation gave a fore-run (1·1 g), b.p. 142–176°/0·03 mm, followed by the *product* (6·2 g) as a pale-yellow oil, b.p. 223–226°/0·03 mm. Found: N, 11·1; titration equivalent 127·2. $C_{22}H_{45}N_3O_2$ requires N, 11·0%; titration equivalent 127·9. The fore-run was identical (infra-red spectrum) with the required product (total yield of triamine 74%). 6-Morpholinohexylethylamine (2·3 g, b.p. 111–112°/0·1 mm) was recovered from the hydrochloride precipitated during the condensation reaction.

Tetrahydropapaverine was obtained from the hydriodide by the extraction method of Pyman (1909), and from papaverine by catalytic hydrogenation (Craig & Tarbell, 1948).

Ethyl N-*adipoyltetrahydropapaverine.* Ethyl adipoyl chloride prepared from ethyl hydrogen adipate (7·5 g), was added in benzene (25 ml) to a stirred solution of tetrahydropapaverine (14·5 g) and triethylamine (10 ml) in benzene (75 ml); the mixture gently refluxed for 45 min. Precipitated triethylamine hydrochloride was filtered off and the filtrate washed with water (20 ml), sodium carbonate (20 ml, 10%) and dried (Na_2SO_4). Evaporation of the solvent gave the *product* as a viscous yellow oil (18 g., 84%) which would not solidify on prolonged drying *in vacuo*. Found: N, 2·9. $C_{28}H_{38}NO_7$ requires N, 2·8%.

N-*Adipoyltetrahydropapaverine* was obtained as a viscous oil by the saponification of the ester (17·5 g) as described for *N*-adipoylpiperidine. The product was contaminated with a little adipic acid which was removed by dissolving in benzene and filtering. The successive addition and evaporation of several volumes of dry ether followed by drying over potassium hydroxide gave a flaky, yellow powder (15·5 g, 94%), m.p. 38–42° (decomp.). Found: N, 3·0; titration equivalent 468·9. $C_{26}H_{34}NO_7$ requires N, 3·0%; titration equivalent 472·6.

N-*Ethyladipamoyltetrahydropapaverine.* The crude acid chloride, prepared by heating *N*-adipoyltetrahydropapaverine (5 g) with thionyl chloride dissolved in benzene (40 ml) was added to a stirred solution of anhydrous ethylamine (4 ml) in benzene (30 ml) at room temperature. The solution

was stirred for 2 hr, filtered, washed with water (10 ml) and sodium carbonate (10 ml, 20%) and dried (Na_2SO_4). Evaporation of the solvent under reduced pressure gave N-*ethyladipamoyltetrahydropapaverine* as an oil (4·75 g, 90%) which was solidified by drying *in vacuo* over potassium hydroxide pellets for several hr. Found: N, 5·2. $C_{28}H_{33}N_2O_6$ requires N, 5·6%.

6,2′-*Tetrahydropapaverinylhexylethylamine* was obtained as a dark-yellow oil (3 g, 71%) by the reduction with lithium aluminium hydride (1·5 g) of *N*-ethyladipamoyltetrahydropapaverine (4·5 g) in ether-tetrahydrofuran (100 ml, 1 : 1). A paper chromatogram (Whatman's No. 1), ascending method, using butanol-acetic acid-water (4 : 1 : 5) gave an Rf value of 0·56 to 0·61. An aliquot portion (1·5 g) of the product in ether-methanol (80 : 1) chromatographed on alumina (8 × 2 cm) gave 6,2′-*Tetrahydropapaverinylhexylethylamine*, Rf 0·56–0·61. Found: N, 5·8; titration equivalent 242·8. $C_{28}H_{43}N_2O_5$ requires N, 5·9%; titration equivalent 236·8.

The *Reineckate* was purified by precipitation from acetone-ethanol-water as a pink powder. Found: N, 16·9. $C_{36}H_{59}Cr_2N_{14}O_6S_8$ requires N, 17·1%.

Bis(6,2′-*tetrahydropapaverinylhexyl*)*ethylamine.* The crude acid chloride, prepared from *N*-adipoyltetrahydropapaverine (6·5 g), was added in benzene (50 ml) to a stirred solution of 6,2′-tetrahydropapaverinylhexylethylamine (5·5 g) and triethylamine (2 ml) in benzene (30 ml), then refluxed for 1 hr. The mixture was allowed to stand overnight, the precipitated triethylamine hydrochloride filtered off and more benzene (100 ml) added to the filtrate which was washed with water (10 ml) and sodium carbonate (10 ml, 20%) and then dried (Na_2SO_4). Removal of the solvent gave a hygroscopic powder (11 g) which on reduction with lithium aluminium hydride (3 g) in a mixture of ether and tetrahydrofuran (100 ml, 1 : 1) gave the crude triamine, as a viscous yellow oil (8 g, 76%). Paper chromatography (Whatman's No. 1) using butanol-acetic acid-water (4 : 1 : 5), showed extensive tailing, with no definite spot discernible. An aliquot portion (6 g) of the oil in ether-methanol (80 : 1) was chromatographed on alumina (15 × 2 cm), and the eluate collected in fractions of 150, 150, 75, 75 and 75 ml. Fractions one and two yielded *bis*(6,2′-*tetrahydropapaverinylhexyl*)*ethylamine* as a pale-yellow oil (3 g and 1·5 g resp.), Rf 0·7, λmax (in ethanol) 284 mμ (ε 10,600). Found: N, 4·8; titration equivalent 298·9. $C_{54}H_{79}N_3O_8$ requires N, 4·7%; titration equivalent 300·7. The third fraction gave a yellow oil (1 g) which deposited a yellow solid on standing; the infra-red spectrum of this showed a hydroxyl band at 3,200 to 3,700 cm^{-1}. The fourth and fifth fractions gave 6-hydroxylhexyl-2′-tetrahydropapaverine as a yellow solid (0·4 g), νmax. 3,200 to 3,700 cm^{-1} (hydroxyl), λmax. 284 mμ (ε 6,540), and Rf 0·85 to 0·89 in butanol-acetic acid-water (4 : 1 : 5). Found: N, 3·1; titration equivalent 435. $C_{26}H_{38}NO_5$ requires N, 3·15%; titration equivalent 444·6.

NNN-*Trisonium compounds* were prepared from either bis-6-dimethylaminohexylmethylamine, bis-6-di-*n*-butylaminohexyl-*n*-butylamine, bis-6-piperidinohexylethylamine, bis-6-morpholinohexylethylamine, or

bis-6,2′-tetrahydropapaverinylhexylethylamine by treating with the appropriate alkyl halide in ethanol, evaporating the solvent and crystallising the product. Reaction conditions and time, crystallisation solvent and yields are indicated for each compound in that order, in parenthesis.

7,7-Dimethyl-7-azoniatridecylenebis(trimethylammonium)tri-iodide (room temperature, 24 hr, water-ethanol-ether, 84%), m.p. 246–248° (decomp.). Found: N, 5·8; I, 53·9. $C_{20}H_{48}I_3N_3$ requires N, 5·9; I, 53·5%.

7-Ethyl-7-methyl-7-azoniatridecylenebis(dimethylethylammonium)tri-iodide (room temperature, 4 days, ethanol-methanol, 61%), m.p. 188–190°. Found: N, 5·6; I, 50·8. $C_{23}H_{54}I_3N_3$ requires N, 5·6; I, 50·6%.

7-Methyl-7-n-butyl-7-azoniatridecylenebis(di-n-butylmethylammonium)-tri-iodide (room temperature 24 hr, refluxed 20 min, ethanol-ethyl acetate-water, 86%), m.p. 176–180° (decomp.). Found: N, 4·5; I, 41·3. $C_{35}H_{78}I_3N_3$ requires N, 4·6; I, 41·3%.

7-Ethyl-7-n-butyl-7-azoniatridecylenebis(di-n-butylethylammonium)tri-iodide (refluxed without solvent 15 min, precipitation from n-propanol-ethyl acetate, 88%), m.p. 163–166° (decomp.). Found: N, 4·3; I, 39·6 $C_{38}H_{84}I_3N_3$ requires N, 4·4; I, 39·5%.

7-n-Butyl-7-n-propyl-7-azoniatridecylenebis(di-n-butyl-n-propylammonium)tri-iodide (refluxed without solvent 80 min, precipitation from n-propanol-ethyl acetate-ether), m.p. 143–146° (decomp.). Found: N, 4·1; I, 37·8. $C_{41}H_{90}I_3N_3$ requires N, 4·2; I, 37·9%.

7,7-Di-n-butyl-7-azoniatridecylenebis(tri-n-butylammonium)tri-iodide (refluxed without solvent 80 min, precipitation from ethanol-acetone-ether), m.p. 140–142° (decomp.). Found: N, 4·0; I, 37·0. $C_{44}H_{96}I_3N_3$ requires N, 4·0; I, 36·3%.

7-Ethyl-7-methyl-7-azoniatridecylenebis(N-methylpiperidinium)tri-iodide (room temperature 24 hr, methanol-light petroleum (b.p. 40–60°), 91%), m.p. 199–211°. Found: N, 5·0; I, 47·2. $C_{27}H_{58}I_3N_3$ requires N, 5·2; I, 47·3%.

7,7-Diethyl-7-azoniatridecylenebis(N-ethylpiperidinium)tri-iodide (room temperature without solvent 24 hr, ethanol-ether, 76%), m.p. 226° (decomp.) with darkening at 214°. Found: N, 4·9; I, 44·6. $C_{30}H_{64}I_3N_3$ requires N, 5·0; I, 44·9%.

Trichloroacetate. Trichloroacetic acid (1 g) in water (1 ml) added dropwise to a vigorously stirred solution of the semi-solid ethiodide (0·12 g) in water (1 ml) precipitated a yellow oil. The supernatant liquid was decanted and the oil triturated with a dilute solution of trichloroacetic acid (5%) giving a fine white powder which was filtered and dried *in vacuo*. The *trichloroacetate* was slightly hygroscopic and was not recrystallised. Found: N, 2·9; titration equivalent 488·9. $C_{42}H_{67}Cl_{18}N_3O_{12}$ requires N, 2·9%; titration equivalent 481.

7-Ethyl-7-methyl-7-azoniatridecylenebis(N-methylmorpholinium)tri-iodide (room temperature, 24 hr, triturated with dry ether, 57%). The *product* was a very hygroscopic yellow powder (1·2 g, 57%) with no definite m.p. Found: N, 5·1; I, 47·3. $C_{25}H_{54}I_3N_3O_2$ requires N, 5·2; I, 47·0%.

Trichloroacetate. A semi-solid sample of the methiodide (0·5 g) treated with a solution of trichloroacetic acid gave the trichloroacetate

as a yellow powder after prolonged trituration. The *product* was washed with water and dried *in vacuo*. Found: N, 3·0; titration equivalent 459·1. $C_{37}H_{57}Cl_{18}N_3O_{14}$ requires N, 3·0%; titration equivalent 468·7.

7,7-Diethyl-7-azoniatridecylenebis(N-*ethylmorpholinium*)*tri-iodide* (room temperature, 24 hr, precipitation with methanol-ethanol-ether, 63%), m.p. 197–200°. Found: N, 4·7; I, 44·9. $C_{28}H_{60}I_3N_3O_2$ requires N, 4·9; I, 44·7%.

7-Ethyl-7-methyl-7-azoniatridecylenebis(N-*methyltetrahydropapaverinium*)-*tri-iodide* (room temperature in ether 24 hr, washed with dry ether, 81%). The melting-point of the methiodide was indefinite. Gladych & Taylor (1962) have also observed that the quaternary salts of other tetrahydropapaverine derivatives melt over a wide range. Found: N, 3·0; I, 28·8. $C_{57}H_{88}I_3N_3O_8$ requires N, 3·2; I, 28·7%.

Pharmacological

METHODS

The methods were as described previously (Edwards, Stenlake, Lewis & Stothers, 1961). All drugs and control solutions were injected in 0·9% sodium chloride solution.

Neuromuscular blockade. The compounds were compared with tubocurarine on the gastrocnemius muscle-sciatic nerve preparation of the pentobarbitone-anaesthetised cat and fowl, on the rabbit by the head-drop assay method; on the mouse by the inclined-screen method; on the frog isolated rectus abdominis muscle, in terms of inhibition of acetylcholine-induced contractures, and on the three-day old chick.

The duration of paralysis after doses causing 40 to 60% inhibition of the gastrocnemius-sciatic preparation in the cat was also estimated.

Sympathetic ganglion block was assessed on the nictitating membrane preparation of the cat, by comparing the drug-induced reduction in the response to preganglionic tetanic stimulation of the cervical sympathetic nerve, with that due to tubocurarine. Doses were four or five times those causing 50% inhibition of twitch height in the gastrocnemius-sciatic preparation.

Respiration and blood pressure. The dose required to paralyse respiration was estimated on the pentobarbitone-anaesthetised cat. Effects on blood pressure were also noted.

RESULTS

Qualitative tests indicated that the compounds possessed no depolarising activity.

In Table 1, the molar potencies are shown as percentages of the molar potency of tubocurarine, and in Fig. 1, the compounds are arranged along the abscissa in order of increasing neuromuscular blocking potency in the cat. For comparison, Table 1 and Fig. 1 contain compounds previously described (Carey & others, 1959).

The highest sensitivity to the compounds when compared with tubocurarine was shown by fowls. Neuromuscular blocking potency was less in the mouse and on the frog rectus abdominis muscle, than in the cat and the rabbit preparations.

TABLE 1. INFLUENCE OF ALKYL ONIUM SUBSTITUENTS UPON NEUROMUSCULAR BLOCKING POTENCY (TUBOCURARINE = 100)

Compound	$R_3R'\overset{+}{N}\cdot[CH_2]_6\cdot\overset{+}{N}\cdot[CH_2]_6\cdot NR_3R'$ (R / R′)			Cat	Rabbit	Mouse	Chick (fowl)	Frog
Ia	Me_3	Me_3	Me_3	6	6	6	20(20)	5
Ib	Me_2Et	MeEt	Me_2Et	22	15	13	86(65)	10
Ic	Et_2Me	EtMe	Et_2Me	50	52	16	*(321)	29
Id	Et_3	Et_3	Et_3	99	78	64	495(495)	52
Ie	Et_2Pr	EtPr	Et_2Pr	97	76	53	144(*)	13
If	Et_2Bu	EtBu	Et_2Bu	139	179	73	210(353)	54
Ig	Pr_2Me	PrMe	Pr_2Me	16	20	12	86(37)	5
Ih	Pr_2Et	PrEt	Pr_2Et	35	46	19	125(157)	17
Ii	Pr_3	Pr_3	Pr_3	24	25	4	298(422)	16
Ij	Bu_2Me	BuMe	Bu_2Me	33	23	8	132(128)	25
Ik	Bu_2Et	BuEt	Bu_2Et	82	56	29	191(197)	38
Il	Bu_2Pr	BuPr	Bu_2Pr	33	19	6	155(155)	20
Im	Bu_3	Bu_3	Bu_3	33	20	5	150(161)	53

* Insufficient material to test.

Respiratory paralysing potency closely paralleled neuromuscular blocking potency for the whole series.

Significant sympathetic ganglion blockade was noted only in compound Ia (Me_3), which was about half as potent as tubocurarine. Compound Ib was about one-tenth as potent as tubocurarine.

The duration of paralysis produced by doses causing 40–60% inhibition

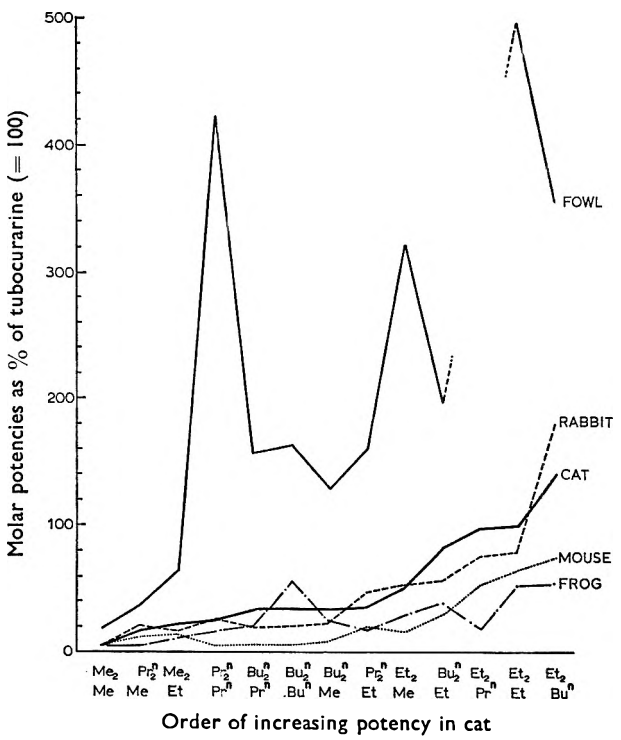
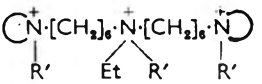


FIG. 1. Comparative neuromuscular blocking potencies of compounds in Table 1. The groups on the abscissa are $R_{(2)}$ (top row) and R′ (bottom row) of the formula.

of the gastrocnemius-sciatic preparation of the cat was in all instances within the limits of 15–29 min found with tubocurarine.

Compounds with heterocyclic substituents. The neuromuscular blocking potencies are shown in Table 2. The most notable features are the low potencies of the morpholino-derivatives, especially in the mammalian species, and the high susceptibility of the fowl to the piperidino- and the tetrahydropapaverino-compounds.

TABLE 2. INFLUENCE OF TERMINAL HETEROCYCLIC SUBSTITUENTS ON NEUROMUSCULAR BLOCKING POTENCY (TUBOCURARINE = 100)



Compound	Alkyl substituent R′	Heterocyclic groups	Cat	Rabbit	Mouse	Fowl	Frog
In	Me	piperidine	47	30	26	360	7
Io	Et	piperidine	99	53	51	450	10
Ip	Me	morpholine	8	•	7	20	•
Iq	Et	morpholine	10	7	6	43	33
Ir	Me	tetrahydropapaverine	103	61	51	400	107

* Insufficient material to test.

Only the morpholino-compounds showed ganglion blocking activity. Respiratory paralysing potency was similar to muscle relaxant potency in all the heterocyclic compounds (Tables 2 and 3).

Discussion

In the *NNN*-trisonium salts investigated, only the alkyl substituents on the onium atoms have been varied, thus allowing an assessment of the effects of alkyl substitution alone.

Compound Ia (Table 1) has tubocurarine-like properties and is an example of a long chain methonium derivative with no depolarising activity. Its significant ganglion blocking activity may therefore indicate that its configuration at the receptor surface in terms of N^+ to N^+ distance, is closer to that of hexamethonium than to that of decamethonium. It also emphasises that for depolarizing activity the dimensions of the molecules must be rigidly defined. The ganglion blocking potency of the Me_3 compound (Ia), and also the Me_2Et compound (Ib) (the other compounds being inactive in this respect), confirms the views of Fakstorp, Pedersen, Poulsen & Schilling (1957) that, for ganglion blockade, more than one methyl substituent on the onium nitrogen is advantageous. Unlike Fakstorp & others (1957), we have found the fully methyl-substituted compound to be a more potent ganglion blocking agent than the dimethylethyl derivative. No general inferences can be made, however, since the two series of compounds are not analogous.

It seems most useful to explain the range of antagonistic potency shown in terms of goodness of fit at the receptor site, the extent of shielding of

TABLE 3. INFLUENCE OF TERMINAL SUBSTITUENTS ON DURATION OF PARALYSIS, RESPIRATORY PARALYSING POTENCY AND SYMPATHETIC GANGLION BLOCK

Compound	Sympathetic ganglion block (cat)*	Respiratory paralysing potency in cat (TC = 100)	Duration of paralysis† (min)	
			Cat	Fowl
Ia	27	7	17–23	26–36
Ib	6	21	15–20	31–36
Ic	0	57	20–41	—
Id	0	59	15–18	—
Ie	0	99	18–29	—
If	0	156	24–26	—
Ig	0	29	17–29	—
Ih	0	60	15–29	—
Ii	0	27	23–29	—
Ij	0	24	15–23	—
Ik	0	78	15–23	—
Il	0	26	18–23	28–37
Im	0	29	20–23	—
In	8	53	15–22	20–27
Io	0	88	15–19	20–28
Ip	29	10	17–18	43–53
Iq	30	10	15–22	37–50
Ir	3	91	20–23	40–50
TC	53	100	15–29	19–28

* Per cent inhibition caused by 4 to 5 times the dose required for 50% inhibition of twitch height in the gastrocnemius-sciatic preparation.
† Time taken for twitch height to recover to control levels following doses causing 40–60% inhibition of the gastrocnemius-sciatic preparation.

the onium nitrogen and perhaps the magnitude of the dissociation and association rate-constants calculated for the drug-receptor reaction (Paton, 1961).

The results suggest that for compounds with alkyl substituents, at least one Et group is required for significant tubocurarine-like muscle relaxant potency in mammals. Larger alkyl substituents, apart from compounds If (Et_2Bu^n) and Ik ($EtBu^n_2$), reduce potency, possibly because they hinder the approach of the onium nitrogen to the anionic site of the receptor. In the chick, an Et group was not essential for high potency (see Ii, Il, Im in Table 1).

The high potency of the Et_2-substituted compounds (Ic, Id, Ie and If) may reflect a combination of optimal receptor fit and minimal shielding of the charged nitrogen atom. The fall in potency in the Me_3 and Me_2Et derivatives would then be due to a poorer fit on the receptor. Reduced potency among compounds with large alkyl groups (e.g. Ii, Ij, Il and Im) may thus indicate a poor fit on to the receptor with shielding of the charged nitrogen atom. In general, it seems that at least two ethyl substituents on the terminal nitrogen atom are required for high potency (compounds Ie and If) provided that the third substituent is not methyl, when potency falls to about half.

Only a limited series of compounds containing heterocyclic substituents was available. When the terminal onium groups were incorporated into a piperidine ring, potency was increased over the Me_3, compound (Ia),

but the corresponding morpholine derivatives were of very low neuromuscular blocking potency. This effect has been ascribed to charge delocalization caused by the oxygen function (Mason & Wien, 1955).

Acknowledgement. We wish to thank the Pharmaceutical Society of Great Britain for a maintenance grant to one of us (C.I.F.).

References

Andrews, K. J. M., Bergel, F. & Morrison, A. L. (1953). *J. chem. Soc.*, 2998–3002.
Avison, A. W. D. (1951). *J. appl. Chem.*, **1**, 469–472.
Bender, M. L. (1957), *J. Amer. chem. Soc.*, **79**, 1258–1259.
Bender, M. L., Chow, Y-L. & Chloupek, F. (1958). *Ibid.*, **80**, 5380–5384.
Carey, F. M., Edwards, D., Lewis, J. J. & Stenlake, J. B. (1959). *J. Pharm. Pharmacol.*, **11**, *Suppl.* 73*T*–86*T*.
Craig, L. E. & Tarbell, D. S. (1948). *J. Amer. chem. Soc.*, **70**, 2783–2785.
Dahlgren, G., & Simmerman, N. L. (1963). *Science*, **140**, 485–486.
Edwards, D., Lewis, J. J., Stenlake, J. B. & Zoha, M. S. (1958). *J. Pharm. Pharmacol.*, **10**, *Suppl.* 106*T*–120*T*.
Edwards, D., Lewis, J. J., McPhail, D. E., Muir, T. C. & Stenlake, J. B. (1960). *Ibid.*, **12**, *Suppl.* 137*T*–152*T*.
Edwards, D., Stenlake, J. B., Lewis, J. J. & Stothers, F. (1961). *J. med. pharm. Chem.*, **3**, 369–399.
Fakstorp, J., Pedersen, J. G. A., Poulsen, E. & Schilling, M. (1957). *Acta pharmacol., scand.*, **13**, 52–58.
Gladych, J. M. Z. & Taylor E. P. (1962), *J. chem. Soc.*, 1481–1487.
Johnson, D. H. (1958). *Ibid.*, 1624–1628.
Leach, S. J. & Lindley, H. (1953). *Trans. Farad. Soc.*, **49**, 921–925.
Mason, D. F. J. & Wien, R. (1955). *Brit. J. Pharmacol.*, **10**, 124–132.
Paton, W. D. M. (1961). *Proc. Roy. Soc.*, **154B**, 21–69.
Pyman, F. L. (1909). *J. chem. Soc.*, 1610–1623.
Prelog, V. (1930). *Coll. Czech. chem. Comm.*, **2**, 712–722.
Stenlake, J. B. (1963). *Progress in Medicinal Chemistry*, Editors, Ellis, G. & West, G. B., **3**, 1–44. London: Butterworths.
Vilsmeier, A. (1958), Ger. Patent, 1,036,859 (1958), from *Chem. Abstr.*, **54**, 19593c.

The paper was presented by MR. LEWIS.

J. Pharm. Pharmacol., 1964, **16**, *Suppl.*, 104*T*–107*T* Received May 1, 1964

SHORT COMMUNICATION

The influence of vehicles on skin penetration

C. W. BARRETT, J. W. HADGRAFT AND I. SARKANY

METHODS which have been devised to estimate the rate of release of medicaments from vehicles and their subsequent penetration and absorption have been reviewed by Gemmel & Morrison (1957). Although *in vitro* methods are useful in assessing rates of release of medicaments, they are too far removed from conditions occurring in normal skin to be of real value. We have, therefore, attempted to assess the influence of vehicles on skin penetration by the use of *in vivo* methods in man.

Malkinson & Rothman (1963) and Barr (1962) have drawn attention to the confusion and in some instances, contradictions, that appear in the literature on the influence of vehicles on penetration. Many of the results obtained are difficult to interpret or to compare due to the inherent variability between test subjects and to species differences. There is obviously a need for more methods which are more closely related to conditions occuring in normal human skin.

It has been shown by McKenzie & Stoughton (1962) that when corticosteroids are applied to the skin in alcoholic solution and occluded for 16 hr, a considerable increase in percutaneous penetration over the non-occluded solution is obtained with the production of a noticeable area of vasoconstriction. This method has been used by McKenzie (1962) to compare the percutaneous absorption of some topical steroids.

Cronin & Stoughton (1962) have used the vasodilatation produced by ethyl nicotinate to determine whether regional differences in percutaneous absorption exist and to assess the effect of hydration on the passage of compounds through the skin. In the present investigation, the vasodilatation produced by the local application of methyl nicotinate and the vasoconstriction produced by applying betamethasone-17-valerate under an occlusive dressing have been used in an attempt to assess the degree of their percutaneous penetration from four vehicles. The effect of altering the osmotic properties of an aqueous cream vehicle containing methyl nicotinate was also determined.

METHODS AND RESULTS

Application of a standard quantity of a topical preparation. A circular hole 8·7 mm in diameter was cut in a piece of tin sheet 0·127 mm thick and approximately 7·5 cm square. The sheet was kept flat by placing between two sheets of plate glass. A piece of polythene sheet 2·5 cm square was placed under the circular hole between the tin and the bottom sheet of glass. A quantity of test vehicle was drawn across the tin sheet with a spatula and the polythene square was then carefully removed so that

From the Departments of Pharmacy and Dermatology, Royal Free Hospital, London, W.C.1.

a discreet disc of vehicle equal in volume to the hole in the tin sheet was removed. Ten test weighings of the disc of vehicle thus produced gave a mean weight of 7·8 ± 0·75 mg.

Experiments with methyl nicotinate. Methyl nicotinate was incorporated into aqueous cream, B.P. and oily cream, B.P. by dissolving in the aqueous phase; it was also incorporated into white soft paraffin B.P. and macrogol ointment B.P.C. (Carbowax 1500) by dispersion in the molten vehicle. The concentrations used are shown in Table 1.

TABLE 1. THE PERCUTANEOUS PENETRATION OF METHYL NICOTINATE FROM FOUR VEHICLES

Concentration of methyl nicotinate	Time of onset of noticeable vasodilatation (min)			
	Aqueous cream B.P.	Oily cream B.P.	White soft paraffin B.P.	Macrogol ointment B.P.
1	3·3	4·1	5·3	8·7
0·5	4·0	4·5	6·6	10·8
0·25	—	—	—	17·1
0·1	4·6	5·2	8·0	22·3
0·05	5·9	6·8	13·1	NR
0·01	7·4	10·5	19·7	NR
0·005	10·9	12·5	NR	NR
0·001	NR	NR	NR	NR

NR = No response
Each figure is an average of 20 readings, two from each of 10 subjects.

A polythene square with the standard quantity of ointment was applied to the flexor aspect of the forearm of each of 5 male and 5 female volunteers and covered with a strip of 1 in. wide cellulose tape. The time for the onset of erythema round the edge of the circle of ointment was recorded. Duplicate tests were made simultaneously for each concentration in each vehicle. Where no erythema was noticeable after 1 hr, "no response" was recorded. In this way, the minimal concentration of methyl nicotinate to produce erythema was found for each vehicle. The results obtained for the complete range of concentrations in the four vehicles are shown in Table 1. There is little difference in the rate of penetration from aqueous cream and oily cream, penetration is slightly slower from white soft paraffin and markedly slower from macrogol ointment.

Twenty readings for each of six concentrations of methyl nicotinate in aqueous cream, B.P. were made on one subject to determine the reproducibility of the results of penetration. Concentrations were %: 1, 0·5, 0·1, 0·05, 0·01, 0·005; the means (± s.d.) for the times of onset (min) were respectively: 4·3 (0·37), 4·7 (0·55), 5·3 (0·62), 5·7 (0·78), 10·5 (1·68), 15·0 (0·39).

The osmotic property of an aqueous cream was altered by adding 40 and 60% of glycerin B.P. and the penetration of methyl nicotinate from these preparations was investigated. An increase in the glycerin content produces a decrease in the rate of penetration of methyl nicotinate (Table 2).

A further series of experiments was conducted in 10 subjects to determine the influence of the vehicle on the rate of penetration of betamethasone-17-valerate. With 0·1 and 0·05% concentrations, no significant

difference in the degree of blanching from the four vehicles was noted. The area of vasoconstriction from the macrogol ointment base was consistently larger in all the subjects indicating greater spread of the medicament from this vehicle.

TABLE 2. THE INFLUENCE OF GLYCERIN B.P. ON THE PERCUTANEOUS PENETRATION OF METHYL NICOTINATE FROM AN AQUEOUS CREAM VEHICLE

Concentration of methyl nicotinate %	Time of onset of noticeable vasodilatation (min)		
	Aqueous cream B.P.	Aqueous cream with 40% glycerin	Aqueous cream with 60% glycerin
1	3·6	4·6	6·3
0·5	4·2	5·6	8·5
0·25	—	—	12·1
0·1	4·7	6·7	16·7
0·05	6·3	11·7	NR
0·01	7·9	17·6	NR
0·005	11·6	NR	NR
0·001	NR	NR	NR

NR = No response.
Each figure is an average of 20 readings as in Table 1.

DISCUSSION

It has been suggested that the penetration of a drug depends essentially on its own lipid and water solubility and that the role of the vehicle is of secondary importance (Malkinson & Rothman, 1963). On theoretical grounds, Higuchi (1960) has shown that the thermodynamic activity and the diffusion coefficient of a medicament in the vehicle are significant factors in its penetration whilst Shelmire (1960) has stated that the hydration of the stratum corneum is also an important factor in percutaneous penetration. Greasy vehicles probably promote hydration by restricting moisture loss from the skin surface. Humectants in aqueous creams would tend to produce the reverse effect by preventing evaporation of the aqueous phase and the subsequent deposition of a continuous oil film on the skin surface. Water-soluble vehicles would cause little change in hydration.

The results with methyl nicotinate show little difference in the rate of penetration from aqueous cream and oily cream. Methyl nicotinate is very soluble in water and is therefore probably released quite readily from these vehicles. The fact that penetration from white soft paraffin is slightly slower may suggest that the stratum corneum has to attain a certain degree of hydration before penetration can occur. Macrogol ointment is water miscible and the significantly slower penetration from this vehicle may be due either to its inability to hydrate, or to an osmotic effect which would tend to dehydrate the stratum corneum, or to an adverse diffusion coefficient of methyl nicotinate in this vehicle.

The decreased penetration of methyl nicotinate from aqueous creams containing glycerin is in agreement with Shelmire's view.

Acknowledgement. We thank Glaxo Laboratories Ltd., for a supply of betamethasone-17-valerate.

References

Barr, M. (1962). *J. pharm. Sci.*, **51**, 395–409.
Cronin, E. & Stoughton, R. B. (1962). *Brit. J. Dermatol.*, **74**, 265–272.

Gemmell, D. H. O. & Morrison, J. C. (1957). *J. Pharm. Pharmacol.*, **9**, 641–656.
Higuchi, T. (1960). *J. Soc. cosmetic Chem.*, **11**, 85–97.
Malkinson, F. D. & Rothman, S. (1963). *Handbuch der Haut. und. Geschlechtskrankheiten. J. Jadassohn.* Publishers, Springer, Berlin.
McKenzie, A. W. (1962). *Arch. Derm. Syph., Chicago*, **86**, 611–612.
McKenzie, A. W. & Stoughton, R. B. (1962), *Ibid.*, **86**, 608–610.
Shelmire, J. B. (1960). *Ibid.*, **82**, 24–31.

The paper was presented by MR. BARRETT.

J. Pharm. Pharmacol., 1964, **16**, *Suppl.*, 108*T*–110*T* Received April 30, 1964

SHORT COMMUNICATION

A physical indicator for sterilisation procedures

D. E. SIMPKINS AND G. R. WILKINSON

THE problem of a suitable time-temperature indicator for sterilising autoclaves has been without a satisfactory solution. Previous methods (Brown & Ridout, 1960; 3M's Co. Ltd.; Propper; Klintex; Browne's Tubes) all have disadvantages. The device described below consists of filter paper laminated to aluminium foil with an adhesive, one end being impregnated with a 2,4-dinitrophenylhydrazone of suitable melting-point. The whole is then sealed in a perforated transparent polypropylene envelope. The time at which the device is maintained above the particular temperature is satisfactorily indicated by the progression of the coloured zone along the paper.

EXPERIMENTAL

2,4-Dinitrophenylhydrazones (DNP) are intensely coloured compounds which can be prepared in a wide range of melting-points. 4-Methylcyclohexanone-2,4-dinitrophenylhydrazone, m.p. 132–134°, was used in the present work when dealing with the sterilisation of fabrics at 134°. The 2,4-DNP of methylethylketone may be used at a temperature of 115°, that of phenylacetaldehyde at 121° and that of acetone at 126°.

To obtain a uniform length of travel of the 2,4-DNP, aluminium foil, 0·02 mm thick, was incorporated to act as a heat distributor. Using a constant area of foil, a reproducible length of flow of the derivative was obtained for a given time. This was found by observing the enveloped laminate in an experimental glass autoclave evolved in these laboratories, and relating time and length of flow to the temperature shown by a thermocouple in contact with the envelope.

Of a range of filter papers investigated, Whatman No. 54 and Green's No. 406 were the most satisfactory. Various adhesives for the lamination were examined. Starch paste least restricted the flow of 2,4-DNP along the paper but was poor as an adhesive. "Q5" (Gerald Young Laboratories, Ltd., London) was satisfactory as an adhesive and the length of flow was but little less than that obtained using starch paste.

A device made from Green's No. 406 filter paper and foil, cut to 0·5 cm × 3 cm, was submitted to the following autoclave procedures uncovered and wrapped in fabrics:

(*a*) Evacuation to 20 mm Hg absolute.
(*b*) Introduction of steam to 2·06 kg cm^{-2}.
(*c*) Maintaining at this pressure for a period.
(*d*) Evacuation to 30 mm Hg absolute.

From Macarthys Pharmaceuticals Ltd., Chesham House, Chesham Close, Romford, Essex.

The 2,4-DNP travelled 12 mm along the paper in 3 min at 134°. With a similar indicator with starch paste replacing the "Q5" adhesive, the indicator travelled 17 mm under identical autoclave conditions.

DISCUSSION

The device described has advantages over others currently available. Flow of the 2,4-DNP along the paper stops if the temperature falls below its melting-point, the indication of time at temperature is thus unequivocal. With a pure chemical, storage conditions are less important, and there is no deterioration similar to that which occurs with spore papers (Sykes, 1958), or Browne's tubes (Darmady, Hughes & Jones, 1958; Sykes, 1958; Brown & Ridout, 1960).

Although the paper and 2,4-DNP used require standardisation, this is easily achieved in the glass autoclave, in which the behaviour can be examined during the whole of the sterilisation cycle and if required the filter paper can be printed with calibration marks.

A series of indicators made for use in dry heat at different temperatures by varying the 2,4-dinitrophenylhydrazones can be used.

By selecting 2,4-DNP's which melt at the recommended sterilisation temperatures (M.R.C. Working Party, 1959) a device can be made which will indicate only at these temperatures. A curve has been superimposed

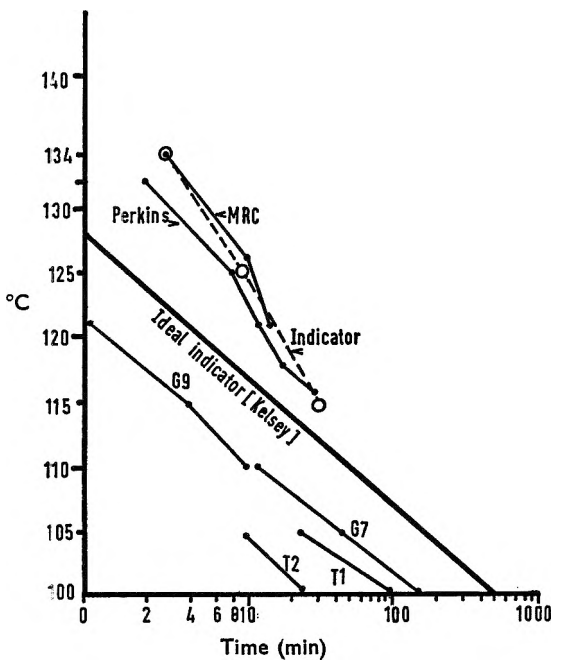


FIG. 1. Graph shewing the thermal death curves of typical gas gangrene organisms (G.7 and G.9) and tetanus organisms (T.1 and T.2) compared with the curves for an ideal indicator and the sterilising times at temperature suggested by Perkins and the M.R.C. Working Party (after Kelsey). The curve for the indicator reported is superimposed.

on those of Perkins (1956) and the M.R.C. (1959) (Fig. 1): it shows that there is a safety margin above the levels suggested as an ideal indicator.

References

Brown, W. R. L. & Ridout, C. W. (1960). *Pharm. J.*, **184**, 5–8.
Darmady, E. M., Hughes, U. E. A. & Jones, J. D. (1958). *Lancet*, **2**, 756–769.
Medical Research Council's Working Party (1959). *Lancet*, **1**, 425–435.
Perkins, J. J. (1956). *Principles and Methods of Sterilisation*, Springfield, Illinois: Chas. C. Thomas.
Sykes, G. (1958). *Disinfection and Sterilisation*, pp. 110–112, London: E. & F. N. Spon Ltd.

The paper was presented by MR. SIMPKINS.

J. Pharm. Pharmacol., 1964, **16**, *Suppl.*, 111*T*–120*T* Received March 9, 1964

Some observations on the effect of lubrication on the crushing strength of tablets

E. SHOTTON AND C. J. LEWIS*

The effect of mean compaction pressure, base particle size, lubricant particle size, and lubricant concentration, on crushing strength of tablets has been determined for crystalline aspirin, hexamine, sucrose and sodium chloride, and simple granulations of sucrose and of hexamine. Magnesium stearate powder was used as a lubricant. The lubricant decreased the strength of all tablets, the reduction being greatest for the crystalline materials. Variation of lubricant particle size had no effect on strength, and the behaviour of the granulations was not predictable from the characteristics of the crystalline materials. The results are compared with those of other workers.

MUCH of the previous research on the problems of pharmaceutical tabletting has involved the use of standard granulations containing adhesive and lubricating agents. Strickland (1956), and Munzel & Kagi (1954) have investigated the distribution of lubricant in a granulation and its role in reducing friction, but paid slight attention to the effect of lubricant on tablet strength. Shotton & Ganderton (1961) found that coating of simple crystalline materials with stearic acid interfered with the mechanism of failure under a crushing load.

We record some observations on the effect of magnesium stearate on the crushing strength of tablets of crystalline materials and of two simple granulations prepared without binders.

Experimental

Crystalline sucrose, hexamine, aspirin and sodium chloride (B.P. or B.P.C. quality) were agitated in small quantities for 15 min on B.S. sieves using an "Inclyno" machine, and 30–40, 40–60, 60–80, 80–100 and −100 mesh fractions were collected.

Granulations of sucrose and of hexamine were prepared by hand from material which had been ball-milled until all passed a 100 mesh sieve. The powder was damped with distilled water and pressed through a 30 mesh sieve. Both granulations were dried at room temperature (2 hr); the hexamine granulation was further dried at 50° and 29 inches mercury vacuum (5 hr); the sucrose granulation at 60° (2 hr). The dried granulations were sieved to give a range of sizes.

A range of lubricant particle sizes was obtained by making a granulation of −100 mesh magnesium stearate (technical grade) using chloroform to produce a coherent mass. The size fractions were obtained by sieving the dried granulation for 15 min. The lubricant was mixed

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

* Present address: Development Department, Boots Pure Drug Co. Ltd., Beeston Works, Nottingham.

with the base material by tumbling in a glass jar (5 min), and, unless otherwise stated, was added as a −100 mesh powder.

All materials were compacted in a hand-operated Lehmann single-punch tablet machine, using a ½ inch plane faced, cylindrical punch and die set instrumented in the manner of Shotton & Ganderton (1960). The upper punch was longer than is normally used (7 cm) to allow a greater degree of adjustment when compressing the granulations. The material to be tabletted was hand filled into the die, the weight of sample being sufficient to produce a tablet 0·4 cm thick at zero porosity. During the compaction cycle the force on the upper punch, the force on the lower punch, and the force necessary to eject the tablet were recorded. The weight and thickness of the tablets were measured before estimation of crushing strength on the apparatus described by Shotton & Ganderton (1960).

The following variables were investigated:

1. The effect of mean compaction pressure on lubricated and unlubricated materials using 40–60 mesh sodium chloride and 30–40 mesh fractions of the other materials. 2% magnesium stearate of −100 mesh was used as lubricant.
2. The effect of base particle size, in the presence and absence of 2% lubricant, the mean compaction pressure being constant.
3. The effect of lubricant concentration, mean compaction pressure remaining constant.
4. The effect of lubricant particle size, mean compaction pressure being constant, and lubricant concentration 2%.

The mean compaction pressure used in (2), (3) and (4) varied with the strength characteristics of the base material. Sucrose and sucrose

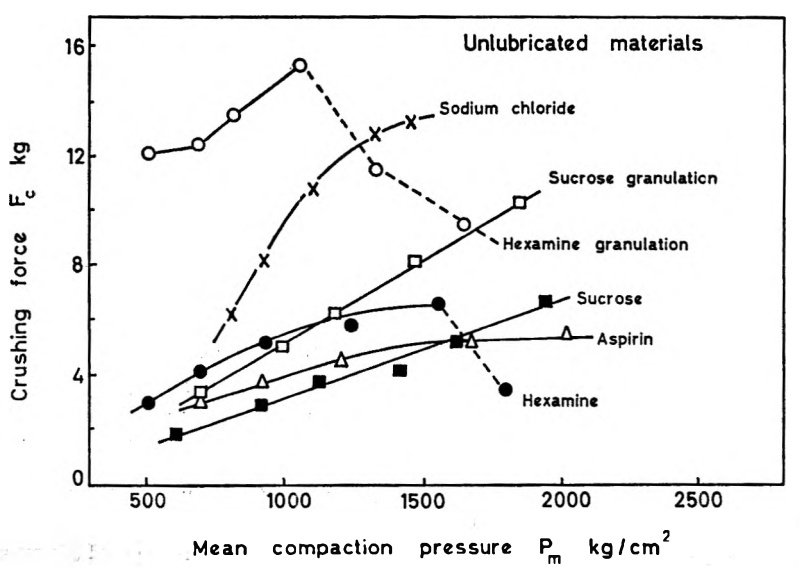


FIG. 1. Effect of mean compaction pressure on crushing strength.

granulation could not be tabletted without some form of die-wall lubrication. The die was lubricated by pressing a tablet containing 30% magnesium stearate in the die before the compaction of each tablet of sucrose or sucrose granulation. At no time was there any lubricant material which could affect particle bonding of unlubricated base.

To obtain reproducible results with hexamine and hexamine granulation the material was heated at 50–55° under 29 inches Hg vacuum for 2½ hr immediately before use.

Results

The results are presented graphically in Figs 1–7. Each point represents the mean of 5 tablets. Particle sizes where given refer to the mean of the sieve apertures by which the size range was classified:

Sieve classification, mesh	20–30	30–40	40–60	60–80	80–100
Mean particle size, μ ..	625	435	315	220	170

1. THE EFFECT OF MEAN COMPACTION PRESSURE ON CRUSHING STRENGTH

The relation between crushing strength and mean compaction pressure for unlubricated materials varies with the material being tabletted (Fig. 1). For sucrose and sucrose granulation there appears to be a direct relationship between crushing strength and the applied pressure, whereas for aspirin and hexamine, and sodium chloride up to 1,450 kg/cm², the relation is logarithmic. Hexamine and hexamine granulation show peak values for crushing strength after which the strength falls off with increasing pressure. This reduction in strength is associated with a pressure at which lamination of the tablet is evident. For hexamine granulation an increase in compaction pressure from approximately 500 kg/cm² to

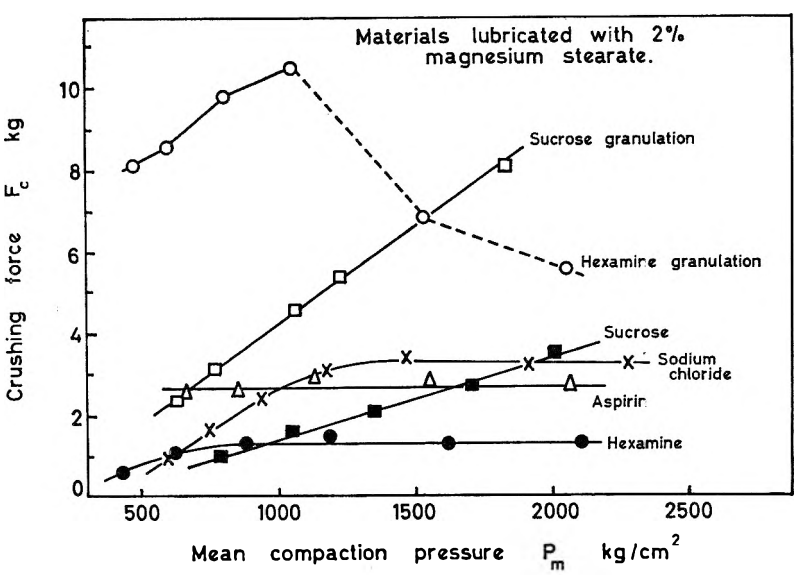


FIG. 2. Effect of mean compaction pressure on crushing strength.

700 kg/cm² has little effect on the crushing strength. Of particular note is the much greater strength of tablets made from granulations compared to those made from the crystalline base materials.

The addition of 2% magnesium stearate powder reduces the strength of all tabletted materials (Fig. 2), the reduction being less for the granulations. Increased compaction pressure has no effect on the crushing strength of lubricated aspirin, and only a slight increase in strength with increased pressure is observed with lubricated hexamine.

For hexamine and hexamine granulation the broken lines link results obtained from laminated compacts.

2. THE EFFECT OF BASE PARTICLE SIZE IN THE PRESENCE OF LUBRICANT

Results for lubricated and unlubricated materials are given in Figs 3, 4 and 5. The crushing strength of sodium chloride tablets, lubricated and unlubricated, compacted at a constant pressure, shows a minimum value over the approximate size range 220–315 μ (Fig. 3). In contrast, tablets of aspirin exhibit maximum strength over this same size range.

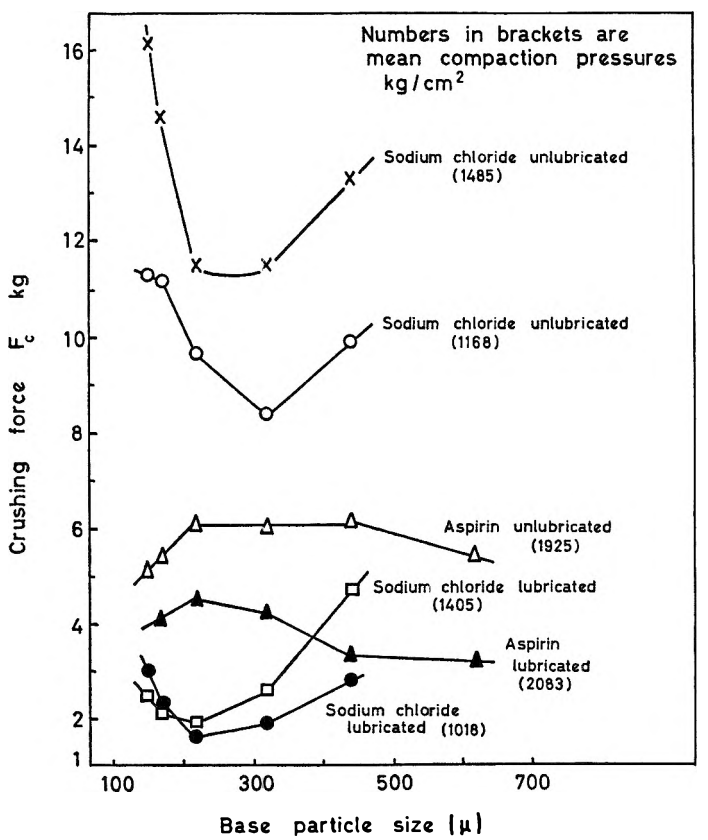


FIG. 3. Effect of base particle size on crushing strength.

For both these materials the presence of lubricant causes a reduction in crushing strength without altering the overall effect of base particle size.

A reduction in the particle size of sucrose, when lubricated, had no effect on crushing strength when the mean compaction pressure was 889 kg/cm² or 1,841 kg/cm² (Fig. 4). Sucrose granulation was also unaffected by changes in particle size when unlubricated, but tablet strength decreased slightly with decreasing particle size in the presence of lubricant.

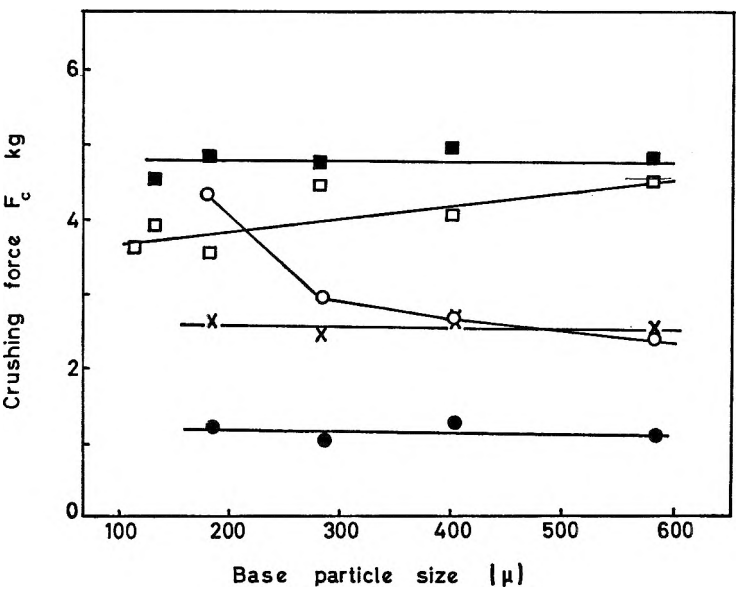


FIG. 4. Effect of base particle size on crushing strength of sucrose compacts.

		P_m (kg/cm²)
■	Sucrose granulation unlubricated ..	981
□	Sucrose granulation lubricated ..	885
○	Sucrose unlubricated	992
●	Sucrose lubricated	889
×	Sucrose lubricated	1841

The effects of particle size on the strength of hexamine tablets are complicated (Fig. 5). With unlubricated hexamine crystals, a reduction in particle size produces an increase in strength to a maximum at 40–60 mesh, any further reduction in size producing weaker tablets. When lubricant is present, the strength decreases with decreasing particle size. The strongest tablets of hexamine granulation were those made with large sizes (20–30 mesh). The presence of lubricant markedly reduces crushing strength as granulation size decreases, the effect being much more pronounced than with the sucrose granulation.

3. THE EFFECT OF LUBRICANT CONCENTRATION

Not unexpectedly (Fig. 6), an increased concentration of lubricant produced weaker tablets, although for all materials used, other than

hexamine granulation, an increase in concentration above 0·5% produced only a slight effect on crushing strength. Results for hexamine granulation are different in form.

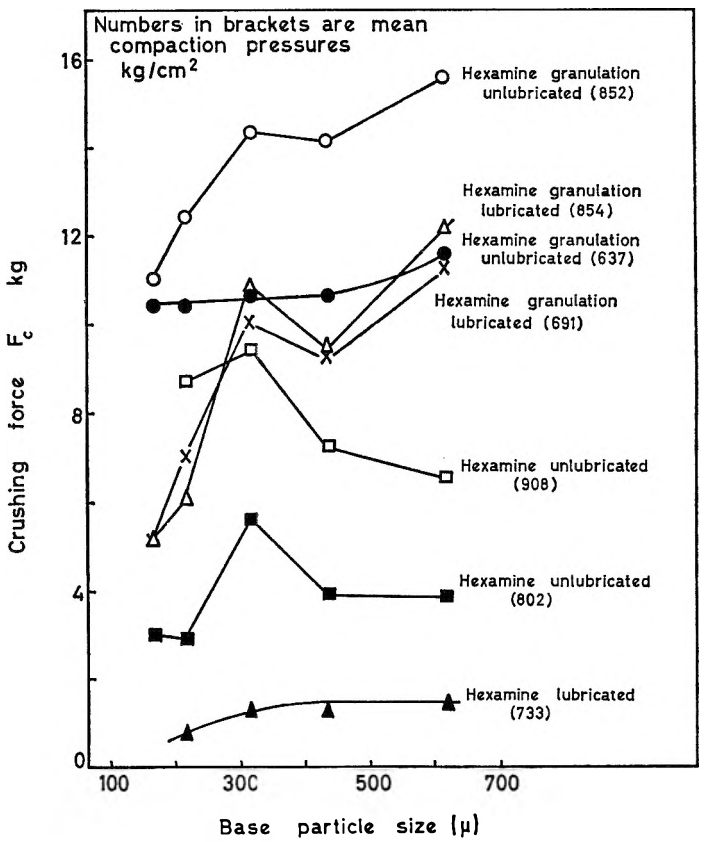


FIG. 5. Effect of base particle size on crushing strength of hexamine compacts.

4. THE EFFECT OF LUBRICANT PARTICLE SIZE (FIG. 7)

A reduction in lubricant particle size below 30–40 mesh (435 μ) has little or no effect on crushing strength.

Discussion

The strength of a tablet depends on the area of intimate contact between particles and the adhesive strength over the whole area. The work of Bowden & Tabor (1954) in the field of friction and lubrication has indicated that the strongest bonds are formed between clean surfaces, so that the addition of a lubricant to a material to be compacted might be expected to interfere with the adhesive bond between particles. The present work indicates that such interference occurs, for apart from the

reduction in values of crushing strength when lubricant is present, visual observations of the fractured surfaces of tablets made from crystalline materials showed little particle-particle bonding.

Apart from interference by the lubricant forming a physical barrier between particles, the reduction of interparticular friction might well reduce the amount of clean reactive surface produced by shear at the sliding contact areas between particles. The fact that lubricated granu-

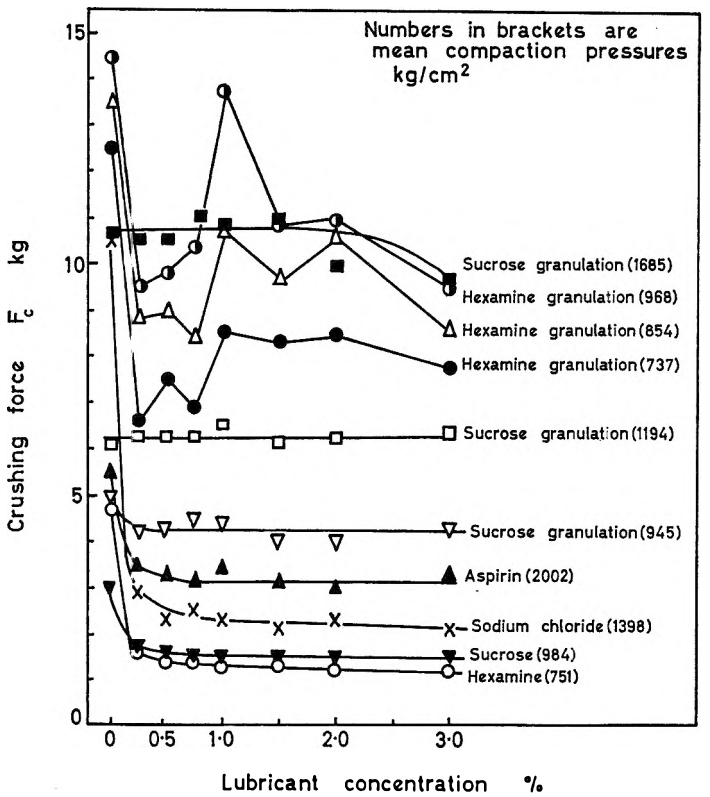


FIG. 6. Effect of lubricant concentration on crushing strength.

lations still produce strong tablets can be accounted for by the observations of Higuchi, Rao, Busse & Swintosky (1953), Higuchi, Elowe & Busse (1954) and Elowe (1954), who found that the surface area of standard granulations increased to a maximum and then decreased as compacting pressure was increased. With a granulation lubricated externally with magnesium stearate powder, most of this new and reactive surface will remain uncontaminated by the lubricant and produce strong bonds. Crystalline substances do not undergo the same degree of fragmentation and will be affected by the lubricant to a greater extent. Table 1 compares the crushing strength of tablets made from lubricated and unlubricated materials at a mean compaction pressure of 1,000 kg/cm².

TABLE 1. THE EFFECT OF LUBRICANT ON CRUSHING STRENGTH
Mean compaction pressure = 1,000 kg/cm²
Lubricant: 2% magnesium stearate — 100 mesh

Material 30–40 mesh except*	Crushing strength kg		Reduction in strength %
	Unlubricated	Lubricated	
Sucrose	2·8	1·4	50·0
Sucrose granulation	4·9	4·0	18·4
Hexamine	5·4	1·3	76·0
Hexamine granulation	14·6	10·5	28·1
Aspirin	3·6	2·7	25·0
Sodium chloride* 40–60 mesh	9·1	2·6	71·4

A linear relationship between strength values and the logarithm of the compaction pressure is quoted for a number of materials (Ganderton, 1962; Higuchi & others, 1953, 1954; Nelson, 1956). We have found unlubricated sodium chloride to deviate from a logarithmic relationship at 1,450 kg/cm², the maximum pressure attainable with the apparatus used. However the presence of a lubricant facilitates compaction to a greater pressure level and Fig. 2 confirms that for sodium chloride the logarithmic

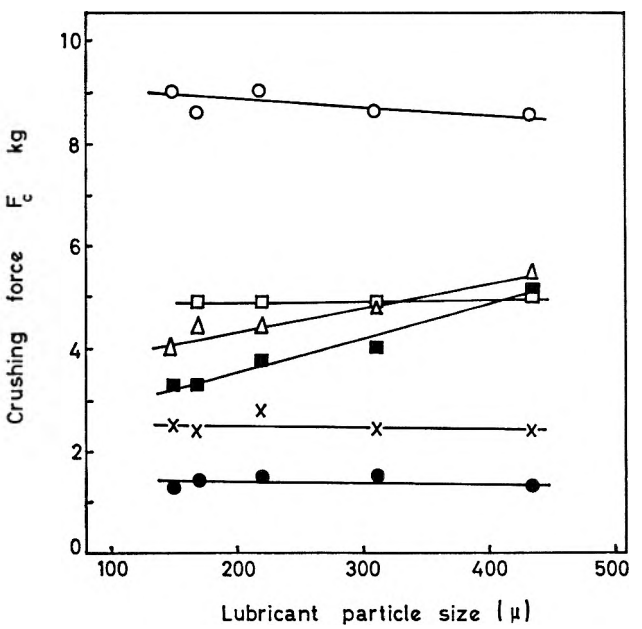


FIG. 7. Effect of lubricant particle size on crushing strength.

		P_m (kg/cm²)
○	Hexamine granulation	728
□	Sucrose granulation	961
△	Aspirin	2025
■	Sucrose	1911
X	Sodium chloride	1418
●	Hexamine	757

relationship breaks down at approximately 1,500 kg/cm². Such a deviation was also noted by Higuchi & others (1954) for sulphadiazine granulation.

On the other hand Huffine (1953) states that the breaking strength of tablets of sodium chloride and of sucrose is directly proportional to the applied pressure, a view supported by the work of Henderson (1962) on sodium chloride and sodium bromide, although his results for tablets of urea, potassium chloride and potassium bromide might be represented equally well by a logarithmic relationship.

That 2% 30–40 mesh lubricant should interfere with bonding to the same extent as −100 mesh powder, was considered surprising (Fig. 7). However, since the lubricant particles were preformed granules of fine powder it is possible that when mixing the lubricant with the base material, sufficient fine powder was transferred to the surface of the base particles to reduce the strength of the bond. A breaking down or spreading of the larger lubricant particles was not evident.

Little work has been reported on the comparative effect of lubricant concentration on the crushing strength of tablets made from crystals and granulations prepared without adhesives. Strickland (1956) states that increasing concentration produces softer tablets but said the effect was negligible at concentrations below 1%. The present work indicates that for crystalline materials 0·25% lubricant produces almost the maximum reduction in strength, and the effect of lubricant concentration on simple granulations varies with the nature of the base material.

The crushing strength of tablets made from the sucrose granulation is only slightly affected by increased lubricant concentration. This is consistent with the hypothesis of appreciable fragmentation producing a clean reactive surface for re-bonding. Using hexamine granulation, 0·25% lubricant produces a marked reduction in strength but as the concentration increases to 1% the strength increases before again decreasing. No explanation is offered for this behaviour which was absent in the crystalline hexamine, but similar behaviour was reported by Ganderton (1962) who lubricated crystalline hexamine with hard paraffin. Work in progress is designed to clarify this point.

The effect of particle size on the crushing strength of tablets made from unlubricated crystalline materials is related to the mechanism of failure (Shotton & Ganderton, 1961). Aspirin has a weak interparticulate bond and when unlubricated shows little particle size effect except for a weakening with 80–100 mesh fractions. In the presence of lubricant, a reduction in size from 20–30 to 60–80 mesh increases the crushing strength of the aspirin compact, with no appreciable effect on particle bonding. This is possibly due to a more uniform distribution of the applied pressure. The lubricant abolishes particle size effects with crystalline hexamine, but has no effect on sodium chloride, which is a harder, cubic, crystal capable of penetrating the lubricant film.

The effect of base particle size on tablets of unlubricated hexamine granulation (Fig. 5) is difficult to interpret; it is unlike that for hexamine crystals or for the granulation of sucrose. The observed behaviour of

hexamine granulation is not due to mechanical defects, e.g., no laminations were found in any tablets of this material at the mean compaction pressures used.

The results from lubricated granules suggest that hexamine granules tend to deform whilst sucrose granules fracture. If, as suggested earlier, fracture of granules is necessary to obtain satisfactory tablet strength in the presence of a lubricant, the bonding of those granules which only deform will be weakened by lubricant films. However, even those granules which fill the void space in the compacting mass largely by deformation, are likely to be subjected to some degree of fragmentation when the granules are large; the importance of fracture and fragmentation becoming less as the granule size decreases. The lubricant powder will have relatively less adverse influence on the bonding of larger granules than on that of smaller ones, because of the improved bonding which arises after fragmentation. Thus where fracture of the hexamine granules is most probable (with 20–30 mesh granules, Fig. 5) the tablet strength is greatest, but the loss of strength is more rapid with the hexamine granulation when granule size is reduced than with sucrose granulation.

Visual observation of the fractured surfaces of all tablets of crystalline materials showed that magnesium stearate powder affected the mode of bonding in the same manner as that recorded by Shotton & Ganderton (1961) for coatings of stearic acid. No visual difference was observed in the fractured surfaces of tablets made from granulations with and without lubricant. Of particular note is that lubrication eliminated the capping effect in crystalline hexamine but not in the hexamine granulation (Cf. Figs 1 and 2). If the elimination of capping is due to stress relief at the crystal boundary, as suggested by Shotton & Ganderton (1961), it would appear that 2% magnesium stearate applied externally to hexamine granules is insufficient to allow stress relief between granules.

References

Bowden, F. P. & Tabor, D. (1954). *The Friction and Lubrication of Solids*, Clarendon Press, Oxford.
Elowe, L. N., Higuchi, T. & Busse, L. W. (1954). *J. Amer. pharm. Ass. Sci. Ed.*, **43**, 718–721.
Ganderton, D. (1962). Ph.D. Thesis, University of London.
Henderson, N. L. (1962). Ph.D. Thesis, Temple University.
Higuchi, T., Rao, A. N., Busse, L. W. & Swintosky, J. V. (1953). *J. Amer. pharm. Ass. Sci. Ed.*, **42**, 194–200.
Higuchi, T., Elowe, L. N. & Busse, L. W. (1954). *Ibid*, **43**, 685–689.
Huffine, C. L. (1953). Ph.D. Thesis, Columbia University.
Munzel, K. & Kagi, W. (1954). *Pharm. Acta Helvet.*, **29**, 53–71.
Nelson, E. (1956). *J. Amer. pharm. Ass. Sci. Ed.*, **45**, 354–355.
Shotton, E. & Ganderton, D. (1960). *J. Pharm. Pharmacol.*, **12**, *Suppl.* 87*T*–92*T*.
Shotton, E. & Ganderton, D. (1961). *Ibid.*, **13**, *Suppl.* 144*T*–152*T*.
Strickland, W. A., Nelson, E., Busse, L. W. & Higuchi, T. (1956). *J. Amer. pharm. Ass. Sci. Ed.*, **45**, 51–55.

The paper was presented by DR. LEWIS.

J. Pharm. Pharmacol., 1964, **16**, *Suppl.*, 121*T*–123*T* Received April 29, 1964

SHORT COMMUNICATION

Tensile strength of sterilised surgical catgut

J. OWEN DAWSON, T. W. ROYLANCE AND T. SMITH

THE test results for tensile strength obtained by us in a recent survey of currently available British-made surgical catgut showed all samples to have a comfortable margin over the B.P. requirements. In tests performed strictly in accordance with B.P. conditions, values ranged from 144% to 178% of the B.P. requirement. These same samples, moreover, had a performance of 92% to 123% of the U.S.P. strength requirement, and the U.S.P. standard therefore more closely reflects performance. No work has been published so far comparing the two test methods in detail, that reported herewith attempts to cover this ground.

EXPERIMENTAL AND RESULTS

The experiment was designed to cover all the conditions specified in the two monographs and all possible combinations.

The testing machines were calibrated by dead weights. The 0–15 lb and 0–75 lb scales were used on the B.P. machine, and the 0–10 lb and 0–20 lb scales on the U.S.P. machine.

The temperature of the testing laboratory was maintained at 21°, and the relative humidity at 65% for testing under B.P. conditions and at 45% for testing under U.S.P. conditions.

After tying the surgeon's knot strictly in the manner specified, a length of rubber tube being used as a former in the U.S.P. test, two knot pull determinations were made on each string and, to balance results, 12 strings were submitted to each test although the U.S.P. calls for only 10. The material used consisted of 5 ft strings from a normal day's raw string production. Thus all the strings of any one gauge were produced from the same supply of sheep casings and from the same ply looping. Chromicised strings of normal manufacture in gauges 3/0, 0 and 2, covering the popular range, were examined. They were wound and sealed in foil packets containing approximately 3·5 ml of tubing fluid and sterilised by gamma irradiation from a ^{60}Co source with a dosage of 2·5 Mrad. The samples were aged for 5 months before testing.

The experiment was run in duplicate with two tubing fluids, 90% isopropanol or 95% industrial methylated spirits. The strings were distributed at random to ensure that no single section of the experiment was unduly biased.

A typical example of the data collected is given in Table 1. The results obtained by calculating the test results strictly in accordance with the respective monographs are abstracted and shown in Table 2.

We found that the U.S.P. machine usually gave a higher value than

From the Laboratories of Ethicon Limited, Bankhead Avenue, Edinburgh, 11.

the B.P. machine, the advantage being about 10% at the lower end of the range and about 5% at the higher end of the range. The B.P. average, i.e., averaging the lower of each of the two pulls determined on each string, gives a value some 5% lower than that obtained by averaging all 24 readings made for each condition examined. The Relative

TABLE 1. GAUGE 0–90% ISOPROPANOL

R.H.	Wait	Knot on former	B.P. machine			U.S.P. machine		
			Diameter thou"	Av. 12 pulls (lb)*	Av. 24 pulls (lb)	Diameter thou"	Av. 12 pulls (lb)*	Av. 24 pulls (lb)
45%	No	No	18·9	7·7 (0·83)	7·8 (0·94)	18·9	8·1 (0·38)	8·5 (0·56)
		Yes	18·9	6·6 (0·42)	6·8 (0·51)	18·9	7·1 (0·50)	7·3U (0·65)
	10 min	No	18·5	8·9 (0·96)	9·5 (1·02)	18·4	8·1 (1·42)	8·7 (1·47)
		Yes	18·6	7·1 (0·64)	7·5 (0·76)	18·4	7·3 (0·93)	7·9 (1·02)
65%	No	No	18·8	7·4 (0·44)	7·7 (0·60)	18·9	7·8 (0·87)	7·7 (0·86)
		Yes	18·7	6·5 (0·31)	6·9 (0·57)	18·9	6·8 (0·43)	7·1 (0·52)
	10 min	No	18·4	8·5B (0·81)	8·9 (0·75)	18·5	8·7 (0·65)	9·1 (0·77)
		Yes	18·5	6·8 (0·63)	7·1 (0·66)	18·5	7·2 (0·49)	7·7 (0·71)

U—Signifies tested strictly in accordance with the U.S.P. XVI.
B—Signifies tested strictly in accordance with the B.P. 1963.
Note.—Figures in parentheses indicate the standard deviation.
* i.e. Average of the lower pull on each of 12 strings.

Humidity, i.e., 45% or 65%, had practically no effect. The waiting time specified in the B.P. leads to a higher value of tensile strength—of about 10% overall.

Finally, the effect of tying the surgeon's knot on a former as in the U.S.P. test is to reduce the value obtained by about 10% although the effect was not so extreme with gut tubed in 95% industrial methylated spirits.

TABLE 2. RESULTS OBTAINED WHEN TESTS MADE STRICTLY IN ACCORDANCE WITH MONOGRAPHS

Sample		B.P. requirement (lb)	U.S.P. requirement (lb)	B.P. test			U.S.P. test	
				Actual (lb)	% B.P. Std.	% U.S.P. Std.	Actual (lb)	% U.S.P. Std.
Gauge 3/0	90% I.P.A.*	1·5	2·5	3·1	207	124	3·4	136
	95% I.M.S.†	1·5	2·5	3·6	240	144	3·2	128
Gauge 0	90% I.P.A.	3·5	5·5	8·5	243	155	7·3	133
	95% I.M.S.	3·5	5·5	7·0	200	127	6·8	124
Gauge 2	90% I.P.A.	6·5	9·0	14·5	223	161	12·2	136
	95% I.M.S.	6·5	9·0	14·0	215	156	12·3	137

* Isopropanol. † Industrial methylated spirits.

DISCUSSION

The differences between the two tests tend to cancel themselves out. Thus the advantage in strength readings obtained on the Incline Plane Tester are compensated to a large extent by the tying of the surgeon's knot on a former (U.S.P.), and the stringency of the B.P. calculation is nullified by the advantage gained by allowing the material to "air off" before testing. Catgut is packaged with a slight excess of water to increase pliability and airing off the suture may reduce the moisture content to that giving peak tensile strength, or it may go beyond it, so giving a lower value of strength. The 10 min period at 60–80% R.H. specified in the B.P. does not achieve equilibrium, as can be demonstrated by the effect of differing tubing fluids on the strength. Furthermore, the residual moisture content may also be affected by the rate of air-flow over the material. The unstable condition is reflected in a larger standard deviation which is observed after waiting; this occurs particularly in the gauge 2 material.

It is obvious from these results that the current B.P. standards do not compare with those of the U.S.P., nor do they reflect the actual performance of British sterilised surgical catgut.

It is recommended that the B.P. raise the existing standards to equal numerically those required by the U.S.P. XVI. Since the Incline Plane Tester is not available in this country, the present method of test and calculation should be retained, although it would be more practical to carry out the test immediately after opening the container. It is then suggested that the atmospheric conditions be revised to a temperature of 18–22° and a relative humidity of not less than 40%.

Table 3 shows the effect of these modifications in relation to the proposed new standard.

TABLE 3. RESULTS OBTAINED WHEN TESTS MADE BY THE PROPOSED METHOD AND COMPARED WITH PROPOSED NEW STANDARD

Sample		Proposed B.P./U.S.P. requirement (lb)	Actual test result by proposed method (lb)	Result expressed as % of standard
Gauge 3/0	90% I.P.A. . .	2·5	3·3	132
	95% I.M.S. . .	2·5	2·6	104
Gauge 0	90% I.P.A. . .	5·5	7·7	140
	95% I.M.S. . .	5·5	6·0	109
Gauge 2	90% I.P.A. . .	9·0	11·4	127
	95% I.M.S. . .	9·0	10·3	115

Acknowledgement. We wish to express our thanks to Mrs. Mary Heigh for assistance in carrying out the many strength determinations involved in this work.

The paper was presented by MR. DAWSON.

J. Pharm. Pharmacol., 1964, **16**, *Suppl.*, 124*T*–126*T* Received April 30, 1964

SHORT COMMUNICATION

Bactericidal effect upon *Pseudomonas aeruginosa* of chemical agents for use in ophthalmic solutions

W. B. HUGO* AND J. H. S. FOSTER†

THE problem of protecting ophthalmic solutions against bacterial recontamination has been the subject of much discussion (Klein, Millwood & Walther, 1954; Brown, Foster, Norton & Richards, 1964; Foster, 1964; Anderson, Lillie & Crompton, 1964).

From an appraisal of available data two facts emerge, firstly that potentially the most dangerous and a frequent contaminant is *Pseudomonas aeruginosa* and secondly that data of valid application to the problem of destroying *Ps. aeruginosa* in eye drops cannot be obtained from the literature of bacteriology.

This communication reports upon the ability of eight bactericides to kill *Ps. aeruginosa* under experimental conditions related directly to the problem.

METHODS

The essentials of the method were that precise and low levels of an inoculum of *Ps. aeruginosa* NCTC 7244 were used to infect 2 ml amounts of solutions of the bactericide under test, contained in 4 ml bijou bottles; these were then held for the desired time and temperature. At the end of the reaction time the whole of the contents of the bottle were sampled into 100 ml of molten cooled agar contained in a 14 cm diameter Petri dish. (This method obviated sampling errors.) The agar contained suitable inactivators or effected inactivation by dilution because of the relative volumes used; control experiments showed that inactivation was achieved (Foster, 1964).

RESULTS AND DISCUSSION

From data obtained from these experiments two sets of results are presented. (1) Table 1 records the concentration of each substance required to reduce the viable count of an inoculum of circa 10 and 100 organisms/ml to zero in 30 min, using three temperatures, 4°, 18° and 30°, representing refrigerator temperature, and room temperature in temperate and tropical conditions. (2) Table 2 lists the times required to kill 100 organisms/ml at 18°, using selected concentrations of the bactericides.

It is clear from Table 1 that solution for eye drops B.P.C. (methyl *p*-hydroxybenzoate 0·0229 and propyl *p*-hydroxybenzoate 0·0114%) cannot be regarded as a satisfactory preservative (see also Hugo & Foster, 1964) and that the concentrations of the constituent esters need to be

* From the Department of Pharmacy, The University, Nottingham, and the
† Department of Pharmacy, Bristol College of Science and Technology.

TABLE 1. CONCENTRATION OF BACTERICIDE (% w/v) TO REDUCE VIABLE COUNT OF AN INOCULUM OF *Ps. aeruginosa* TO ZERO IN 30 MIN

	4°		18°		30°	
	10 orgs/ml	100 orgs/ml	10 orgs/ml	100 orgs/ml	10 orgs/ml	100 orgs/ml
Chlorocresol	0·07	0·10	0·05	0·07	0·04	0·05
Thiomersal	0·00175	0·00225	0·001	0·002	0·001	0·0015
Phenylmercuric nitrate	0·002	0·00225	0·00125	0·002	0·00075	0·001
Methyl *p*-hydroxybenzoate 2 parts propyl *p*-hydroxybenzoate 1 part	0·20	0·25	0·125	0·20	0·075	0·1
2-Phenylethanol	1·0	1·0	0·9	0·9	0·5	0·7
Benzalkonium chloride	0·08	0·1	0·05	0·08	0·01	0·02
Chlorhexidine	0·005	0·007	0·003	0·005	0·002	0·002
Chlorbutol	0·7	0·7	0·6	0·7	0·3	0·5

raised to between four and eight times the B.P.C. levels (dependent on the level of contamination) to destroy *Ps. aeruginosa* in 30 min at 18°.

The results also show that thiomersal and phenylmercuric nitrate are satisfactory bactericides for the organism although there are reports, as yet unpublished, that prolonged use of these mercurial compounds can lead to deposition of mercury in the cornea. Chlorocresol, which also has a satisfactory killing action, Table 2, was adopted in the 1963 B.P.C. but has since been rejected in favour of solution for eye-drops because of reports that it caused damage to the anterior chamber of the eye (Crompton & Anderson, 1963).

TABLE 2. TIMES TO STERILISE AN INOCULUM OF *Ps. aeruginosa* (100 ORG/ML) AT 18°

Bactericide %	(Time, min)
Chlorocresol (0·1)	10
Thiomersal (0·01)	10
Phenylmercuric nitrate (0·001)	40
Solution for Eye Drops B.P.C.	>60
2-Phenylethanol (0·6)	45
Benzalkonium chloride (0·01)	45
Chlorhexidine (0·05)	15
Chlorhexidine (0·005)	30
Chlorbutol (0·5)	45

Chlorhexidine is also efficient in killing the test organism (Table 2) but it is precipitated by bicarbonates, borates, phosphates and sulphates as well as by fluorescein and physostigmine (Mitchell, Keane & Scotis, 1961); this limits its usefulness in ophthalmic preparations. Benzalkonium chloride is satisfactory but has had the criticism levelled against it that it causes solubilisation of the cement of the corneal epithelium (Klein, Millwood & Walther, 1954). This concept has arisen from a paper by Ginsberg & Robson (1949) who reported such an effect with anionic and non-ionic detergents. These authors, however, did not use a quaternary ammonium (cationic) detergent and Buschke (1949) reports no adverse effect on corneal epithelium by benzalkonium chloride in

concentrations up to 10%. At 18°, 2-phenylethanol (0·6%) kills an inoculum of 100 organisms/ml in 45 min (Table 2), while a concentration of 0·9% is required to achieve a kill in 30 min. In recommending 0·5%, Brewer, Goldstein & McLaughlin (1953) report that this concentration would kill an inoculum of $3{\cdot}6 \times 10^4$ organisms/ml in seven days. A seven-day endpoint is not a satisfactory choice for the problem and our findings support those of Klein & others (1954) that 2-phenylethanol should be used at concentrations greater than 0·5%. It is used with benzalkonium chloride in certain B.P.C. formulations.

Chlorbutol is satisfactory only at concentrations near saturation (0·8%) and has the disadvantage of losing its effectiveness on storage.

This work must clearly be supported by clinical appraisal but it would appear that, provided the question of deposition of mercury is solved satisfactorily, the mercurial compounds are of value as preservatives of ophthalmic solutions, and, where no incompatibility occurs, chlorhexidine has an effective lethal action.

Benzalkonium, too, is a satisfactory compound; the suggestions concerning its solubilising effect on the corneal epithelium have been an obvious bar to its more general adoption.

2-Phenylethanol is satisfactory provided the concentration is higher than 0·5%. It has the additional merit that otherwise it has not given rise to adverse criticism.

References

Anderson, K., Lillie, Susan & Crompton, D. (1964). *Pharm. J.*, **192**, 593–594.
Brewer, J. H., Goldstein, S. W. & McLaughlin, C. B. (1953). *J. Amer. pharm. Ass. Sci. Ed.*, **42**, 584–585.
Brown, M. R. W., Foster, J. H. S., Norton, D. A. & Richards, R. M. E. (1964). *Pharm. J.*, **192**, 8.
Buschke, W. (1949). *J. cell. comp. Physiol.*, **33**, 145–176.
Crompton, D. O. & Anderson, K. (1963). *Lancet*, **2**, 1279.
Foster, J. H. S. (1964). M.Pharm. Thesis. Nottingham University.
Ginsberg, M. & Robson, J. M. (1949). *Brit. J. Ophthalmol.*, **33**, 574–579.
Hugo, W. B. & Foster, J. H. S. (1964). *J. Pharm. Pharmacol.*, **16**, 209.
Klein, M., Millwood, E. G. & Walther, W. W. (1954). *Ibid.*, **6**, 725–732.
Mitchell, J. A., Keane, R. M. & Scotis, N. G. (1961). *Aust. J. Pharm.*, **42**, 1284–1292.

The paper was presented by MR. FOSTER.

J. Pharm. Pharmacol., 1964, **16**, *Suppl.*, 127*T*–130*T* Received April 27, 1964

SHORT COMMUNICATION

Some properties of bronopol, a new antimicrobial agent active against *Pseudomonas aeruginosa*

BETTY CROSHAW, M. J. GROVES AND B. LESSEL

MARKED antimicrobial activity was noted in a series of aliphatic halogeno-nitro compounds (unpublished observations of Clark, Croshaw, Leggetter & Spooner). Of these, 2-bromo-2-nitropropane-1,3-diol (bronopol) has been further investigated because it is more stable in aqueous media than other members of the series.

This preliminary communication describes some properties of bronopol.

EXPERIMENTAL AND RESULTS

Physical properties. Bronopol is a colourless, odourless, crystalline solid m.p. 121°. It is slightly hygroscopic, readily soluble in water, lower alcohols and glycols but only slightly soluble in oils; the distribution coefficient water: chloroform is 14·7: 1 at 22–24°. The pH of an aqueous solution varies between 5·1 and 5·5 according to the concentration and falls slowly on storage, the rate of fall increasing with increased temperature.

Antimicrobial activity. The inhibitory activity of bronopol (Table 1) was determined *in vitro* by serial dilution in agar and surface-inoculation using a multi-point inoculator (Hale & Inkley, 1964). The inoculum was 0·01 ml of 18 hr broth cultures of the test bacteria or yeasts, or 0·01 ml of spore suspensions prepared from 7 day cultures of the fungi. The minimum inhibitory concentrations were noted after 24 hr at 37° for the bacteria, 48 hr at 26° for the yeasts and 120 hr at 26° for the fungi.

Bronopol is more active against bacteria than against fungi or yeasts; all bacteria tested, including *Pseudomonas aeruginosa*, were inhibited at 12·5–50 μg/ml. A comparison of the bacteriostatic activity of bronopol and other antibacterial agents against some strains of *Ps. aeruginosa* is shown in Table 2. This inhibitory activity falls two- to eight-times when the pH increases from pH 5·3 to pH 7 or 8. The inhibitory activity is decreased three to four-times in the presence of 75% ox serum or 0·1% cysteine hydrochloride, whilst 75% oxalated horse blood reduces the activity at least thirty-two-fold. Tween 80 1%, suramin 1%, Lubrol W 1% or lecithin 0·1% had no effect on activity.

The bactericidal activity of bronopol was determined using bacterial suspensions prepared by washing off the organisms from an 18 hr agar slope culture and standardising by opacity to approximately 1×10^6 organisms per ml. 1 ml of such a suspension was added to 9 ml of aqueous solution of the compound at 22° and plate counts were made on samples after 15, 30 and 60 min intervals. Bactericidal activity is greater

From the Research Department, Boots Pure Drug Co. Ltd., Nottingham.

against Gram-negative bacteria than against Gram-positive cocci (Table 3), and it shows little variation over a pH range of 5–8. The addition of 50% ox serum has little effect on the bactericidal activity and solutions containing 1·6 mg/ml of bronopol in 50% blood gave at least a 90% reduction in the number of viable cells of *Ps. aeruginosa* (10S) and *Salmonella typhosa* (TY2) in 1 hr.

Twelve daily passages *in vitro* in liquid medium in the presence of bronopol of *Ps. aeruginosa* and *Staphylococcus aureus* have not increased the resistance of these organisms to the compound.

TABLE 1. THE INHIBITORY ACTIVITY OF BRONOPOL IN AGAR*

Organisms	No of strains tested	MIC μg/ml†
Gram-positive bacteria		
Staphyloccus aureus‡	30	12.5–50
Streptococcus pyogenes	2	25
Corynebacterium pyogenes	4	25–50
Bacillus subtilis	1	25
Gram-negative bacteria		
Pseudomonas aeruginosa (see Table 2)	22	25–50
Proteus vulgaris	17	12·5–25
P. rettgeri	1	25
P. morganii	1	25
P. inconstans	1	25
P. mirabilis	3	25–50
Escherichia coli	13	12·5–50
Aerobacter aerogenes	1	25
Salmonella typhosa	2	25
Salm. typhimurium	3	25
Salm. gallinarum	2	25
Salm. enteritidis	2	25
Salm. ser. *dublin*	1	25
Salm. ser. *heidelberg*	2	25
Shigella sonnei	2	25
Klebsiella pneumoniae	2	25
Fungi and yeasts		
Trichophyton mentagrophytes	1	200
T. rubrum	1	100–200
T. tonsurans	1	100
Microsporum canis	1	100–200
Cladosporium herbarum	1	400
Penicillium roqueforti	1	400
Candida albicans	3	400–>400

* "Oxoid" blood agar base (CM 55) for the bacteria (with the addition of 2·5% glucose and 10% ox serum for *Str.* and *Corynebacterium pyogenes*) and "Oxoid" Sabouraud dextrose agar (CM 41) for the fungi and yeasts.
† MIC after 24 hr at 37° for bacteria, 48 hr at 26° for yeasts and 120 hr at 26° for fungi.
‡ Benzylpenicillin-resistant and -sensitive organisms tested.

TABLE 2. THE BACTERIOSTATIC ACTIVITY OF BRONOPOL AND OTHER ANTIBACTERIAL AGENTS AGAINST 22 STRAINS OF *Pseudomonas aeruginosa*

Compound	No of strains with MIC* (μg/ml)			
	25	50	100	>100
Bronopol	14	8		
Chlorhexidine digluconate B.P.	0	0	2	20
Hexachlorophane B.P.	0	9	13	
Cetrimide B.P.	0	0	0	22
Domiphen bromide B.P.	0	0	0	22
Phenoxyethanol B.P.C.	0	0	0	22
Dequalinium chloride B.P.	0	0	0	22

* Serial dilution in agar with surface inoculation incubated for 24 hr at 37°.

Using *Ps. aeruginosa* as test organism, preparations of the compound were assayed by agar diffusion at pH 5·3 (the zones of inhibition were greater at this pH). There is a marked loss of microbiological activity in the presence of cetomacrogol B.P.C., pure propylene glycol or polyethylene glycol 300 although 20% aqueous dilutions of the two glycols appear to have no effect on the compound. Unbuffered aqueous solutions are relatively stable at temperatures up to 50° but solutions buffered at pH > 5 are less stable. Concentrated solutions buffered at pH > 5 turn brown when exposed to heat or light; this discolouration, which can be prevented by the addition of sodium metabisulphite, is not always associated with loss of microbiological activity.

TABLE 3. THE BACTERICIDAL ACTIVITY OF BRONOPOL IN AQUEOUS SOLUTION AGAINST THREE SPECIES OF BACTERIA

Concentration of bronopol mg/ml	% reduction* in number of viable organisms at time in min (Temp. 22°)								
	Ps. aeruginosa (10S)			*Salm. typhosa* (TY2)			*Staph. aureus* (NCTC 8452)		
	15	30	60	15	30	60	15	30	60
1·6	99·9	>99·9	>99·9	99·9	>99·9	>99·9	90	92	99
0·8	99	99·9	99·9	99	99·9	99·9	30	51	80
0·4	40	98	99·9	50	99	99·9	<10	<10	40

* Compared with a water control tube with initial inoculum level of approx. 10^6 organisms per ml.

Toxicity. In aqueous solution the acute LD50 of bronopol (based on small numbers of animals and estimated graphically) to mice is 350 mg/kg orally and 20 mg/kg intraperitoneally; to rats the figure is 400 mg/kg orally and 200 mg/kg subcutaneously.

Male and female albino rats (5–6 weeks old) were fed concentrations of 100 and 1,000 p.p.m. in the diet for twelve weeks, so that the average daily doses ingested were roughly equivalent to one-fortieth and one-quarter of the LD50 respectively; control rats were fed plain diet. Both dose levels were tolerated by the animals without any effect on growth, food consumption, blood picture, liver and kidney weights or histopathology of major organs.

Concentrations of 0·5 and 2·0% in an emulsion base and in solution were tested in rabbits for irritation to skin and to the mucous membrane of the eye. The 2·0% concentration was irritant to skin and to the eye after one application while there was no observable difference in the local reaction between the 0·5% concentration and the vehicle alone after application on four successive days.

Guinea-pigs were injected intradermally with 0·05% aqueous solution on alternate days for a total of ten doses (one of 0·1 ml followed by nine of 0·05 ml). This was followed two weeks later by injection of a challenging dose of 0·05 ml; no evidence of skin sensitisation was observed.

DISCUSSION

Unlike many antibacterial agents, bronopol has activity against *Pseudomonas aeruginosa* as well as against other Gram-negative and Gram-positive bacteria and to a lesser extent against fungi.

Its wide spectrum of activity makes it suitable as a preservative, and its relatively low toxicity on chronic oral administration to rats suggests that it may be safe for use in the preservation of oral medicaments.

The absence of local irritancy to the skin and mucous membranes of experimental animals at a concentration of 0·5%, together with the fact that lower concentrations (0·1 or 0·2%) are bacteriostatic and bactericidal, also suggests that bronopol could be used as a topical antibacterial agent. It shows optimum antibacterial activity and optimum stability in aqueous vehicles at a slightly acid pH; such properties are desirable in a topical skin formulation.

Reference

Hale, L. J. & Inkley, G. W. (1964). *Lab. Pract.*, in the press.

The paper was presented by MR. GROVES.

J. Pharm. Pharmacol., 1964, **16**, *Suppl.*, 131*T*–135*T* Received May 1, 1964

Umbelliferous fruit identification by thin-layer chromatography

T. J. BETTS

Unknown umbelliferous fruits, including powders, may be identified by thin-layer chromatography of petroleum extracts on silicic acid containing fluorescein. The constituents of the essential oils thus extracted are usually diagnostic. The method is particularly useful in distinguishing Indian dill (*Anethum sowa* Roxb.) from dill (*A. graveolens* L.), and will detect an admixture of one part of the former in four parts of the latter.

WHILST whole umbelliferous fruits can be distinguished more or less readily, one from another, powdered fruits present a much more difficult problem, and in the case of the two dills, *Anethum graveolens* L. and *A. sowa* Roxb., they appear to be indistinguishable. As a method of examining quickly the constituents of such fruits, thin-layer chromatography seemed a suitable method. Silicic acid—starch "chromatostrips" (Kirchner, Miller & Keller, 1951) provided one of the earliest forms of thin-layer chromatograms; these were devised to study terpenes including limonene and carvone, and were subsequently used for essential oils. By such means the oils distilled from the following umbelliferous fruits have been examined: anise (Paris & Godon 1961, Schrantz, Lopmeri, Strömmer, Salonen & Brunni 1962, Wellendorff 1963); caraway (El-deeb, Karawya & Wahba 1962); coriander (Pertsev & Pivnenko 1962, Wellendorff 1963); and fennel (Schrantz & others 1962; Wellendorff, 1963). The method has been applied here to petroleum extracts of these and other umbelliferous fruits.

Experimental

Silicic acid ("Kieselgel G" Macherey, Nagel) was spread 0·25 mm thick after mixing with 0·05% aqueous fluorescein sodium (25 g to 50 ml). The plates were heated at 105° for 30 min, then stored over a desiccant. The running solvent, chloroform: benzene, 1 : 1 by volume was used in a tank having the walls lined with absorbent paper wetted with the solvent.

The umbelliferous fruits (500 mg) were extracted with light petroleum* (5 ml) at room temperature. Powdered fruits were shaken with the solvent and used as soon as the suspension had settled. Whole fruits were broken in a mortar, the tougher ones being triturated with a little washed sand, before extraction. Approximately 0·03 ml of the extract was applied to the thin-layer plate by ten successive applications with a glass rod to produce a spot which was about 1 cm in diameter. The chromatographic solvent was allowed to rise about 15 cm from the starting line; this took about 40 min at 20°.

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

* Where light petroleum is used this refers to the fraction b.p. 60–80°.

Spot detection. The plate was first examined in ultra-violet light ("Hanovia" lamp with 366 mμ filter) and the presence of any dark, fluorescence-quenching spots noted against the bright yellow fluorescein background. After brief treatment with bromine vapour to convert the fluorescein to eosin, the plate was re-examined in ultra-violet light to note any persistent fluorescein fluorescence, indicating spots of unsaturated substances against the dull background. The plate was then sprayed with a saturated solution of 2,4-dinitrophenylhydrazine in N hydrochloric acid, which revealed some ketones and aldehydes as orange spots. When the plate had air-dried, it was finally sprayed with sulphuric acid containing 1% vanillin. With this, some substances gave coloured spots immediately or in a few minutes (e.g. khellin, thymol), but others (e.g. fenchone) were only properly visible in up to 12 hr at room temperature.

Results and discussion

Table 1 records observations made on reference substances known to be present in certain umbelliferous fruits. Table 2 lists the results obtained with several different samples of various umbelliferous fruits, extracted and chromatographed by the method above. Table 3 is in the form of a key derived from Table 2, and provides an aid to identifying an unknown umbelliferous fruit.

TABLE 1. RESULTS OBTAINED WITH REFERENCE SUBSTANCES

Substance (and source)	Average Rf value	Fluoresc.-quenching	See after bromination	Dinitro ph. hydrazine	Vanillin/ sulph. acid
(+)-Limonene (B)	0·79	−	+	−	yellow then brown
Anethole "pure 21/22°" (B)[1] ..	0·72	+	+	(+)	−
	0·36	−	−	+	−
p-Cuminaldehyde (L)	0 59	−	−	+	−
Thymol (M & B)	0 39	+	+	−	red-mauve
Anisaldehyde (P & S)	0 38	−	−	+	−
(+)-Carvone (B)	(0·36–0·47)	−	+	+	−
Linalöl (P & S)	0·26	−	+	−	mauve-brown
Fenchone[2]	0 19	−	−	−	slow mauve-brown
Khellin[3]	0 02	+	−	−	bright yellow

[1] Anethole gave two spots, the faster moving one being the principal constituent, and the slower moving one corresponding to anisaldehyde, presumably being a decomposition product. The reaction of the faster moving spot to 2,4-dinitrophenylhydrazine is anomalous, and is probably due to other decomposition products appearing at this position. The extracts of umbelliferous fruits known to contain anethole gave similar results.

[2] Fenchone was prepared from (+)-fenchyl alcohol by chromic acid oxidation. A light petroleum extract of the crude reaction mixture was used; this was chromatographically distinct from fenchyl alcohol. This saturated ketone is unreactive, and did not form a hydrazone on spraying with dinitrophenylhydrazine.

[3] Khellin was prepared from *Ammi visnaga* L. by extraction with ether, and crystallisation from water, m.p. 155°.

With the exception of carvone the Rf values quoted above are constant to ±0·02 if the solvent mixture is allowed to rise exactly 15 cm from the starting line. For carvone the range of values observed is given in brackets.

Compounds such as anethole and thymol, which exhibit fluorescence-quenching, are visible after bromination of the plate as a fluorescing halo.

The reference substances were obtained from (B) W. J. Bush & Co. Ltd., and (P & S) Polak & Schwarz Ltd. (International Flavors & Fragrances Ltd.) and are here acknowledged. Commercial samples were from (L) L. Light & Co. Ltd. and (M & B) May & Baker Ltd.

The essential oils of umbelliferous fruits contain true terpenes such as limonene, α-phellandrene and α-pinene which are visible as a bright fluorescent spot after bromination of the plate. They also give a slight

dark purple, or a brief yellow-brown response to vanillin in sulphuric acid. With the solvent system used here they appear together as the fastest moving spot (Rf ~ 0·80). They appear to have been largely lost from powdered samples. As these components appear to have no diagnostic value, they are not included in Tables 2 and 3. The light petroleum extracts in addition contained fixed oil from the seed endosperm. This was as readily extracted from the broken whole fruits as from the powder. Like arachis and cottonseed oils, this provided a spot of Rf approximately 0·60 to 0·70, visible as a bright fluorescence after bromination of the plate. Only with parsley fruits is this spot superimposed on a constituent of the essential oil, and as the latter can still be seen it is not necessary to eliminate the fixed oil by examining a steam distillate. Again, fixed oil spots have no diagnostic value and are not

TABLE 2. RESULTS OF DIAGNOSTIC SIGNIFICANCE OBTAINED WITH UMBELLIFEROUS FRUIT EXTRACTS

Umbelliferous fruit (and source of samples)	Average spot Rf	Reaction and comments (see footnotes)
Ajowan, *Trachyspermum ammi* (L.) Sprague. (P/S Mus, S/P Mus)	0·41	As thymol; q, f, v red-mauve.
Angelica, *Angelica archangelica* L. (B & S)	0·45	?; intense blue fluorescence; others, less intense, follow.
	0·28	?; q, v brown.
Anise, *Pimpinella anisum* L. (B & S, Sc, S/P st)	0·71	As anethole; q, f, (h). With fixed oil tail.
	0·39	As anisaldehyde; h.
Caraway, *Carum carvi* L. (B & S, S/P st)	0·43	As carvone: f, h.
Coriander, *Coriandrum sativum* L. (B & S, Sc, S/P st)	0·26	As linalol; f, v mauve.
Cumin, *Cuminum cyminum* L. (B.D.H., B & S, Sc, S/P st)	0·58	As cuminaldehyde; h.
	0·55	?; f.
Dill, *Anethum graveolens* L. (S/P Mus, S/P st)	0·42	As carvone; f, h.
Fennel, *Foeniculum vulgare* Mill. (Sc, S/P st)	0·72	As anethole; q, f, (h). With fixed oil tail.
	0·38	As anisaldehyde; h.
Indian dill, *Anethum sowa* Roxb. (P/S Mus, S/P Mus, S/P st)	0·57	Dillapiole?; q, f, v mauve-brown.[1]
	0·46	?; f.
	0·41	As carvone; f, h.
Parsley, *Petroselinum crispum* (Mill.) Airy-Shaw. (B & S, C "Double Curled")	0·64	Apiole?; q, f, v brown. Horse-shoe shape, superimposed on fixed oil.
	0·47	?; f.
	0·33	?; q, f.
Visnaga, *Ammi visnaga* L. (B & S, S/P st)	0·04	As khellin; q, v bright yellow.

[1] The tentative identification of the spot from Indian dill with Rf 0·57 as dillapiole is based on its fluorescence-quenching property and other reactions. These are similar to a constituent of parsley, which is known to contain apiole. They are also identical to the reactions of a constituent of the fruits of *Ligusticum scoticum* L. (also umbelliferous, kindly supplied by Chelsea Physic Garden) which contain dillapiole (Kariyone & Teramoto, 1939).

f = visible as yellow fluorescent spot after bromination of the plate.
h = orange spot produced by spraying with dinitrophenylhydrazine solution.
q = visible initially in ultra-violet light as a dark spot against the fluorescein background.
v = response indicated to spraying with vanillin 1% in sulphuric acid.

The sources of the samples used is indicated above as (B & S) Brome & Schimmer Ltd. and (P/S Mus) Museum of the Pharmaceutical Society of Great Britain, to whom acknowledgements are here made for specimens. Fruits were purchased, packed by (C) Carters Tested Seeds Ltd., and (Sc) W. H. Schwartz & Sons Ltd. Other samples were taken from the School of Pharmacy, University of London stock (S/P st) or Museum (S/P Mus) and were of unknown origin apart from one marked British Drug Houses Ltd., (B.D.H.)

Rf values given above are less constant than for the reference substances, varying in some cases by ±0·06. The constituents of an extract, however, are all advanced or retarded together. Only obvious constantly appearing spots are recorded as various minor spots are apparent from time to time.

All the above fruits give a terpene spot Rf approximately 0·80, a fixed oil spot Rf approximately 0·65, and a fatty acid streak from the starting line.

included in Table 2 and 3. The extracts also contained varying amounts of free fatty acids, which formed a streak from the starting line to a position about Rf 0·30. These were visible initially in ultra-violet light as a brighter yellow streak against the fluorescein background, and also as a bright fluorescent streak after bromination. Like the fixed oil spots, these streaks can be stained with a spray of 0·05% aqueous rhodamine 6G. Most whole fruits other than ajowan gave only short streaks of fatty acid, but powdered fruits, unless freshly powdered, gave longer streaks which interfered with essential oil components of Rf value less than 0·30, such as linalöl in coriander. Most of the fatty acid streak could be eliminated by steam distilling the powdered umbelliferous fruit and chromatographing a light petroleum extract of the condensate. The increased amount of

TABLE 3. IDENTIFICATION OF UNKNOWN UMBELLIFEROUS FRUIT

Fluorescence-quenching		Rf 0·70. Anise or Fennel. Confirmed by spot Rf 0·40 (−f, +h). 0·65. Parsley. Distinguished by absence of spot Rf 0·40 (+f, +h). 0·60. Indian dill. Confirmed by spot Rf 0·40 (+f, +h). 0·40. Ajowan. (Red-mauve with v). 0·25. Angelica. Confirmed by spot Rf 0·45 (intense blue fluorescence). 0·05. Visnaga. (Yellow with v).
No fluorescence-quenching	Visible after bromine	Rf 0·55. Cumin. Confirmed by spot Rf 0·60 (−f, +h). 0·45–0·40. Caraway or Dill. (+h). 0·25. Coriander. (Mauve-brown with v).
	Not visible after bromine	Other common umbelliferous fruit, e.g., carrot, celery, hemlock, parsnip.

f = following bromination of the plate, the spot does (+) or does not (−) appear as a yellow fluorescent area.
h = spraying with dinitrophenylhydrazine solution does (+) or does not (−) give the spot an orange colour.
v = response indicated to spraying with vanillin 1% in sulphuric acid.
The above Rf values are averages, and given in round figures.
Terpene, fixed oil and fatty acid spots are present in all the extracts, but are ignored here, as are minor spots.

fatty acid in powders compared with whole fruits suggests that powdering facilitates the action of an esterase on the fixed oil. The two samples of ajowan examined were distinct in containing as much free fatty acid in the whole as other umbelliferous fruits did in powdered form. Both came from museums, and during prolonged storage an esterase may have taken effect.

Light petroleum was selected as the extracting solvent because it is reasonably selective in dissolving essential oils but not many other constituents. Some principles other than oils are occasionally obtained; khellin is extracted from visnaga and thus serves to identify this fruit. Some pigments are extracted, but remain on the starting line. With a few fruit extracts, often those obtained from new supplies of material, substances which gave bright blue fluorescing spots in ultra-violet light were noted: this was observed with visnaga and parsley. The brilliant blue fluorescent streak from angelica fruits was distinctive; it was probably due to coumarin and umbelliferone derivatives which, Guenther (1950a) records, have been found in the extract.

Guenther (1950b) and Schrantz & others (1962) have recorded anisaldehyde as being a constituent of fennel oil. A spot corresponding chromatographically to this compound, which is likely to be a decomposition product of anethole, was observed in the extracts of both anise and fennel.

The fluorescence-quenching of substances such as thymol and anethole is readily observed and of diagnostic value, especially in distinguishing Indian dill from dill. A sample of "dill" from a drug broker, and one from a retail source were both shown to be Indian dill by the method described here, the identification being supported by the macroscopical characters of the fruit. It is interesting to note the claim that Indian dill contains a toxic principle dillapiole and that it should not be used in place of European dill (Wallis 1960). The chromatographic method will detect an admixture of one part Indian dill with four parts dill.

According to Brockmann & Volpers (1947) the fluorescence-quenching of aromatic compounds is due to their absorbing part of the ultra-violet illumination. Eugenol exhibits fluorescence-quenching against fluorescein, and since dillapiole and apiole are chemically similar they too may be expected to exhibit fluorescence-quenching. The relevant spots from Indian dill and parsley fruits (Table 2) are assigned on this basis.

Although thin-layer chromatography serves to identify many umbelliferous fruits, other methods are still required to distinguish caraway from dill, and anise from fennel. The anethole spot from anise is normally more intense than that from fennel, and this may be a guide. In theory, medicinal fennel, which is *F. vulgare* var. *vulgare* (Mill.) Thelung, should be distinguished from anise (and from sweet fennel) by its content of fenchone. This very unreactive ketone, however, was not noted in any sample of fennel examined.

References

Brockmann, H. & Volpers, F. (1947). *Chem. Ber.*, **80**, 77–82.
El-deeb, S. R., Karawya, M. S. & Wahba, S. K. (1962). *J. Pharm. Sci. U.A.R.*, **3**, 81–88.
Guenther, E. (1950). *The Essential Oils*, Vol. 4, p. 562 (a) & p. 643 (b), New York: van Nostrand.
Kariyone, T. & Teramoto, H. (1939). *J. pharm. Soc. Japan.*, **59**, 313–314.
Kirchner, J. G., Miller, J. M. & Keller, G. J. (1951). *Analyt. Chem.*, **23**, 420–25.
Paris, R. R. & Godon, M. (1961). *Ann. pharm. franç.*, **19**, 86–93.
Pertsev, I. M. & Pivnenko, G. P. (1962). *Farmatsevt. Zh.*, **17**, 35–40.
Schrantz, M., Lopmeri, A., Strömmer, E., Salonen, R. & Brunni, S. (1962). *Farm. Aikakauslehti*, **71**, 52–88.
Wallis, T. E. (1960). *Textbook of Pharmacognosy*, 4th ed., p. 241, London: Churchill
Wellendorff, M. (1963). *Dansk. Tidsskr. Farm.*, **37**, 145–177.

The paper was presented by THE AUTHOR.

J. Pharm. Pharmacol., 1964, **16**, *Suppl.*, 136*T*–139*T* Received April 27, 1964

The assay of nux vomica and its preparations

H. M. PERRY AND M. L. SHEPPARD

A method is proposed for the extraction of strychnine and brucine, from nux vomica and some of its preparations. An ammoniacal suspension of the drug is extracted with chloroform in a downward displacement liquid-liquid extractor. The strychnine and brucine are then extracted from the chloroform with normal sulphuric acid and the strychnine determined spectrophotometrically. In general the results are in good agreement with those obtained by official methods and the method effects a considerable saving of time.

THE assay of nux vomica and its preparations essentially involves the separation of the strychnine, together with brucine, followed by the isolation and estimation of the strychnine. Solvent extraction, followed by separation and nitration (B.P. 1963), column chromatography (B.P.C. 1959; 1963), or ion-exchange resins (Elvidge & Proctor 1957) are the usual methods involved in the isolation step and the use of any of these is both difficult and time consuming. We have overcome this by the simple extraction of an ammoniacal aqueous suspension of the sample with chloroform in a downward displacement liquid-liquid extractor. In the preparations examined, no substances which interfere with the spectrophotometric assay at 262 and 300 mμ (Elvidge & Proctor, 1957; B.P.C. 1959; 1963) are present in the final solution.

Experimental

Apparatus. A downward displacement liquid-liquid extractor (Quickfit and Quartz Type EX 10/23, nominal capacity 60 ml) and disc baffle (Type EX 10/20). The apparatus was completed by a condenser and a 100-ml flat bottomed flask. 3 Conical separating funnels 250 ml capacity.

Reagents. Ammonia solution: 10% v/v solution of AR ammonia in water. AR chloroform. 70% v/v ethanol in water. N Sulphuric acid.

GENERAL METHOD

Using distilled water (25 ml) and ammonia solution (5 ml) transfer the sample (as prepared below) to a liquid-liquid extractor containing chloroform (80 ml). Reflux for 4 hr, cool, and transfer the chloroform in the flask to a separating funnel. Successively extract the chloroform with N sulphuric acid (4 × 20 ml) combine the acid solutions, and wash once with chloroform (10 ml). Transfer the acid solution to a flat dish and warm gently with stirring to remove the dissolved chloroform. Cool, transfer to a 100 ml graduated flask (filtering if necessary), and make up to volume with N sulphuric acid. This is Solution S.

From a knowledge of the approximate strychnine content of the original sample, dilute solution S with N sulphuric acid so that it contains approximately 1 mg strychnine per 100 ml of solution. (Optimum absorbance

From Stafford Allen & Sons Ltd., Wharf Road, London, N.1.

between 0·6 and 0·9). Measure the absorbance at 262 mμ and 300 mμ in a 1 cm cell against N sulphuric acid as a blank.

If the formula of Elvidge & Proctor (1957) is applied to the present case,

$$\text{the percentage strychnine} = \frac{0{\cdot}318\ a - 0{\cdot}460\ b}{\%\ \text{concentration}}$$

Where a = absorbance at 262 mμ

b = absorbance at 300 mμ

$$\%\ \text{concentration} = \frac{\text{ml or g sample} \times c}{100}$$

c = ml of solution S used in final dilution.

SPECIFIC METHODS

Liquid extract of nux vomica B.P. Extract 2·0 ml of the sample and dilute 5·0 ml of solution S to 100 ml before measurement. Results are given in Table 1.

TABLE 1. STRYCHNINE % W/V IN LIQUID EXTRACT OF NUX VOMICA AND TINCTURE OF NUX VOMICA B.P.

	Proposed method		B.P. 1963	
Sample No.	No. of tests	Range of results	No. of tests	Range of results
Liquid extract				
1	6	1·45–1·52	2	1·44–1·54
2	3	1·43–1·48	3	1·43–1·44
3	3	1·53–1·54	2	1·51–1·61
4	3	1·63	2	1·60–1·67
5	3	1·67	4	1·63–1·67
6	4	1·55–1·57	3	1·47–1·54
Tincture				
1	4	0·116–0·118	3	0·109–0·125
2	2	0·136	2	0·125
3	3	0·123–0·125	2	0·123–0·128
4	3	0·128–0·130	2	0·128–0·133

Tincture of nux vomica B.P. Extract 5·0 ml of the sample and dilute 20·0 ml of solution S to 100 ml before measurement. Results are given in Table 1.

Nux vomica beans B.P. and prepared nux vomica B.P. 1953. Two methods were tried on these materials and both are given below.

Method 1. Accurately weigh about 400 mg of the finely powdered sample into a centrifuge tube and mix well with ethanol solution (25 ml). Centrifuge, and transfer the supernatant liquid to an evaporating dish. Repeat the above extraction three times. Evaporate the combined

extracts to about 5 ml and using the ammonia solution, water and chloroform (20 ml) transfer to a liquid-liquid extractor containing chloroform (60 ml). Extract and dilute 25 ml of solution S to 100 ml before measurement.

Method 2. Accurately weigh about 400 mg of the finely powdered sample into a beaker and mix with ethanol solution (2 ml). Add ammonia solution (5 ml), mix, and using water (25 ml) and chloroform (20 ml) transfer to a liquid-liquid extractor containing chloroform (50 ml). Extract and dilute 25 ml of solution S to 100 ml before measurement.

With Method 2, lumps of sample must be broken down with a glass rod. When transferring to the extractor no sample should be left adhering to the top of the disc baffie before the rest of the chloroform is added. With solid samples it is advisable to swirl the contents of the extractor gently from time to time during the extraction period. Failure to observe these conditions usually leads to incomplete extraction. Results are given in Table 2.

TABLE 2. STRYCHINE % W/W IN NUX VOMICA BEANS, PREPARED NUX VOMICA (B.P. 1953) AND NUX VOMICA DRIED EXTRACT B.P.C.

	Proposed method		B.P. 1963 B.P. 1953	
Sample No.	No. of tests	Range of results	No. of tests	Range of results
Nux vomica beans and prepared nux vomica				
1	3	1·21–1·26	2	1·14–1·16
2	2	1·20–1·22	2	1·22–1·26
3	2	1·07–1·11	2	1·09–1·10
4	4	1·11–1·19	4	1·06–1·12
5	3	1·93–1·96	3	1·84–1·98
6	4	1·83–1·91	2	1·84–1·88
Dried extract				
1	10	6·18–6·74	5	5·64–6·76
2	3	5·25–5·30	3	5·17–5·66
3	3	5·13–5·17	2	4·90–5·12
4	4	5·83–5·95	2	5·65–5·95
5	4	7·44–7·92	5	7·39–8·10
6	2	5·32–5·36	2	5·40

Dry extract of nux vomica B.P.C. As for nux vomica B.P. and prepared nux vomica B.P. 1953, diluting 5 ml of Solution S to 100 ml before measurement. Results are given in Table 2.

Results and discussion

Tables 1 and 2 show the results obtained. The standard deviation was calculated for the first sample of dried extract only. By the proposed method a mean of 6·38% (s.d. $\pm$ 0·20) was obtained whilst by official methods the mean was 6·19 (s.d. $\pm$ 0·49).

The proposed method gives results as reproducible as those obtained by existing methods. For liquid extracts and tinctures it is more readily and quickly carried out with no loss of accuracy.

The assay of solid products particularly dry extract of nux vomica B.P.C. is subject to wider variation irrespective of the method used. By Method 2 the results were more reproducible and the assay technique was trouble free provided the conditions of operation were strictly observed. For the assay of nux vomica beans and prepared nux vomica (B.P. 1953) Method 2 gave comparable results in less than half the time taken by the B.P. methods.

References

British Pharmacopoeia 1963, p. 536. London: Pharmaceutical Press.
British Pharmaceutical Codex 1959, p. 971. London: Pharmaceutical Press.
British Pharmaceutical Codex 1963, p. 1,065. London: Pharmaceutical Press.
Elvidge & Proctor (1957). *J. Pharm. Pharmacol.*, **9**, 974–980.

The paper was presented by MISS PERRY.

J. Pharm. Pharmacol., 1964, **16**, *Suppl.*, 140*T*–146*T* Received April 24, 1964

Preparation and biological activity of some complexes of trypanocidal phenanthridinium compounds

BY M. J. GROVES AND E. C. WILMSHURST

The preparation and *in vivo* testing is described of complexes of some phenanthridinium trypanocides with polysaccharides and polymeric materials possessing strong anionic groups. The laminarin sulphate complex of Prothidium bromide [2-amino-7-(2-amino-6-methyl-4-pyrimidylamino)-9-*p*-aminophenylphenanthridium 10,1′-dimethobromide] shows enhanced prophylactic activity in mice challenged with *Trypanosoma congolense* (F.N.). When tested in cattle in East Africa the complex produced less local reaction at the site of injection but prophylactic activity was similar to that obtained with the equivalent dose of Prothidium bromide itself. Possible reasons for these results are discussed.

A NUMBER of new phenanthridinium trypanocides have been produced in recent years. One of the main disadvantages of these has been the excessive tissue reactions at the site of injection, resulting in large swellings which sometimes ruptured (Robson & Cawdry, 1958; Stephen, 1958; Smith, 1959; Smith & Brown, 1960; Cawdry & Knight, 1961).

Several attempts have been made to prolong the period of protection afforded by prophylactic trypanocides, and to reduce the local and systemic toxicities of both prophylactic and curative agents. These have usually taken the form of depot preparations from which the active substance is gradually released. Examples are the relatively insoluble chloride of quinapyramine (Davy, 1950), insoluble complexes of trypanocides with suramin (Stephen, 1958; Stephen, 1960; Stephen & Williamson, 1958; Stephen & Grey, 1960), and incorporation of the active substance in an oil or grease base (Cawdry & Knight, 1961). The soluble complex of dimidium bromide with thymus nucleic acid has been used to reduce systemic toxicity (Seaman & Woodbine, 1955).

Phenanthridinium compounds of value in the treatment of bovine trypanosomiasis include homidium bromide (B. Vet. C.) and Prothidium bromide; the latter also possesses considerable prophylactic activity. Our attention was directed particularly to these compounds and to a lesser extent to other phenanthridinium compounds, all of which possess quaternary nitrogen groups. Antibacterial quaternary ammonium compounds react with many polysaccharides to produce insoluble products (Groves, 1958). Accordingly, an attempt was made to prepare complexes of phenanthridinium compounds which would delay the release of the active component from an intramuscular or subcutaneous injection site. We hoped thereby to reduce the intensity of the local reaction, and to confer prolonged prophylactic activity.

Experimental

MATERIALS

Acacia, agar, carboxyvinyl polymer (Carbopol 934), isometamidium

From the Research Department, Boots Pure Drug Company Limited, Station Street, Nottingham.

[7-(*m*-amidinophenyldiazoamino)-2-amino-10-ethyl-9-phenylphenanthridinium chloride hydrochloride], sodium alginate, sodium carboxymethylcellulose (Edifas B), stearic acid, sterculia and tragacanth were all pharmaceutical materials of commercial origin. Prothidium bromide [2-amino-7-(2-amino-6-methyl-4-pyrimidylamino)-9-*p*-aminophenylphenanthridinium 10,1′-dimethobromide]; homidium bromide B. Vet. C.; R.D. 2787 [2-amino-7-(2-amino-6-methylpyrimidyl-4-amino)-9-*p*-nitrophenylphenanthridinium 10,1′-dimethochloride, $4H_2O$ or $6H_2O$]; R.D. 2902 [2-amino-7-(2-amino-6-methylpyrimidyl-4-amino)-9-phenylphenanthridinium 10-ethomethanesulphonate-1′-methomethane sulphonate], (Watkins & Woolfe, 1956) and laminarin sulphate LM 111 (degraded material as used by Adams, Heathcote & Walker, 1962; Black & Dewar, 1954) were all available in the laboratory.

Degraded carrageen. Commercial carrageen extract proved unsuitable for complexing. A 5·0% w/w dispersion of commercial carrageen extract in 0·01N sulphuric acid was autoclaved at 10 lb in.^{2} for 30 min. When cool the product was clarified by centrifugation and dialysed against distilled water at 5° for 3 days until free of sulphate ions. The filtered solution was a clear colourless liquid (containing 0·7% solids dried to constant weight at 105°): this produced a copious precipitate with Prothidium bromide solutions.

PREPARATION OF COMPLEXES

Insoluble complexes. The following method yielded reproducible quantities of insoluble precipitates easy to collect and in a form suitable for subsequent testing.

An excess of 1·0% solution of the phenanthridinium salt in distilled water was added slowly, with stirring, to a 1·0% solution or gel of the complexing substance, also in distilled water. The mixture was stirred at room temperature for 5 min and allowed to stand for 1 hr before collecting the precipitate by centrifugation. The material was resuspended in an equal volume of distilled water, and again centrifuged. The "wet" solid was stored at 5°, assayed by total nitrogen analysis, then resuspended in sterile 2·0% hydroxyethylcellulose (Cellosize QP 15,000). In some instances the precipitated complex was dried in an air oven at 105° or at 60° under vacuum and the solid resuspended in hydroxyethylcellulose solution. Equilibrium dialysis (Graham & Thomas, 1962) gave no evidence of complex formation between any of the phenanthridinium compounds tested and hydroxyethylcellulose. In no instance did the colour of the phenanthridinium solutions change.

Soluble complexes. When saturated aqueous solutions of Prothidium or homidium bromide were added to solutions of agar or pectin no precipitation occurred but the change in colour indicated that a reaction had taken place. This was confirmed by equilibrium dialysis (Graham & Thomas, 1962). Agar complexes of Prothidium and homidium were prepared by the general method described above, evaporated to dryness at 105°, assayed, then redissolved in hot water at the required concentration. Pectin complexes were prepared likewise and precipitated with

an excess of ethanol. For testing purposes the solid was redissolved in water, and the pH adjusted to 3·3 with 0·01M sodium bicarbonate solution.

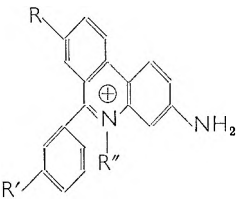
Some properties of the complexes are listed in Table 1.

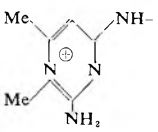
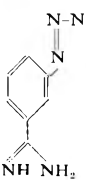
MEASUREMENT OF PROPHYLACTIC ACTIVITY IN MICE

Mice were injected subcutaneously with 1·0 or 0·2 mg/kg of phenanthridinium or with doses of complex containing 1·0 or 0·2 mg/kg of phenanthridinium. The dose was always contained in 0·2 ml of suspension

TABLE 1. PROPERTIES OF THE PHENANTHRIDINIUM COMPLEXES

Phenanthridinium nucleus:



Phenanthridinium	Structure of cation R	R	R''	Complexing moiety (I = insoluble complex) (S = soluble complex)		Total phenanthridinium content of complex dried to constant weight at 105° (total nitrogen) determination)
Prothidium bromide (cation λ_{max} 466 mμ)		NH_2	Me	Acacia	(I)	35·2
				Agar	(S)	56·5
				Carbopol 934	(I)	55·4
				Carboxymethylcellulose	(I)	57·1
				Degraded carrageen	(I)	44·6
				Heparin	(I)	87·6
				Laminarin sulphate	(I)	53·5
				Pectin	(S)	—
				Sodium alginate	(I)	69·2
				Stearic acid	(I)	68·4
				Sterculia	(I)	62·2
				Tragacanth	(I)	32·9
R.D. 2787 (cation λ_{max} 465 mμ)	As for Prothidium	NO_2	Me	Laminarin sulphate	(I)	65·3
				Degraded carrageen	(I)	57·3
R.D. 2902 (cation λ_{max} 465 mμ)	As for Prothidium	H	Et	Laminarin sulphate	(I)	68·5
				Degraded carrageen	(I)	58·5
Isometamidium (cation λ_{max} 474 mμ)		H	Et	Laminarin sulphate	(I)	65·5
				Degraded carrageen	(I)	53·7
Dimidium (cation λ_{max} 480 mμ)	NH_2–	H	Me	Laminarin sulphate	(I)	81·4
				Degraded carrageen	(I)	65·2
Homidium bromide (cation λ_{max} 482 mμ)	NH_2–	H	Et	Agar	(S)	61·5
				Carbopol 934	(I)	23·6
				Carboxymethylcellulose	(I)	50·2
				Degraded carrageen	(I)	68·6
				Heparin	(I)	97·4
				Laminarin sulphate	(I)	87·1
				Pectin	(S)	20·5
				Sodium alginate	(I)	75·8
				Sterculia	(I)	51·7
				Tragacanth	(I)	38·9

or solution. The dosed mice were challenged by intraperitoneal inoculation of 20,000 *Trypanosoma congolense* (strain FN) per mouse. The inocula were obtained by harvesting infected blood from mice in which the trypanosome was maintained by serial blood passage. Challenges were normally made at 2, 4 and 8 weeks after the mice had been dosed; but for substances unlikely to be prophylactic the first challenge was made 1 week after the dose. The tail blood of inoculated mice was examined for trypanosomes every 3 days. As it takes a minimum of 4 days for a mouse to die after the first *T. congolense* (FN) appear in its peripheral blood, examination every 3 days means that no mouse could die from trypanosomiasis without trypanosomes being seen.

Results

In some instances complexing the phenanthridinium compounds effected marked changes in the prophylactic properties of the drugs in mice. For the greater part of this work Prothidium was used as it was then the only phenanthridinium with prophylactic properties proven in the field in Africa. Of all the complexes of Prothidium, only that with laminarin sulphate clearly showed enhanced activity in mice. Complexes with heparin and degraded carrageen may have had some slight advantage compared with the drug itself (Table 2). Complexes with acacia, agar,

TABLE 2. THE PROPHYLACTIC TRYPANOCIDAL ACTIVITY OF PHENANTHRIDINE COMPLEXES

Phenanthridinium	Complex	Dose mg/kg†	Number of surviving uninfected mice from groups of 10 challenged at		
			2 weeks	4 weeks	8 weeks
Prothidium ..	Uncomplexed control (results of 5 tests)	0·2	0–5	0–2	0
		1·0	10	7–10	0–3
	Degraded carrageen	0·2	4	2	0
		1·0	9	5	2
	Laminarin sulphate	0·2	10	10	3
		1·0	10	9/9*	10
	Heparin	0·2	4	3	0
		1·0	9	9	5
R.D. 2787.. ..	Uncomplexed control	0·2	0	0	0
		1·0	9	0	0
	Degraded carrageen	0·2	0	0	0
		1·0	9	6	0
	Laminarin sulphate	0·2	0	0	0
		1·0	9	9	9
R.D. 2902.. ..	Uncomplexed control	0·2	0	0	0
		1·0	1	0	0
	Degraded carrageen	0·2	2	0	0
		1·0	0	0	0
	Laminarin sulphate	0·2	0	0	0
		1·0	8	8	2
Isometamidium ..	Uncomplexed control	0·2	2	0	0
		1·0	10	10	0
	Degraded carrageen	0·2	9	6	0
		1·0	9	10	8
	Laminarin sulphate	0·2	4	4	0
		1·0	9	10	8
Dimidium ..	Al complexes and uncomplexed control	1·0	No survivors in any group challenged one week after dosing		
Homidium ..	Al complexes and uncomplexed control	1·0	No survivors in any group challenged one week after dosing		

* The 10th mouse in this group died from causes other than trypanosomiasis.
† Doses are quoted as their equivalent of prothidium.

Carbopol, carboxymethylcellulose, sodium alginate and sterculia, showed reduced activity, while those with pectin, sodium alginate and tragacanth showed similar activity to the drug itself.

When the Prothidium-laminarin sulphate complex was dried and the solid resuspended just before administration it was less active than the original suspension, although it was still more active than Prothidium, Table 3.

TABLE 3. COMPARISON OF THE ACTIVITIES OF DRIED AND FRESHLY PREPARED PROTHIDIUM-LAMINARIN SULPHATE COMPLEXES

Substance	Equivalent dose of Prothidium in mg/kg sc.	Numbers of surviving, uninfected mice after being challenged 8 weeks after dosing
Prothidium	1	0/9
	2	2/9
Prothidium-laminarin sulphate, dried	1	2/9
	2	3/9
Prothidium	1	0/5
	2	2/5
Prothidium-laminarin sulphate, freshly prepared	1	5/5
	2	5/5

Prophylactic phenanthridinium compounds other than Prothidium were complexed only with laminarin sulphate and degraded carrageen. Those compounds possessing prophylactic activity in their own right, all showed enhanced activity in mice after complexing with one or other of these complexing moieties. The activities of isometamidium and R.D. 2787 were enhanced after coupling with either, that of R.D. 2902 only after coupling with laminarin sulphate. Two phenanthridiniums possess mainly curative activity, viz dimidium and homidium, and these were complexed with all of the agents used with Prothidium. In mice, no prophylactic activity was apparent.

The complexes of homidium and Prothidium were compared with the parent compounds for local irritancy by intradermal injection into guinea-pigs. The assessment of differences in reaction proved difficult and inconclusive but the laminarin sulphate complexes appeared to be no more irritant than either homidium or Prothidium bromide alone (R. Bough, personal communication).

Evaluation of the Prothidium-laminarin sulphate complex in cattle in East Africa showed that the reaction at the site of injection had been considerably reduced (R. Fairclough & E. F. Whiteside, personal communications). The standard dose to cattle of Prothidium bromide (2·0 mg/kg) injected into the dewlap can produce a necrotic lesion up to 20 to 30 cm in diameter which may burst. When the same dose of Prothidium bromide was given as the complex the size of the lesion was reduced to between a tenth and a fifth, and did not burst. The period of protection against trypanosomiasis, however, was almost exactly the same as that given by a dose of the bromide equivalent to the dose of complex.

Discussion

The formation of complexes of Prothidium may result in an increase, a decrease or no change in prophylactic activity against *T. congolense* in mice, according to the complexing substance used (Table 3). These changes in activity presumably reflect the ease with which each complex is split under physiological conditions to liberate its trypanocidal moiety into the blood. The complex most active in mice, that with laminarin sulphate, presumably releases phenanthridinium at an optimum rate, which is sufficient to maintain trypanocidal activity in the blood without allowing unduly rapid excretion. When a complex like that with Carbopol is less active than its equivalent of Prothidium, the binding of the two molecules would seem to be so strong, that trypanocidal levels of the drug cannot be maintained in the blood.

Because it apparently had the greatest biological interest, particular attention was paid to the properties of the Prothidium-laminarin sulphate complex. Precipitation of this complex under a wide variety of conditions and concentrations showed that approximately 11 parts of the bromide reacted with 10 parts of laminarin sulphate. The complex was evidently loose since the supernatant washing liquids were invariably slightly coloured. The wet complex dried on a glass plate as translucent reddish brown scales, but there was a reduction in the prophylactic activity in the dried material (Table 3). When the complex was precipitated from dilute solution and concentrated by centrifugation the precipitate varied in consistency from a suspension to a thixotropic gel according to the concentration of solid.

It was disappointing that the increased duration of prophylaxis obtained in mice with the Prothidium bromide-laminarin sulphate complex was not matched by any increase in prophylaxis in cattle in Africa. This may be explained by comparing the situations at the site of injection in mice and cattle. In mice the complex presumably forms a depot at the site of injection, while the bromide alone does not. The latter supposition is supported by observations that no depot has been seen in mice at post-mortem examination after subcutaneous or intramuscular injection of the bromide, and that the period of prophylaxis in mice was the same whether the drug was given by subcutaneous, intramuscular or intravenous injection. In addition, when the site of injection in mice of the Prothidium bromide-laminarin sulphate complex is examined 8 weeks after the injection traces of coloured complex can still be seen. Thus the complex in mice is probably effecting prolonged prophylaxis mainly by liberating the drug slowly from its local depot, in contrast to the bromide, which is apparently bound in tissues generally in the body and slowly released from them.

When Prothidium bromide alone is administered to cattle by subcutaneous or intramuscular injection, the local reaction around the site of injection would appear to have the effect of converting the sealed-off drug into a local depot. If so the injection of cattle with a ready formed depot would be unlikely to confer longer prophylaxis.

The ready formed depot complex in cattle, however, markedly reduces the local tissue reaction, an effect which may be due to a slow release of drug from the complex, thus preventing too sudden an exposure of tissues round the injection site to high concentrations.

Acknowledgements. We wish to thank Mr. A. W. Bull and Dr. G. Woolfe for help in discussion.

References

Adams, S. S., Heathcote, B. V. & Walker, D. (1962). *J. Atheroscler. Res.*, **2**, 314–316.
Black, W. A. P. & Dewar, G. T. (1954). *J. Sci. Fd. Agric.*, **5**, 176.
Cawdry, M. J. H. & Knight, R. H. (1961). *Vet. Rec.*, **73**, 982–984.
Davey, D. G. (1950). *Trans. roy. Soc. trop. Med. Hyg.*, **43**, 583–616.
Groves, M. J. (1958). M.Pharm. Thesis, University of Nottingham.
Graham, H. D. & Thomas, L. B. (1962). *J. pharm. Sci.*, **51**, 988–992.
Robson, J. & Cawdry, M. J. H. (1958). *Vet. Rec.*, **70**, 870–876.
Seaman, A. & Woodbine, M. (1955). *J. appl. Bact.*, **18**, 397–400.
Smith, I. M. (1959). *J. comp. Path.*, **69**, 105–115.
Smith, I. M. & Brown, K. N. (1960). *Ibid.*, **70**, 161–175.
Stephen, L. E. (1958). *Ann. trop. Med. Parasit.*, **52**, 417–426.
Stephen, L. E. (1960). *Vet. Rec.*, **72**, 80–84.
Stephen, L. E. & Gray, A. R. (1960). *Ann. trop. Med. Parasit.*, **54**, 493–507.
Stephen, L. E. & Williamson, J. (1958). *Ibid.*, **52**, 427–442.
Watkins, T. I. & Woolfe, G. (1956). *Nature, Lond.*, **178**, 368.

The paper was presented by MR. GROVES.

J. Pharm. Pharmacol., 1964, **16**, *Suppl.*, 147*T*–149*T* Received April 29, 1964

SHORT COMMUNICATION

A temperature dependent micellar change

J. E. ADDERSON AND H. TAYLOR

AS part of a broader programme of investigation of problems of emuslification, the variation of critical micelle concentration with temperature for aqueous solutions of dodecylpyridinium bromide (DPB) has been studied (Adderson & Taylor, 1964) and thermodynamic quantities have been calculated on the basis of the phase change model of micellisation (see e.g. Matijevic & Pethica, 1958; Shinoda & Hutchinson, 1962). In this way variations with temperature, of the entropy change and heat capacity change resulting from micelle formation have been interpreted as indicating a change in the nature of the micelles at about 55° (Adderson & Taylor, 1964). To test this hypothesis, other properties of the solutions have been studied.

EXPERIMENTAL

The surfactants were prepared and purified as described previously (Adderson & Taylor, 1964). The solubilisation of Waxoline Yellow IS* by 0·02 molal solution of DPB has been studied from 25–70°. The solution of DPB was placed in a sealed spectrophotometric cell with an excess of the dye twice recrystallised from benzene. The cell was kept at constant temperature in a Unicam S.P.500 spectrophotometer and the absorption of the solution at 485 mμ measured against (*a*) water, (*b*) a similar solution saturated with dye at 25° as reference. The results obtained for (*a*) are shown graphically in Fig. 1 (a), those for (*b*) were qualitatively the same, showing two straight lines intersecting at about 55°.

The CMC of DPB had previously been obtained by the conductivity method (Adderson & Taylor, 1964). The slope of the conductivity *versus* concentration curve for solutions (*a*) below the CMC (*b*) above the CMC have now been calculated at each temperature of measurement.

The solubilisation of Waxoline Yellow IS by 0·01 molar tetradecylpyridinium bromide (TPB) was studied in the same way as for DPB. The slopes of the conductivity *versus* concentration curves for TPB were obtained in the same way as those for DPB.

DISCUSSION

Figs 1 (a) and 2 (b) show a sharp break in the curve at about 55°. By contrast Figs 1 (c), 2 (c) and 2 (d) are almost linear.

The sharp change in the solubilising power of DPB solution at about 55° is consistent with the suggested change in the nature of the micelle from a gel-like to a liquid state (Adderson & Taylor, 1964). A change in

From the Pharmacy Department, Bradford Institute of Technology, Bradford, 7 Yorkshire.

* α-Benzeneazo-β-naphthol (C.I. 12,055).

the structure of a phase is likely to be accompanied by a change in its solvent powers. The absence of a break in the curve for TPB and the presence of a break for DPB when measured against the dye solution as reference, indicates that the effect observed with DPB is a property of DPB solution and not of the dye.

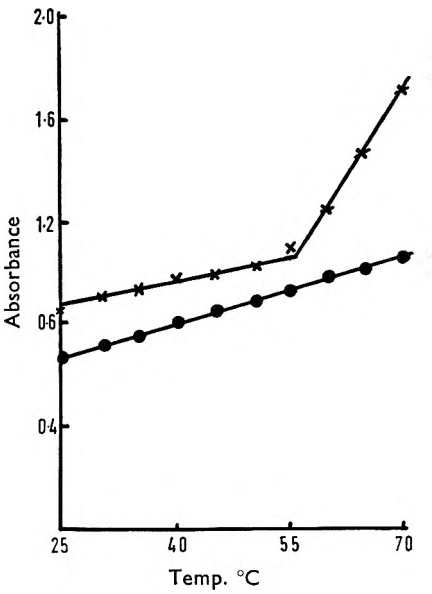


FIG. 1. Solubilisation of Waxoline Yellow IS. ×, 0·02M dodecylpyridinium bromide. ●, 0·01M tetradecylpyridinium bromide.

Again a break in the conductivity slope curve 2 (b), absent in 2 (a), 2 (c) and 2 (d) indicates a change in the micelles of DPB at about 55°. The slope of the conductivity versus concentration curve for a surfactant solution at concentrations above the CMC is a function in part of the mobility of the micelle. Evans (1956) derived the following equation (modified here for DPB):

$$1000S_2 = p^2(1000S_1 - \wedge_{Br} -)/n^{4/3} + p \wedge_{Br} - /n$$

where S_2 is the slope of the specific conductivity against concentration curve above the CMC, S_1 the slope below the CMC, p the micellar charge and n the number of DPB ions in the micelle. To obtain this expression Evans eliminated the micelle mobility by assuming the micelle to be spherical and introducing Stokes' law. He was then able to calculate p and n. However the mobility of an ion in a unit field depends on its charge, shape and size, and a change in the slope of the conductivity plot may result as much from change of shape as from change of size and charge. For this reason no attempt is made to calculate size and charge until further information has been obtained about micelle shape. Fig. 2 does indicate a change in the mobility of the DPB micelles at about 55° additional

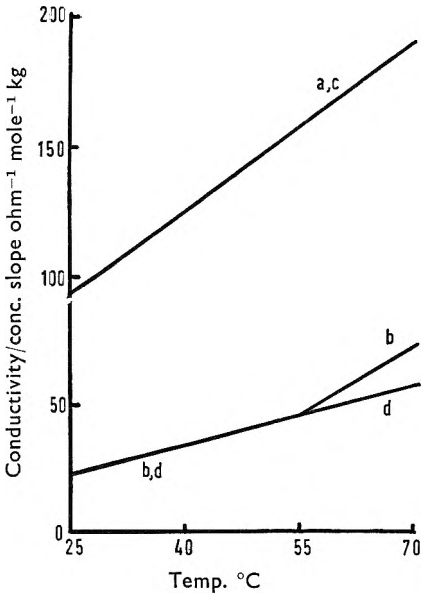


FIG. 2. Variation with temperature of slope of curve of conductivity *v.* concentration. (a) and (c) dodecylpyridinium bromide and tetradecylpyridinium bromide below CMC. (b) and (d) same above CMC.

to that normally expected from a change in temperature; this change moreover is not shown by the single ions. The results again are consistent with a change in the nature of the micelle, a change not shown by TPB.

References

Adderson, J. E. & Taylor, H. (1964). *J. Colloid Sci.*, in the press.
Evans, H. C. (1956). *J. chem. Soc.*, 579–586.
Matijevic, E. & Pethica, B. A. (1958). *Trans. Faraday Soc.*, **54**, 587–592.
Shinoda, K. & Hutchinson, E. (1962). *J. Phys. Chem.*, **66**, 577–582.

The paper was presented by MR. ADDERSON.

J. Pharm. Pharmacol., 1964, **16**, *Suppl.*, 150*T*–155*T* Received May 1, 1964

Reproducibility of extinctions measured on the slopes of absorption curves

M. ISMAIL AND A. L. GLENN

Errors in setting the wavelength scale of an ultra-violet spectrophotometer cause an increase in the variance of extinctions measured on the slope of an absorption curve. For a given instrument and operator, the coefficient of variation of extinctions measured on a steep slope was ten times the normal value for zero slope. A method is described for obtaining reasonably precise estimates of ($\delta \log E/\delta S$), where "S" refers to the displacement of the wavelength scale. The coefficient of variation bears a linear relationship to ($\delta \log E/\delta S$), which is relevant to the choice of wavelengths for Vierordt's method.

WHEN measuring the extinction at a wavelength of maximum or minimum absorption, a small error in setting the wavelength scale or a small change in the spectrophotometer's wavelength calibration, such as may occur with time, produces a negligible effect on the measured extinction. However, as is well known, the situation is very different for measurements on the slope of an absorption curve, which are made frequently in multicomponent spectrophotometric analysis. The reproducibility of this technique may in fact be more dependent upon wavelength setting than upon photometric errors (von Halban & Ebert, 1924; Twyman & Lothian, 1933).

In the course of an experimental evaluation of the precision of Vierordt's method (Glenn, 1960), it proved necessary to develop a weighting system, which would account for the difference between assays in the matter of "wavelength setting errors." An experimental study of the dependence of such errors upon the slope of the absorption curve was essential to this purpose. Hence, for different values of the slope ($\delta \log E/\delta \lambda$), it was necessary to obtain estimates of c.v.(λE), the coefficient of variation* of extinction measurements which include wavelength setting errors in addition to the inevitable photometric error. The results are also of general interest in view of the dearth of experimental information relating to optimum conditions of spectrophotometric analysis (Crawford, 1959).

c.v.(λE) was obtained from eight successive extinction measurements, each of which entailed a prior re-setting of the wavelength scale. Chlorocresol in 0·1N aqueous H_2SO_4 provided a suitable system of measurement, which readily lent itself to the choice of a set of wavelengths, for which the slopes ranged from "gentle" to "very steep." In view of the decision to study c.v.(λE) as a function of ($\delta \log E/\delta \lambda$), this choice was made from a graph of log$E$ against wavelength.

The wavelength setting errors of a careful observer arise from minute variations in the setting of the wavelength scale, so that graphical estimation of slopes from an absorption curve determined at 2 or 3 mμ

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

* Throughout the paper, "coefficient of variation" denotes 100 (standard deviation/mean).

intervals would have been grossly inadequate for the present quantitative study. It was therefore necessary to use a device (Fig. 1), which gave a 20-fold magnification of the wavelength scale displacement. A further problem arose from a characteristic of the prism monochromator, whereby

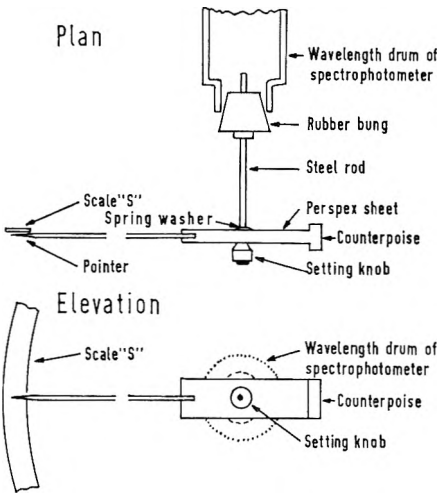


FIG. 1. Device for obtaining a magnified indication of the setting of the wavelength scale.

the wavelength scale becomes increasingly cramped in going from smaller to greater wavelengths. Thus, as can be seen from Table 1, a given wavelength change involves a scale displacement at 220 mμ, which is more than three times that at 300 mμ. Hence, the slope of an absorption curve can be expressed either as ($\delta \log E/\delta\lambda$), the change in $\log E$ for a given change of wavelength, or ($\delta \log E/\delta S$), the change in $\log E$ for a given displacement of the wavelength scale. The operator's error in setting the scale to a given mark, should be independent of wavelength, so that c.v.(λE) ought to bear a more simple relationship to ($\delta \log E/\delta S$) than to ($\delta \log E/\delta\lambda$).

Mean values of c.v.(λE) were determined at seven chosen wavelengths in a sequence of 29 blocks and the results recorded in Table 2 together

TABLE 1. MEAN VALUES OF ($\Delta\lambda/\Delta S$) FOR THE UVISPEK PHOTOELECTRIC SPECTROPHOTOMETER

λ_m (mμ)	$\Delta\lambda$ (mμ)	ΔS (cm)	($\Delta\lambda/\Delta S$) (mμ cm^{-1})
225	1	13·4	0·075
230	2	24·6	0·081
240	2	20·5	0·098
250	2	17·5	0·114
260	3	22·5	0·133
270	3	19·7	0·152
280	4	23·0	0·174
290	4	19·8	0·202
295	5	23·9	0·209

TABLE 2. SLOPE PARAMETERS AND C.V.(λE)

λ_m (mμ)	($\Delta S/\Delta\lambda$)	($\delta \log E/\delta S$)	($\delta \log E/\delta\lambda$)	c.v.(λE)
245	9·52	0·0193	0·183	0·292
270	6·58	0·0115	0·075	0·146
285	5·41	0·0032	0·017	0·088
290	5·00	0·0326	0·163	0·333
291	5·00	0·0396	0·198	0·515
293·5	4·83	0·0556	0·268	0·711
295	4·78	0·0696	0·332	0·725

with the relevant values of ($\delta \log E/\delta S$) and ($\delta \log E/\delta\lambda$). ($\delta \log E/\delta S$) was measured directly at each of the chosen wavelengths and ($\delta \log E/\delta\lambda$) calculated therefrom, using the appropriate values of ($\Delta\lambda/\Delta S$) interpolated from Fig. 2. To maintain a basis of comparison, the solutions of chlorocresol used to measure c.v.(λE) and ($\delta \log E/\delta S$) were of such concentration that E(1 cm) was close to 0·50 at λ_m, the wavelength of measurement. In this way, the contribution of photometric error was kept constant throughout.

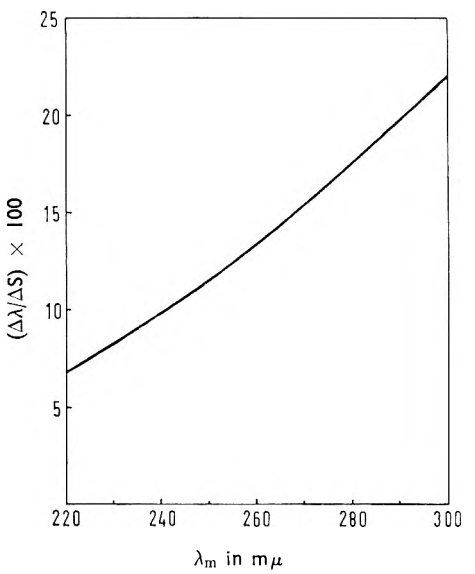


FIG. 2. Mean values of ($\Delta\lambda/\Delta S$) versus wavelength for the Uvispek photoelectric spectrophotometer.

Experimental

The measurements described in (*a*) and (*c*) below, employed solutions of chlorocresol (in 0·1N aqueous H_2SO_4) of such strength that E(1 cm) was close to 0·50 at all values of λ_m. In setting the wavelength scale to a given mark, the final movement was invariably made in the direction of higher wavelengths.

(*a*) DETERMINATION OF MEAN VALUES OF c.v.(λE). At a particular wavelength, λ_m, nine successive measurements were made of E(1 cm) of

the appropriate solution, the first measurement being rejected on account of the considerable re-adjustment of the instrument controls, which normally preceded it. By re-setting the wavelength scale to λ_m before each measurement, the latter became subject to both wavelength setting and photometric errors. (No special device was used in setting the scale). The work was carried through in a series of blocks, each taking about 2 hr to complete and resulting in one estimate of $V(\lambda E)$, the variance of (λE), for each of the seven wavelengths. Each value of the mean c.v.(λE) was then calculated from the mean $V(\lambda E)$ for the wavelength in question throughout the 29 blocks. In this way, fluctuations of operator and instrument performance contributed very little to the regression in Fig. 3.

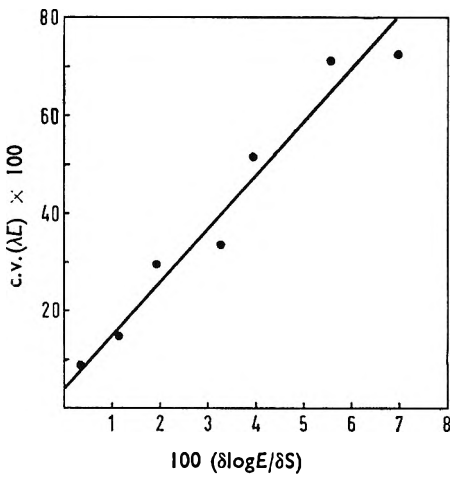


FIG. 3. Regression of mean c.v.(λE) upon $(\delta \log E/\delta S)$.

(*b*) MEASUREMENT OF $(\Delta\lambda/\Delta S)$ FOR THE UVISPEK PHOTOELECTRIC SPECTROPHOTOMETER. A magnified indication of the setting of the wavelength scale was obtained by means of the simple device shown in Fig. 1. It was free from backlash and easily adapted for measurements at any point on the wavelength scale. It consisted of a long thin duralumin pointer attached to one end of a small sheet of perspex, the opposite end of which bore a counterpoise. At its centre of gravity the whole assembly was mounted on a steel rod, and was kept in position by a setting knob and rubber washers. The steel rod was held in a rubber bung which could be tightly fitted into the hollow end of the spectrophotometer wavelength drum. In this way, the pointer was rigidly connected to the wavelength drum during measurements over a small interval of wavelength. It could be released by means of the setting knob, when the drum had to be rotated by more than a few degrees. Great care was taken not to strain the wavelength drum and so cause damage to the micrometer screw, which moves the prism table in the monochromator. For the same reason, the weight of the device was kept as low as possible. The pointer was 13 inches long and ended in a fine blade, which moved over a

scale attached to the front of the monochromator. *The symbol*, "S," *refers to measurements along this scale.* The latter was made by cutting out the scale of a thin plastic millimetre rule and forming it into an arc of radius 13 in. To obtain $(\Delta\lambda/\Delta S)$, the wavelength drum was set to the wavelength, λ_m, at which $(\Delta\lambda/\Delta S)$ was to be measured and the pointer clamped into position, so that it rested at about the middle of the scale. The position of the pointer was then noted after the drum had been accurately set to (i), $\lambda_m - (\Delta\lambda/2)$ and (ii) $\lambda_m + (\Delta\lambda/2)$, both wavelengths coinciding with scale graduations. ΔS was kept to the same order of magnitude throughout by suitable adjustment of $\Delta\lambda$. The data presented in Table 1 and Fig. 2 refer to means of 20 estimates of ΔS at each value of λ_m.

(*c*) MEASUREMENT OF $(\delta\log E/\delta S)$ FOR CHLOROCRESOL (IN 0·1N AQUEOUS H_2SC_4) AT VARIOUS λ_m. To determine $(\delta\log E/\delta S)$ at a particular wavelength, λ_m, the wavelength drum was set to λ_m and the pointer adjusted so that it rested at a point, S_0, near the centre of the scale. Extinctions were then measured at 5 mm intervals along the scale, starting at $S_0 - 15$ mm and concluding at $S_0 + 15$ mm. In this way, a small section of the absorption curve (E against λ) was plotted out in the vicinity of λ_m and the slope, $(\delta E/\delta S)$, obtained therefrom. $(\delta\log E/\delta S)$ was then equal to $0{\cdot}868(\delta E/\delta S)$ since $\delta\log E \approx 0{\cdot}434\delta E/E$ and all solutions were of such concentration that E(1 cm) was close to 0·50 at λ_m. The same procedure was followed in triplicate for all values of λ_m quoted in Table 2. The readings which related to a particular λ_m were accumulated on one graph and the mean slope determined, the plots of E against λ being substantially linear in all cases. These measurements were of modest precision in view of: (i) small concentration differences between batches of solution; (ii) difficulty of reproducing λ_m in the initial setting of the pointer on the scale, and (iii) small drifts in the wavelength calibration, which occurred during the few days that were occupied with these measurements. For these reasons, the uncertainty in $(\delta\log E/\delta S)$ was probably of the same order as the uncertainty in the mean value of c.v.(λE). However, to have improved the precision of $(\delta\log E/\delta S)$ would have entailed an unjustifiable amount of additional labour, in view of the final objective.

Discussion

The use of c.v.(λE) instead of $\sigma(\lambda E)$ or V(λE) stems from the fact that for almost every analytical purpose, it is the *relative* extinction error, rather than the absolute error, that is important. This is most certainly true of Vierordt's method, which depends either implicitly or explicitly on the measurement of three extinction ratios (Glenn, 1960).

A graph of mean c.v.(λE) against $(\delta\log E/\delta S)$ is shown in Fig. 3 together with a regression line calculated by least squares. The value, 0·04, of c.v.(λE) at the intercept of this graph refers to c.v.(E), the coefficient of variation of extinction measurements which are devoid of wavelength setting error, and is lower than actually experienced in this laboratory (0·07 for $E = 0{\cdot}5$).

The graph of mean c.v.(λE) against ($\delta \log E/\delta\lambda$) bore a close resemblance to that of Fig. 3, mainly because six of the seven wavelengths fell between 270 mμ and 295 mμ, over which interval the change in ($\Delta S/\Delta\lambda$) is small. Nevertheless, an indication of what might have happened, if the wavelengths had been evenly spread throughout the range, 220 mμ to 300 mμ, is given by the point for 245 mμ. This was a notable outlier in the plot of mean c.v.(λE) against ($\delta \log E/\delta\lambda$) but not in Fig. 3, which supports the view that an operator's error in setting the wavelength scale to a given mark is independent of wavelength.

It is evident that c.v.(λE) bears an almost linear relationship to ($\delta \log E/\delta S$). Furthermore, relative to the usual value of c.v.(E), 0·07 for $E = 0{\cdot}5$, c.v.(λE) is about ten times c.v.(E) for measurements on the steepest slopes and about five times c.v.(E) for measurements on average slopes—a statement, which refers to a particular combination of operator and instrument. Although a linear relationship between c.v.(λE) and ($\delta \log E/\delta S$) probably applies to all combinations, the regression constant may be subject to variation.

The steep linear slope of the relationship between c.v.(λE) and ($\delta \log E/\delta S$) is of no small relevance to the choice of wavelengths for Vierordt's method, for it is obviously desirable to avoid very steep slopes, even at the cost of an increase in purely photometric error. By ignoring the important matter of slope, one of the methods suggested by Stearns (1950) is not altogether satisfactory since a graph of

$$[(E\ 1\%,\ 1\ \text{cm})\ \text{of A}]\ /\ [(E\ 1\%,\ 1\ \text{cm}\ \text{of B}]$$

against wavelength obscures the slopes of the absorption curves which contribute thereto. The second method recommended by Stearns and described in greater detail by Glenn (1960), involves a super-position of the log$E$ vs. wavelength curves for the two components and so gives a clear indication of the slopes which will be encountered at any given wavelength. Furthermore, this second method usually involves less labour than the first.

Acknowledgements. The authors thank Prof. L. Saunders and Dr. A. R. Rogers for helpful discussion. M. Ismail also thanks the Colombo Plan Authority for the award of a grant.

References

Crawford, C. M. (1959). *Analyt. Chem.*, **31**, 343–348.
Glenn, A. L. (1960). *J. Pharm. & Pharmacol.*, **12**, 595–608.
von Halban, H. & Ebert, L. (1924). *Z. f. Phys. Chem.*, **112**, 373.
Stearns, E. I. (1950). *Analytical Absorption Spectroscopy*, Editor, Mellon, M. G. p. 371. London: Chapman & Hall.
Twyman, F. & Lothian, G. F. (1933). *Proc. Phys. Soc.*, **45**, 643.

J. Pharm. Pharmacol., 1964, 16, *Suppl.*, 156*T*–162*T* Received May 1, 1964

Mathematical treatment of oral sustained release drug formulations

M. ROWLAND AND A. H. BECKETT

A mathematical model is described for an ideal sustained release dosage form in which there is a constant rate of release of drug from the maintenance dose. Kinetic equations related to this model are derived. The implications of these equations in the calculation of the dosage regimen for a sustained release product are considered.

THE objectives of oral sustained release drug formulations: (*i*) to give rapidly, blood concentrations of the drug, sufficient to elicit the desired therapeutic effect; (*ii*) to maintain these concentrations at an essentially constant level for a suitable period of time; (*iii*) to reduce the frequency of administration of the drugs compared with those in conventional forms; (iv) to give a more uniform biological response and a reduced incidence and intensity of side-effects. These last result from high drug concentration peaks which obtain after administration of conventional dosage forms (Mulligan 1954; Freed, Keatings & Hays, 1956; O'Connor 1958). In general, the total dose used in a sustained release formulation is the sum of the amount of the drug in the "free form," determined by conventional dosage, and that estimated to be required in the "maintenance form."

Despite the widespread use of sustained release drug formulations, little has been published on the method of calculation of the total dose required for the products or on the mathematical treatment of the drug

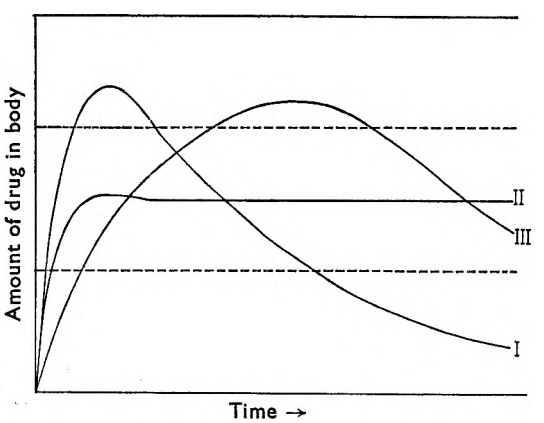


FIG. 1. Hypothetical drug level—time curves of a drug in various dosage forms. I. Drug in conventional dosage form. II. Sustained release formulation, the release of drug from the "maintenance form" occurring at a constant rate. III. Formulation in which the release of the drug from the "maintenance form" is by a first-order rate (c.f. applying the equation used by Weigand & Taylor, 1960.) Solid line marks limits of side-effects. Broken lines define limits of therapeutic range.

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

biokinetic data. Nelson (1957) gave a method of deriving the "maintenance form" of the drug from data based on its biological half-life. Weigand & Taylor (1960) presented a mathematical model and derived equations based on a first order release rate of the drug from the maintenance dose. Nelson (1963) derived an equation for the variation in the body level of a drug after administration assuming a constant rate of release from the "maintenance form" of the drug. Although the derived equations are based on a hypothetical system, some guide to the formulation of oral sustained release dosage forms may be obtained.

In Fig. 1 are presented body drug level versus time curves for a drug presented in different forms. Curve (I) indicates that the conventional dosage form may result in concentrations above the therapeutic range, curve (II) is that resulting from an ideal sustained release formulation which is not achieved if the drug release from this product follows a first order rate (curve III).

A model system is represented in Fig. 2.

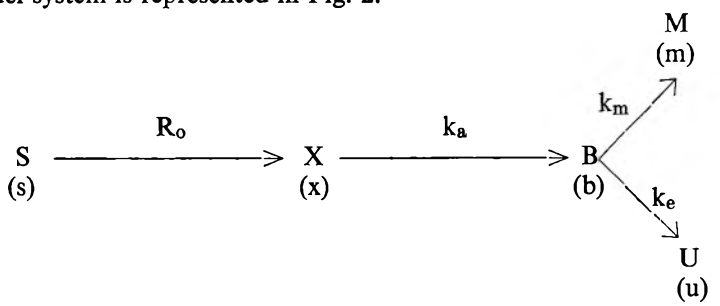


FIG. 2.

S, is the sustained release preparation, X, the gastrointestinal tract, B, the body (excluding the gut and urine), U, the urine, and M, the total metabolites in all compartments, whilst (s), (x), (b), (u) and (m) represent the amount of drug in each compartment. The assumptions made are that

(1) The transfer from one compartment to another is irreversible.

(2) The rate of transfer of drug from a compartment is directly proportional to the concentration or amount of drug in that compartment. Thus absorption, excretion and metabolism are first order processes, with rate constants k_a, k_e and k_m respectively.

(3) There is a fixed rate of release of drug from the "maintenance form" of the product (R_o).

(4) The release of drug from the "maintenance form" is rate determining in the absorption process.

(5) There is no decomposition of the drug at the absorption site. There is no enterohepatic recycling or any diffusion of the drug from the blood into the stomach.

(7) The drug is completely absorbed.

(8) The rate constant for the absorption is unchanged along the gastrointestinal tract.

The following equations then apply:

$$-\frac{ds}{dt} = R_o \quad .. \quad .. \quad .. \quad .. \quad .. \quad (1)$$

$$\frac{dx}{dt} = R_o - k_a.x \quad .. \quad .. \quad .. \quad .. \quad (2)$$

$$\frac{db}{dt} = k_a.x - k_e.b - k_m.b \quad .. \quad .. \quad .. \quad (3)$$

$$= k_a.x - k_d.b$$

$$\frac{du}{dt} = k_e.b \quad .. \quad .. \quad .. \quad .. \quad .. \quad (4)$$

Each compartment is now considered separately.

THE SUSTAINED RELEASE PREPARATION (S)

Let D_o be the amount of "free" drug and S_o the "maintenance form" in the sustained release dose. Integrating equation (1) gives:

$$s = S_o - R_o.t \quad .. \quad .. \quad .. \quad .. \quad (5)$$

The total drug in the "maintenance form" is then given by:

$$S_o = R_o.T$$

where T is the period of time over which the drug is released from the formulation, in order to maintain the desired body level of drug, for some number of hours, h.

$$\text{Total dose} = D_o + S_o \quad .. \quad .. \quad .. \quad (6)$$

THE GASTROINTESTINAL TRACT (X)

Integrating equation (2); since the amount of drug in the tract at zero time is that in the readily available form, D_o then

$$x = \frac{R_o}{k_a}\left[1 - e^{-k_a t}\right] + D_o e^{-k_a t} \quad .. \quad .. \quad (7)$$

THE BODY COMPARTMENT (B)

Putting k_d equal to k_e plus k_m where k_d is the overall rate constant for loss of drug from the body and substituting x from equation (7) into equation (3). Then:

$$\frac{db}{dt} = R_o\left[1 - e^{-k_a t}\right] + k_a D_o e^{-k_a t} - k_d b \quad .. \quad .. \quad (8)$$

Integrating and putting $b = 0$ when $t = 0$ then:

$$b = \frac{R_o}{k_d}\left[1 - e^{-k_d t}\right] - \frac{R_o}{k_d - k_a}\left[e^{-k_a t} - e^{-k_d t}\right]$$

$$+ \frac{k_a D_o}{k_d - k_a}\left[e^{-k_a t} - e^{-k_d t}\right] \quad .. \quad .. \quad .. \quad .. \quad (9)$$

The concentration of drug (c) in the plasma is then given by:

$$c = \frac{b}{V_d} \quad .. \quad .. \quad .. \quad .. \quad .. \quad (10)$$

where V_d is the apparent volume of distribution of the drug.

THE URINE (U)

Substituting b, from equation (9) into equation (4) and integrating, putting $u = 0$ when $t = 0$, gives:

$$u = \frac{k_e R_o}{k_d^2}\left[k_d t + e^{-k_c t} - 1\right]$$

$$+ \frac{k_e}{k_d - k_a}\left\{\left[\frac{1}{k_a}(e^{-k_a t} - 1) - \frac{1}{k_d}(e^{-k_d t} - 1)\right]\left[R_o - k_a D_o\right]\right\} \quad (11)$$

If the drug is in solution or conventional dosage form $R_o = 0$. Then substituting b from equation (9) in equation (10) the latter equation reduces to

$$c = \frac{k_a D_o}{V_d(k_d - k_a)}\left[e^{-k_a t} - e^{-k_d t}\right] \quad .. \quad .. \quad (12)$$

which is of the same form derived perviously (Gehlen, 1933; Teorell, 1937; Dost, 1953; Bray & White, 1957) for "free" drug forms.

Similarly equation (11), for conventional dosage forms reduces to

$$u = \frac{k_e\, k_a\, D_o}{k_d - k_a}\left[\frac{1}{k_d}(e^{-k_d t} - 1) - \frac{1}{k_a}(e^{-k_a t} - 1)\right] \quad .. \quad (13)$$

and when

$t = \infty$, then:

$$u_\infty = \frac{k_e}{k_d}.D_o \quad .. \quad .. \quad .. \quad .. \quad .. \quad (14)$$

Equations (13) and (14) are of the same form as those derived by Weigand & Taylor (1960) for non-sustained drug forms.

In calculating the total dose and the "maintenance form" of the drug required to give the desired therapeutic level Nelson (1957) relates all equations to the dose which gives the therapeutic response in the conventional dosage form. This is in the general form.

$$W_t = W_o + \frac{0{\cdot}693\ W_o.f.h.}{t^1/_2} \quad .. \quad .. \quad .. \quad (15)$$

in which W_t is the total dose in the sustained release product; W_o is the dose giving clinical response in the conventional dosage form; $t^1/_2$ is the biological half-life of the drug in the body; h is the number of hours required for a sustained body level of drug; and f relates the optimum therapeutic body level of the drug with the peak body level obtained with the conventional dosage form. This equation can lead to a higher drug quantity than is actually required, if no account is taken of the effect of the "maintenance form" of the drug, which is released from zero time;

this makes its contribution along with the "free form" of the drug. In consequence the initial dose will be too large. Since this value is used to calculate the "maintenance form" of the drug (equation 15), instead of being based on the optimum therapeutic level in the body, this form will also be excessive. The following example is used to demonstrate the above considerations. Consider a drug with the following properties, $W_o = 1g$, $k_d = 0{\cdot}173\ hr^{-1}$ ($t^1/_2 = 4$ hr) $k_a = 2{\cdot}0\ hr^{-1}$, $f = 0{\cdot}7$, and $h = 10$ hr.

Applying equation (15)

$$W_t = 1{\cdot}0 + \frac{0{\cdot}693 \times 1{\cdot}0 \times 0{\cdot}7 \times 10}{4}$$

$$= 1{\cdot}0 + 1{\cdot}21$$

$$= 2{\cdot}21g$$

Using the same parameters as above and adopting the equations outlined in the present article, the values for the initial dose in the "free form" (D_o) and maintenance dose (S_o) are given in Table 1.

TABLE 1. CALCULATION OF INITIAL DOSE AND MAINTENANCE DOSE FOR OPTIMUM THERAPEUTIC LEVEL

Parameter required	Value	Equation used
1g Conventional dosage form (*a*) Peak body level (b_{max}) .. .. (*b*) Time to attain peak level	 0·79 g 1·43 hr	 (12) (8)
Optimum therapeutic level (b_{opt})	0·56 g	$b_{opt} = f.b_{max}$ ($f = 0{\cdot}7$)
Rate constant (R_o)	0·096 g/hr	Since require rate in = rate out of body for sustained level, $R_o = k_d . b_{opt}$
Initial dose in the "free form" in the sustained release preparation (D_o)	0·60 g	(9) by: substituting $b = 0{\cdot}56$ g, $t = 1{\cdot}43$
Total maintenance dose (S_o)	1·09 g	$S_o = R_o . T = R_o(h + 1{\cdot}43)$
Total dose	1·69 g	

Thus there is a difference in the value of the total dose (W_t) from that calculated using equation (15): the results are shown graphically in Fig. 3. Curves are derived from equation (12) for a drug in solution, and equation (9) for sustained release dosage forms. Fig. 3 shows that the values given in Table 1, produce a constant body level of the drug, and maintain it at this level (b_{opt}) for the time required. On the other hand, using the values derived from equation (15) and substituting them in equation (9), the body level of drug rises higher than the initial dose in solution and could increase the incidence of side effects, the level always being above the therapeutic optimum.

Other workers have used equations similar to equation (15) and based W_0 on clinical data i.e. the dose which gives an optimum therapeutic level in the body (Robinson & Swintosky, 1959; Swintosky, 1960). Calculation of the "maintenance form" is still based on W_0 and not on the amount of the drug in the body at therapeutic level. The difference between calculation of the "maintenance form" presented here, and that

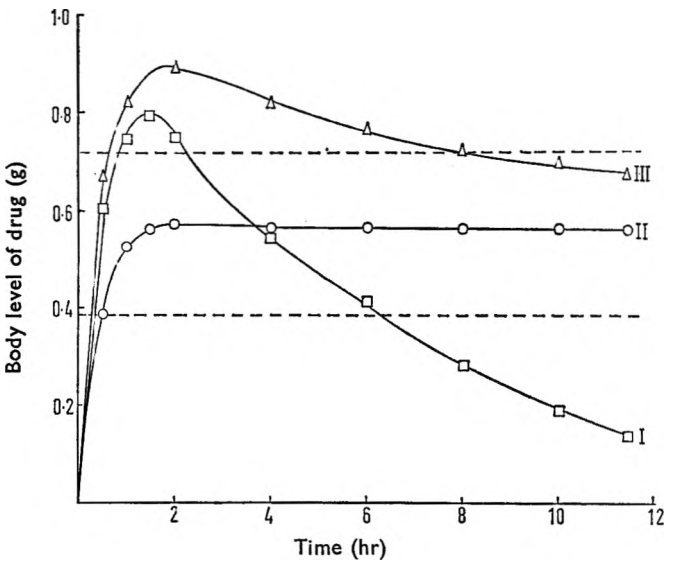


FIG. 3. Calculated plot for drug in solution, and for sustained release dosage forms, showing change in the body level of the drug with time. I. 1·0 g drug in solution (Eq. 12). II. Sustained release dosage form using values given in Table 1 (total dose 1·69 g; Eq. 9). III. Dosage form using values derived from equation 15. (Total dose 2·21 g; Eq. 9.) Broken lines define limits of therapeutic range.

using equation (15), will not be significantly different if there is little loss of drug from the body during attainment of the optimum therapeutic level. Hence the correction in calculating the maintenance form of the drug will only become large if the drug is rapidly eliminated from the body.

Acknowledgement. One of us (M.R.) thanks The Medical Research Council for a grant held while carrying out this research.

References

Bray, H. G. & White, K. (1957). *Kinetics and Thermodynamics in Biochemistry*, p. 166. New York: Academic Press Inc.
Dost, F. H. (1953). *Der Blutspiegel*, p. 41. Leipzig: Arbeitsgemeinschaft medizinischer Verlag.
Freed, S. C., Keatings, J. S. & Hays, E. E. (1956). *Ann. intern. Med.*, **44**, 1136–1141.
Gehlen, W. (1933). *Arch. exp. Path. Pharmak.*, **171**, 541.
Mulligan (1954). *J. Allerg.*, **12**, 366.

Nelson, E. (1957). *J. Amer. pharm. Ass. Sc. Ed.*, **46**, 572–573.
Nelson, E. (1963). *Clin. Pharmacol. Ther.*, **4**, 283–292.
O'Connor (1958). *Lancet*, 609.
Robinson, M. J. & Swintosky, J. V. (1959). *J. Amer. pharm. Ass. Sc. Ed.*, **48**, 473–478.
Swintosky, J. V. (1960). *Drug. Cosmet. Ind.*
Teorell, T. (1937). *Archs. int. Pharmacodyn.*, **57**, 205–225.
Weigand, R. G. & Taylor, J. D. (1960). *Biochem. Pharmacol.*, **3**, 256–263.

The paper was presented by MR. ROWLAND.

J. Pharm. Pharmacol., 1964, **16**, *Suppl.*, 163*T*–165*T* Received May 1, 1964

Alkaloids of the leaves of *Rauwolfia vomitoria* Afz.

M. B. PATEL, C. POISSON, J. L. POUSSET AND J. M. ROWSON

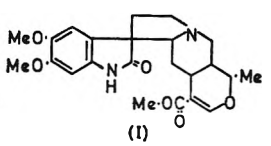
Leaves of *Rauwolfia vomitoria*, collected in Nigeria, contained at least six alkaloids, three are indole derivatives (aricine, tetrahydroalstonine, isoreserpiline) and three are isomers of α-oxindole type (carapanaubine, rauvoxine, rauvoxinine). Rauvoxine and rauvoxinine are new alkaloids. The leaves contained no reserpine.

THE tree *Rauwolfia vomitoria* Afz., family Apocynaceae, is found throughout West Africa. It is very abundant in Nigeria, especially in the southern parts, and is up to about 70 ft in height. Its root, root-bark, and, to a lesser extent, its stem bark are used as native medicines but the leaves and fruits are less frequently taken internally because of their powerful emetic properties.

Some investigations of the alkaloidal content of this species have been made but only the roots have been studied in detail and about 20 indole or indoline alkaloids have been identified (Poisson, 1958; Schlittler, 1964). Stem bark was shown to contain rauvanine (Goutarel, Gut & Parello, 1961). The fruits, rich in carotenoids, contain only traces of alkaloids and these are located in the seeds (Poisson, private communication). As yet no investigations of the alkaloidal content of the leaves have been published and hence this present work was undertaken.

The alkaloids of a leaf sample collected in Ibadan, Nigeria, from trees taxonomically identified as *Rauwolfia vomitoria* Afz., have been extracted and were fractionated by chromatography. Four crystalline alkaloids have been isolated: aricine, found in different *Rauwolfia* spp. and in *Cinchona pelletieriana* Wedd. (Goutarel, Janot, Le Hir, Corrodi & Prelog, 1954; Stoll, Hofman & Brunner, 1955), and carapanaubine, found in *Aspidosperma carapanauba* Pichon (Gilbert, Aguayo Brissolese, Finch, Taylor, Budzikiewicz, Wilson & Djerassi, 1963): the two others are new and have been named rauvoxine and rauvoxinine. In addition, small quantities of tetrahydroalstonine (Hochstein, 1955) and of isoreserpiline (Stoll, Hofmann & Brunner, 1955) have been isolated and identified. Reserpine was not found in this material.

Rauvoxine and rauvoxinine are isomers of carapanaubine or isoreserpiline-oxindole B (Finch, Gemenden, Hsiu-Chu Hsu & Taylor, 1963, Gilbert & others, 1963) and a close relationship is suggested by their similar ultra-violet and infra-red spectra. Thus, like carapanaubine, they probably possess a dimethoxyheteroyohimbane α-oxindole nucleus I.



(I)

From the Department of Pharmacy, Nigerian College of Technology, Ibadan, the Faculté de Pharmacie de Paris, and the Department of Pharmacy, Institute of Technology, Bradford.

These three alkaloids are the first members of the oxindole group to be reported in *Rauwolfia* spp. Their structure and stereochemistry are now being studied (Poisson & Pousset, in preparation).

Experimental

Melting-points were determined by the Köffler microscopic method; unless otherwise stated, optical rotations were in chloroform containing 0·5% ethanol using a Zeiss electronic polarimeter at 578 mμ; ultra-violet spectra were obtained in ethanol solution.

Total alkaloids were extracted from 4 kg of powdered dried leaves of *Rauwolfia vomitoria* by percolation to exhaustion with ethanol containing 1% of acetic acid. Ethanol was removed from the extract by evaporation at low temperature under reduced pressure. The residue was taken up in 2·5 litres of 2% hydrochloric acid and the resins removed by filtration. The filtrate was washed several times with light petroleum (b.p. 60–80°), made alkaline with ammonium hydroxide (0·88) and extracted with chloroform. This extract was washed with a solution of sodium bicarbonate, dried and evaporated to yield 45 g of crude bases (11·2 g/kg dried leaf). When dissolved in ether 5 g of insoluble residue remained.

Separation of alkaloids. Ether-soluble bases (40 g) were dissolved in benzene (insoluble fraction 9·2 g) and were chromatographed on alumina. Elution was as follows:

(*a*) Benzene (4,900 ml) yielded 67 mg.

(*b*) Benzene : ether (99 : 1; 2,100 ml) yielded 25 mg. When run on thin-layer plates (see below), one spot had the same Rf value as an authentic specimen of tetrahydroalstonine.

(*c*) Benzene : ether (90 : 10; 3,500 ml) yielded 831 mg. Crystallisation from ether gave aricine (529 mg), m.p. 187°, giving no depression on admixture with an authentic specimen. $[\alpha]_{578}^{20}$ — 58·4° (*c*, 0·93 in ethanol). Its ultra-violet and infra-red spectra were likewise identical with those of authentic aricine.

(*d*) Ether (700 ml) yielded 2·8 g which on crystallisation from ether yielded aricine (1·57 g).

(*e*) Ether (8,400 ml) yielded 3·14 g. Chromatography on thin-layer plates showed the presence of aricine and small quantities of a second alkaloid with the same Rf value as isoreserpiline.

(*f*) Ether : methanol (99 : 1; 2,800 ml) gave 1·54 g which crystallised from methanol to yield rauvoxine, 355 mg, m.p. 210° $[\alpha]_{578}^{20}$ + 98° (*c*, 1·34). Found: C, 64·1; H, 6·7; N, 6·5. $C_{23}H_{28}O_6N_2$ requires C, 64·5; H, 6·6; N, 6·5%. Molecular weight (mass spectrometer) 428 (calc. 428·5). λ_{max} 218 mμ (ε 26,300), 280 mμ (ε 5,300), shoulders at 245 and 300 mμ; ν_{max} (in chloroform) 2,820 cm^{-1}, 1,714 cm^{-1}, 1,627 cm^{-1}.

(*g*) Ether : methanol (99 : 1; 10,500 ml) gave carapanaubine (2·67 g), m.p. 218–219°, giving no depression on admixture with an authentic specimen. $[\alpha]_{578}^{20}$ — 115° (*c*, 1·05). Found: C, 64·5; H, 6·9; N, 6·4. Calc. for $C_{23}H_{28}O_6N_2$, C, 64·5; H, 6·6; N, 6·5%. Molecular weight (mass spectrometer) 428 (calc. for $C_{23}H_{28}O_6N_2$ 428·5). λ_{max} 218 mμ (ε 28,800),

280 mμ (ϵ 5,000), shoulders at 245 and 300 mμ; ν_{max} (in chloroform) 2,800 cm^{-1}, 1,710 cm^{-1}, 1,635 cm^{-1}. The infra-red spectrum was identical with an authentic sample of carapanaubine.

(*h*) Ether : methanol (98 : 2; 4,900 ml) gave 820 mg shown by thin-layer chromatography to be a mixture of carapanaubine and rauvoxine.

(*i*) Ether : methanol (95 : 5; 3,500 ml) gave rauvoxinine (520 mg), m.p. 203–204° $[\alpha]_{573}^{20}$ + 64·6 (*c*, 1·17). Found: C, 64·5; H, 6·6. $C_{23}H_{28}O_6N_2$ requires, C, 64·5; H, 6·6%. Molecular weight (mass spectrometer) 428 (calc. 428·5). λ_{max} 218 mμ (ϵ 24,500), 280 mμ (ϵ 5,600), shoulders at 245 and 300 mμ; ν_{max} (in chloroform) 2,820 cm^{-1}, 1,712 cm^{-1}, 1,630 cm^{-1}.

The ultra-violet absorption spectra of carapanaubine, rauvoxine and rauvoxinine are superimposable; the infra-red spectra of the three compounds are similar.

Further elution with ether containing more methanol, then with pure methanol gave uncrystallisable residues.

Tetrahydroalstonine, aricine and isoreserpiline were detected on plates of alkaline kieselgel using dichloromethane containing 1 or 2% methanol, also on plates of alumina using benzene : acetone (3 : 1) and employing authentic samples as controls. Carapanaubine, rauvoxine and rauvoxinine were separated on plates of alkaline kieselgel using dichloromethane containing 3% of methanol also on plates of alumina using benzene : acetone (3 : 2). Separations on alumina were less sharp than on kieselgel.

Acknowledgements. We thank Professor M. M. Janot and Dr. R. Goutarel for their interest in this work. We also thank Mlle. M. Rouat for technical assitance, Mme. Houelle for infra-red spectra, Mme. Orley and M. Varenne (I.C.S.N. Gif) for mass spectra and Mlle. Muller (Gif) for the analyses. Our thanks are due to D. E. Schlittler for the manuscript of his article (Schlittler, 1964). We are indebted to Drs. Finch and Taylor for the supply of a reference sample of carapanaubine.

References

Finch, N., Gemenden, C. W., Hsiu-Chu Hsu, I. & Taylor, W. I. (1963). *J. Amer chem. Soc.*, **85**, 1520–1523.

Gilbert, B., Aguayo Brissolese, J., Finch, N., Taylor, W. I., Budzikiewicz, H., Wilson, J. M. & Djerassi, C. (1963). *Ibid.*, **85**, 1523–1528.

Goutarel, R., Gut, M. & Parello, J. (1961). *C.R. Acad. Sci., Paris*, **253**, 2589–2591.

Goutarel, R., Janot, M. M., Le Hir, A., Corrodi, N. & Prelog, V. (1954). *Helv. Chim. Acta*, **37**, 1805–1814.

Hochstein, F. A. (1955). *J. Amer. chem. Soc.*, **77**, 5744–5.

Poisson, J. (1958). Thèse de Doctorat-ès-Sciences, Paris.

Schlittler, E. (1964). "*Rauwolfia Alkaloids with special reference to the Chemistry of Reserpine*" in Manske & Holmes, *The Alkaloids*, Vol. 8, Acad. Press (in press).

Stoll, A., Hofman, A. & Brunner, R. (1955). *Helv. Chim. Acta*, **38**, 270–283.

The paper was presented by DR. ROWSON.

J. Pharm. Pharmacol., 1964, **16**, *Suppl.*, 166*T*–168*T* Received May 2, 1964

SHORT COMMUNICATION

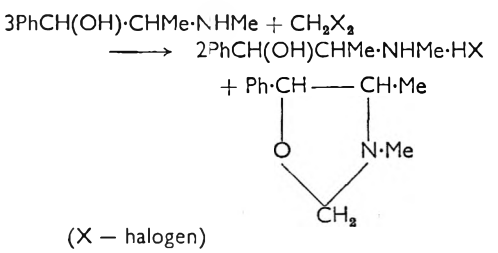
Reaction of ephedrine with chloroform

H. WILLIAMS

IN an official test for ephedrine (British Pharmacopoeia, 1958; British Pharmaceutical Codex, 1963) a solution of the alkaloid in chloroform is allowed to evaporate spontaneously to give a crystalline solid, identical in properties with ephedrine hydrochloride.

It has now been shown that when (—)-ephedrine is refluxed with chloroform B.P. the resulting ephedrine hydrochloride is contaminated with bromide ion. This arises from the impurity, bromochloromethane, which together with dichloromethane is present in chloroform B.P. (Caws & Foster, 1957). When (—)-ephedrine is refluxed with pure bromochloromethane a mixture of the hydrobromide and hydrochloride salts of the alkaloid is formed in equimolecular proportions. (—)-Ephedrine apparently reacts more readily with chloroform B.P. than with purified chloroform in which bromochloromethane is absent.

In addition to the alkaloidal salts a colourless oil is obtained whose infra-red spectrum is identical with 3,4-dimethyl-5-phenyloxazolidine. This is formed from the dihalomethane in accordance with the equation:



The formation of 3,4-dimethyl-5-phenyloxazolidine is in line with the reported formation of an oxazolidone from ephedrine and carbon tetrachloride (Hyne & Calosing, 1963; Pesez & Bartos, 1963).

EXPERIMENTAL

Chloroform B.P. was purified by the method of Caws & Foster (1957). This yielded chloroform (b.p. 60·5–61·0° n_D^{20} 1·4455) free from bromochloromethane and dichloromethane. (—)-Ephedrine, anhydrous: b.p. 132°/5 mm was obtained by vacuum distillation of (—)-ephedrine B.P. 1958. This was used immediately. Infra-red spectra were recorded in potassium bromide discs.

Comparison of reaction rates. The reaction of ephedrine with chloroform was followed by measuring the amount of halide ion liberated after

From the Welsh School of Pharmacy, the Welsh College of Advanced Technology, Cardiff.

definite time intervals at 60°. Proportions of base to chloroform of 0·01 to 0·03 moles gave measurable quantities of halide ion using the technique of Williams (1959). The results (Fig. 1) indicate (*a*) that both (—)-ephedrine B.P. (1958, hemihydrate) and (—)-ephedrine, anhydrous, are much more reactive towards chloroform B.P. than to purified chloroform and (*b*) that (—)-ephedrine B.P. is more reactive than (—)-ephedrine, anhydrous, towards both samples of chloroform.

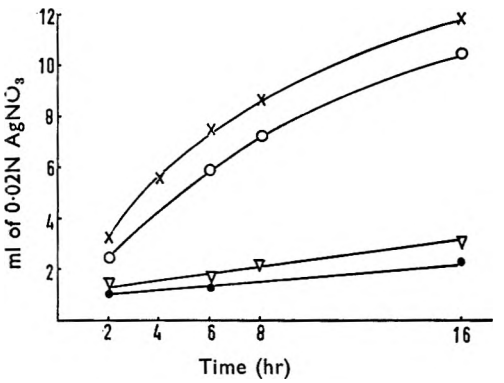


FIG. 1. Reaction between ephedrine, 0·01 moles, and chloroform, 0·03 moles. Temperature 60°. ×, Ephedrine B.P. and chloroform B.P. ○, Ephedrine, anhydrous and chloroform B.P. ▽, Ephedrine B.P. and purified chloroform. ●, Ephedrine, anhydrous and purified chloroform.

Investigation of reaction products. The alkaloid was refluxed (48 hr) with excess chloroform (B.P. or purified). The precipitate was filtered off and the filtrate refluxed further up to 48 hr when additional precipitate was formed. Concentration of the filtrate under vacuum gave further small amounts of solid and finally, unchanged ephedrine. The bulked crystalline product was washed with solvent and dried in a vacuum.

The results from various experiments are as follows:

(*a*) Ephedrine B.P. 1958 (17·4 g, 0·1 mole) in chloroform B.P. (1·5 moles) gave a product (2·3 g) m.p. 213°. Found: Cl, 14·5; Br, 8·8%.

(*b*) (—)-Ephedrine, anhydrous (16·5 g, 0·1 mole) in chloroform B.P. (1·5 mole) gave a product (1·6 g) m.p. 215°. Found: Cl, 10·4; Br, 14·7%.

(*c*) Ephedrine B.P. 1958 (8·7 g, 0·05 mole) in purified chloroform (0·5 mole) gave a product (0·47 g) m.p. 219°. Found: Cl, 17·8. Calc. for $C_{10}H_{15}NO.HCl$; Cl, 17·6%.

(*d*) (—)-Ephedrine, anhydrous (16·5 g, 0·1 mole) in purified chloroform (0·5 mole) gave a product (0·17 g) m.p. 217°. Found: Cl, 17·9. Calc. for $C_{10}H_{15}NO.HCl$; Cl, 17·6%.

In (*a*) and (*b*) the product had an infra-red spectrum identical with the ephedrinium ion. The product in (*c*) and (*d*) gave no depression on admixture with authentic ephedrine hydrochloride.

(*e*) Ephedrine B.P. 1958 (0·5 g) was dissolved in redistilled bromochloromethane (10 ml) and the solution allowed to stand at room temperature for several hr. The solid mass was filtered off and washed with chloroform to give a white crystalline solid (0·34 g) m.p. 212°. Found: C,53·6; H, 6·8; Br, 18·0; Cl, 8·0; N, 6·3. Calc. for an equimolecular mixture of $C_{10}H_{15}NO.HCl$ and $C_{10}H_{15}NO.HBr$; C, 53·6; H, 7·2; Br, 17·9; Cl, 7·9; N, 6·3%.

(*f*) (−)-Ephedrine, anhydrous (16·5 g, 0·1 mole) in dry benzene (75 ml) was refluxed (48 hr) with redistilled bromochloromethane (65 g, 0·5 mole). After removal of the crystalline solid the filtrate was further refluxed (12 hr) when more solid separated. Total yield: 13·6 g, m.p. 213°, producing no depression on admixture in equal proportions with authentic ephedrine hydrochloride and hydrobromide. The infra-red spectrum was identical with that of ephedrinium ion. Found: Br, 17·1; Cl, 8·4; N, 6·2. Calc. for an equimolecular mixture of $C_{10}H_{15}NO.HCl$ and $C_{10}H_{15}NO.HBr$: Br, 17·9; Cl, 7·9; N, 6·3%. After removal of all solids the filtrate was concetrated to yield a colourless oil (A) (5·48 g) b.p. 122°/8 mm, n_D^{20} 1·5248, $[\alpha]_D^{20}$–5·0 (EtOH).

(*g*) (−)-Ephedrine, anhydrous (16·5 g, 0·1 mole) in dry benzene (75 ml) was refluxed (48 hr) with redistilled dibromomethane (87 g, 0·5 mole). After removal of the precipitate the filtrate was further refluxed (12 hr) when the solid formed was again filtered off. Total yield: 14·63 g, m.p. 211°, giving no depression on admixture with ephedrine hydrobromide. The infra-red spectrum was identical with that of ephedrine hydrobromide. Found: Br, 32·5. Calc. for $C_{10}H_{15}NO.HBr$: Br, 32·5%. Concentration of the filtrate gave a colourless oil (B) (4·89 g) b.p. 118°/6 mm, n_D^{20} 1·5242, $[\alpha]_D^{20}$ − 5·2 (EtOH).

(−)-3,4-Dimethyl-5-phenyloxazolidine was prepared from (−)-ephedrine and formaldehyde (Bergmann, Zimkin & Pinchas, 1952). This was a colourless oil b.p. 125°/12 mm, n_D^{20} 1·5242, $[\alpha]_D^{20}$ − 5·6 (EtOH). Found: C, 74·0; H, 8·5; N, 8·0. Calc. for $C_{11}H_{15}NO$: C, 74·6; H, 8·5; N, 7·9%.

The infra-red spectrum (liquid) of this compound was identical with (A) and (B) above.

References

Bermann, E. D., Zimkin, E. & Pinchas, S. (1952). *Rec. Trav. Chim. Pays-Bas.*, **71**, 237–242.
British Pharmacopoeia (1958), p. 246, London: The Pharmaceutical Press.
British Pharmaceutical Codex (1963), p. 287, London: The Pharmaceutical Press.
Caws, A. C. & Foster, G. E. (1957). *J. Pharm. Pharmacol.*, **9**, 824–833.
Hyne, J. B. & Calosing, R. (1963). *Chem. & Ind.*, 488–489.
Pesex, M. & Bartos, J. (1963). *Bull. Soc. Chim.*, 1122–1124.
Williams, H. (1959). *J. Pharm. Pharmacol.*, **11**, 400–410.

The paper was presented by THE AUTHOR.

PRINTED BY W. HEFFER & SONS LTD., CAMBRIDGE, ENGLAND.