THE JOURNAL OF GENERAL MICROBIOLOGY

EDITED FOR
THE SOCIETY FOR GENERAL MICROBIOLOGY

VOLUME 42, 1966

CAMBRIDGE
AT THE UNIVERSITY PRESS
1966

PUBLISHED BY THE CAMBRIDGE UNIVERSITY PRESS
Bentley House, 200 Euston Road, London, N.W. 1
American Branch: 32 East 57th Street, New York, N.Y. 10022

Printed in Great Britain at the University Printing House, Cambridge
(*Brooke Crutchley, University Printer*)

Contents

No. 1 *issued January* 1966

No. 2 *issued February* 1966

No. 3 *issued March* 1966

THE JOURNAL OF GENERAL MICROBIOLOGY

The *Journal* will publish accounts of original research in general microbiology, i.e. the study of bacteria, microfungi, microscopic algae, protozoa, and viruses in their biological activities and, more particularly, the fundamental aspects of the study of these forms, including structure, development, physiology, genetics, cytology, systematics and ecology. Writers of papers on a specialized aspect of their subject should describe their work so that its relevance to their own science and to microbiology in general will be apparent to readers who may be unfamiliar with the particular aspect.

THE PREPARATION OF PAPERS

'Easy writing's curst hard reading.'—*Richard Brinsley Sheridan.*

'Easy reading's curst hard writing.'—*The Editors, J. gen. Microbiol.*

The Editors wish to emphasize ways in which contributors can help to avoid delays in publication.

(1) Papers must be written in English with the utmost conciseness consistent with clarity. The best English for the purpose of the *Journal* is that which gives the sense in the fewest short words.

(2) A paper should be written only when a piece of work is rounded off. Authors should not be seduced into writing a series of papers on the same subject *seriatim* as results come to hand. It is better, for many reasons, to wait until a concise and comprehensive paper can be written.

(3) Authors should state the objects they had in view when the work was undertaken, the means by which they carried it out and the conclusions they draw. A section labelled 'Discussion' should be strictly limited to discussing, if this be necessary, and not to recapitulating. Many papers when first sent to the *Journal* are too long for the crucial information they contain. It is unnecessary to describe preliminary or abortive experiments.

(4) Figures and tables should be selected to illustrate the points made, to summarize, or to record important quantitative results. Well-designed tables or graphs should need little explanatory letterpress. Photographs or drawings should not be submitted unless they illustrate facts that cannot be conveniently described in the text.

(5) Authors should remember that in preparing their typescript they are giving instructions to the printer (about layout, etc.), as well as attempting to convey their meaning to their readers. The latter object will be the better attained the more carefully authors consider how their typescripts will be converted to the printed page. Ink corrections on a typescript greatly prolong the type-setter's work; the final version of a paper must if necessary be retyped to provide a clean copy for the printer. Typescripts which do not conform to the conventions of the *Journal* will be returned to authors for revision.

(6) Special attention should be given to the details below in 'Directions to Contributors'. Strict observance of these requirements will help to shorten the interval between the receipt of a paper and its publication. Where relevant the 'Suggestions to Authors, Symbols and Abbreviations and Notes on Usage and Conventions' published in the *Biochemical Journal* (1957), **66**, 1–16 should be followed. The pamphlet, *General Notes on the Preparation of Scientific Papers*, published by the Royal Society, Burlington House, Piccadilly, London, W. 1 (2*s.* 6*d.*; post free, 2*s.* 10*d.*) will be found useful.

Editors do not alter authors' typescripts except to increase clarity and conciseness, or to bring them into line with the *Journal*'s conventions. If an editorial alteration changes an author's meaning one explication is that it was expressed ambiguously. When an editor can grasp the meaning of a sentence unequivocally it may be assumed that anyone can.

DIRECTIONS TO CONTRIBUTORS

Communications. Papers submitted for publication should be sent to A. F. B. Standfast (*The Journal of General Microbiology*), Lister Institute of Preventive Medicine, Elstree, Hertfordshire, England. Communications about offprints should be addressed to The University Press, Cambridge.

General. Submission of a paper to the Editors will be held to imply that it reports unpublished work, that it is not under consideration for publication elsewhere, and that if accepted for the *Journal* it will not be published again in the same form, either in English or in any other language, without the consent of the Editors.

Form of papers Submitted for Publication. The onus of preparing a paper in a form suitable for sending to press lies in the first place with the author. Authors should consult a current issue in order to make themselves familiar with the *Journal*'s typographical and other conventions, use of cross-headings, layout of tables, etc.

Papers should be headed with the title of the paper, the names of the authors (male authors use initials, female authors use one given name in full) and the name and address of the laboratory where the work was performed.

A paper should be submitted in double-spaced typing (top copy) with a 1½ in. left-hand margin, and on paper suitable for ink corrections. The paper should in general be divided into the following parts in the order indicated: (*a*) Summary: brief and self-contained; (*b*)

Introduction; (*c*) Methods; (*d*) Results (illustrative protocols only should be included); (*e*) Discussion (if any), and general conclusions; (*f*) Acknowledgements; (*g*) References.

The position of Tables and Figures should be indicated in the typescript.

Typescripts should carry the name and address of the person to whom proofs are to be sent, and a shortened version of the paper's title, not exceeding forty-five letters and spaces in length, suitable for a running title in the published pages of the work.

References. References in the text are cited thus: Brewer & Stewer (1942), (Brewer & Stewer, 1942). Where a paper to be cited has more than two authors, the names of all the authors should be given when reference is first made in the text, e.g. (Brewer, Stewer & Gurney, 1944), and subsequently as (Brewer *et al.* 1944); but papers with more than four authors may be cited, e.g. (Cobley *et al.* 1940) in the first instance. Where more than one paper by the same author(s) has appeared in one year the references should be distinguished in the text and the bibliography by the letters *a*, *b*, etc. following the citation of the year (e.g. 1914*a*, 1914*b*, or 1914*a*, *b*).

References at the end of the paper should be given in alphabetical order according to the name of the first author of each publication, and should include the title of the paper. Titles of journals should be abbreviated in accordance with the *World List of Scientific Periodicals*, 4th edn. (1965). References to books and monographs should include year of publication, title, edition, town of publication and publisher, in that order. It is the *duty of the author to check his references* and see that the correct abbreviations are used.

Illustrations. Illustrations and diagrams should be approximately *twice the size of the finished block*, each on a separate sheet, bearing the author's names, short title of the paper and Plate or Figure numbers on the back. Diagrams should be drawn in indian ink on plain white paper, Bristol board, faintly *blue*-lined paper, or tracing linen (but not plastic tracing linen) with letters, numbers, etc. written lightly in pencil. Lettering should be clear of the diagram and indicate by blue pencilled lines the desired position. Caption and legend should be typed on a sheet separate from the illustration and numbered to correspond. Drawings and photographs should include a statement of magnification. Photographs should be well-contrasted prints on glossy paper, and should be chosen for size and number, bearing in mind layout on the finished Plate; layout should be indicated. Coloured plates must be paid for by the author.

Tables. Tables should carry headings describing their content and be comprehensible without reference to the text. Each table should be typed on a separate sheet and its approximate position in the text indicated on the typescript.

Symbols and Abbreviations. Authors should refer to current issues of *The Journal of General Microbiology* for information in this connection. Attention is particularly drawn to the following points: degrees Centigrade are written, e.g. 100°, not 100° C.; hr, min., sec. (singular and plural); M = molar; m (milli-) = 10^{-3} and μ (micro-) = 10^{-6}; ml. (millilitre) should be used instead of c.c., and μg. (microgram) instead of γ; N = normal (of solutions); No. or no. = number. Ratios should be written 1:10; dilutions, 1/10.

Chemical Formulae. These should be written as far as possible on one line. The chemical nomenclature adopted is that followed by the Chemical Society (*J. chem. Soc.* 1936, p. 1067). With a few exceptions the symbols and abbreviations are those adopted by a committee of the Chemical, Faraday, and Physical Societies in 1937 (see *J. chem. Soc.* 1944, p. 717). Care should be taken to specify exactly whether anhydrous or hydrated compounds were used, i.e. the correct molecular formation should be used, e.g. $CuSO_4$, $CuSO_4.H_2O$ or $CuSO_4.5H_2O$.

Descriptions of Solutions. The concentrations of solutions are preferably defined in terms of normality (N) or molarity (M). The term '%' must be used in correct sense, i.e. g./100 g. of solution. For 'per cent of volume', i.e. ml./100 ml., the term '% (v/v)' should be used, and for weight of a substance in 100 ml. of solution, the term '% (w/v)'.

Proprietary Substances and Materials. At first mention, the correct designation of a manufacturer should be given in the text, with address to enable other workers to obtain the product mentioned.

Nomenclature of Amino Acids and Vitamins. The rules published in the *Biochemical Journal* (1952), **52**, 1–2, should be followed.

Nomenclature and Descriptions of Micro-organisms. Binomial Latin names of micro-organisms, the generic name only with a capital, must be used in accordance with International Rules of Nomenclature; in full at the first mention in each paragraph and in the Summary but in subsequent mention with the generic name abbreviated. Single initial letter abbreviations a used where they are not ambiguous. Binomials shou be underlined in the typescript. Scientific epithets trivial names are not underlined and should be witho capitals.

Descriptions of new species of cultivable microb should not be submitted unless an authentic specime of a living culture has been deposited in a recognize culture collection

The word 'generation' should not be used synon mously with 'subculture'. For an agreed use of tern like strain, type, variant, phase, etc., see the Inte national Bacteriological Code of Nomenclature, Se tion 1, Rules 7 and 8.

Except for good reasons, micro-organisms should designated by the names used in the works listed belo When other authorities are followed, they should b cited whenever obscurity might result from their use.

MICROFUNGI. *Ainsworth & Bisby's Dictionary of t Fungi*, 1961, 5th ed. (Kew: Commonwealth Myc logical Institute.)

PLANT PATHOGENIC FUNGI AND PLANT DISEASES. *List Common British Plant Diseases*, 1944. (Cambrid University Press.)

PLANT VIRUSES AND VIRUS DISEASES (1957). *Rev. ap Mycol.* 35, Suppl. 1–78.

BACTERIA. Author's preferences in naming are at prese accepted provided that the designation is unamb guous and conforms with the International Bacteri logical Code of Nomenclature (1949; *J. gen. Microbi* 3, 444) and the Opinions issued by the Internation Committee on Bacteriological Nomenclature. If desired, a synonym may be added in brackets when a name is first mentioned.

J. gen. Microbiol. (1966), **42**, 1–5
Printed in Great Britain

Inhibition by *o*-Aminoazotoluene of the Incorporation of Certain Radioactive Amino Acids into Lipids of *Mycobacterium smegmatis*

By M. TSUKAMURA, S. MIZUNO and S. TSUKAMURA

The Obuso National Sanatorium, Obu (near Nagoya), Aichi-Prefecture, and the Department of Bacteriology, Medical Faculty of Nagoya University, Nagoya, Japan

SUMMARY

A carcinogenic substance, *o*-aminoazotoluene (AAT), inhibited the incorporation of [1-^{14}C]glycine and [1-^{14}C]leucine into the lipid fraction of *Mycobacterium smegmatis*. It inhibited transiently the incorporation of [1-^{14}C]glutamic acid into lipids, but it did not inhibit the incorporation of [1-^{14}C]acetate into lipids. AAT did not inhibit the incorporation into the protein and nucleic acid fractions of [1-^{14}C]glycine, [1-^{14}C]leucine, [1-^{14}C]glutamic acid, and [1-^{14}C]acetate.

INTRODUCTION

Yoshida (1932, 1934*a*, *b*) demonstrated that *o*-aminoazotoluene (AAT) produced proliferation of hepatic tissue and hepatomas in mice. Biochemical observations on carcinogenesis, including reaction of the azo dyes with proteins, have been carried out by many investigators (reviews by Miller & Miller, 1953; Rondoni, 1955; Busch, 1962; Reid, 1962). Recently we observed that the growth of mycobacteria was inhibited by low concentrations of AAT in chemically defined media, but that this inhibition was abolished in egg media. As the action of AAT on mycobacterial cells is of interest in relation to its action on the cells of higher organisms, we have studied biochemical changes produced in mycobacteria by AAT.

METHODS

Mycobacterium smegmatis strain Jucho was used throughout. The following radioactive compounds were employed for the study: [1-^{14}C]glycine (specific radioactivity, 57·3 μc./mg.), DL-[1-^{14}C]leucine (specific radioactivity, 44·0 μc./mg.), DL-[1-^{14}C]glutamic acid (specific radioactivity, 21·0 μc./mg.), and sodium [1-^{14}C]-acetate (specific radioactivity, 148 μc./mg.); all supplied by the Radiochemical Centre, Amersham, Buckinghamshire.

The test organism was cultivated at 37° for 5 days in a modified Sauton medium, in which sodium glutamate was substituted for asparagine. The bacteria were harvested, washed three times in saline, and the washed bacteria incubated in reaction mixtures containing a radioactive compound. At different times, the reaction mixtures were cooled in ice and centrifuged. The bacteria were washed once with a solution of 1 % (w/v) glycine, 1 % leucine, 1 % sodium glutamate, or 1 % sodium acetate. They were then washed three times in distilled water and fractionated according to the procedure of Schneider (1945). The method was described previously (Tsukamura, Tsukamura & Mizuno, 1964).

Vol. 41, *No.* 3 *was issued* 21 *January* 1966

Samples (0·2 ml.) of each fraction were placed in stainless steel planchets and heated to dryness. The radioactivity was measured by a 2π gas flow counter and expressed as 'counts/minute/mg. dry weight organism' (the radioactivity in the sample was multiplied according to the amount of the fraction and divided by the dry weight). The radioactivity was recorded after subtracting the radioactivity at time 0.

Portions of the trichloroacetic acid (TCA)-soluble fractions were extracted five times with ethyl ether to remove the TCA and concentrated by heating under reduced pressure. Portions of the protein fractions were hydrolysed by heating in 6 N-HCl at 100° for 24 hr and concentrated by heating. These concentrates were subjected to ascending paper chromatography, using Toyo filter paper no. 50 and solvents consisting of tertiary butanol + formic acid + water (70 + 15 + 15 by vol.) and isobutanol + formic acid + water (70 + 15 + 15 by vol.). The R_F values given in this paper were obtained with the former solvent system unless it is specifically stated otherwise. The radioactivity in paper chromatograms was recorded on an automatic paper chromatogram scanner (Nihon Musen, Tokyo; running: 300 mm./hr; recording: 150 mm./hr; slit, 3 mm.; range: 300 or 1000 counts/5 sec.).

AAT (Takeda Chemical Co., Osaka) was dissolved in propyleneglycol at a concentration of 2 mg./ml. and 0·1 ml. samples of this solution were added to 4·0 ml. of the reaction mixtures. Thus, the final concentration in the reaction mixtures was 50 μg. AAT/ml., which was two to four times higher than the minimal inhibitory concentration. Control reaction mixtures contained 0·1 ml. propyleneglycol without AAT.

RESULTS

Effect of AAT on the incorporation of [1-^{14}C]glutamic acid

AAT did not inhibit the incorporation of [1-^{14}C]glutamic acid into the protein fraction, but it inhibited incorporation into the lipid and nucleic acid fractions during the first 6 hr. The effects of the inhibition had disappeared after 24 hr of incubation (Table 1).

Table 1. *Effect of o-aminoazotoluene on the incorporation of [1-^{14}C]glutamic acid into the cellular fractions of Mycobacterium smegmatis strain Jucho*

		Radioactivity as counts/min./mg. dry wt. bacteria			
		Fraction			
Reaction mixture	Time (hr)	TCA-soluble	Lipid	Nucleic acid	Protein
Control	3	1070	73	77	131
AAT	3	502	49	40	65
Control	6	740	159	102	152
AAT	6	532	49	56	170
Control	24	606	275	193	686
AAT	24	302	226	207	805

Reaction mixtures consisted of 4·0 ml. of 0·067 M-phosphate buffer (pH 7·1) containing 35 mg. wet wt. bacteria/ml., 2·5 μc. DL-[1-^{14}C]glutamic acid/ml., 5 μg., sodium glutamate/ml., 125 μg. glucose/ml. and 0 or 50μg. o-aminoazotoluene/ml.

Effect of AAT on the incorporation of DL-[*1*-^{14}C]*leucine*

Incorporation of [1-^{14}C]leucine into the lipid fraction was depressed in the presence of AAT. On the other hand, its incorporation into the protein fraction was not inhibited, but rather accelerated, by AAT (Table 2).

The only radioactive peak found in paper chromatograms of the TCA-soluble and protein fractions had an R_F (0·80) identical to that of leucine, whether AAT was present or not.

Table 2. *Effect of o-aminoazotoluene on the incorporation of* DL [*1*-^{14}C]*-leucine into the cellular fractions of Mycobacterium smegmatis strain Jucho*

		Radioactivity as counts/min./mg., dry wt. bacteria			
		Fraction			
Reaction mixture	Time (hr)	TCA-soluble	Lipid	Nucleic acid	Protein
Control	6	614	110	171	6,280
AAT	6	450	76	109	5,710
Control	24	545	520	348	10,800
AAT	24	588	325	337	17,900

Reactions mixture consisted of 4·0 ml. of 0·067 M-phosphate buffer (pH 7·1) containing 30 mg. wet wt. bacteria/ml., 2·0 μc. DL-[1-^{14}C]leucine/ml., 5 μg. L-leucine/ml., 125 μg. glucose/ml., and 0 or 50 μg. *o*-aminoazotoluene/ml.

Table 3. *Effect of o-aminoazotoluene on the incorporation of* [*1*-^{14}C]*glycine into the cellular fractions of Mycobacterium smegmatis strain Jucho*

		Radioactivity as counts/min./mg. dry wt. bacteria			
		Fraction			
Reaction mixture	Time (hr)	TCA-soluble	Lipid	Nucleic acid	Protein
Control	3	6,110	653	2,140	3,040
AAT	3	6,380	736	3,090	7,070
Control	6	3,700	920	4,680	6,970
AAT	6	4,360	870	5,270	10,000
Control	24	6,840	4,600	17,300	37,000
AAT	24	4,900	1,680	15,900	44,000

Reaction mixtures consisted of 4·0 ml. of 0·067 M-phosphate buffer (pH 7·1) containing 14 mg. wet wt. bacteria/ml., 2·5 μc. [1-^{14}C]glycine/ml., 5 μg. glycine/ml., 125μ g. glucose/ml., and 0 or 50 μg. *o*-aminoazotoluene/ml.

Effect of AAT on the incorporation of [*1*-^{14}C]*glycine*

[1-^{14}C]Glycine was incorporated into the lipid, nucleic acid and protein fractions. The incorporations into the nucleic acid and protein fractions were not inhibited by AAT. On the other hand, the incorporation into the lipid fraction was inhibited by AAT, and showed most clearly after 24 hr of incubation (Table 3).

A radioactive peak, identified as serine (R_F 0·36–0·37) by comparison with authentic serine, was found in paper chromatograms of the TCA-soluble and protein fractions. Since other radioactive peaks were not found in the paper chromatograms, presumably glycine was converted into serine and incorporated into protein (Fig. 1). However, in what form glycine is incorporated into lipids remains unknown.

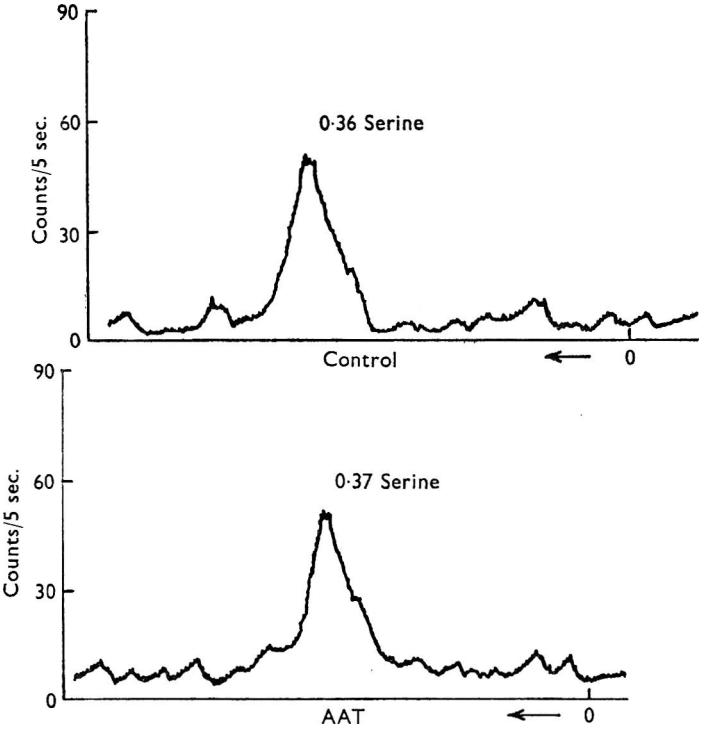


Fig. 1. Radioactivity in paper chromatograms of the acid hydrolysates of the protein fraction obtained after incubation with [1-^{14}C]glycine for 24 hr in the presence and absence of AAT.

Table 4. *Effect of o-aminoazotoluene on the incorporation of sodium [1-^{14}C]-acetate into the cellular fractions of Mycobacterium smegmatis strain Jucho*

Reaction mixture	Time (hr)	Radioactivity as counts/min./mg., dry wt. bacteria: Fraction: TCA-soluble	Lipid	Nucleic acid	Protein
Control	6	300	697	146	830
AAT	6	505	705	100	725
Control	24	492	958	188	1130
AAT	24	474	858	171	1070

Reaction mixtures consisted of 4·0 ml. of 0·067 M-phosphate buffer (pH 7·1) containing 70 mg. wet wt. bacteria/ml., 1·0 μc. sodium [1-^{14}C]acetate/ml., 100 μg. ammonium chloride/ml., and 0 or 50 μg. *o*-aminoazotoluene/ml.

Effect of AAT on the incorporation of sodium [1-^{14}C]acetate

[1-^{14}C]Acetate was incorporated into the lipid, nucleic acid and protein fractions. Almost no inhibitory effect of AAT on incorporation into these fractions was found (Table 4).

DISCUSSION

Incorporation of [1-^{14}C]glutamic acid, [1-^{14}C]leucine, [1-^{14}C]glycine, and [1-^{14}C]-acetate into the cellular fractions was observed both in the presence and absence of AAT. The concentration of AAT used was 2 to 4 times higher than the minimal growth-inhibitory concentration. Since the generation time of this organism was more than ten times longer than that of *Escherichia coli*, cultures were observed for a period of 24 hr.

The results showed that AAT inhibited the incorporation of [1-^{14}C]glutamic acid, [1-^{14}C]leucine and [1-^{14}C]glycine into the lipid fraction, the incorporation of the latter two amino acids being inhibited more markedly. On the other hand, incorporation of [1-^{14}C]acetate into the same fraction was not inhibited significantly by this agent.

It has been reported recently that serine is incorporated into lipids of *Escherichia coli* in the form of phosphatidylserine (Kanfer & Kennedy, 1964). Although it has been demonstrated in the present study that glycine is converted to serine, it remains to be tested in future whether or not glycine is incorporated into lipids via serine.

It was noted that AAT did not inhibit the incorporation of glutamic acid, leucine, glycine, or acetate into the protein and nucleic acid fractions.

The authors wish to express their appreciation to Professor K. Ogasawara for his kind advice. They thank also Dr R. Katsunuma for his kind help for this study.

REFERENCES

Busch, H. (1962). *An Introduction to the Biochemistry of the Cancer Cell*, p. 197. New York: Academic Press.

Kanfer, J. & Kennedy, E. P. (1964). Metabolism and function of bacterial lipids. II. Biosynthesis of phospholipids in *Escherichia coli*. *J. biol. Chem.* **239**, 1720.

Miller, J. A. & Miller, E. C. (1953). The carcinogenic dyes. *Advanc. Cancer Res.* **1**, 339.

Reid, E. (1962). Significant biochemical effects of hepatocarcinogens in the rat: a review. *Cancer Res.* **22**, 398.

Rondoni, P. (1955). Some aspects of carcinogenesis. *Advanc. Cancer Res.* **3**, 171.

Schneider, W. C. (1945). Phosphorus compounds in animal tissues. I. Extraction and estimation of desoxypentose nucleic acid and of pentose nucleic acid. *J. biol. Chem.* **161**, 293.

Tsukamura, M., Tsukamura, S. & Mizuno, S. (1964). Mode of action of chromomycin A3 on a Mycobacterium. *J. Antibiot. Tokyo*, A, **17**, 246.

Yoshida, T. (1932). Hepatoma induced by feeding with *o*-aminoazotoluene. *Proc. imp. Acad. Japan*, **8**, 464.

Yoshida, T. (1934*a*). Studies on the course of the development of experimental production of hepatoma induced by ortho-aminoazotoluol. *Gann*, **28**, 225.

Yoshida, T. (1934*b*). Experimental production of tumours by subcutaneous injection of an olive oil solution of *o*-aminoazotoluene. *Gann*, **28**, 441.

J. gen. Microbiol. (1966), **42**, 7–12
Printed in Great Britain

Mycobacterium parafortuitum: a New Species

By M. TSUKAMURA

The Obuso National Sanatorium, Obu near Nagoya, Aichi-Prefecture, Japan

(*Received* 13 *May* 1965)

SUMMARY

A new species *Mycobacterium parafortuitum* is described. A number of strains of the genus *Mycobacterium* isolated from soil resembled *M. fortuitum* in their rapid growth, in their pattern of utilization of organic acids and in their pattern of amidase tests. However, these strains differed from *M. fortuitum* in the following points: they showed negative 3-day arylsulphatase test, they showed negative salicylate degradation and PAS degradation, they did not utilize nitrite as sole nitrogen source, and they formed acid from pentoses, arabinose, xylose, inositol and mannitol. This group of strains has been named as *M. parafortuitum* sp.nov.

INTRODUCTION

In the course of a study on 'atypical' mycobacteria isolated in Japan, the author isolated many acid-fast bacilli from the soil for comparison with the 'atypical' mycobacteria isolated from humans. Organisms isolated from soil, after treatment of soil samples with 4% (w/v) NaOH and 4% (v/v) H_2SO_4, were studied. Strains which were considered to be mycobacteria, on morphological grounds, were then classified according to their amidase pattern (Boenicke, 1962). Strains which showed the pattern characteristic for *Mycobacterium fortuitum* were then tested for 3-day arylsulphatase activity (Wayne, 1961), nitrite utilization (Tsukamura & Tsukamura, 1965), and salicylate and *p*-aminosalycilate (PAS) degradation (Tsukamura, 1965). A group of strains showing positive reactions in three or four of these tests was classified as *M. fortuitum*. However, several strains which showed negative reactions in all of these tests were also found. These strains are considered to be a new species and their characters are considered in this paper.

METHODS

Growth rate and *temperature response* were observed on Löwenstein–Jensen medium.

Amidase tests were carried out according to the method of Boenicke (1962).

Three-day arylsulphatase test was carried out according to the method of Wayne (1961), and *3-week arylsulphatase test* according to the method of Kubica & Vestal (1961).

Utilization of organic acid as sole carbon source was observed in the following medium: $(NH_4)_2SO_4$, 2·64 g.; KH_2PO_4, 0·5 g.; $MgSO_4.7H_2O$, 0·5 g.; purified agar (Wako Chemical Co., Osaka), 30·0 g.; distilled water, 1000 ml. This was adjusted to pH 7·0 by addition of 10% (w/v) KOH and the medium sterilized by autoclaving at 115° for 30 min. Organic acids (sodium salts) were sterilized at 100° for 15 min. and

added to medium at a concentration of 0·01 M. The medium was readjusted to pH 7·0 and sterilized at 100° for 10 min. Growth was observed after 2 weeks incubation at 37°.

Acid formation from sugars was tested in the following medium: NH_4Cl, 2·0 g.; KH_2PO_4, 0·5 g.; $MgSO_4.7H_2O$, 0·5 g.; 0·2 % (w/v) bromothymol blue in 0·02 M-NaOH. 20·0 ml.; purified agar, 30·0 g.; distilled water, 1000 ml. (pH 7·2). Sugar solutions were sterilized separately by heating at 100° for 5 min. and added to medium aseptically to a final concentration of 0·5 % (w/v). Acid formation was observed after 2 weeks incubation at 37°.

Utilization of nitrogen compounds as sole nitrogen source (including sodium nitrite) was tested on the following medium: glycerol, 50·0 ml.; KH_2PO_4, 0·5 g.; $MgSO_4.7H_2O$, 0·5 g.; sodium citrate, 2·0 g.; purified agar, 30·0 g.; distilled water, 950 ml. (pH 7·0). Nitrogen compounds were sterilized by heating at 100° for 5 min. or Seitz filtration (sodium nitrite) and added to medium aseptically to a final concentration of 0·02 M. Growth was observed after 2 weeks incubation at 37°.

Salicylate degradation was tested on Sauton agar medium containing 0·5 mg. and 1·0 mg. sodium salicylate/ml. Blackening of the medium after 7 days incubation at 37° was recognized as positive degradation.

PAS degradation was observed on Löwenstein–Jensen medium or Ogawa egg medium containing 1 mg. sodium *p*-aminosalicylate (PAS)/ml. Blackening of the medium after 7 days incubation at 37° was recorded as a positive reaction.

Pathogenicity to mice (CF1 strain) was tested by intravenous injection of the test strains in a dose of about 2 mg. (wet weight) per mouse. The viable numbers in the lungs and spleen were recorded as an index of pathogenicity. For the determination of the viable numbers in organs, three mice were killed every week and the lungs and spleens were homogenized (in a motor homogenizer) and mixed with 10 vol. saline containing 100 units/ml. penicillin G. The organ homogenates were diluted with saline to make $1/1$–$1/10^4$ dilutions: 0·02 ml. of each dilution was inoculated to Löwenstein–Jensen medium (five tubes for a dilution) and incubated at 37°. After 4 days incubation, the number of colonies was counted and viable numbers per organ calculated.

RESULTS

Characters of seven representative strains are shown in Table 1.

Morphology. Rods, 2–3 μ in length after cultivation for 3–4 days on Löwenstein–Jensen medium at 37°; acid-fast short bacillary to coccoid forms in old cultures. Non-motile. No cord formation in Dubos liquid medium without Tween. No mycelium was observed in slide culture even at early stage of incubation when acid-fast rods were present.

Löwenstein–Jensen medium. At 3–4 days growth was smooth, wet, thin, soft, creamy or pale yellow; at 10–14 days it became yellowish. A strain of group B (see below) was scotochromogenic. Strains 310 and 311 were mucoid.

Sauton agar medium. At 6–7 days growth was smooth, wet, soft, creamy or pale yellow. A strain of the group B was yellowish orange. In tables, non-photochromogenic strains were designated by 'N' and scotochromogenic (only one strain) by 'S'.

Temperature relations. Some strains showed good growth at 28–45°, but no growth at 52°. These strains were named group A. Other strains showed little or

no growth at 45° (Table 1). This group was named group B. Neither group A nor group B strains survived 60° for 4 hr.

Salt tolerance. Growth variable in glycerol broth containing 5% (w/v) NaCl.

Nitrate reduction: variable.

Table 1. *Physiological characteristics of Mycobacterium parafortuitum*

	Strain						
	Group A				Group B		
Property	N310	N311	N304	N314	N305	N307	S312
Growth at							
52°	−	−	−	−	−	−	−
45°	+	+	+	+	±	−	−
37°	+	+	+	+	+	+	+
28°	+	+	+	+	+	+	+
Acid from							
Glucose	+	+	+	+	+	+	+
Mannose	+	+	+	+	+	+	+
Galactose	−	−	−	−	−	−	−
Arabinose	+	+	+	+	+	−	+
Xylose	+	+	+	+	+	+	+
Rhamnose	−	−	−	−	−	+	+
Trehalose	+	+	+	−	+	+	+
Lactose	−	−	−	−	−	−	−
Raffinose	−	−	−	−	−	−	−
Inositol	+	+	+	−	+	+	+
Mannitol	+	+	+	+	+	+	+
Sorbitol	−	−	−	−	−	−	−
Utilization of							
Acetate	+	+	+	+	+	+	+
Citrate	+	+	+	+	+	+	+
Succinate	+	+	−	+	+	+	+
Malate	+	+	+	+	+	+	+
Pyruvate	−	−	−	−	−	−	−
Benzoate	±	−	−	−	±	−	−
Utilization of							
Glycerol	+	+	+	+	+	+	+
Glucose	+	+	+	+	+	+	+
Fructose	+	+	+	+	+	+	+
Sucrose	+	+	−	−	+	+	−
Propyleneglycol	+	+	+	−	+	+	+
Amidase for							
Acetamide	+	+	+	−	+	+	−
Benzamide	−	−	−	−	−	−	−
Urea	+	+	+	+	+	+	+
Isonicotinamide	−	−	−	−	−	−	−
Nicotinamide	+	+	+	+	+	+	+
Pyrazinamide	+	+	+	+	+	+	+
Salicylamide	−	−	−	−	−	−	−
Allantoin	+	+	+	−	+	+	−
Succinamide	−	−	−	−	−	−	−
Malonamide	−	−	−	−	−	−	−
$NaNO_3$ reduction	−	+	−	+	+	+	+
3-day arylsulphatase test	−	−	−	−	−	−	−
3-week arylsulphatase test	+	+	+	+	+	+	+
Salicylate degradation	−	−	−	−	−	−	−
PAS degradation	−	−	−	−	−	−	−
$NaNO_2$ utilization	−	−	−	±	−	−	−

Three-day arylsulphatase test: negative.

Three-week arylsulphatase test: positive.

Salicylate degradation: negative.

PAS degradation: negative.

Amidase tests. Amidase positive for acetamide, urea, nicotinamide, pyrazinamide, and allantoin. Amidase negative for benzamide, isonicotinamide, salicylamide, succinamide, and malonamide. Incubation: 15 hr. Temperature: 37°.

Utilization of organic acids. Acetate, citrate, succinate and malate were utilized as sole carbon sources. Pyruvate was not utilized. No growth or scanty growth on medium containing benzoate as sole carbon source.

Acid formation from sugars. Acid was formed from glucose, mannose, arabinose, xylose, trehalose, inositol, and mannitol. No acid from galactose, lactose, raffinose, and sorbitol; rhamnose variable. Good growth or moderate growth on media containing most sugars, but scanty growth on media containing lactose or raffinose.

Table 2. *Virulence to mice of strains of Mycobacterium parafortuitum*

Strain	Inoculum	Organ	Viable numbers per organ*			
			1 week	2 weeks	3 weeks	4 weeks
N 310	$1{\cdot}3 \times 10^8$	Lungs	< 18	< 16	< 16	.
		Spleen	760	715	< 12	.
N 311	$2{\cdot}0 \times 10^8$	Lungs	< 18	229	< 16	< 18
		Spleen	6300	3630	1350	24
N 304	$9{\cdot}7 \times 10^8$	Lungs	< 23	< 20	< 18	.
		Spleen	440	< 30	< 22	.
N 314	$1{\cdot}0 \times 10^9$	Lungs	< 10	< 15	< 17	.
		Spleen	< 17	124	< 25	.
N 305	$3{\cdot}5 \times 10^8$	Lungs	< 15	< 26	< 27	< 20
		Spleen	6320	5330	545	< 24

* Average in three mice. Inoculation was made intravenously. At different intervals, the mice inoculated were sacrificed and viable numbers of bacteria were counted by dilution plating technique.

Utilization of nitrogen compounds as sole nitrogen source. L-Glutamate, L-leucine, L-glycine, L-serine, L-methionine, ammonium sulphate, ammonium chloride, sodium nitrate, nicotinamide, and urea were utilized. Isonicotinamide and pyrazinamide variable. Sodium nitrite not utilized.

Pathogenicity to mice. Intravenous injection of the strains at a dose of 2 mg. wet weight did not kill mice within 1 month. No macroscopic lesions were observed in the mice and intravenously injected bacilli were eliminated rapidly from mouse organs. Among the strains tested, N 311 and N 305 were eliminated more slowly than the other strains (Table 2).

DISCUSSION

In view of the results, the most useful characteristics for recognizing the strains described in this paper are: (1) rapid growth on egg media; (2) good growth at 45° for group A—this characteristic most easily differentiates this group from *M. fortuitum*; (3) negative 3-day arylsulphatase reaction; (4) inability to decompose salicylate or PAS; (5) failure to utilize sodium nitrite as sole nitrogen source within 1 week; (6) amidase positive for acetamide, urea, nicotinamide, pyrazinamide, and

allantoin; amidase negative for benzamide, isonicotinamide, salicylamide, succinamide, and malonamide after incubation at 37° for 15 hr; (7) ability to form acid from glucose, mannose, arabinose, xylose, trehalose, inositol, and mannitol; inability to form acid from galactose, raffinose, and sorbitol; (8) failure to utilize

Table 3. *Comparison of physiological characteristics of Mycobacterium fortuitum, M. parafortuitum, M. smegmatis, and M. rhodochrous*

Property	*M. fortuitum**	*M. parafortuitum*	*M. smegmatis**	*M. rhodochrous**
Growth at				
at 52°	−	−	−	−
at 45°	−	Variable	+	Variable
at 37°	+	+	+	+
Acid from				
Glucose	+	+	+	+
Mannose	+	+	+	+
Galactose	Rare	−	+	Rare
Arabinose	Rare	+	+	−
Xylose	Rare	+	+	Rare
Rhamnose	−	Variable	+	Rare
Trehalose	+	+	+	+
Lactose	−	−	−	−
Raffinose	−	−	−	−
Inositol	Rare	+	+	Rare
Mannitol	Variable	+	+	+
Sorbitol	Rare	−	+	+
Utilization of benzoate	−	−	+	+
3-day arylsulphatase test	+	−	−	−
Salicylate degradation	+	−	−	.
PAS degradation	+	−	−	.
$NaNO_2$ utilization	+	−	(+)	.

* Includes data taken from Gordon & Mihm (1959).

Table 4. *Comparison of physiological characteristics among Mycobacterium fortuitum, M. parafortuitum, and M. smegmatis*

Property	*M. fortuitum**	*M. parafortuitum*	*M. smegmatis**
Amidase for			
Acetamide	+	+	− or +
Benzamide	−	−	+
Urea	+	+	+
Isonicotinamide	−	−	+
Nicotinamide	+ or −	+	+
Pyrazinamide	+ or −	+	+
Salicylamide	−	−	− or +
Allantoin	+ or −	+	−
Succinamide	−	−	+
Malonamide	−	−	−

* Data taken from those of Boenicke (1962).

benzoate; (9) colonies slightly pigmented or non-pigmented; (10) rapid elimination from mouse body

By comparison of these data with the descriptions of Gordon & Mihm (1959), Gordon & Smith (1955), and Boenicke (1962), the strains described in this paper are differentiated from *Mycobacterium fortuitum*, *M. smegmatis*, *M. rhodochrous* and *M. phlei* Tables 3, 4).

The most important reactions separating these strains from those of *Mycobacterium fortuitum* are: negative 3-day arylsulphatase test; negative salicylate and PAS degradation tests; failure to utilize nitrite as sole nitrogen source; acid production from arabinose, xylose, and inositol; growth at 45° (group A).

The most important reactions separating the strains from *Mycobacterium smegmatis* are: failure to form acid from galactose or sorbitol; failure to utilize benzoate as sole carbon source; negative reactions for benzamidase, isonicotinamidase, and succinamidase; positive reaction for allantoinase.

The most important reactions separating the strains from those of *Mycobacterium rhodochrous* are: acid production from arabinose, xylose and inositol; inability to form acid from sorbitol; inability to utilize benzoate as sole carbon source.

The most important reactions separating the strains from *Mycobacterium phlei* are: no growth at 52°; failure to survive 4 hr at 60°; acid production from inositol; inability to form acid from sorbitol; positive reaction for allantoinase.

By these comparisons, the strains described in this study were differentiated from the known species and considered to belong to a new species. The author proposes to name this group of strains *Mycobacterium parafortuitum* sp.nov.

Strains 310 and 311 have been deposited in the National Collection of Type Cultures, Colindale Avenue, London. N.W. 9.

Strain 310 is NCTC 10410.
Strain 311 is NCTC 10411.

REFERENCES

BOENICKE, R. (1962). L'identification des mycobactéries à l'aide des méthods biochimiques. *Bull. Un. int. Tuberc.* **32**, 13.

GORDON, R. E. & MIHM, J. M. (1959). A comparison of four species of mycobacteria. *J. gen. Microbiol.* **21**, 736.

GORDON, R. E. & SMITH, M. M. (1955). Rapidly growing, acid fast bacteria. II. Species description of *Mycobacterium fortuitum* Cruz. *J. Bact.* **69**, 502.

KUBICA, G. P. & VESTAL, A. L. (1961). The arylsulfatase activity of stock cultures of acid-fast bacilli. *Am. Rev. resp. Dis.* **83**, 728.

TSUKAMURA, M. (1965). Salicylate degradation test for differentiation of *Mycobacterium fortuitum* from other mycobacteria. *J. gen. Microbiol.* **41**, 309.

TSUKAMURA, M. & TSUKAMURA, J. (1965). Differentiation of mycobacteria by utilization of nitrite as sole nitrogen source. *J. Bact.* **89**, 1442.

WAYNE, L. G. (1961). Recognition of *Mycobacterium fortuitum* by means of a three-day phenolphthalein sulfatase test. *Am. J. clin. Path.* **36**. 185.

J. gen. Microbiol. (1966), **42**, 13–23
Printed in Great Britain

Continuous Culture of Yeast on Phenol

By D. A. J. WASE and J. S. HOUGH
Department of Biochemistry, University of Birmingham

(*Received* 8 *June* 1965)

SUMMARY

Debaryomyces subglobosus (NCYC 459) was grown in continuous culture at 30° in media containing 3, 6·65, 9 and 12 μmoles phenol/ml. as sole source of carbon. With each medium, wash-out of organisms occurred at dilution rates in the range 0·33–0·36 hr^{-1}. The yeasts became quickly adapted to the medium with lowest phenol content and grew readily at dilution rates between 0·1 and 0·3 hr^{-1}. At lower dilution rates, however, the endogenous metabolism of the yeasts tended to outstrip the rate at which phenol was supplied. With 9 μmoles phenol/ml. the best rate of output of yeast was achieved but at 6·65 μmoles phenol/ml., was most fully utilized, utilization being complete up to a dilution rate of 0·3 hr^{-1}. At the highest contents of phenol in the medium, the yeast adapted less readily and produced considerable amounts of riboflavin. The population became heterogeneous, with some yeasts clumping and others large and fragile. There was some evidence that the latter were unable to metabolize phenol but could utilize catechol, a product of metabolism of the normal organisms.

The appendix provides a comparison between the reported results and those calculated from the growth constants using classical mathematical treatment of continuous culture. Substantial deviations are attributed to high maintenance energy requirements, flocculation of the yeast and toxicity of the medium.

INTRODUCTION

The breakdown of phenols by micro-organisms has received considerable attention because of its biochemical interest and its industrial importance in effluent treatment. In metabolic studies, attention has been principally focused on pseudomonads and vibrioform bacteria (Evans, 1963), while studies on industrial effluents have usually involved undefined mixtures of micro-organisms (Evans & Kite, 1961). Recently, the ability of yeasts to degrade phenol has been investigated (Harris & Ricketts, 1962) and, at least on pilot plant scale has been exploited commercially (Reiche, Hilgetag, Lorenz & Martini, 1962). The metabolic pathways involved have been shown to be similar to methods of breakdown of phenols by bacteria (Wase & Hough, to be published).

Special problems attend the use of toxic materials as substrates for micro-organisms and this is particularly apparent when phenols are used as the sole source of carbon. It has been shown in batch culture by Wase & Hough (1965) that selected yeasts will grow on low concentrations of phenol after a period of adaptation. With such limited supplies of carbon, however, the period of growth is very short in comparison with the lag period. After the phenol has been exhausted, the

yeast de-adapts quickly and a further lengthy period of adaptation is necessary before a fresh supply of phenol can be attacked. Continuous culture was therefore used to supply organisms fully adapted to phenol.

METHODS

Organism. *Debaryomyces subglobosus* (Zach) Lodder & Kreger-van Rij, strain NCYC 459, was obtained from the National Collection of Yeast Cultures, Brewing Industry Research Foundation, Nutfield, Redhill, Surrey. Stock cultures were maintained by monthly subculture on medium (*a*).

Media. (*a*) 10 % (w/v) malt-wort was steamed with 2 % (w/v) agar for 1 hr. The medium was dispensed in screw-cap bottles which were sloped after final sterilization.

(*b*) The medium used for continuous culture is given in Table 1. It was only effective for periods under 10 days because the pyridoxine was unstable. Phenol was always the limiting nutrient.

Table 1. *Composition of the growth medium*

(i) *Basal solution*

The following were dissolved in order in distilled water and made up to 7990 ml.: $(NH_4)_2HPO_4$, 19·0 g.; KH_2PO_4, 16·0 g.; $MgSO_4.7H_2O$, 8·0 g.; HCl (conc.), 4 ml.; $CaCl_2.6H_2O$, 2·0 g.

(ii) *Trace element solutions*

Solution A: KI (0·1 %, w/v, aqueous solution), 0·8 ml. added to basal solution.

Solution B: the following were dissolved in order in distilled water and made up to 100 ml.: $FeSO_4.7H_2O$, 0·25 g.; boric acid, 0·10 g.; ammonium molybdate, 0·02 g.; $MnSO_4.4H_2O$, 0·04 g.; $ZnSO_4.4H_2O$, 0·04 g.; $CuSO_4.5H_2O$, 0·045 g.; HCl (conc.), 2 drops. 1 ml. of solution B was added to the basal solution. The combined solutions were adjusted to pH 5·5 with A. R. KOH solution (40 %, w/v) and sterilized in a 10 l. pyrex aspirator at 15 lb. for 15 min.

(iii) *Growth factor solution*

The following were dissolved in distilled water and made up to 100 ml.: inositol, 1·0 g.; pyridoxine hydrochloride, 250 mg.; calcium D-pantothenate, 240 mg.; thiamine hydrochloride, 200 mg.; nicotinic acid, 200 mg.; folic acid, 10 mg.; D-biotin, 0·22 mg. This solution was separately sterilized in 8 ml. portions at 10 lb. instantaneously and then added aseptically to the bulk of the medium.

(iv) *Phenol* (A.R.)

Apparatus. Medium was fed by a metering pump to the 250 ml. culture vessel which was stirred magnetically and fitted with an overflow tube which maintained the culture volume at 150 ml. The vessel was surrounded by a glass jacket through which water at 30° was pumped. Aeration was provided by a small aquarium pump sparging air at about 15 l./hr through a sintered glass diffuser. A sampling device (Heatley, 1950) was initially used to transfer an active culture of the yeast into the vessel, and the pump rate was then adjusted to correspond with a dilution rate of 0·2 hr^{-1}. Equilibrium was judged to be achieved when the yeast content and the phenol concentrations were similar on consecutive daily tests.

Yeast concentration. Yeast concentration was measured as extinction in a Unicam SP 500 spectrophotometer operating at 650 mμ. Results were expressed as dry weights by means of a standard curve relating extinctions to weight of organisms dried at 120° for 16 hr.

Phenol concentration. Phenol content within the culture vessel was estimated by a method based on that of Snell & Snell (1937) using a 0·4% (w/v) solution of 2,6-dibromoquinonechloroimide in absolute ethanol. The reagent was stable for a week at about −2°. For the assay, 4·5 ml. solution was diluted with 95·5 ml. distilled water and a 2 ml. portion added to 1 ml. M-bicarbonate buffer (pH 9·4) and 1 ml. of test solution in a test-tube. The tube was shaken and held at 37° in a water bath for precisely 1 hr and the extinction of the solution at 625 mμ was recorded with a Unicam SP 500 spectrophotometer against a diluted reagent blank. Phenol concentration was directly proportional to the extinction, whereas similar concentrations of catechol (which was often present in the culture vessel) gave very low readings.

Manometric measurements. Oxygen uptake of yeast suspensions was determined by the direct method described by Umbreit, Burris & Stauffer (1957).

RESULTS

Four continuous cultures *a*, *b*, *c* and *d* were done with media containing 3, 6·65, 9 and 12 μmoles phenol/ml. as the sole source of carbon and equilibrium conditions were established for a range of dilution rates (relative flow rates.) With the cultures at equilibrium, phenol content, yeast content and yeast output were estimated. Phenol contents of *a* were low for dilution rates up to 0·2 hr^{-1}, but at higher dilution rates phenol content rose abruptly (Fig. 1). Culture *b* gave similar results except that the abrupt rise occurred after 0·3 hr^{-1}. In contrast, *c* gave a long steady rise after 0·2 hr^{-1}, while *d* provided low values until 0·1 hr^{-1}, followed by a steady rise.

For yeast content, *a* and *b* gave a long steady rise with increasing dilution rate and then an abrupt fall (Fig. 2). This contrasts with the short rise and long steady fall of *c* and *d*. The values for yeast output are shown in Fig. 3; *a* and *b* achieved maximum output at comparatively high dilution rates but *c* and *d* at much lower dilution rates. Except for *d*, maximum yeast output and maximum yeast content occurred at about the same dilution rate.

The lowest dilution rate needed for complete washing out of yeasts in the four experiments was within the narrow range 0·33–0·36 hr^{-1}. Phenol was supplied under these conditions for *a*:*b*:*c*:*d* in the ratio 1·00:2·33:3·24:4·32. In contrast, at maximum yeast output, the corresponding ratio for phenol supply was 1·00:2·66:2·40:2·00.

In *a* and *b* the organisms were mainly discrete, single and small, only a small proportion being large and fragile. The organisms of *c* were also of the two sizes but many were aggregated and this clumping was even more marked in *d*. The same aggregation occurred when organisms from *a* were transferred to a batch of medium containing 10·6 μmoles phenol/ml. and had grown after a 50 hr lag period. Transfer of yeast from *a* to a batch of medium with 1·06 μmole/ml. phenol, however, resulted in immediate growth and the organisms remained separate.

At dilution rates below 0·2 hr^{-1}, the *a* and *b* cultures had developed a slight fluorescent yellow coloration but in the *c* and *d* cultures this pigmentation of the medium was much more intense and persistent, being produced over a much wider range of dilution rates. The material imparting the colour had well marked

absorption peaks at 375 and 445 mμ and corresponded with authentic riboflavin in these and other spectrophotometric characteristics.

An inoculum of yeasts grown on 1 μmole phenol/ml. was used to initiate culture *c* with a dilution rate of 0·029 hr^{-1} being maintained. Slowly the yeast became adapted to the higher concentration of phenol and its ability to take up oxygen in the presence of phenol was measured manometrically (Table 2). As the culture

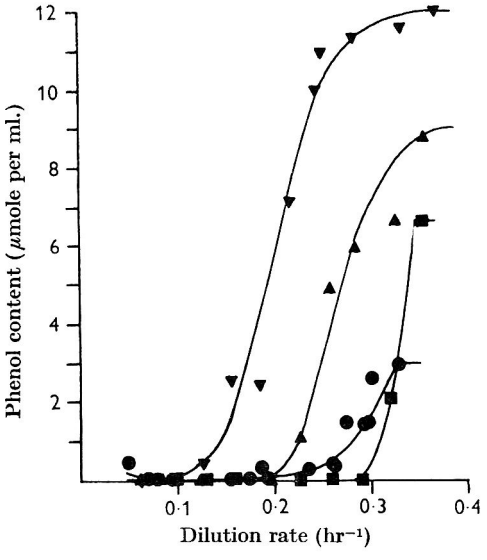


Fig. 1

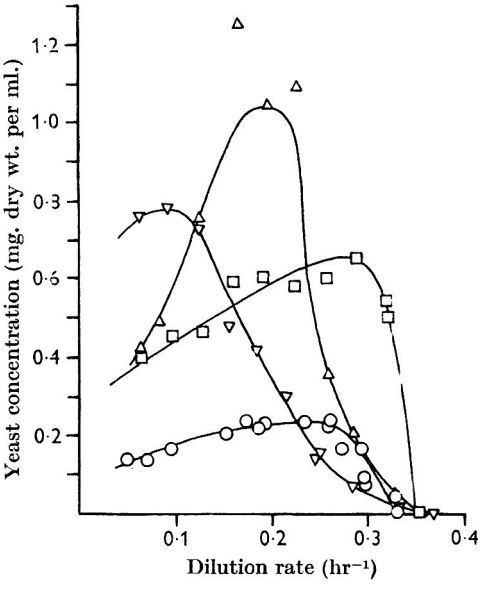


Fig. 2

Fig. 1. Phenol contents maintained in culture vessel at various dilution rates. Experiments: *a*, ●—●; *b*, ■—■; *c*, ▲—▲; *d*, ▼—▼; using media containing 3, 6·65, 9 and 12 μmoles phenol/ml. respectively.

Fig. 2. Yeast contents maintained in culture vessel at various dilution rates. Symbols (as in Fig. 1): *a*, ○—○; *b*, □—□; *c*, △—△; *d*, ▽—▽.

became heterogeneous, however, the manometric results showed that the ability to metabolize phenol was decreased. Furthermore, while the original adapted homogeneous population took up less oxygen in the presence of catechol than in the presence of phenol, the position was reversed for the heterogeneous population.

DISCUSSION

The results indicate that *Debaryomyces subglobosus* was able to metabolize substantial amounts of phenol as sole source of carbon. With 6·65 μmoles phenol/ml. organisms became adapted relatively easily in either batch or continuous culture but less readily at higher concentrations, and other differences observed were the disproportionately greater quantity of riboflavin produced and clumping, particularly at high dilution rates. Moreover, yeast grown in a low concentration of phenol and transferred to batch cultures with comparatively high concentrations grew only after a long period. The extended lag period and the clumping which

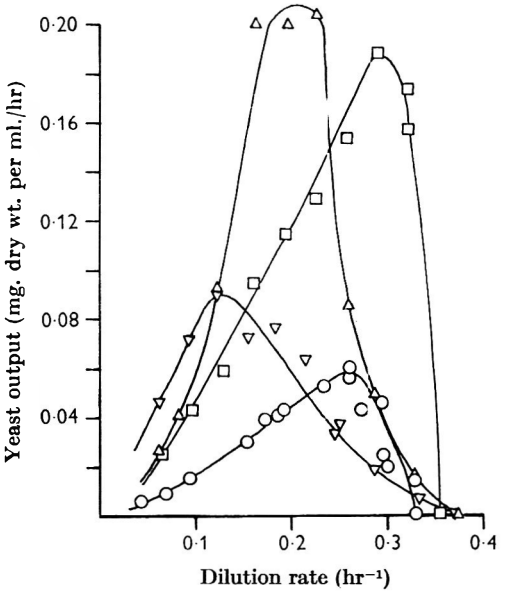


Fig. 3

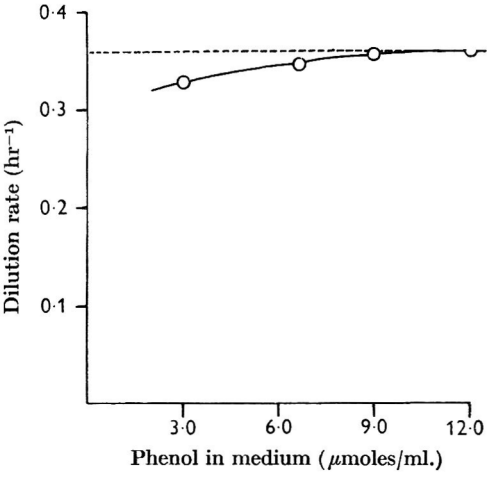


Fig. 4

Fig. 3. Yeast output from the culture vessel at various dilution rates. Symbols as in Fig. 2.

Fig. 4. Relationship between dilution rate just giving washout (critical dilution rate) and the concentration of phenol in the medium supplied.

Table 2. *Oxygen uptake of yeasts growing continuously on 9 μmoles phenol/ml.*

Yeasts grown batchwise on 1 μmole phenol/ml. were used in the continuous culture, with dilution rate of 0·029 hr^{-1}, sampled early and at the end of the adaptation period (10 and 80 hr after inoculation). Samples were offered either phenol (5 μmoles), catechol (5 μmoles) or medium without phenol in the Warburg respirometer.

Substrate	Oxygen uptake after 80 min (μl.) Early sample	Late sample
Medium without phenol (endogenous respiration)	63	185
Catechol	242	550
Phenol	507	243

occurred in batch and continuous cultures could be interpreted as an adaptation to the presence of relatively high concentrations of a metabolic inhibitor. But associated with this adaptive change the yeast population became less able to utilize the phenol. Some of the organisms may have retained their original ability in spite of the higher phenol content, while the rest became either less efficient at metabolizing phenol or were unable to utilize it at all. Because there is strong evidence that phenol is oxidized by yeasts to catechol and because the heterogeneous population metabolized catechol more strongly than phenol, it is possible that many of

the organisms were attacking extracellularly the catechol produced from phenol by the rest of the population.

Maximum yeast output (mg./ml./hr) for cultures *a*, *b*, *c* and *d* occurred at dilution rates (D_M) of 0·26, 0·30, 0·21 and 0·13 hr^{-1}, a reflexion of the somewhat toxic properties of the media in *c* and *d* and the relatively poor supply of substrate in *a*. The medium used for *b* gave both the highest dilution rate and rate of input of phenol for maximum yeast output (Table 3). It was the more toxic medium *c*, however, which gave the highest values for yeast concentration in the vessel and the

Table 3. *Comparison of continuous cultures using four levels of phenol concentration*

Expt.	Phenol conc. in medium (μmole/ml.) (S_R)	Dilution rate giving max. output (D_M)	Phenol input at D_M ($S_R.D_M$)	Max. yeast conc. achieved (mg. dry wt./ml.)	Dilution rate giving max. yeast conc. (Dx_M)	Phenol input at max. yeast conc. ($S_R.Dx_M$)	Apparent yield at max. yeast conc. (mg. dry wt./μmole)	Dilution rate at washout (D_c)
a	3·0	0·26	0·78	0·23	0·26	0·78	0·09	0·33
b	6·65	0·30	2·00	0·65	0·29	1·93	0·10	0·35
c	9·0	0·21	1·89	1·05	0·20	1·80	0·12	0·36
d	12·0	0·13	1·56	0·78	0·09	1·08	0·07	0·36

highest output: input ratio (that is the apparent yield values). This may have been because of the greater clumping of organisms in *c*. With discrete yeasts there was both regular division and regular loss of a proportion of the organisms in the continuous culture, but with clumps such regularity was impaired. The clumps varied both in size and in the age of their organisms; they fragmented either because of mechanical damage or growing larger than a critical size. This led to a spread of mean generation times of the organisms, discrete and aggregated, and to a low average growth rate.

With low dilution rates small yeast concentrations were maintained, particularly for media containing little phenol. This may have resulted from the very high endogenous metabolism of the population which accounted for the utilization of much of the phenol supplied. The amount of substrate left for growth therefore became progressively smaller at lower dilution rates, giving a progressively smaller population.

In contrast with bacteria, yeasts grow poorly at very low dilution rates because of the high endogenous metabolism, and are also washed out of the culture vessel at lower dilution rates. The yeasts at the point of washout are under considerable selection pressure with only the fastest growing surviving but surprisingly *a*, *b*, *c* and *d* washed out at dilution rates ranging only from 0·33 to 0·36 hr^{-1} (Table 3). Certain organisms adapted in *d* must therefore have grown as quickly as those in *a*, despite the large difference in phenol content of the media.

In this connexion, Ware & Evans (1959) were able to cultivate continuously bacteria in phenolic media at dilution rates as high as 0·55 hr^{-1} and with a concentration of 3750 mg. phenol/l. (40 μmoles/ml.) in the medium and 15 mg./l. in the effluent. Moreover Evans & Kite (1961), with *Pseudomonas aeruginosa*, were able to increase the dilution rates for phenol media to *c.* 0·66 hr^{-1} and to *c.* 0·77 hr^{-1}

with mixed bacterial cultures. With a non-toxic source of carbon present, rates of about 3 hr^{-1} were mentioned. On these grounds it would seem that yeasts are unlikely to be important commercially in the removal of phenol from effluents because of their slowness in adaptation, growth and uptake of phenol. It may be, however, that yeasts are more acceptable as a source of protein for animal feeding than pseudomonads. From the point of view of effluent utilization, the more slowly growing yeasts might therefore be worth investigation.

APPENDIX: THEORETICAL CONSIDERATIONS

Simple mathematical descriptions of continuous cultures have been produced where organisms are dispersed and fed with a medium in which one essential foodstuff is in limiting concentration (Monod, 1950; Novick & Szilard, 1950). Experimental results agree with calculated values in some cases (Herbert, Elsworth & Telling, 1956) but in others the yield of organisms per unit substrate varies with dilution rate (Herbert, 1958). For instance at low dilution rates, certain micro-organisms produce poor yields because the supply of nutrients is barely sufficient to meet the requirements for maintenance. At higher dilution rates, these demands are fully met and any additional nutrient available is used for growth. This means that the highest concentration of organisms is achieved, not at the lowest dilution rate but at a rate between zero flow and the point of washout.

Results of the present work are of interest because of the toxicity of the medium, and therefore values based on mathematical relationships derived by Herbert *et al.* (1956) were calculated. For this purpose, values for maximum rate of growth ($\mu_{max.}$), saturation constant (K_s) and yield constant (Y) are needed.

In batch culture of adapted yeasts grown on a medium containing 0·01 % phenol as sole source of carbon, $\mu_{max.}$ was 0·1133 hr^{-1}. It might be expected that a similar value would apply to continuous culture, but the following considerations refute this. On the basis of theory (Herbert *et al.* 1956)

$$\mu_{max.} = D_c \frac{(K_s + s_R)}{s_R}, \tag{1}$$

where D_c is the dilution rate at which washout occurs and s_R is the concentration of limiting substrate in the medium supplied. In practice, K_s is normally small compared with s_R and therefore the expression $(K_s + s_R)/s_R$ is almost unity, especially when s_R is large.

If this be so, then $\mu_{max.}$ should almost equal D_c which ranges from 0·328 hr^{-1} in *a* to 0·360 in *d*. When D_c is plotted against s_R, the relationship can be shown to be roughly asymptotic to the value 0·360. This value for D_c, and hence $\mu_{max.}$, is very different from $\mu_{max.}$ calculated from batch culture work. Because $\mu_{max.}$ and K_s are intimately related, it might be expected that similar discrepancies would occur in values calculated for K_s from batch and continuous cultures. Again on the basis of theory, the cell concentration,

$$x = Y\left[s_R - K_s\left(\frac{D}{\mu_{max.} - D}\right)\right], \tag{2}$$

and therefore

$$D_x = DY\left[s_R - K_s\left(\frac{D}{\mu_{max.} - D}\right)\right]. \tag{3}$$

As D, the dilution rate, increases from zero to D_c, x falls to zero. There will be a value of D where x is greatest and it is convenient to term this D_M. By differentiating the above equation and equating to zero, we can arrive at an expression for D_M. Thus

$$D_M = \mu_{max}\left\{1 - \sqrt{\frac{K_s}{K_s + s_R}}\right\}. \tag{4}$$

The values for D_M in *a* and *b* were recorded as 0·262 and 0·290 hr^{-1} respectively, but in *c* and *d* were considerably lower (0·21 and 0·13 respectively), presumably because of clumping and other toxicity effects. Values for K_s calculated on the basis of the above D_M results for *a* and *b*, are 0·2401 and 0·2613 μmole/ml. respectively. An alternative approach is to calculate values for K_s by substituting in equation (1). This gives K_s as 0·292 for *a* and 0·269 μmolc/ml. for *b*. These four estimates of K_s were averaged to give 0·266 μmole/ml. or 24·96 mg./l.

To arrive at a satisfactory value for Y, each recorded yield value in *a*, *b*, *c* and *d* was plotted against the appropriate dilution rate. The results (Fig. 5) give high correlation coefficients of +0·644 and +0·983 for *a* and *b* respectively, but a low degree of correlation for *c* and *d* (−0·110 and +0·194 respectively). The lines of best fit for *a* and *b* were calculated and extrapolated to give Y values at D_c. At D_c it might be expected that a large proportion of the phenol taken up by the organism would be directed to cell division. The Y values arrived at on this basis were 0·118 for *a* and 0·115 for *b*, giving a mean value of 0·1165 mg. dry wt./μmole.

Having arrived at values for $\mu_{max.}$, K_s and Y, it is possible to derive values for x and s for a range of values of D based on the following equations:

$$x = Y(s_R - s), \tag{5}$$

$$s = K_s\left(\frac{D}{\mu_{max.} - D}\right). \tag{6}$$

A comparison between the calculated values for x and s and the corresponding experimental results is shown in Fig. 6. For x values there are clearly considerable discrepancies at low dilution rates, with the experimental results being considerably ower. At higher dilution rates there is reasonable conformity in *a* and *b*, but wide deviations from the expected results in *c* and *d*. This is probably due to the toxic effects of phenol in *c* and *d* which lead to clumping and heterogeneity of the yeasts.

For s values, it is clear that in *a* and *b* rather more phenol is taken up than would be expected at high dilution rates. This may arise because the metabolic step of phenol to catechol is not rate limiting, while some of the later metabolic products might perhaps be lost, for instance 3 oxoadipate. With *c* and *d*, less phenol is taken up than expected and this again is presumably due to the toxic effects of the medium on the yeast population.

Returning to the low x values experienced at reduced dilution rates in *a*, *b* and *c*, it is probable that the expected results were not realized because of the high maintenance energy requirements of the yeast strain. Some allowance has been suggested by Herbert (1958) for these requirements by modifying the basic growth equation

$$dx/dt = \mu x \tag{7}$$

to

$$dx/dt = (\mu - k)x, \tag{8}$$

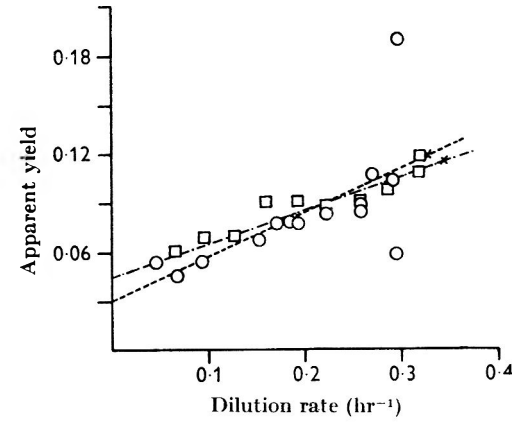


Fig. 5

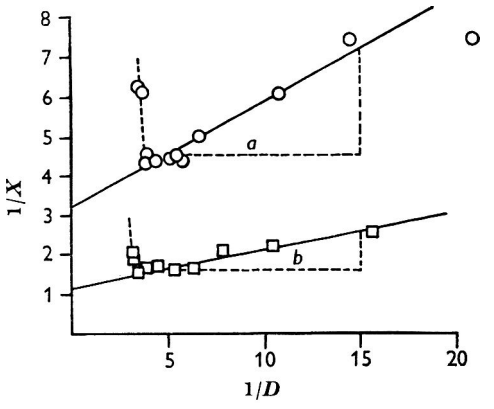


Fig. 7

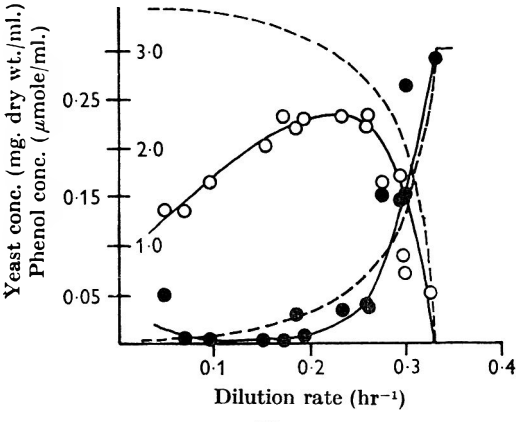


Fig. 6 (*a*)

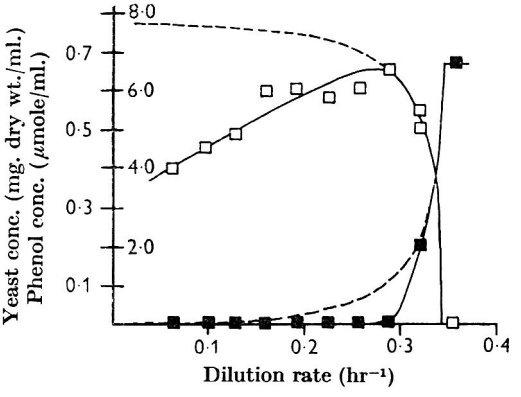


Fig. 6 (*b*)

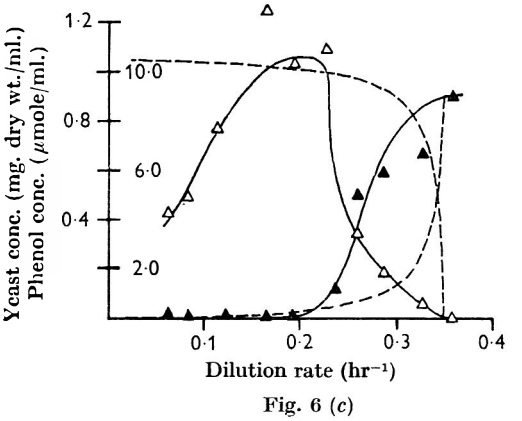


Fig. 6 (*c*)

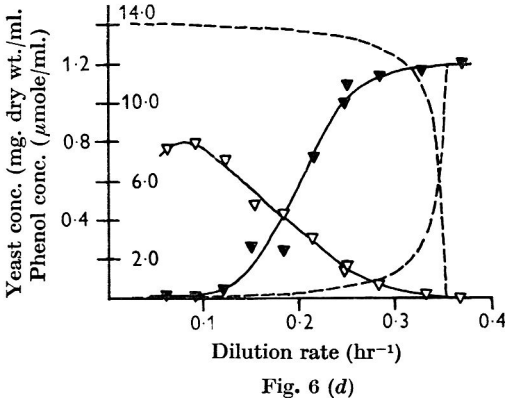


Fig. 6 (*d*)

Fig. 5. Relationship between apparent yield (mg. dry wt. of yeast per μmole phenol supplied) and dilution rate. Experiments: *a*, ○—○; *b*, □--□; using media containing 3, and 6·65, μmoles/phenol ml. respectively.

Fig. 6. Experiment *a*: comparison between observed (solid lines) and expected (broken lines) results for yeast and phenol concentrations at different dilution rates. —●—●, phenol content; —○—○, yeast content. Experiment *b*: —■—■, phenol content; —□—□—, yeast content. Experiment *c*: ▲—▲, phenol content; —△—△, yeast content. Experiment *d*: ▼—▼, phenol content; —▽—▽—, yeast content.

Fig. 7. Plot of inverse of yeast concentration against inverse of dilution rate in order to derive a value for k. Experiment *a*, ○—○; experiment *b*, □—□; $k = (\tan A)$ (max. yeast concentration).

where k is a constant cell maintenance requirement and hence

$$\mu = D + k. \tag{9}$$

Therefore

$$D(s_R - s) = x\left(\frac{D+k}{Y}\right). \tag{10}$$

If there are no maintenance requirements, then the cell concentration in the vessel would be $x_{max.}$ which is equal to the expression $Y(s_R - s)$.

Therefore

$$D(s_R - s) = x(D+k)\left(\frac{s_R - s}{x_{max.}}\right), \tag{11}$$

and thus

$$D = x\left(\frac{D+k}{x_{max.}}\right),$$

and hence

$$x = x_{max}\left(\frac{D}{D+k}\right),$$

or

$$\frac{1}{x} = \frac{k}{x_{max.}D} + \frac{1}{x_{max.}}. \tag{12}$$

If $1/x$ is plotted against $1/D$ (Fig. 7) a linear relationship would be expected. Where the ordinate is intercepted, the value $1/x_{max.}$ is given. (The slope of the line represents the value $k/x_{max.}$). An estimate of k can therefore be derived from these two values. In the case of *a* it is 0·0818 hr^{-1} and for *b*, 0·0842. These values are considerably greater than that calculated by Marr, Nilson & Clark (1963) for a strain of *Escherichia coli*, namely *c.* 0·02 and may reflect generally greater maintenance energy requirements for yeasts compared with bacteria.

The authors are grateful to Professor S. V. Perry for his help and encouragement and to the Institute of Brewing for a generous grant towards equipment. One of the authors (D.A.J. Wase) held a Ministry of Agriculture, Fisheries and Food Research Studentship during the work.

REFERENCES

EVANS, C. G. T. & KITE, S. (1961). Application of homogeneous continuous culture to the treatment of spent liquor. S.C.I. *Monograph No.* 12, '*Continuous Culture of Micro-organisms*'.

EVANS, W. C. (1963). Microbiological degradation of aromatic compounds. *J. gen. Microbiol.* **32**, 177.

HARRIS, G. & RICKETTS, R. W. (1962). Metabolism of phenolic compounds by yeast. *Nature, Lond.*. **195**, 473.

HEATLEY, N. G. (1950). A versatile fermentation sampling arrangement. *J. gen. Microbiol.* **4**, 410.

HERBERT, D. (1958). Some principles of continuous culture. In *Recent Progress in Microbiology. Symp. 7th, int. Congr. Microbiol.*, Stockholm.

HERBERT, D., ELSWORTH, R. & TELLING, R. C. (1956). The continuous culture of bacteria; a theoretical and experimental study. *J. gen. Microbiol.* **14**, 601.

MARR, A. G., NILSON, E. H. & CLARK, D. J. (1963). The maintenance requirement of *Escherichia coli*. *Ann. N.Y. Acad. Sci.* **102**, 536.

MONOD, J. (1950). La technique de culture continue. Théorie et applications. *Ann. Inst. Pasteur*, **79**, 390.

NOVICK, A. & SZILARD, L. (1950). Experiments with the Chemostat on the spontaneous mutation of bacteria. *Proc. natn. Acad. Sci., U.S.A.* **36**, 708.

REICHE, A., HILGETAG, G., LORENZ, M. & MARTINI, A. (1962). Continuous production of yeast from phenol-containing substrates. In *Continuous Cultivation of Micro-organisms, Proc. 2nd Symp., Prague.* Czechoslovak Academy of Science.

SNELL, F. D. & SNELL, C. T. (1937). *Colorimetric Methods of Analysis.* New York: Van Nostrand.

UMBREIT, W. W., BURRIS, R. H. & STAUFFER, J. F. (1957). *Manometric Techniques.* Minneapolis: Burgess.

WARE, G. C. & EVANS, C. G. T. (1959). Some preliminary experiments on the treatment of phenolic trade effluents by continuous culture. *Folia Microbiologica*, **4**, 62.

J. gen. Microbiol. (1966), 42, 25–37
Printed in Great Britain

Numerical Taxonomy of Bacteria — Some Published Data Re-examined

By D. W. GOODALL

C.S.I.R.O. Division of Mathematical Statistics, Western Australian Regional Laboratory, Nedlands, Western Australia

(*Received* 21 *June* 1965)

SUMMARY

A probabilistic similarity index has been calculated from published data for the genera *Chromobacterium* and *Mycobacterium*, and for the Enterobacteriaceae. The resulting similarity matrices have been used as a basis for classification with significance tests.

The results show very good general agreement with the original analyses using simple Jaccard similarity indices, but the new index has proved rather more sensitive, and has enabled certain additional subdivisions to be made.

INTRODUCTION

Eight years ago Sneath (1957*a*) published a first account of the use of computer methods for the classification of bacteria, and since that time a large number of papers have appeared in which the same or similar methods have been applied to other groups (see, for instance, Sneath's 1964 review of the subject).

Sneath used a very simple similarity index first introduced by Jaccard (1901) for the study of plant distribution and ecology, namely $100a/(a+b+c)$, where a was the number of positive two-value attributes shared by two strains, and b and c were the numbers of positive values shown by each strain separately, and not shared with the other. Most other workers since have used a modification of the same index (sometimes called the Simple Matching Coefficient) in which positive and negative records are treated alike, so that the expression becomes $[100(a+d)]/(a+b+c+d)$, where d is the number of attributes absent from both strains; this inclusion of negative matches was agreed by Sneath (Sokal & Sneath, 1963) to be a desirable improvement.

Once a matrix of similarity indices has been calculated, the question of how to use them in establishing a classification arises. The usual solution has been to group in a single taxon a set of OTUs (operational taxonomic units—a useful expression proposed by Sokal & Sneath, 1963, to designate the entities whose classification is in question—individuals, strains, or low-rank taxa already recognized), each of which is linked to one or all others of the set by a similarity index exceeding a specified arbitrary limit. A lower limit is then set, and any new groups, accretions to the existing groups, or combinations of groups, recorded at this new level. Or the association of a new OTU with a group already recognized may be made to depend on the mean value of its similarity indices with all members of the group.

Many workers using these methods of 'cluster analysis' have done so only with misgivings, because the clustering results could vary widely according to the particular arbitrary limits selected. Moreover, these methods did not lend themselves to significance tests, because the distribution of the indices under appropriate null hypotheses was not known.

'Natural' classification depends on correlation between the different classes of the values observed for a number of different attributes. If the question is raised whether a particular set of OTUs should be subdivided, an appropriate null hypothesis is consequently that the attribute values are assorted at random among them. If, on this null hypothesis, the probability of the observed degree of non-independence in distribution of attribute values within the set is less than the chosen significance level, the null hypothesis is rejected and the set is subdivided. Such a significance test has not been developed in connexion with the usual similarity indices. Recently, however (Goodall, 1964, 1966), a new type of similarity index has been proposed which is the complement of the probability that two OTUs would have the observed (or a greater) degree of similarity if attribute values were assorted at random. In principle, this new index should lend itself to a classification controlled by a specified significance level.

A probabilistic similarity index of 0·95, considered in isolation, is clearly just significant at the 5 % level—there is a probability of exactly 0·05, on the null hypothesis, of obtaining a value as large or larger. But when a whole similarity matrix is under consideration its collective probability rather than that of a single index may be required. For instance, a similarity matrix for 20 individuals containing 190 indices would, on the null hypothesis of random assortment of attributes (implying a rectangular distribution of the indices) be expected to contain 19 indices $> 0{\cdot}90$ and nearly two $> 0{\cdot}99$; consequently a single pair of individuals with a similarity index of 0·99 is not to be regarded as constituting a cluster at the 1 % significance level. For this, an index of 0·99995 would be required, which is expected just once in 100 such matrices.

If, on the other hand, a set of 20 individuals included a subset of six, every pair of which had a similarity index of 70 or more, this subset must be regarded as constituting a cluster at a significance level of 1 %,* for so large a subset linked by similarity indices all as high as this would be expected by chance less than once in 100 sets of twenty. This would be true even if none of the pairs individually had the index value required for significance.

The fact that significance of a larger cluster does not imply that smaller subsets within it are also significant clusters complicates the procedure for establishing a hierarchy of taxa. For this purpose several courses are open. One could, for example, apply a fixed significance level to the original similarity matrix, identify clusters of different sizes at this level, and regard the smaller clusters as subclasses at a lower hierarchic level within those larger clusters which include them. Or one could use a hierarchy of significance levels, smaller clusters identifiable at a higher significance level being included as subgroups within larger clusters at a lower

* This, together with similar statements elsewhere in this paper, is based on an assumption of independence of the various similarity indices in the matrix—an assumption never fully valid in a practical situation. It will be shown, however, in a paper shortly to be published, that this is often an acceptable approximation.

significance level. Neither of these procedures seems fully satisfactory, since within a significant cluster relations among similarity indices based on the whole population cannot be expected to follow expectations from a model of random assortment of attributes. On the other hand, if a new similarity matrix is calculated from the frequencies of attribute values within the cluster only (the indices in which will not necessarily be closely related to those between the same pairs in the original matrix), a new null hypothesis of random assortment of attributes within this more limited population may be set up and tested. Accordingly, this is the procedure which has been provisionally adopted. If significant clustering is detected, the largest significant clusters are separated, new similarity matrices calculated, and tests for clusters of subordinate rank performed. A detailed description of this procedure will be published shortly.

Some of the papers proposing bacterial classifications based on computer analysis have included full data on the attributes studied. It has seemed of interest to re-analyse these by the new methods proposed, and compare the results with those obtained by using simple similarity indices and arbitrary levels for distinguishing clusters. Three such sets of data have been re-analysed, with the results described below.

RESULTS

Enterobacteriaceae

In the study by Lysenko & Sneath (1959) of fourteen species or species groups in the Enterobacteriaceae, thirty-one characters were included, in the main biochemical. Of these, twenty-five were described as either positive or negative (or as varying between strains in the group, in which case they were ignored), while the other six—sucrose, trehalose, H_2S, citrate, glucose utilization, and glutamic acid—were recorded in more than one grade of positivity, and coded by the authors as two or more binary features. Since the probabilistic similarity index can take direct account of multi-value attributes, this binary coding was unnecessary, and the values were treated as ordered in the following sequence: prompt positive > delayed positive > late, irregular or mutated positive > negative.

As Lysenko and Sneath said, the characters reported had been selected, so that these data could not show whether the groups are valid taxa. It may nevertheless be of interest to see whether these selected data support the accepted taxonomic relationships when probabilistic similarity indices are calculated.

The indices are reported in Table 1. The large concentration of high values in the bottom right-hand corner of the table will be noted. The groups from 9 to 14 are all linked by similarity indices greater than 0·83, and only one of the fifteen is below 0·95. The chance that any such subset of groups could occur if the characters recorded had been randomly assorted among the groups is vanishingly small. The first eight bacteria also show a good deal of affinity, with nine (out of 28) similarity indices greater than 0·9, and 17 greater than 0·7, whereas between these two subsets (1–8 and 9–14) only 2 of the 48 similarity indices exceed 0·5.

These results, then, suggest a first division into two subsets, one including the groups from *Escherichia* to *Serratia* inclusive, the other the remainder including the two Proteus species. If now the process is repeated on these two subsets, the resulting similarity indices are those shown in Table 2.

Table 1. *Similarity indices for Enterobacteriaceae*

Group name	Group no. 1	2	3	4	5	6	7	8	9	10	11	12	13
1. *Escherichia*	—	—	—	—	—	—	—	—	—	—	—	—	—
2. *Salmonella*	0·924	—	—	—	—	—	—	—	—	—	—	—	—
3. *Arizona*	0·892	0·996	—	—	—	—	—	—	—	—	—	—	—
4. *Citrobacter*	0·872	0·867	0·920	—	—	—	—	—	—	—	—	—	—
5. *Cloaca*	0·608	0·622	0·769	0·793	—	—	—	—	—	—	—	—	—
6. *Klebsiella*	0·576	0·411	0·742	0·744	0·973	—	—	—	—	—	—	—	—
7. *Hafnia*	0·158	0·626	0·901	0·396	0·852	0·981	—	—	—	—	—	—	—
8. *Serratia*	0·104	0·322	0·473	0·158	0·932	0·968	0·938	—	—	—	—	—	—
9. *Proteus vulgaris*	0·136	0·034	0·153	0·059	0·063	0·040	0·052	0·825	—	—	—	—	—
10. *P. mirabilis*	0·034	0·139	0·381	0·092	0·096	0·001	0·134	0·758	1·000	—	—	—	—
11. *Morganella*	0·421	0·026	0·014	0·004	0·001	0·000	0·352	0·145	0·997	0·999	—	—	—
12. *Providencia*	0·373	0·011	0·002	0·008	0·006	0·048	0·040	0·537	0·974	0·961	1·000	—	—
13. *Rettgerella*	0·143	0·001	0·000	0·004	0·027	0·919	0·012	0·027	0·997	0·832	0·999	1·000	—
14. *Shigella* type A1	0·822	0·448	0·509	0·148	0·017	0·315	0·012	0·061	0·768	0·947	0·999	0·999	0·950

Table 3. *Similarity indices between Mycobacterium strains*

	(a)	(b)	35	24	10	(c)	41	33	(d)	39	(e)	27	34	42
(a) 1, 2, 3, 4, 5, 6, 8, 9, 36, 37	0·896	—	—	—	—	—	—	—	—	—	—	—	—	—
(b) 13, 16, 17, 18, 19, 20, 22, 23, 38	0·691	0·778	—	—	—	—	—	—	—	—	—	—	—	—
35	0·843	0·578	—	—	—	—	—	—	—	—	—	—	—	—
24	0·821	0·537	0·742	—	—	—	—	—	—	—	—	—	—	—
10	0·821	0·535	0·741	0·710	—	—	—	—	—	—	—	—	—	—
(c) 6a, 11, 12, 14, 15, 21, 25	0·578	0·686	0·836	0·404	0·408	0·889	—	—	—	—	—	—	—	—
41	0·753	0·421	0·654	0·704	0·615	0·287	—	—	—	—	—	—	—	—
33	0·804	0·505	0·719	0·686	0·800	0·371	0·588	—	—	—	—	—	—	—
(d) 7, 26, 28	0·404	0·531	0·731	0·874	0·227	0·810	0·221	0·200	0·977	—	—	—	—	—
39	0·351	0·480	0·620	0·852	0·182	0·721	0·176	0·157	0·961	—	—	—	—	—
(e) 29, 30, 31, 32	0·166	0·280	0·431	0·742	0·054	0·556	0·051	0·041	0·923	0·994	1·000	—	—	—
27	0·716	0·366	0·902	0·568	0·958	0·704	0·455	0·955	0·553	0·414	0·220	—	—	—
34	0·152	0·004	0·426	0·047	0·719	0·089	0·063	0·706	0·019	0·011	0·010	0·997		
42	0·411	0·081	0·290	0·235	0·871	0·032	0·270	0·864	0·003	0·002	0·002	0·994	1·000	—
40	0·108	0·010	0·050	0·070	0·673	0·001	0·913	0·658	0·000	0·310	0·302	0·978	1·000	1·000

Within the eight groups forming the first subset, there is a strong suggestion that two smaller subsets (1–4 and 5–8) are more closely related. The twelve similarity indices within these subsets are all more than 0·66, while only two of the sixteen indices between the two subsets exceed this value. The chance that a particular partition of eight species groups into two subsets of four should give twelve values all more than 0·66 is $(1-0{\cdot}66)^{12} = 2{\cdot}4 \times 10^{-6}$, on the assumption of independence

Table 2. *Similarity indices within subsets of Enterobacteriaceae*

Group no....	1	2	3	4	5	6	7
Group name							
1. *Escherichia*							
2. *Salmonella*	0·905	—	—	—	—	—	—
3. *Arizona*	0·809	0·994	—	—	—	—	—
4. *Citrobacter*	0·759	0·821	0·883	—	—	—	—
5. *Cloaca*	0·305	0·179	0·829	0·906	—	—	—
6. *Klebsiella*	0·176	0·147	0·115	0·436	0·721	—	—
7. *Hafnia*	0·054	0·550	0·504	0·345	0·663	0·821	—
8. *Serratia*	0·008	0·165	0·346	0·102	0·897	0·748	0·986

Group no....	9	10	11	12	13
9. *Proteus vulgaris*					
10. *P. mirabilis*	1·000	—	—	—	—	—	—
11. *Morganella*	0·494	0·864	—	—	—	—	—
12. *Providencia*	0·244	0·048	0·787	—	—	—	—
13. *Rettgerella*	0·425	0·214	0·743	0·895	—	—	—
14. *Shigella* type A1	0·001	0·188	0·761	0·821	0·078	—	—

Group no....	1	2	3
1. *Escherichia*			
2. *Salmonella*	0·631	—	—
3. *Arizona*	0·070	0·842	—
4. *Citrobacter*	0·334	0·744	0·642

Group no....	5	6	7
5. *Cloaca*			
6. *Klebsiella*	0·584	—	—
7. *Hafnia*	0·307	0·932	—
8. *Serratia*	0·306	0·082	0·906

Group no....	11	12	13
11. *Morganella*			
12. *Providencia*	0·835	—	—
13. *Rettgerella*	0·921	0·976	—
14. *Shigella* type A1	0·450	0·344	0·052

of the similarity indices. Since there are seventy possible partitions of a set of eight into two subsets of four, the overall probability of the observed partition of similarity indices is less than 0·0002, and the distinction between the two subsets must be adjudged highly significant.

Within the second subset (groups 9–14), the very high similarity index between the two *Proteus* species (more accurately, 0·999809) sets them apart from the rest, for the chance that at least one out of fifteen similarity indices derived from randomly assorted characters would be so high is only 0·003.

The next stage in the analysis is accordingly to test whether the subsets 1–4, 5–8, and 11–14 show further internal relationships when they are treated separately. The results, also in Table 2, give no firm grounds for a positive answer. The high similarities included are no higher, and no more numerous, than one could reason-

ably expect in sampling from a rectangular distribution, nor do they give evidence of clustering.

In comparing the results of this analysis with that of Lysenko & Sneath (1959), one first notes agreement in the close affinity of the two *Proteus* species, and in inclusion of *Morganella*, *Providencia* and *Rettgerella* in a broader group (*Proteus* sensu lato). *Providencia* and *Rettgerella* were placed very close together in both analyses. *Shigella* was placed in an isolated position by Lysenko & Sneath, whereas here it is placed within the broader *Proteus* group; the difference arises because the Jaccard index used by Lysenko & Sneath only takes presence of characters into account, whereas the index used here gives equal weight to their absence. The affinity of *Shigella* with *Escherichia* is however reflected in a fairly high similarity index (0·822), so that *Shigella* may to some extent form a bridge between the two main groups. Lysenko & Sneath comment on the close relationship between *Salmonella* and *Arizona*, reflected here in similarity indices of 0·996, 0·994 and 0·842 in successive stages of the analysis. But the distinction between the first two subsets of four groups did not emerge from their treatment of the data.

Mycobacterium

Bojalil & Cerbón (1961) studied 43 strains of *Mycobacterium*, recording 27 two-value biochemical characters for each of them. There were five groups (including respectively 10, 9, 7, 4 and 3 strains) within each of which these characters were identical, while the other ten strains had sets of characters not shared with any other.

Probabilistic similarity indices for the whole set of data are recorded in Table 3. It will be noted that there is a concentration of high values in the bottom right-hand corner of the table. The isolated strains 27, 34, 40 and 42 form a subset (C) within which all similarity indices exceed 0·97; and groups (d) and (e), with strain 39, form another subset (B) with all indices greater than 0·92. The occurrence of sets of similarity indices like this by random assortment of attributes is a highly unlikely event, so these may be regarded as groups of strains distinct from the remainder. Incidentally, all the other 31 strains (subset A) are linked by 465 similarity indices of which only 23 fall below 0·5, whereas *between* the three subsets thus recognized 281 out of 404 indices are less than 0·5.

If these three subsets are separated and similarity indices recalculated within each subset, the results of Table 4 are obtained. Within subset A, six of the eight differing characters are recorded in single strains only—different in each case. The distribution of the other two characters so as to leave a group of seven strains (c) forming acid on neither mannitol nor salicin, a group of nine (b) forming it on salicin but not mannitol, and a third group of ten (a) forming it on both substrates is no reason for rejecting the null hypothesis of homogeneity.

Subset B also need not be divided further, for the strains in this are distinguished by two attributes only (utilization of citrate, and formation of acid from fructose). Group (d) alone have the first attribute, and the chance that these three strains should also agree (as they do) in respect of the fructose response is 0·143—well above the usually accepted significance level. In subset C, consisting of four strains, the highest similarity index recorded is likewise not such as to lead one to regard the subset as heterogeneous.

Table 4. *Similarity indices within subsets of Mycobacterium strains*

Subset A

	(a)	(b)	35	24	10	(c)	41
(a)	0·885	—	—	—	—	—	—
(b)	0·544	0·697	—	—	—	—	—
35	0·771	0·271	—	—	—	—	—
24	0·765	0·259	0·570	—	—	—	—
10	0·769	0·464	0·577	0·567	—	—	—
(c)	0·271	0·468	0·907	0·035	0·186	0·946	—
41	0·750	0·233	0·547	0·750	0·545	0·024	—
33	0·638	0·261	0·523	0·375	0·625	0·131	0·349

Subset B

	(d)	39	(e)
(d)	0·983	—	—
39	0·631	—	—
(e)	0·158	0·411	0·822

Subset C

	27	34	42
27	—	—	—
34	0·762	—	—
42	0·467	0·980	—
40	0·153	0·494	0·685

In this set of data, then, we are left with three homogeneous groups of strains, the variations within which may (as far as these data go) reasonably be ascribed to chance. These results are in accordance with the conclusions of Bojalil & Cerbón (1961) themselves, using the Jaccard similarity index. They distinguished the same three groups of strains, the first and largest under the name of *Mycobacterium fortuitum*, while the other two were unnamed.

Table 5. *Chromobacterium*

Sneath's feature nos.	Character	Number of ordered values
1–5	Length	4
6–9	Breadth	3
22–23	Presence of fat	3
25–26	Gelatinous consistency	3
28, 29, 63	Violacein production	4
32–35	Liquefaction in gelatin stab cultures	4
36–37	Digestion of Löffler serum slope	3
39–40	Colouration on potato slope	3
41–43	Heat resistance	3
44–46	Phenol resistance	3
50–53	Upper temperature limit for growth	4
54–57	Lower temperature limit for growth	3
58–59	Citrate utilization	3
60–61	Tolerance of NaCl	3
91–92	Nitrite destruction	3
94–95	Phosphatase test	3
97–98	Casein hydrolysis	3
99–100	Haemolysis of horse-blood agar	3
101–102	Egg-yolk reaction	3
104–105	Gluconate test	3

Chromobacterium

Finally we have as our largest set of data that published by Sneath (1957*b*), which he used in his original exposition of the application of similarity indices in bacterial classification. For 45 strains of *Chromobacterium* he recorded 70 characters,

Table 6. *Probabilistic similarity indices: Sneath's data for Chromobacterium*

Strains	2	15	24	23	8	12	13	14	3	4	16	28	6	5	9	11	10	1	32	21	22	27	25	29	38	36	30	35	20	31	37	33	17	18	19	34	26	7	39	40	41	42	45	43	44
15	100	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
24	100	100	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
23	100	100	100	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
8	100	100	100	100	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
12	100	100	100	100	100	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
13	100	100	99	99	99	100	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
14	100	99	100	100	100	100	100	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
3	100	100	100	97	100	100	100	100	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
4	100	100	100	99	100	100	99	100	100	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
16	97	98	98	95	94	100	100	100	100	100	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
28	99	100	100	98	100	100	99	100	100	100	99	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
6	100	100	100	100	99	98	100	99	100	99	99	100	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
5	93	95	99	97	99	97	96	97	99	90	99	99	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
9	92	92	88	78	97	97	98	93	98	93	98	100	91	92	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
11	88	99	75	85	49	86	100	77	100	93	99	93	99	98	97	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
10	88	99	87	92	62	87	94	88	94	92	98	99	99	91	100	100	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
1	98	99	100	96	100	99	100	96	100	100	100	100	100	99	100	98	95	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
32	1	2	14	34	4	2	8	5	6	2	16	9	17	61	5	62	66	2	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
21	0	1	1	9	0	0	11	2	0	0	1	2	4	32	4	72	41	7	99	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
22	1	1	2	1	0	0	8	4	0	0	1	3	0	27	7	37	15	4	99	100	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
27	0	0	1	10	7	0	0	2	0	0	3	2	3	65	4	16	10	3	94	93	100	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
25	2	3	4	2	1	0	2	0	0	5	1	7	1	34	10	18	20	1	94	93	100	100	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
29	2	3	4	2	1	0	3	11	0	6	2	5	9	11	8	9	2	2	83	90	93	96	97	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
38	3	4	4	2	1	0	5	10	1	5	3	7	2	25	11	14	4	2	87	92	95	97	95	99	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
36	7	9	4	2	1	2	13	1	3	12	5	3	1	10	9	8	2	2	86	83	88	77	89	94	99	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
30	1	2	3	1	1	0	1	7	0	0	1	4	20	31	6	14	13	1	98	99	99	99	99	100	95	89	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
35	0	1	1	8	1	0	2	4	0	1	1	3	17	29	4	46	39	1	99	99	98	98	99	99	97	92	100	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
20	1	3	4	1	3	0	2	7	0	0	1	6	1	37	11	14	21	0	86	94	98	99	99	82	83	69	98	99	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
31	4	6	14	7	5	7	14	23	13	7	35	50	35	29	4	53	24	1	99	92	94	71	84	95	74	74	100	97	69	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
37	12	3	4	14	1	4	3	1	4	3	2	1	9	13	15	17	28	1	95	95	95	91	88	66	90	89	81	84	93	44	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
33	17	21	31	20	15	19	17	19	26	18	49	17	36	40	10	26	5	5	85	41	48	80	81	96	80	74	99	84	50	99	48	—	—	—	—	—	—	—	—	—	—	—	—	—	—
17	7	26	27	36	68	2	13	4	6	34	7	33	40	6	13	16	14	29	92	78	81	72	83	97	91	91	95	95	49	96	61	84	—	—	—	—	—	—	—	—	—	—	—	—	—
18	6	24	27	5	24	1	10	16	8	6	5	45	44	11	6	8	7	12	97	81	91	84	89	91	91	81	96	83	84	97	81	87	100	—	—	—	—	—	—	—	—	—	—	—	—
19	1	10	12	1	10	0	1	5	1	1	1	17	39	5	6	1	2	3	92	89	96	92	89	93	96	90	97	91	91	83	89	71	97	100	—	—	—	—	—	—	—	—	—	—	—
34	2	4	6	2	5	0	5	2	2	0	32	6	44	21	28	10	12	32	77	68	76	96	81	94	89	88	99	98	92	84	81	90	88	85	92	—	—	—	—	—	—	—	—	—	—
26	17	22	26	50	24	6	20	5	5	0	17	11	25	8	7	71	35	44	92	83	87	80	61	88	94	68	77	91	67	91	83	76	89	97	89	91	—	—	—	—	—	—	—	—	—
7	0	0	0	6	0	0	4	1	1	1	5	20	31	15	2	72	28	25	89	98	99	96	92	94	89	52	95	97	92	98	85	60	69	80	97	70	99	—	—	—	—	—	—	—	—
39	95	74	79	86	99	100	95	93	100	92	88	76	53	37	73	17	8	78	0	0	0	0	0	6	0	0	3	1	3	0	2	3	9	7	1	9	6	14	—	—	—	—	—	—	—
40	94	78	78	85	99	100	94	93	100	93	70	49	53	25	46	5	1	52	0	0	0	1	0	8	0	0	4	1	5	0	3	5	5	9	1	5	8	17	100	—	—	—	—	—	—
41	60	12	22	59	64	23	47	30	91	39	81	15	81	48	58	42	47	75	14	15	1	41	3	52	11	9	51	58	41	8	61	30	25	26	31	89	51	58	100	99	—	—	—	—	—
42	60	18	31	65	56	32	1	21	26	5	17	10	73	34	37	7	27	52	14	10	1	43	2	42	6	55	36	45	38	4	66	17	21	18	31	87	61	30	100	99	100	—	—	—	—
45	12	14	49	10	55	28	56	23	87	34	89	86	72	80	75	88	63	100	36	34	26	44	1	60	44	8	35	23	26	72	32	53	74	83	71	68	95	90	92	78	100	100	—	—	—
43	50	49	43	10	28	42	26	75	85	48	31	88	37	24	39	17	14	52	0	1	4	64	9	14	8	0	4	13	46	0	19	2	0	9	60	31	15	43	81	71	95	93	92	—	—
44	15	13	8	1	4	8	49	30	91	39	52	55	22	2	22	28	4	89	1	17	37	30	5	2	5	3	3	12	39	1	44	0	5	13	31	41	51	98	87	85	98	85	100	100	—

coded as 105 two-value features. For the present analysis, some of these characters have been recoded. The presence and arrangement of polar flagella (Sneath's features 16 and 18) were rescored as a three-value qualitative attribute, while twenty other characters, scored by Sneath as groups of binary features, were rescored each as a single attribute taking a number of ordered values, as shown in Table 5.

Table 7. *Similarity indices for Chromobacterium after first subdivision*

Group A

Strain	1	2	3	4	5	6	8	9	10	11	12	13	14	15	16	23	24
2	3	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
3	64	30	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
4	21	74	100	—	—	—	—	—	—	—	—	—	—	—	—	—	—
5	98	7	15	1	—	—	—	—	—	—	—	—	—	—	—	—	—
6	86	59	9	23	98	—	—	—	—	—	—	—	—	—	—	—	—
8	63	74	55	60	70	36	—	—	—	—	—	—	—	—	—	—	—
9	99	23	21	6	94	55	46	—	—	—	—	—	—	—	—	—	—
10	76	16	4	6	83	85	1	100	—	—	—	—	—	—	—	—	—
11	71	14	27	9	98	80	0	98	100	—	—	—	—	—	—	—	—
12	6	95	97	94	4	5	72	21	4	3	—	—	—	—	—	—	—
13	85	67	46	6	26	65	23	74	37	93	66	—	—	—	—	—	—
14	2	59	80	30	18	50	37	34	21	5	59	53	—	—	—	—	—
15	16	91	43	58	8	53	78	21	60	57	74	69	28	—	—	—	—
16	69	13	82	39	76	59	3	62	61	84	37	73	48	15	—	—	—
23	31	85	2	23	59	69	100	13	32	16	60	33	62	67	8	—	—
24	52	76	14	26	71	44	98	22	16	3	70	41	52	91	14	98	—
28	75	36	23	63	43	77	61	97	90	39	32	23	86	34	40	23	30

Group B

	7	17	18	19	20	21	22	25	26	27	29	30	31	32	33	34	35	36	37
17	9	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
18	15	100	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
19	51	82	98	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
20	38	4	50	64	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
21	76	12	33	44	74	—	—	—	—	—	—	—	—	—	—	—	—	—	—
22	79	14	38	55	86	98	—	—	—	—	—	—	—	—	—	—	—	—	—
25	56	48	49	30	91	64	94	—	—	—	—	—	—	—	—	—	—	—	—
26	96	44	71	32	7	19	4	7	—	—	—	—	—	—	—	—	—	—	—
27	37	22	17	42	92	45	87	92	27	—	—	—	—	—	—	—	—	—	—
29	73	70	46	39	27	27	36	77	55	72	—	—	—	—	—	—	—	—	—
30	17	40	56	54	65	64	66	70	16	56	90	—	—	—	—	—	—	—	—
31	86	74	84	19	10	31	31	37	89	2	70	92	—	—	—	—	—	—	—
32	18	76	65	29	29	91	74	49	41	26	13	49	88	—	—	—	—	—	—
33	52	92	84	16	5	3	5	76	88	36	90	86	100	92	—	—	—	—	—
34	19	87	26	49	52	6	10	20	74	89	70	70	34	36	76	—	—	—	—
35	34	40	11	18	96	72	40	81	40	27	72	91	49	53	21	72	—	—	—
36	1	72	24	40	21	37	45	60	13	17	60	29	30	55	68	57	42	—	—
37	42	28	36	48	90	90	77	58	22	59	6	18	4	90	26	47	30	70	—
38	21	36	29	46	26	44	51	65	57	71	93	34	6	19	45	20	47	86	36

Group C

	39	40	41	42	43	44
40	100	—	—	—	—	—
41	38	26	—	—	—	—
42	39	28	100	—	—	—
43	0	0	25	12	—	—
44	0	0	23	5	100	—
45	13	7	79	81	73	89

The similarity indices calculated from these recoded data are presented in Table 6. To facilitate comparison with Sneath's table 7, they are arranged in the same way and expressed in the same terms (as percentages rounded to the nearest unit).

Table 8. *Similarity indices for Chromobacterium after second subdivision*

Group A1

Strain	1	5	6	9	10	11	16
5	61	—	—	—	—	—	—
6	54	57	—	—	—	—	—
9	82	40	19	—	—	—	—
10	20	15	42	87	—	—	—
11	20	68	30	33	100	—	—
16	54	66	44	25	21	62	—
28	56	19	78	88	53	19	67

Group A2

Strain	2	8	12	15	23
8	9	—	—	—	—
12	88	15	—	—	—
15	81	38	48	—	—
23	48	93	9	14	—
24	24	82	42	88	85

Group A3

Strain	3	4	13
4	95	—	—
13	16	6	—
14	28	54	93

Group B1

Strain	20	22	25
22	47	—	—
25	60	87	—
27	36	53	33

Group B2

Strain	7	17	18	19	21	26	29	30	31	32	33	34	35	36	37
17	2	—	—	—	—	—	—	—	—	—	—	—	—	—	—
18	5	100	—	—	—	—	—	—	—	—	—	—	—	—	—
19	35	61	97	—	—	—	—	—	—	—	—	—	—	—	—
21	92	14	55	62	—	—	—	—	—	—	—	—	—	—	—
26	92	26	60	29	18	—	—	—	—	—	—	—	—	—	—
29	64	64	40	23	27	49	—	—	—	—	—	—	—	—	—
30	24	38	56	41	78	11	89	—	—	—	—	—	—	—	—
31	90	62	76	11	40	79	57	93	—	—	—	—	—	—	—
32	30	63	74	55	97	30	16	70	87	—	—	—	—	—	—
33	50	90	80	9	4	78	89	87	100	87	—	—	—	—	—
34	9	75	21	37	5	61	64	64	24	30	69	—	—	—	—
35	39	35	9	12	77	22	70	92	54	65	24	45	—	—	—
36	1	72	30	42	39	18	57	24	28	55	60	52	46	—	—
37	64	29	51	58	93	20	8	24	4	94	19	38	27	68	—
38	22	43	36	48	52	65	94	39	5	31	42	20	50	90	46

A first inspection of these figures suggests the following groupings:

A. Strains 1–6, 8–16, 23, 24 and 28 with 87 indices above 99%, and only 5 less than 80%.

B. Strains 7, 17–22, 25–27 and 29–38 with 22 indices above 99%, and only 33 (out of 190) less than 80%.

C. Strains 39–45 with 10 indices above 99%, and only 4 less than 80%.

Groups A and B here are identical with those separated by Sneath as mesophilic (a) and psychrophilic (b), while C includes all his five other small groups (c)–(g).

The groups listed above were analysed separately, with the results shown in Table 7.

Within group A, two distinct subgroups may be recognized:

A1. Strains 1, 5, 6, 9, 10, 11, 16 with all indices above 55%. To these may perhaps be added no. 28—close to strains 1, 6, 9 and 10 but less so to the others. Further similarity analysis shows no significant clustering within A1 (see Table 8)—even the highest value (99·68) is such as might occur about once in ten such sets of data.

A2. Strains 2, 8, 12, 15, 23 and 24, with similarity indices all exceeding 59%. Again, no significant groupings within A2 emerge (Table 8).

The remaining strains, 3, 4, 13 and 14, may be placed together as a residue (group A3) after removing these more closely related groups. The index between the first two approaches significance at the 5% level, but apart from this these four may not be very closely related. Further analysis of this residue (Table 7) does not show any one pair as significantly similar. However, the indices for 3 and 4 and for 13 and 14 both exceed 0·93, and the chance that four strains with random sets of attributes will be divisible into two pairs with similarities at this level is only 0·015. We may accordingly regard these as two small subgroups within A, and designate them as A3 and A4.

We may note that the order in which the strains of group A are placed by Sneath accords exactly with the classification derived here, although he includes them all in his group (a). He places the strains of my A2 first, then A4, A3 and finally A1 as showing some affinity with group B. In a subsequent publication (Sneath, 1960) he described three 'indistinct subgroups' of the mesophilic strains, two of which were identical with my A2 and A3, while the third consisted of three strains (9, 10, 11) included in my A1.

Within group B, only strains 20, 22, 25 and 27 are significantly related, with a minimum index of 86% (Table 7); these are separated as B1, while the remainder forms group B2. Further analysis of these subgroups (Table 8) does not reveal any further internal relationships. Sneath (1960) tentatively recognized three subgroups among the psychrophilic strains. Their existence as distinct taxonomic entities is not supported by the figures of Table 7, though three of the four strains in my B1 were included in the first of his subgroups.

Group C (Table 7) is clearly heterogeneous. The similarity indices between 39 and 40, and between 43 and 44, both exceed 99·99999%. Strains 41 and 42 are also closely related. Sneath likewise concluded that 39 and 40, 41 and 42 formed two distinct groups (c and d), but regarded 43 and 44 (for which his similarity index was only 63%) as each representing a distinct group. The present analysis, however, leaves no doubt that, within the context of the strains he studied, the relationship of this pair was very close.

An example of overlapping is provided by strains 39 and 40; though included here in group C they also show some affinity with all of group A except strains 10 and 11. If, for instance, strains 39 and 40 had been combined with the six strains of group A2, the minimum similarity index within the group may be seen from Table 6 to be 74%. Thus we may again regard strains 39 and 40 as bridging the gap between group A and the rest of group C, and the strains may perhaps be placed in a sequence as follows: (10, 11), (1, 5, 6, 9, 16, 28), (3, 4, 13, 14), (2, 8, 12, 15, 23, 24), (39, 40), (41, 42), 45, (43, 44).

Particularly when one is studying the structure of closely knit groups, it seems likely that overlapping and interlocking relations of this sort may be commoner than the discrete clusters assumed in the conventional taxonomic approach.

DISCUSSION

It is a tenet of most schools of numerical taxonomy that the various attributes considered should be given equal weight. At first glance, the use of a probabilistic similarity index might seem to offend this principle, since it gives differential weighting to attributes according to the commonness or rarity of the values observed. The objections to differential weighting, however, apply rather to *a priori* weights based on preconceptions as to the relative taxonomic importance of different attributes, and can have little force when the different weights are inherent in the data themselves. Similar procedures are countenanced by some of those advocating equal weighting. For instance, Sokal & Sneath (1963), in relation to distance techniques, write of 'the automatic and quite appropriate weighting of mismatches by their magnitude'.

It must be understood that the similarity indices calculated here explicitly refer to a specified universe of discourse, and have no meaning in isolation. They are indeterminate for a particular pair of strains, until these strains are considered as two of a larger collection. This is also true according to one common convention, for the better-known indices. Many users (e.g. Hill, Tuiri, Gilardi & Silvestri, 1961; Silvestri, 1962) feel that an attribute present in all the organisms considered contributes nothing to the similarity of any pair, and should be excluded in calculating the index. If this rule is followed consistently, the index will again be indeterminate when a pair is considered in isolation, and can only express the similarity of a pair in the context of a larger set.

When matrices of the simpler similarity indices are used for taxonomic purposes, a dendrogram (Sokal & Sneath, 1963) is often prepared showing the minimum values of the indices at which successively larger groups are linked. The same could be done with a matrix of probabilistic similarity indices. This could at least ensure that within different phena recognized at any given level every pair of individuals was similar at a specified level of significance. But in doing this one would be throwing away a large part of the advantage of probabilistic treatment; the significance of a cluster depends on its size, as well as on the values of the indices and the size of the whole set under consideration. It is consequently not possible to use constant values of the index for the separation of phena without sacrificing a constant significance level. In the same way, a hierarchic treatment based only on the complete similarity matrix would not make the best use of the technique. Once the data have been found to include clusters the null hypothesis on which the probabilities are calculated is negatived, and new null hypotheses need to be set up if significance tests are to be valid.

The time required for the technique proposed here is obviously much longer than that needed for preparing dendrograms, for instance, based on matching coefficients. But the requirements are well within the powers of a modern electronic computer.

Apart from the obvious advantages that it facilitates special treatment of multivalue attributes and that it permits significance tests of hypotheses concerning the

classifications proposed, the similarity index used here is shown to be more sensitive in enabling smaller groupings to be distinguished. Both in the data for *Chromobacterium* and for the Enterobacteriaceae subdivisions have been recognized additional to those of the original authors. By and large, though, the results in the two analyses are remarkably similar, and the results obtained with the Jaccard similarity index have in general been confirmed. It has often been suggested that, provided large numbers of unrelated attributes are taken into account, the results of different classificatory procedures may be expected to approach one another. The results reported here provide further support for this view.

I am indebted to Dr C. A. Parker and Dr P. H. A. Sneath for helpful comments. and to Mr D. W. G. Moore of the University of Western Australia and Dr G. N, Lance of the C.S.I.R.O. Computing Research Section in Canberra for computer facilities.

REFERENCES

Bojalil, L. F. & Cerbón, J. (1961). Taxonomic analysis of nonpigmented, rapidly growing Mycobacteria. *J. Bact.* **81**, 338.

Goodall, D. W. (1964). A probabilistic similarity index. *Nature, Lond.* **203**, 1098.

Goodall, D. W. (1966). A new similarity index based on probability. *Biometrics* (in the Press).

Hill, L. R., Turri, M., Gilardi, E. & Silvestri, L. G. (1961). Quantitative methods in the systematics of Actinomycetales. II. *G. Microbiol.* **9**, 56.

Jaccard, P. (1901). Distribution de la flore alpine dans le Bassin des Dranses et dans quelques régions voisines. *Bull. Soc. Sci. nat. vaud.* **37**, 241.

Lysenko, O. & Sneath, P. H. A. (1959). The use of models in bacterial classification. *J. gen. Microbiol.* **20**, 284.

Silvestri, L. G. (1962). Computer correlation of microrganisms. In *Data Acquisition and Processing in Biology and Medicine.* Proceedings of Conference in New York, 1962, p. 43. Oxford: Pergamon Press.

Sneath, P. H. A. (1957*a*). Some thoughts on bacterial classification. *J. gen. Microbiol.* **17**, 184.

Sneath, P. H. A. (1957*b*). The application of computers to taxonomy. *J. gen. Microbiol.* **17**, 201.

Sneath, P. H. A. (1960). A study of the bacterial genus *Chromobacterium*. *Iowa St. J. Sci.* **34**, 243.

Sneath, P. H. A. (1964). New approaches to bacterial taxonomy: use of computers. *A. Rev. Microbiol.* **18**, 335.

Sokal, R. R. & Sneath, P. H. A. (1963). *Principles of Numerical Taxonomy.* San Francisco and London: W. H. Freeman & Co.

J. gen. Microbiol. (1966), **42**, 39–42
Printed in Great Britain

Autolytic Enzymes in Fungal Cell Walls

R. MITCHELL* AND NAAMA SABAR
Weizmann Institute of Science, Biochemistry Section, Rehovoth, Israel

(*Received* 25 *June* 1965)

SUMMARY

Autolytic glucanase and protease activity was detected in cell walls of some pythium species prepared by ultrasonic treatment. The glucanase activity was correlated with mycelial development. Maximal glucose release was detected in growing cultures during the linear growth period of the fungus. Assays of glucanase activity in cell walls prepared from mycelium harvested at different times showed that the enzymic activity increased with the age of mycelium from which the cell walls were prepared. The relationship between autolytic enzymes and mycelial development is discussed.

INTRODUCTION

Enzymic lysis of fungal cell walls by other micro-organisms has been reported for a number of fungi. Horikoshi & Iida (1958) associated lysis of aspergillus by some bacillus species with the action of a chitinase. Mitchell & Alexander (1963) showed the activity of a chitinase and protease in bacterial degradation of Fusarium cell walls. With these fungi the source of the lytic enzymes was another micro-organism. Bacterial lysis of pythium has been investigated in our laboratory (Mitchell & Hurwitz, 1965). During these investigations preparations were made of fungal cell walls and evidence was obtained which suggested that autolytic enzymes were active in the cell-wall preparations (Mitchell & Sabar, 1966). This report will describe the action of autolytic enzymes on Pythium cell walls.

METHODS

Cultures of *Pythium butleri* and *Pythium myriotylum* for cell-wall preparation were incubated at 30° for 3 days. The medium used was a potato glucose liquid medium (Mitchell & Hurwitz, 1965). For measurements of glucose release from actively developing cultures of pythium corn meal was substituted for glucose. Cell walls were prepared by ultrasonic treatment of 20 ml. batches with a Branson 'Sonifier' ultrasonic oscillator in an ice bath for 3 min. The walls were washed six times until free from cytoplasmic material by centrifugation in a refrigerated centrifuge at 5000*g* for 10 min.; the cytoplasmic material remained in suspension. Walls were washed with 0·05 M-potassium phosphate buffer (pH 7), until shown to be free from cytoplasmic material by phase-contrast microscopy, and by examining the extinction at 260 mμ in a Zeiss spectrophotometer.

* Present address: Laboratory of Applied Biology, Division of Engineering and Applied Physics, Harvard University, Cambridge, Mass., U.S.A.

Glucanase activity was measured by determination of glucose release by the glucose oxidase method (Keston, 1956; the reagent was obtained from Worthington Biochemical Corp. Freehold, N.J.). Proteolysis was determined by the method of Moore & Stein (1954).

Double-layer plates were used to assay for the presence of bacteriophage from culture filtrates during cell-wall autolysis. The bottom layer consisted of potato glucose agar (Difco). A mixture of autolytic filtrate and an inoculum of pythium mycelium was added to 0·6% agar at 45°; this medium was then spread on the surface of the solidified bottom layer. Petri dishes were incubated at 30°. Ultracentrifuge analyses were done with a Spinco ultracentrifuge. Samples were suspended in 0·5 M potassium phosphate buffer (pH 7). All samples analysed in the ultracentrifuge had extinction readings greater than 1·0 when measured in a Zeiss ultraviolet spectrophotometer at 260 mμ.

RESULTS

During studies of the chemical structure of pythium cell walls (Mitchell & Sabar, 1966) it was observed that large quantities of glucose were released in control tubes containing cell walls alone during incubation at 37°. These observations suggested a tendency of the mycelium of this fungus to undergo autolysis. To study this phenomenon in greater detail cell walls of *Pythium butleri* and *P. myriotylum* were prepared, precautions being taken to prevent destruction of enzymes which might be present in the cell-wall material. These two pythium species have been shown to contain a glucan as the only polysaccharide. *P. butleri* glucan was found to be β 12 linked, whereas the linkage of *P. myriotylum* was β 14 (Mitchell & Sabar, 1966).

Autolytic activity was determined by measuring release of glucose and amino groups. Both species tested showed strong glucanase activity against their own glucans, as shown by the rapid release of glucose. Proteolysis was also detected in cell-wall preparations of both species of the fungus. There was little difference in the rate of release of amino groups between the two species. Both autolytic glucans and protease activity were inhibited by heating the cell-wall preparations at 90° for 30 min.

Tests were made to determine whether autolytic activity was related to phage infection of the fungus. Double-layer plates were prepared and inoculated with growing mycelium together with culture filtrate from actively growing mycelium. No plaques were observed. Culture filtrates were tested for the presence of nucleotide material by measurement of extinction in the u.v. spectrophotometer. No significant peak was found at 260 mμ, indicating the absence of nucleotide material. Neither ultracentrifugal nor electron microscope analyses yielded evidence of phage particles.

The relationship between autolytic activity and mycelial growth was examined. To determine the rate of growth of pythium mycelium, Erlenmeyer flasks were inoculated with the *Pythium butleri* and incubated at 25°. The growth rate was determined by measurement of the weight of dried mycelium harvested at various times. The mean of weights obtained from three flasks was used at each sampling time. These data showed a linear rate of growth for 10 days; between 10 and 14 days the dry weight did not increase.

Measurements of quantities of glucose released into the medium during growth of *Pythium butleri* in liquid culture showed a linear rate of release during the period of most active growth of the fungus. A potato corn meal medium was used. Typical data for *P. butleri* are shown in Fig. 1. The glucose concentration in the medium reached a maximum after 6 days of incubation and then began to decline.

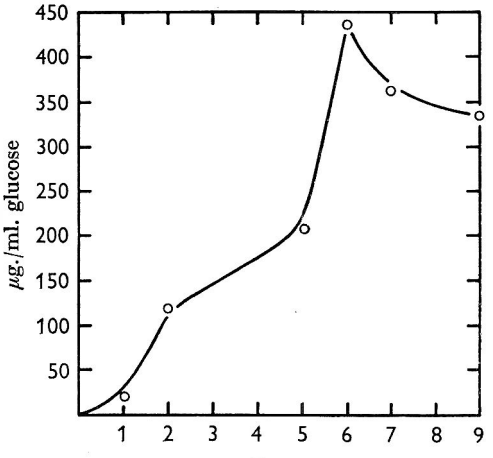


Fig. 1

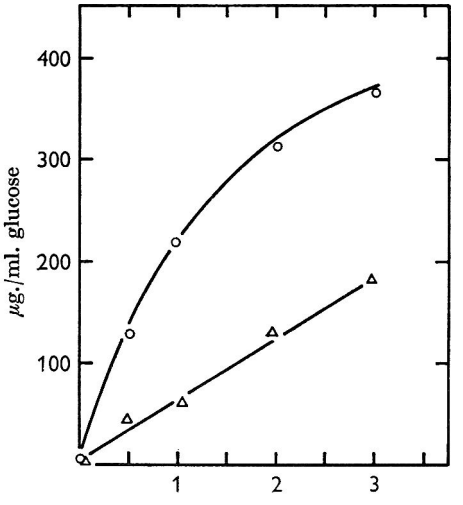


Fig. 2

Fig. 1. Relationship between the age of the Pythium culture from which cell walls were prepared and autolytic glucanase activity.

Fig. 2. Relationship between mycelial growth of Pythium and release of glucose into the medium by whole mycelium. △, 3-day culture; ○, 7-day culture.

Cell walls were prepared from *Pythium butleri* mycelium harvested at different incubation times, to test the relationship between mycelial age and autolytic glucanase activity of the cell walls. Some of the data obtained are shown in Fig. 2. The cell walls prepared from 7-day mycelium yielded glucose 120 μg. /ml. cell-wall preparation/hr. as compared to 60 μg./ml. by cell walls from 3-day cultures. Walls prepared from 24 hr cultures displayed autolytic activity but values were not higher than glucose 15 μg./ml. culture released/hr.

DISCUSSION

Weidel, Frank & Leutgeb (1963) reported autolysis of salmonella cell-walls and pointed out that precautions should be taken against autolysis in the study of bacterial cell walls. Results obtained in the current investigation show that similar care must be taken in the study of fungal cell-walls. Both cell-wall glucan and protein of Pythium were susceptible to degradation by autolytic enzymes. Young, Spizizen & Crauford (1963) showed autolytic activity in *Bacillus subtilis* cell walls, and associated autolysis with competence for transformation. Genetic transfer in fungi occurs either by parasexuality or by karyogamy. These sexual processes are rare in the Phycomycetes (Raper, 1955); sexual forms were not observed in the cultures used in the present work. Cell-wall lysis has also been observed during release of

bacterial spores (Greenberg & Halvorson, 1955). The small numbers of spores observed in our pythium cultures excluded this possibility. Park (1956) observed that klebsiella cell walls were lysed by enzymes carried by the phage during bacteriophage infection. No evidence of phage infection of pythium was detected here.

Cell-wall autolysis of *Staphylococcus aureus* has been shown to occur during cell division (Mitchell & Moyle, 1954). It has been suggested (Strange, 1959) that these autolytic enzymes may either have a synthetic function or be related to the change in state of the dividing bacteria. Our results show a direct relationship between fungal growth and autolytic activity. The strong activity observed in cell walls prepared from old pythium mycelium may be a result of the combined activity of autolytic enzymes associated with growth and senescence. The rapid rate of branching which occurs during development of young hyphae of pythium suggests that branching points may be sites of maximum lytic activity. However, both mycelial growing tips and cell-wall synthesis may equally account for the relationship between autolysis and growth.

REFERENCES

GREENBERG, R. A. & HALVORSON, O. H. (1955). Studies on an autolytic substance produced by an aerobic sporeforming bacterium. *J. Bact.* **69**, 45.

HORIKOSHI, K. & IIDA, S. (1958). Lysis of fungal mycelia by bacterial enzymes. *Nature, Lond.* **181**, 917.

KESTON, A. S. (1956). Specific colorimetric reagents for glucose. *Abstr. 129th Meeting, Am. Chem. Soc.* p. 31*c*.

MITCHELL, P. & MOYLE, J. (1957). Autolytic release and osmotic properties of protoplasts from *Staphylococcus aureus*. *J. gen. Microbiol.* **16**, 184.

MITCHELL, R. & ALEXANDER, M. (1963). Lysis of soil fungi by bacteria. *Can. J. Microbiol.* **9**, 169.

MITCHELL, R. & HURWITZ, E. (1965). Suppression of *Pythium debaryanum* by lytic rhizosphere bacteria. *Phytopathology*, **55**, 156.

MITCHELL, R. & SABAR, N. (1966). Hyphal cell wall structure of two species of Pythium. *Can. J. Microbiol.* (in the Press).

MOORE, S. & STEIN, W. H. (1954). A modified ninhydrin reagent for the photometric determination of amino acids and related compounds. *J. biol. Chem.* **211**, 907.

PARK, B. H. (1956). An enzyme produced by a phage-host-cell system. I. The properties of a Klebsiella phage. *Virology*, **2**, 711.

RAPER, J. R. (1955). Heterokaryosis and sexuality in fungi. *Trans. N. Y. Acad. Sci.* Ser. II, **17**, 627.

STRANGE, R. E. (1959). Cell wall lysis and the release of peptides in Bacillus species. *Bact. Rev.* **23**, 1.

WEIDEL, W., FRANK, H. & LEUTGEB, W. (1963). Autolytic enzymes as a source of error in the preparation and study of Gram-negative cell walls. *J. gen. Microbiol.* **30**, 127.

YOUNG, F. E., SPIZIZEN, J. & CRAUFORD, I. P. (1963). Biochemical aspects of competence in the *Bacillus subtilis* transformation system. I. Chemical composition of cell walls. *J. biol. Chem.*, **238**, 311.

J. gen. Microbiol. (1966), **42**, 43–56
Printed in Great Britain

DNA Homology and Taxonomy of *Pseudomonas* and *Xanthomonas*

By J. DE LEY, I. W. PARK, R. TIJTGAT, and J. VAN ERMENGEM

Laboratory for Microbiology, Faculty of Sciences, State University, Gent, Belgium

(*Received* 5 *July* 1965)

SUMMARY

The relatedness between several nomen species of the *Pseudomonas–Xanthomonas* group and some other organisms was numerically fixed through DNA homology and DNA base composition. For Pseudomonas the numerically analysed strains proposed by Lysenko as neotypes were used. The mean % (G+C) was in the range 60–67·5. ^{14}C-DNA from either *Pseudomonas fluorescens* or *P. putida* was hybridized with DNA from 17 different species centres and the DNA homology was in the range 50–100 %. Genetic species differentiation in the genus *Pseudomonas* seems justified. In three border cases (*P. iodinum*, *P. diminuta* and *P. atlantica*) DNA homology was only 28–50 %, so the inclusion of these organisms in the genus *Pseudomonas* is uncertain. The species centres *P. pavonacea* and *P. rubescens* are omitted from the genus *Pseudomonas* because of their very low DNA homology and aberrant DNA base composition. Twenty-eight nomen species of *Xanthomonas* all form a narrow group in the range 63·5–69 % (G+C). With two exceptions DNA homology with a median strain *Xanthomonas pelargonii* was always over 75 % and frequently nearly complete. ^{14}C-DNA from *P. fluorescens* hybridized with *Xanthomonas*-DNA to the same extent as with the pseudomonads proper. The *Xanthomonas* cluster overlapped perfectly with part of the *Pseudomonas* group. It is proposed therefore to gather all xanthomonads in a single genetic species *P. campestris*. This is such a dense cluster that the preservation of separate species names for the border cases seems undesirable. About one half to two-thirds of *Pseudomonas*- and *Xanthomonas*-DNA is identical. The genera *Rhizobium*, *Azotobacter* and *Azomonas* appeared to be rather closely related to *Pseudomonas* since they shared some 40–50 % of their DNA. The genus *Serratia* appeared to be more closely related to *Pseudomonas* than to *Escherichia*. The genera *Gluconobacter*, *Acetobacter*, *Serratia* and *Escherichia* shared some 20–30 % DNA with *Pseudomonas*, but *Bacillus*-DNA was almost entirely different. From a comparison between DNA homology and taximetric similarity, it appeared that most pseudomonads would not contain unused genes. The advantages of a classification based on % (G+C) and DNA homology are obvious.

INTRODUCTION

Assuming a molecular weight of 6×10^9 for bacterial chromosomal DNA and about 10^3 base pairs per cistron, it can be estimated that a bacterial genome contains some 10^4 cistrons. The present orthodox classification, as exemplified in

Bergey's Manual (1957), is built on phenotypic data, quite often on very few, thus encompassing only a minute fraction of the total genome. Numerical analysis is a much better approach to determine relatedness: because up to 200 characters may be used, and assuming that an average of 5–10 cistrons is involved in each character, it can be estimated that some 10–20 % of the genome is detected. Many properties still remain hidden and classification could be improved considerably by determining the degree of identity of chromosomal DNA. This is possible through the DNA-hybridization method of McCarthy & Bolton (1963). The present paper is a molecular biological approach to a more rational classification based on two DNA parameters: (i) base composition, expressed as a mean molar (guanine + cytosine) content (% (G+C)), and (ii) homology. It is likewise an attempt for a better understanding of the ill-defined concept of species and genus in bacteriology. As a model system we sought to clarify the relatedness between several nomen species within *Pseudomonas* and *Xanthomonas*, as well as the relatedness between the two groups. We selected the collection of Pseudomonas strains from Lysenko (1961) because their taximetric similarity is well known. By comparing DNA homology and taximetric similarity of the same strains, one might also detect whether a fraction of the genome remains unused. For *Xanthomonas* some 60-odd nomen species are listed in *Bergey's Manual* (1957). With nine of them 80–100 % of their DNA is identical and they can be gathered in one genetic species, *X. campestris* (Friedman & De Ley, 1965; De Ley & Friedman, 1965). These investigations were extended in the present paper with a total of 28 different nomen species and their DNA homology with a representative Pseudomonas was determined. The degree of DNA homology was determined between the pseudomonads and representatives of a few other genera in the same % (G+C) range (*Gluconobacter*, *Acetobacter*, *Serratia*, *Rhizobium*, *Azotobacter* and *Azomonas*) and in a different % (G+C) range (*Escherichia* and *Bacillus*).

METHODS

Organisms used. All organisms used are shown in Table 1. The strains of *Pseudomonas* (except strain PR 107) were received from Dr O. Lysenko, Laboratory of Insect Pathology, Prague; the strain numbers are from the Culture Collection of Entomogenous Bacteria (CCEB), Prague. Strain 293 was originally thought to be *P. synxantha* (Lysenko, 1961) but was later identified as *Serratia marcescens* (*Catalogue of Cultures*, 1964). Strain 607 was only tentatively labelled as *P. synxantha* (O. Lysenko, personal communication). All xanthomonads were received from Professor M. P. Starr from the International Collection of Phytopathogenic Bacteria, Department of Bacteriology, University of California, Davis, California. The strain PR 107 was received as *Pseudomonas* (*Xanthomonas*?) *rimaefaciens*. The results given in the present paper show it to belong in *Pseudomonas*. The Rhizobium strains were received from Professor C. Bonnier, Institut Agronomique de l'État, Gembloux, Belgium, and the Azotobacter strain was obtained from the National Collection of Industrial Bacteria (NCIB), Aberdeen, Scotland. In a future paper we shall bring evidence to show why the generic name *Azomonas* (Baillie, Hodgkiss & Norris, 1962), which is used in Table 1, is a better choice for strain 9218. The strains of Gluconobacter, Acetobacter, Escherichia and Bacillus were from our departmental collection.

Cultivation techniques. All organisms were grown on solid media in Roux flasks. The Pseudomonas and Serratia strains were grown for about 2 days at 30° on a medium containing (%, w/v): Proteose peptone 1, yeast extract (Nederl. Gist-en Spiritusfabriek, Brugge, Belgium) 0·1, NaCl 0·5, glucose 1 and agar 2.5. The xanthomonads were grown at 25° for 3–4 days on a medium, selected for good growth and low polysaccharide production, and containing (%, w/v): beef extract (Oxoid) 1, Difco-peptone 1, NaCl 0·5, agar 2·5. The acetic acid bacteria were grown for 3 days at 30° on (%, w/v): glucose 5, $CaCO_3$ 3, yeast extract 1, agar 2·5. Rhizobium was grown for 3 days at 25° on (%, w/v): mannitol 1, NaCl 0·02, K_2HPO_4 0·05, $MgSO_4.7H_2O$ 0·02, $CaSO_4.2H_2O$ 0·01, $CaCO_3$ 0·01, yeast extract 0·1, agar 2·5. Azotobacter and Azomonas were grown for 2 days at 30° on (%, w/v): glucose 1, K_2HPO_4 0·1, $MgSO_4.7H_2O$ 0·02, $CaCO_3$ 0·1, NaCl 0·02, Na molybdate 0·0005, agar 2·5. *Escherichia coli* and *Bacillus subtilis* were grown for 1–2 days at 30° on (%, w/v): peptone 0·5, yeast extract 0·25 and agar 2·5.

Cultivation of ^{14}C-labelled strains. Xanthomonas pelargonii P 121 was selected because its % (G+C) is in the middle of the group. For the same reason it had been used in previous hybridization experiments (Friedman & De Ley, 1965; De Ley & Friedman, 1965). *Pseudomonas putida* 520 and *P. fluorescens* 488 were selected because their % (G+C) (Table 1) and taximetric similarity (Lysenko, 1961) put them centrally in the *Pseudomonas* group. The organisms were grown at 30° on a reciprocal shaker in liquid medium in 1 l. Erlenmeyer flasks containing 200 ml. each of the medium. The medium contained (%, w/v) proteose peptone (Oxoid) 1, yeast extract 0·1, NaCl 0·5, glucose 1, at pH 7·2. At the beginning of the log phase a sterile solution with 100 μc. uracil-2-^{14}C/200 ml. of medium was added and the organisms were allowed to grow until the end of the log phase. They were harvested and washed several times in 0·01 M-phosphate buffer (pH 6).

SSC buffer contained 0·15 M-NaCl and 0·15 M-trisodium citrate at pH 7·0. It was also made up at quarter strength (0·25 × SSC) and double strength (2 × SSC) etc.

Preparation of DNA was done according to Marmur (1961). Its molecular weight was in the range 5–10 × 10^6. High molecular weight denatured DNA agar was prepared according to Bolton & McCarthy (1962), with the exception that it was heated at 107–110° in 0·25 × SSC buffer instead of at 100°, because the 'melting point' of Pseudomonas- and Xanthomonas-DNA is rather high. Depending on the strain 1 g. of wet agar contained about 500 μg. DNA. ^{14}C-DNA was sheared by passing it through the French pressure cell at 12,000 p.s.i. Its molecular weight was about 3 × 10^5. It was denatured at 107–110° in 0·1–0·2 × SSC buffer for 5 min. followed by quick cooling in ice. Its final concentration was 50 μg./ml. 2 × SSC buffer. The specific activity was 680 cpm./min/μg. ^{14}C-DNA from *X. pelargonii*, 336 cpm./min./μg. ^{14}C-DNA from *P. fluorescens* 488 and 70 cpm./min./μg. ^{14}C-DNA from *P. putida* 520.

Hybridization experiments; phosphodiesterase treatment. The method of McCarthy & Bolton (1963) was used. An amount of agar containing 350 μg. of high-molecular denatured DNA from different strains and 35 μg. of sheared denatured ^{14}C-DNA from each of the three labelled strains was used. Disintegrations were counted as previously described (De Ley & Friedman, 1965). The correction factor for self-absorption in 2 × SSC buffer in the conditions used was 14·4 %; for 0·01 × SSC it was negligible.

To determine the percentage of unhybridized ^{14}C-DNA which adhered as loose single-stranded ends to the DNA-agar after washing with 2 × SSC buffer, a treat-

Table 1. *Some physical properties ('melting point' T_m; average (guanine + cytosine) content % (G+C)) of purified DNA and degree of DNA homology of several pseudomonads, xanthomonads and a few other groups.*

The pseudomonads are the neotype species centres as proposed by Lysenko (1961). The values with * are taken from De Ley & Friedman (1965), the values for T_m and % (G + C) for the acetic acid bacteria are from De Ley & Schell (1963), Rhizobium from De Ley & Rassell (1965) and *Escherichia coli* from De Ley (1965). The 'melting point' T_m was determined with the automatic recording thermal spectrophotometer (De Ley & Van Muylem, 1963). The % (G + C) was calculated from T_m according to Marmur & Doty (1962). The method of McCarthy & Bolton (1963) was followed for the hybridization experiments.

				Hybridization with ^{14}C-DNA from *P. fluorescens* 488		Hybridization with ^{14}C-DNA from *P. putida* 520		Hybridization with ^{14}C-DNA from *X. pelargonii* P121	
Strain number	Organism	T_m	% (G + C)	% ^{14}C-DNA bound	% ^{14}C-DNA bound relative to *P. fluorescens*	% ^{14}C-DNA bound	% ^{14}C-DNA bound relative to *P. putida*	% ^{14}C-DNA bound	% ^{14}C-DNA bound relative to *X. pelargonii*
	Pseudomonas								
338	*geniculata*	97·0	67·5	35·8	64	28·5	51	—	—
513	*diminuta*	96·9	67·3	21·5	38	21·2	38	—	69*
481	*aeruginosa*	96·7	66·8	35·4	63	34·0	61	—	—
506	*atlantica*	96·6	66·5	15·7	28	16·2	29	—	75*
PR107	*rimaefaciens*	96·6	66·5	35·8	64	—	—	33·5	55
380	*ovalis*	95·9	64·8	65·0	116	37·7	67·5	—	63*
522	*stutzeri*	95·8	64·5	42·4	75·5	33·5	60	—	—
520	*putida*	95·4	63·5	39·6	70·5	55·8	100	32·8	54
559	*chlororaphis*	95·3	63·3	36·5	65	34·6	62	—	—
518	*aureofaciens*	95·1	62·8	30·8	55	27·8	50	—	—
488	*fluorescens*	94·95	62·4	56·1	100	46·3	83	40·8	67
525	*denitrificans*	94·9	62·3	35·4	63	43	77	—	81*
607	*synxantha*	94·35	61·0	52·3	93·5	35·7	64	—	—
387	*fragi*	94·2	60·6	41·0	73	35·7	64	—	62*
512	*iodinum*	94·05	60·2	25·3	45	27·3	49	—	—
381	*taetrolens*	93·85	59·8	43·8	78	32·6	58·5	—	—
519	*rubescens*	88·25	46·1	1·7	3	3·3	6	—	—
533	*pavonacea*	88·05	45·6	3·9	7	5·5	10	—	—
	Xanthomonas								
C144	*celebensis*	97·7	69·2	18·5	33	—	—	32·8	54
H110	*hyacinthi*	97·4	68·5	33·1	59	—	—	45·6	75
C129	*campestris*	97·3	68·2	—	—	—	—	—	91*

Table 1 (*continued*)

Strain number	Organism	T_m	% (G+C)	Hybridization with ^{14}C-DNA from *P. fluorescens* 488: % ^{14}C-DNA bound	Hybridization with ^{14}C-DNA from *P. fluorescens* 488: % ^{14}C-DNA bound relative to *P. fluorescens*	Hybridization with ^{14}C-DNA from *P. putida* 520: % ^{14}C-DNA bound	Hybridization with ^{14}C-DNA from *P. putida* 520: % ^{14}C-DNA bound relative to *P. putida*	Hybridization with ^{14}C-DNA from *X. pelargonii* P121: % ^{14}C-DNA bound	Hybridization with ^{14}C-DNA from *X. pelargonii* P121: % ^{14}C-DNA bound relative to *X. pelargonii*
T7	*translucens*	97·2	68·0	34·8	62	—	—	47·2	77·5
M16	*maculafoliagardeniae*	97·1	67·7	39·8	71	—	—	50·6	83
T20	*tamarindi*	97·1	67·7	—	—	—	—	—	104*
A121	*alfalfae*	96·9	67·3	33·7	60	—	—	54·1	89
H1	*hederae*	96·9	67·3	—	—	—	—	—	87*
B3	*begoniae*	96·85	67·1	—	—	—	—	—	92*
C104	*carotae*	96·8	67·0	—	—	—	—	—	114*
P10	*pruni*	96·7	66·8	28·6	51	—	—	48·7	80
L1	*lespedezae*	96·65	66·6	33·7	60	—	—	56	92
C5	*corylina*	96·65	66·6	41·0	73	—	—	52·3	86
P121	*pelargonii*	96·6	66·5	31·4	56	27·6	49·5	60·9	100
V136	*vesicatoria*	96·55	66·4	25·0	44·5	—	—	52·3	86
J107	*juglandis*	96·5	66·3	—	—	—	—	—	93*
I3	*incanae*	96·5	66·3	27·5	49	—	—	48·7	80
P137	*poinsettiaecola*	96·4	66·0	29·7	53	—	—	50·9	83·5
P162	*phaseoli*	96·4	66·0	29·7	53	—	—	45·6	75
C110	*cassavae*	96·35	65·9	37·6	67	—	—	55·3	91
V118	*vignicola*	96·3	65·7	32·5	58	—	—	58·3	96
P5	*papavericola*	96·3	65·7	25·8	46	—	—	48·7	80
B109	*beticola*	96·3	65·7	26·4	47	—	—	46·9	77
M2	*malvacearum*	96·25	65·6	25·8	46	—	—	37·2	61
L4	*lactucae*	96·0	65·1	27·0	48	—	—	56·7	93
V24	*vasculorum*	95·9	64·8	25·3	45	—	—	46·3	76
T11	*taraxaci*	95·7	64·3	27·0	48	—	—	54·4	89·5
G1	*geranii*	95·4	63·5	25·8	46	—	—	53·2	87·5
8131	*Gluconobacter oxydans* (*viscosus*)	95·35	63·4	11·2	20	10·1	18	—	23*
20	*Acetobacter aceti* (*liquefaciens*)	95·6	64·0	19·1	34	15·6	28	—	37*
293	*Serratia marcescens*	93·9	59·9	20·2	36	17·3	31	—	—
I5	*Rhizobium meliloti*	95·0	62·5	21·6	38·5	—	—	—	—
6.2	*Rhizobium leguminosarum*	93·6	59·1	31·4	56	—	—	—	—
9125	*Azotobacter chroococcum*	96·4	66·0	—	—	27·9	50	—	—
9128	*Azomonas macrocytogenes*	93·3	58·4	—	—	23·5	42	—	—
B	*Escherichia coli*	90·9	52·5	16·8	30	9·5	17	—	19*
BQ2	*Bacillus subtilis*	88·9	47·7	3·4	6	6·1	11	—	—
	Agar	—	—	0·5	1	—	—	1·2	2

ment with dogfish phosphodiesterase was carried out as follows. Hybridization of DNA and washing of the agar column with ten 10 ml. fractions of 2 × SSC buffer at 60° was carried out as usual. All buffer was then eliminated with slight air pressure until the agar was still just moist. The temperature of the column was brought down to 37°. The agar was washed with 5 ml. of PD buffer (containing $1{\cdot}5 \times 10^{-3}$ M-$MnCl_2$ 0·01 M-tris, 0·14 M-NaCl; pH 8·0) and the eluate was collected. 1/50 diluted enzyme in 1 ml. buffer was added to the DNA agar and incubated at 37° for 1 hr, upon which the column was again washed at 60° with ten 10 ml. fractions 2 × SSC buffer and samples taken for counting. The last step consisted in increasing the column temperature to 75° and washing with 10–15 10-ml. fractions of 0·01 × SSC buffer.

Determination of the average base composition and the compositional distribution of the DNA molecules. Both values were calculated from the thermal denaturation curve of pure DNA. The latter was determined with the automatic recording thermal spectrophotometer as previously described (De Ley & Van Muylem, 1963). The mean base composition, expressed as % (G+C), was calculated from the 'melting point' T_m with Marmur & Doty's (1962) formula % (G+C) = $(T_m - 69{\cdot}3)/0{\cdot}41$. A previous paper chromatographic check on this formula (De Ley & Schell, 1963) showed it to be valid in the range used. The standard deviation σ of the compositional distribution around the average % (G+C) was calculated according to Doty, Marmur & Sueoka (1959).

RESULTS

The main results are summarized in Table 1.

DNA base composition. The pseudomonads are divided into two groups. The largest has a T_m in the range of 93·8° to 97·0° or a % (G+C) from 59·8 to 67·5. The other group comprises only *P rubescens* 519 and *P. pavonacea* 533 with a quite different % (G+C) of about 46 %. The latter strains are thus genetically different from the main group. The % (G+C) range of the main group agrees very well with values of a few strains reported by Colwell & Mandel (1964), De Ley & Friedman (1965), Marmur, Falkow & Mandel (1963).

The 28 strains of *Xanthomonas* are all in the narrow range of T_m 96·6° ± 1·2° or a % (G+C) from 63·5 to 69·2. These values confirm the previous conclusion from this laboratory, that these organisms form a narrow group. It may be noted that the % (G+C) values of *Xanthomonas* overlap with the higher range of *Pseudomonas* and that no distinction can be made between these groups on the basis of base composition alone. The T_m of *Serratia marcescens* 293 falls completely within the range of other serratias (Colwell & Mandel, 1965), although it might belong to a slightly aberrant subgroup. It is noticeable that the % (G+C) from *Serratia* overlaps with the lower range of *Pseudomonas.*

Compositional distribution of DNA molecules. Most of the pseudomonads and all xanthomonads have a σ value between 0 and 1 with an average of 0·3, a value which confirms our previous findings (De Ley & Van Muylem, 1963). The unusually high value of 2·5 for *Pseudomonas iodinum* is another argument that this strain possibly does not belong in *Pseudomonas.* The high σ value of 3·0 for *Serratia marcescens* 293 conforms to the values of 1·3–3 for other Enterobacteriaceae (De Ley & Van Muylem, 1963; De Ley, 1965). A word of caution is required on the calculation of

Table 2. *Determination of the fraction of single-stranded ^{14}C-DNA, attached to DNA agar, which remains unhybridized.*

Species	Total c.p.m.* in wash	Total c.p.m. released by phospho-diesterase†	Total c.p.m. retained on DNA-agar after phospho-diesterase	% labelled DNA released by phospho-diesterase	With phosphodiesterase: % labelled DNA bound	With phosphodiesterase: % labelled DNA bound relative to *P. fluorescens*	Without phosphodiesterase: % labelled DNA bound	Without phosphodiesterase: % labelled DNA bound relative to *P. fluorescens*
P. fluorescens	4736	744	4975	7·1	47·6	100‡	54·7	100‡
P. aeruginosa	7150	458	3315	4·2	30·3	63·7	34·5	61·4
P. taetrolens	6212	755	3930	6·9	36·1	75·8	43·0	76·5
P. chlororaphis	7184	355	3090	3·3	29·1	61·1	32·4	57·7
P. stutzeri	5800	194	3825	2·0	39·0	81·9	41·0	73·0

* C.p.m. = counts per minute.
† The phosphodiesterase treatment is described in the text.
‡ All values relative to *P. fluorescens* 488, which is taken as 100 %.
The method of McCarthy & Bolton (1963) was followed for the hybridization experiments.

the σ value according to Doty *et al.* (1959). When the compositional distribution is calculated from this σ value with, for example, the strains H110, P121 and G1, it appears that these strains would have very few DNA molecules in common, but the hybridization experiments show that most of the DNA molecules are identical. This means that the overlapping of the compositional distribution curves must be considerable, and thus that the σ values are too small because the correction factor of 3° 0, introduced by Doty *et al.* (1959), is too great. The best fit with our experimental data is obtained when no correction at all is applied; this means also that all σ values, calculated from thermal denaturation curves, including those mentioned above, would be some 7·5 % (G+C) too small.

Table 3. *Comparison between DNA homology and taximetric similarity of several strains of Pseudomonas.*

The former values are from Table 1 and the latter, for the same strains, are from Lysenko (1961). Values indicated by * are put as 100 %.

	Relative to *P. putida* 520		Relative to *P. fluorescens* 488	
Species	% DNA homology	% taximetric similarity	% DNA homology	% taximetric similarity
P. geniculata	51	44	64	62
P. diminuta	38	61	38	62
P. aeruginosa	61	73	63	72
P. atlantica	29	50	28	56
P. ovalis	67·5	81	116	77
P. stutzeri	60	52	77·5	68
P. putida	100*	100*	70·5	78
P. chlororaphis	62	80	65	86
P. aureofaciens	50	75	55	79
P. fluorescens	83	78	100*	100*
P. denitrificans	77	65	63	59
P. fragi	64	66	73	69
P. iodinum	49	65	45	59
P. taetrolens	58·5	58	78	68
P. rubescens	6	46	3	64
P. pavonacea	10	50	7	48

DNA-hybridization experiments. Hybridization with either *Pseudomonas fluorescens* 488 or *P. putida* 520 shows that all pseudomonads fall into two distinct groups. The low % (G+C) strains *P. rubescens* and *P. pavonacea* hybridize for only 3–10 %. The main group, made up of all the other strains, contains a few doubtful cases: *P. diminuta*, *P. atlantica* and *P. iodinum*, which hybridize to the extent of 28–50 %; all other strains show a high degree of relatedness. DNA of *P. fluorescens* 488, *P. ovalis* 380 and *P.* '*synxantha*' 607 appears to be almost, if not completely, identical. The degree of DNA homology of many strains is about the same with both *P. fluorescens* and *P. putida*. All strains of *Xanthomonas*, except *X. celebensis* C144, have a DNA homology of 45–73 % with *P. fluorescens*, with an average of 54 %. This agrees closely with our previous conclusion based on a smaller collection of strains that about two-thirds of *Xanthomonas*-DNA is equal to *Pseudomonas*-DNA (De Ley & Friedman, 1965).

From the hybridization of DNA from all xanthomonads with *X. pelargonii* P121,

it follows that all strains, except *X. celebensis* C144 and *X. malvacearum* M2, hybridize for 75% or more, with an average for the group of 87%. Nine strains out of 27 hybridize for 90–100% with *X. pelargonii*; eleven strains hybridize for 80–90% and the remaining 5 for 76–79%.

Reproducibility of the method. The values for DNA homology were in most cases reproducible to within 1–6%, although in four cases errors of 9–13% were observed. The number of erroneous results depends on the age of the DNA preparations. High-molecular, double-stranded DNA can be preserved in good condition in concentrated solution (about 1 or more mg. DNA/ml. SSC buffer) for several months at 4° with a drop of chloroform added. For reproducible results it is advisable to use freshly prepared DNA agar and sheared denatured ^{14}C-DNA solution that is not over 2 weeks old.

The effect of phosphodiesterase. One set of experiments, representative for several others, is summarized in Table 2. It was invariably found that with every strain used, about 5% of the total recovery is not hybridized; this corresponds to about one-tenth of the length of a ^{14}C-DNA fragment. Since this value is about the same for both the reference strain and the others under investigation, it does not affect the final conclusion on DNA homology.

DISCUSSION

An approach to a rational classification, based on two independent parameters from the genome (mean base composition and DNA homology) is represented in Figs. 1–3.

Xanthomonas

With respect to the central reference strain *X. pelargonii* P121, all strains (except M2 and C144, see below) form one tight cluster (Fig. 1). These results throw a new light on the biological relatedness between organisms and on the species concept. They show that artificial pigeon-holing in the usual orthodox classifications of this genus do not reflect reality but they corroborate the concept of a genetic species. The genus *Xanthomonas* appears not to contain about 60 sharply separated species, but to be a tight cluster of strains, each one having 75–100% of its DNA identical with that of the central reference strain, and probably also amongst themselves. It seems to us that at least the ten strains which have over 90% DNA similarity (campestris, tamarindi, begoniae, carotae, lespedezae, pelargonii, juglandis, cassavae, vignicola and lactucae) can no longer be regarded as separate species and should be gathered into one. In view of the tightness of the group, it seems better to regard the entire group as one biological unit, one geno-species, for which the specific epithet 'campestris' is proposed (the generic name will be discussed below). This supports a previous suggestion (Friedman & De Ley, 1965; De Ley & Friedman, 1965), which was made after a similar study of a smaller group. The most widely divergent strains, e.g. pelargonii and phaseoli, differ by 25% of their genome or about 2500 cistrons. Whether these organisms deserve separate species recognition is more a matter of opinion than of fact. For nomenclatural purposes it should be recalled that other organisms which differ by some 25% of their genome, such as rhesus monkey and man (Hoyer, McCarthy & Bolton, 1964), may differ by as many as 8×10^5 cistrons ('total' mol. weight of mammalian DNA 2×10^{12} and assuming

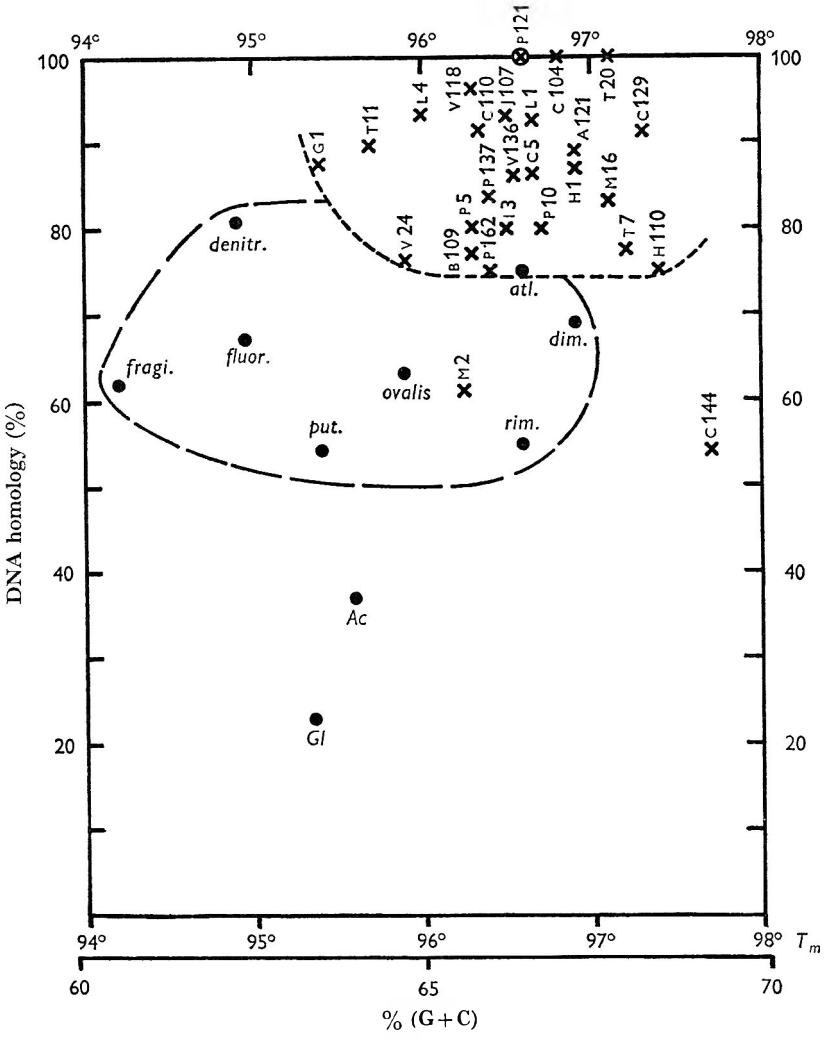


Fig. 1. Relatedness within the Xanthomonas group and between the xanthomonads and the pseudomonads, on the basis of DNA base composition and DNA homology (hybridization relative to *X. pelargonii* P 121). The organisms, whose position on the map is indicated by × followed by a strain number, are named xanthomonads. The line ---- shows the approximate border of the proposed genetic group *Pseudomonas campestris*. It is seen that strain M 2 and C 144 are aberrant from the main group. The position of the named pseudomonads is indicated by ● and the abbreviation of the species name. The named *Pseudomonas* group is delineated by — — —. Relatedness with a strain of *Acetobacter* (Ac) and *Gluconobacter* (Gl) is given for comparison. All data from Table 1.

10^3 base pairs per cistron) and that a few thousand genes are now thought to account for the differences within the species man.

The strains malvacearum M 2 and celebensis C 144 seem to fall outside the *Xanthomonas* group. The former strain, however, still groups with the other xanthomonads, with respect to *P. fluorescens* 488. Strain C 144 is the most remote one. We believe it to be still a xanthomonad because of its alleged phytopathogenicity and because we detected the typical xanthomonas-carotenoid (absorption maxima at 464 and

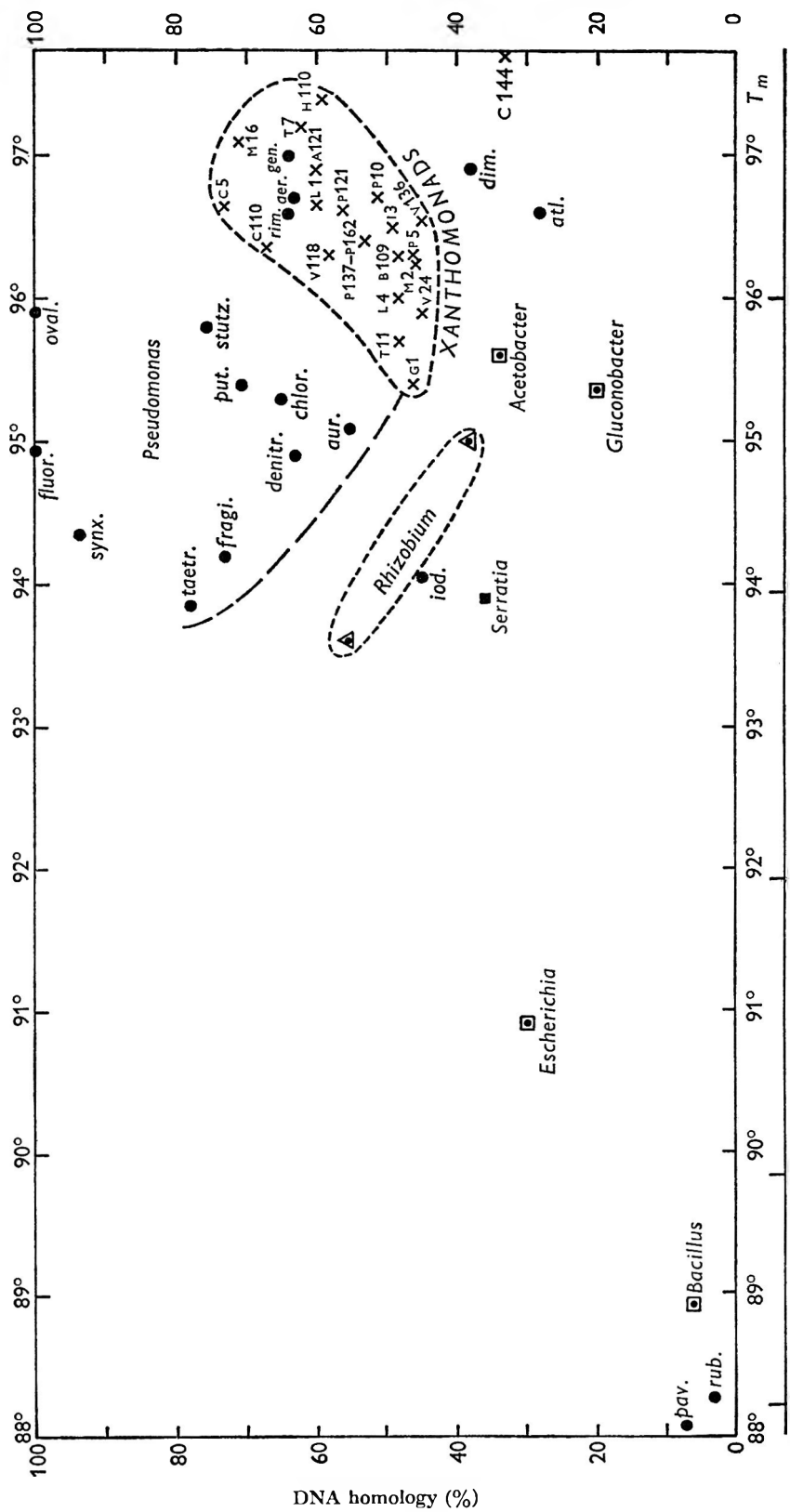


Fig. 2. Relatedness within the Pseudomonas group and between the pseudomonads, the xanthomonads and some other groups on the basis of DNA base composition and DNA homology (hybridization relative to *Pseudomonas fluorescens* 488). Symbols and abbreviations as in Fig. 1. The approximate relatedness of other genera is indicated by one or two strains only.

437 mμ and a shoulder at 418 mμ) after extraction according to Starr & Stephens (1964). Both strains might belong to separate species-centres, although it seems more likely that they are aberrant variations on the main theme. Other strains aberrant in % (G+C) are known, in *Chromobacterium* and in *Agrobacterium*. (De Ley & Van Muylem, 1963; De Ley, 1964).

Pseudomonas

The strains 'rubescens' 519 and 'pavonacea' 533 fall completely outside the main group, since their DNA is almost completely different. They differ more from *Pseudomonas* than does, for example, *Escherichia*. We propose to remove them from the genus *Pseudomonas*. Their % (G+C) and physiological properties suggest a

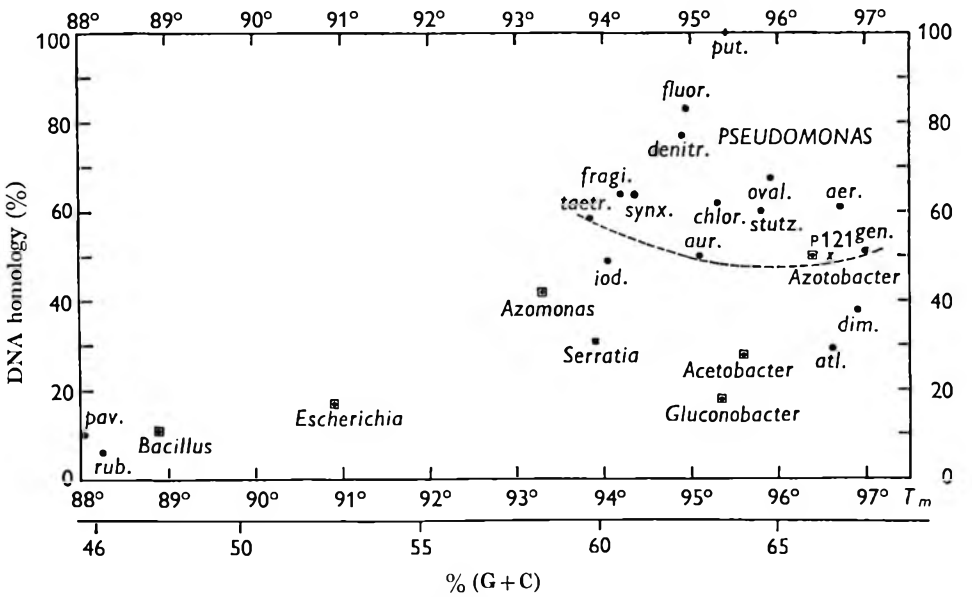


Fig. 3. Relatedness within the pseudomonas group and between the pseudomonads and a few other genera on the basis of DNA base composition and DNA homology (hybridization relative to *P. putida* 520). Symbols and abbreviations as in Figs. 1 and 2.

possible relationship with *Vibrio* which might be worth investigating. All the other strains form one broad group in which the strains are clearly separated. Since each strain is proposed as a species centre on the basis of numerical analysis (Lysenko, 1961), the present results indicate that—in contrast to *Xanthomonas* species differentiation in *Pseudomonas* may be justified. Since DNA-homology of the three doubtful cases (*P. iodinum*, *P. atlantica* and *P. diminuta*) is small, their inclusion in *Pseudomonas* is open to doubt. In fact they are about as far removed from *P. fluorescens* as, for example, *Serratia*, *Acetobacter*, *Rhizobium* and *Azotobacter*. The strain 'rimaefaciens' PR 107, previously of uncertain generic status, appears to belong to *Pseudomonas*.

Previously we found that about 60–80 % of Xanthomonas-DNA is identical to Pseudomonas-DNA (De Ley & Friedman, 1965). The reverse is also true since 45–73 % of *Pseudomonas fluorescens*-DNA is identical with Xanthomonas-DNA.

Figure 2 confirms Fig. 1: in relation to *P. fluorescens* all xanthomonads form one tight cluster. They share about as much DNA with *P. fluorescens* as do, for example, *P. geniculata* or *P. aeruginosa*. Therefore, there seems to be no reason to keep the xanthomonads in a separate genus and we propose to gather them in a single genetic species *Pseudomonas campestris*. It may be recalled that the creation of the genus-name *Xanthomonas* (Dowson, 1939) was not inspired by genetic data, but by the desire to group together the yellow-pigmented, necrotic phytopathogenic pseudomonads.

Phenotypically non-expressed DNA in Pseudomonas. It is not known whether all or only part of bacterial DNA is phenotypically expressed. It is conceivable that a certain fraction would have no genetic function as for example, nonsense DNA, and unused genes. Comparison of taximetric similarity of characters and DNA homology of the same strains might provide an answer. If homologous, phenotypically non-expressed DNA is present, DNA homology will be higher than the similarity value. Such a comparison is made in Table 3. In 21 out of 30 cases DNA homology is not higher than the taximetric similarity. In many of the remaining 9 cases the difference lies within the limit of sensitivity of the methods. Therefore it seems that in most cases all DNA is used for phenotypic expression and that unused genes either do not exist or are rather scarce.

Imperfection of numerical analysis. In spite of its considerable advance over usual classification, taximetric analysis still deals with only a small part of the genome. Therefore occasional imperfections in its conclusions can be expected. It was previously (De Ley & Van Muylem, 1963) pointed out that the yellow-pigmented marine Cytophaga-like bacteria fall into widely divergent % (G+C) groups. The present paper provides another example with the strains rubescens and pavonacea. A classification based on % (G+C) and DNA homology appears to us to be more logical.

Relatedness of some other genera to Pseudomonas (Figs. 2 and 3). Genera such as *Gluconobacter*, *Acetobacter*, *Serratia* and *Escherichia* have some 20–30% DNA similarity with *Pseudomonas*. They would have some 2000 cistrons in common with it. *Rhizobium*, *Azotobacter* and *Azomonas* appear to be more closely related to *Pseudomonas* than expected, since 40–56% of their DNA seems to be *Pseudomonas*-like. Similarly, *Serratia* might be more closely related to *Pseudomonas* than expected. The chromosome of *Bacillus* is almost entirely different.

One of us (J.D.L.) is indebted to the Centre National de Biochimie et de Biologie Moléculaire for research and personnel grants. Another (I.W.P.) is indebted to the Patrimonium of the University of Gent for a study grant. We are indebted to the institutes and individuals who kindly sent us the strains, and to Dr M. Mandel, Texas, Medical Centre Houston, Texas, for a gift of phosphodiesterase.

REFERENCES

Baillie, A., Hodgkiss, W. & Norris, J. R. (1962). Flagellation of Azotobacter spp. as demonstrated by electron microscopy. *J. appl. Bact.* **25**, 116.

Bergey's Manual of Determinative Bacteriology. (1957). Ed. by R. S. Breed, E. G. D. Murray and N. Smith. Baltimore: Williams and Wilkins Co.

Bolton, E. T. & McCarthy, B. J. (1962). A general method for the isolation of RNA complementary to DNA. *Proc. natn. Acad. Sci., U.S.A.* **48**, 1390.

Catalogue of Cultures, Czechoslovak Collections of Micro-organisms, Brno, 1964.

COLWELL, R. R. & MANDEL, M. (1964). Adansonian analysis and deoxyribonucleic acid base composition of some Gram-negative bacteria. *J. Bact.* **87**, 1412.

COLWELL, R. R. & MANDEL, M. (1965). Adansonian analysis and deoxyribonucleic acid base composition of *Serratia marcescens*. *J. Bact.* **89**, 454.

DE LEY, J. (1964). Effect of mutation on DNA-composition of some bacteria. *Antonie van Leeuwenhoek*, **30**, 281.

DE LEY, J. (1965). DNA base composition of *Klebsiella rubiacearum*. *Antonie van Leeuwenhoek*, **31**, 203.

DE LEY, J. & FRIEDMAN, S. (1965). Similarity of Xanthomonas and Pseudomonas deoxyribonucleic acid. *J. Bact.* **89**, 1306.

DE LEY, J. & RASSEL, A. (1965). DNA base composition, flagellation and taxonomy of the genus *Rhizobium*. *J. gen. Microbiol.* **41**, 85.

DE LEY, J. & SCHELL, J. (1963). Deoxyribonucleic acid base composition of acetic acid bacteria. *J. gen. Microbiol.* **33**, 243.

DE LEY, J. & VAN MUYLEM, J. (1963). Some applications of deoxyribonucleic acid base composition in bacterial taxonomy. *Antonie van Leeuwenhoek*, **29**, 344.

DOTY, P., MARMUR, J. & SUEOKA, N. (1959). The heterogeneity in properties and functioning of deoxyribonucleic acids. *Brookhaven Symp. Biol.* **12**, 1.

DOWSON, W. J. (1939). On the systematic position and generic names of the Gram negative bacterial plant pathogens. *Zentbl. Bakt.* (2 *Abt. Ong.*), **100**, 177.

FRIEDMAN, S. & DE LEY, J. (1965). 'Genetic species' concept in Xanthomonas. *J. Bact.* **89**, 95.

HOYER, B. H., MCCARTHY, B. J. & BOLTON, E. T. (1964). A molecular approach in the systematics of higher organisms. *Science*, **144**, 959.

LYSENKO, O. (1961). *Pseudomonas*—an attempt at a general classification. *J. gen. Microbiol.* **25**, 379.

MCCARTHY, B. J. & BOLTON, E. T. (1963). An approach to the measurement of genetic relatedness among organisms. *Proc. nat. Acad. Sci., U.S.A.* **50**, 156.

MARMUR, J. (1961). A procedure for the isolation of deoxyribonucleic acid from micro-organisms. *J. mol. Biol.* **3**, 208.

MARMUR, J. & DOTY, P. (1962). Determination of the base composition of deoxyribonucleic acid from its thermal denaturation temperature. *J. mol. Biol.* **5**, 109.

MARMUR, J., FALKOW, S. & MANDEL, M. (1963). New approaches to bacterial taxonomy. *Annu. Rev. Microbiol.* **17**, 329.

STARR, M. P. & STEPHENS, W. L. (1964). Pigmentation and taxonomy of the genus *Xanthomonas*. *J. Bact.* **87**, 293.

J. gen. Microbiol. (1966), **42**, 57–69
Printed in Great Britain

Chemistry of Hyphal Walls of Phytophthora

By S. BARTNICKI-GARCIA
Department of Plant Pathology, University of California, Riverside, California, U.S.A.

(*Received 7 July* 1965)

SUMMARY

Hyphal walls of two phytopathogenic moulds, *Phytophthora cinnamomi* and *P. parasitica*, were isolated essentially free from cytoplasmic contamination. They have a complex chemical structure consisting of polysaccharide, protein and lipid. D-Glucose was the main monosaccharide detected in acid hydrolysates. Chromatographic evidence suggested the presence of small amounts of mannose (0·6 %), glucosamine (0·3 %) and traces of galactosamine and ribose. Glucans constituted nearly 90 % of the wall but only about a maximum 25 % of the wall could be regarded as cellulose I on the basis of solubility, resistance to hydrolysis and X-ray diffraction. Most of the wall glucan exhibited chemical and physical properties unlike typical cellulose.

The spectrum of amino acids commonly found in fungal walls was detected; hydrolysates also contained hydroxyproline and two minor unidentified ninhydrin-positive components. Protein comprised 3–5 % of the wall. A small amount of lipid (1–3 %), mostly of the bound type, was found, and also traces of phosphorus, and compounds with absorption maxima at 263 mμ. Hyphal walls of *Phytophthora cinnamomi* and *P. parasitica* differed only slightly in quantitative composition.

INTRODUCTION

Customarily the cell walls of filamentous fungi are described as chitinous or cellulosic. The grouping of fungi based on the presence of either one or, in some cases, both of these polysaccharides, parallels taxonomic demarcations (Wettstein, 1921; Frey, 1950; Fuller & Barshad, 1960; Aronson & Preston, 1960). With the availability of physical methods for isolating cell walls, the chemical complexity of the walls of fungi has now been clearly realized. In addition to chitin or cellulose, various carbohydrates, proteins, lipids, and other components have been found in the cell walls of moulds (Kreger, 1954; Aronson & Machlis, 1959; Bartnicki-Garcia & Nickerson, 1962; Crook & Johnston, 1962; Hamilton & Knight, 1962; Bartnicki-Garcia & Reyes, 1964; Horikoshi & Iida, 1964; Russell, Sturgeon & Ward, 1964; Potgieter & Alexander, 1965; Wessels, 1965; etc.). The majority of filamentous fungi have chitinous walls. Quantitative studies of wall chemistry in several such fungi have already been reported, but hitherto no comparable studies on cellulosic fungi had been made.

Phytophthora, a notorious genus of plant pathogens, is a prominent example of the relatively few fungi with cellulosic walls. The presence of cellulose in the phytophthora mycelium, long claimed (Dastur, 1913), was definitely established through X-ray analysis by Frey (1950). More recently Crook & Johnston (1962)

made a qualitative survey of sugars and amino acids in the cell walls of fungi, including *Phytophthora cactorum*. The present communication, describing the quantitative composition of hyphal walls of two phytophthora species, is an initial study designed to gain insight into the morphogenetic role of cell-wall chemistry in the life-cycle of these plant pathogens. A strain of *Phytophthora cinnamomi* parasitic on avocado, and a strain of *P. parasitica* pathogenic to citrus trees, were selected from among various other phytophthora strains and species tested; the choice was made on the basis of the relative facility with which hyphal walls could be isolated with a minimum of cytoplasmic contamination.

METHODS

Microbiological techniques. *Phytophthora cinnamomi* Rands strain SB 216–1 and *Phytophthora parasitica* Dastur strain T 89 were isolated and kindly given by Drs G. Zentmyer and P. Tsao from this Department. Stock cultures were maintained on V-8 juice agar slants. Liquid V-8 medium was prepared with the clear supernatant fluid of centrifuged V-8 juice (Campbell Soup Co., Camden, New Jersey), diluted 1/4 with glass-distilled water and its pH adjusted to 6·3 with KOH. V-8 juice agar plates were inoculated and grown for about a week at 27°. Disc plugs were removed from the periphery of growth with a sterile stainless-steel cork borer and inoculated into 350 ml. prescription bottles containing 40 ml. of the following asparagine glucose medium: KH_2PO_4, 3 g.; $CaCl_2$, 3·4 mg.; $FeSO_4.7H_2O$, 1·0 mg., with citric acid, 1·4 mg.; $ZnSO_4.7H_2O$, 1·8 mg.; $MnSO_4.H_2O$, 0·3 mg.; $CuSO_4.5H_2O$, 0·4 mg.; $(NH_4)_6Mo_7O_{24}.4H_2O$, 0·3 mg.; $MgSO_4.7H_2O$, 0·5 g.; thiamine HCl, 1·0 mg.; L-asparagine, 2 g.; glucose, 20 g.; glass-distilled water to 1000 ml. Glucose in concentrated solution was autoclaved separately and added to the rest of the ingredients which had been previously adjusted to pH 6·0 with KOH and autoclaved for 20 min. at 121°. Inoculum bottles were incubated at 27° for about 5–7 days. Mycelial mats from 5 bottles were placed in a sterile cup of a Servall Omni-mixer and homogenized at medium speed for a few minutes. The homogenized mycelium was centrifuged, washed once with culture medium solution and inoculated into 8 2-l. flasks containing 600 ml. asparagine glucose medium. These flasks were placed on a rotary shaker and agitated 5 days at 27°. The mycelium was filtered through a sintered glass filter, washed with distilled water and lyophilized.

Isolation of cell walls. The mycelium of *Phytophthora cinnamomi* prepared as above was suspended in water (0·5–1·0 g./35 ml.) and blended in a Virtis '45' homogenizer at full speed and centrifuged at 1400 *g*; the supernatant fluid containing cytoplasmic debris was removed. The residues were combined and suspended in 20 ml. water/g. initial mycelium. Samples (10 ml.) were withdrawn and placed in the cup of a 10 kcyc. Raytheon Sonic Oscillator with 15·5 g. glass beads (150 μ average diameter). The resulting paste was treated at full power for 5 min. Sample temperature was maintained at 0–20° by circulating coolant at −5°. Two to four treatments sufficed to obtain satisfactory breakage. Glass beads were separated by decantation. Cell walls were then spun down at 1400 *g* for 5 min. and the supernatant fluid, consisting chiefly of cytoplasmic debris, was discarded. The residue containing wall fragments of different size and cytoplasmic

debris was divided into 8 portions, suspended in 30 ml. water each, and homogenized in an Omni-mixer for 30 sec. at half speed. These wall suspensions were fractionated by a series of consecutive 1 min. centrifugations at 50, 210, 500, 900 and 2000 *g*. Each residue was resuspended and centrifuged at the same speed as when first sedimented and the resulting supernatant fluid combined with the residue sedimenting at the next higher speed. The entire procedure was repeated twice. In this way, wall fractionation according to size was achieved; the heavier fractions, sedimenting at 50 and 210 *g*, contained large wall fragments with some hyphal segments partially filled with cytoplasm and glass particles. The lightest pellet (2000 *g*) contained minute wall pieces mixed with fine uncharacterized particles; the fractions sedimenting at 500 and 900 *g* contained the purest walls as revealed by staining with Lugol's iodine solution. Walls were lyophilized and stored in a freezer. The hyphal walls of *P. parasitica* were more difficult to prepare because of a tendency of wall fragments to agglutinate in big clumps after the first washings, making subsequent differential centrifugation impossible. Instead, walls were subjected to repeated treatments in the sonic oscillator (without glass beads) rapidly followed by centrifugation at 210 *g* for 1 min. In this manner, cytoplasmic contaminants were gradually discarded with the supernatant fluids. Treatments were continued until light microscopy revealed practical disappearance of cytoplasmic debris.

Chemical analyses. Three types of hydrolysates were prepared. (*a*) N-H_2SO_4 hydrolysate. Wall suspensions (2 mg./ml.) in N-H_2SO_4 were sealed in glass ampules under N_2 and heated at 105° for various specified times. (*b*) 22·5 N-0·85 N-H_2SO_4 hydrolysate. Dry wall samples were soaked with 22·5 N-H_2SO_4 and incubated at 30° for 3 hr; the acid was diluted to 0·85 N and the suspension heated at 97° for 4 hr. (*c*) 6 N-HCl hydrolysate. Walls were suspended in 6 N-HCl, sealed in glass ampoules under N_2, and hydrolysed at 105° for 8 hr; excess HCl was removed in a desiccator over NaOH pellets under vacuum.

Neutral sugars were separated in descending paper chromatograms (Whatman no. 1) irrigated with *n*-butanol + pyridine + water (6 + 4 + 3, by vol.) for 30 hr or longer. Sugars were revealed (aniline phthalate), eluted and measured (Wilson, 1959). Before chromatography, H_2SO_4 hydrolysates were neutralized with $BaCO_3$ and de-ionized through a double-bed column of Dowex-1 (acetate form) and Dowex-50 (hydrogen form).

Amino sugars were determined in 6 N-HCl hydrolysates by the Elson–Morgan method as described by Tracey (1955). Hexoses were estimated by the anthrone procedure of Dreywood as described earlier (Bartnieki-Garcia & Nickerson, 1962). Reducing sugars were estimated by the method of Somogyi & Nelson (Hodge & Hofreiter, 1962). Glucose was assayed with a purified glucose oxidase reagent (Glucostat 'special', Worthington Biochemical Corp., Freehold, New Jersey, U.S.A.).

Search for uronic acids was made in H_2SO_4 hydrolysates by adsorption on a 10 ml. column of Dowex-1 (acetate form). A stepwise elution with increasing concentrations of acetic acid up to 4 N was used. A modified carbazole reagent was used to detect uronic acid in each of the 2 ml. fractions collected (Bitter & Muir, 1962). Protein-N was estimated in 6 N-HCl hydrolysates by a ninhydrin method described earlier (Bartnieki-Garcia & Nickerson, 1962). Individual amino acids

were resolved on two-dimensional paper chromatograms irrigated first with *n*-butanol + pyridine + water (1 + 1 + 1, by vol.); second, with phenol saturated with a 0·2 M solution of K_2HPO_4 in water to which 0·01 % (w/v) 8-hydroxyquinoline was added. The solvent pair prescribed by Crook & Johnston (1962) was also tested: *n*-butanol + acetic acid + water (100 + 22 + 50, by vol.) followed by phenol + water (80 + 20, by vol.) in the presence of NH_3 but without NaCN. Quantitative amino acid analyses (Spackman, Stein & Moore, 1958) were made in a Spinco 120B Automatic Amino Acid Analyser. Total phosphate was measured colorimetrically (Dryer, Tammes & Routh, 1957). Lipids were extracted in two categories, free and bound, as described earlier (Bartnicki-Garcia & Nickerson, 1962). Hyphal walls were chemically fractionated with acid and alkaline solutions as detailed previously (Bartnicki-Garcia & Nickerson, 1962).

Physical techniques. Infrared spectra were determined in KBr pellets with a Perkin–Elmer Infracord spectrophotometer.

X-ray diagrams of samples spread on a glass microslide were obtained with a Norelco Phillipps recording diffractometer using CuK_α radiation.

RESULTS

Chemical analyses

Carbohydrates. The anthrone procedure, with glucose as standard, served to assess the total amount of hexosan present in intact walls. To improve sample uniformity, dilute wall suspensions were homogenized by sonic oscillation immediately before pipetting. Nearly 90 % of the hyphal wall dry weight of both species of Phytophthora was found to be anthrone-positive carbohydrate (Table 1).

Table 1. *Hyphal wall composition of phytophthora*

Component	Method	% wall dry weight	
		P. cinnamomi	*P. parasitica*
Glucan*	Anthrone	88 ± 2	86 ± 2
Mannose	Paper chromatography	0·6	0·7
Glucosamine	Elson–Morgan	0·3	0·3
Protein	Micro-Kjeldahl N × 6·25	3·6	4·3
Protein	Ninhydrin	3·4	4·1
Free lipids	Direct extraction	0·3	0·2
Bound lipids	Hydrolysis and extraction	2·1	0·9
Phosphorus (as H_2PO_3)	Semidine	0·3	0·4

* Range of values obtained in 3 determinations.

Measurement of monosaccharide constituents of hyphal-wall polysaccharides was made in two types of H_2SO_4 hydrolysates; in neither case was sugar recovery complete. Direct hydrolysis in N-H_2SO_4 was insufficient to dissolve walls completely, even after prolonged digestion for 48 hr; by which time about a quarter of the wall carbohydrate remained insoluble, a quarter had been destroyed, and a half was in solution (Table 2). The soluble carbohydrate was composed almost exclusively of glucose as shown by determination of total and reducing sugar and by paper chromatography.

The 22·5–0·85 N-acid hydrolysis was much more efficient (Table 3); respectively, 93 and 87 % of the initial polysaccharide of *Phytophthora cinnamomi* and *P. parasitica* was recovered in solution and only 0·1 % remained insoluble. Paper chromatograms of this hydrolysate revealed glucose as the chief component. There was also a much smaller amount of two disaccharides subsequently identified as cellobiose and gentiobiose and traces of another one identified as laminaribose (Bartnicki-Garcia & Lippman, to be published), plus a faint streak of unresolved oligosaccharides.

Table 2. *Hydrolysis of hyphal walls of phytophthora in* N-H_2SO_4

Carbohydrate in hydrolysate	% of initial total carbohydrate			
	P. cinnamomi		*P. parasitica*	
	18 hr	48 hr	18 hr	48 hr
Total*	97·8	76·1	91·8	74·4
Soluble*	59·0	51·1	61·7	49·0
Insoluble†	38·8	25·0	30·1	25·4
Destroyed‡	2·2	23·9	8·2	25·6

* Determined with anthrone using glucose as standard. Values are % anhydroglucose of initial glucan content in the wall (Table 1).
† Calculated: total−soluble; ‡ Calculated: 100−total.

Table 3. *Hydrolysis of hyphal walls of phytophthora with 22·5–0·85* N-H_2SO_4

Carbohydrate*	*P. cinnamomi*		*P. parasitica*	
	% wall	% initial carbohydrate†	% wall	% initial carbohydrate†
Total soluble (anthrone)	80·7	92·6	75·6	87·5
Glucose (glucostat)	70·9	81·4	64·5	75·6

* As anhydroglucose. † Initial glucan content values are shown in Table 1.

Respectively, 81·4 and 75·6 % of the total wall polysaccharide of *Phytophthora cinnamomi* and *P. parasitica* were conclusively identified as D-glucose by the glucose oxidase assay. There are minimum values which do not include glucose di- and oligo-saccharides present in the hydrolysates and do not take into account some glucose inevitably destroyed during hydrolysis. A trace of mannose, 0·6–0·7 % of the wall, was also found in both *P. cinnamomi* and *P. parasitica*. Inasmuch as the hydrolysates of wall polysaccharides of *P. cinnamomi* and *P. parasitica* revealed almost exclusively glucose and disaccharides thereof, the determination in intact walls of total carbohydrate by the anthrone method may be regarded as the best approximation to the actual glucan content of the wall (Table 1). In heavily loaded chromatograms (3·1 mg. walls) a vestigial spot appeared which had the colour of a pentose (aniline phthalate spray), the R_F value of ribose, and was visually estimated to be less than 0·1 % wall dry wt.

Uronic acids in H_2SO_4 hydrolysates were not detected.

Proteins. The protein content of mycelial walls of phytophthora was estimated by determining amino acid-N in 6 N-hydrolysates and by micro-Kjeldahl determination of total nitrogen in intact walls (Table 1). Similar values were obtained

in both determinations; the sum of individual amino acids determined in the automatic amino acid analyser (Table 4) also agreed with the above values. Amino acid quantities (Table 4) are given with the reservation that some destruction took place during hydrolysis as shown by humin formation.

Both two-dimensional paper chromatography and automatic column chromatography of hyphal wall hydrolysates revealed the common spectrum of protein amino acids, threonine being the most abundant. Hydroxyproline, an amino acid not usually found in fungal wall proteins, was shown in both paper and column chromatograms. Two weak unidentified ninhydrin-violet spots appeared in amino acid paper chromatograms of *Phytophthora cinnamomi* and *P. parasitica*. $R_{alanine}$ values were respectively 1·09 and 0·74 in *n*-butanol + acetic acid + water; 1·64 and 1·23 in phenol + water + NH_3. They probably correspond to the unknown amino acid spots of *P. cactorum* reported by Crook & Johnston (1962). ($R_{alanine}$ values in this publication were misprinted; they should be 1·03 and 0·76 in *n*-butanol + acetic acid + water and 1·40 and 1·30 in phenol + water + NH_3; I. R. Johnston, private communication).

Table 4. *Overall amino acid composition of protein from hyphal walls of Phytophthora cinnamomi*

	% of total amino acids*		% of total amino acids*
Threonine	14·8	Glycine	4·5
Glutamic acid	11·1	Arginine	4·2
Serine	9·8	Ammonia	3·7
Aspartic acid	9·0	Tyrosine	2·6
Alanine	9·0	Phenylalanine	2·1
Proline	5·5	Isoleucine	1·8
Hydroxyproline	5·0	Histidine	1·1
Valine	5·0	Cysteine	1·1
Lysine	4·7	Methionine	1·1
Leucine	4·5		

* The sum of amino acids recovered was 3·79 % of the initial wall dry weight.

Amino sugars. A glucosamine peak, representing 0·20 % of the wall of *Phytophthora cinnamomi*, and a much smaller peak, presumably galactosamine, were detected during the automatic analysis of amino acids. Elson–Morgan determinations on 6 N-hydrolysates confirmed the existence of a small amount of amino sugar in both phytophthora species (Table 1).

Lipids. A small amount of lipid, mainly of the bound type, was detected in the walls of both phytophthora species (Table 1).

Various. Determination of total phosphorus indicated phosphate content of about 0·3 % (Table 1). Hot N-KOH extracts of walls showed sharp absorption peaks at 263 mμ, suggesting the possible presence of 0·8 % nucleic acid components (calculated at an average ϵ of 8800).

Chemical fractionation. Hyphal walls were fractionated by two different methods. The distribution of carbohydrates (Table 5) and protein (Table 6) was investigated by anthrone and ninhydrin assays. Fractionation method I indicated that even at high temperatures the hyphal-wall glucans were alkali-insoluble; likewise, hot acetic acid did not dissolve any appreciable quantity of glucan. In the same

fractionation, over half of the wall protein was dissolved by cold alkali and most of the remainder dissolved in hot alkali. A small proteinaceous residue was left associated with the insoluble glucan. Fractionation method II revealed that a large portion of the wall glucan (61–66%) was soluble in hot dilute acid. Consecutive extraction of hyphal walls with hot acid and hot alkali left only about 20% of insoluble glucan. Recovery of carbohydrate in this method was incomplete, probably due to destruction of glucan by hot alkali after walls had first been treated with hot acid, a situation also observed for the glucan of *Mucor rouxii* spore walls (Bartnicki-Garcia & Reyes, 1964).

Table 5. *Distribution of carbohydrates in hyphal wall fractions of phytophthora*

Method	Fraction	% of initial carbohydrate*	
		P. cinnamomi	*P. parasitica*
I	Cold N-KOH	2·7	2·1
	Hot N-KOH	3·2	3·3
	Hot N-acetic acid	0·2	0·3
	Residue	93·2	95·6
	Sum	99·3	101·3
II	Cold N-HCl	0·2	0·3
	Hot N-HCl	61·3	65·9
	Hot N-KOH	3·2	3·0
	Residue	21·2	19·7
	Sum	85·9	88·9

* Determined with anthrone reagent and calculated as % anhydroglucose of the initial glucan values shown in Table 1.

Table 6. *Distribution of protein in hyphal walls of phytophthora fractionated according to Method I (Table 5)*

Fraction	% of total protein*	
	P. cinnamomi	*P. parasitica*
Cold N-KOH	59·5	63·1
Hot N-KOH	27·2	21·4
Hot N- acetic acid	5·1	5·9
Residue	8·2	9·6

* Determined by the ninhydrin method and calculated on the basis of the total protein values (ninhydrin) from Table 1.

Extraction with 17·5% NaOH, under N_2, is commonly used in cellulose technology to determine and separate α-cellulose from low molecular weight celluloses and other carbohydrates (Corbett, 1963). Accordingly, only 3·4% of *Phytophthora cinnamomi* walls dissolved as anthrone-positive carbohydrate. It may not be concluded, however, that the portion of wall glucan insoluble in 17·5% NaOH is α-cellulose since it is known that other glucans, notably yeast cell-wall glucan (Chung & Nickerson, 1954), are highly insoluble in concentrated alkali.

Infrared spectra. Intact walls of *Phytophthora cinnamomi* and *P. parasitica* showed nearly identical spectra, corroborating their chemical similarity. They showed absorption peaks at about 890 cm.$^{-1}$, indicative of β-linked polysaccharide.

There was no evidence of absorption in the α-glycoside region at 844 cm.$^{-1}$ (Barker, Bourne & Whiffen, 1956). The spectra of isolated walls was compared with that of crystalline cellulose I (Whatman α-cellulose powder for chromatography); there was a general resemblance, but the wall spectra differed noticeably in the blurring of peak sharpness in the 1000–1500 cm.$^{-1}$ region (Fig. 3). Extraction of walls with cold N-KOH for 1 hr, followed by 0·5 N-KOH at 100° for 30 min., improved the resemblance slightly; minor peaks at 1510–1540 cm.$^{-1}$ (amines and/or aromatics) disappeared, and other small peaks were enhanced. The treatment removed most of the protein and lipid, leaving a wall composed of glucan almost entirely. Infrared spectra of Phytophthora walls showed a much greater similarity to the spectra of amorphous cellulose (cf. O'Connor, DuPre & McCall, 1957) than to that of cellulose I.

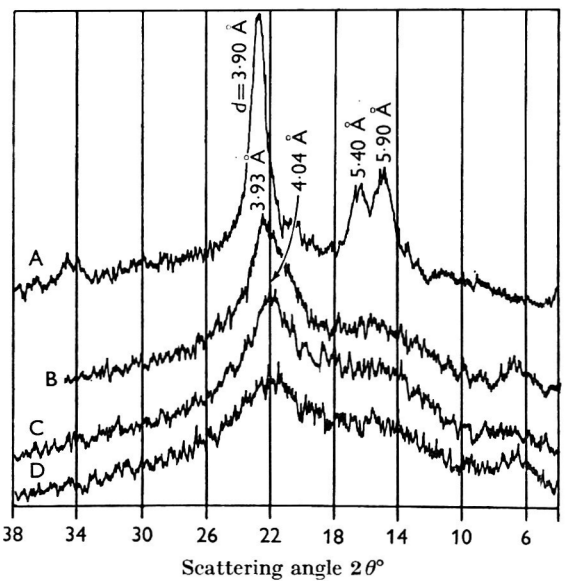


Fig. 1

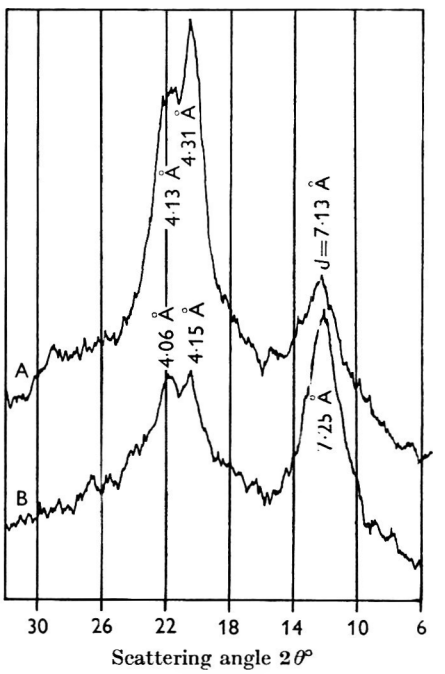


Fig. 2

Fig. 1. X-ray diagrams. A, Cellulose I; B, insoluble residue after extraction of *Phytophthora cinnamomi* hyphal walls with hot N-HCl; C, intact hyphal walls of *P. cinnamomi*; D, isolated hyphal walls of *P. parasitica*. Tracings vertically displaced for clarity.

Fig. 2. X-ray diagrams. A, Cellulose II regenerated from Whatman cellulose I; B, material regenerated from *Phytophthora cinnamomi* hyphal walls. Tracings vertically displaced for clarity.

X-ray diagrams. Intact walls showed very weak reflexions suggestive of cellulose I (Fig. 1). Reflexion sharpness did not improve after extraction with KOH as specified above for infrared spectra. Since walls thus treated consist almost entirely of glucan polymers, cellulose crystallinity in the phytophthora walls is either poor or is masked by amorphous glucans. Definite evidence for the presence of cellulose was obtained by twice extracting walls of *Phytophthora cinnamomi*

with Schweizer's cellulose solvent (Jayme & Lang, 1963) for 24 hr under N_2. Upon acidification of the solvent, a precipitate was obtained which showed X-ray spacings similar to that of cellulose II prepared from Whatman cellulose powder by identical handling (Fig. 2). There was, however, a divergency in the relative intensity of the reflexions. This discrepancy may be an artifact due to lack of randomness in the spatial orientation of cellulose crystallites in the specimen slides.

Anthrone determinations showed that only 11% of the wall regenerated as cellulose II and that 63% did not dissolve at all in Schweizer's reagent; the unaccounted glucan (14% of the wall) probably remained in solution.

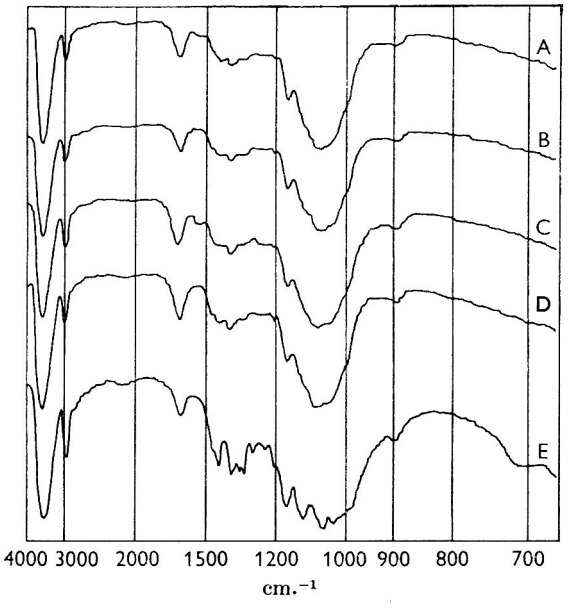


Fig. 3

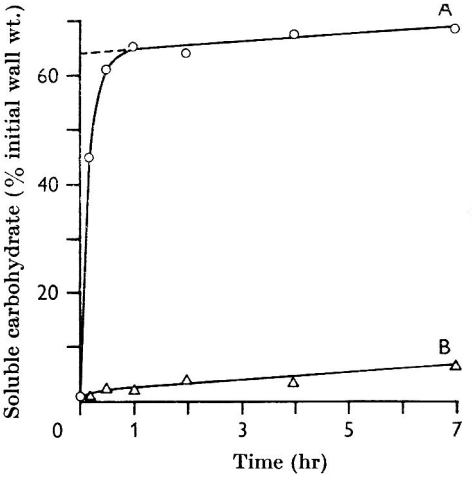


Fig. 4

Fig. 3. Infrared spectra: A, isolated hyphal walls of *Phytophthora cinnamomi*; B, as A but extracted with KOH; C, isolated hyphal walls of *P. parasitica*; D, as C but extracted with KOH; E, cellulose I. Tracings vertically displaced for clarity.

Fig. 4. Comparative rate of hydrolysis of isolated hyphal walls of *Phytophthora cinnamomi* (curve A) and Whatman cellulose powder (curve B) in N-HCl at 97°.

The single, broad, X-ray peak of negative walls (4·04 Å) approximated to the main reflexion of authentic cellulose I (3·90 Å) and disappeared upon extraction with Schweizer's solvent, leaving an isotropic glucan residue. When, on the other hand, the amorphous glucan was first removed from walls, by N-acid digestion (see below), a residue was left which exhibited a slightly more pronounced X-ray spectrum than did native walls, with its main reflexion (3·93 Å) closer to that of cellulose I. It may, therefore, be concluded that cellulose I of relative low crystallinity exists in the phytophthora walls.

Acid-extraction of wall glucans. Since hot dilute mineral acid dissolved a major portion of the glucan from hyphal walls of phytophthora (Table 5), the kinetics of hyphal wall solution of *Phytophthora cinnamomi* by N-HCl at 97° was studied. As depicted in Fig. 4, 61·2% of the wall (i.e. 70% of the total polysaccharide) dis-

solved within 30 min. Thereafter the extraction rate decreased sharply to a slow constant rate. The experiment was ended at 7 hr, by which time 69·5% of the wall had dissolved (i.e. 80% of the total polysaccharide). On the other hand, only 6·5% Whatman cellulose dissolved under the same conditions. To calculate the amount of glucan readily extracted by HCl, the upper portion of the curve corresponding to the hydrolysis of a resistant polysaccharide was extrapolated to zero time. Thus, 63% of the wall consists of a glucan readily soluble in hot dilute mineral acid. Hence, about 25% of the wall corresponded to a relatively acid-insoluble glucan whose X-ray spectrum (Fig. 1) indicated that it was partly, if not entirely, cellulose I.

The acid-soluble glucan was also rapidly hydrolysed; thus 84% of the total carbohydrate extracted by N-HCl in 30 min., was dialysable. Under less drastic conditions, using several brief extractions with hot 0·1 N-HCl, depolymerization was considerably decreased and a soluble non-dialysable glucan(s) was isolated. Preliminary measurement of optical rotation in water $[\alpha]_D^{23} - 33°$ suggests it was predominantly a β-glucan.

DISCUSSION

As in the case for the chitinous cell walls of moulds, the cellulosic hyphal walls of *Phytophthora cinnamomi* and *P. parasitica* also consist of polysaccharide, protein, and lipid; they are, however, somewhat simpler than the hyphal walls of a chitinous Phycomycete such as *Mucor rouxii*, which contains a wider variety of building blocks (Bartnicki-Garcia & Nickerson, 1962); in phytophthora, glucose polymers account for nearly 90% of the hyphal walls. As expected, cellulose was identified in the hyphal walls of Phytophthora but its exact content is not known. Qualitative evidence for cellulose includes the following observations. Release of large amounts (about 20%) of cellobiose during enzymic digestion of *P. cinnamomi* walls with a crude cellulase from Streptomyces (Bartnicki-Garcia & Lippman, to be published); very limited solubility of walls in 17·5% NaOH; partial solubility of hyphal walls in Schweizer's reagent and subsequent regeneration of a polymer with an X-ray pattern similar to cellulose II; X-ray diagrams of intact walls suggestive of cellulose I.

A quantitative measurement of cellulose would be arbitrary and largely dependent on which physical or chemical properties were used to define cellulose (Ott & Tennent, 1954). Furthermore, the notion of having true cellulose as a chemical entity separate from the other wall glucans may be artificial, for it is possible that the hyphal walls of phytophthora consist of one single glucan species with amorphous regions and a cellulose-like core. More work obviously is needed to clarify this point.

Separation of two glucan fractions from hyphal walls of *Phytophthora cinnamomi* was accomplished by treatment with hot dilute mineral acid. Thus were obtained a readily soluble, rapidly hydrolysable amorphous glucan comprising 63% of the wall, and a much more acid-resistant polymer representing 25% of the wall. It is not yet known whether the acid treatment cleaved a single native glucan into these two types. In any event, the acid-resistant glucan showed X-ray reflexions approximating that of cellulose I and may thus be considered to represent that portion of the phytophthora wall most closely resembling native crystalline cellulose of higher plants.

Release of gentiobiose and laminaribose during chemical or enzymic hydrolysis

(Bartnicki-Garcia & Lippman, to to be published) indicated the presence of β (1 → 6) and β (1 → 3) linkages and opens the possibility that two types of glucan exist in the wall—a homogenous β (1 → 4) cellulose-like polymer, and an amorphous glucan containing an as yet undetermined number of β (1 → 3) and β (1 → 6) linkages.

Although *Phytophthora cinnamomi* was examined in greater detail, the data on *P. parasitica* is sufficient to conclude that both species have a similar hyphal-wall composition. Yet, differences in certain properties such as the propensity of *P. parasitica* walls to agglutinate and the more intense staining of *P. cinnamomi* walls with Lugol's solution may correspond to significant chemical differences. Hyphal wall analyses of *P. cinnamomi* and *P. parasitica* agree with the qualitative findings of Crook & Johnston (1962) on the sugars and amino acids present in hydrolysates of *P. cactorum*, except for ribose which was only vaguely detectable in the former species.

Glucosamine, major component of most mould walls, was found in the cellulosic walls of phytophthora in such a small quantity as to question its relevance as a structural wall element. This finding agrees with Frey's (1950) failure to obtain X-ray evidence for chitin in various phytophthora cultures. Thomas (1943) claimed the presence of chitin in *Phytophthora cinnamomi* on the basis of a positive chitosan staining reaction; this conclusion was challenged by Frey (1950) and seems untenable in view of the present work. Interestingly, much earlier Dastur (1913), who also used cytochemical tests, found staining-reaction evidence for cellulose, but not for chitin, in *P. parasitica*.

Hydroxyproline is not known to occur in the walls of chitinous fungi. Its presence in fungi with cellulosic walls such as phytophthora and saprolegnia (Crook & Johnston, 1962) may have special significance since a similar concurrence is conspicuously apparent in the cell wall of higher plants, where hydroxyproline is a characteristic component (Lamport & Northcote, 1960). Although assumed to be minimal, the exact extent to which the preparative procedure altered the composition of the hyphal walls of phytophthora by addition or deletion of components is not known. Undoubtedly, the physical technique used in this study for cell-wall preparation is superior to the old method of exhaustive chemical extraction of whole organisms which only the most inert substances endured. Nevertheless, upon hyphal breakage, even though the mycelium was coenocytic, cytoplasmic contents did not flow out entirely; solid plugs remained whose dislodgement necessitated the strong action of a sonic oscillator. By this procedure, all visible cytoplasmic debris was essentially, but not entirely, eliminated. Minute residues, whose contribution to the composition of the walls has been assumed to be negligible, were occasionally seen. On the other hand, the extensive action of sonic oscillation may have selectively removed or modified some wall components to an undetermined degree.

I wish to acknowledge the excellent technical assistance of Miss Dorothy Campos and Mrs Eleanor Lippman, and the kind co-operation of Dr J. B. Mudd in the automatic analysis of amino acids. This investigation was supported in part by a research grant (AI-06205) from the National Institutes of Health, Bethesda, Maryland, U.S.A.

REFERENCES

ARONSON, J. M. & MACHLIS, L. (1959). The chemical composition of the hyphal walls of the fungus Allomyces. *Am. J. Bot.* **46**, 292.

ARONSON, J. M. & PRESTON, R. D. (1960). An electron microscopic and X-ray analysis of the walls of selected lower Phycomycetes. *Proc. R. Soc.* B, **152**, 346.

BARKER, S. A., BOURNE, E. J. & WHIFFEN, D. H. (1956). Use of infrared analysis in the determination of carbohydrate structure. *Meth. biochem. Analysis*, **3**, 213.

BARTNICKI-GARCIA, S. & NICKERSON, W. J. (1962). Isolation, composition, and structure of cell walls of filamentous and yeast-like forms of *Mucor rouxii*. *Biochim. biophys. Acta*, **58**, 102.

BARTNICKI-GARCIA, S. & REYES, E. (1964). Chemistry of spore wall differentiation in *Mucor rouxii*. *Archs Biochem. Biophys.* **108**, 125.

BITTER, T. & MUIR, H. M. (1962). A modified uronic acid carbazole reaction. *Analyt. Biochem.* **4**, 330.

CHUNG, C. W. & NICKERSON, W. J. (1954). Polysaccharide synthesis in growing yeasts. *J. biol. Chem.* **208**, 395.

CORBETT, W. M. (1963). Determination of alpha-cellulose content of cotton and wood cellulose. In *Methods in Carbohydrate Chemistry*. Ed. by R. L. Whistler. Vol. 3. p. 27. New York: Academic Press.

CROOK, E. M. & JOHNSTON, I. R. (1962). The qualitative analysis of the cell walls of selected species of fungi. *Biochem. J.* **83**, 325.

DASTUR, J. F. (1913). On *Phytophthora parasitica* nov.spec. A new disease of the castor oil plant. *Mem. Dep. Agric. India bot. ser.* **5**, 177.

DRYER, R. L., TAMMES, A. E. & ROUTH, J. I. (1957). Determination of phosphorus in body fluids. *J. biol. Chem.* **225**, 177.

FREY, R. (1950). Chitin and Zellulose in Pilzzellwänden. *Ber. schweiz. bot. Ges.* **60**, 199.

FULLER, M. S. & BARSHAD, I. (1960). Chitin and cellulose in the cell walls of *Rhizidiomyces* sp. *Am. J. Bot.* **47**, 105.

HAMILTON, P. B. & KNIGHT, S. G. (1962). An analysis of the cell walls of *Penicillium chrysogenum*. *Archs Biochem. Biophys.* **99**, 282.

HODGE, J. E. & HOFREITER, B. T. (1962). Determination of reducing sugars and carbohydrates. In *Methods in Carbohydrate Chemistry*. Ed. by R. L. Whistler & M. L. Wolfram. Vol. 1. p. 380. New York: Academic Press.

HORIKOSHI, K. & IIDA, S. (1964). Studies of the spore coats of fungi. I. Isolation and composition of the spore coats of *Aspergillus oryzae*. *Biochim. biophys. Acta*, **83**, 197.

JAYME, G. & LANG, F. (1963). Cellulose solvents. In *Methods in Carbohydrate Chemistry*, Ed. by R. L. Whistler. Vol. 3. p. 75. New York: Academic Press.

KREGER, D. R. (1954). Observations on cell walls of yeasts and some other fungi by X-ray diffraction and solubility tests. *Biochim. biophys. Acta*, **13**, 1.

LAMPORT, D. T. A. & NORTHCOTE, D. H. (1960). Hydroxyproline in primary cell walls of higher plants. *Nature, Lond.* **188**, 665.

O'CONNOR, R. T., DUPRE, E. F. & MCCALL, E. R. (1957). Infrared spectro-photometric procedure for analysis of cellulose and modified cellulose. *Analyt. Chem.* **29**, 998.

OTT, E. & TENNENT, H. G. (1954). Introduction, in *Cellulose and Cellulose Derivatives*. Ed. by E. Ott, H. M. Spurlin & M. W. Grafflin. Part I, p. 1. New York: Interscience Publishers.

POTGIETER, H. J. & ALEXANDER, M. (1965). Polysaccharide components of *Neurospora crassa* hyphal walls. *Can. J. Microbiol.* **11**, 122.

RUSSELL, D. W., STURGEON, R. J. & WARD, V. (1964). Preparation and properties of a hyphal wall fraction from *Pithomyces chartarum*. *J. gen. Microbiol.* **36**, 289.

SPACKMAN, D. H., STEIN, W. H. & MOORE, S. (1958). Automatic recording apparatus for use in the chromatography of amino acids. *Analyt. Chem.* **30**, 1190.

THOMAS, R. C. (1943). Composition of fungus hyphae. IV. Phytophthora. *Ohio J. Sci.* **43**, 135.

TRACEY, M. V. (1955). In *Modern Methods of Plant Analysis.* Ed. by K. Paech and M. V. Tracey. Vol. 2, p. 264. Berlin: Springer-Verlag.

WESSELS, J. G. H. (1965). Morphogenesis and biochemical processes in *Schizophyllum commune. Wentia*, **13**, 1.

WETTSTEIN, F. VON (1921). Das Vorkommen von Chitin und seine Verwertung als systematischphylogenetisches Merkmal im Pflanzenreich. *Sber. Akad. Wiss. Wien*, Abt. I, **130**, 3.

WILSON, C. M. (1959). Quantitative determination of sugars on paper chromatograms. *Analyt. Chem.* **31**, 1199.

J. gen. Microbiol. (1966), **42**, 71–82
Printed in Great Britain

Flagellar Synthesis in *Salmonella typhimurium*: Factors Affecting the Formation of the Flagellar ε-*N*-Methyllysine

By D. KERRIDGE

Sub-Department of Chemical Microbiology, Department of Biochemistry, Tennis Court Road, Cambridge

(*Received* 8 *July* 1965)

SUMMARY

In growing cultures of *Salmonella typhimurium*, flagellar ε-*N*-methyllysine (NML) can be isotopically labelled with either L-[Me-^{14}C]methionine or L-[U-^{14}C]lysine. This isotopic labelling of flagellar NML was unaffected by the addition of DL-NML to the incubation medium. The methionine analogues, D-methionine and DL-ethionine, competitively inhibited the incorporation of the methyl group from methionine into flagellar NML, but had no effect on the incorporation of L-[U-^{14}C]lysine. Although it is probable that methylation of lysine occurs after it has been incorporated into the flagellin molecule, it has not been possible by using antibiotics and purine and pyrimidine analogues to differentiate between flagellar synthesis and the formation of flagellar NML. Attempts to obtain a cell-free preparation capable of synthesizing NML have so far been unsuccessful.

INTRODUCTION

The presence of ε-*N*-methyllysine (NML) in flagellar protein (flagellin) of *Salmonella typhimurium* strain SW 1061 was first reported by Ambler & Rees (1959), and since then this amino acid has been found in flagellins from other serotypes of *S. typhimurium* (Stocker, McDonough & Ambler, 1961) and in calf thymus nuclear histones (Murray, 1964). The methyl donor for the synthesis of NML in both bacterial flagella (Kerridge, 1963) and nuclear histone (Murray, 1964; Allfrey, Faulkner, & Mirsky, 1964) is methionine, and it was first suggested by Stocker *et al.* (1961) that this methylation might occur after the incorporation of lysine into the protein molecule. More recently, Allfrey *et al.* (1964) have found that, although puromycin inhibits the synthesis of protein by isolated calf thymus nuclei, this antibiotic has little or no effect on the methylation of the histone lysine.

The incorporation of labelled precursors into NML of the flagellar protein by *Salmonella typhimurium* was used by Kerridge (1963) as a measure of flagellar synthesis, and further studies on the factors affecting the synthesis of flagellar NML are reported in this paper. A preliminary account of some of the results has already been given (Kerridge, 1962).

METHODS

Organisms and growth media. Salmonella typhimurium strain SW 1061, obtained from Dr Iino (National Institute of Genetics, Misimo, Shizuoko-ken, Japan), was used for the majority of the experiments reported in this paper. Other strains of

S. typhimurium used were SW 1153 (paralysed), SW 1154 (non-flagellate) and SL 871 (*his*$^-$, NML$^-$): these were obtained from Professor B. A. D. Stocker, The Lister Institute, London. The cultures were maintained on Dorset egg slopes at room temperature. For the preparation of washed suspensions the bacteria were grown in an ammonium salts medium (DO) supplemented with 0·2 % (w/v) glucose (Kerridge, 1959). After 16 hr growth at 37° the culture was diluted with 20 vol. of DO medium + 0·2 % (w/v) glucose and the incubation continued for a further 4 hr. The bacteria were harvested by centrifugation and resuspended in DO at a density of 5 mg. dry wt./ml.

The dry weights of bacteria in suspension were determined from extinction measurements made with a Hilger 'Spekker' absorptiometer and by a calibration curve prepared by drying bacterial suspensions of known extinction to constant weight at 105°.

Incorporation of ^{14}C-labelled amino acids into Salmonella typhimurium. Suspensions of *Salmonella typhimurium* (0·5 mg. dry wt./ml.) were incubated statically at 37° in DO + 0·2 % (w/v) glucose. L-[Me-^{14}C]methionine (specific activity 6·6 mC./mmole) or L-[U-^{14}C]lysine (specific activity 8·3 mC./mmole) was added to give a final concentration of 0·1 μC./ml. and the medium was supplemented with other amino acids or inhibitors as required. 5 ml. samples were taken at intervals and pipetted into centrifuge tubes containing 5 ml. of an aqueous solution of the unlabelled amino acid (100 μg./ml.). After cooling rapidly to 0°, the bacteria were harvested by centrifugation at 1300*g* for 20 min. in the cold, and resuspended in 2·5 ml. cold distilled water. The flagella were detached mechanically by treatment in a M.S.E. microhomogenizer and separated from the bacteria by centrifugation at 1300*g* for 20 min. The fractionation of the bacteria and the determination of the isotope incorporation into the various cellular fractions were done by the methods described by Kerridge (1963). The radioactivity of the flagellar NML was determined using a thin end-window gas flow counter (Nuclear Chicago Corp., 333 East Howard Avenue, Des Plaines, Illinois, U.S.A.), the efficiency of which is approximately three times that of the Panax type D 657 Scaler (Panax Equipment Ltd., Mitcham, Surrey) and associated mica end-window Gieger-Muller tube used for the assay of radioactivity in the other cellular fractions.

RESULTS

The effects of methionine, lysine and NML on the incorporation of ^{14}C-labelled amino acids by Salmonella typhimurium. The rate of incorporation of an isotopically labelled amino acid into flagella by *Salmonella typhimurium* under normal conditions of growth parallels the rate of incorporation into other cellular fractions and provides a satisfactory measure of flagellar formation. A more specific method of studying flagellar synthesis in *S. typhimurium* is to follow the incorporation of ^{14}C-labelled precursors into flagellar NML, and studies have been made on the factors affecting the labelling of flagellar NML in an attempt to differentiate between protein synthesis and NML formation.

The incorporation of L-[Me-^{14}C]methionine into general cell protein was slightly stimulated by adding lysine to the incubation medium, but there was a more marked increase in the incorporation of ^{14}C into the flagellar NML (Table 1). This was

detectable at fairly low concentrations of lysine and tended towards a plateau value as the concentration of lysine was increased.

The incorporation of ^{14}C from L-[Me-^{14}C]methionine into lipid, nucleic acid, and protein by *Salmonella typhimurium* was unaffected by the addition of NML to the incubation medium, and there was no reduction in the methyl labelling of the

Table 1. *The effect of lysine on the incorporation of* L-[*Me*-^{14}C]*methionine into Salmonella typhimurium strain SW 1061*

Suspensions of *S. typhimurium* (0·5 mg. dry wt./ml.) were incubated statically at 37° in DO medium containing glucose (0·2 %, w/v) and L-[Me-^{14}C]methionine (0·1 μC./ml.; specific activity 6·6 mC./mmole). L-Lysine was added as required. After 15 min. incubation the bacteria were harvested by centrifugation, fractionated, and the radioactivity of the various fractions determined. *a* and *b* represent separate experiments.

	L-Lysine added (μg./ml.)				
	0	10	50	100	200
	Radioactivity (counts/min./mg. bacteria)				
Expt. *a*					
Protein	3500	—	4200	4000	4540
Flagellar NML	52	124	164	148	150
Expt. *b*					
Protein	4100	4060	4900	4330	4540
Flagellar NML	230	260	310	370	320

Table 2. *The effect of NML on the incorporation of* L-[*Me*-^{14}C]*methionine into Salmonella typhimurium strain SW 1061*

Suspensions of *S. typhimurium* (0·5 mg. dry wt./ml.) were incubated statically at 37° in DO medium containing glucose (0·2 %, w/v) and L-[Me-^{14}C]methionine (0·1 μC./ml.; specific activity, 6·6 mC./mmole). DL-NML was added as required. After 10 min. incubation the bacteria were harvested by centrifugation, fractionated, and the radioactivity in the various fractions determined.

	DL-NML added (μg./ml.)			
	0	10	30	100
	Radioactivity (counts/min./mg. bacteria)			
Cell fraction				
Lipid	1870	1870	2050	2090
Nucleic acid	300	264	330	350
Protein (acid insoluble residue)	7280	7120	6750	7800
'NML'				
Flagella	276	260	314	342
Protein (acid-insoluble residue)	330	340	332	—

flagellar NML even when the molar ratio of NML/methionine was approximately 40/1 (Table 2). In the presence of exogenous NML there appeared to be no reduction in the incorporation of ^{14}C from L-[Me-^{14}C]methionine into cellular 'NML'.

L-[U-^{14}C]lysine is also incorporated into flagellin by *Salmonella typhimurium* and the label is found in both the NML and lysine residues, but it is not such a satisfactory precursor as L-[Me-^{14}C]methionine for studying formation of bacterial flagella (Kerridge, 1963). Addition of carrier-free L-[U-^{14}C]lysine to a suspension of *S. typhimurium* in the glucose ammonium salts medium resulted in an immediate

linear incorporation of the amino acid into the bacteria. After 15 min. incubation, the rate of lysine incorporation into the flagellar NML fell rapidly and after 30 min. was practically zero, but the radioactivity of the lysine in the flagellar fraction continued to rise for at least an hour (Fig. 1). This increase may have been due to

Table 3. *The effect of methionine on the incorporation of* L-[*U*-^{14}C]*lysine into Salmonella typhimurium strain* SW *1061*

Suspensions of *S. typhimurium* (0·5 mg. dry wt./ml.) were incubated statically at 37° in DO medium containing glucose (0·2 %, w/v) and L-[U-^{14}C]lysine (0·1 μC./ml.; specific activity 8·3 mC./mmole). L-Methionine was added as required. After 15 min. the bacteria were harvested by centrifugation, fractionated and the radioactivity of the various fractions determined.

	Methionine (μg./ml.)				
	0	10	50	100	200
	Radioactivity (counts/min./mg. bacteria)				
Protein fraction	17,900	13,700	19,000	19,100	17,600
Flagella					
(*a*) NML	780	870	880	880	880
(*b*) Lysine	1,535	1,430	1,540	1,740	1,430

Table 4. *The effect of NML on the incorporation of* L-[*U*-^{14}C]*lysine into Salmonella typhimurium strain* SW *1061*

Suspensions of *S. typhimurium* (0·5 mg. dry wt./ml.) were incubated statically at 37° in DO medium containing glucose (0·2 %, w/v) and L-[U-^{14}C]lysine (0·1 μC./ml.; specific activity, 8·3 mC./mmole). DL-NML was added as required. After 10 min. incubation the bacteria were harvested by centrifugation, fractionated, and the radioactivity in the various fractions determined.

	DL-NML (μg./ml.)			
	0	10	30	100
	Radioactivity (counts/min./mg. bacteria)			
Cell fraction				
Lipid	27	20	24	10
Nucleic acid	515	625	520	350
Protein (acid-insoluble residue)	12,300	13,950	13,700	12,200
'NML'				
Flagella	140	144	148	132
Protein (acid-insoluble residue)	212	264	140	140

the presence of proteins other than flagellin in this fraction, probably 'released' from the bacterial cells. The presence of methionine in the medium had little or no effect on the incorporation of L-[U-^{14}C]lysine into flagellar NML and lysine (Table 3). The incorporation of L-[U-^{14}C]lysine into flagellar NML and lysine was unaffected by the addition of NML to the incubation medium, but there was some reduction in the isotopic labelling of the cellular 'NML' (Table 4).

The incorporation of precursors into NML by Salmonella strains other than SW *1061*

NML is not a constituent of all Salmonella flagellins, and methionine incorporation has been studied in a number of strains of *Salmonella typhimurium* which either do not have this amino acid in their flagella or are non-motile as a result of 'paralysis'

or absence of flagella. The paralysed strain sw 1153 behaved almost identically to the wild-type organism (strain sw 1061) and the ^{14}C-labelled methyl group from methionine was incorporated into the flagellar NML after a short lag (Fig. 2). The isotope was also incorporated into a constituent of the residual protein fraction which behaved electrophoretically like NML. *S. typhimurium* strain SL 871, a strain having no NML in its flagellin, and the flagella-less strain sw 1154 behaved exactly as expected, and no radioactivity was detected in the NML region after electrophoresis of an acid hydrolysate of the 'flagellar fractions'. These results demonstrated satisfactorily that other cellular constituents did not contribute significantly to the radioactivity of the NML in the flagellar fraction. In both SL 871 and sw 1154 some radioactivity was detected in the NML region after electrophoresis of an acid hydrolysate of the residual protein.

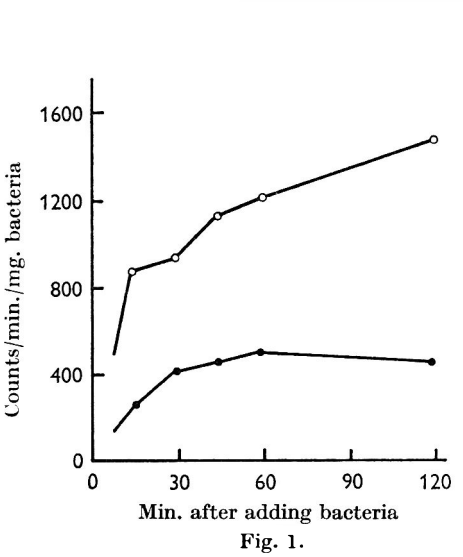


Fig. 1.

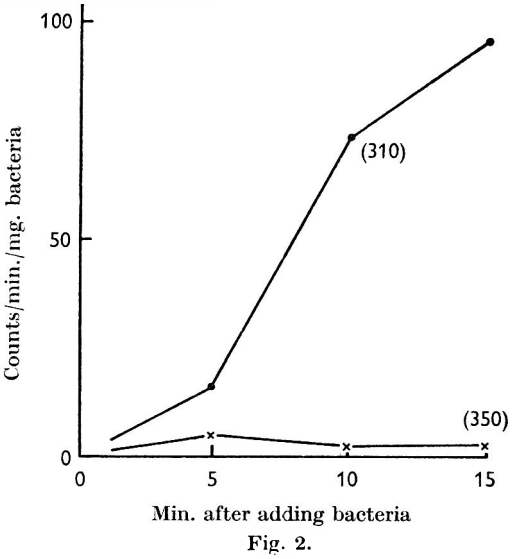


Fig. 2.

Fig. 1. The incorporation of L-[U-^{14}C]lysine into flagellar protein by *Salmonella typhimurium*, strain sw 1061. A suspension of *S. typhimurium* (0·5 mg. dry wt./ml.) was incubated in DO medium containing glucose (0·2 %, w/v) and L-[U-^{14}C]lysine (0·1 μC./ml.; specific activity 8·3 μmC./mmole). Samples were taken at intervals and the radioactivity of the flagellar NML and lysine determined. ●—●, NML; ○—○, lysine.

Fig. 2. The incorporation of L-[Me-^{14}C]methionine into non-motile strains of *Salmonella typhimurium*. Suspensions of strains sw 1153 (*mot*$^-$) and sw 1154 (*fla*$^-$) (0·5 mg. dry wt./ml.) were incubated in DO medium containing glucose (0·2 %, w/v) and L-[Me-^{14}C]-methionine (0·1 μC./ml.; specific activity 6·6 mC./mmole). Samples were taken at intervals, the bacteria harvested by centrifugation, fractionated, and the radioactivity of the various cellular fractions determined. ●—●, Flagellar NML, sw 1153; × — ×, 'flagellar NML', sw 1154. The figures in parentheses give the radioactivity of the 'NML' in the residual protein fraction at the time indicated.

The effects of methionine analogues on the incorporation of L-[*Me*-^{14}*C*]*methionine and* L-[*U*-^{14}*C*]*lysine into Salmonella typhimurium.* The methionine analogues, D-methionine, methionine sulphone, methionine sulphoxide and ethionine, did not prevent bacterial growth in defined media or the regeneration of flagella by previously deflagellated bacteria. However, these analogues decreased the incorporation of isotope from L-[Me-^{14}C]methionine into *S. typhimurium* (Figs. 3, 4) and there was

no obvious differential effect on the methylation of the flagellar lysine and the incorporation of methionine into the residual protein fraction. D-Methionine and DL-ethionine were shown to inhibit competitively both the incorporation of methionine into the residual protein fraction and the methylation of the flagellar lysine.

D-Methionine and DL-ethionine had no effect on the incorporation of L-[U-^{14}C]-lysine into either the residual protein fraction or into flagellar NML (Table 5) so, although these analogues prevent the incorporation of the methyl group from exogenous methionine into the flagellar NML, they do not prevent the synthesis of this compound by *Salmonella typhimurium*.

The effects of antibiotics on the synthesis of flagellar NML. If methylation of lysine occurs after the amino acid has been incorporated into the flagellin molecule, then antibiotics known to inhibit protein synthesis might have a differential effect on the methylation of lysine and on the incorporation of methionine into cell protein.

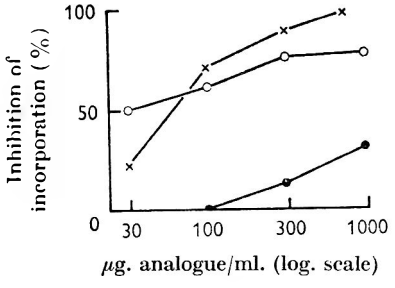


Fig. 3*a*.

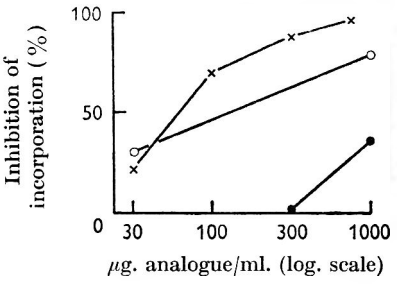


Fig. 3*b*.

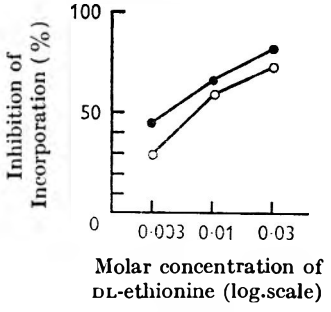


Fig. 4.

Fig. 3. The effect of methionine analogues on the incorporation of L-[Me-^{14}C]methionine into *Salmonella typhimurium*. Suspensions of *S. typhimurium* (0·5 mg. dry wt./ml.) were incubated at 37° in DO medium containing glucose (0·2 %, w/v) and L-[Me-^{14}C]methionine. The methionine analogues were added as required. After 15 min. the bacteria were harvested by centrifugation, fractionated, and the radioactivity in the various fractions determined. (*a*) Incorporation into the acid-insoluble material (protein). (*b*) Incorporation into flagellar NML. ×—×, D-methionine; ●—●, DL-methionine sulphone; ○—○, DL-methionine sulphoxide.

Fig. 4. The effect of DL-ethionine on the incorporation of L-[Me-^{14}C]methionine into *Salmonella typhimurium*. Suspensions of *S. typhimurium* strain SW 1061 were incubated in DO medium containing glucose (0·2 %, w/v) and L-[Me-^{14}C]methionine (0·1 μC./ml.; specific activity 6·6 mC/mmole). Ethionine was added as required. After 15 min. the bacteria were harvested by centrifugation, fractionated, and the radioactivity of the various fractions determined. ○—○, flagellar NML; ●—●, residual protein fraction.

Allfrey *et al.* (1964) were able to demonstrate such an effect with puromycin on the methylation of lysine residues in histones and the synthesis of protein by isolated calf thymus nuclei. Similar experiments were therefore carried out with *Salmonella typhimurium*. At the minimum growth inhibitory concentrations the antibiotics chloramphenicol, terramycin, and puromycin inhibited the regeneration of flagella by deflagellated bacteria (Table 6), and so smaller concentrations were used in attempts to show a differential effect on the methylation of lysine and the synthesis of protein. The effects of the antibiotics on the incorporation of ^{14}C from L-[Me-^{14}C]-methionine into nucleic acid and protein were in good agreement with the results of other workers (see Gale, 1963). At all concentrations of the antibiotics tested,

the inhibition of methyl labelling of flagellar NML paralleled the inhibition of ^{14}C-incorporation into the residual protein fractions, and no differential effects were detected (Table 7). The incorporation of methyl C into the material present in the residual protein fraction which behaved electrophoretically as NML was

Table 5. *The effects of* DL-*ethionine and* D-*methionine on the incorporation of* L-[*U*-^{14}*C*]*lysine into Salmonella typhimurium strain sw 1061*

Suspensions of *S. typhimurium* (0·5 mg./ml.) were incubated statically at 37° in DO medium containing glucose (0·2 %, w/v) and L-[U-^{14}C]lysine (0·1 μC./ml.; specific activity 8·3 mC./mmole). DL-Ethionine or D-methionine was added as required. After 10 min. incubation at 37° the bacteria were harvested by centrifugation, fractionated, and the radioactivity of the various fractions determined.

	Experiment 1		Experiment 2	
	Control	+ 100 μg. DL-ethionine/ml.	Control	+ 1 mg. D-methionine/ml.
Cell fraction				
Lipid	15	30	130	40
Nucleic acid	300	310	680	490
Protein (acid-insoluble residue)	12,900	13,300	22,500	22,600
'NML'				
Flagella	450	435	570	660
Acid-insoluble residue	530	280	370	405

Table 6. *The effects of inhibitors on the regeneration of flagella by Salmonella typhimurium strain sw 1061*

Suspensions of mechanically deflagellated bacteria (0·2 mg. dry wt./ml.) were incubated statically at 37° in DO medium containing glucose (0·2 %, w/v) and Difco vitamin-free casamino acids (0·2 %, w/v). The inhibitors were added as required. The regeneration was followed microscopically by motility determinations and flagella staining (Kerridge, 1959).

	Regeneration after 1 hr. incubation	
	Motility (%)	Flagellated bacteria (%)
Control	90	90
Puromycin, 10 μg./ml.	70	70
Puromycin, 30 μg./ml.	10	10
Puromycin, 100 μg./ml.	1	Very few short stumps of flagella
Terramycin, 0·3 μg./ml.	1	A few very short stumps
Terramycin, 1·0 μg./ml.	1	A few very short stumps
Terramycin, 3 μg./ml.	1	A few very short stumps
6-Thioguanine, 10 μg./ml.	90	90
6-Thioguanine, 100 μg./ml.	90	90

apparently not as sensitive to inhibition by chloramphenicol as the methyl labelling of the flagellar NML; for example, at 10 μg. chloramphenicol/ml. the methyl labelling of the flagellar NML was 5 % of the control value, whereas that of the residual protein 'NML' was 30 % and the incorporation of ^{14}C into the residual protein fraction was 8 %.

Dr R. J. Martinez, in an unpublished symposium, said that flagella synthesis in *Spirillum serpens* can occur in the presence of growth-inhibiting concentrations of chloramphenicol, suggesting that there may be an intermediary pool of

flagellin molecules in this organism. Previous results suggest that if such a pool of flagellin molecules occurs in *Salmonella typhimurium* it must be small (Kerridge, 1963). The addition of chloramphenicol to a culture of *S. typhimurium* incubated in the presence of L-[Me-^{14}C]methionine immediately inhibited ^{14}C incorporation into flagellar NML (Fig. 5). This and the earlier results support the idea that *S. typhimurium* unlike *S. serpens* does not have a large pool of flagellin molecules that can be used for flagellar synthesis.

Table 7. *The effects of antibiotics on the incorporation of* L-[*Me*-^{14}*C*]*methionine into Salmonella typhimurium strain* SW *1061*

Suspensions of *S. typhimurium* (0·5 mg./ml.) were incubated in DO medium containing glucose (0·2 %, w/v) and L-[Me-^{14}C]methionine (0·1 μC./ml.; specific activity 6·6 mC./mmole). The antibiotics were added as required. After 10 min. at 37° the bacteria were harvested by centrifugation, fractionated, and the radioactivity in the various fractions determined.

	Radioactivity (counts/min./mg. bacteria)			
	Chloramphenicol (μg./ml.)			
	0	1	3	10
Fraction				
Lipid	1230	1180	1380	1320
Nucleic acid	380	350	310	280
Protein (acid-insoluble material)	7400	2670	1210	610
'NML'				
Flagella	310	60	19	4
Residual protein	150	—	—	46
	Puromycin (μg./ml.)			
	0	10	30	100
Lipid	720	760	720	680
Nucleic acid	320	380	310	300
Protein (acid-insoluble residue)	5180	6700	4900	2420
'NML'				
Flagella	190	170	67	22
	Terramycin (μg./ml.)			
	0	0·03	0·1	0·3
Lipid	1390	1240	1920	2350
Nucleic acid	330	325	293	240
Protein (acid-insoluble residue)	6200	3170	2030	1350
'NML'				
Flagella	93	30	21	24
Acid-insoluble residue	76	56	28	40

If the methylation of lysine in *Salmonella typhimurium* occurs before the amino acid is incorporated into the flagellin molecule it should be possible to detect NML in the low molecular weight fraction of the bacteria. So far this has not proved possible, and even under conditions where protein synthesis was inhibited by the addition of antibiotics, ^{14}C-methyl labelled NML could not be detected in either the culture supernatant fluid or in the low molecular weight fraction of the bacteria.

Actinomycin D inhibits RNA synthesis *in vivo* in certain Gram-positive bacteria, but not in *Escherichia coli* (Kirk, 1960; Hurwitz, Furth, Malamy & Alexander, 1962), but it has recently been reported that pre-treatment of *E. coli* with 10^{-3} M

ethylenediaminetetraacetic acid (EDTA) at pH 8·0 renders the bacteria completely sensitive to the inhibitory action of actinomycin D (Leive, 1965). *Salmonella typhimurium* strain sw 1061 was unaffected by incubation in tris + HCl buffer (pH 8·0) containing EDTA (10^{-2} M) for 2 min., and the subsequent addition of 50 μg. actinomycin D had no effect on the rates of incorporation of either L-[U-^{14}C]leucine or [8-^{14}C]adenine into the acid-insoluble material. Actinomycin could therefore not be used to study the stability of the messenger-RNA involved in the synthesis of flagella by *S. typhimurium* strain sw 1061.

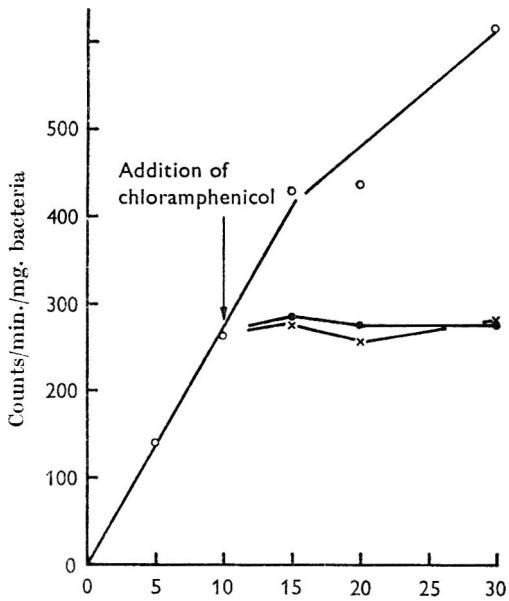


Fig. 5.

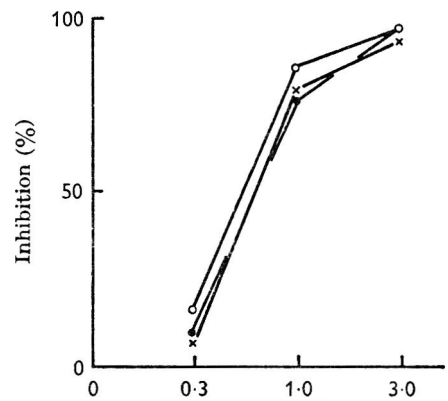


Fig. 6.

Fig. 5. The effect of chloramphenicol on methyl labelling of flagellar NML. A suspension of *Salmonella typhimurium* strain sw 1061 (0·5 mg. dry wt./ml.) was incubated in DO medium containing glucose (0·2 %, w/v) and L-[Me-^{14}C]methionine (0·1 μC./ml.; specific activity, 6·6 mC./mmole). After 10 min. incubation at 37° samples were pipetted into conical flasks containing chloramphenicol and incubation continued. The cultures were sampled at intervals and the radioactivity of the flagellar NML determined. O—O, Control; ●—●, with 1 μg./ml. chloramphenicol; ×—×, with 10 μg./ml. chloramphenicol.

Fig. 6. The effect of phenol on the incorporation of L-[Me-^{14}C]methionine into *Salmonella typhimurium* strain sw 1061. Suspensions containing 0·5 mg. dry wt./ml. were incubated at 37° in DO medium supplemented with glucose (0·2 %, w/v) and L-[Me-^{14}C]-methionine. Phenol was added as required. After 15 min. incubation the bacteria were harvested by centrifugation, fractionated, and the radioactivity of the various fractions determined. ●—●, Residual protein; ×—×, residual protein 'NML'; O—O, flagellar NML.

The effects of inhibitors other than antibiotics on the synthesis of flagellar NML. For many years the addition of phenol (0·1 %, w/v) to solid media has been used to obtain non-flagellated bacteria (see, for example, Kramer & Koch, 1931), and recently Iino & Mitani (1962) suggested that phenol inhibits the multiplication of the 'flagella-forming apparatus' but not the formation of flagellar protein. Experi-

ments have been carried out to see if there is a differential effect on the synthesis of flagellar NML and growth in *Salmonella typhimurium* (Fig. 6). There was no obvious differential inhibition by phenol of the incorporation of ^{14}C into flagellar NML and into general cell protein during incubation for 15 min. This result is quite consistent with those of Iino & Mitani, and would suggest that phenol does not immediately inhibit the functioning of the existing 'flagella-forming apparatus'. There is no evidence as to the nature of this apparatus, which could be an integral part of the 'cell wall, membrane' complex.

Table 8. *The effects of analogues of purines and pyrimidines on the incorporation of* L-[*Me*-^{14}C]*methionine into Salmonella typhimurium strain sw 1061*

Suspensions containing 0·5 mg. dry wt./ml. were incubated statically at 37° in DO medium containing glucose (0·2 %, w/v) and L-[Me-^{14}C]methionine (0·1 μC./ml.; specific activity 6·6 mC./mmole). The analogues were added as required. After 10 min. the bacteria were harvested by centrifugation, and the radioactivity of the various fractions determined.

	Radioactivity (counts/min./mg. dry wt. bacteria)	
	Cell protein	Flagellar NML
Control	3960	145
Thiouracil (3×10^{-3} M)	2450	135
5-Flurouracil ($3{\cdot}8 \times 10^{-4}$ M)	3260	138
6-Azauracil ($2{\cdot}7 \times 10^{-4}$ M)	3600	121
6-Azauracil ($8{\cdot}8 \times 10^{-4}$ M)	2370	96
FDUR (10^{-5} M)	3660	130
8-Azaguanine (10^{-3} M)	3240	132
6-Thioguanine (6×10^{-4} M)	3800	155

FDUR = fluorodeoxyuridine.

A number of purine and pyrimidine analogues are known not to affect the synthesis of existing flagella by *Salmonella typhimurium*, but certain of them, for example 8-azaguanine, apparently prevent the formation of new flagella (Kerridge, 1963). However, Mandel, Latimer & Riis (1965) reported that growth of *Bacillus cereus* in the presence of 6-thioguanine resulted in the production of non-flagellated bacteria, and they suggested that the analogue preferentially inhibited the formation of flagella. In *S. typhimurium* strain sw 1061 6-thioguanine at $0{\cdot}6 \times 10^{-3}$ M did not prevent the regeneration of functional flagella after mechanical removal (Table 6), and growth in the presence of the analogue did not produce a predominantly non-flagellated culture. Thioguanine and other analogues were tested for their effects on the incorporation of L-[Me-^{14}C]methionine into *S. typhimurium* (Table 8). Even at the highest concentrations used there was incomplete inhibition of incorporation of ^{14}C into the residual protein fraction, and none of the analogues showed any marked differential effect on the methyl labelling of flagellar NML and the incorporation of ^{14}C into the residual protein fraction during the incubation for 10 min.

Synthesis of NML by cell-free extracts of Salmonella typhimurium. Suspensions were disrupted using either the ultrasonic disintegrator or the French pressure cell, and the broken bacteria fractionated by centrifugation (Kerridge, 1963). S-Adenosyl

methionine or L-[Me-^{14}C]methionine with ATP and an energy generating system were used as the methyl donors and lysine or flagellin from *S. typhimurium* strain SL 871 (NML$^-$) were used as methyl acceptors, but in no case was it possible to show any synthesis of NML.

DISCUSSION

These results confirm the earlier finding that in *Salmonella typhimurium* the incorporation of the methyl group from L-[Me-^{14}C]methionine into flagellar NML is a satisfactory method of following the synthesis of flagella in this organism. The increased incorporation of ^{14}C into flagellar NML following the addition of lysine to the incubation medium was a little surprising. Exogenously added lysine is unlikely to alter the NML content of the flagellin and one possible explanation is that the cells can synthesize more flagellin in the presence of lysine. The number of flagella per bacterium does depend to a large extent on the complexity of the growth medium (Stocker & Campbell, 1959; Kerridge, 1959) and in a minimal medium the rate of lysine synthesis, although sufficient to support formation of essential proteins, may possibly be limiting for the formation of the non-essential flagella. Exogenous lysine would overcome this limitation on flagella synthesis.

The methylation of 'flagellar' lysine by exogenously added methionine was not separated from the synthesis of flagellin molecules except by the addition of methionine analogues to the incubation medium. These did not themselves inhibit the synthesis of flagellar NML, since L-[U-^{14}C]lysine was still incorporated into flagellar NML in their presence. If the methylation of lysine in flagellin is specific and the ratio lysine:NML is constant for this protein, it is not surprising that inhibitors of protein synthesis have a similar effect on the incorporation of Me-^{14}C into flagellar NML. The effect on the 'NML' present in the residual protein fraction was not so clear-cut, and in the presence of inhibitors of protein synthesis this methylation was perhaps not inhibited to the same extent as the methylation of flagellar NML, a result in agreement with the findings of Allfrey *et al.* (1964) for the methylation of calf thymus histones. However, the reduction in labelling of the residual protein 'NML' by exogenous NML conflicts with this interpretation, and further studies are needed on the localization and formation of this material.

The purine and pyrimidine analogues tested had no obvious effects on the methylation of lysine by *Salmonella typhimurium*, but, as the incorporation of ^{14}C into flagellar NML was followed for only 15 min., any long-term effects which might result from the incorporation of these compounds into nucleic acid would not be detected. The absence of a differential effect of thioguanine on flagella formation by *S. typhimurium*, similar to that found in *Bacillus cereus* by Mandel *et al.* (1965), might well be due to the different bacteria studied.

The stage in the synthesis of flagella at which methylation of lysine occurs has still to be determined. The failure to detect NML in the low molecular weight fraction of *Salmonella typhimurium* and the absence of any effect of exogenously added NML on the incorporation of either L-[Me-^{14}C]methionine or L-[U-^{14}C]lysine into flagellar NML are further evidence in favour of methylation occurring after the lysine has been incorporated into flagellin. Formation of flagellar NML does not occur in the presence of agents known to inhibit protein synthesis in *S. typhimurium*, but this cannot be considered as evidence for NML being incorporated as

such into the flagellin molecule. Until a satisfactory system for the cell-free methylation of lysine is developed, our information on the stage in the formation of flagella at which lysine is methylated must of necessity be circumstantial. Cell-free methylating systems have been developed for the methylation of individual amino acids (Blumenstein & Williams, 1963), polynucleotides (Fleissner & Borek, 1963) and nuclear histone (Allfrey *et al.* 1964), and unless the structural integrity of the cell is necessary for the synthesis of flagellar NML by *S. typhimurium* it should be possible to obtain an enzyme system from this organism capable of methylating lysine.

The author wishes to thank Dr L. M. Babineau for a gift of ϵ-*N*-methyllysine, Miss P. Furber for skilled technical assistance, and Professor E. F. Gale, F.R.S., for his continued interest in this work.

REFERENCES

ALLFREY, V. G., FAULKNER, R. & MIRSKY, A. E. (1964). Acetylation and methylation of histones and their possible role in the regulation of RNA synthesis. *Proc. natn. Acad. Sci. U.S.A.* **51**, 789.

AMBLER, R. P. & REES, M. W. (1959). ϵ-*N*-methyl-lysine in bacteria flagellar protein. *Nature, Lond.* **184**, 56.

BLUMENSTEIN, J. & WILLIAMS, G. R. (1963). Glycine methyltransferase. *Can. J. Biochem. Physiol.* **41**, 201.

FLEISSNER, E. & BOREK, E. (1963). Studies on the enzymatic methylation of soluble RNA. I. Methylation of the s-RNA polymer. *Biochemistry, N.Y.* **2**, 1093.

GALE, E. F. (1963). Mechanisms of antibiotic action. *Pharm. Rev.* **15**, 481.

HURWITZ, J., FURTH, J. S., MALAMY, M. & ALEXANDER, M. (1962). The role of deoxyribonucleic acid in ribonucleic acid synthesis. III. The inhibition of the enzymatic synthesis of ribonucleic acid and deoxyribonucleic acid by Actinomycin D and proflavin. *Proc. natn. Acad. Sci. U.S.A.* **48**, 1222.

IINO, T. & MITANI, M. (1962). Effect of phenol on the flagellation of Salmonella cell. *Rep. natn. Inst. Genet., Misima*, **13**, 74.

KERRIDGE, D. (1959). Synthesis of flagella by amino acid requiring mutants of *Salmonella typhimurium*. *J. gen. Microbiol.* **21**, 168.

KERRIDGE, D. (1962). The incorporation of ^{14}C labelled methionine into bacterial flagella. *J. gen. Microbiol.* **31**, xxiv.

KERRIDGE, D. (1963). Flagellar synthesis in *Salmonella typhimurium*. The incorporation of isotopically labelled amino acids into flagellin. *J. gen. Microbiol.* **33**, 63.

KIRK, J. M. (1960). The mode of action of Actinomycin D. *Biochim. biophys. Acta*, **42**, 167.

KRAMER, E. & KOCH, F. E. (1931). Hemmung des Schwärmens der Proteuskolonien durch Chloralhydrat, verglichen mit der Hemmung durch Dextrose, 42° -Züchtung, Phenol, Natrium-chloride, Wismut, Esbach Reagenz, Malachitgrün, Galle und Kupfersulphat. *Zentbl. Bakt. (Orig. Abt.* 1), **120**, 452.

LEIVE, L. (1965). Actinomycin sensitivity in *Escherichia coli* produced by EDTA. *Biochem. biophys. Res. Com.* **18**, 13.

MANDEL, H. G., LATIMER, R. G. & RIIS, M. (1965). The actions of thioguanine in *Bacillus cereus*. *Biochem. Pharmacol.* **14**, 661.

MURRAY, K. (1964). The occurrence of ϵ-*N*-methyllysine in histones. *Biochemistry, N.Y.* **3**, 10.

STOCKER, B. A. D. & CAMPBELL, J. C. (1959). The effect of non-lethal deflagellation on bacterial motility and observations on flagellar regeneration. *J. gen. Microbiol.* **20**, 670.

STOCKER, B. A. D., MCDONOUGH, M. W. & AMBLER, R. P. (1961). A gene determining presence or absence of ϵ-*N*-methyllysine in Salmonella flagellar protein. *Nature, Lond.* **189**, 556.

J. gen. Microbiol. (1966), **42**, 83–92
With 1 *plate*
Printed in Great Britain

Characteristics of Cell Walls from Morphological Variants of *Escherichia coli*

By G. WEINBAUM

Research Laboratories, Department of Biochemistry, Albert Einstein Medical Centre, Philadelphia, Pennsylvania, U.S.A.

(*Received* 12 *July* 1965)

SUMMARY

Two strains (E-26 and B) of *Escherichia coli* were examined for their growth response in a medium containing 0·75% (w/v) NaCl, 1% (w/v) L-lysine hydrochloride, 2% (w/v) glucose, 2·4% (w/v) nutrient broth, and 5% (w/v) casein hydrolysate. Strain E-26 grew as a rod which was slightly larger than when it was grown in a defined medium. Strain B grew as a filamentous and branched form; while in defined medium it grew as a rod of normal dimensions. Cell walls were isolated from both strains. Chemical analysis showed that the filamentous and branched forms had one-third the amount of hexosamine and one-fifth the amount of reducing sugar as compared with the morphologically normal form. The abnormal cell walls were more susceptible to trypsin action, more resistant to lysozyme action and more completely disaggregated by sodium dodecyl sulphate than the walls from the morphologically normal organisms.

INTRODUCTION

Many organisms exhibit morphological variation under a variety of conditions. It has been suggested (Hughes, 1956) that there are three main classes of agents which can cause aberrant long forms to appear in culture: (1) certain poisons, including antibiotics, (2) starvation, and (3) physical traumata. *Mycobacterium acapulcensis* grows as filamentous and globular forms in the presence of D-cycloserine (Mora & Bojalil, 1965). Hughes (1956) has shown that low concentrations of penicillin can cause long forms of *Proteus vulgaris* to predominate in the culture. Other bacteriostatic agents such as 6-diazo-5-oxo-L-norleucine (Coggin & Martin, 1965) or penicillin (Bartmann & Höpken, 1956) can induce aberrant forms in *Escherichia coli*.

Holden & Holman (1957) have shown by omitting pyridoxamine from cultures of *Lactobacillus arabinosus* that removal of an essential nutrient from the medium can cause aberrant forms. It was also reported that *L. delbrueckii* elongated abnormally when vitamin B_{12} was eliminated from the medium (Kitahara & Kusaka, 1959).

Anaerobiosis can cause 'thread' formation in *Escherichia coli* (Hughes, 1953), and ZoBell & Cobet (1964) demonstrated that increased hydrostatic pressure (200–500 atm.) caused filament formation in three strains of the same organism. Adler & Hardigree (1964) showed that another physical trauma, exposure to ultraviolet radiation, caused non-septate filaments in *E. coli* K-12. A culture of *E. coli* resistant to gamma-irradiation was isolated, and a most striking change was observed in its gross morphology (Pontefract & Thatcher, 1965). There developed, as a permanent

feature of the culture, long filamentous organisms with formation of vesicles or 'buds' from the sides or ends. It was suggested that these swellings were due to physical weaknesses in the cell wall, or that these 'buds' were abortive attempts at forming daughter organisms tangential to the parent.

Most investigators make use of some bacteriostatic agent to induce morphological variation. Recently, we reported a series of experiments which indicated that *Escherichia coli* B could be induced to form filamentous and branched forms in a medium devoid of any known bacteriostatic agent (Weinbaum, 1965). The results of these experiments can be briefly summarized as follows: (1) Three strains of *E. coli* (E-26, K-12, and B) were morphologically similar when grown in a minimal salts+glucose medium. However, when the three strains were grown in a medium containing 0·75 % (w/v) NaCl, 1 % (w/v) L-lysine hydrochloride, 2 % (w/v) glucose, 2·4 % (w/v) nutrient broth, and 5 % (w/v) Hycase (Sheffield Chemical Co., Norwich, N.Y.) they each showed a different morphological response. Strain E-26 retained its appearance as a normal short rod; strain K-12 appeared much larger with many organisms appearing branched or triangular shaped. Strain B showed greater morphological variation; most organisms were filamentous and much wider than strain B grown in defined medium. Many of the organisms were also branched, and some were amorphous or spheroplast like. (2) The morphological variants of strain B could be continually propagated as filaments and branched forms by repeated serial subculturing in the Hycase-containing medium. However, within 5 hr after the abnormal form of strain B was diluted 1/100 into 1 % (w/v) nutrient broth, the majority of the organisms developed a normal short rod appearance. Therefore, the change in structure of strain B was environment-dependent, and was readily reversible as soon as the environment was changed. (3) It is known that magnesium deficiency causes filamentous growth in *E. coli* (Brock, 1962), consequently the Hycase-containing medium was supplemented with Mg^{2+}, Ca^{2+} and/or PO_4^{3-}. However, these additions had no effect on strain B filament formation. Other modifications of the medium showed that filament formation was induced by the casein hydrolysate (either Hycase or enzymically hydrolysed casein) and both L-lysine and nutrient broth were required to induce branching.

It was of interest, therefore, to examine the chemical composition of isolated cell walls of normal and aberrant forms of *Escherichia coli* for compositional differences to note whether the aberrant forms were due to aberrant wall synthesis, as opposed to inhibition of cell division by unknown mechanisms. Part of these results have been reported in preliminary form (Weinbaum & Fischman, 1965).

METHODS

Organisms. Two strains of wild-type *Escherichia coli* were used in this study. Strains B and E-26 were generously supplied by Dr M. F. Mallette, Department of Biochemistry, Pennsylvania State University, and Dr H. C. Reeves, Department of Biochemistry, Albert Einstein Medical Centre, respectively. The cultures were maintained on slants of trypticase soy agar and subcultured weekly. Stock cultures were stored at 4°.

Conditions for growth and harvest. Cultures were loop inoculated from slants into 20 ml. of a minimal salts-glucose defined medium containing 0·4 % (w/v)

glucose, 0·05% (w/v) NaCl, 0·041% (w/v) $MgSO_4.7H_2O$, 0·1% (w/v) NH_4Cl, 0·6% (w/v) Na_2HPO_4 and 0·3% (w/v) KH_2PO_4 (Weinbaum & Mallette, 1959) in a 125 ml. flask and shaken vigorously at 36° overnight. A 1/400 dilution of this culture was made into inducing (Hycase) medium containing 0·75% (w/v) NaCl, 1% (w/v) L-lysine hydrochloride, 2% (w/v) glucose, 2·4% (w/v) nutrient broth (Difco), and 5% (w/v) casein hydrolysate (Weinbaum, 1965) and the organisms were again allowed to grow aerobically at 36°. Within 6 hr the morphological variants of strain B appeared, and the culture continued to grow for at least 16 hr. The organisms were harvested by centrifugation.

Isolation of cell walls. Cell walls from strains E-26 and B were isolated by vigorous shaking with glass beads according to the method of Shockman, Kolb & Toennies (1957). Packed bacterial paste was suspended to a concentration of 10% (w/v) in either water or 0·4% (w/v) sodium dodecyl sulphate (SDS) and 10 times the bacterial wet weight of glass beads (HD-E, 0·2 mm., Prismo Potters, Huntingdon, Pa.) was added. The shaker head on the International centrifuge was operated at −10° and 1900 rev./min. and complete breakage was obtained after four 5-min. runs. The cell walls were isolated by differential centrifugation and were washed 8–10 times with glass-distilled water. The SDS was initially used to inhibit any lytic enzymes (Weidel, Frank & Leutgeb, 1963) but, since similar results were obtained by breakage in water, the SDS was usually omitted.

Enzyme treatment of cell walls. Lysozyme treatment of purified cell walls was performed according to the method of Repaske (1958) using 50 μg./ml. lyzozyme and 1×10^{-3}M-EDTA. Changes in turbidity were measured using a Cary Model 14 recording spectrophotometer at 570 mμ (Hirs, 1955). Trypsin treatment of purified cell walls was performed with 0·005% (w/v) trypsin previously stabilized with 1×10^{-3}M-Ca^{2+} (Bailey, 1962). Changes in turbidity were determined at 570 mμ.

Analytical methods. The purified cell walls were disaggregated with various concentrations of sodium dodecyl sulphate (pH 7·0) (Shafa & Salton, 1960) and the percentage decrease in turbidity at 570 mμ recorded after a 10 min. treatment with the detergent. Quantitative determination of cell-wall yields was accomplished by drying samples of cell-wall suspensions to constant weight using an infra-red heat lamp, and weighing on tared planchets. Each determination was prepared in triplicate. Total reducing sugar content was measured by the anthrone procedure of Scott & Melvin (1953) with glucose as the standard. Hexosamines were determined by two procedures, the indole-HCl procedure of Dische & Borenfreund (1950) and the Elson-Morgan procedure (Winzler, 1955) using glucosamine as the standard. Two procedures were used since certain hydrolysis products react with one but not the other technique (Dische, 1955). Protein was determined by the method of Lowry, Rosebrough, Farr & Randall (1951) with a standard curve obtained for human serum albumin. All results given are averages of at least three determinations. The reduced NAD oxidase activity of purified cell walls or dialysed supernatants was assayed at pH 6·8 (0·05M-phosphate buffer) by measuring the decrease in extinction at 340 mμ with a recording spectrophotometer. The initial slope was used to give a measure of reaction rate.

RESULTS

Modification of inducing medium

During an earlier investigation of the induction of aberrant cell formation in *Escherichia coli* B (Weinbaum, 1965), it was found that acid or enzymically hydrolysed casein caused filament formation when present at concentrations above 3% (w/v). Modification of the inducing medium by substituting other protein extracts or hydrolysates gave variable results. The aberrant organisms seen in Pl. 1, figs. 1–3 were induced to form filamentous and branched forms in medium in which Hycase was replaced by the same concentration of casitone (Difco, a vitamin-free peptone) or yeast extract (Difco). Substitution by Bacto-peptone (Difco), trypticase or polypeptone (both Baltimore Biological Laboratories) gave poorer induction of abnormal forms. It appeared, then, that certain protein extracts were more effective in causing *E. coli* B to grow as morphologically bizarre forms. *E. coli* E-26, when grown in any of the inducing media described above, showed no propensity to form aberrant types, as can be seen in Pl. 1, fig. 4.

Yield of cell walls isolated from strains B and E-26

Cell walls were isolated from *Escherichia coli* B and E-26 grown in defined medium as well as from the same organisms grown in inducing medium. The results are shown in Table 1. When the isolation procedure involved only cell breakage and

Table 1. *Effect of trypsin on the yield of cell walls from two strains of Escherichia coli*

Expt. no.	*E. coli* strain	Growth medium*	Bacteria dry wt. (g./100 ml. of culture)	Trypsin treatment	Cell wall dry wt. (mg./100 ml. of culture)	Cell walls as % dry wt. of bacteria
1	E-26	IM	0·198	+	30·9	15·6
2	E-26	IM	0·189	−	33·1	17·5
3	E-26	DM	0·191	+ or −	32·4	16·9
4	B	IM	0·199	+	9·0	4·5
5	B	IM	0·204	−	41·4	20·3
6	B	DM	0·211	+ or −	37·3	17·7

* IM = inducing medium; DM = defined medium.

exhaustive washing with water the two strains yielded approximately the same amount of cell walls/g. dried bacteria (17·0–20·3%) independent of the growth medium. However, when cell wall isolation included trypsin treatment, as suggested by Salton (1964), the yield of strain E-26 cell walls, regardless of growth medium, remained constant while that of strain B walls from organisms grown in inducing medium decreased by 75%. The walls of strain B grown in defined medium were resistant to trypsin. The marked decrease in strain B cell-wall yields after trypsin treatment suggested the possibility of using various enzymes as aids in characterizing the structural difference observed.

Trypsin sensitivity of isolated cell walls

The effect of trypsin on the isolated cell walls of strain B can be seen in Fig. 1. Trypsin studies are presented only on cell walls isolated from organisms grown in

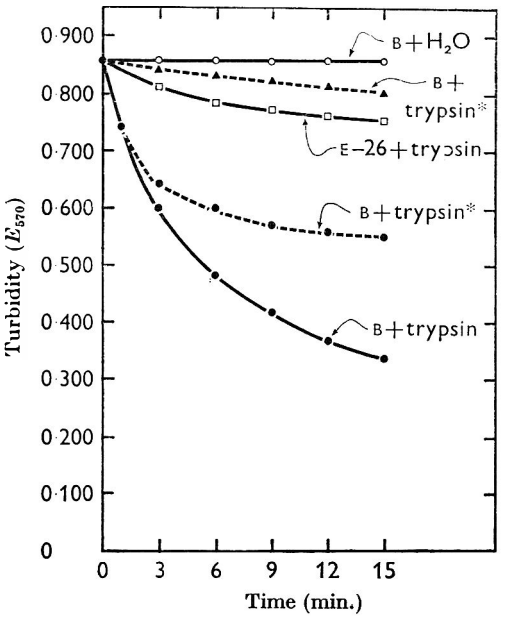


Fig. 1

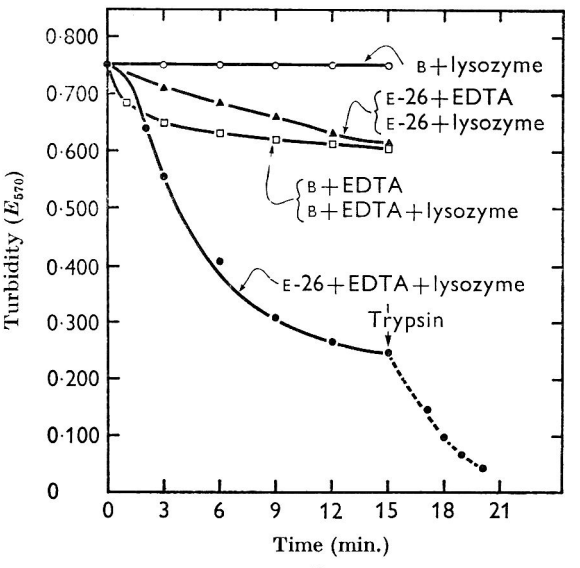


Fig. 2

Fig. 1. The effect of trypsin on isolated cell walls from *E. coli* B and E-26. Trypsin is present at 0·005 % (w/v) and Ca^{2+} at 1×10^{-3} M final concentration. Trypsin* indicates soy bean trypsin inhibitor added at 100 μg./ml. final concentration. ▲---▲ soy bean trypsin inhibitor added at zero time, ●---● soy bean trypsin inhibitor added 1·5 min. after trypsin addition.

Fig. 2. The effect of lysozyme on isolated cell walls from *Escherichia coli* B and E-26. Lysozyme final concentration is 50 μg./ml. and EDTA final concentration 1×10^{-3} M. Trypsin is added under the same conditions as described for Fig. 1.

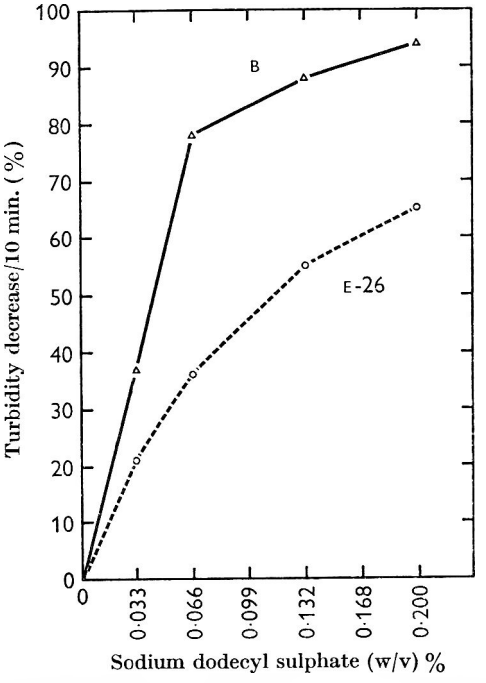


Fig. 3. The effect of various concentrations of sodium dodecyl sulphate (SDS) on isolated cell walls of *E. coli* B and E-26. All SDS treatment was at pH 7·0.

inducing medium since walls from strains B and E-26 grown in defined medium were resistant to trypsin treatment. If soy bean trypsin inhibitor was present with cell walls from strain B, trypsin caused little turbidity change. Cell walls from strain E-26 appeared resistant to trypsin. However, it appeared that trypsin rapidly attacked cell walls from strain B. Substitution of the proteolytic enzyme pronase for trypsin gave essentially identical results. The apparent tryptic lysis of the aberrant strain B cell walls may be due to trypsin liberation of a lytic enzyme, attached to the cell walls and not removed by the washing process. This possibility, however, was rendered very unlikely by the effect of soy bean trypsin inhibitor added 1·5 min. after trypsin addition. The inhibition of the rate and extent of lysis suggests that trypsin is the causative agent in turbidity reduction.

Lysozyme sensitivity of isolated cell walls

Lysozyme was used as an additional aid in characterizing the aberrant cell walls of strain B. Figure 2 shows that strain E-26 cell walls were sensitive to lysozyme in the presence of EDTA (1×10^{-3} M). There was no difference in cell-wall lysozyme sensitivity when E-26 were grown in either defined medium or inducing medium. Under identical conditions, the strain B cell walls were unaffected by treatment with lysozyme and EDTA. The slight change in turbidity in the presence of EDTA alone was also noted by Repaske (1958), and has not been adequately explained. It is interesting to note in Fig. 2 that once E-26 cell walls have been attacked by lysozyme, the walls become sensitive to trypsin. An attempt to interpret this observation will be presented in the Discussion.

Sensitivity of isolated cell walls to SDS dissolution

Shafa & Salton (1960) have shown that sodium dodecyl sulphate (SDS) at 0·2 % (w/v) and pH 7·0 can cause dissolution of *Escherichia coli* cell walls resulting in a 70 % decrease of the original turbidity. The results of a similar experiment are seen in Fig. 3. Various concentrations of SDS were used against cell wall suspensions of strains E-26 and B. The walls were obtained from organisms grown in inducing medium. The results show that at all concentrations of SDS the aberrant cell walls of strain B are more susceptible to disaggregation than those of strain E-26. At 0·2 % (w/v) SDS approximately 33 % of the original turbidity of strain E-26 cell walls remained; by comparison, less than 5 % of the turbidity due to strain B cell walls was demonstrable.

Chemical analysis of whole bacteria and isolated cell walls

Preliminary experiments were performed to determine growth variation in the morphologically aberrant forms in the rigid layer (as measured by hexosamine concentration), or in whole cell lipopolysaccharide (as measured by reducing sugar concentration) content. It was found that the aberrant forms had much lower total hexosamine and reducing sugar values—about one-third to one-fifth of the content in morphologically normal organisms. In order to determine if these variations were specifically located in the cell walls, chemical analysis of isolated cell walls was done. These data are presented in Table 2. Cell walls from abnormal strain B cells had a hexosamine concentration only one-third of that present in strain E-26 cell walls. Normal strain B cell walls had a hexosamine content

similar to that of strain E-26 walls, using either the indole+HCl or Elson-Morgan procedure. Reducing sugar determinations showed that the abnormal strain B cell walls had one-fifth the amount of reducing substance of strain E-26 cell walls. Again, the normal strain B cell walls had reducing sugar values similar to those obtained for strain E-26 cell walls. This was true regardless of whether the bacteria were initially boiled or treated with a 0·4% (w/v) SDS prior to cell-wall isolation in order to inhibit lytic enzyme activity. The protein determinations showed that cell walls from strain B grown in inducing medium had more protein associated with them than strain E-26 walls isolated from inducing medium grown organisms. Qualitative paper chromatography of acid-hydrolysed cell walls from each strain showed no difference in the amino acid pattern. Also, the amino acid composition of rigid layer isolated from walls showed no strain variation.

Table 2. *Chemical analysis of cell walls from two strains of Escherichia coli grown in inducing medium*

	% dry wt. cell walls	
Component analysed	E-26	B
Hexosamine (Indole-HCl method)	2·9	1·1
Hexosamine (Elson-Morgan method)	4·5	1·9
Reducing sugars (Anthrone method)	5·0	1·1
Protein (Lowry method)	62·4	74·2

Table 3. *Reduced NAD oxidase activity in cell walls and supernatants from two strains of Escherichia coli grown in inducing medium*

Fraction analysed	E-26	B
Reduced NAD oxidase of cell walls (S.A.*)	14·0	79·0
Reduced NAD oxidase of cell supernatants (S.A.*)	7·2	4·5
Total units† in cell walls	1,890	12,195
Total units† in cell supernatants	3,456	1,598

* Specific activity is expressed as mμ moles/min./mg. protein.
† Total units of enzyme activity is expressed as mμ moles/min./g. dry wt. of bacteria.

Reduced NAD oxidase activity in isolated cell walls

Cell wall+cell membrane complexes are isolated from Gram-negative cell-wall preparations (Salton, 1964). Studies have established that many enzymes are associated with the cell membrane (Salton, 1964) and reduced NAD+cell oxidase was chosen as a stable representative of a typical enzyme present in the cell membrane. The data in Table 3 show that aberrant strain B cell wall+membrane complexes have 5 times more of this enzyme activity than cell wall+cell membrane complexes from strain E-26. Dialysed supernatants combined with the washings from cell-wall isolations were also assayed for the reduced NAD oxidase. The difference in the values of enzyme in the two cell-wall preparations could not be accounted for by the amount of enzyme released into the soluble fraction. The total amount of enzyme activity was 2·6 times higher in the strain B than in the E-26. The distribution showed that 88% of the reduced NAD oxidase activity was in the cell wall+cell membrane of strain B, but only 35% of the enzyme activity was associated with the cell wall+cell membrane complex of strain E-26.

DISCUSSION

The morphological variants of *Escherichia coli* described here are unusual only because they are formed in response to a supposedly non-bacteriostatic environment. It has been shown that cell-wall composition, as determined by lysozyme sensitivity, can be altered by the growth medium (Litwack & Pramer, 1956). It is possible that the inducing medium, containing a very high concentration of casein hydrolysate (5%, w/v), may be acting in a manner similar to high concentrations of certain amino acids—notably glycine. It is well established that 3% (w/v) glycine will induce spheroplast formation in a suitable osmotically protective medium (Jeynes, 1961). This spheroplast formation is thought to be mainly due to inhibition by glycine of the reaction in which L-alanine is added to UDP-acetylmuramic acid (Strominger & Birge, 1965). The spheroplasts formed in such an environment are capable of propagation in serial culture, but become progressively smaller and require increasing concentrations of glycine to maintain the spheroplast form. Arguing against the possibility that the inducing medium acted here in a similar manner were the observations that (1) growth and morphological variation during propagation in serial subculture were identical to the characteristics observed in the original culture, (2) multiple factors were required for the observed morphological variation (i.e. lysine, nutrient broth and Hycase were required for branched forms and Hycase alone for filament formation), and (3) substitution of the casein hydrolysate in the inducing medium with components rich in amino acids did not give uniform induction of aberrant forms, showing that amino acid concentration was not the sole factor in causing the abnormal cell formation. The role of the lysine in the medium is quite complex. It is possible that lysine is feedback-inhibitory or represses the synthesis of diaminopimelate, thus regulating mucopeptide completion. Substitution of other amino acids (glycine or alanine), for lysine reduced branched cell formation. However, filament formation was unimpaired. The chemical analysis of the cell walls of the morphological variants showed that while the quantity of hexosamine was greatly decreased, it was still present in sufficient concentration to support the suggestion that some rigid layer is a component of the isolated cell walls. This is in agreement with the findings of Vaituzis & Doetsch (1965) showing that aberrant filamentous and branched forms result during recovery of *Salmonella typhimurium* from spheroplast induction by penicillin. In other words, it is only during the *recovery*, or intermediate, phase (i.e. rigid layer synthesizing period) that the aberrant organisms are observed. When sufficient rigid layer has been synthesized the organisms regain their normal morphology. One interpretation presently available is that the aberrant forms of *E. coli* B have been induced by partial inhibition of cell-wall synthesis and that these forms have only a small portion of the normal rigid layer.

This interpretation is further strengthened in these studies by the use of trypsin and lysozyme. *Escherichia coli* E-26 cell walls appear to be resistant to trypsin attack since the continuous rigid layer is able, by covalent or ionic bonding, to prevent the tryptic fragments from disaggregating. On the other hand, the rigid layer of strain B is thought to be discontinuous and therefore not capable of holding the tryptic fragments. Consequently, the walls disaggregate during trypsin treatment. Once the strain E-26 rigid layer has been made discontinuous by the action

of lysozyme these cell-walls become highly susceptible to trypsin disaggregation. The results of detergent (SDS) dissolution of strain E-26 cell walls shows that 30% of the initial turbidity remains, supposedly due to non-solubilized intact rigid layer. However, dissolution of strain B cell walls with SDS was almost complete, increasing the possibility that the rigid layer is partially absent or in some way discontinuous. Finally, the observation that filamentous and branched forms of *E. coli* B had 5 times more reduced NAD oxidase activity associated with their cell wall + cell membrane complex than strain E-26 can be explained by the fact that strain B walls have more protein and also more enzyme. It is also possible that the two enzymes (and hence the membranes) are different and the strain B oxidase is five times more active. This can only be clarified by purification of the enzyme from both wall preparations.

This work was supported by a grant, AM 07418, from the National Institute of Arthritis and Metabolic Diseases, U.S. Public Health Service.

REFERENCES

ADLER, H. I. & HARDIGREE, A. A. (1964). Analysis of a gene controlling cell division and sensitivity to radiation in *Escherichia coli*. *J. Bact.* **87**, 720.

BAILEY, J. L. (1962). *Techniques in Protein Chemistry*. Amsterdam: Elsevier Publishing Co.

BARTMANN, K. & HÖPKEN, W. (1956). Zur Frage des sogenannten unvollständigen L-Zyklus. *Zeutbl. Bakt.* (*I Orig.*), **166**, 30.

BROCK, T. D. (1962). Effects of magnesium ion deficiency on *Escherichia coli* and possible relation to the mode of action of novobiocin. *J. Bact.* **84**, 679.

COGGIN, J. H., JUN. & MARTIN, W. R. (1965). 6-Diazo-5-oxo-L-norleucine inhibition of *Escherichia coli*. *J. Bact.* **89**, 1348.

DISCHE, Z. (1955). New color reactions for determination of sugars in polysaccharides. *Meth. biochem. Analysis*, **2**, 313.

DISCHE, Z. & BORENFREUND, E. (1950). A spectrophotometric method for the microdetermination of hexosamines. *J. biol. Chem.* **185**, 517.

HIRS, C. H. W. (1955). Chromatography of enzymes on ion exchange resins. *Meth. Enzymol.* **1**, 113.

HOLDEN, J. T. & HOLMAN, J. (1957). Abnormal cellular morphology associated with a vitamin B_6 deficiency in *Lactobacillus arabinosus*. *J. Bact.* **73**, 592.

HUGHES, W. H. (1953). The origin of the L-form variants in anaerobic cultures of *Bacterium coli*. *J. gen. Microbiol.* **8**, 307.

HUGHES, W. H. (1956). The structure and development of the induced long forms of bacteria. *Symp. Soc. gen. Microbiol.* **6**, 341.

JEYNES, M. H. (1961). The growth and division of bacterial protoplasts. *Exp. Cell Res.* **24**, 255.

KITAHARA, K. & KUSAKA, I. (1959). Pleomorphism in bacterial cells. I. Filament formation in *Lactobacillus delbrueckii* and its relation to vitamin B_{12}. *J. gen. appl. Microbiol.* (*Tokyo*), **5**, 78.

LITWACK, G. & PRAMER, D. (1956). Growth of *Micrococcus lysodeikticus* as a substrate for lysozyme. *Proc. Soc. exp. Biol. Med.* **91**, 290.

LOWRY, O. H., ROSEBROUGH, N. J., FARR, A. L. & RANDALL, R. J. (1951). Protein measurement with the Folin phenol reagent. *J. biol. Chem.* **193**, 265.

MORA, J. & BOJALIL, L. F. (1965). Antagonism of the D-alanine reversal of D-cycloserine action by L-alanine in *Mycobacterium acapulcensis*. *Proc. Soc. exp. Biol. Med.* **119**, 49.

PONTEFRACT, R. D. & THATCHER, F. S. (1965). A cytological study of normal and radiation-resistant *Escherichia coli*. *Can. J. Microbiol.* **11**, 271.

REPASKE, R. (1958). Lysis of Gram-negative organisms and the role of versene. *Biochim. biophys. Acta*, **30**, 225.

SALTON, M. R. J. (1964). *The Bacterial Cell Wall.* Amsterdam: Elsevier Publishing Co.

SCOTT, T. A. & MELVIN, E. H. (1953). Determination of dextran with anthrone. *Anal. Chem.* **25**, 1656.

SHAFA, F. & SALTON, M. R. J. (1960). Disaggregation of bacterial cell walls by anionic detergents. *J. gen. Microbiol.* **23**, 137

SHOCKMAN, G. D., KOLB, J. J. & TOENNIES, G. (1957). A high speed shaker for the disruption of cells at low temperature. *Biochim. biophys. Acta*, **24**, 203.

STROMINGER, J. L. & BIRGE, C. H. (1965). Nucleotide accumulation induced in *Staphylococcus aureus* by glycine. *J. Bact.* **89**, 1124.

VAITUZIS, Z. & DOETSCH, R. N. (1965). Flagella of *Salmonella typhimurium* spheroplasts. *J. Bact.* **89**, 1586.

WEIDEL, W., FRANK, H. & LEUTGEB, W. (1963). Autolytic enzymes as a source of error in the preparation and study of Gram-negative cell walls. *J. gen. Microbiol.* **30**, 127.

WEINBAUM, G. & MALLETTE. M. F. (1959). Enzyme biosynthesis in *Escherichia coli. J. gen. Physiol.* **42**, 1207.

WEINBAUM, G. & FISCHMAN, D. (1965). Morphologic variation in *Escherichia coli. Bact. Proc.* p. 35.

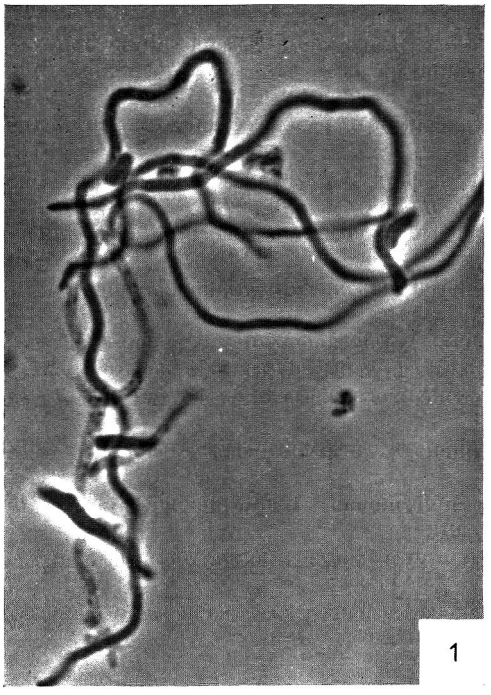
WEINBAUM, G. (1965). Cellular response to environment. I. Morphologic variation in *Escherichia coli. J. Einstein med. Cent.* **13**, 176.

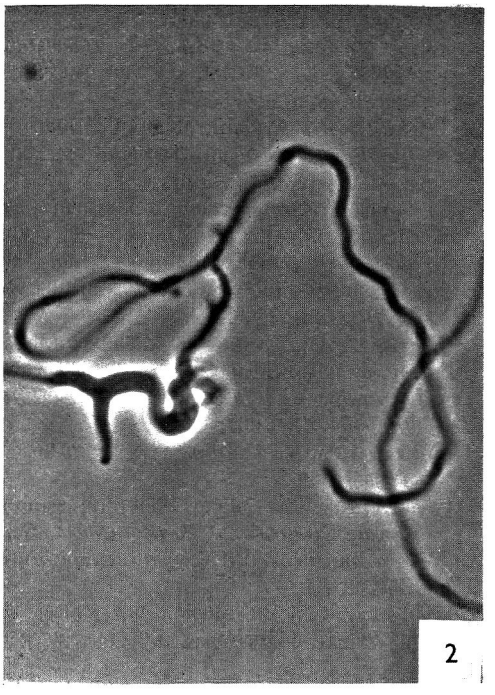
WINZLER, R. J. (1955). Determination of serum glycoproteins. *Meth. biochem. Analysis*, **2**, 279.

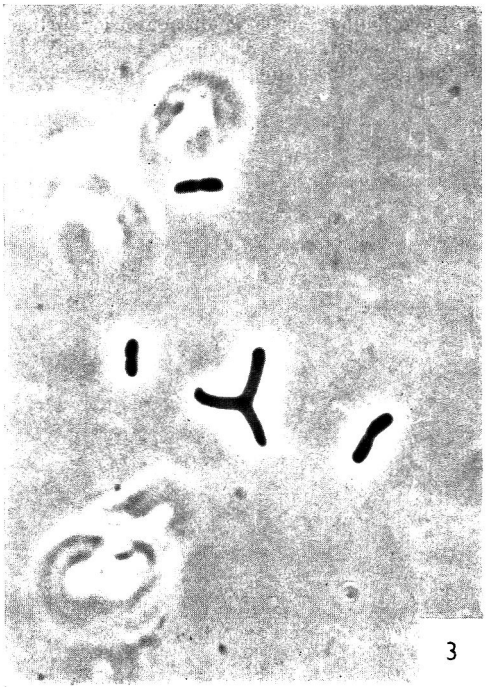
ZOBELL, C. E. & COBET, A. B. (1964). Filament formation by *Escherichia coli* at increased hydrostatic pressures. *J. Bact.* **87**, 710.

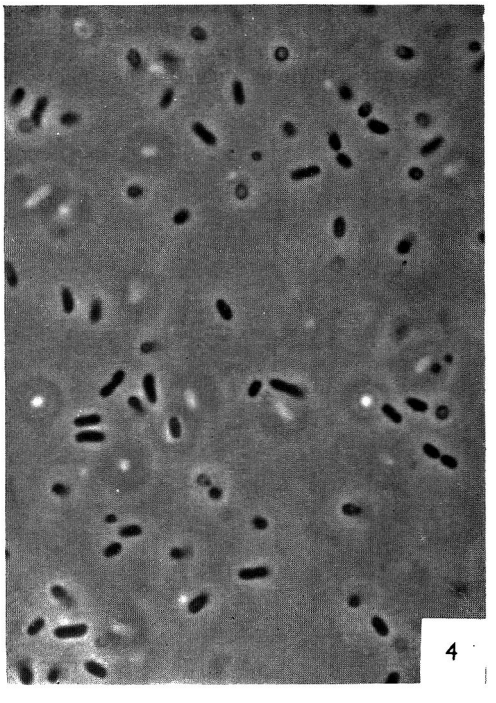
EXPLANATION OF PLATE

Escherichia coli B grown in inducing medium showing fig. 1, filamentous, fig. 2, filamentous branched, and fig. 3 branched cells. Phase micrographs at magnification, ×4000. Figure 4, *E. coli* E-26 grown in inducing medium showing normal rod shapes. Phase micrograph magnification is ×4000.









(*Facing p.* 92)

J. gen. Microbiol. (1966), **42**, 93–106
Printed in Great Britain

Studies on the Mode of Action of the Streptogramin Antibiotics

By D. VAZQUEZ

Sub-Department of Chemical Microbiology, Department of Biochemistry, Tennis Court Road, Cambridge

(*Received* 20 *July* 1965)

SUMMARY

Streptogramin is a complex antibiotic containing two components, streptogramin A and streptogramin B. The individual components are bacteriostatic whereas the mixture is bactericidal. At growth-limiting concentrations streptogramin A and the streptogramin complex inhibit *Staphylococcus aureus* by blocking protein synthesis but not nucleic acid and cell wall synthesis. Protein, nucleic acid and cell wall synthesis were all reduced in the presence of streptogramin B.

Streptogramin A, but not streptogramin B, inhibits the binding of radioactive chloramphenicol to bacterial ribosomes. However, streptogramin B enhances this effect of streptogramin A in a cell-free system containing both bacterial soluble fraction and ribosomes. The results suggest that streptogramin A inhibits protein synthesis by bacteria at the ribosome level, and that this effect is enhanced by streptogramin B.

INTRODUCTION

The antibiotic streptogramin was first obtained from culture filtrates of a species of Streptomyces, now classified as *Streptomyces graminofaciens* (Charney *et al.* 1953). Other antibiotics closely related to streptogramin have been described, for example, virgimycin or staphylomycin (De Somer & Van Dijck, 1955), E 129 or ostreogrycin (Garrod & Waterworth, 1956; Ball *et al.* 1958), synergistin or PA 114 (Celmer & Sobin, 1955/1956), mikamycin (Arai *et al.* 1956), pristinamycin or pyostacin (Benazet *et al.* 1962) and vernamycin (Bodanszky & Ondetti, 1963). All these antibiotics are mixtures of two or more different active compounds, and there are numerous reports on the isolation and properties of the individual components from PA 114, staphylomycin, ostreogrycin, mikamycin and vernamycin. All these complex antibiotics consist of at least two components belonging to two major groups, A and B. The antibiotics in group A show a marked synergism with those in group B in their activity against Gram-positive bacteria and consequently the complex antibiotics of the streptogramin family have a markedly higher activity than the individual components. The classification of the antibiotics of the streptogramin family has been reviewed by Lester Smith (1963).

The antibiotics in group A are mainly active against Gram-positive cocci and the antibiotics in group B against Gram-positive bacilli. Although there is a strong synergism between the antibiotics of groups A and B in their antibacterial effect against Gram-positive bacteria in general, there is no such effect on Gram-negative organisms; consequently, the antibiotic complexes are effective mainly against Gram-positive bacteria.

This work describes investigations of the mode of action of streptogramin and its components in an attempt to clarify the mechanism of the remarkable synergism shown by mixtures of the individual antibiotics. Preliminary reports of this work have been published (Vazquez, 1962, 1963*a*, 1964*b*).

METHODS

Organisms and growth conditions. The organisms used in this work were *Staphylococcus aureus* strain Duncan, *Bacillus megaterium* strain KM (NCIB 9521) and *Escherichia coli*, strain B. *Staphylococcus aureus* was grown overnight at 37° in medium A of Gale (1947). Next morning the culture was diluted 10 times with fresh medium and incubation continued. For studies of synthesis of cellular constituents or isotope incorporation, *S. aureus* was resuspended in the defined medium of Gale & Folkes (1953*a*) containing glucose, amino acids, purines, pyrimidines and the buffered salt solution of Gale (1947). *Bacillus megaterium* and *E. coli* were grown overnight aerobically in CG medium containing 0·03% (w/v) glucose (McQuillen & Roberts 1954) at 30° and 37° respectively. In the morning, excess glucose was added and incubation continued. Growth of bacteria was followed by measuring the optical density as previously described (Vazquez, 1965*a*).

Viable counts. Viable counts were made in triplicate on medium A+2% (w/v) agar using the method described by Miles & Misra (1938) after serial dilution of the bacteria in saline.

Synthesis of cellular constituents

(*a*) *Net synthesis.* Suspensions of *Staphylococcus aureus* at an initial concentration equivalar 1 mg. dry wt./ml. were incubated statically at 37° in the defined medium. Samples of 3 ml. were taken at intervals and cooled rapidly. Bacteria were harvested by centrifugation and washed with the buffered salt solution. The bacterial pellet was extracted twice with 2 ml. of 0·2 N-perchloric acid at 4° for 60 min. and the supernatant fluids from these extractions combined. This constituted the pool fraction. The nucleic acids were extracted from the pellet with 3 ml. 0·5 N-perchloric acid at 70° for 20 min. (three extractions were sufficient). The nucleic acids were estimated by measuring the absorption at 260 mμ in a Unicam SP 500 spectrophotometer. A solution of 100 μg. nucleic acid/ml. was assumed to have an absorption of 2·8 (Gale & Folkes, 1953*a*). The DNA in the 0·5 N-perchloric extracts was estimated by the diphenylamine method of Burton (1956).

The residual pellet was taken up in N-NaOH and protein in this solution was estimated by the method of Lowry *et al.* (1951).

(*b*) *Isotope incorporation.* Suspensions of *Staphylococcus aureus* at an initial cell concentration equivalar 1 mg. dry wt./ml. were incubated at 37° statically in the defined medium to which a ^{14}C-labelled compound had been added. Samples of 1 ml. were taken at intervals and cooled rapidly. The bacteria were harvested by centrifugation and washed with 5 ml. of the buffered salt solution. The bacterial pellet was extracted with 3 ml. 5% (w/v) trichloroacetic acid for 1 hr at 4°; nucleic acids were extracted from the pellet with 3 ml. of 5% (w/v) trichloroacetic acid at 90° for 15 min. The insoluble material remaining after this extraction was washed once with 2 ml. 0·01 M-NH_4HCO_3, resuspended in 2 ml. of this solution, and digested with 2 mg. trypsin to separate the protein and the cell-wall fractions (Hancock & Park, 1958).

Samples of every fraction were transferred to aluminium planchets and radioactivity determined as previously described (Vazquez, 1965*b*).

Uptake of chloramphenicol. The effect of the streptogramin antibiotics on the uptake of ^{14}C-labelled chloramphenicol (methylene ^{14}C; 9·90 μC./μmol; [^{14}C]CAP) by *Staphylococcus aureus* suspended in medium A was studied as described by Vazquez (1965*b*) for *Bacillus megaterium*. The non-radioactive antibiotics were mixed in the tubes with [^{14}C]CAP prior to the addition of bacterial suspensions.

Preparation of bacterial cell sap and ribosomes. Suspensions of *Staphylococcus aureus* (100 mg. dry wt./ml.) in the buffered salt solution were disrupted in a Mullard ultrasonic disintegrator (model SL 82) for 10 min. Broken cell walls and membranes were separated by centrifugation at 40,000*g* for 20 min. and discarded. The supernatant cell sap was diluted with buffered salt solution when required. The buffered salt solution contained 0·002 M-Mg^{2+} and under these conditions the ribosomes were split into 30S and 50S ribosomal subunits. Ribosomes from *Bacillus megaterium* and *Escherichia coli* were obtained as previously described (Vazquez, 1965*a*). These ribosomes were resuspended in 0·01 M-tris + HCl buffer, pH 7·4, containing 0·2 M-K^+.

[^{14}C]CAP binding to ribosomes. The effect of the streptogramin antibiotics on [^{14}C]CAP binding to *Staphylococcus aureus* ribosomes was studied without separating the ribosomes from the bacterial 'soluble fraction' using bacterial cell sap. Suspensions of *Bacillus megaterium* ribosomes in 0·01 M-tris-HCl buffer, pH 7·4, containing 0·2 M-K^+ were also used for binding experiments. The experiments were carried out as described by Vazquez (1965*b*). The non-radioactive antibiotics were mixed in the tubes with [^{14}C]CAP before adding of ribosome suspensions. *B. megaterium* and *S. aureus* ribosomes were harvested by centrifugation at 150,000*g* for 100 min. and 150 min., respectively.

Antibiotics. Streptogramin, PA 114-A-1 and PA 114-B-1 were kindly supplied by Dr H. B. Woodruff. The two major components of streptogramin (streptogramin A and streptogramin B) were separated and purified following the methods described by Vanderhaeghe, Van Dijck, Parmentier & De Somer (1957) for the separation of the components of staphylomycin. Streptogramin A was further purified by the method described by Sarin (1962) for the purification of ostreogrycin A. The ultraviolet and infrared spectra of the streptogramins A and B were determined and compared with those of PA 114-A-1 and PA 114-B-1. There were no significant differences between streptogramin A and PA 114-A-1, or streptogramin B and PA 114-B-1. The chemical structures of the individual components of the streptogramin complex have not been published, but Dr Woodruff and Dr Lester Smith (personal communications) have suggested that streptogramins A and B are identical with PA 114-A-1 (synonym, ostreogrycin A) and PA 114-B-1 (synonym, ostreogrycin B), respectively.

PA 114-A-1 and PA 114-B-1 were used in this work, and will be referred to as component A and component B of streptogramin. In many experiments the individual components isolated from streptogramin were also used to confirm results obtained.

The sources of other antibiotics used in this work are given by Vazquez (1965*b*).

Isotopically labelled compounds were obtained from the Radiochemical Centre, Amersham, Buckinghamshire.

RESULTS

Effect on the viability of Staphylococcus aureus. The limiting growth-inhibitory concentrations for *Staphylococcus aureus* of streptogramin, component A and component B were respectively, 0·6, 6, and 10 μg./ml. Streptogramin was bactericidal whereas the individual components A and B were bacteriostatic, even when tested at three times the minimum growth-inhibitory concentrations. The effect of mixtures of component A with other antibiotics on the viability of *S. aureus* was studied with the results shown in Table 1. Bactericidal effects were found when component A was mixed with either viridogrisein (Garcia-Mendoza, 1965) or component B and to a lesser extent when mixed with aureomycin or terramycin. On the other hand, component A prevented the lethal effect of streptomycin but not that of echinomycin.

Table 1. *The effect of component A with other antibiotics on the viability of Staphylococcus aureus*

Antibiotics	Colony count 0 min.	75 min.	150 min.
None	$3{\cdot}0 \times 10^7$	$8{\cdot}0 \times 10^7$	$1{\cdot}5 \times 10^8$
Component A ('A')		$3{\cdot}3 \times 10^7$	$3{\cdot}5 \times 10^7$
'A' + Component B		$1{\cdot}2 \times 10^5$	$< 10^5$
Viridogrisein		$3{\cdot}5 \times 10^7$	$4{\cdot}5 \times 10^7$
'A' + Viridogrisein		$1{\cdot}0 \times 10^5$	$< 10^5$
Aureomycin		$2{\cdot}5 \times 10^7$	$2{\cdot}1 \times 10^7$
'A' + Aureomycin		$9{\cdot}2 \times 10^6$	$3{\cdot}0 \times 10^6$
Terramycin		$3{\cdot}0 \times 10^7$	$2{\cdot}8 \times 10^7$
'A' + Terramycin		$1{\cdot}2 \times 10^7$	$8{\cdot}0 \times 10^6$
Chloramphenicol		$5{\cdot}4 \times 10^7$	$9{\cdot}0 \times 10^7$
'A' + Chloramphenicol		$3{\cdot}4 \times 10^7$	$3{\cdot}5 \times 10^7$
Streptomycin		$8{\cdot}0 \times 10^6$	$2{\cdot}5 \times 10^6$
'A' + Streptomycin		$3{\cdot}0 \times 10^7$	$3{\cdot}1 \times 10^7$
Echinomycin		$7{\cdot}5 \times 10^6$	$2{\cdot}5 \times 10^6$
'A' + Echinomycin		$7{\cdot}2 \times 10^6$	$3{\cdot}0 \times 10^6$

Staphylococcus aureus ($3{\cdot}0 \times 10^7$ bacteria/ml.) was incubated at 37° for 150 min. in medium A. Antibiotics were added simultaneously (final concentrations 2×10^{-6} M), at the beginning of the incubation period. The colony count was determined by the method of Miles & Misra (1938).

Chloramphenicol, aureomycin, terramycin, erythromycin and carbomycin prevent the bactericidal action of penicillin and streptomycin on many bacteria (Jawetz, Gunnison, Bruff & Coleman, 1952; Coleman, Gunnison & Jawetz, 1953; Anand & Davis, 1960; Hurwitz & Rosano, 1962). A similar protective effect of chloramphenicol was found when it was added with streptogramin to an exponentially growing culture of *Staphylococcus aureus*. The protective effect of chloramphenicol occurred to the same extent when it was added to the bacterial culture before, or at the same time as, streptogramin, and to some extent when added after streptogramin (Table 2).

Penicillin and streptomycin are bactericidal only when the bacteria are growing (Kirby & Burnell, 1954; Prestidge & Pardee, 1957; Anand & Davis, 1960), and it was thought that chloramphenicol might protect by preventing growth. However,

this may not be the complete explanation in the case of streptogramin, as chloramphenicol at 3 μg./ml. inhibited the growth of *Staphylococcus aureus* by only 60% but still prevented the bactericidal effect of streptogramin (Fig. 1).

Erythromycin, puromycin, aureomycin and terramycin are bacteriostatic and inhibit growth of *Staphylococcus aureus* at concentrations of 1, 50, 1 and 1 μg./ml. respectively. At growth inhibitory concentrations erythromycin also prevented the bactericidal effect of streptogramin, and puromycin had no such effect, while aureomycin and terramycin increased its bactericidal effect. When growth of *S. aureus* was prevented by using a defined medium with glutamic acid omitted, the bactericidal effect of streptogramin was not significantly decreased. These findings suggested that streptogramin can kill *S. aureus*, even when growth is lessened, and that the effect of chloramphenicol and erythromycin in preventing killing by streptogramin cannot be explained simply by their effect in inhibiting bacterial growth.

Table 2. *The effect of time of addition of chloramphenicol on the bactericidal action of streptogramin*

Time of addition of antibiotics (min.)					Colony count after 90 min. incubation	% Decrease in colony count
0	1	3	5	10		
—	—	—	—	—	$6{\cdot}3 \times 10^6$	—
CAP	—	—	—	—	$2{\cdot}7 \times 10^6$	—
S	—	—	—	—	$8{\cdot}1 \times 10^4$	96
CAP+S	—	—	—	—	$2{\cdot}5 \times 10^6$	—
S	CAP	—	—	—	$2{\cdot}2 \times 10^6$	12
S	—	CAP	—	—	$1{\cdot}5 \times 10^6$	40
S	—	—	CAP	—	$9{\cdot}0 \times 10^5$	64
S	—	—	—	CAP	$6{\cdot}0 \times 10^5$	76
CAP	S	—	—	—	$2{\cdot}6 \times 10^6$	—
CAP	—	S	—	—	$2{\cdot}2 \times 10^6$	12
CAP	—	—	S	—	$2{\cdot}2 \times 10^6$	12
CAP	—	—	—	S	$2{\cdot}4 \times 10^6$	6

Staphylococcus aureus ($2{\cdot}5 \times 10^6$ bacteria/ml.) was incubated at 37° in medium A for 90 min. Chloramphenicol at 10 μg./ml. (CAP) and streptogramin at 0·6 μg./ml. (S) were added at the times shown in the Table. The colony count was determined by the method of Miles & Misra (1938).

Respiration and fermentation. Streptogramin and the individual components A and B at concentrations of up to 60 μg./ml., had no effect on endogenous respiration, oxidation of ethanol or glucose, or anaerobic fermentation of glucose when added to washed suspensions of *Staphylococcus aureus* in the Warburg vessels. The studies were carried out using the manometric techniques described by Umbreit, Burris & Stauffer (1949).

Synthesis of cellular constituents. The effect of component A on net synthesis and on the incorporation of radioactive isotopes into the different cell fractions is shown in Tables 3 and 4. These results indicate that the antibiotic inhibits bacterial growth by blocking protein synthesis. The inhibitory effect of the antibiotic on the incorporation of [U-^{14}C]glycine into the protein fraction was detected within 30 sec. of addition of the antibiotic (Fig. 2). Similar results were obtained with L-[U-^{14}C]-lysine. The increased rate of glutamic acid accumulation in the pool in the presence

of component A was probably due to the amino acid not being incorporated in the protein fraction, and has been observed in the presence of other inhibitors of protein synthesis like chloramphenicol (Hancock, 1960). The increased nucleic acid content in the presence of component A was due to an increase of RNA accumulation; the synthesis of DNA was unaffected. An increase in the rate of accumulation of RNA has also been observed in bacteria treated with chloramphenicol (see Gale, 1963).

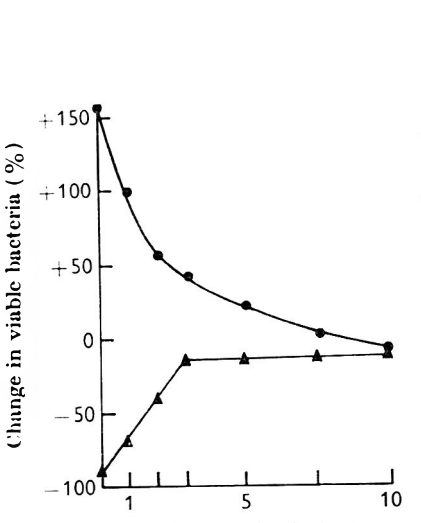


Fig. 1.

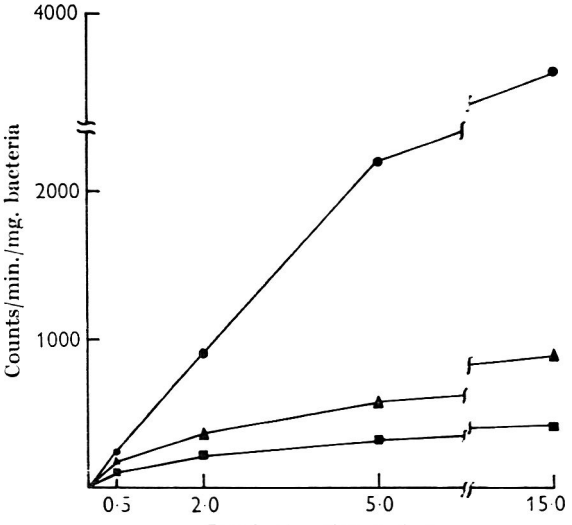


Fig. 2.

Fig. 1. The effect of chloramphenicol on the bactericidal action of streptogramin. Suspensions of *Staphylococcus aureus* (7×10^5 bacteria/ml.) were incubated in medium A at 37° for 90 min. and viable bacteria were counted at the beginning and at the end of the incubation period by the method of Miles & Misra (1938). ●—●—, No streptogramin added; —▲—▲—, 0·6 μg. streptogramin/ml.

Fig. 2. The effect of component A on the incorporation of [U-^{14}C] glycine into the protein fraction of *Staphylococcus aureus*. Suspensions of 1 mg. *S. aureus*/ml. were incubated at 37° for 15 min. in the defined medium without adenine or guanine and supplemented with [U-^{14}C]glycine (36 μg./ml.; 0·4 μc./ml.). Samples were taken at intervals and fractionated by the method of Hancock & Park (1958). —●——●—, Control; ▲—▲—, 6 μg. component A/ml.; —■—■—, 20 μg. component A/ml.

Similar experiments with component B suggest that it has a complex effect on *Staphylococcus aureus* (Tables 3 and 4). Protein, nucleic acid, and cell-wall synthesis were all reduced. However, the incorporation of glycine into the protein fraction was affected by component B within 30 sec., whereas the effect on nucleic acid and cell wall fractions was not established until after 1–2 min. Similar results were obtained for the incorporation of L-[U-^{14}C]lysine.

These results are very similar to those reported by Garcia-Mendoza (1965) for the effect of viridogrisein on *Staphylococcus aureus*. Viridogrisein is included in group B of the streptogramin family of antibiotics and is more active than the other members of this group in inhibiting bacterial growth and protein synthesis. The effects of equal concentrations of viridogrisein and component B on nucleic acid and cell-wall synthesis were found to be very similar.

Effect of the streptogramin complex. The effect of the streptogramin complex at limiting growth-inhibitory concentrations on the net synthesis and incorporation of radioactive isotopes in the different cell fractions is shown in Tables 3 and 4, and resembles those obtained with component A (at 10 × concentration). The main

Table 3. *The effect of the streptogramin antibiotics on synthesis of cellular constituents*

Antibiotic	μg./ml.	Net synthesis of control: 260 mμ absorbing material: Pool	Net synthesis of control: 260 mμ absorbing material: NA* fraction	Protein estimated in the fraction insoluble in hot perchloric acid
Component A	6·0	100	173	32
	20·0	100	158	16
Component B	10·0	87	61	56
	30·0	80	57	42
Streptogramin	0·6	100	174	38
	2·0	100	163	15

NA*: total nucleic acid. Suspensions of 1 mg. *Staphylococcus aureus*/ml. were incubated at 37° for 15 min. in the defined medium. Samples were taken at the beginning and at the end of the incubation period, fractionated by perchloric acid treatment, and protein estimated in the hot perchloric acid-insoluble fraction.

Table 4. *The effect of the streptogramin antibiotics on synthesis of cellular constituents*

Antibiotic	μg./ml.	Radioactive compound	Incorporation of radioactive compounds: % control incorporation: Pool	NA*	Protein	Wall
Component A	6·0	[U-^{14}C]Glycine	100	100	26	100
	20·0		100	100	12	100
	6·0	L-[U-^{14}C]Glutamic acid	120	100	27	100
	20·0		128	100	11	100
	6·0	[8-^{14}C]Adenine	126	204	—	—
	20·0		121	189	—	—
Component B	10·0	[U-^{14}C]Glycine	77	59	54	57
	30·0		73	49	43	46
	10·0	L-[U-^{14}C]Glutamic acid	83	57	52	58
	30·0		76	46	40	48
	10·0	[8-^{14}C]Adenine	74	61	—	—
	30·0		69	58	—	—
Streptogramin	0·6	[U-^{14}C]Glycine	100	100	34	100
	2·0		100	100	11	100
	0·6	L-[U-^{14}C)Glutamic acid	118	100	35	100
	2·0		127	100	10	100
	0·6	[8-^{14}C]Adenine	130	206	—	—
	2·0		120	176	—	—

*NA: total nucleic acid. [U-^{14}C]Glycine added 0·4 μC./ml.; 36 μg./ml. L-[U-^{14}C]Glutamic acid added 0·1 μC./ml.; 100 μg./ml. [8-^{14}C]Adenine added 0·05 μC./ml.; 10 μg./ml.

Suspensions of 1 mg. *Staphylococcus aureus*/ml. were incubated at 37° for 15 min. in the defined medium supplemented with the required radioactive compound. Samples were taken at the end of the incubation period, fractionated by the method of Hancock & Park (1958), and the radioactivity in the fractions determined.

difference is that component A had an immediate effect on protein synthesis, whereas a lag of about 2 min. was observed before the synergistic mixture exerts its effect.

Gale & Folkes (1953*a*) showed that the action of chloramphenicol on bacterial protein is reversible; synthesis begins again immediately the antibiotic is removed. A comparison was made of the inhibitory effects of chloramphenicol and streptogramin on net protein synthesis as measured by increase in the perchloric acid-insoluble fraction and their reversal in *Staphylococcus aureus*. The inhibitory effect of streptogramin was not readily reversible, and removal of the antibiotic was followed by only a slight increase in the rate of protein synthesis.

The effect of a mixture of chloramphenicol and streptogramin on L-[U-^{14}C]-glutamic acid incorporation into the protein fraction was studied, and showed that chloramphenicol at a concentration which prevented the lethal effect of streptogramin did not also block its inhibitory action on protein synthesis (Table 5).

Results similar to those found with streptogramin were obtained when an equivalent mixture of component A (80%) and B (20%) was used. (Fig. 3).

Table 5. *Incorporation of ^{14}C-labelled glutamic acid into protein. The effect of streptogramin with chloramphenicol*

	Bacteria (counts/min./mg.)
Control	1135
+CAP (3·5 μg./ml.)	485
+Streptogramin (0·9 μg./ml.)	161
+CAP (3·5 μg./ml.)+Streptogramin (0·9 μg./ml.)	150

Suspensions of 1 mg. *Staphylococcus aureus*/ml. were incubated at 37° for 30 min. in the defined medium without adenine nor guanine in the presence of ^{14}C-labelled glutamic acid 0·1 μC./ml.; 100 μg./ml. Samples were taken and fractionated by the method of Hancock & Park (1958).

Effect of the streptogramin antibiotics on the binding of chloramphenicol. Studies of the uptake of antibiotics by bacteria and their binding to different structures or components within the cell have been very helpful in elucidating their initial site of action.

As radioactive components A and B were not available, the uptake and binding of these antibiotics could not be studied directly. However, the results described above showed that component A had an overall effect resembling that of chloramphenicol, and it was thought that component A and chloramphenicol might compete for the same binding site(s) in sensitive bacteria. The binding of ^{14}C-labelled chloramphenicol [^{14}C]CAP has been used to test this hypothesis. Component A significantly reduced the uptake of [^{14}C]CAP by *Staphylococcus aureus* and the effect was somewhat enhanced by component B (Table 6). Component B also reduced [^{14}C]CAP uptake, but to a lesser extent than component A. When cells are exposed to [^{14}C]CAP and then broken and fractionated in the centrifuge, all the radioactivity is associated with the ribosomal and soluble fraction (Vazquez, 1963*b*).

The effect on the binding of chloramphenicol by ribosome preparations was next studied. In a cell-free system consisting of a mixture of ribosome subunits (30 S and 50 S) and soluble fraction in the buffered salt solution, the ribosome bound

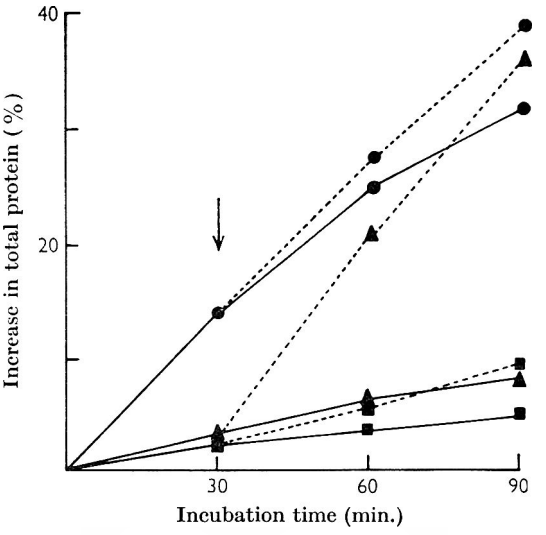


Fig. 3. Net protein synthesis in *Staphylococcus aureus* after streptogramin or chloramphenicol treatment. Suspensions of 1 mg. *Staphylococcus aureus*/ml. were incubated at 37° for 90 min. in the defined medium without antibiotic (—●——●), with 30 μg. chloramphenicol/ml. (—▲—▲—), or with 3 μg. streptogramin/ml. (—■—■—). At time 30 min. (at arrow) portions of the bacteria were spun down, washed twice and resuspended in fresh medium free from antibiotic. Broken lines show increase in total protein synthesis after resuspending *S. aureus* in fresh medium.

Table 6. *The effect of the streptogramin antibiotics on [^{14}C]chloramphenicol uptake by Staphylococcus aureus*

Antibiotic concentration		$\mu\mu$moles [^{14}C]CAP taken up, mg. bacteria
Component A	Component B	
—	—	56
1×10^{-6} M	—	48
3×10^{-6} M	—	33
1×10^{-5} M*	—	21
3×10^{-5} M	—	14
1×10^{-4} M	—	9
—	1×10^{-6} M	52
—	3×10^{-6} M	42
—	1×10^{-5} M*	34
—	3×10^{-6} M	27
—	1×10^{-4} M	23
$(1 \times 10^{-6}$ M $+ 1 \times 10^{-7}$ M)*		32
$(1 \times 10^{-5}$ M $+ 1 \times 10^{-6}$ M)		12

* Minimum growth-inhibitory concentration of the antibiotic(s).

Suspensions of *Staphylococcus aureus* (0·25 mg./ml.) in medium A were incubated at 37° in the presence of (3×10^{-6} M) [^{14}C]chloramphenicol with the required antibiotic. After 15 min. the bacteria were harvested by centrifugation and the surface of the bacterial pellet rinsed with buffered salt solution. Finally bacteria were resuspended in water, and the radioactivity determined. Corrections were made for [^{14}C]chloramphenicol present in the intercellular fluid as previously described (Vazquez, 1965*b*).

[^{14}C]CAP. This binding was inhibited by component A but not by component B. However, the addition of components A and B to ribosomes slightly reduced [^{14}C]CAP binding still further. Nevertheless, the effect of component B was too small to explain the strong synergistic effect of the two streptogramin components on intact cells. Addition of components A and B before or after [^{14}C]CAP did not significantly change the results. In further experiments suspensions of *Staphylococcus aureus* were incubated with streptogramin or mixtures of components A

Table 7. *Binding of* [^{14}C]*chloramphenicol to bacterial ribosomes. Intact bacteria pretreated with the streptogramin antibiotics*

Conditions of incubation				Antibiotic concentration		[^{14}C]CAP binding
Organism	Medium	Temperature	Time (min.)	Component A	Component B	(% of control)
S. aureus	A	37°	20	1×10^{-6} M	—	97
				5×10^{-6} M	—	78
				1×10^{-5} M	—	54
				3×10^{-5} M	—	45
				—	5×10^{-7} M	100
				1×10^{-6} M	1×10^{-7} M	24
				5×10^{-6} M	5×10^{-7} M	4
	Buffer*	4°	10	1×10^{-5} M	—	50
	A	4°	10	1×10^{-5} M	—	56
	A	37°	10	1×10^{-5} M	—	53
	Buffer*	4°	10	1×10^{-6} M	1×10^{-7} M	83
	A	4°	10	1×10^{-6} M	1×10^{-7} M	92
	A	37°	10	1×10^{-6} M	1×10^{-7} M	38
E. coli	Peptone†	37°	20	1×10^{-5} M	—	58
				—	2×-0^{-6} M	100
				1×10^{-5} M	2×10^{-6} M	55

* The buffered salt solution of Gale (1947).

† Medium containing 5 mg. Difco Bacto peptone/ml.

Suspensions of bacteria (3 mg./ml.) were incubated under the specified conditions. At the end of the incubation period, cells were harvested by centrifugation and the surface of the bacterial pellet rinsed with the buffered salt solution. The bacteria were resuspended in the buffered salt solution, prior to ultrasonic breakage and addition of (3×10^{-6} M) [^{14}C]chloramphenicol to the cell-sap containing the bacterial soluble fraction and ribosome subunits. After 5 min. at 4° the ribosome subunits were harvested by centrifugation at 150,000 *g.* for 150 min. and the radioactivity in the pellet estimated. Ribosomes and soluble fraction from 60 mg. bacteria were added per tube (2 ml. tubes).

and B, before breakage of the cocci and the addition of [^{14}C]CAP to the cell sap; the amount of chloramphenicol bound to ribosomes was then markedly decreased (Table 7), and component B enhanced the effect of component A on [^{14}C]CAP binding. Component A alone was also effective when cocci were incubated at 4° during the pretreatment with this antibiotic but the additional inhibition due to component B only occurred under conditions where growth would normally occur in the absence of the antibiotics (Table 7). Components A and B had no synergistic inhibitory effect on the growth of *Escherichia coli* and, in this organism, pre-treatment of intact cells with A+B did not enhance the inhibitory effect of component A in the binding of [^{14}C]CAP to ribosomes.

Since chloramphenicol protects *Staphylococcus aureus* against the bactericidal

effect of streptogramin, chloramphenicol might have inhibited the association of streptogramin with ribosomes. This was tested by treating *S. aureus* with a mixture of streptogramin and chloramphenicol before breakage of the cells and treatment of the cell-cap with [^{14}C]CAP (Table 8), but this did not annul the inhibitory effect of

Table 8. *Binding of [^{14}C]chloramphenicol to Staphylococcus aureus ribosomes after pre-treatment of the bacteria with the streptogramin antibiotics*

Antibiotics added during the initial incubation	[^{14}C]chloramphenicol binding (% of control)
Chloramphenicol (3·3 μg./ml.)	64
Streptogramin (0·6 μg./ml.)	36
Chloramphenicol and (3·3 μg./ml.) + streptogramin (0·6 μg./ml.)	21

Suspensions of *Staphylococcus aureus* (3 mg. bacteria/ml.) in medium A were incubated at 37° for 15 min. in the presence of the required antibiotic(s). At the end of the incubation period, cells were harvested by centrifugation and the experiment continued as described in Table 7.

Table 9. *The effect of the streptogramin antibiotics on the binding of [^{14}C]chloramphenicol to Bacillus megaterium ribosomes*

Soluble fraction		M conc. of antibiotic		[^{14}C]CAP binding
Amount	Treatment	Component A	Component B	(% of control)
—	—	6×10^{-8}	—	90
—	—	6×10^{-7}	—	38
—	—	6×10^{-6}	—	19
—	—	—	6×10^{-6}	100
—	—	6×10^{-8}	6×10^{-6}	92
From 50 mg. bacteria	—	6×10^{-8}	6×10^{-6}	68
From 100 mg. bacteria	—	6×10^{-8}	6×10^{-6}	56
From 50 mg. bacteria	Incubated 15 min. at 30° with the antibiotics	6×10^{-8}	6×10^{-6}	69
From 50 mg. bacteria	Boiled 10 min. and coagulated protein removed	6×10^{-8}	6×10^{-6}	71
From 50 mg. bacteria	Dialysed against buffer 24 hr. at 4°	6×10^{-8}	6×10^{-6}	92

The different experimental conditions set out above produced no alteration in the separate effects of component A (6×10^{-8} M) or component B (6×10^{-6} M) on the binding of ^{14}C-chloramphenicol.

Ribosome suspensions in 0·01 M-tris + HCl buffer, pH 7·4, containing 0·01 M-Mg^{2+} and 0·2 M-K^{+} were incubated for 5 min. at 4° with a mixture of (3×10^{-6} M) [^{14}C]chloramphenicol and the required antibiotic(s) and the additions as shown in the table. The ribosomes were harvested by centrifugation at 150,000 g for 100 min. and the radioactivity in the pellet estimated. 2 mg. of ribosomes were added per tube (2 ml. tubes).

streptogramin on the binding of [^{14}C]CAP by ribosomes. Similar results to those found with streptogramin were obtained with the equivalent mixture of components A and B.

Bacillus megaterium is more easily broken than *Staphylococcus aureus*, and further studies on the interaction of the streptogramin components have been carried out mainly with ribosomes from *B. megaterium*. The binding of [^{14}C]CAP to bacterial

ribosomes is not dependent on the presence of the soluble fraction; maximum binding to 70 S ribosomes occurs in 0·01 M-tris+HCl buffer, pH 7·4, containing 0·01 M-Mg^{2+} and 0·2 M-K^+ or NH_4^+ (Vazquez, 1964*b*). The previous finding that component B enhanced the inhibitory effect of component A on the binding of [^{14}C]CAP to ribosomes was not confirmed with ribosomes from *B. megaterium*, *E. coli* or *S. aureus* when these were suspended in 0·01 M-tris-HCl buffer containing either 0·01 M-Mg^{2+} and 0·2 M-K^+ (70 S ribosomes) or 0·001 M-Mg^{2+} and 0·06 M-K^+ (30 S and 50 S ribosome subunits). It was then found that enhancement of inhibition required the presence of the soluble fraction and that its extent depended upon the amount of soluble fraction added (Table 9).

Treatment of components A and B with soluble fraction before the addition of ribosomes did not increase the inhibitory effect on [^{14}C]CAP binding and the effect of the soluble fraction is not likely to be due to enzymic modification of either component A or B (Table 9). Removal of protein by boiling and centrifugation did not alter the effect of the supernatant fluid. After dialysis against buffer the non-dialyzable part of the soluble fraction was inactive (Table 9); ashed soluble fraction taken up as chloride or nitrate was inactive.

These results were obtained with ribosomes from *Bacillus megaterium* and have been confirmed with *Escherichia coli* ribosomes. Since enhancement can be demonstrated in cell-free systems from *E. coli*, but not with whole organisms, component B may be unable to penetrate the intact cells of Gram-negative bacteria.

DISCUSSION

These results show that the overall effects of streptogramin components A and chloramphenicol are very similar. The inhibitory effect of component A on [^{14}C]CAP binding suggests that it acts at the ribosome level. This suggestion is supported by the inhibitory effect of the antibiotic on amino acid incorporation (from aminoacyl-s-RNA into protein) in cell-free systems (Vazquez, 1965*c*). Inhibition of amino acid incorporation by other antibiotics of group A has also been observed in bacterial cell-free systems by other workers (Yamaguchi & Tanaka, 1964; Laskin & May Chan, 1964). However, chloramphenicol has a greater effect on the incorporation of proline and lysine than on the incorporation of phenylalanine, whereas component A inhibits the incorporation of the three amino acids to a similar extent (Vazquez, 1965*c*), which suggests that component A does not attach to ribosomes at precisely the same site as chloramphenicol. If so, the inhibitory effect of component A on [^{14}C]CAP binding to ribosomes might be due to an allosteric effect.

The site of action of streptogramin component B cannot be deduced from the present results. It has an immediate inhibitory effect on protein synthesis, and a delayed effect on cell wall and nucleic acid synthesis. The inhibitory effect of component B on protein synthesis has been confirmed in cell-free systems (Vazquez, 1965*c*).

The overall effects of streptogramin, or of equivalent mixtures of component A and component B, are similar to those of component A. There are, however, three important differences: (*a*) the minimum growth-inhibitory concentration of the mixture for *Staphylococcus aureus* is 10% of that of component B alone; (*b*) component A has an immediate effect on protein synthesis, whereas a lag period of about two minutes is observed before the synergistic mixture exerts its effect; (*c*) com-

ponent A is bacteriostatic, whereas the mixture of components A and B is bactericidal. No synergism between component A and component B has been found for inhibition of incorporation of proline, lysine and phenylalanine in cell-free systems directed respectively by polycytidylic, polyadenylic and polyuridylic acids (Vazquez, unpublished data). The above results suggest that the synergism of components A and B can be explained by an enhancement by component B of the binding of component A on ribosomes. A dialysable, low molecular weight, heat stable constituent of the soluble fraction, whose nature is at present unknown, is required for this enhancement.

I wish to express my gratitude to Dr D. Kerridge for valuable criticism and encouragement, to Professor E. F. Gale, F.R.S., for his advice and interest and to my colleagues of the Sub-Department of Chemical Microbiology for their helpful discussions. My thanks are due to The British Council and to The Ramsay Memorial Fellowship Trust for Fellowships and to all the colleagues who provided me with samples of different antibiotics.

REFERENCES

ANAND, N. & DAVIS, B. D. (1960). Effect of streptomycin on *Escherichia coli*. Damage by streptomycin to the cell membrane of *E. coli*. *Nature, Lond.* **185**, 22.

ARAI, M., NAKAMURA, S., SAKAGAMI, Y., FUKUHARA, K. & YONEHARA, H. (1956). A new antibiotic, mikamycin. *J. Antibiot. (Japan)*, Ser. A, **9**, 193.

BALL, S., BOOTHROYD, B., LEES, K. A., RAPER, A. H. & LESTER SMITH, E. (1958). Preparation and properties of an antibiotic complex E 129. *Biochem. J.* **68**, 24*P*.

BODANSZKY, M. & ONDETTI, A. (1963). Structures of the vernamycin B group of antibiotics. *Antimicrobial Ag. & Chemother.* p. 360.

BENAZET, F., COSAR, C., DUBOST, M., JULOU, L. & MANCY, D. (1962). Un nouvel antibiotique, la pristinamycine (7293 R.P.). *Sem. Hôp. Paris*, **38**, 13.

BURTON, K. (1956). A study of the conditions and mechanisms of the diphenylamine estimation of desoxyribonucleic acid. *Biochem. J.* **62**, 315.

CELMER, W. D. & SOBIN, B. A. (1955/56). The isolation of two synergistic antibiotics from a single fermentation source. *Antibiotics Ann.* p. 437.

CHARNEY, J., FISHER, W. P., CURRAN, C., MACHLOWITZ, R. A. & TYTELL, A. A. (1953). Streptogramin, a new antibiotic. *Antiobiotics & Chemother.* **3**, 1283.

COLEMAN, V. R., GUNNISON, J. B. & JAWETZ, E. (1953). Participation of erythromycin and carbomycin in combined antibiotic action *in vitro*. *Proc. Soc. exp. Biol. Med.* **83**, 668.

DE SOMER, P. & VAN DIJCK, P. (1955). A preliminary report on antibiotic number 899, a streptogramin-like substance. *Antibiotics & Chemother.* **5**, 632.

GALE, E. F. (1947). The assimilation of amino acids by bacteria. 1. The passage of certain amino acids across the cell wall and their concentration in the internal environment of *Streptococcus faecalis*. *J. gen. Microbiol.* **1**, 53.

GALE, E. F. (1963). Mechanisms of antibiotic action. *Pharmacol. Rev.* **15**, 481.

GALE, E. F. & FOLKES, J. P. (1953*a*). The assimilation of amino acids by bacteria. 14. Nucleic acid and protein synthesis in *Staphylococcus aureus*. *Biochem. J.* **53**, 483.

GALE, E. F. & FOLKES, J. P. (1953*b*). The assimilation of amino acids by bacteria. 15. Actions of antibiotics on nucleic acid and protein synthesis in *Staphylococcus aureus*. *Biochem. J.* **53**, 493.

GARCIA-MENDOZA, C. (1965). Studies on the mode of action of etamycin (viridogrisein). *Biochim. biophys. Acta*, **97**, 394.

GARROD, L. P. & WATERWORTH, P. M. (1956). Behaviour *in vitro* of some new antistaphylococcal antibiotics. *Brit. med. J.* ii, 61.

HANCOCK, R. (1960). Accumulation of pool amino acids in *Staphylococcus aureus* following inhibition of protein synthesis. *Biochim. biophys. Acta*, **37**, 47.

HANCOCK, R. & PARK, J. T. (1958). Cell wall synthesis by *Staphylococcus aureus* in the presence of chloramphenicol. *Nature, Lond.* **181**, 1050.

HURWITZ, C. & ROSANO, C. L. (1962). Chloramphenicol sensitive and insensitive phases of the lethal action of streptomycin. *J. Bact.* **83**, 1202.

JAWETZ, E., GUNNISON, J. B., BRUFF, J. B. & COLEMAN, V. R. (1952). Studies on antibiotic synergism and antagonism. *J. Bact.* **64**, 29.

KIRBY, W. M. M. & BURNELL, J. M. (1954). Effect of combinations of antibiotics on lysis of *Staphylococcus aureus* by penicillin. *J. Bact.* **67**, 50.

LASKIN, A. I. & MAY CHAN, W. (1964). Inhibition by vernamycin A of amino acid incorporation in *Escherichia coli* cell-free systems. *Antimicrobial Ag. & Chemother.* p. 485.

LESTER SMITH, E. (1963). The ostreogrycins. A family of synergistic antibiotics. *Proc. Soc. gen. Microbiol.* **33**, iii.

LOWRY, O. H., ROSEBROUGH, N. J., FARR, A. L. & RANDALL, R. J. (1951). Protein measurement with the Folin-phenol reagent. *J. biol. Chem.* **193**, 265.

McQUILLEN, K. & ROBERTS, R. B. (1954). The utilization of acetate for synthesis in *Escherichia coli*. *J. biol. Chem.* **207**, 81.

MILES, A. A. & MISRA, S. S. (1938). The estimation of the bactericidal power of the blood. *J. Hyg., Camb.* **38**, 732.

PRESTIDGE, L. S. & PARDEE, A. B. (1957). Induction of bacterial lysis by penicillin. *J. Bact.* **74**, 48.

SARIN, P. S. (1962). Structural studies on the antibiotics ostreogrycin A and G. Ph.D. Thesis. Cambridge University.

UMBREIT, W. W., BURRIS, R. H. & STAUFFER, J. F. (1949). *Manometric Techniques and Tissue Metabolism.* Minneapolis, U.S.A. Burgess Publishing Co.

VANDERHAEGHE, H., VAN DIJCK, P., PARMENTIER, G. & DE SOMER, P. (1957). Isolation and properties of the components of staphylomycin. *Antibiotics & Chemother.* **7**, 606.

VAZQUEZ, D. (1962). Studies on the mode of action of streptogramin. *Biochim. biophys. Acta*, **61**, 849.

VAZQUEZ, D. (1963*a*). The effect of streptogramin and related compounds on *Staphylococcus aureus*. *Proc. Soc. gen. Microbiol.* **33**, ix.

VAZQUEZ, D. (1963*b*). Antibiotics which affect protein synthesis: the uptake of ^{14}C-chloramphenicol by bacteria. *Biochem. biophys. Res. Commun.* **12**, 409.

VAZQUEZ, D. (1964*a*). The effect of streptogramin A and B on the binding of ^{14}C-labelled chloramphenicol to ribosomes. *Proc. Soc. gen. Microbiol.* **35**, v.

VAZQUEZ, D. (1964*b*). The binding of chloramphenicol by ribosomes from *Bacillus megaterium*. *Biochem. biophys. Res. Commun.* **15**, 464.

VAZQUEZ, D. (1965*a*). A method for the preparation of bacterial 50 S ribosomal subunits. *Biochim. biophys. Acta*, **108**, 154.

VAZQUEZ, D. (1965*b*). Binding of chloramphenicol to ribosomes. The effect of a number of antibiotics. *Biochim. biophys. Acta* (in the Press).

VAZQUEZ, D. (1965*c*). Antibiotics affecting chloramphenicol uptake by bacteria. Their effect on amino acid incorporation in a cell-free system. *Biochim. biophys. Acta* (in the Press).

YAMAGUCHI, H. & TANAKA, N. (1964). Selective toxicity of mikamycins inhibitors of protein synthesis. *Nature, Lond.* **201**, 409.

J. gen. Microbiol. (1966), **42**, 107–113
Printed in Great Britain

Micro-electrophoresis of Cowpox and Vaccinia Viruses in Molar Sucrose

By H. W. DOUGLAS, C. J. M. RONDLE and B. L. WILLIAMS

Departments of Bacteriology and Chemistry,
The University of Liverpool

(*Received* 27 *July* 1965)

SUMMARY

Preparations of cowpox and vaccinia viruses, grown on rabbit skin and on chick chorioallantois, purified by methods involving Arcton treatment or centrifugation in sucrose density gradients gave electrophoretically reproducible materials. A micro-electrophoresis technique is described with which measurements may be made conveniently and reproducibly on individual virus particles suspended in buffered molar sucrose solutions. The viruses had a mobility of approximately $-0{\cdot}4$ μ/sec./V./cm. in molar sucrose at pH 7 and ionic strength 0·05, their negative charge decreasing with decreasing pH value to give isoelectric points at pH 4·3 for cowpox, 3·7 for egg-grown vaccinia and 3·0 for rabbit-grown vaccinia; in greater acidities the viruses were positively charged. The results showed close reproducibility for the final preparations of cowpox of different origin and treatment, suggesting that the virus suspensions were relatively pure and free from adsorbed or extraneous material. Similar reproducibility was found for rabbit-grown vaccinia virus preparations, but there was some variation among different preparations of egg-grown vaccinia virus.

INTRODUCTION

So far as is known to the authors all previous electrophoretic studies on virus particles have been carried out either with the Tiselius moving-boundary method or with virus adsorbed on larger carrier particles. Thus Shedlovsky & Smadel (1940) showed that under carefully controlled conditions, purified preparations of vaccinia virus migrated as single boundaries in the Tiselius cell. Similar experiments by McFarlane (1940) were hampered by endo-osmotic disturbances, a difficulty which Shedlovsky & Smadel were able to eliminate by the addition of a small amount of protein to their suspensions. Both groups of workers arrived at about the same value, $-1\,\mu$/sec./V./cm., for the mobility of the virus at pH 7. No doubt the relatively large amount of purified virus required for moving-boundary experiments and the limited dispersion of virus particles at some pH values have been a deterrent to further studies.

In micro-electrophoretic methods, on the other hand, individual particles are observed and smaller volumes at low virus concentrations generally suffice so that, as suggested by Brinton & Lauffer (1959), the electrophoretic behaviour of virus particles may be more conveniently and economically studied. Over many years the micro-electrophoretic method has been applied with considerable success to a variety of cells in the size range 1–10 μ, e.g. bacteria and erythrocytes. No account

has been published however of a direct application of the technique to particles approximately 0·2 μ in size, though influenza virus adsorbed on much larger collodion particles was studied by Miller, Lauffer & Stanley (1944).

There are particular difficulties with virus particles. Thus their size and refractivity limit the applicability of the general methods of illumination (bright-field, phase-contrast) and only with a critically focused dark-field condenser arrangement may they be conveniently seen. Further, their Brownian movement in water is so large that they move out of a particular focal plane with frustrating rapidity and frequency, so that continued observation is not practicable. Increasing the viscosity of the suspending medium decreases this movement, and in molar sucrose, which has approximately three times the viscosity of water, continuous observation for a sufficient period for accurate mobility determinations (about 10 sec.) becomes readily possible by using a dark-field arrangement.

The present paper describes the technique used and results obtained in an initial study of preparations of cowpox and vaccinia viruses. These viruses are of the order of 0·25 μ and various methods have been devised for the preparation of purified suspensions for study of their physical and chemical properties.

METHODS

Viruses. The Brighton strain of cowpox and the Lister Institute strain of vaccinia were used. Each virus was grown on chick chorioallantois and on rabbit dermis. Virus passed at least 36 times on chick chorioallantois was used in the preparation of egg-grown virus. Virus maintained solely by dermal infection of rabbit was used in the preparation of rabbit-grown virus.

Virus purification. Virus was recovered from extracts of infected tissue by differential centrifugation and this crude material purified either by the use of Arcton (Epstein, 1958) or by sucrose density gradient centrifugation (Zwartouw, Westwood & Appleyard, 1962). In both instances the original methods were somewhat modified. In the Arcton method, the crude virus material was shaken once only with an equal volume of Arcton 113 for 5 min. at room temperature, and the aqueous layer containing suspended virus material then further purified by differential centrifugation for 30 min. at 10,000 rev./min. in a Spinco 40 rotor. In purifying the virus by the sucrose density gradient technique 2 ml. of crude virus suspension was layered on top of a 1 cm. band of 40% (w/v) sucrose, overlying similar bands of 50 and 60% sucrose contained in a 5 ml. Spinco tube, and the whole spun at 20,000 rev./min. for 20 min. in an SW 39 head. The 50% layer, which contained the bulk of the virus, was removed, mixed with 2 volumes of sterile water and the virus then deposited by centrifugation at 20,000 rev./min. for 30 min. in the Spinco 40 rotor. The supernatant fluid was replaced by molar sucrose solution, buffered to pH 7 with phosphate buffer of ionic strength 0·001, and the virus redispersed by ultrasonic treatment for 1 min. with an M.S.E.-Mullard ultrasonic disintegrator. Dilution, centrifugation and resuspension were repeated until the suspended particles appeared uniform by dark-field examination, uniform after staining by the silver technique of Gispen (1952) and constant in their electrophoretic behaviour in molar sucrose at pH 7. This was achieved after not more than 3 cycles in phosphate-buffered molar sucrose. The final virus suspensions had

infectivity titres of 10^9–10^{10} pock-forming units (p.f.u.)/ml. when titrated on chick chorioallantois. They were agglutinated by rabbit hyperimmune anti-vaccinial sera. Hence for each virus four kinds of final stock preparations in molar sucrose buffered at pH 7 were obtained; egg-grown purified with Arcton (EA); egg-grown purified by density gradient centrifugation (ES); and the corresponding materials for rabbit-grown virus (RA) and (RS).

Buffers and virus suspensions for electrophoresis. The buffers used were the acetate + veronal + HCl mixtures described by Michaelis (1931). For each electrophoretic experiment volumes of buffered virus suspension and of plain buffer, both molar to sucrose and of ionic strength 0·05, were prepared from the same intermediate buffer. This intermediate buffer was made from 5 ml. stock sodium acetate + sodium veronal solution (0·143 M each) + the volume of 0·1 M-HCl to give the required pH value, together with 5 ml. 0·714 M-NaCl to adjust the ionic strength, 57 ml. 1·66 M-sucrose, and sterile distilled water added to a final volume of 95 ml. Since the micro-cell used for electrophoresis required only 10 ml. to fill it and it was important to conserve the limited volumes of final stock suspension from each batch (about 10 ml. at 10^9–10^{10} p.f.u./ml.), only 20 ml. of diluted suspension (at about 10^8 p.f.u./ml.) were made for each mobility determination; 1 ml. of the virus stock in M-sucrose being added to 19 ml. of the intermediate buffer of the required pH value and ionic strength 0·0525. In addition to the 20 ml. of diluted virus, volumes of plain buffer ionic strength 0·05 and molar to sucrose were used for flushing one suspension out of the cell before introducing the next and also for the separate measurement of pH and electrical conductivity.

Micro-electrophoresis cell and optics. The electrophoresis cells used were of the pattern described by Douglas (1955, 1957), having a channel of rectangular section, 10 mm. wide by 0·2 mm. deep. The cell was mounted and operated on the vertical stage of a Watson 'Service' microscope as described in the earlier papers on bacterial systems. In replacing the phase-contrast optics by the dark-field arrangement used here, a Cooke, Troughton and Simms dark-field condenser mounted in a suitable adapter replaced the normal substage condenser and a Zeiss ×40 objective and ×15 eyepiece (with a linear scale) were used. The internal focusing adjustment of the condenser proved most convenient in obtaining optimum dark field at the top and bottom surfaces of the cell and the associated stationary levels, where there was no movement of fluid and at which the particle velocities were determined. A useful reserve in the intensity of the light scattered by the virus particles was obtained by supplying the power to the Watson transformer and ribbon-filament lamp (6 V, 108 W) through a Variac transformer so that the lamp could be somewhat over-run. The determination of cell depth and location of the stationary levels were made by using a Mercer gauge (graduated to 2 μ) rigidly mounted on the barrel of the microscope and with the probe bearing against the upper surface of the microscope stage.

Measurement of particle velocities. As in previous work (Douglas, 1957), the current in the micro-cell was adjusted so that virus particles in focus at one of the stationary levels of the cell took some 10 sec. to traverse 20 divisions of the eyepiece scale, equivalent to 52 μ in the focal plane of the objective. Under these conditions only a small proportion of particles initially in focus went out of focus before completing the course under the applied field; these were discounted. Particles were observed

at both stationary levels and travelling in both directions (current reversal), and at least 32 observations were made on different particles to obtain mean mobility values. In determining the frequency distribution of particle velocities for particular suspensions, the speeds of at least 128 individuals were measured.

Electrical measurements. The current in the cell giving the chosen rate of particle movement was measured using a Cambridge Unipivot d.c. microammeter. The potential gradient in the channel of the cell was calculated from this current together with the measured cross-sectional area of the cell and the electrical conductivity of equivalent virus-free buffer determined at 25° by using a Mullard dip-type cell and a Wayne–Kerr B221 bridge. The pH values of the buffers were checked by using a Beckman model 72 meter and glass electrode.

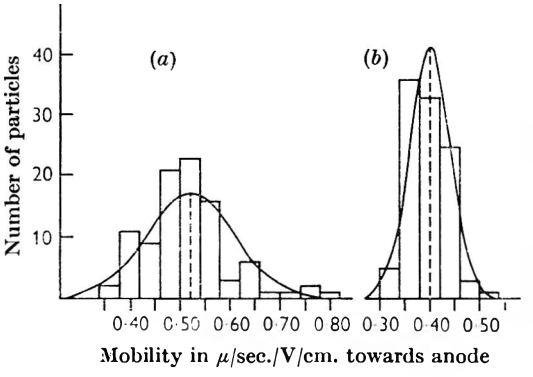


Fig. 1

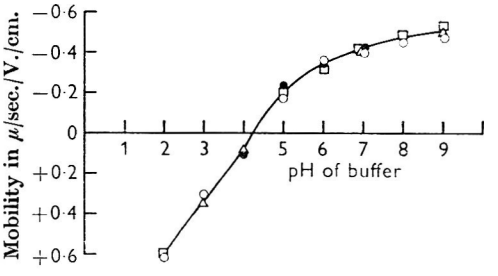


Fig. 2

Fig. 1. Histograms of frequency of individual particle mobilities in μ/sec./V/cm. (towards anode) for egg-grown cowpox preparations Br Ea 32 together with the corresponding normal distributions calculated from the standard deviation (S.D.) for (*a*) initial preparation, S.D. = 0·09, and (*b*) a final preparation, S.D. = 0·04 mobility units.

Fig. 2. Mean mobility against pH behaviour for final preparations of egg-grown and rabbit-grown cowpox at 25°, in media molar to sucrose and of ionic strength 0·05. ○, RS preparation, 32; ●, RA, 36; △, ES, 35; □, EA, 32, 34.

RESULTS

All particle velocities and mean mobility values presented here refer to buffered media, ionic strength 0·05 and molar to sucrose, at 25°.

Cowpox virus

It was essential to remove all host-cell debris during purification otherwise the light it scattered seriously hampered the observation of virus particles. Further, particulate material of different surface composition could alter the distribution of observed particle velocities and hence the mean mobility calculated for a given virus preparation. The two histograms shown in Fig. 1 illustrate this point, being typical of initial and final preparations. Washing the virus preparations narrowed the distribution of particle velocities until finally a limiting standard deviation and mean mobility value was reached. The smooth curves superimposed on the histograms are the corresponding Normal (Gaussian) distributions calculated from

the experimental standard deviations. Mean mobility values obtained for final preparations over a range of pH are plotted in Fig. 2, and show the agreement between different preparations obtained by one method and by different methods of purification.

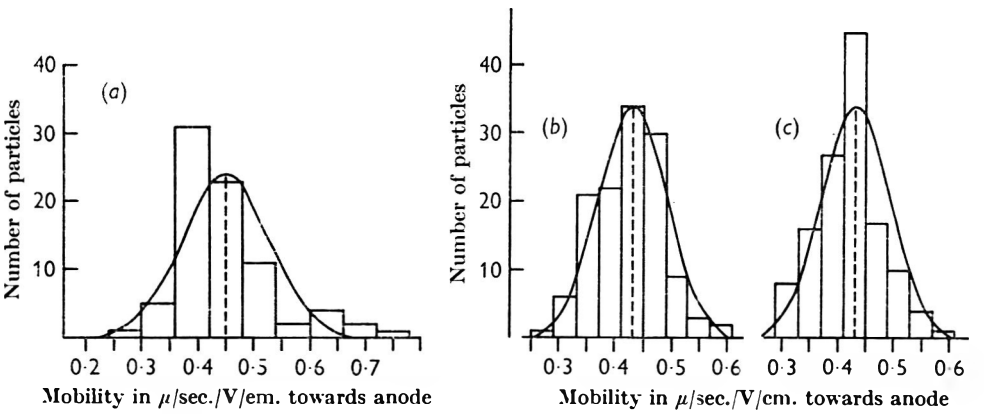


Fig. 3. Histograms of frequency of individual particle mobilities in μ/sec./V/cm. (towards anode) in egg-grown vaccinia preparations together with the corresponding normal distributions calculated from the standard deviation (S.D.) for (*a*) initial preparation, S.D. 0·08, (*b*) an intermediate preparation, S.D. 0·06 (2 phosphate-sucrose cycles), and (*c*) a final preparation, S.D. = 0·06 (3 phosphate-sucrose cycles).

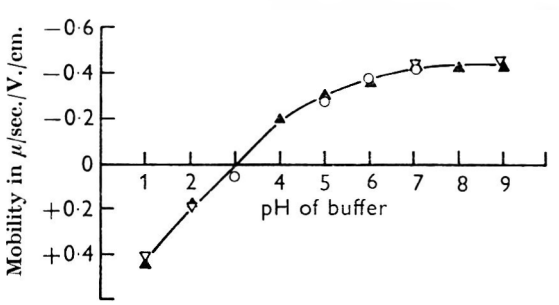


Fig. 4

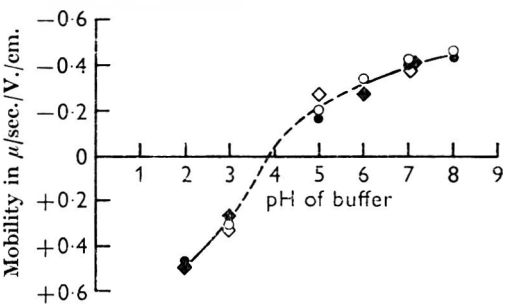


Fig. 5

Fig. 4. Mean mobility against pH behaviour for final preparations of rabbit-grown vaccinia at 25°, in media molar to sucrose and of ionic strength 0·05. ○, RA 14, S.D. 0·05; △, RS 15, S.D. 0·05; ▲, RS Porton, S.D. 0·05.

Fig. 5. Mean mobility against pH behaviour for final preparations of egg-grown vaccinia at 25°, in media molar to sucrose and of ionic strength 0·05. Mobility in μ/sec./V./cm., −ve towards anode, +ve towards cathode, S.D. 0·06 at pH 7. ○, ES 13; ●, ES 9; ◆, EA 14; ◇, EA 10.

Vaccinia virus

Preparations of this virus also were purified to limiting values for the standard deviation and the mean mobility at pH 7. Crude virus material again showed a wider distribution of particle velocities than final preparations and this is illustrated by the histograms and calculated Normal distributions of Fig. 3. Final preparations of rabbit-grown vaccinia virus, including material kindly supplied by Dr H. T. Zwartouw and purified by the method of Zwartouw *et al.* (1962) gave closely reproducible results and a definitive mobility against pH. The results for

preparations of egg-grown vaccinia agreed well *inter se* and also agreed with rabbit-grown material at pH 7. At more acid pH values some variation was shown and the egg-grown material differed from rabbit-grown virus (Fig. 5). Limiting standard deviations for final preparations of egg-grown and rabbit-grown vaccinia virus are included in the legends to these figures.

DISCUSSION

Except for some of the crudest virus preparations, the distributions of frequency of particle velocities were always unimodal and narrowed with purification. The narrowness of the distributions for final preparations and the reproducibility of results at pH 7 gives confidence that the particles in their populations were all of the same kind. This general result would be unlikely had any appreciable host-cell substance remained on the surface of the particles.

The mobility/pH curves are consistent with the surfaces of cowpox and vaccinia viruses being composed of lipoprotein or protein material which is slightly different for the 2 viruses. By interpolation and observation the isoelectric point in molar sucrose lies close to pH 4·3 for cowpox virus (both egg-grown and rabbit-grown material), to pH 3·0 for rabbit-grown vaccinia virus and pH 3·8 for egg-grown vaccinia virus. These values may have been determined in part by the sucrose added to reduce Brownian movement.

In the case of vaccinia the mobility at pH 7 ($-0{\cdot}42-\pm 0{\cdot}05\mu$/sec./V/cm.) may be compared with values obtained by the Tiselius method in buffers alone. Thus Shedlovsky & Smadel (1940) reported a value of $-1{\cdot}0\mu$/sec./V/cm. in buffer of ionic strength 0·02 and in the presence of a small amount of protein, while McFarlane (1940) also obtained an approximate mobility of -1μ/scc./V/cm., but in phosphate buffer of ionic strength 0·05 with no protein added. Since the viscosity of water at the temperature of Tiselius measurement is about 1·7 centipoise and molar sucrose has a viscosity of about 3·0 centipoise at 25°, McFarlane's value would correspond to a mobility of approximately $-0{\cdot}55\mu$/sec./V/cm. in molar sucrose at 25° if all other factors, including particle charge and size remained constant. However it is clear, both from the ultracentrifuge studies of Smadel, Pickels & Shedlovsky (1938) and from the partition experiments of McFarlane *et al.* (1939), that vaccinia particles are hydrated in aqueous suspension and that this hydration is reduced in the presence of sucrose. On the minimal hydration consistent with the findings of Smadel *et al.* there would be a decrease of about 8% in the effective particle radius on going from water to molar sucrose. This would tend to increase the mobility in molar sucrose above $-0{\cdot}55$ unless compensated by a decrease in effective charge, which might occur in the presence of molar sucrose and/or the change in the buffer system, or in some of the other factors detailed in the general equation relating particle velocity and charge as derived by Henry (1931).

The chemist authors are indebted to the War Office and the Microbiological Research Establishment (Porton) for a grant which made this work possible. They are also indebted for hospitality to Professor A. W. Downie, F.R.S., from

whom the authors have received helpful criticism. The authors appreciate Dr H. T. Zwartouw's helpfulness in supplying a sample of rabbit-grown vaccinia virus purified by his technique.

REFERENCES

BRINTON, C. C. & LAUFFER, M. A. (1959). The electrophoresis of viruses, bacteria and cells, and the microscope method of electrophoresis. In *Electrophoresis*. Ed. by M. Bier New York: Academic Press.

DOUGLAS, H. W. (1955). Electrophoretic studies on bacterial spores. *Trans. Faraday Soc.* **51**, 146.

DOUGLAS, H. W. (1957). Electrophoretic studies on spores and vegetative cells of certain strains of *B. megaterium*, *B. subtilis* and *B. cereus*. *J. appl. Bact.* **20**, 390.

EPSTEIN, M. A. (1958). Investigation into the purifying effect of a fluorocarbon on vaccinia virus. *Br. J. exp. Path.* **39**, 436.

GISPEN, R. (1952). Silver impregnation of smallpox elementary bodies after treatment with xylol. *Antonie van Leeuwenhoek*, **18**, 107.

HENRY, D. C. (1931). Cataphoresis of suspended particles. I. The equation of cataphoresis. *Proc. roy. Soc.* A, **133**, 106.

MCFARLANE, A. S. (1940). The electrical double layer and virus stability. *Trans. Faraday Soc.* **36**, 257.

MCFARLANE, A. S., MCFARLANE, M. G., AMIES, C. R. & EAGLES, G. H. (1939). A physical and chemical examination of vaccinia virus. *Br. J. exp. Path.* **20**, 485.

MICHAELIS, L. (1931). Der Acetat-Veronal Puffer. *Biochem. Z.* **234**, 139.

MILLER, G. L., LAUFFER, M. A. & STANLEY, W. M. (1944). Electrophoretic studies on PR8 influenza virus. *J. exp. Med.* **80**, 548.

SHEDLOVSKY, T. & SMADEL, J. E. (1940). Electrophoretic studies on elementary bodies of vaccinia. *J. exp. Med.* **72**, 511.

SMADEL, J. E., PICKELS, E. G. & SHEDLOVSKY, T. (1938). Ultracentrifugal studies on the elementary bodies of vaccine virus. II. Influence of sucrose, glycerol and urea solutions on the physical nature of the vaccine virus. *J. exp. Med.* **68**, 607.

ZWARTOUW, H. T., WESTWOOD, J. N. C. & APPLEYARD, G. (1962). Purification of pox virus by density gradient centrifugation. *J. gen. Microbiol.* **29**, 523.

J. gen. Microbiol. (1966), 42, 115–122
Printed in Great Britain

Cell-Wall Deficiencies in L-forms of *Staphylococcus aureus*

By B. C. PRATT*

Department of Bacteriology, Wright–Fleming Institute of Microbiology, St Mary's Hospital Medical School, London, W.2

(*Received* 3 *August* 1965)

SUMMARY

Three strains of penicillin-induced and one strain of D-cycloserine-induced L-forms of *Staphylococcus aureus* were investigated for components of the normal bacterial cell wall. None of the 4 strains was found to contain more than 0·008 % of its dry weight as muramic acid, indicating the lack of mucopeptide. These forms also lacked the cell-wall ribitol teichoic acid as shown by the failure to yield any material containing ribitol. Neither muramic acid nor ribitol was detected as acid-soluble nucleotides in the L-forms, and teichoic acid was not released into the supernatant medium. Serological evidence for polysaccharide A was not found, but there was serological identity between extracts of membranes obtained from staphylococci and extracts of membranes isolated from the L-forms.

INTRODUCTION

Since the report of Dienes & Sharp (1956) on the isolation of various organisms in the L-phase by using a medium containing a high salt concentration, serum and penicillin, there have been several studies on the biology of the L-forms, including those of *Staphylococcus aureus* (Schönfeld, 1959, 1961; Marston, 1961*a*, *b*; Williams, 1963). Although these reports, particularly the last, gave indirect evidence, such as osmotic fragility, phage and antibiotic insensitivity, for the lack of cell-wall material, no direct evidence was presented. Only one report has appeared on cell-wall components of staphylococcal L-forms; Sharp (1963) found a very low level of muramic acid in an L-form from staphylococcus H.

The mucopeptide of the cell wall has been shown to be absent or greatly decreased in amount both in streptococcal L-forms (Panos, Barkulis & Hayashi, 1959) and stable Proteus L-forms (Kandler, Hund & Zehender, 1958; Morrison & Weibull, 1962), and in the doubtfully related Mycoplasma group (Plackett, 1959). Further evidence for a deficiency in cell-wall components from L-forms has been provided by serological techniques; for example, the L-forms of group A streptococci were found to lack group-specific polysaccharide (Sharp, Hijmans & Dienes, 1957). Similar results had earlier been obtained in the cases of L-forms of *Streptobacillus moniliformis* (Klieneberger, 1942) and Proteus (Dienes, Weinberger & Madoff, 1950), although these studies antedated much important work on cell-wall structures. The present paper reports some attempts to demonstrate cell-wall components in L-forms of *Staphylococcus aureus*.

* Present address: Chemistry Department, University College, Swansea.

METHODS

Organisms and media. Three strains of staphylococcal L-form, 1444L, 6L and 42EL, previously described by Williams (1963), were used. A fourth strain, L Rogers, isolated from a penicillinase-producing staphylococcus (phage type 42E) by growth in the presence of D-cycloserine, was also used.

The solid and liquid media used for growth of the penicillin-induced L-forms were those described by Williams (1963). The media used for L Rogers were essentially the same except that more sodium chloride was added to give a final concentration of 4·5% (w/v) and the penicillin was replaced by D-cycloserine (Lilly) 500 μg./ml.

Broth cultures of the L-forms were harvested after 48 hr incubation at 37° by centrifugation at 27,000 *g* for 10 min. The pellet was washed once with ice-cold salt solution of the appropriate strength and then extracted by grinding for 5 min. with cold 10% (w/v) trichloroacetic acid (TCA). The residue was recovered by centrifugation and dialysed in the cold overnight against distilled water and lyophilized.

The parent staphylococci were grown in Difco brain heart infusion broth overnight at 37°, centrifuged, washed once with physiological saline, once with distilled water and either lyophilized or used directly for the preparation of cell walls.

Preparation of cell fractions. Cell walls of the parent staphylococci were isolated by the method of Salton & Horne (1951), by using a Mickle tissue disintegrator and no. 12 ballotini beads.

Staphylococcal cell membranes and membranes from the L-form strains were isolated essentially as described by Freimer (1963) for similar structures from group A streptococci and osmotically fragile protoplasts. The supernatant fluids remaining after removal of cell walls and membranes from suspensions of disrupted staphylococci (or membranes from suspensions of lysed L-forms) were centrifuged at 100,000 *g* for 1 hr and the resulting 'ribosomal' gel (Davison & Baddiley, 1963) was lyophilized.

Mucopeptide fractions from whole staphylococci were isolated by the method of Park & Hancock (1960) and by extraction with hot formamide (Fuller, 1938). Whole L-form was also extracted using these methods and the resulting residues lyophilized.

Attempts to isolate teichoic acids from staphylococci and L-forms were made by extraction with cold 10% (w/v) TCA (Armstrong *et al.* 1958).

Paper chromatography. Samples for the chromatographic detection of amino acids and amino sugars were hydrolysed in 4N-HCl at 100° for 16 hr in sealed tubes. The compounds were separated on Whatman no. 1 paper by an ascending two-dimensional technique using the solvents: (*a*) *n*-butanol + acetic acid − water (120 + 30 + 50, by vol.); (*b*) 2, 4/2, 5 lutidine + water (130 + 70, by vol.).

The dried papers were dipped in ninhydrin reagent (0·2% (w/v) in acetone) and heated for 5 min. at 90°. Sugar alcohols were released by hydrolysis in 2N-HCl at 100° for 3 hr and were separated by descending chromatography on Whatman no. 1 paper with solvent *n*-butanol + acetic acid + water (120 + 30 + 50, by vol.) Periodic acid + benzidine reagent (Smith, 1960) was used to detect the sugar alcohols.

Hexosamines. Hydrolysis of samples for the quantitative estimation of hexosamines was done in 4 N-HCl at 100° for 4 hr. The hydrolysates were freed from interfering chromogens according to Boas (1953) and the total hexosamine content estimated by the method of Rondle & Morgan (1955). Colours were read at 530 mμ in a 1 cm. optical cell and compared with a glucosamine standard.

The individual amino sugars were separated on charcoal + celite columns (Perkins & Rogers, 1959) and muramic acid estimated by a micro modification of the Rondle & Morgan test. The reagents used in the micro modification were those described for the original test, but the final test volume was only 1·5 ml. instead of 10 ml. Extinctions were read at a wavelength of 505 mμ in a 2 cm. micro-cell after 24 hr and compared with a standard of authentic muramic acid (kindly supplied by Dr H. R. Perkins).

Agar-gel diffusion. Tests were made in a gel consisting of 1 % (w/v) Ionagar no. 2 (Oxoid) in M/30 phosphate buffer (pH 7·2). Antigen preparations were suspended or dissolved in the same buffer. Antisera to staphylococci and L-forms were prepared in rabbits by a series of intravenous injections of whole organisms.

RESULTS

Hexosamines

Preliminary chromatographic evidence suggested that the amino sugar content of the L-forms was very much less than that of the parent staphylococci. In none of the four L-form strains was either muramic acid or glucosamine detected chromatographically, even when large (up to 10 mg.) samples of starting material were used. Large samples of L-form, which had not been extracted with cold 10 % TCA prior to drying, yielded on hydrolysis and chromatography a minute quantity of a substance corresponding in R_F values to glucosamine. On acid-washing of the harvested L-form, however, this chromatographic spot was not detectable.

Separation of the total hexosamines from hydrolysates and quantitative estimation confirmed the results obtained by chromatography. Whereas the total hexosamine separated from hydrolysates of bacterial cells accounted for 2·79–3·8 % of the dry weight of the organism, in hydrolysates of L-forms the amino sugars were not present in sufficient quantity to allow estimation. Consequently, the lower level of sensitivity of the test was taken as the maximum possible amino sugar content (Table 1).

Estimation of muramic acid content

The results above give total hexosamine content, but for the purposes of this investigation, estimation of the muramic acid content was of prime importance. Hydrolysates of both cocci and L-forms were treated on charcoal + Celite columns and the eluates that could be expected to contain muramic acid were collected. Estimation of the muramic acid contents of the eluates from L-forms gave very low values in the conventional Rondle & Morgan test, with glucosamine as a standard. Greater sensitivity was gained, however, by using the micro modification of the Rondle & Morgan test and by using authentic muramic acid as standard. In an effort to concentrate muramic acid further, the fractions of whole organisms,

Table 1. *Total hexosamine content of staphylococci and L-forms*

	Sample (mg.)	Hexosamine* content (μg.)	Hexosamine as % dry weight
1444 cocci	10·18	284	2·79
L-form 1	12·20	<10	<0·082
2	26·50	<10	<0·038
PS6 cocci	10·62	309	2·91
L-form 1	7·10	<10	<0·141
2	31·20	<10	<0·032
PS42E cocci	9·65	279	2·89
L-form 1	6·70	<10	<0·149
2	28·80	<10	<0·035
Rogers cocci	2·50	95	3·80
L-form 1	6·70	<10	<0·149
2	12·50	<10	<0·080

* Material eluted by 2N-HCl from Dowex 50 H^+, estimated in Rondle & Morgan (1955) test at 530 mμ and expressed as glucosamine base.

Table 2. *Muramic acid contents of isolated fractions from whole staphylococci and L-forms*

Organism	Residue from extraction	Equiv. dry wt. organisms (mg.)	Muramic acid recovered (μg.)	Muramic acid in residue (%)	Muramic acid in whole organisms (%)
1444 cocci	S+H†	11·1	278*	13·9	2·50
	P+H	8·1	260	13·0	3·20
	Form	14·2	415	20·7	2·92
L-form	S+H	67·2	<4	<0·08	<0·006
	P+H	113·0	<4	<0·14	<0·004
	Form	56·1	<4	<0·12	<0·007
PS6 cocci	S+H	13·2	363	18·1	2·75
	P+H	17·8	593	24·7	3·33
L-form	S+H	79·0	<4	<0·07	<0·005
	P+H	55·6	<4	<0·21	<0·007
PS42E cocci	S+H	12·2	327	16·3	2·68
	P+H	15·9	459	22·4	2·89
L-form	P+H	47·9	<4	<0·24	<0·008
Rogers cocci	S+H	11·6	322	16·1	2·78
	P+H	12·5	406	20·3	3·25
	Form	10·5	317	15·9	3·02
L-form	S+H	53·4	<4	<0·25	<0·007
	P+H	55·5	<4	<0·04	<0·007

* Extraction methods used: Salton & Horne (1951), S+H: Park & Hancock (1960), P+H; Fuller (1938), Form.

† Estimated using the micro modification of Rondle & Morgan (1955) method; expressed in terms of a muramic acid standard.

whether cocci or L-forms, likely to contain the amino sugar were isolated. Hydrolysis of these fractions and estimation of their muramic acid contents gave the results shown in Table 2.

Since the results shown in Table 2 were obtained with preparations of L-forms which had been extracted with 10% (w/v) TCA, there remained the possibility that

the L-forms might have contained small amounts of muramic acid in an acid-soluble form. Trichloroacetic acid was removed from the extracts with ether, and the aqueous solutions dried. Hydrolysis of the acid-soluble material and subsequent amino sugar estimation yielded no evidence of muramic acid.

Although the level of estimation of muramic acid was considerably decreased by using the micro modification, no detectable colour at 505 mμ was found in hydrolysates of fractions from L-forms. The maximum amount of muramic acid which could be present was taken as 4 μg. By virtue of the characteristic spectrum of muramic acid in this test, lower values than 4 μg. were detectable but could not be measured with certainty. Even so, the results obtained serve to show the absence of muramic acid and so of cell-wall mucopeptide from the L-forms of *Staphylococcus aureus*.

Cell-wall teichoic acid

The loss of mucopeptide during induction to the L-phase raises the question of the fate of the other major cell-wall polymer, the ribitol teichoic acid. If it were still produced, this teichoic acid could be found in two possible sites, still attached to the cell even in the absence of rigid mucopeptide, or it could be released into the medium as produced. Whole L-form preparations were hydrolysed to release any sugar alcohols that they might contain and the hydrolysates subjected to paper chromatography. At the same time, samples of whole cocci were similarly treated. Staining of the developed chromatograms with the periodic acid + benzidine reagent revealed the presence of spots corresponding to ribitol and anhydroribitol in hydrolysates of cocci. Small amounts of glycerol were also detected. Faint traces of glycerol but no spots corresponding to ribitol or anhydroribitol were evident in hydrolysates of L-forms.

Prolonged extraction of samples of L-forms with cold 10% (w/v) TCA, the normal procedure for extraction of teichoic acids, did not remove any material containing either ribitol or glycerol. The same treatment, when applied to the parent staphylococci, extracted an ethanol-insoluble substance, which on hydrolysis and chromatography yielded spots characteristic of both ribitol- and glycerol-teichoic acids.

Hydrolysates of freeze-dried supernatant medium from L-form cultures contained no detectable ribitol or anhydroribitol, but did contain traces of glycerol. In spite of this finding, prolonged extraction of the broth residue with cold TCA and attempts to precipitate glycerol teichoic acid proved fruitless.

Agar-gel diffusion

Further evidence for the lack of ribitol teichoic acid from the L-form strains was provided by gel diffusion experiments. Whereas suspensions of whole staphylococci or cell walls produced a characteristic 'polysaccharide A' precipitation line when diffused against antiserum to *Staphylococcus aureus*, suspensions of the L-forms did not. No 'polysaccharide A' was extracted from any of the L-form strains by the technique of Haukenes, Losnegard & Oeding (1961). The L-forms produced only what appeared to be a single line with the staphylococcal antiserum; and this line showed identity with one produced by intracellular contents isolated from the staphylococci.

Since the normal cell wall of the coccus had been so drastically altered during induction to the L-form, the normal means of serological identification were lost. However, trypsin extracts of membranes isolated from the four L-form strains, when diffused against L-form antiserum produced apparently single precipitation lines (Fig. 1). These lines showed fusion with each other, indicating the serological identity of the original membranes. Similar preparations of 42E staphylococcal cell membranes also produced a single precipitation line with L-form antiserum. Fusion of this line with that produced by a trypsin preparation of 42E L-form membranes (Fig. 2) established the similarity between the membrane structures of the staphylococcus and its L-form. This relationship held for all four strains of staphylococci and L-forms, and cross-reaction between the strains was complete.

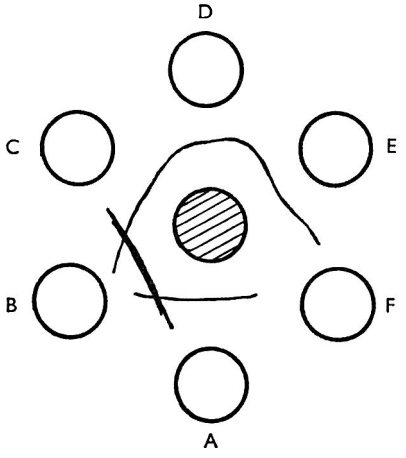


Fig. 1

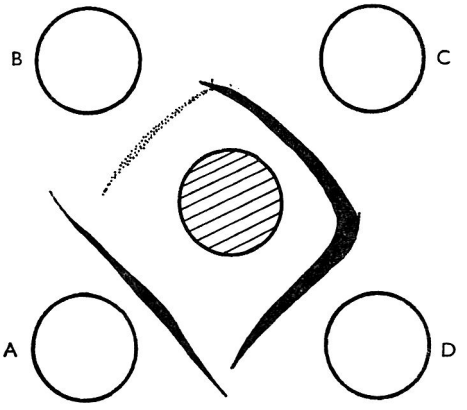


Fig. 2

Fig. 1. Agar-gel precipitin reactions of 1444 L-form antiserum (centre well) with trypsin extracts of membranes isolated from (A) 1444L, (C) 42EL, (D) 6L and (E) L Rogers. Well B contains a buffer suspension of whole 1444L-form and F a trypsin-buffer control.

Fig. 2. Precipitin reactions of 42E L-form antiserum (centre well) with (A) whole 42 E L-form, (B) trypsin extract of 42E cell walls, (C) trypsin extract of 42E staphylococcal cell membranes and (D) trypsin extract of 42E L-form membranes.

DISCUSSION

The nature of the change produced in the cell wall of *Staphylococcus aureus* during induction to the L-form was indicated by Williams (1963), who described the osmotic sensitivity and insusceptibility to phage action in the staphylococcal L-form. These characters, together with the evidence of antibiotic sensitivities, presented in the same paper, pointed to an alteration in surface structure in the L-form, but did not of themselves provide proof of total lack of cell wall material. Sharp (1963) presented evidence for the absence of muramic acid from one strain of staphylococcal L-form, that of *S. aureus* H. The results of the chemical analyses for muramic acid content of the four staphylococcal L-forms agree with the findings reported by Sharp. However, a further indication of the extensive loss of cell-wall material from the L-forms is evident from the failure to find ribitol teichoic

acid in these organisms. Since the precise structural relationship between the cell-wall teichoic acid and the muramic-acid-containing mucopeptide is unknown, it is impossible to judge whether the non-production of ribitol teichoic acid might be caused by the absence of rigid mucopeptide or is a more direct consequence of antibiotic action.

In L-forms of group A streptococci, the M protein, which is normally found in the cell wall, is still produced but is liberated into the culture medium (Sharp, Hijmans & Dienes, 1957). The possibility was considered that the teichoic acid of the staphylococci might behave in the same way but no evidence for this was found.

With the absence of cell wall and the loss of characters such as cell-wall-associated antigens, Gram-staining properties and typical morphology, the identification of the L-forms as being derived from *Staphylococcus aureus* is difficult. Williams (1963) described the continued coagulase production by, and lysogenicity of, the L-forms. The present finding that the surface membrane of the L-form bears a close serological relationship to the cell membrane of the staphylococcus provides additional evidence on the origin of the L-form strains.

The author gratefully acknowledges the technical assistance of Miss J. Corse during this investigation. The work was supported by a grant from Pfizer Ltd.

REFERENCES

ARMSTRONG, J. J., BADDILEY, J., BUCHANAN, J. G., CARSS, B. & GREENBERG, G. R. (1958). Isolation and structure of ribitol phosphate derivatives (teichoic acids) from bacterial walls. *J. chem. Soc.* p. 4344.

BOAS, N. F. (1953). Method for the determination of hexosamines in tissues. *J. biol. Chem.* **204**, 553.

DAVISON, A. L. & BADDILEY, J. (1963). The distribution of teichoic acids in staphylococci. *J. gen. Microbiol.* **32**, 271.

DIENES, L. & SHARP, J. T. (1956). The role of high electrolyte concentration in the production and growth of L-forms of bacteria. *J. Bact.* **71**, 208.

DIENES, L., WEINBERGER, H. J. & MADOFF, S. (1950). Serological reactions of L-type cultures isolated from Proteus. *Proc. Soc. exp. Biol. Med.* **75**, 409.

FREIMER, E. H. (1963). Studies of L-forms and protoplasts of group A streptococci. II. Chemical and immunological properties of the cell membrane. *J. exp. Med.* **117**, 377.

FULLER, A. T. (1938). The formamide method for the extraction of polysaccharides from haemolytic streptococci. *Br. J. exp. Path.* **19**, 130.

HAUKENES, G., LOSNEGARD, N. & OEDING, P. (1961). Extraction and fractionation of antigenic material from *Staphylococcus aureus*. *Acta path. microbiol. scand.* **53**, 84.

KANDLER, O., HUND, A. & ZEHENDER, C. (1958). Cell wall composition in bacterial and L-forms of *Proteus vulgaris*. *Nature, Lond.* **181**, 572.

KLIENEBERGER, E. (1942). Some new observations bearing on the nature of the pleuropneumonia-like organism known as L1 associated with *Streptobacillus moniliformis*. *J. Hyg., Camb.* **42**, 485.

MARSTON, J. (1961*a*). Observations on L-forms of staphylococci. *J. infect. Dis.* **108**, 75.

MARSTON, J. (1961*b*). Cultivation of staphylococcal L-forms in a liquid medium. *J. Bact.* **81**, 832.

MORRISON, T. H. & WEIBULL, C. (1962). The occurrence of cell wall constituents in stable Proteus L-forms. *Acta path. microbiol. scand.* **55**, 475.

PANOS, C., BARKULIS, S. S. & HAYASHI, J. A. (1959). Streptococcal L-forms. II. Chemical composition. *J. Bact.* **78**, 863.

PARK, J. T. & HANCOCK, R. (1960). A fractionation procedure for studies of the synthesis of cell-wall mucopeptide and of other polymers in cells of *Staphylococcus aureus*. *J. gen. Microbiol.* **22**, 249.

PERKINS, H. R. & ROGERS, H. J. (1959). The products of the partial acid hydrolysis of the mucopeptide from cell walls of *Micrococcus lysodeikticus*. *Biochem. J.* **72**, 647.

PLACKETT, P. (1959). On the probable absence of 'mucocomplex' from *Mycoplasma mycoides*. *Biochim. biophys. Acta*, **35**, 260.

RONDLE, C. J. M. & MORGAN, W. T. J. (1955). The determination of glucosamine and galactosamine. *Biochem. J.* **61**, 586.

SALTON, M. R. J. & HORNE, R. W. (1951). Bacterial cell wall. II. Methods of preparation and some properties. *Biochim. biophys. Acta*, **45**, 355.

SCHÖNFELD, J. K. (1959). 'L'-Forms of staphylococci; their reversibility; changes in the sensitivity pattern after several intermediary passages in the 'L' phase. *Antonie van Leeuwenhoek*, **25**, 325.

SCHÖNFELD, J. K. (1961). 'L'-Forms of *Staphylococcus*. II. Studies on the morphology of the transformation and on the reversibility. *Antonie van Leeuwenhoek*, **27**, 139.

SHARP, J. T. (1963). Amino sugars in L-forms of bacteria and pleuropneumonia-like organisms. *J. Bact.* **86**, 692.

SHARP, J. T., HIJMANS, W. & DIENES, L. (1957). Examination of the L-forms of group A streptococci for the group-specific polysaccharide and M protein. *J. exp. Med.* **105**, 153.

SMITH, I. (1960). *Chromatographic Techniques*, Vol. I. London: Heinemann.

WILLIAMS, R. E. O. (1963). L-forms of *Staphylococcus aureus*. *J. gen. Microbiol.* **33**, 325.

J. gen Microbiol. (1966), 42, 123–131
Printed in Great Britain

On the Nature of Mixed Cultures of *Chlorella pyrenoidosa* TX 71105 and Various Bacteria

By G. R. VELA* and CLESTE N. GUERRA
United States Air Force School of Aerospace Medicine, Brooks Air Force Base, Texas, U.S.A.

(*Received* 4 *August* 1965)

SUMMARY

The growth of several selected micro-organisms in rapidly dividing cultures of *Chlorella pyrenoidosa* TX 71105 was studied. Bacterial proliferation was a function of algal growth and bacterial growth occurred, at least in part, as a result of the excretion of organic substances into the culture medium by rapidly dividing algae. These substances capable of supporting bacterial oxidation and growth were varied in kind and were utilized selectively by the different bacteria. Only a small fraction of the soil and air bacteria grew in the algal cultures. The majority of soil and air bacteria survived in mixed culture for several days but did not increase in numbers. On the other hand, 6 out of 8 bacteria pathogenic for man died promptly in cultures of *Chlorella pyrenoidosa*; but *Salmonella typhi* and *S. paratyphi* grew well for extended periods of time. Fungi capable of producing macrocolonies on potato glucose agar at pH 3·5 did not increase in numbers during 8 days. Yeasts and actinomycetes were not detected by the methods used; bacteriophages were observed with some regularity.

INTRODUCTION

Recent reports proposing the use of microalgae in 'closed ecological systems' suitable for space vehicles have described the inherent advantages of these organisms as gas exchangers, transformers of human wastes, and as food material (Eley & Myers, 1964; Myers, 1964; Brown, Kennedy & Tischer, 1964; McDowell & Leveille, 1964; Krauss, 1962). It is well recognized that the utilization of algae as symbionts with man in a closed space will result not in a two-organism system but rather in a very complex biological universe. Because of the impracticality of maintaining pure cultures of algae for long periods of time, even in the laboratory, it is assumed that algal populations mixed with selected bacteria will be used. In the opinion of the authors, the feasibility of using mass cultures of algae with or without bacterial contaminants is at present precluded by a dearth of knowledge of the effects of microbial contamination on the algal culture systems. The problem of bacterial contamination of algal cultures has been noted (Krauss & Thomas, 1954; Eley & Myers, 1964; Ward, Moyer & Vela, 1964; Blasco, 1965) but, unfortunately, there are few studies which elucidate the ecological and physiological relationships that must exist in cultures of algae and other micro-organisms living together. A search of the literature showed that previous investigations of the microbiology of algal cultures were concerned primarily with population

* Present address: Department of Biology, North Texas State University, Denton, Texas.

densities and with the gross effects of bacterial contamination on the algal populations. Myers, Phillips, & Graham (1951) stated that the presence of bacteria had little effect on the growth rates of *Chlorella pyrenoidosa*. Myers (1957) reported that bacterial and mould contaminants usually have no effect on algae in rapidly growing steady-state cultures. Mayer, Zuir, Shain & Ginzburg (1964) stated that bacterial contamination of mass cultures (2000 l.) of algae was not a serious complicating factor and apparently had little or no effect on the productivity of the culture. Ward *et al.* (1964) presented data that indicated that the yield of mass of *C. pyrenoidosa* TX 71105 in steady-state cultures was substantially decreased as a result of bacterial contamination, while Nakamura (1963) proved that selected bacterial species enhanced the growth of algae in nutritionally deficient cultures. Nakamura postulated that mixed cultures of Chlorella and bacteria form a true symbiotic relationship in nature and that this is also the case in laboratory cultures. To the contrary, Vladimirova (1960) showed that *C. pyrenoidosa* Pringh. suppressed bacterial growth. Further studies on the bacteriology of algal cultures were clearly indicated. The purpose of the present report is to present data which may help to characterize the relationships that exist in mixed cultures of algae and other micro-organisms and to evaluate the feasibility of using these in closed systems for the support of man.

METHODS

Organisms. All work was done with the strain of *Chlorella pyrenoidosa* designated TX 71105 by Sorokin & Myers (1953). Bacteria utilized included *Bacterium anitratum* (BAT), *B. anitratum* atypical (BAA), *Mima polymorpha* (MP), and a Gram-negative organism tentatively assigned to the genus *Flavobacterium* (GNB). Cultures of fourteen other species of bacteria capable of growing in algal cultures were obtained from mass cultures of algae growing in open non-sterile systems. In addition to the above, a collection of soil and air bacteria was made in locales far removed from laboratory algal cultures. All organisms were selected on the basis of colony morphology and were probably all different one from the other and from BAT, BAA, MP and GNB.

Recently isolated pathogenic organisms of proven virulence used in several experiments were: *Salmonella typhi*, *S. paratyphi*, *Shigella flexneri*, *S. dysenteriae*, *Proteus vulgaris*, *Streptococcus pyogenes*, *Staphylococcus aureus* and *Corynebacterium diphtheriae*.

Cultures and analytical methods. The medium used in all flask culture experiments was Knop's medium (Phillips & Myers, 1954). It was prepared by adding the following, in order, to 950 ml. water: 50 ml. solution I, 10 ml. solution II, 0·05 g. $FeSO_4.7H_2O$; 10 ml. solution III, 2·5 g. KNO_3, adjusted to pH 6·0 before autoclaving.

Solution I: 25·0 g. $MgSO_4.7H_2O$, 20·0 g. KNO_3, 10·0 g. KH_2PO_4, 2·1 g. $CaCl_2$, 2·85 g. H_3BO_3, and water to 1 l.

Solution II: 8·82 g. $ZnSO_4.7H_2O$, 1·44 g. $MnCl_2.4H_2O$, 0·924 g. H_2MoO_4 (85%), 1·57 g. $CuSO_4.5H_2O$, 0·49 g. $Co(NO_3)_2.6H_2O$, and water to 1 l.

Solution III: 50 g. ethylenediamine tetra-acetic acid (EDTA), 31 g. KOH, and water to 1 l.

The same Knop medium was used in mass culture experiments except that

KNO_3 was replaced by urea in a final concentration of 1·5 g./l. (1·1 times equivalent nitrogen). This substitution was made since it is felt that mass cultures of algae in space vehicles would probably use urine urea as nitrogen source.

Small flask cultures were grown in an incubator shaker fitted with fluorescent lights. The gaseous atmosphere of the incubator was maintained at 2% (v/v) CO_2 by introducing a mixture of CO_2 and air at the rate of 1 l./min.; incubation temperature was 37°, shaker excursion 5 cm. and fast enough to cause a 'splash' against the flask side but not a 'spray' up to the stoppers. One-litre Erlenmeyer flasks containing 100 ml. medium and capped with beakers were used as culture vessels.

Studies on the nature of microbial populations in mass cultures (32 l.) were done in a photosynthetic gas-exchange culture apparatus of the type described by Ward, Wilks & Craft (1963). 5% (v/v) CO_2 in air was introduced into the culture and the temperature maintained at 39°.

Algal populations in all experiments were monitored by replicate counts with a haemocytometer. Bacterial numbers were estimated by triplicate plate counts on Trypticase Soy agar or, in the case of the pathogenic bacteria, Difco brain heart infusion agar. Yeasts and fungi were enumerated on Difco potato glucose agar (pH 3·5) according to the American Public Health Association Manual on Standard Methods for the Examination of Dairy Products (11th ed. 1960).

Actinomycetes and certain pigmented bacteria were grown in a solution containing the following ingredients (g./l.): 2·0 g. $NaNO_3$, 1·0 g. K_2HPO_4, 0·5 g. $MgSO_4.7H_2O$, 0·5 g. $FeSO_4.5H_2O$, 20 g. reagent grade agar, and 10 g. glucose added as a sterile 10% (w/v) aqueous solution to the sterile basal medium.

Culture filtrates. Soluble algal excretion products were separated from organisms by centrifugation and filtration through 0·45 μ washed (Winneberger, Austin & Klett, 1963) filter membranes. Further separation was obtained by dialysis and extraction as outlined in Fig. 3. Warburg manometry was used to determine which of the fractions obtained were capable of supporting bacterial oxidation. Substances isolated by extraction with organic solvents were taken to dryness, redissolved in water, and taken to dryness again for three complete cycles in an effort to avoid bacterial oxidation of residual organic solvents. Further analyses of the fractions containing oxidizable substances were done by paper and thin-layer chromatography.

RESULTS

Flask culture studies

In previous reports (Ward *et al.* 1963; Ward *et al.* 1964) workers at this laboratory had noted that the growth of bacteria in algal cultures closely paralleled algal growth. The data presented in the present report confirm and extend those findings. Small flask cultures of chlorella TX 71105 contaminated with pure cultures of selected bacteria were sampled repeatedly during the algal growth phase and well into the stationary phase. Fig. 1 represents an experiment typical of a large number of studies designed to reveal the kinetics of growth of mixed cultures of chlorella TX 71105 and various bacteria. It was evident that the bacterial populations increased only while the algal component of the culture was actively dividing. It was also noted that bacterial populations at stationary

phase of algal growth were always of about the same numerical magnitude as the algal populations. This equilibrium was reported previously by Mayer *et al.* (1962) in large open culture tanks. Since other investigations (Fogg, 1952) have shown

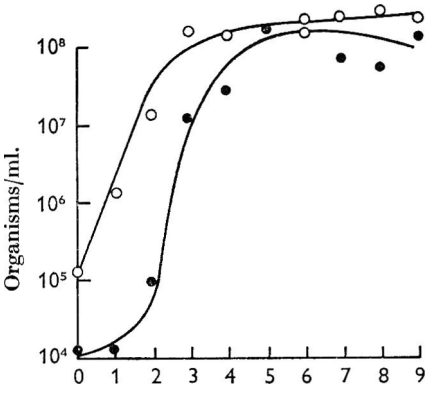


Fig. 1

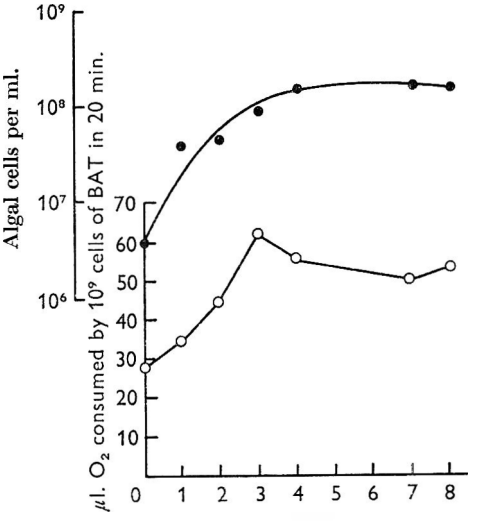


Fig. 2

Fig. 1. Growth curves of *Chlorella pyrenoidosa* TX 71105 and the Gram-negative bacterium (GNB) added to the culture as a bacterial contaminant. Algal cells, ○; viable bacterium GNB, ●.

Fig. 2. Excretion of oxidizable metabolites by axenic *Chlorella pyrenoidosa* TX 71105 (lower) as a function of growth (upper). Oxidizable metabolites were measured by classical Warburg manometry.

Table 1. *Selective utilization of oxidizable substances by bacteria growing in algal cultures*

	Ratio of oxygen consumed by these test bacteria*			
	BAT†	MP†	BAA†	GNB†
Filtrates from 4-day cultures of the following:				
Axenic chlorella TX 71105	0	0·47	0·58	0·68
TX 71105 + BAT	0	0	0	0·16
TX 71105 + MP	0	0·03	0·03	0·16
TX 71105 + BAA	0	0·02	0	0·37
TX 71105 + GNB	0·12	0·07	0·15	0
TX 71105 + all 4	0	0	0·01	0

* Ratio of μl. 0_2 consumed by 10^9 starved bacteria in 20 min. Ratio represents the amount of oxygen utilized by test bacteria using culture filtrate as substrate and 0·025 M-glucose as substrate. The results were corrected for endogenous respiration as measured by using Knop's medium i.e.;

$$\frac{\text{Culture filtrate} - \text{Knop's medium}}{\text{0·025 M-glucose} - \text{Knop's medium}}$$

† BAT, *Bacterium anitratum*; MP, *Mima polymorpha*; BAA, *Bacterium anitratum* atypical; GNB, Gram-negative rod possibly *Flavobacterium* sp.

that some algae excrete larger amounts of organic material during active growth periods than they do at resting stages, it was assumed that the same phenomenon occurred in cultures of chlorella TX 71105. To prove that rapidly dividing TX 71105 excreted large amounts of substances capable of supporting bacterial respiration and growth, axenic cultures of the chlorella were sampled daily and assayed for these materials. Figure 2 shows that the excretion of water-soluble substances capable of supporting bacterial respiration was a function of algal growth. These data were confirmed by studies which showed that substances capable of supporting bacterial growth were also excreted in the same fashion. The data (Fig. 2) also indicated that these substances accumulated in the culture fluid and were not removed completely by algal activities or autolytic reactions.

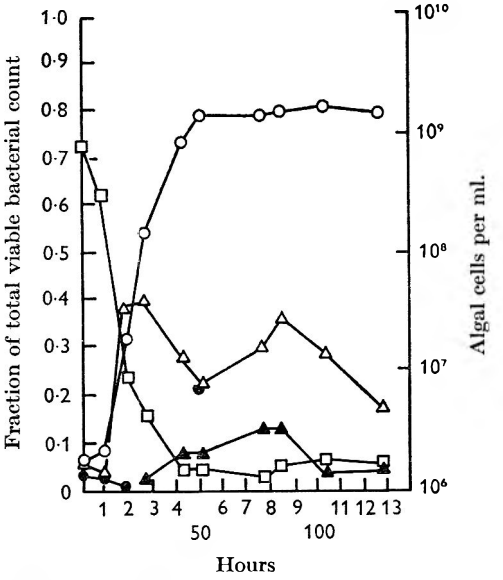


Fig. 3. Shifts in bacterial populations as a function of culture age. Algal cells, ○; 'white colonies', △; 'brown colonies', □; 'green colonies', ●; and 'red colonies', ▲. Each colony type listed is plotted as a fraction of the total viable bacterial count.

It was then reasoned that if bacteria grew on the excreted metabolites, filtrates from contaminated algal cultures should be free from these metabolites. The data in Table 1 verify this and show that more than one substance was excreted by the chlorella since bacterium GNB could utilize substances not oxidizable by bacteria BAT, BAA and MP.

The products of algal metabolism that can be oxidized by bacteria were dialysable in distilled water when placed in Visking membranes (Union Carbide Corporation, 6733 West 65th Street, Chicago, Illinois). Fractionation of the dialysate (Fig. 4) proved that various classes of chemical compounds capable of supporting bacterial respiration were excreted by chlorella TX 71105. The progressive increase in capacity to support respiration of each fraction as purification proceeded cannot be explained at this time. It can, however, be stated that the phenomenon was reproducible. Several experiments have shown that it was not a matter of

antagonism since mixing of the separated fractions did not result in lowered oxidative capacity of the purer fractions. That is, mixing equal parts of the non-dialysable residue and the final extract (or any other combination) gave oxygen uptakes proportional to the quantity of ether-extractable material present, and no inhibition was observed. Also, mixtures of, say, non-dialysable residue and glucose did not result in lowered rates of oxygen consumption by bacteria utilizing the glucose.

Table 2. *Growth of bacteria unrelated to algal communities in flask cultures of Chlorella pyrenoidosa* TX *71105*

Isolate number	Viable bacteria/ml.	
	Day 0	Day 4
R-PD 1	$1{\cdot}1 \times 10^5$	$1{\cdot}2 \times 10^4$
C-KS 1	$1{\cdot}9 \times 10^6$	$<1 \times 10^3$
R-BR	$4{\cdot}5 \times 10^4$	$1{\cdot}2 \times 10^4$
C-SN 2	$7{\cdot}7 \times 10^4$	$1{\cdot}0 \times 10^3$
C-AP	$4{\cdot}7 \times 10^5$	$<1 \times 10^3$
R-GR 1	$1{\cdot}6 \times 10^5$	$1{\cdot}0 \times 10^3$
C-HD 2	$5{\cdot}2 \times 10^5$	$1{\cdot}8 \times 10^4$
R-YL	$1{\cdot}0 \times 10^6$	$6{\cdot}0 \times 10^3$
R-GR 4	$7{\cdot}8 \times 10^5$	$2{\cdot}1 \times 10^4$
B-RE	$8{\cdot}8 \times 10^4$	$1{\cdot}4 \times 10^4$
C-SN 1	$2{\cdot}4 \times 10^5$	$2{\cdot}0 \times 10^3$
R-GR 2	$4{\cdot}4 \times 10^5$	$<1 \times 10^3$
R-DD	$2{\cdot}8 \times 10^5$	$1{\cdot}0 \times 10^4$
C-FA 1	$4{\cdot}1 \times 10^3$	$1{\cdot}2 \times 10^3$
C-FA 2	$4{\cdot}0 \times 10^2$	$1{\cdot}9 \times 10^5$
C-SP 3	$2{\cdot}0 \times 10^2$	$3{\cdot}3 \times 10^3$
C-SP 4	$1{\cdot}6 \times 10^3$	$2{\cdot}1 \times 10^4$
R-PD 2	$1{\cdot}5 \times 10^3$	$>1 \times 10^7$
C-KS 2	$4{\cdot}9 \times 10^3$	$6{\cdot}0 \times 10^3$
R-GR 3	$6{\cdot}0 \times 10^3$	$1{\cdot}0 \times 10^2$
C-HD 1	$3{\cdot}1 \times 10^3$	$5{\cdot}5 \times 10^3$
C-SP 2	$4{\cdot}0 \times 10^3$	$9{\cdot}0 \times 10^2$

Chromatographic analyses of the two terminal fractions (Fig. 4) revealed the presence of certain organic acids and certain ninhydrin-reactive materials in pure cultures of chlorella TX 71105 that were not found in cultures mixed with bacteria.

It is inferred from these studies that bacteria grow in cultures of chlorella TX 71105 by utilizing substances excreted during the algal growth. It is further inferred that algae/bacterial population equilibria resulted from the establishment of steady-state rates of algal division and cell death at stationary phase. It has been proven that algal metabolites were utilized selectively by different bacteria (Table 1) and that various classes of chemical substances were excreted (Fig. 4).

The literature reviewed implies that algal cultures are subject to bacterial contamination but no reports were found which attempted to describe the nature of the compatible bacteria. Table 2 shows that a few select bacteria could grow well in cultures of chlorella TX 71105 but that the majority of soil and air bacteria could not proliferate in mixed culture with the algae. It was shown that those organisms incapable of proliferating in algal cultures were also incapable of multiplying in filtrates of axenic cultures or to oxidize these filtrates. Also, when filtrates of axenic cultures of chlorella were inoculated and incubated with bacteria

incapable of growing in algal cultures and the bacteria subsequently removed, it was plainly evident that the filtrates still contained substances oxidizable by bacteria BAT and GNB. These data indicate that the algae determined the kind of bacteria with which they will grow, according to the nature of the organic substances they excreted.

Table 3 shows that 6 out of 8 bacteria pathogenic for man died out rapidly in cultures of chlorella TX 71105 but that *Salmonella typhi* and *S. paratyphi* grew quite well for prolonged periods of time. It is assumed that other human pathogens might also grow in cultures of chlorella TX 71105.

Table 3. *Growth and survival of some pathogens in cultures of Chlorella pyrenoidosa TX 71105*

	Days						
	0	1	2	3	5	6	7
Organism	Viable organisms/ml.						
Salmonella typhi	$2{\cdot}0\times10^5$	$6{\cdot}9\times10^6$	$4{\cdot}7\times10^6$	$2{\cdot}9\times10^3$	$5{\cdot}4\times10^4$	$3{\cdot}5\times10^4$	4×10^2
S. paratyphi	$8{\cdot}9\times10^4$	$1{\cdot}0\times10^6$	$8{\cdot}0\times10^2$	$3{\cdot}9\times10^2$	$1{\cdot}8\times10^4$	$3{\cdot}0\times10^2$	$7{\cdot}9\times10^4$
Shigella flexneri	$2{\cdot}1\times10^5$	$8{\cdot}3\times10^4$	$>1<10$	0	—	—	—
S. dysenteriae	$2{\cdot}2\times10^5$	$1{\cdot}0\times10^4$	$>1<10$	0	—	—	—
Proteus vulgaris	$1{\cdot}5\times10^5$	$1{\cdot}3\times10^6$	$9{\cdot}4\times10^3$	$>1<10$	0	—	—
Streptococcus pyogenes	$1{\cdot}7\times10^4$	0	0	—	—	—	—
Staphylococcus aureus	$1{\cdot}2\times10^5$	0	0	—	—	—	—
Corynebacterium diphtheriae	$1{\cdot}3\times10^4$	0	0	—	—	—	—

Mass culture studies

Studies of bacterial growth in non-sterilized open mass cultures of chlorella TX 71105 revealed that the same algae/bacteria population relationships existed there as were observed in flask cultures. All mass culture studies utilized the natural microbial flora normally encountered in non-sterilized algal culture systems. It was possible to monitor the growth of heterotrophic and autotrophic bacteria, fungi, yeasts and actinomycetes. Table 4 (representative of the data obtained from 5 experiments) shows that bacteria proliferated as a function of algal growth in mass cultures of chlorella TX 71105. It also shows that heterotrophic bacteria outgrew bacteria capable of utilizing KNO_3 as sole nitrogen source. This indicates that most bacterial growth resulted from the utilization of organic nitrogenous substances excreted by the algae rather than from the use of KNO_3 from the algal medium. Table 4 presents data which show that the fungi survived but did not grow in cultures of chlorella TX 71105. No significant fungal populations were detected in algal cultures maintained for 8 days and neither yeasts nor actinomycetes were found in these cultures by the methods used.

It was evident that constituents of the detectable microbial populations varied as the algal cultures aged. New cultures generally contained a low but widely varied bacterial population, while ageing cultures in the stationary phase reached equilibrium with a few bacterial species only. Figure 3 shows the shifting population balances as a function of culture age. These population changes were also observed during stationary phase; it is assumed that they were the result of a

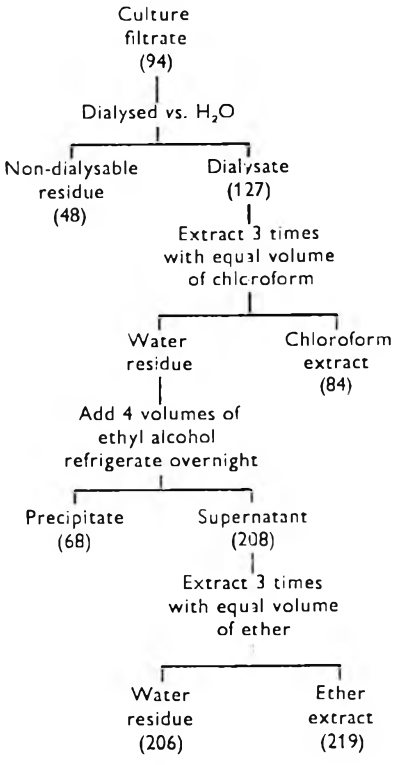


Fig. 4. Fractionation of culture filtrate. Numbers in parentheses give μl. O_2 consumed by 10^9 viable *Bacterium anitratum* in 40 min. All fractions were reconstituted to original quantity with water.

Table 4. *The growth of various micro-organisms in mass cultures of Chlorella pyrenoidosa* TX *71105*

		Bacteria			
		Trypticase	KNO_3	TSA-KNO_3	
	Algae	soy agar	agar	KNO_3	Fungi
Time (hr)	Organisms/ml.				
0	$2{\cdot}5 \times 10^6$	$1{\cdot}5 \times 10^6$	$9{\cdot}5 \times 10^5$	0·5	$1{\cdot}1 \times 10^2$
8	$3{\cdot}1 \times 10^6$	$2{\cdot}0 \times 10^7$	$1{\cdot}4 \times 10^6$	13	$1{\cdot}0 \times 10^2$
18	$2{\cdot}9 \times 10^7$	$4{\cdot}7 \times 10^7$	$5{\cdot}9 \times 10^5$	77	$1{\cdot}2 \times 10^2$
26	$1{\cdot}5 \times 10^8$	$1{\cdot}0 \times 10^8$	$>10^8$	—	$3{\cdot}3 \times 10^2$
42	$9{\cdot}4 \times 10^8$	$2{\cdot}7 \times 10^8$	$3{\cdot}2 \times 10^7$	8	$4{\cdot}0 \times 10^2$
50	$1{\cdot}4 \times 10^9$	$2{\cdot}7 \times 10^8$	$1{\cdot}8 \times 10^7$	14	$4{\cdot}0 \times 10^2$
76	$1{\cdot}5 \times 10^9$	$2{\cdot}4 \times 10^8$	$1{\cdot}4 \times 10^7$	16	$1{\cdot}8 \times 10^2$
84	$1{\cdot}7 \times 10^9$	$1{\cdot}6 \times 10^8$	$2{\cdot}1 \times 10^7$	7	$2{\cdot}0 \times 10^2$
104	$2{\cdot}2 \times 10^9$	$2{\cdot}2 \times 10^8$	$2{\cdot}4 \times 10^7$	8	$1{\cdot}8 \times 10^2$
128	$1{\cdot}8 \times 10^9$	$9{\cdot}7 \times 10^7$	$4{\cdot}1 \times 10^6$	23	$1{\cdot}1 \times 10^2$

Counts were also made of yeasts and actinomycetes but in no sample did the count of either of these organisms exceed 10 organisms/ml.

given organism's ability to utilize substances not available to a faster growing organism.

Throughout these experiments it was obvious that bacteriophages were present in some mass algal cultures. The presence of these phages was confirmed by serial passage on host bacteria isolated from the cultures.

The technical assistance of Mr B. T. Anderson is gratefully appreciated. We also thank the Bacteriology Section of the U.S.A.F. Epidemiology Laboratory, Lackland AFB, Texas, U.S.A., for providing the pathogenic organisms employed.

The research reported in this paper was funded in part by 'In-House Laboratory Independent Research Funds' U.S.A.F. School of Aerospace Medicine, A.F.S.C., and N.A.S.A. Defense Project no. R-99.

REFERENCES

Blasco, R. J. (1965). Nature and role of bacterial contaminants in mass culture of thermophilic *Chlorella pyrenoidosa*. *Appl. Microbiol.* **13**, 473.

Brown, L. R., Kennedy, M. V. & Tischer, R. G. (1964). An algal medium produced from human wastes. *Devs ind. Microbiol.* **5**, 245.

Eley, J. H., Jun. & Myers, J. (1964). Study of a photosynthetic gas exchanger, a quantitative repetition of the Priestley experiment. *Texas J. Sci.* **16**, 296.

Fogg, G. E. (1952). The production of extracellular nitrogenous substances by a blue-green alga. *Proc. Roy. Soc.* B, **139**, 372.

Krauss, R. W. (1962). Mass culture of algae for food and other organic compounds. *Am. J. Bot.* **49**, 425.

Krauss, R. W. & Thomas, W. H. (1954). The growth and inorganic nutrition of *Scenedesmus obliquus* in mass culture. *Pl. Physiol.* **29**, 205.

Mayer, A. M., Zuri, U., Shain, Y. & Ginzburg, H. (1964). Problems of design and ecological considerations in mass culture of algae. *Biotech. Bioengin.* **6**. 173.

Myers, J. (1957). *Algal cultures.* In *Encyclop. Chem. Tech.* (1st suppl. vol.) New York: The Interscience Encyclopedia, Inc.

Myers, J. (1964). *Use of algae for support of the human in space.* In *4th Int. Space Sci. Symp. Warsaw.*

Myers, J., Phillips, J. N., Jun. & Graham, J-R. (1951). On the mass culture of algae. *Pl. Physiol.* **26**, 539.

McDowell, M. E. & Leveille, G. A. (1964). Algae systems. In *National Aeronautics and Space Administration conference on Nutrition in Space and Related Waste Problems.* Tampa, Florida.

Nakamura, H. (1963). Studies on the eco-system of Chlorella. *Jap. Soc. Pl. Physiol.*, p. 197. University of Tokyo Press.

Phillips, J. N., Jun. & Myers, J. (1954). Measurement of algal growth under controlled steady-state conditions. *Pl. Physiol.* **29**, 148.

Sorokin, C. & Myers, J. (1953). A high-temperature strain of Chlorella. *Science.* **117**, 330.

Vladimirova, M. G. (1960). Dynamics of the bacterial microflora growth in Chlorella cultures. Translated from *Mikrobiologiya*, **30**, 374.

Ward, C. H., Moyer, J. E. & Vela, G. R. (1964). Studies on bacteria associated with *Chlorella pyrenoidosa* TX 71105 in mass culture. *Devs ind. Microbiol.* **5**. 213.

Ward, C. H., Wilks, S. S. & Craft, H. L. (1963). Use of algae and other plants in the development of life support systems. *Am. Biol. Teach.* **25**, 512.

Winneberger, J. H., Austin, J. H. & Klett, C. A. (1963). Membrane filter weight determinations. *J. Wat. Pollut. Control Fed.* **35**, 807.

J. gen. Microbiol. (1966), **42**, 133–138
Printed in Great Britain

The Role of Cellular Lipid in the Resistance of Gram-positive Bacteria to Penicillins

By W. B. HUGO and R. J. STRETTON
Department of Pharmacy, The University, Nottingham

(*Received* 6 *August* 1965)

SUMMARY

It has been shown that when the Gram-positive micro-organisms *Bacillus subtilis*, *Staphylococcus aureus* and *Streptococcus faecalis* were grown under conditions in which their cellular lipid was increased a corresponding increase in their resistance to the penicillins benzylpenicillin, cloxacillin, methicillin and quinacillin was produced. Likewise cell-wall lipid depletion caused a corresponding increase in sensitivity.

INTRODUCTION

Amongst the so-called semi-synthetic penicillins produced by treating a suitable acyl derivative with 6-aminopenicillanic acid the recently described 3-carboxy-2-quinoxalinyl penicillin or quinacillin possesses some unusual properties. Thus, whereas quinacillin is active against staphylococci it is, unlike other penicillins both natural and semi-synthetic, relatively inactive against streptococci, *Sarcina lutea* and members of the genus *Bacillus*. Gram-negative organisms were completely resistant to quinacillin (Richards, Housley & Spooner, 1963). In a previous study Hugo & Stretton (1964) showed that quinacillin had a typical penicillin-like action in that it promoted the accumulation of acetylamino sugars in growing cultures of *Staphylococcus aureus* strain Oxford.

Distribution studies using water and chloroform at various pH values showed that, whereas between pH 5·5 and 7·0 benzylpenicillin and phenoxymethylpenicillin were appreciably soluble in chloroform, quinacillin was undetectable in the chloroform layer until pH 2·0 was reached. This finding led to a study of the role of cellular lipids on the sensitivity of bacteria to quinacillin and, for comparison, to benzylpenicillin, methicillin and cloxacillin. The experimental approach adopted consisted in measuring the minimum inhibitory concentration of four penicillins against three species of Gram-positive bacteria in which the lipid content had been varied by appropriate treatments.

METHODS

Organisms. *Staphylococcus aureus* strain Oxford, penicillin-sensitive; NCTC 8452, phage type 80, penicillinase producer; strain 100110, isolated at Queen Mary's Hospital for Children, Carshalton, and reported to be methicillin-resistant; *Bacillus subtilis*, Marburg strain, NCTC3601; *Streptococcus faecalis*, NCIB8553.

Materials. Quinacillin was a sample kindly given by Boots Pure Drug Co. Ltd., cloxacillin and methicillin were a gift from Beecham's Research Laboratories Ltd.

Benzylpenicillin was a commercial sample. All other chemicals used were of A.R. B.P., or B.P.C. quality when available or of laboratory reagent grade. Two samples of lipase were used: one of animal origin (pancreatic lipase, steapsin, L. Light and Co. Ltd.) and one of plant origin (wheat-germ lipase, British Drug Houses Ltd.). Silica gel for thin-layer chromatography was as supplied by E. Merck A.G. Darmstadt. A more detailed chromatographic investigation was made by using Whatman no. 1 paper impregnated with silicic acid (S.G.81).

Media and conditions of growth. Culture media were prepared from Oxoid material with appropriate additions, sterilized 10 lb/30 min., final pH, 7·2.

The nutrient broth used contained (g./l.): peptone (Oxoid no. 1) 10, meat extract (Lemco, Oxoid) 5, sodium chloride 5; to provide adequate growth for *Streptococcus faecalis* yeast extract 5 was added. To these media the following single additions were made as required: (*a*) glycerol to a final concentration of 3%, (*b*) sodium acetate 0·5%, (*c*) sodium oleate 20 μg./ml., (*d*) coenzyme A 1300 μM, (*e*) acriflavine 10^{-5} M, (*f*) flavine mononucleotide 10^{-4} M, (*g*) pancreatic lipase 0·01 mg./ml., (*h*) wheat-germ lipase 0·10 mg./ml. When a solid medium was required Ion-agar no. 2, 1·2%, was incorporated.

Media (*a*)–(*c*) were used to enhance the lipid content of the organism, (*d*)–(*h*) were designed for diminishing lipid content. All incubation was at 37°.

For lipase treatment, in addition to growth in the presence of the enzyme, washed suspensions were treated for 2 hr at 37° with the enzyme at a concentration of 0·01 (pancreatic) and 0·1 (wheat germ) mg./ml. before examination for possible lipid depletion.

Determination of minimum inhibitory concentrations. The minimum inhibitory concentration (MIC) was determined using a tube dilution method with a final inoculum size of 10^3 organisms/ml. in the test system. All readings were taken exactly 24 hr from commencement of incubation. The media used were the basal media with the addition, where appropriate, of reagents to maintain the depleted or enhanced lipid content.

Determination of lipid. 1. *Total lipid by extraction.* Organisms grown on solid medium for 18 hr were harvested by washing with distilled water, centrifuged at low speed to remove agar and the bacteria collected by centrifugation at 8000*g* and thrice washed by the same procedure. The washed bacteria were dried on an aluminium dish to constant weight in a partial vacuum over phosphoric oxide, transferred quantitatively to a Soxhlet apparatus and extracted with chloroform + methanol (2 + 1 by vol.). All solvents used with bacterial lipid contained 0·01% butylated hydroxytoluene as an antioxidant. The extract so obtained was quantitatively transferred to an aluminium dish, the solvent allowed to evaporate spontaneously and residual solvent allowed to evaporate in a desiccator under vacuum over phosphoric oxide.

2. *Chromatography of the lipid extracts.* Preliminary identification of the components of the extracted lipid was made by thin-layer chromatography on silica gel using chloroform + methanol + water (14 + 6 + 1) for phosphatid components and *n*-hexane + ether + acetic acid (50 + 50 + 1) for non-phosphatide fractions (Nichols, 1964). Visualization of the spots in all cases was with iodine vapour (Whitehouse, Bresler & Staple, 1958). Standards were run at the same time as an aid to identification. A more detailed chromatographic investigation was made by on silicic-

acid-impregnated paper developed with di-isobutylketone + acetic acid + water (40 + 20 + 3 by vol.; Marinetti, 1964).

Identification of the various components was accomplished by the use of appropriate spray reagents: periodate–Schiff's reagent for vicinal-glycol-containing lipids, Dragendorff's reagent for choline, ninhydrin for amino lipids and rhodamine 6G (0·05% in 96% ethanol) followed by examination under ultraviolet light, a procedure which detects lipids generally, but produces different colours according to the type of lipid present. Thus: yellow, lysolecithin; orange, phosphatidyl- and lysophosphatidylethanolamine, neutral lipid; blue, lysophosphatidylserine, phosphatidylglycerol, bis (phosphatidic) acid and phosphatidic acid.

Phosphatidylserine (Koch–Light Ltd.), phosphatidyl ethanolamine (Sigma Chemical Co.) and lecithin, 95–100% (British Drug Houses Ltd.), were used as markers or standards. The two latter compounds although not chromatographically pure contained a major component in excess which was taken as the authentic spot. Published R_F values were also used as a further aid to identification (Matches, Walker & Ayres, 1964; Kates, Adams & Martin, 1964).

Detection of lipid by electrophoresis. Dyar & Ordal (1946), Dyar (1948) and Hill, James & Maxted (1963) showed that if the electrophoretic mobility of bacteria suspensions was measured and compared with the mobility in the presence of an anionic surface-active agent such as sodium dodecyl sulphate (SDS) an increase in negative mobility was observed which could be related quantitatively to surface lipid. Measurements were made using the cell and circuit described by McQuillen (1952). The bacteria, after washing three times in buffer, were suspended in phosphate (NaH_2PO_4/Na_2HPO_4) buffer pH 7·2 ($I = 0{\cdot}01$) at a final concentration of 0·01 mg. dry wt./ml. Mobilities were measured in this system as described by McQuillen and in the same system containing sodium dodecyl sulphate 10^{-6}, 10^{-5} and 10^{-4} M. Where there was a tendency for the bacteria to form clumps the suspension was shaken for a short time with 2 mm. diameter glass beads to obtain a suspension of single organisms necessary for the test.

The overall reproducibility of duplicate mobility measurements made on the same day was ± 1% and on different days ± 3% ($P = 0{\cdot}05$). Samples in which the mobility differed by ± 10% were taken as being significantly different. The lipid content of the bacteria, determined by extraction and/or by mobility measurements in the presence of SDS, increased with each subculture in the presence of glycerol, reaching a maximum at between 10 and 15 subcultures. A subculture régime of 10 was adopted for all experiments.

Bacteria in which the lipid content had been decreased by lipase or enhanced by glycerol reverted to their normal value after one subculture in the basal medium. Consequently, all measurements on penicillin inhibition were made in the presence of either lipase or glycerol, with appropriate controls which indicated that these additions did not themselves affect penicillin action or growth.

RESULTS

Addition of glycerol 3% to the basic culture medium resulted in an increase in cellular lipids with all the organisms listed under Methods. Treatment with lipase reduced the lipid content of the bacteria. Pancreatic lipase at the concentration used,

Table 1. *Effect of cellular lipid on the resistance of Gram-positive bacteria to penicillins*

Organism	Treatment	Lipid content (%, w/w, of whole dried bacteria)	Mobility (μ sec.$^{-1}$ V.$^{-1}$ cm.$^{-1}$ in the presence of 10^{-1} M sodium dodecyl sulphate)	Minimum inhibitory concentration (μg./ml.)			
				Quinacillin	Benzylpenicillin	Methicillin	Cloxacillin
Staphylococcus aureus (Oxford)	Control	6·4	−2·84	0·3	0·03	8·0	0·35
	Grown with glycerol	18·3	−4·56	20·0	2·0	20·0	8·0
	Lipase treated	6·4	−2·40	0·1	0·01	6·0	0·25
	Grown with oleate	6·5	.	1·0	0·5	9·0	0·5
S. aureus NCTC 8452	Control	6·5	−3·21	0·7	*	1·0	0·5
	Grown with glycerol	7·0	−3·68	2·0	*	2·0	0·8
	Grown with oleate	.	.	1·5	*	1·5	0·75
S. aureus 100110	Control	.	−3·69	5·0	100	26	1·0
	Grown with glycerol	.	−3·68	5·0	100	26	1·0
	Lipase treated	.	−3·0	3·5	100	24	0·8
Streptococcus faecalis	Control	5·3	−2·42	110	3·2	45	40
	Grown with glycerol	5·9	−3·01	350	10·0	100	200
Bacillus subtilis	Control	8·6	−4·56	8·0	0·6	0·4	0·01
	Grown with glycerol	19·0	−5·48	40	5·0	1·0	0·03
	Lipase treated	8·3	−3·45	5·0	0·3	0·2	0·008

* Destroyed by penicillinase.

0·01 mg./ml., was not inhibitory, but was effective in diminishing cellular lipid; wheat-germ lipase inhibited the growth of *Staphylococcus aureus* unless the concentration was decreased to 0·05%. In the case of staphylococci producing an extracellular lipase cellular lipid could be increased by adding a lipase inhibitor to the growth medium. It was found that sodium oleate, 20 μg./ml., inhibited pancreatic lipase and the lipase present in the culture medium in which *S. aureus* (Oxford) had been grown. When this amount of sodium oleate was added to culture media in which *S. aureus* (Oxford) or 8452 were growing, the lipid content and resistance to penicillins was increased. *Staphylococcus aureus* 100110 did not produce an extracellular lipase.

Robinson, Brady & Bradley (1963) reported that acriflavine, coenzyme A and flavine mononucleotide inhibited the biosynthesis of fatty acids in enzymes from cat brain and liver. In our hands these reagents at appropriate concentrations had no detectable effect on the lipid content of the bacterial species used in this study.

Acetate was less effective than glycerol in inducing cellular lipid and was, in fact, inhibitory towards the growth of *Staphylococcus aureus* even at concentrations of 0·5%. Without exception, but in varying degrees, increasing cellular lipid resulted in an increase in the dose of penicillin required to inhibit growth whereas decrease in lipid produced precisely the opposite effect (Table 1).

DISCUSSION

Quinacillin has been shown to cause the accumulation of *N*-acetylamino sugars at concentrations which inhibit cell growth (Hugo & Stretton, 1964). However, the different pattern of solubility of quinacillin in chloroform when compared with benzylpenicillin or phenoxymethylpenicillin led to the suggestion that cellular lipid may be a factor in determining the susceptibility of bacteria to penicillins or the toxicity of penicillins towards bacteria. Lipid enhancement resulted in a marked increase in the dose to inhibit growth of all the penicillins tested; lipid depletion caused a less well marked but none the less significant increase in sensitivity. Changes in lipid content were also reflected in the appearance of the culture. In general, with increase in lipid content pellicle formation was either induced or, where already present as in *Bacillus subtilis*, became thicker and more wax-like. Treatment with lipase abolished pellicle formation in this organism. It was not possible to attribute increase in penicillin resistance to any particular lipid component, since all appeared to increase equally, as assessed by chromatography, during growth in glycerol.

Some evidence that the cellular location of the lipid was the controlling factor was obtained from experiments in which lipid extracted from fattened bacteria was added to cultures of non-fattened bacteria of the same species to give, as near as could be achieved, the same overall lipid content in the system. In these circumstances no increase in resistance to the penicillins could be detected.

The role of drug lipid solubility as measured by chloroform buffer distribution seems to be of small importance as compared with the marked general effect of cell lipid in penicillin resistance. Support for the general hypothesis on the role of lipid comes from a consideration of the difference in sensitivity of Gram-positive and Gram-negative bacteria to penicillin. The former are, in general, sensitive, the

latter are not, yet the mode of action is the same (Gale, 1964); but, whereas Gram-negative cell walls contain a large amount of lipid (up to 25%), Gram-positive cell walls normally contain little or none (Salton, 1964).

It is possible that an increase or decrease in cellular lipid of the type reported in this paper might occur *in vivo* leading to a change in resistance to the penicillins; in this connexion it is interesting to note that Hill *et al.* (1963) have shown that freshly isolated pathogenic streptococci possess more cell lipid than laboratory strains. Both serum glycerol and possibly serum lipases may play a role in changing the sensitivity of the Gram-positive bacteria to the penicillins.

REFERENCES

DYAR, M. T. (1948). Electrokinetic studies on bacterial surfaces. II. Studies on surface lipids, amphoteric material and some other surface properties. *J. Bact.* **56**, 821.

DYAR, M. T. & ORDAL, N. J. (1946). Electrokinetic studies on bacterial surfaces. I. The effects of surface active agents on the electrophoretic mobilities of bacteria. *J. Bact.* **51**, 149.

GALE, E. F. (1964). The mode of action of antibiotics. *Pharmacol. Rev.* **15**, 481.

HILL, M. J., JAMES, A. M. & MAXTED, W. R. (1963). Some physical investigations of the behaviour of bacterial surfaces. X. The occurrence of lipid in the streptococcal cell wall. *Biochim. biophys. Acta*, **75**, 414.

HUGO, W. B. & STRETTON, R. J. (1964). Action of quinacillin on *Staphylococcus aureus*. *Nature, Lond.* **202**, 1217.

KATES, M., ADAMS, G. A. & MARTIN, S. M. (1964). Lipids of *Serratia marcescens*. *Canad. J. Biochem.* **42**, 461.

McQUILLEN, K. (1952). *Electrophoresis of bacteria.* Ph.D. thesis, University of Cambridge.

MARINETTI, G. V. (1964). Chromatographic analysis of polar lipids on silica acid impregnated paper. In *New Biochemical Separations*, ed. A. M. James and L. J. Morris. London: Van Nostrand.

MATCHES, J. R., WALKER, H. W. & AYRES, J. C. (1964). Phospholipids in vegetative cells and spores of *Bacillus polymyxa*. *J. Bact.* **87**, 16.

NICHOLS, B. W. (1964). The separation of lipids by thin layer chromatography. *Lab. Pract.* **13**, 299.

RICHARDS, H. C., HOUSLEY, J. R. & SPOONER, D. F. (1963). Quinacillin: a new penicillin with unusual properties. *Nature, Lond.* **199**, 354.

ROBINSON, J. D., BRADY, R. O. & BRADLEY, R. M. (1963). Biosynthesis of fatty acids: IV. Studies with inhibitors. *J. Lipid Res.* **4**, 144.

SALTON, M. R. J. (1964). *The Bacterial Cell Wall.* Amsterdam: Elsevier.

WHITEHOUSE, M. W., BRESLER, A. E. & STAPLE, E. (1958). The use of iodine for the detection of phospholipids. *J. Chromatog.* **1**, 385.

J. gen. Microbiol. (1966), **42**, 139–145
With 4 plates
Printed in Great Britain

Variations in Mycoplasma Morphology Induced by Long-chain Fatty Acids

By S. RAZIN,* B. J. COSENZA and M. E. TOURTELLOTTE
Department of Bacteriology and Department of Animal Diseases, University of Connecticut, Storrs, U.S.A.

(*Received* 12 *August* 1965)

SUMMARY

Addition of unsaturated long-chain fatty acids to tryptose broth improved growth of *Mycoplasma laidlawii* and induced the formation of very long and highly branched filaments by this organism. Most filaments were composed of small coccoid bodies. The saturated long-chain fatty acids, in a similar concentration, did not produce this effect; palmitic and stearic acids inhibited growth. Similar, but less striking results were obtained with *M. gallisepticum* and *Mycoplasma* sp. strain 14. The possibility that the morphological variations reflect changes in the lipid composition of the cell membrane is discussed.

INTRODUCTION

Several mycoplasma species including *Mycoplasma mycoides* var. *mycoides*, *M. gallisepticum* and *M. laidlawii* require long-chain fatty acids for growth (Rodwell & Abbot, 1961; Tourtellotte, Jensen, Gander & Morowitz, 1963; Razin & Rottem, 1963). Evidence for the incorporation and use of the fatty acids of the growth medium for the biosynthesis of mycoplasma lipids is accumulating (Smith & Boughton, 1960; Tourtellotte *et al.* 1963; Argaman & Razin, 1965). Since practically all mycoplasma lipids are part of the cell membrane (Razin, Argaman & Avigan, 1963) it is evident that the long-chain fatty acids are important constituents of this structure. Growth of *M. mycoides* var. *mycoides* in a medium deficient in essential long-chain fatty acids was accompanied by profound morphological changes in the cells, reflecting apparently defective biosynthesis of the limiting cell membrane (Rodwell & Abbot, 1961).

The dependence of biosynthesis of membrane lipids on an external supply of long-chain fatty acids prompted us to investigate the possibility of changing the fatty acid composition of the membrane by varying the fatty acid composition of the growth medium. The method chosen to approach this problem consisted of adding increasing amounts of one long-chain fatty acid at a time to the growth medium and observe its effects on cell morphology, osmotic fragility and fatty acid composition of membrane lipids. This report describes the morphological changes occurring in mycoplasma cells grown in the presence of various long-chain fatty acids.

* Present address: Department of Clinical Microbiology, The Hebrew University, Hadassah Medical School, Jerusalem, Israel.

METHODS

Organisms. *Mycoplasma laidlawii* strain B (PG 9) was obtained from Dr D. G. ff. Edward (Wellcome Research Laboratories, Beckenham, Kent). *M. gallisepticum* strain A 5969 was taken from the culture collection of the Department of Animal Diseases, University of Connecticut. *Mycoplasma* sp. strain 14 (goat strain) was obtained from Dr H. E. Adler (School of Veterinary Medicine, University of California, Davis, U.S.A.).

Growth conditions. The basal growth medium was tryptose broth consisting of (g./l.): Bacto-tryptose, 20; sodium chloride, 5; tris (2-amino-2-hydroxymethyl-propane-1,3-diol), 5; glucose, 7. The pH value of the medium was 8·2–8·4 without adjustment. The medium was supplemented with 4 g. lipid-extracted bovine albumin. fraction V (Calbiochem, Los Angeles, U.S.A.)/l. The albumin was extracted with acetone as described by Razin & Rottem (1963). The saprophytic *Mycoplasma laidlawii* could grow well in this medium after an adaptation period of 10–20 daily transfers in the medium. The parasitic *M. gallisepticum* and the goat strain could not grow in this medium unless supplemented with Bacto-PPLO serum fraction in a final concentration of 1% (v/v). Experiments to replace the need for PPLO serum fraction with mixtures of cholesterol and long-chain fatty acids failed to yield satisfactory growth of the two parasitic strains.

Long-chain fatty acids were added to the growth medium in ethanolic solution. The concentration of ethanol in the medium never exceeded 0·5% (v/v). The presence of lipid-extracted albumin in the medium enabled growth of Mycoplasma with rather high concentrations of fatty acids. Growth was usually done in 100 ml. quantities of medium dispensed in 200 ml. screw-capped bottles. Each bottle received 5 ml. of a 24 hr culture in the medium with no fatty acids added. The bottles were incubated statically at 37° for 24 hr or longer and samples were taken for microscopic examinations.

Assessment of growth. The organisms were harvested by centrifugation at 9000*g* for 15 min. and washed twice in β-buffer (Pollack, Razin, Pollack & Cleverdon, 1965) consisting of: sodium chloride, 0·15 M; tris, 0·05 M; 2-mercaptoethanol, 0·01 M in de-ionized water, adjusted to pH 7·4 with HCl. The washed organisms were resuspended in 10 ml. buffer and the amount of cell protein determined in a sample of this suspension according to Lowry, Rosebrough, Farr & Randall (1951).

Chemicals. Laurie acid (A grade, gas chromatography > 99·5% pure), palmitic acid (A grade, gas chromatography homogeneous), stearic acid (A grade, gas chromatography homogeneous) and oleic acid (A grade, > 99% pure) were the products of Calbiochem (Los Angeles, California, U.S.A.). Myristic acid (99·8% pure), linoleic acid (90 + % pure), linolenic acid (99 + % pure) and arachidonic acid (90 + % pure) were purchased from Mann Research Laboratories Inc. (New York 6, N.Y., U.S.A.).

Microscopy and photomicrography. Drops taken from the liquid cultures of the tested Mycoplasma were put on glass slides, covered with coverslips and examined with a Wild M20 microscope. The Wild Fluotar phase objective HI 50/1·00 and a ×10 or ×20 eyepiece were routinely used. The illumination source was a Bausch and Lomb low-voltage lamp. Photographs were taken with a Leitz Wetzlar camera using Kodak Contrast Process panchromatic film. The total microscopic magnifica-

tion was 625. Prints were made on Kodabromide (F-3) paper to a final magnification of 1250, 2700 or 3850.

RESULTS

Mycoplasma laidlawii. Growth of *M. laidlawii* in the tryptose medium was characterized by a steep decline phase involving lysis of the organisms within 48–72 hr incubation at 37°. Addition of increasing concentrations of oleic acid to this medium improved growth and lengthened the decline phase. The concentration of oleic acid giving optimal growth was about 50 μg./ml.; higher concentrations decreased growth. Growth promotion was also shown with the other unsaturated fatty acids linoleic acid and linolenic acid, and to a lesser extent with arachidonic acid (Table 1). While lauric acid promoted growth, the other saturated long-chain fatty acids inhibited growth; the growth-inhibitory effect increased with the increase in the chain length (Table 1).

Table 1. *Effect of long-chain fatty acids on growth of Mycoplasma laidlawii strain* B

Organisms were grown in 100 ml. quantities of tryptose broth containing 0·4 % (v/v) lipid-extracted bovine albumin, fraction V, and 50 μg./ml. of one of the fatty acids. The organisms were harvested after 24 hr incubation at 37°, washed and resuspended in β-buffer. Total cell protein in the suspension was determined according to Lowry *et al.* (1951).

Fatty acid added to medium	Cell protein (mg.)	Fatty acid added to medium	Cell protein (mg.)
Oleic	11·0	Myristic	5·1
Linoleic	12·9	Palmitic	3·7
Linolenic	12·9	Stearic	2·0
Arachidonic	8·4	No fatty acid added	5·2
Lauric	9·8		

The different effects of the long-chain fatty acids on the growth of *Mycoplasma laidlawii* were also expressed by profound morphological variations in the organisms. *M. laidlawii* grown in the tryptose medium with no fatty acid added appeared as spherical bodies, either single, in pairs or arranged in short, frequently branching chains (Pl. 1, fig. 1). Addition of any one of the four unsaturated fatty acids caused the organisms to grow as long and branched filaments. Most filaments were composed of small spheres (Pl. 1, fig. 3). In many cases microcolonies, composed of entangled filaments, were formed in the liquid culture (Pl. 2, fig 4). In small microcolonies the filaments were seen to originate from a central region (Pl. 2, fig. 5). The dimensions of the organisms were very variable, especially of those appearing singly or in pairs. The diameter of many of these organisms, as measured on the photomicrographs, was 0·6–0·7 μ, while the thickness of the filaments was usually more constant, averaging 0·35 μ. Growth of *M. laidlawii* in the presence of 50 μg./ml. of any of the saturated long-chain fatty acids was characterized by very few filaments; most organisms appeared either singly, in pairs or in very short chains (Pl. 1, fig. 2; Pl. 2, fig. 6). The organisms, especially those grown with palmitic or stearic acid, were bigger than those grown with the unsaturated fatty acids, many reaching a diameter of 1 μ.

Mycoplasma gallisepticum. This mycoplasma could not grow in tryptose broth without PPLO serum fraction, even when cholesterol was supplied to the medium.

The organisms grown in tryptose broth supplemented with 1% (v/v) PPLO serum fraction had the typical coccobacillary form (Klienberger-Nobel, 1962). Many organisms appeared as short rods, very frequently arranged in clusters (Pl. 3, fig. 7). The organisms were usually smaller than those of *M. laidlawii*. The width of the bacillary forms averaged 0·3 μ. Addition of oleic acid to the growth medium caused the appearance of some short and branched filaments (Pl. 3, fig. 8), but the phenomenon was much less pronounced than with *M. laidlawii*. Addition of 25–50 μg. oleic acid/ml. to the tryptose broth increased the growth of *M. gallisepticum* by about 30% as measured by increase in cell protein. Palmitic acid, when added to the medium, decreased growth by 28% at a level of 50 μg./ml. and by 66% at a level of 100 μg./ml. Organisms grown in the presence of 50 μg. palmitic acid/ml. were usually spherical and bigger than those grown without the addition of fatty acids (Pl. 3, fig. 9). It is of importance to notice in this photomicrograph the growth of *M. gallisepticum* in short branched chains of cocci.

Mycoplasma sp. strain 14 (goat strain). As with *M. gallisepticum*, the goat strain required the addition of PPLO serum fraction to the tryptose medium. This strain showed a tendency to form short and branching filaments without the addition of unsaturated fatty acids to the medium (Pl. 4, fig. 10). However, filamentation was more pronounced when oleic acid was added to the medium to a final concentration of 50 μg./ml. Addition of palmitic acid to the growth medium in the same concentration decreased growth very markedly, the organisms appeared either singly or in pairs and their diameter was larger than that of organisms growing with oleic acid. A striking difference between organisms grown with or without the addition of oleic acid was seen after incubation of the cultures for 48 hr at 37°. The culture growing in the presence of added oleic acid showed intense filamentation. The thickness of the filaments was about 0·3–0·4 μ (Pl. 4, fig. 11). The organisms grown without the addition of oleic acid were very swollen and distorted, many appearing as empty ghosts (Pl. 4, fig. 12).

DISCUSSION

Growth of *Mycoplasma laidlawii* in long and branched filaments resembles very much that of the type species *M. mycoides* var. *mycoides* (Edward & Freundt, 1956). The claim of Freundt (1958, 1960) that filament formation is a characteristic of all Mycoplasma species, including *M. laidlawii*, has been questioned by several authors (Kandler & Kandler, 1954; Liebermeister, 1960; Dienes, 1960). Liebermeister (1960) states that branching filaments are very seldom seen in *M. laidlawii* strains, and when they are found they are artifacts of the microscopic procedure. Weibull & Lundin (1963), using phase-contrast microscopy, could not see filaments in *M. laidlawii* under conditions which enabled filamentation in human mycoplasma strains. Even the electron micrographs presented by Freundt (1960) to prove his point that *M. laidlawii* does produce filaments are not too convincing, because at least some of the very thin filaments (less than 0·1 μ in width) might be the result of cell destruction occurring during the drastic treatment of drying and fixation for electron microscopy. Our observations leave no doubt that *M. laidlawii* is capable of growing in long and branching filaments. The technique used, phase-contrast microscopy, does not involve any pre-treatment of the extremely plastic organisms. The photographs were taken just when the organisms and filaments settled

on the slide. The images obtained in the photomicrographs corresponded closely to those seen moving freely in the liquid.

Formation of filaments by mycoplasma is at least in part a function of the amount of unsaturated fatty acids in the growth medium. This has been already shown for *Mycoplasma mycoides* var. *mycoides* (Rodwell & Abbot, 1961). Cholesterol has apparently no role in filament formation in *M. laidlawii*, because in our experiments this organism was grown in a medium with no PPLO serum fraction and the organisms contained negligible amounts of cholesterol (Razin & Cleverdon, 1965). The dependence of filament formation on constituents of the growth medium should be kept in mind when using this property as a taxonomic criterion. The use of different media by various authors may explain the divergent views about filament formation by *M. laidlawii*.

The effects of long-chain fatty acids on morphology of *Mycoplasma gallisepticum* and the goat mycoplasma were less striking than with *M. laidlawii*. This might be due to the presence of 1 % (v/v) of PPLO serum fraction in the growth medium for the parasitic strains. The variety of fatty acids in this component may have masked the effects of the unsaturated fatty acids added. However, our preliminary observations indicate that basically the mode of multiplication of *M. gallisepticum* resembles that of other mycoplasma, and short branching filaments can be formed by this organism. The goat strain, which seems to be related to *M. mycoides* var. *capri* (Razin, unpublished results), produces longer and more branching filaments when oleic acid is added to the growth medium. Moreover, the organisms grown with oleic acid do not show the rapid degeneration and lysis typical for the rapid decline phase of this organism grown in the presence of 1 % PPLO serum fraction alone.

Inhibition of filament formation and growth by the saturated fatty acids may be caused by interference by these acids, supplied to the medium in excess, with the incorporation of the essential unsaturated fatty acids by the organisms. The need for a proper balance of fatty acids in growth media for mycoplasma and other micro-organisms has been stressed (Shorb & Lund, 1959; Rodwell & Abbot, 1961).

The interpretation of the present morphological observations must depend on chemical and consequently physical alterations in the mycoplasma membrane. Preliminary findings in our laboratory show that the morphological variations are accompanied by changes in osmotic fragility of the organisms indicating variations in membrane elasticity. Other observations indicate a difference in the fatty acid composition of membrane lipids of mycoplasma grown with different fatty acids. It is hoped that with the accumulation of more data an interpretation relating the morphological variations with changes in membrane composition can be postulated.

Contribution no. 123 of the Institute of Cellular Biology, University of Connecticut. One of the authors (S.R.) held a Senior Foreign Scientist Fellowship of the National Science Foundation during the work.

REFERENCES

ARGAMAN, M. & RAZIN, S. (1965). Cholesterol and cholesterol esters in Mycoplasma. *J. gen. Microbiol.* **38**, 153.

DIENES, L. (1960). Controversial aspects of the morphology of PPLO. *Ann. N.Y. Acad. Sci.* **79**, 356.

EDWARD, D. G. FF. & FREUNDT, E. A. (1956). The classification and nomenclature of organisms of the pleuropneumonia group. *J. gen. Microbiol.* **14**, 197.

FREUNDT, E. A. (1958). *The Mycoplasmataceae (the Pleuropneumonia Group of Organisms): Morphology, Biology and Taxonomy.* Copenhagen: Munksgaard.

FREUNDT, E. A. (1960). Morphology and classification of the PPLO. *Ann. N.Y. Acad. Sci.* **79**, 312.

KANDLER, G. & KANDLER, O. (1954). Untersuchungen über die Morphologie und die Vermehrung der pleuropneumonieähnlichen Organismen und der L-phase der Bakterien. I. Lichtmikroskopische Untersuchungen. *Arch. Mikrobiol.* **21**, 178.

KLIENEBERGER-NOBEL, E. (1692). *Pleuropneumonia-like Organisms (PPLO), Mycoplasmataceae.* London: Academic Press

LIEBERMEISTER, K. (1960). Morphology of the PPLO and L forms of Proteus. *Ann. N.Y. Acad. Sci.* **79**, 326.

LOWRY, O. H., ROSEBROUGH, N. J., FARR, A. L. & RANDALL, R. J. (1951). Protein measurement with the Folin phenol reagent. *J. biol. Chem.* **193**, 265.

POLLACK, J. D., RAZIN, S., POLLACK, M. E. & CLEVERDON, R. C. (1965). Fractionation of *Mycoplasma* cells for enzyme localization. *Life Sciences*, **4**, 973.

RAZIN, S. & CLEVERDON, R. C. (1965). Carotenoids and cholesterol in membranes of *Mycoplasma laidlawii*. *J. gen. Microbiol.* **41**, 409.

RAZIN, S. & ROTTEM, S. (1963). Fatty acid requirements of *Mycoplasma laidlawii*. *J. gen. Microbiol.* **33**, 459.

RAZIN, S., ARGAMAN, M. & AVIGAN, J. (1963). Chemical composition of *Mycoplasma* cells and membranes. *J. gen. Microbiol.* **33**, 477.

RODWELL, A. W. & ABBOT, A. (1961). The function of glycerol, cholesterol and long-chain fatty acids in the nutrition of *Mycoplasma mycoides*. *J. gen. Microbiol.* **25**, 201.

SHORB, S. & LUND, P. G. (1959). Requirements of trichomonads for unidentified growth factors, saturated and unsaturated fatty acids. *J. Protozool.* **6**, 122.

SMITH, P. F. & BOUGHTON, J. E. (1960). Role of protein and phospholipid in the growth of pleuropneumonia-like organisms. *J. Bact.* **80**, 851.

TOURTELLOTTE, M. E., JENSEN, R. G., GANDER, G. W. & MOROWITZ, H. J. (1963). Lipid composition and synthesis in the pleuropneumonia-like organism *Mycoplasma gallisepticum*. *J. Bact.* **86**, 370.

WEIBULL, C. & LUNDIN, B. M. (1963). Morphology of pleuropneumonia-like organisms and bacterial L-forms grown in liquid media. *J. Bact.* **85**, 440.

EXPLANATION OF PLATES

PLATE 1

Mycoplasma laidlawii

Fig. 1. Organisms grown for 24 hr at 37° in tryptose broth with no fatty acid added. ×2700.

Fig. 2. Organisms grown for 24 hr at 37° in tryptose broth supplemented with 50 μg. palmitic acid./ml. ×2700.

Fig. 3. Organisms grown for 24 hr at 37° in tryptose broth supplemented with 50 μg. oleic acid./ml. ×2700.

PLATE 2

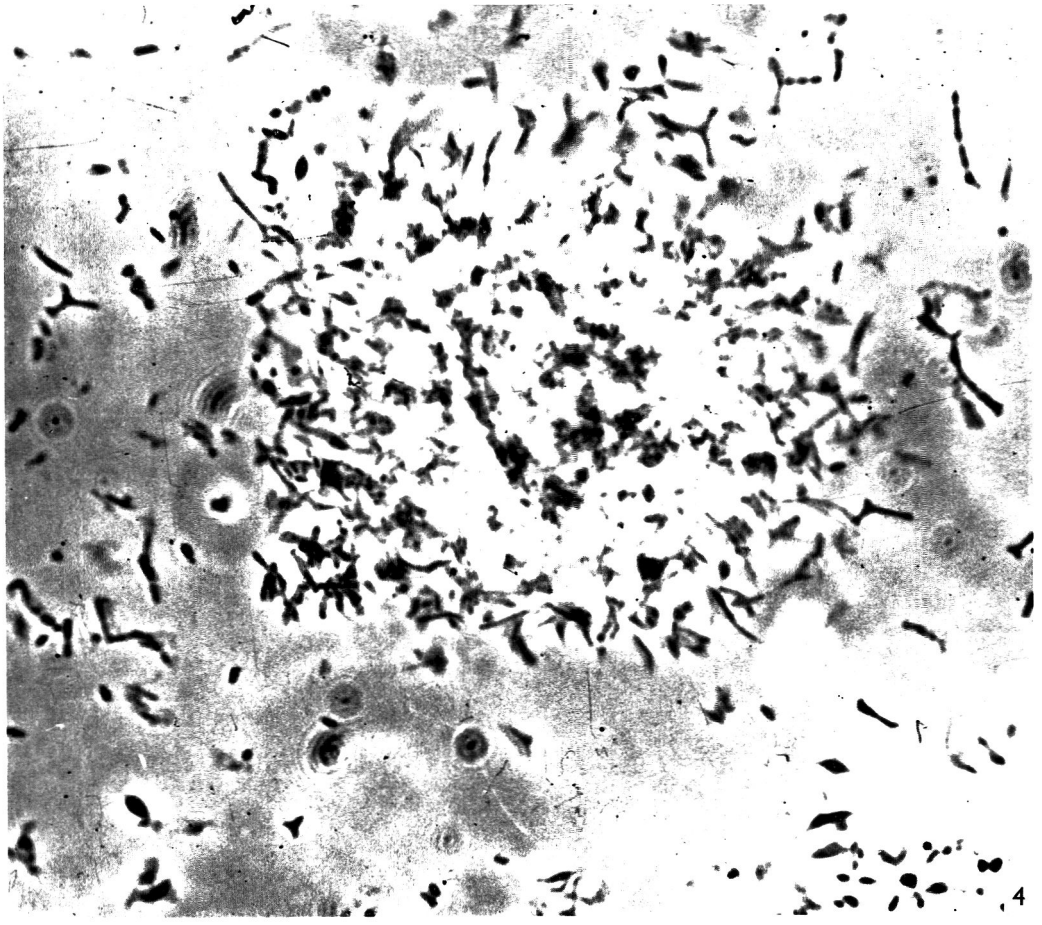
Mycoplasma laidlawii

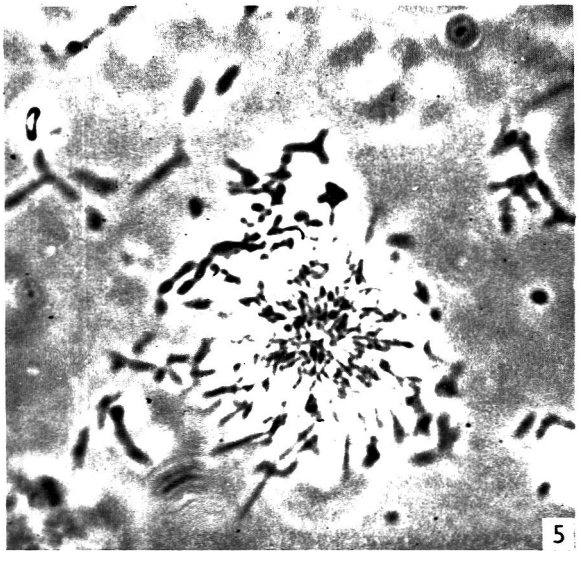
Fig. 4. A microcolony formed after 24 hr incubation at 37° in tryptose broth supplemented with 50 μg. oleic acid./ml. ×1250.

Fig. 5. A microcolony formed after 24 hr incubation at 37° in tryptose broth supplemented with 50 μg. linolenic acid./ml. ×1250.

Fig. 6. Organisms grown for 24 hr at 37° in tryptose broth supplemented with 50 μg. stearic acid./ml. ×2700.

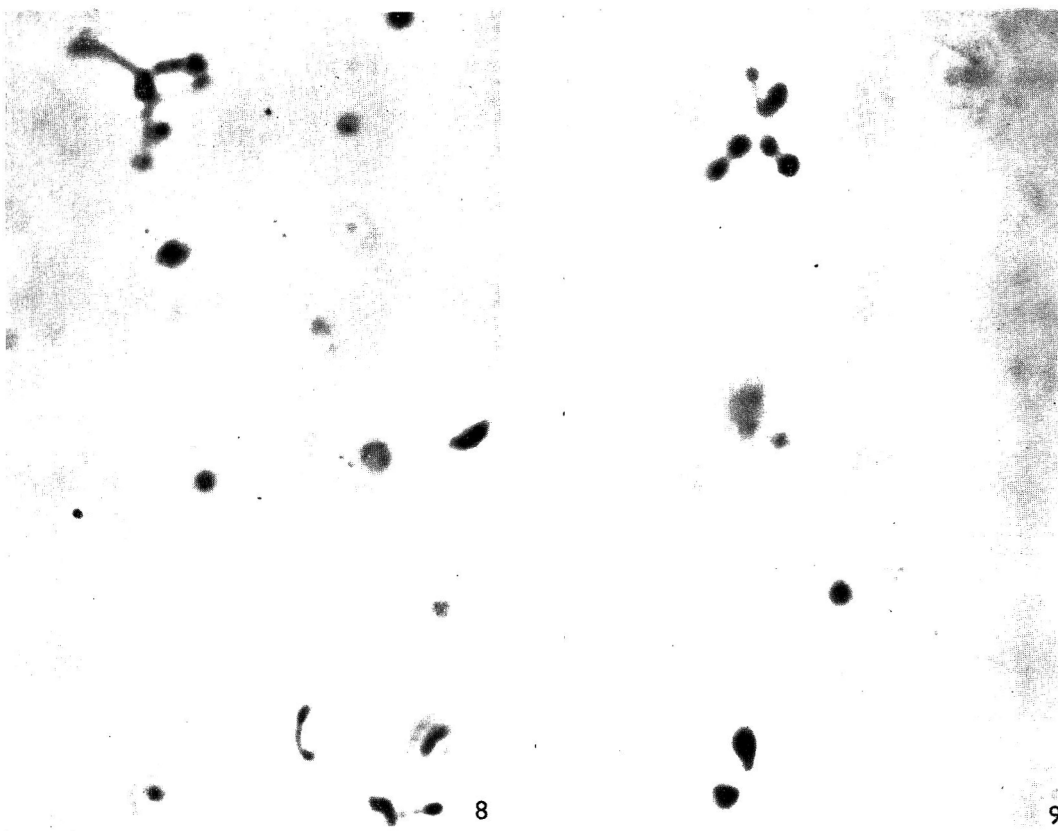
S. RAZIN, B. J. COSENZA AND M. E. TOURTELLOTTE (*Facing p.* 144)


4


5


6


7


8
9

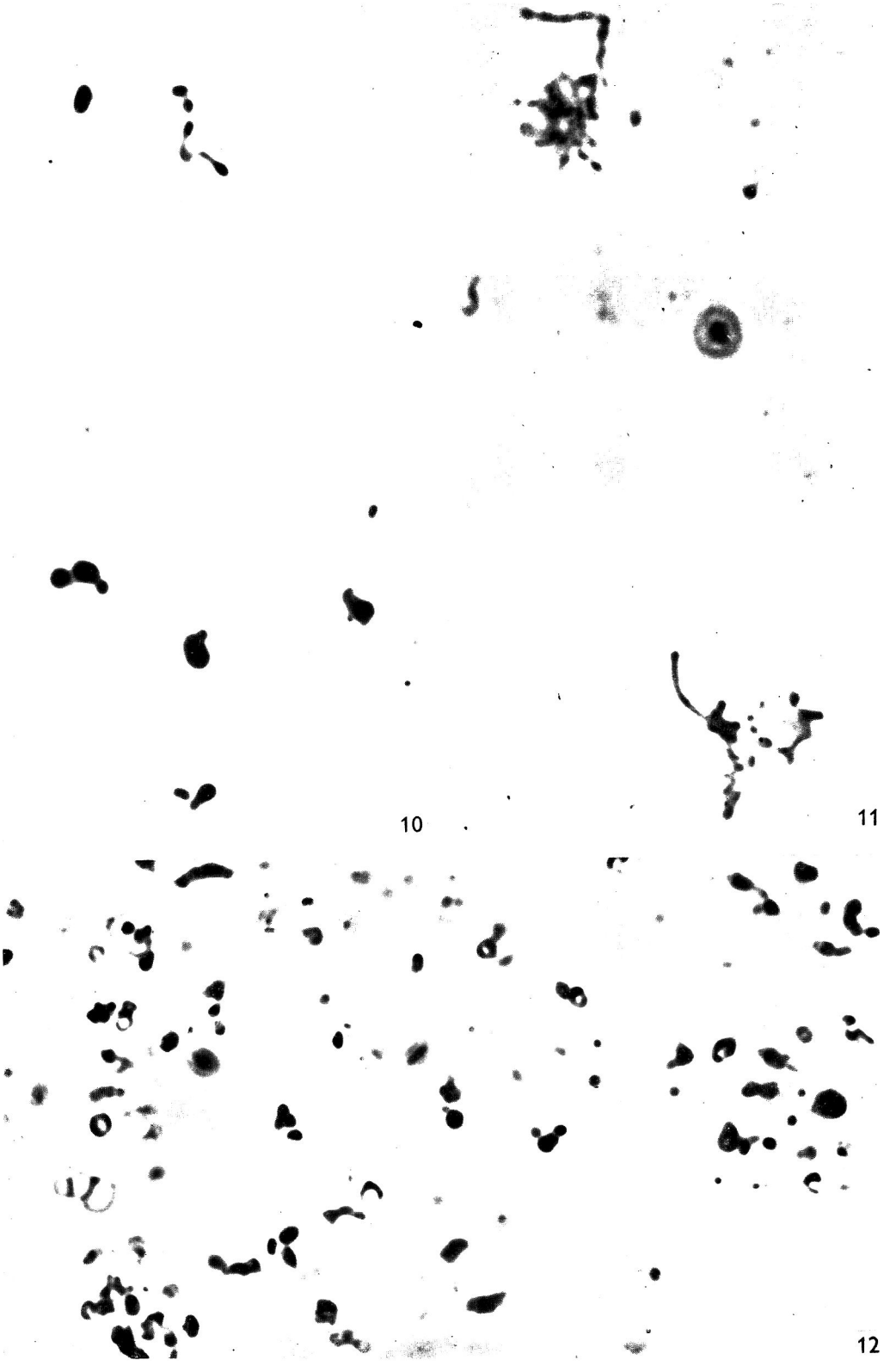

10
11
12

PLATE 3

Mycoplasma gallisepticum

Fig. 7. Organisms grown for 24 hr at 37° in tryptose broth containing 1 % (v/v) PPLO serum fraction. × 2700.

Fig. 8. Organisms grown for 24 hr at 37° in tryptose broth supplemented with 1 % (v/v) PPLO serum fraction and 50 μg. oleic acid./ml. × 2700.

Fig. 9. Organisms grown for 24 hr at 37° in tryptose broth supplemented with 1 % (v/v) PPLO serum fraction and 50 μg. palmitic acid./ml. × 3850.

PLATE 4

Mycoplasma sp. strain 14 (goat strain)

Fig. 10. Organisms grown for 24 hr at 37° in tryptose broth supplemented with 1 % PPLO serum fraction. × 2700.

Fig. 11. Organisms grown for 48 hr at 37° in tryptose broth supplemented with 1 % (v/v) PPLO serum fraction and 50 μg. oleic acid./ml. × 2700.

Fig. 12. Organisms grown for 48 hr at 37° in tryptose broth supplemented with 1 % (v/v) PPLO serum fraction only. × 1250.

J. gen. Microbiol. (1966), 42, 147–154
Printed in Great Britain

The Chemical Composition of the Cell Walls of Some Thermophilic Bacilli

By I. T. FORRESTER and A. J. WICKEN
Department of Biochemistry, Lincoln College, University of Canterbury, Canterbury, New Zealand

(*Received* 6 *September* 1965)

SUMMARY

The chemical compositions of the cell walls of one strain each of *Bacillus stearothermophilus* and *B. coagulans* prepared from organisms grown at 37° and 55° are compared. The higher temperature of cultivation resulted in an increased proportion of mucopeptide and a decreased proportion of teichoic acid in the walls of both organisms. A higher lipid content than is usual in Gram-positive organisms was found in these walls. The teichoic acid from *B. stearothermophilus* walls was a glycerophosphate polymer substituted with glucose and alanine; evidence suggesting the presence of 2,3-phosphodiester linkages in this polymer is presented. The teichoic acid from *B. coagulans* walls was also a glycerophosphate polymer, substituted with two neutral sugars, glucose and galactose, but lacking amino acid substituents hitherto reported as characteristic of teichoic acids.

INTRODUCTION

The average size of the bacterial cell appears to be a direct function of the growth rate. The relationship between cell-wall composition and growth rate has been studied in *Bacillus megaterium* (Sud & Schaechter, 1964), where the content of wall hexosamine and diaminopimelic acid, and of membrane lipid phosphorus, on a whole organism dry-weight basis, was found to be inversely proportional to the rate of growth. Nothing appears to be known of the effect of temperature of growth on cell-wall composition. With the exception of the determination of the mole ratios of wall amino acids in a strain of *Bacillus stearothermophilus* (Salton & Pavlik, 1960) the chemical composition of the walls of thermophilic bacilli is unknown. In the present study we have analysed the walls of two facultatively thermophilic bacilli prepared from organisms grown at 37° and 55°, respectively.

METHODS

Organisms. The following two organisms were used. *Bacillus stearothermophilus* (numbered B65 in our own collection and isolated originally from a 'Kilit Autoclave Control' spore suspension of *B. stearothermophilus* obtained from Baltimore Biological Laboratories, Inc., Baltimore 18, Maryland, U.S.A.) and *B. coagulans* (NRS no. T2007). Cultures were maintained on nutrient agar slopes. Both organisms were facultatively thermophilic in broth culture under the conditions used.

Preparation of cell walls. Both organisms were grown in a broth medium based on that of Campbell & Williams (1953) and composed of 1% (w/v) trypticase

(BBL), 0·5 % (w/v) yeast extract (BBL), 0·25 % (w/v) K_2HPO_4, 0·2 % (w/v) glucose, 0·1 % (v/v) salts solution (0·5 g. of each of $MgSO_4.5H_2O$, $FeCl_3$, $CaCl_2$/100 ml. water). 2-l. batches of medium in 4-l. flasks were inoculated with 20 ml. of an 18 hr culture, grown in the same medium at 37° or 55° as required in a Gallenkamp shaking water-bath and incubated at either 37° or 55°, respectively, with sterile air sparging at a rate of 1 l. air/l. culture/min. Excessive foaming was controlled by periodic additions of 1 ml. of a 10 % (v/v) silicone antifoam solution (Dow–Corning formula B). Growth was stopped near the end of the exponential phase of growth ($E^{1cm.}_{650m\mu}=1·00$) by adding chipped ice directly to the culture vessels. The organisms (no spores were seen) were collected by centrifugation in the cold, washed twice with cold 0·9 % (w/v) saline and finally resuspended (20 % w/v) in 0·9 % saline. Mean generation times in the exponential phase of growth of 32 and 20 min. were obtained for *Bacillus stearothermophilus* and 60 and 18 min. for *B. coagulans* at 37° and 55°, respectively.

Volumes of suspension (45 ml.) were mixed with an equal volume of ballotini beads (English Glass Co., grade 11) and disintegrated at 0–5° at 14,000 rev./min. in a M.S.E. homogenizer until 90–95 % of the organisms were broken, as judged by phase-contrast microscopy (25–35 min.). The cell-wall suspension was separated from glass beads by filtration through a No. 1 sintered glass filter and the beads washed twice with minimal volumes of cold saline. All subsequent operations were performed on an International Refrigerated Centrifuge (model HR 1 angle head no. 856). Unbroken organisms were separated from cell walls at 1000*g* for 10 min. (3 times). Walls were precipitated at 10,000*g* for 10 min. and washed by resuspension and recentrifugation in ice-cold M-KCl (3 times) and ice-cold distilled water (12 times). Final suspensions were freeze-dried and stored over P_2O_5. This procedure yielded 35–50 mg. dry-wt. cell wall/l. original culture. Electron-microscope examination of the isolated walls showed them to be free from cytoplasmic material. The absence of nucleic acids and proteins from these preparations was established by spectrophotometric examination at 260 mμ (Mitchell, 1950) and paper chromatography of acid hydrolysates as described in the text, respectively.

Paper chromatography. The following solvent systems were used: A, propan-1-ol + aq. ammonia (sp.gr. 0·88) + water (6 + 3 + 1, by vol.; Hanes & Isherwood, 1949), Whatman no. 4, ascending; B, pyridine + ethylacetate + water + acetic acid (5 + 5 + 3 + 1, by vol.; Fischer & Nebel, 1956), Whatman no. 1, descending; C, butan-1-ol + acetic acid + water (4 + 1 + 5, by vol.), upper layer (Partridge, 1948), Whatman 3MM, descending; D, butan-1-ol + pyridine + water (6 + 4 + 3, by vol.; Jeanes, Wise & Dimler, 1951), Whatman no. 4, descending; E, butan-2-ol + formic acid + water (7 + 1 + 2, by vol.; Roberts *et al.* 1955), Whatman no. 1, descending; F, phenol + water + aq. ammonia (sp.gr. 0·88) (80 g. + 20 ml. + 0·3 ml.; Roberts *et al.* 1955), Whatman no. 1, descending. Whatman no. 4 paper was washed before use with 2 N-acetic acid containing EDTA (0·1 %, w/v) and then water.

The following spray reagents were used where appropriate: periodate + Schiff reagent for polyols (Baddiley, Buchanan, Handschumacher & Prescott, 1956); perchloric acid + molybdate reagent for phosphates (Hanes & Isherwood, 1949); alkaline silver nitrate (Trevelyan, Procter & Harrison, 1950) and aniline phthalate (Partridge, 1949) for sugars; ninhydrin (Consden & Gordon, 1948) for amino acids; Elson–Morgan reagent for amino sugars (Partridge, 1949).

Hydrolysis conditions and analytical methods. For the analysis of amino acids 2 mg. dry-wt. cell wall were heated with 200 μl. 6N-HCl under N_2 in sealed tubes at 105° for 16 hr. After cooling, humin was removed by centrifugation and washed twice with water, the washing being pooled with the supernatant fluid. HCl was removed by repeated evaporation from water in vacuum over P_2O_5 and NaOH pellets. Two-dimensional chromatography in solvents E and F, by the technique of running standard substances and unknown mixture on the same paper (Saxena & Gandhi, 1962) was used to identify amino acids. For the quantitative estimation of diaminopimelic acid, glutamic acid, glycine and alanine, hydrolysates were dissolved in 100 μl. water and duplicate chromatograms were developed from 25 μl. portions in solvent D. Known concentrations of the four amino acids were chromatographed under identical conditions. The cadmium acetate + ninhydrin method of Heilmann, Barollier & Watzke (1958) was used to estimate amino acids on the developed chromatograms.

For the analysis of neutral sugars, 3 mg. dry-wt. cell wall were heated with 200 μl. 2N-H_2SO_4, under N_2 in sealed tubes at 100° for 2 hr. Hydrolysates were neutralized with 0·2N-$Ba(OH)_2$, the precipitate of $BaSO_4$ removed by centrifugation and washed twice with water, and supernatant fluid + washings passed through a short column of Dowex 50 (H^+) resin (1–2 ml.). The resin was washed with 5 column-volumes of water and the combined eluates concentrated by rotary evaporation. Paper chromatography in solvent B was used to identify the sugars present. For quantitative analysis eluates from the resin columns were made to 5 ml. with water and suitable portions used for analysis of total reducing sugar by the methods of Dubois *et al.* (1956) and Park & Johnson (1949). Glucose was estimated by the glucose oxidase method of Huggett & Nixon (1957) and galactose by the method of Park & Johnson (1949) after destruction of glucose by glucose oxidase.

For the detection of hexosamines, 2 mg. dry-wt. cell walls were heated in 200 μl. 4N-HCl in sealed tubes for 4 hr at 100°. Excess HCl was removed in vacuum over P_2O_5 and NaOH and the residue taken up in water. Neutral sugars were removed by passage of the hydrolysate through short columns of Dowex 50 (H^+) resin (1–2 ml.). After washing the resin with water (5 column-volumes) hexosamines were eluted with M-NH_4OH and evaporated to dryness in vacuum. Paper chromatography in solvent C was used to identify the hexosamines. Glucosamine was distinguished from galactosamine by ninhydrin oxidation and chromatography in solvent D (Ellwood, Kelemen & Baddiley, 1963). Quantitative estimations of amino sugar were made on hydrolysates prepared as above from 5 mg. dry wt. cell wall. After cooling, the hydrolysates were diluted quantitatively to 5 ml. with water. Portions (2 ml.) were neutralized with a slight excess of NH_4OH, freeze-dried and finally dissolved in 1 ml. water. Samples (50 μl.) were used to estimate hexosamines, as glucosamine, essentially by the procedure of Strominger, Park & Thompson (1959). The values obtained were corrected for acid destruction of hexosamines under the hydrolytic conditions used, by the method of Czerkawski, Perkins & Rogers (1963), ammonia in the diluted acid hydrolysates being estimated by Conway microdiffusion (Jacobs, 1956, 1960).

Phosphorus was estimated by the method of Fiske & SubbaRow (1925).

The lipid content of cell walls was determined, after heating with methanol by exhaustive extraction, under reflux, with ether, and then chloroform + methanol (1 + 1 by vol.).

The configuration of diaminopimelic acid in wall hydrolystes was determined by a combination of the chromatographic methods of Rhuland, Work, Denman & Hoard (1955) and Jusic, Roy, Schocker & Watson (1963).

Extraction of teichoic acids. Teichoic acids were extracted from 25 mg. portions of wall preparations with 10% (w/v) trichloracetic acid at 0–5° during 48 hr. After sedimentation by centrifugation, teichoic acid was precipitated from the supernatant solution by the addition of 5 vol. cold ethanol, and the precipitate washed with ethanol, ether, then dried in vacuum. The chemical composition of the teichoic acids was determined by hydrolysis with 2N-HCl or N-NaOH salution for 3 hr at 100°, followed by examination of the products by chromatography in solvents A and B. Alanine ester residues were detected by reaction with aqueous ammonia and chromatography of the resulting alanine and its amide in solvent A. Alkaline phosphomonoesterase (Sigma, type II) was used to degrade further the products of alkaline hydrolysis (see Wicken & Baddiley, 1963).

RESULTS

Acid hydrolysates from all of the cell-wall preparations of both organisms contained alanine, $\alpha\epsilon$-diaminopimelic acid, glutamic acid, glycine, muramic acid, glucosamine, glucose, glycerol and glycerol mono- and diphosphates; hydrolysates of *Bacillus coagulans* walls contained in addition galactose. The configuration of $\alpha\epsilon$-diaminopimelic acid in these walls was *meso-* as shown by paper chromatography of the isolated acid and its dinitrophenyl derivatives.

Table 1. *Composition of the cell walls of Bacillus stearothermophilus and B. coagulans grown at 37° and 55°*

	B. stearothermophilus		*B. coagulans*	
	37°	55°	37°	55°
	μmoles/mg. dry wt. cell wall			
Diaminopimelic acid	0·24	0·35	0·25	0·34
Glutamic acid	0·32	0·45	0·35	0·37
Alanine	1·14	0·96	0·50	0·57
Glycine	0·04	0·11	0·06	0·06
Hexosamine*	0·60	0·70	0·63	0·81
Glucose	0·98	0·88	0·67	0·53
Galactose	0·00	0·00	0·67	0·53
Phosphorus	1·27	1·28	0·83	0·71

* Calculated as glucosamine and corrected for destruction under the conditions of hydrolysis used as described in text.

Table 1 lists the results obtained from quantitative analyses of acid hydrolysates of cell walls; the relative mole ratios of amino acids and hexosamine are shown in Table 2.

Ether extractable, chloroform + methanol extractable and total extractable lipid from the cell walls of *Bacillus stearothermophilus* grown at 55° were 4·3, 3·7 and 8·0% of the dry-wt. of wall, respectively. Corresponding figures for *B. coagulans* were 8·3, 4·3 and 12·6%. Insufficient material was available for comparable

analyses of walls from organisms grown at 37°. Chromatographic examination of acid hydrolysates of extracted lipid material in solvents B, E and F did not show neutral sugars, hexosamines or amino acids.

The chromatographically identified products of acid and alkali hydrolysis of teichoic acids extracted from the walls of both organisms are listed in Table 3. Ammonolysis of the teichoic acid from, and whole cell walls of, *Bacillus stearothermophilus* gave alanine and alanine amide, indicating the presence of alanyl ester linkages. The absence of similar residues from *B. coagulans* walls or extracted

Table 2. *Mole ratios* of amino acids and hexosamines in the cell walls of Bacillus stearothermophilus and B. coagulans grown at 37° and 55°*

	Hexosamines	Glutamic acid	DAP†	Alanine	Glycine
B. stearothermophilus, 37°	1·89	1·00	0·76	3·57	0·01
B. stearothermophilus, 55°	1·50	1·00	0·77	2·10	0·02
B. coagulans, 37°	1·80	1·00	0·70	1·43	0·02
B. coagulans, 55°	2·17	1·00	0·93	1·55	0·02

* Relative to glutamic acid = 1·00. † DAP = *meso*-αε-diaminopimelic acid.

Table 3. *Hydrolysis products of teichoic acids extracted from Bacillus stearothermophilus and B. coagulans*

	B. stearothermophilus			*B. coagulans*	
	Acid hydrolysis	Alkali hydrolysis	Alkali hydrolysis + alkaline phospho-mono-esterase	Acid hydrolysis	Alkali hydrolysis*
Glycerol	+	Trace	+	+	–
Glyceromonophosphates	+	+	–	+	Trace
Glucosylglycerophosphates	–	+	–	–	–
Glycerodiphosphates	+	+	–	+	Trace
Glucosylglycerol	–	Trace	+	–	–
Glucose	+	–	–	+	–
Galactose	–	–	–	+	–
Inorganic phosphate	+	Trace	+	+	–
Alanine	+	+	.	–	–

* Highly resistant to alkali hydrolysis.

teichoic acid was confirmed by ammonolysis. No ninhydrin-positive material was found in acid hydrolysates of the teichoic acid from *B. coagulans*; this acid was highly resistant to alkali hydrolysis.

The teichoic acid of *Bacillus stearothermophilus* on alkali hydrolysis followed by treatment with alkaline phosphomonoesterase gave glycerol and a compound that reacted rapidly with the periodate Schiff reagent and had an R_F value of glucosylglycerol in solvent A. Acid hydrolysis of this material gave glucose and glycerol as the sole products.

DISCUSSION

The cell walls of *Bacillus coagulans* and *B. stearothermophilus* appear to be composed of three main components: glycosaminopeptide (mucopeptide), teichoic acid, lipid. The constituents of the glycosaminopeptide fractions, (glucosamine, muramic acid, meso-$\alpha\epsilon$-diaminopimelic acid, glutamic acid, alanine, small amounts of glycine) are typical of many Gram-positive bacteria (Salton, 1964). In both organisms a significantly greater proportion of glycosaminopeptide components to other wall constituents was found for cell walls prepared from organisms grown at 55° as compared with those grown at 37°. Variations in the molar proportions of hexosamines and wall amino acids with temperature of growth were seen and these may reflect differences in glycosaminopeptide structure within the same organism. A greater resistance to mechanical rupture during cell-wall preparation was noted with organisms grown at 37° as compared with those grown at 55°. Salton & Pavlik (1960) reported mole ratios of glutamic acid:$\alpha\epsilon$-diaminopimelic acid:alanine:glycine of 1·0:0·50:1·9:0·0 for the cell walls of a strain of *B. stearothermophilus* but did not state the conditions of culture used. The higher content of alanine in *B. stearothermophilus* walls than in *B. coagulans* found in the present work can be accounted for by the presence of alanyl ester substitution of the teichoic acid in the former organism and the absence of such substitution in *B. coagulans*.

The lipid content of the walls of these thermophilic organisms was surprisingly high as compared with that of many mesophilic Gram-positive bacteria, which seldom exceed 1–2% (Salton, 1964). The absence of amino acids other than typical cell-wall amino acids in acid hydrolysates of these wall preparations suggests that the extracted lipid is a true wall component and not due to contamination with lipoprotein cell membrane. The nature of the lipid was not investigated other than to establish the absence, in acid hydrolysates of extracted lipid, of neutral sugars, amino sugars or amino acids.

The teichoic acids in these organisms show some unusual features. That from *Bacillus stearothermophilus* walls gave on acid hydrolysis products which may be considered typical of a glycerol teichoic acid substituted with glucose in glycosidic linkage to a polyol hydroxyl group and with alanine in ester linkage to the polymer (see Wicken & Baddiley, 1963). The course of alkali hydrolysis of glycerol teichoic acids was discussed by Kelemen & Baddiley (1961). It has been shown that increasing glycosidic substitution of a 1,3-phosphodiester-linked glycerophosphate polymer increases the resistance of the polymer to alkali degradation, substitution of the 2-hydroxyl group of the glycerol moieties with a sugar preventing the necessary intermediate formation of a cyclic phosphate (see Wicken & Baddiley, 1963). A 2,3-phosphodiester-linked glycerol teichoic acid, on the other hand, would be expected to be degraded in alkali, even if highly substituted with sugar, to a mixture of glyceromonophosphates, glycosylglyceromonophosphates and small amounts of glycerol and glycerol diphosphates. The glycerol teichoic acid extracted from *B. stearothermophilus* walls was completely degraded in alkali to such a mixture of phosphates. Treatment of an alkali hydrolysate with alkaline phosphomonoesterase gave glycerol as a minor product and a major component which had the R_F value of glucosylglycerol in solvent A. Acid hydrolysis of this material gave glucose and glycerol only. The fast reaction obtained between this material and the periodate

Schiff reagent is indicative of the presence of an α-glycol group within the molecule (Roberts, Buchanan & Baddiley, 1963), which in turn suggests that the glycosidic linkage is to a primary hydroxyl group of glycerol. It is therefore suggested that this teichoic acid contains 2,3-phosphodiester linkages rather than the more usual 1,3-phosphodiester linkages. The possible existence of a similarly linked glycerol teichoic acid from *B. licheniformis* was reported by Burger (1963).

The glycerol teichoic acid isolated from *Bacillus coagulans* showed two unusual features. No substitution by alanine or another amino acid was found and this was confirmed by ammonolysis of intact cell walls. The presence of both glucose and galactose in acid hydrolysates of this teichoic acid and the high degree of resistance of the polymer to alkali degradation suggests either a single 1,3-phosphodiester-linked glycerophosphate polymer substituted with both sugars, or a mixture of two polymers each substituted with a single sugar. More recent work has confirmed the former possibility (Forrester & Wicken, unpublished observations). The possible linkage of glycerophosphate moieties through sugar residues in this polymer is not precluded by these observations. Further investigations into the complete structures of these two teichoic acids is in progress. The increased glycosaminopeptide content of the walls of organisms when grown at the higher temperature was accompanied by a decrease in total neutral reducing sugars. This might be equated with a decrease in teichoic acid content at the higher growth temperature, or alternatively a decrease in the degree of glycosidic substitution of the polymers. Corresponding decreases in total phosphorus content were not as marked but it has been established that teichoic acid phosphorus does not necessarily represent the total cell-wall phosphorus (Ellwood *et al.* 1963). In *Bacillus stearothermophilus* walls a lower total alanine content and lower mole ratio of this amino acid to other wall constituents was noted for walls from 55°-grown organisms. This, too, would be consistent with a lower teichoic acid content of these walls.

REFERENCES

Baddiley, J., Buchanan, J. G., Handschumacher, R. E. & Prescott, J. F. (1956). Biosynthesis of purine nucleotides. I. Preparation of N-glycylglycosylamines. *J. chem. Soc.* p. 2818.

Burger, M. M. (1963). The enzymic glucosylation of polyglycerophosphate. *Biochim. biophys. Acta*, **71**, 495.

Campbell, L. L. & Williams, O. B. (1953). The effect of temperature on the nutritional requirements of facultative and obligate thermophilic bacteria. *J. Bact.* **65**, 141.

Consden, R. & Gordon, A. H. (1948). Effect of salt on partition chromatograms. *Nature, Lond.* **162**, 180.

Czerkawski, J. W., Perkins, H. R. & Rogers, H. J. (1963). A study of the composition and structure of the cell wall mucopeptide of *Micrococcus lysodeikticus*. *Biochem. J.* **86**, 468.

Dubois, M., Gilles, K. A., Hamilton, J. K., Rebers, P. A. & Smith, F. (1956). Colorimetric method for determination of sugars and related substances. *Analyt. Chem.* **28**, 350.

Ellwood, D. C., Kelemen, M. V. & Baddiley, J. (1963). The glycerol teichoic acid from the walls of *Staphylococcus albus* NCTC 7944. *Biochem. J.* **86**, 213.

Fischer, F. G. & Nebel, H. G. (1956). Detection and estimation of glucosamine and galactosamine on paper chromatograms. *Analyt. abstr.* **3**, 1101.

Fiske, C. H. & SubbaRow, W. (1925). The colorimetric determination of phosphorus. *J. biol. Chem.* **66**, 375.

HANES, C. S. & ISHERWOOD, F. A. (1949). Separation of phosphoric esters on the filter paper chromatogram. *Nature, Lond.* **164**, 1107.

HEILMANN, J., BAROLLIER, J. & WATZKE, E. (1958). Determinations of amino acids on paper chromatograms. *Z. physiol. chem.* **309**, 219.

HUGGETT, A. ST G. & NIXON, D. A. (1957). Use of glucose oxidase, peroxidase and o-dianisidine in determination of blood and urinary glucose. *Lancet*, **273**, 368.

JACOBS, S. (1956). An improved method for the quantitative determination of amino acids by means of indanetrione hydrate. *Analyst*, **81**, 502.

JACOBS, S. (1960). The determination of nitrogen in organic compounds by the indanetrione hydrate method. *Analyst.* **85**, 257.

JEANES, A., WISE, C. S. & DIMLER, R. J. (1951). Improved techniques in paper chromatography of carbohydrates. *Analyt. Chem.* **23**, 415.

JUSIC, D., ROY, C., SCHOCKER, A. J. & WATSON, R. W. (1963). Chromatographic separation of isomeric dinitrophenyl derivatives of α-ϵ-diaminopimelic acid. *Canad. J. Biochem. & Phys.* **41**, 817.

KELEMEN, M. V. & BADDILEY, J. (1961). Structure of the intracellular glycerol teichoic acid from *Lactobacillus casei* ATCC 7469. *Biochem. J.* **80**, 246.

MITCHELL, P. (1950). Spectrophotometric estimation of nucleic acid in bacterial suspensions. *J. gen. Microbiol.* **4**, 399.

PARK, J. T. & JOHNSON, M. J. (1949). A submicrodetermination of glucose. *J. biol. Chem.* **181**, 149.

PARTRIDGE, S. M. (1948). Filter-paper partition chromatography of sugars. *Biochem. J.* **42**, 238.

PARTRIDGE, S. M. (1949). Aniline hydrogen phthalate as a spraying reagent for chromatography of sugars. *Nature, Lond.* **164**, 443.

RHULAND, L. E., WORK, E., DENMAN, R. F. & HOARE, D. S. (1955). The behaviour of the isomers of α, ϵ-diaminopimelic acid on paper chromatograms. *J. Am. Chem. Soc.* **77**, 4844.

ROBERTS, W. K., BUCHANAN, J. G. & BADDILEY, J. (1963). The specific substance from *Pneumococcus* type 34 (41). *Biochem. J.* **88**, 1.

ROBERTS, R. B., COWIE, D. B., ABELSON, P. H., BOLTON, E. T. & BRITTEN, R. J. (1955). In *Studies of Biosynthesis in Escherichia coli.* Publs Carnegie Instn, no. 607.

SALTON, M. R. J. (1964). *The Bacterial Cell Wall.* London: Elsevier Publishing Co.

SALTON, M. R. J. & PAVLICK, J. G. (1960). Studies of the bacterial cell wall. VI. Wall composition and sensitivity to lysozyme. *Biochim. biophys. Acta*, **39**, 398.

SAXENA, K. N. & GANDHI, J. R. (1962). Separation and identification of amino acids by two-dimensional paper chromatography. *J. Chromat.* **8**, 546.

STROMINGER, J. L., PARK, J. T. & THOMPSON, R. E. (1959). Composition of the cell wall of *Staphylococcus aureus*: its relation to the mechanism of action of penicillin. *J. biol. Chem.* **234**, 3263.

SUD, I. J. & SHAECHTER, M. (1964). Dependence of the content of cell envelopes on the growth rate of *Bacillus megaterium*. *J. Bact.* **88**, 1612.

TREVELYAN, W. E., PROCTER, D. P. & HARRISON, J. S. (1950). Detection of sugars on paper chromatograms. *Nature, Lond.* **166**, 444.

WICKEN, A. J. & BADDILEY, J. (1963). Structure of intracellular teichoic acids from group D streptococci. *Biochem. J.* **87**, 54.

J. gen. Microbiol. (1966), **42**, 155–163
Printed in Great Britain

The Regulation of Cystathionine Formation in *Escherichia coli*

By R. J. ROWBURY and the late D. D. WOODS

Department of Botany, University College London, and Microbiology Unit, Department of Biochemistry, University of Oxford

(*Received* 16 *September* 1965)

SUMMARY

The amounts of cystathionine-forming enzymes (homoserine *O*-trans-succinylase and cystathionine synthetase) of *Escherichia coli* were decreased by growth with methionine, and therefore enzymic repression applied to all the enzymes of the methionine biosynthetic pathway. Enzyme resynthesis occurred when 'inactive' organisms (organisms grown with methionine) were suspended in methionine-free culture fluid. Although the formation of all the enzymes of the pathway appears to be controlled by a single regulator gene, co-ordinate repression and de-repression were not observed. Methionine also regulates its own synthesis by inhibition of the activity of homoserine *O-trans*-succinylase, the inhibitory effect being more marked with intact organisms than with cell-free extracts. Methionine did not influence the activity of cystathionine synthetase or the subsequent methionine-forming enzymes.

INTRODUCTION

The biosynthetic pathways of amino acids and other metabolites in bacteria are generally controlled by two regulatory mechanisms. Enzymic repression (Vogel, 1957), the diminution of enzyme synthesis by the end-product, frequently affects all the enzymes of a pathway, whereas feedback inhibition, the diminution of the activity of enzymes by end-product, usually applies only to the first enzyme (Yates & Pardee, 1956; Wormser & Pardee, 1958) although there may be exceptions, especially in complex pathways (Stadtman, 1963). The present work forms part of a study of the regulation of methionine biosynthesis. Previous work showed that the homocysteine methylase complex and its constituent reactions and cystathionase were subject to enzymic repression by methionine (Wijesundera & Woods, 1953; Rowbury & Woods, 1961*a*, *b*; Foster, Rowbury & Woods, 1963). The effect of methionine on the formation and activity of enzymes catalysing the synthesis of cystathionine is examined in the present work. A brief report of part of this work was presented by Rowbury (1962*a*).

METHODS

Organisms. All the strains of *Escherichia coli* used in this work were maintained on slopes of Oxoid nutrient agar, subcultured monthly and stored at 4° after incubation for 18 hr at 37°. *E. coli* strain 26/18 responded to homocysteine or methionine and *E. coli* strains 7/9, 2/2 and 122/33 responded also to cystathionine.

Growth of organisms. Organisms were grown on a glucose lactate salts (GL) medium supplemented with DL-homocysteine (0·25 mM) except where otherwise stated. Conditions for growth, harvesting and preparation of enzymic extracts were as described by Rowbury & Woods (1961*a*). In certain experiments the resynthesis of enzymes after repression by methionine was studied as described by Rowbury & Woods (1961*a*).

Assay of homocysteine methylase. The overall homocysteine methylase complex was assayed in some extracts by the method of Rowbury & Woods (1961*a*) with a heated extract of organisms as source of folic acid co-factor and serine as donor of the one-carbon unit.

Cystathionase assay. Cystathionase activity was assayed by measuring pyruvate production from cystathionine as described by Wijesundera & Woods (1962). In certain experiments pyruvate production from cysteine was measured by the same method.

Assay of the cystathionine-forming enzymes. The overall conversion of homoserine + cysteine to cystathionine by extracts from *Escherichia coli* strain 26/18 was assayed as described by Rowbury & Woods (1964*b*). Incubation was for 3–4 hr at 37° in solution *A* which contained (in 3 ml. of 80 mM-phosphate buffer (pH 7·5): DL-homoserine (5 mM), L-cysteine (3·3 mM), sodium succinate (50 mM), ATP (3·3 mM), glucose (6·6 mM), coenzyme A (0·06 mM) with 3·3 mg./ml. ultrasonic extract.

Homoserine *O-trans*-succinylase was assayed by two methods. In the first (method *a*), different amounts of extract from *Escherichia coli* strain 7/9 were incubated in solution *A* with an excess of extract from *E. coli* strain 2/2. Activity was assessed from the amount of cystathionine formed. In the second method (method *b*), extract from *E. coli* strain 7/9 was incubated in solution *A* (cysteine omitted) with 5 μc. of $^{14}C_{2,3}$-succinate added. Activity was assessed by the incorporation of ^{14}C into *O*-succinylhomoserine (separated from succinate on columns of Dowex-1 resin as described by Rowbury & Woods, 1964*b*). The synthesis of *O*-succinylhomoserine by whole organisms of *E. coli* strain 7/9 (incubated in medium GL) was also assayed by uptake of ^{14}C-succinate.

The reaction of *O*-succinylhomoserine with cysteine (cystathionine synthetase) was assayed either by measuring the cystathionine formed by various amounts of extract from *Escherichia coli* strain 2/2 incubated in solution *A* with excess extract from *E. coli* strain 7/9 (method *a*), or by following cystathionine formation from cysteine + synthetic *O*-succinylhomoserine (method *b*), or by measuring the disappearance of isotope from ^{14}C-*O*-succinylhomoserine and the appearance of ^{14}C in free succinate on incubation with the appropriate extract plus cysteine (method *c*).

Assay of cystathionine. Assays were done as described by Rowbury & Woods (1964*b*) with *Escherichia coli* strain 122/33.

Chromatographic methods. In certain experiments reaction products were run on paper chromatograms in *n*-butanol + propionic acid + water (47 + 22 + 31, by vol.) and the chromatograms either scanned for radioactivity directly with the Geiger–Müller tube of a Ratemeter Unit (type 1355B, Fleming Radio Development Ltd.) or after drying were cut up and specific regions assayed for cystathionine after elution with buffer.

Column chromatography used the acetate form of Dowex-1 resin (X8, 200–400

mesh) and the procedure was as described by Rowbury & Woods (1964*b*). In certain experiments the radioactivity of the eluate was measured by spotting samples on uniform ground-glass discs and counting at infinite thinness with a Scaler 1700 (Isotope Developments Ltd., Reading, Berkshire) for 1000 sec. Corrections were made for background activity.

Amino acids were detected on paper chromatograms by spraying with ninhydrin (0·2% w/v in *n*-butanol saturated with water) and heating at 80° for 10 min. The eluate from columns of Dowex-1 resin was assayed for amino-nitrogen by the method of Moore & Stein (1954).

Chemicals. Most of the chemicals used were as described previously (Rowbury & Woods, 1964*a*, *b*). $^{14}C_{2,3}$-succinate was obtained from the Radiochemical Centre, Amersham, Bucks. ^{14}C-*O*-succinylhomoserine was obtained from ^{14}C-succinate by growth of *Escherichia coli* strain 7/9 on medium GL with added DL-homocysteine (0·25 mM) and DL-homoserine (2·5 mM) followed by chromatographic separation as described by Rowbury & Woods (1964*b*).

RESULTS

Enzymic repression of the cystathionine-forming enzymes by growth with methionine

A cystathionaseless strain of *Escherichia coli* (strain 26/18) has been used previously to study cystathionine synthesis from homoserine and cysteine (Rowbury, 1961). Homocysteine methylase was known to be repressed by methionine in this strain (Rowbury & Woods, 1961*a*). Overall cystathionine synthesis from homoserine + cysteine was also decreased in extracts from organisms grown with methionine, the extent of repression produced by 3 mM-DL-methionine being about the same as for homocysteine methylase (Table 1) although at lower methionine concentrations the extent of repression was greater for homocysteine methylase.

Table 1. *The repression of cystathionine formation by methionine in Escherichia coli strain 26/18*

Ultrasonic extracts were prepared from organisms grown on medium GL with the stated amount of methionine. Methionine synthesis (from homocysteine) was assayed as described by Rowbury & Woods (1961*a*) and cystathionine formation from homoserine + cysteine measured in solution *A*. Incubation was for 3 hr at 37° in each case.

DL-methionine in growth medium (mM)	Methionine formed (μmmoles/mg. protein/hr)	Cystathionine formed (μmmoles/mg. protein/hr)
0	66	76
0·1	30	67
0·3	13	17
1	10	13
3	8	11

The formation of cystathionine requires two enzymes: homoserine *O-trans*-succinylase and cystathionine synthetase (Rowbury, 1962*a*; Rowbury & Woods, 1964*b*). Growth with methionine represses the formation of both of these enzymes. When *Escherichia coli* strain 7/9 was grown with DL-methionine (3 mM) the formation of homoserine-*O-trans*-succinylase was decreased by about 80%; homo-

cysteine methylase, cystathionase and cysteine deaminase were repressed by 85–90% under these conditions (Table 2). Growth of *E. coli* strain 2/2 with methionine (3 mM) decreased the amount of cystathionine synthetase by 85% while the amounts of homocysteine methylase and cystathionase decreased by about 90% (Table 3).

Table 2. *Repression of homoserine O-trans-succinylase by methionine in Escherichia coli strain 7/9*

Extracts were prepared from *E. coli* strain 7/9 after growth with the stated amount of methionine. Homocysteine methylase was assayed as described by Rowbury & Woods (1961*a*), cystathionase and cysteine deaminase were measured as described by Rowbury & Woods (1964*a*) and homoserine *O-trans*-succinylase was assayed by method *a* (see Methods) with a 3 hr incubation at 37°.

DL-methionine in growth medium (mM)	Homocysteine methylase (μmmoles/mg. protein/3 hr)	Cystathionase (μmoles pyruvate/mg. protein/hr)	Cysteine deaminase (μmoles pyruvate/mg. protein/hr)	Homoserine *O-trans*-succinylase (μmmoles/mg. protein/hr)
0	48	0·8	0·10	50
0·3	15	0·18	0·02	15
1·0	13	0·12	0·015	12
3·0	8	0·10	0·013	9

Table 3. *Repression by methionine of the formation of cystathionine synthetase of Escherichia coli strain 2/2*

Homocysteine methylase and cystathionase were assayed as for Table 2 in ultrasonic extracts prepared from *E. coli* strain 2/2 grown with the stated concentration of methionine. Cystathionine synthetase (i.e. the enzyme forming cystathionine from *O*-succinylhomoserine plus cysteine) was assayed by method *a* in solution *A* (see Methods) with a 3 hr incubation time at 37°.

DL-methionine in growth medium (mM)	Homocysteine methylase (μmmoles/mg. protein/3 hr)	Cystathionase (μmoles pyruvate/mg. protein/hr)	Cystathionine synthetase (μmmoles/mg. protein/3 hr)
0	54	0·98	570
0·3	9	0·18	170
1·0	6	0·12	95
3·0	6	0·11	83

Enzyme resynthesis after repression by methionine

Homocysteine methylase and cystathionase are rapidly reformed when 'inactive' organisms (organisms which have been grown with methionine) are suspended in culture fluid free from methionine (Rowbury & Woods, 1961*a*, *b*). Recovery from repression also occurs with the cystathionine-forming enzymes. Organisms of *Escherichia coli* strain 26/18 when transferred from methionine medium to methionine-free medium rapidly regained the ability to form cystathionine from homoserine + cysteine and from *O*-succinylhomoserine + cysteine, although the latter reaction reached maximal values more quickly (Fig. 1). Homocysteine methylase increased at a similar rate to that of the overall reaction.

'Inactive' organisms of *Escherichia coli* strain 7/9 had much decreased amounts of homoserine *O-trans*-succinylase (Table 2) but on resuspension in methionine-

free medium, enzyme activity rose to the de-repressed value in $3\frac{1}{2}$ hr; cystathionase activity increased during this period and reached the normal value in $2\frac{1}{2}$ hr (Fig. 2). Enzyme resynthesis also occurred when 'inactive' organisms from *E. coli* strain 2/2 were suspended in methionine-free medium, and cystathionine synthetase reached maximal values more rapidly than cystathionase (Fig. 3).

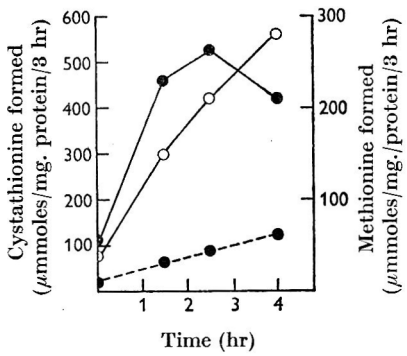


Fig. 1

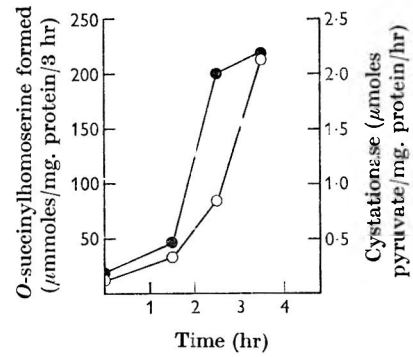


Fig. 2

Fig. 1. Resynthesis of the methionine-forming enzymes in *Escherichia coli* strain 26/18. Organisms grown in medium GL with DL-methionine (3·3 mM) were transferred to methionine-free culture fluid and samples taken at the stated intervals for the preparation of ultrasonic extracts. Homocysteine methylase activity (○—○) was assayed as described by Rowbury & Woods (1961*a*), the overall synthesis of cystathionine from homoserine plus cysteine (●···●) measured in solution *A* (see Methods) and the formation of cystathionine from *O*-succinylhomoserine (2 mM) and cysteine (3·3 mM) assayed by method *b* (●—●). In each case incubation was for 3 hr at 37°.

Fig. 2. Resynthesis of homoserine *O-trans*-succinylase after repression by methionine. Organisms of *Escherichia coli* strain 7/9 were harvested from medium GL containing DL-methionine (3 mM) and resuspended in methionine-free culture fluid. Ultrasonic extracts were prepared at the stated intervals. Homoserine *O-trans*-succinylase (○—○) was assayed by method *b* with a 3 hr incubation period at 37°, and cystathionase (●—●) measured as described by Rowbury & Woods (1964*b*) with a 30 min. incubation period at 37°.

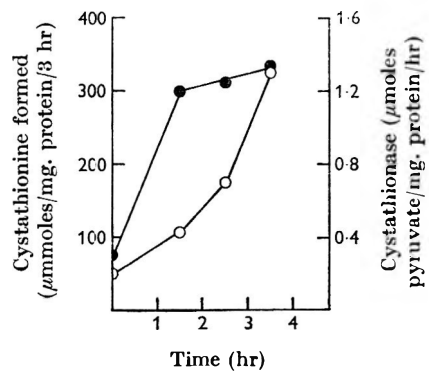


Fig. 3. Recovery from repression by methionine in *Escherichia coli* strain 2/2. Organisms were grown in medium GL with methionine (3 mM), and after harvesting resuspended in culture fluid free of methionine. Ultrasonic extracts were prepared initially and at the stated intervals and cystathionase (○—○) assayed as described for Fig. 2 and cystathionine synthetase (●—●) by method *b* (see Methods).

The effect of methionine on the activity of the cystathionine-forming enzymes

The effect of methionine on overall cystathionine synthesis from homoserine and cysteine was treated by adding various amounts of methionine to reaction mixtures containing extract from *Escherichia coli* strain 26/18. After incubation the cystathionine formed was separated from the added methionine by paper chromatography and assayed with *E. coli* strain 122/33. The overall reaction was markedly inhibited by methionine; 60% inhibition was produced by 2 mM-DL-methionine (Table 4).

Table 4. *Feedback inhibition by methionine of cystathionine synthesis in Escherichia coli strain 26/18*

Cystathionine formation by extract from *E. coli* strain 26/18 was assayed by incubation in solution *A* with the stated amount of methionine, separation of the product from the added methionine by paper chromatography in *n*-butanol + acetic acid + water (2 + 1 + 1, by vol.) and bioassay of the eluted cystathionine with *E. coli* strain 122/33.

DL-methionine added to reaction mixture (mM)	Cystathionine formed (μmmoles/mg. protein/3 hr)
0	150
2	51
6	14
10	6

Table 5. *Feedback inhibition by methionine of homoserine O-trans-succinylase activity in intact organisms and cell-free extracts of Escherichia coli strain 7/9*

Enzymic activity of whole organisms was assayed by measuring the incorporation of ^{14}C-succinate into *O*-succinylhomoserine by suspensions (about 1 mg. dry wt./ml.) incubated for 1 hr at 37° in medium GL with DL-homoserine (2·5 mM) and ^{14}C-succinate (5 μc.) added and with the stated concentration of methionine present. Activity of ultrasonic extracts was assessed by incubation in solution *A* (with cysteine omitted) with the addition of ^{14}C-succinate (5 μc.) and the stated amount of methionine; incorporation of ^{14}C into *O*-succinylhomoserine was measured. In both cases values are given as a % of the control incubation (without added methionine).

	Homoserine *O-trans*-succinylase	
	Intact organisms	Ultrasonic extracts
DL-methionine added (mM)	Activity (% of control)	
0	100	100
0·01	35	—
0·05	8	100
0·5	0	55
1·0	—	31
5·0	—	4

Methionine also inhibited homoserine *O-trans*-succinylase, the first reaction of the biosynthetic pathway. Synthesis of *O*-succinylhomoserine (assayed by incorporation of ^{14}C-succinate) was decreased to 50% by 0·5 mM-DL-methionine and to 5% by 5 mM-methionine in extracts of *Escherichia coli* strain 7/9 (Table 5). Whole organisms of *E. coli* strain 7/9 accumulate *O*-succinylhomoserine in the

culture fluid (Rowbury, 1964*c*) and this accumulation is also subject to feedback inhibition by methionine. Accumulation was decreased to 35% by 0·01 mM-DL-methionine and to 8% by 0·05 mM-methionine (Table 5).

The effect of methionine on cystathionine synthetase (the enzyme forming cystathionine from *O*-succinylhomoserine + cysteine) was also tested with extracts from *Escherichia coli* strains 26/18 and 2/2. With extract from *E. coli* strain 26/18 cystathionine formation (assayed by the disappearance of *O*-succinylhomoserine and the formation of ^{14}C-succinate) from *O*-succinylhomoserine was not decreased by the presence of 20 mM-DL-methionine (Table 6). Similarly the activity of cystathionine synthetase from *E. coli* 2/2 was unaffected by the presence of methionine in reaction mixtures. The formation of cystathionine from *O*-succinylhomoserine in the presence of methionine was confirmed by paper chromatography and by isotope experiments with *O*-succinylhomoserine labelled with ^{14}C in the homoserine residue.

Table 6. *The effect of methionine on the formation of cystathionine and succinate from O-succinylhomoserine + cysteine by Escherichia coli strain 26/18*

Extract from *E. coli* strain 26/18 was incubated for 2 hr at 37° with ^{14}C-*O*-succinylhomoserine (2 mM) + cysteine (3·3 mM). The products were separated by paper chromatography in *n*-butanol + propionic acid + water (47 + 22 + 31, by vol.) and the radioactivity assessed as described in methods.

DL-methionine added to reaction mixture (mM)	Disappearance of *O*-succinylhomoserine (μmoles/ml.)	Formation of succinate (μmoles/ml.)
0	0·72	0·69
6	0·70	0·70
20	0·75	0·72

DISCUSSION

Previous work on the regulation of methionine biosynthesis in *Escherichia coli* has shown that growth with methionine repressed the formation of homocysteine methylase and cystathionase (Rowbury & Woods, 1961*a*, *b*). The methylation of homocysteine involves the functioning of two enzymes concerned, respectively, with the synthesis, and the transfer of methyl groups (as derivatives of folic acid coenzymes). Both enzymes are repressed by growth with methionine (Foster, Rowbury & Woods, 1963). After growth with cobalamin, a cobamide enzyme functions in the methyl-group transfer; this enzyme also is subject to enzymic repression by methionine (Rowbury, 1962*b*). The present work shows that the cystathionine-forming enzymes are also subject to repression, so that methionine regulates the synthesis of at least six enzymes. Two observations suggest, however, that co-ordinate repression (Ames & Garry, 1959) does not occur. First, enzyme A of homocysteine methylase (Foster *et al.* 1963) is normally present in excess and can be repressed by concentrations of methionine which do not influence enzyme B. Secondly, during enzyme resynthesis after repression the methionine-forming enzymes are not re-formed at the same rate (Figs. 1, 2, 3). The genes concerned with methionine synthesis in *E. coli* do not constitute a single group on the chromosome and consequently co-ordinate repression which appears to be de-

pendent on such close linkage (Monod, Jacob & Gros, 1962) would not be expected. Although the methionine-synthesis genes do not appear to constitute a single operon in *E. coli* their function is controlled by a single regulator gene. Thus certain norleucine-resistant mutants of *E. coli* are not repressible by methionine; the mutation appears to lead to failure to form an apo-repressor with consequent oversynthesis (and non-repressibility) of all the enzymes for methionine synthesis (Rowbury, 1965).

The present work also shows that methionine inhibits the activity of the first enzyme peculiar to the methionine synthesis pathway, homoserine *O-trans*-succinylase, but does not affect cystathionine synthetase or the other biosynthetic enzymes (Wijesundera & Woods, 1962; Rowbury & Woods, 1964*a*). Homoserine *O-trans*-succinylase of whole organisms was much more sensitive to feedback inhibition by methionine than the enzyme in cell-free extract, possibly due to the high substrate levels used with *in vitro* experiments. Although homoserine *O-trans*-succinylase was so sensitive to methionine, feedback inhibition alone does not adequately regulate methionine formation for non-repressible strains excrete methionine while still sensitive to feedback inhibition (Rowbury, 1965).

The present work further confirms the rôle of cystathionine as a methionine precursor in *Escherichia coli* and the pathway and mode of regulation appears to be the same as in *Salmonella typhimurium* (Rowbury, 1964*a*). The other product of cystathionine synthetase is free succinate (Rowbury, 1964*b*; and Table 6) so that the scheme for cystathionine biosynthesis suggested by Rowbury & Woods (1964*b*) may be considered to be established.

REFERENCES

AMES, B. N. & GARRY, B. (1959). Coordinate repression of the synthesis of four histidine biosynthetic enzymes by histidine. *Proc. natn. Acad. Sci. U.S.A.* **45**, 1453.

FOSTER, M. A., ROWBURY, R. J. & WOODS, D. D. (1963). Control of the methylation of homocysteine in *Escherichia coli*. *J. gen. Microbiol.* **31**, xix.

MONOD, J., JACOB, F. & GROS, F. (1962). Structural and rate-determining factors in the biosynthesis of adaptive enzymes. In *The Structure and Biosynthesis of Macromolecules. Biochem. Soc. Symp.* **20**, 104.

MOORE, S. & STEIN, W. H. (1954). A modified reagent for the photometric determination of amino-acids and related compounds. *J. biol. Chem.* **211**, 907.

ROWBURY, R. J. (1961). The synthesis of cystathionine by *Escherichia coli*. *Biochem. J.* **81**, 42P.

ROWBURY, R. J. (1962*a*). Control of cystathionine formation in *Escherichia coli* by methionine. *Biochem. J.* **82**, 24P.

ROWBURY, R. J. (1962*b*). Factors influencing the control of methionine synthesis in micro-organisms. *D.Phil. Thesis, Oxford University.*

ROWBURY, R. J. (1964*a*). Synthesis of cystathionine and its control in *Salmonella typhimurium*. *Nature, Lond.* **203**, 977.

ROWBURY, R. J. (1964*b*). The metabolism of *O*-succinylhomoserine by *Escherichia coli*. *Biochem. J.* **93**, 20P.

ROWBURY, R. J. (1964*c*). The accumulation of *O*-succinylhomoserine by *Escherichia coli* and *Salmonella typhimurium*. *J. gen. Microbiol.* **37**, 171.

ROWBURY, R. J. (1965). Resistance to norleucine and control of methionine synthesis in *Escherichia coli*. *Nature, Lond.* **206**, 962.

ROWBURY, R. J. & WOODS, D. D. (1961*a*). Further studies on the repression of methionine synthesis in *Escherichia coli*. *J. gen. Microbiol.* **24**, 129.

ROWBURY, R. J. & WOODS, D. D. (1961*b*). Repression of cystathionase production in *Escherichia coli*. *Biochem. J.* **79**, 36P.

ROWBURY, R. J. & WOODS, D. D. (1964*a*). Repression by methionine of cystathionase formation in *Escherichia coli*. *J. gen. Microbiol.* **35**, 145.

ROWBURY, R. J. & WOODS, D. D. (1964*b*). *O*-succinylhomoserine as an intermediate in the synthesis of cystathionine by *Escherichia coli*. *J. gen. Microbiol.* **36**, 341.

STADTMAN E. R. (1963). Enzyme multiplicity and function in the regulation of divergent metabolic pathways. *Bact. Rev.* **27**, 170.

VOGEL, H. J. (1957). Repression and induction as control mechanisms of enzymic biogenesis: the 'adaptive' formation of acetylornithinase. In *The Chemical Basis of Heredity* Ed. W. D. McElroy and B. Glass, p. 276. Baltimore: The Johns Hopkins Press.

WIJESUNDERA, S. & WOODS, D. D. (1953). The effect of growth on a medium containing methionine on the synthesis of this amino acid by *Bacterium coli*. *Biochem. J.* **55**, viii.

WIJESUNDERA, S. & WOODS, D. D. (1962). The catabolism of cystathionine by *Escherichia coli*. *J. gen. Microbiol.* **29**, 353.

WORMSER, E. H. & PARDEE, A. B. (1958). Regulation of threonine biosynthesis in *Escherichia coli*. *Arch. Biochem. Biophys.* **78**, 416.

YATES, R. A. & PARDEE, A. B. (1956). Control of pyrimidine biosynthesis in *Escherichia coli* by a feedback mechanism. *J. biol. Chem.* **221**, 757.