THE JOURNAL OF GENERAL MICROBIOLOGY

EDITED FOR
THE SOCIETY FOR GENERAL MICROBIOLOGY

Editors
B. C. J. G. KNIGHT   A. F. B. STANDFAST

Editorial Board
P. W. BRIAN
R. DAVIES
T. H. FLEWETT
B. A. FRY
T. GIBSON
J. R. GUEST
N. G. HEATLEY
C. KAPLAN
J. W. LIGHTBOWN
K. McQUILLEN
G. G. MEYNELL
P. MITCHELL
R. H. NIMMO-SMITH
J. R. POSTGATE
L. J. ZATMAN
K. ZINNEMANN

VOLUME 41, 1965

CAMBRIDGE
AT THE UNIVERSITY PRESS
1965

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* 23 *November* 1965

	PAGE
Interspecific Transformation in Azotobacter BY M. SEN and S. P. SEN	1
The Genetics of *Bacillus licheniformis* Penicillinase: a Preliminary Analysis from studies on Mutation and Inter-strain and Intra-strain Transformations BY D. A. DUBNAU and M. R. POLLOCK	7
Utilization of Sodium Acetate by Shigella and Escherichia BY I. D. COSTIN	23
The Morphology of Proteus Bacteriophages BY O. W. PROZESKY, H. C. DE KLERK and J. N. COETZEE	29
RNA Metabolism of *Rhodopseudomonas spheroides* during Preferential Photopigment Synthesis. BY T. G. LESSIE	37
Complementation of Non-flagellate Salmonella Mutants BY T. M. JOYS and B. A. D. STOCKER	47
Inheritance of the O Antigens of Salmonella Groups B and D BY P. H. MÄKELÄ	57
Effects of Nutrients on Self-Inhibition of Germination of Conidia of *Glomerella cingulata* BY B. T. LINGAPPA and Y. LINGAPPA	67
Differentiation of Porcine Mycoplasma Strains BY Z. DINTER, D. DANIELSSON and K. BAKOS	77
DNA Base Composition, Flagellation and Taxonomy of the Genus *Rhizobium* BY J. DE LAY and A. RASSEL	85
Chromosome Behaviour at Meiosis in *Saccharomyces cerevisiae* BY H. TAMAKI	93
Metal-Complexing Agents as Metal Buffers in Media for the Growth of *Aspergillus niger* BY A. Q. CHOUDHARY and S. J. PIRT	99
The Effect of Calcium Nutrition on the Production of Diffusible Antigens by *Rhizobium trifolii* BY B. A. HUMPHREY and J. M. VINCENT	109
An Induced Enzyme in X-irradiated *Escherichia coli*: Comparison with Lethal Effects BY J. L. MOORE	119
Nutritional Factors in Relation to Growth and Fat Synthesis in *Mortierella vinacea* BY C. G. C. CHESTERS and J. F. PEBERDY	127
Isolation of Variant Strains from Foot-and-Mouth Disease Virus Propagated in Cell Cultures Containing Antiviral Sera BY N. ST G. HYSLOP	135
Effect of Dilution Rate and Growth-Limiting Substrate on the Metabolic Activity of *Torula utilis* Cultures BY D. W. TEMPEST and D. HERBERT	143
Proceedings of the London Meeting of the Society on 7 and 8 January 1965	i

No. 2 *issued* 17 *December* 1965

PAGE

Electronmicroscopy of the Anti-Bacterial Agent Produced by *Escherichia coli* 15 BY H. D. MENNIGMANN 151

Effects of Certain Penicillins on Growth and Cell Division in a Species of *Erwinia* BY M. M. GRULA and E. A. GRULA 155

Paper Chromatography as an Aid to the Identification of *Nocardia* Species BY I. G. MURRAY and A. G. J. PROCTOR 163

The Urease Activity of Fluorescent Pseudomonads BY D. J. STEWART . 169

Modes of Resistance to Benzylpenicillin and Ampicillin in Twelve Klebsiella Strains BY J. M. T. HAMILTON-MILLER 175

γ-Glutamyl Transfer Reactions in Bacteria BY R. MILBAUER and N. GROSSOWICZ 185

Antigenic variation in a strain of *Trypanosoma brucei* transmitted by *Glossina morsitans* and *G. palpalis* BY A. R. GRAY 195

Effects of Meso-inositol Deficiency on Some Important Biological and Chemical Characteristics of Yeast BY L. M. LEWIN 215

An Alternative Method of Isolating the Membrane of a Halophilic Bacterium BY A. D. BROWN, C. D. SHOREY and H. P. TURNER 225

The Isolation and morphology of Some New Bacteriophages Specific for *Bacillus* and *Acetobacter* species BY D. E. BRADLEY 233

Composition of Cell Walls of Some Gram-Negative Cocci. BY R. K. GRAHAM and J. W. MAY 243

Electron Microscopy Studies on the Immobilization Antigens of *Paramecium aurelia* BY M. R. MOTT 251

A Note on the Taxonomic Status of Strains Like 'Campo,' Hitherto Classified as *Mycoplasma hominis*, Type 2 BY D. G. FF. EDWARD and E. A. FREUNDT 263

The Influence of Temperature and pH Value on the Macromolecular Composition of Magnesium-limited and Glycerol-limited *Aerobacter aerogenes* Growing in a Chemostat BY D. W. TEMPEST and J. R. HUNTER . 267

The Fine Structure of Sporangia of *Phytophthora erythroseptica* Pethyb. BY J. A. CHAPMAN and R. VUJIČIĆ 275

Studies on the Haemagglutinin Present in Coxsackie A7 Virus-Infected Suckling Mouse Tissue BY J. D. WILLIAMSON and N. R. GRIST . . 283

No. 3 *issued January* 1966

The Antigenic Structure of Haemophilus and Corynebacterium Species from the Human Genital Tract Claimed to be Associated with or Derived from *Mycoplasma hominis* BY P. PEASE and N. LAUGHTON . . . 293

The Antigenic Structure of PPLO (*Mycoplasma hominis*) and Related Bacteria BY P. PEASE 299

PAGE

Salicylate Degradation Test for Differentiation of *Mycobacterium fortuitum* from Other Mycobacteria BY M. TSUKAMURA 309

Conversion of Salicylate to Catechol by *Mycobacterium fortuitum* BY M. TSUKAMURA 317

Thymine Incorporation and Metabolism by Various Classes of Thymine-less Bacteria BY A. P. HARRISON, JUN. 321

Deoxyribonucleic Acid Base Compositions among Thermophilic Actinomycetes: the Occurrence of two Strains with Low GC Content BY R. CRAVERI, L. R. HILL, P. L. MANACHINI and L. G. SILVESTRI . . 335

Carotenoids in Mutants of *Verticillium albo-atrum* BY L. R. G. VALADON and J. B. HEALE 341

Temperate Phages Influencing Lipase Production by *Staphylococcus aureus* BY K. ROSENDAL and P. BÜLOW 349

The Taxonomy of certain Thiobacilli BY M. HUTCHINSON, K. I. JOHNSTONE and D. WHITE 357

Efflux of Macromolecules from Washed *Dictyostelium discoideum* BY M. I. KRICHEVSKY and L. L. LOVE 367

The cell walls of streptococci BY G. COLMAN and R. E. O. WILLIAMS . 375

Self-heating of Hay and Grain in Dewar Flasks and the Development of Farmer's Lung Antigens BY G. N. FESTENSTEIN, J. LACEY, F. A. SKINNER, P. A. JENKINS and J. PEPYS 389

Carotenoids and cholesterol in membranes of *Mycoplasma laidlawii* BY S. RAZIN and the late R. C. CLEVERDON 409

Proceedings of the London Meeting of the Society on 16, 17 and 18 September 1965 xv

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 implication 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 $CuSO_4.5H_2O$.

Description 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 are used where they are not ambiguous. Binomials should be underlined in the typescript. Scientific epithets or trivial names are not underlined and should be without capitals.

Descriptions of new species of cultivable microbes should not be submitted unless an authentic specimen of a living culture has been deposited in a recognized culture collection.

The word 'generation' should not be used synonymously with 'subculture'. For an agreed use of terms like strain, type, variant, phase, etc., see the International Bacteriological Code of Nomenclature, Section 1, Rules 7 and 8.

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

MICROFUNGI. *Ainsworth & Bisby's Dictionary of the Fungi*, 1961, 5th ed. (Kew: Commonwealth Mycological Institute.)

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

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

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

J. gen. Microbiol. (1965), **41**, 1–6
Printed in Great Britain

Interspecific Transformation in Azotobacter

BY MIRA SEN AND S. P. SEN
Microbiology Laboratory, Department of Botany,
Kalyani University, Nadia, West Bengal, India

(*Received* 5 *February* 1965)

SUMMARY

This paper describes the results obtained in experiments on interspecific transformation in Azotobacter. When *Azotobacter chroococcum* was transformed with the DNA of *A. vinelandii* 6–10 % of the colonies differed from the parent strain in colour, colony character and biochemical characteristics. Some of the transformant strains had higher nitrogen-fixing capacities than had the receptor strain. Transformants obtained by treatment of *A. vinelandii* with the DNA of *A. chroococcum* also had different colour, colony characteristics and capacity to grow in nutrient broth and peptone, but their nitrogen-fixing capacity was decreased.

INTRODUCTION

Genetic transformation in micro-organisms has numerous possibilities apart from its utility in genetic analysis. The characters transferred hitherto by this process include resistance to antibiotics and certain enzymes concerned with the production of capsule, specific polysaccharides, antigens and several cellular metabolites. Ravin (1961) listed some 20 bacterial species which have been found to be transformable. We record here observations concerning interspecific transformation between two species of the free-living nitrogen-fixers *Azotobacter chroococcum* and *A. vinelandii.*

METHODS

The strain of *Azotobacter vinelandii* used in this work was originally obtained through the courtesy of Professor R. H. Burris (University of Wisconsin). In most of the work described here a dark brown non-segregating strain AZCF of *A. chroococcum* was used. This was isolated from local soil samples by the enrichment plate method. The cultures were usually grown on Ashby's nitrogen-free mannitol medium having the following composition: mannitol, 10·0 g.; K_2HPO_4, 0·5 g.; $MgSO_4.7H_2O$, 0·2 g.; NaCl, 0·2 g.; $MnSO_4$, trace; $FeCl_3$, trace; distilled water, 1000 ml.

DNA extraction. In preliminary experiments several methods of DNA extraction were tried. The methods of McCarty & Avery (1946), Anagnostopoulos & Spizizen (1961), Marmur (1961) and Colter, Brown & Ellem (1962) all yielded good results. Lysozyme, sodium deoxycholate and sodium lauryl sulphate were useful for the disruption of the bacteria. The method finally adopted was as follows: the bacteria were ground with alumina in 50 ml. of saline + EDTA (containing 0·15 M-NaCl and 0·1 M-EDTA, pH 8·0); 1·5 g. sodium citrate and 2·5 ml. of a 1 % (w/v) solution of

Vol. 40, *No.* 3 *was issued* 17 *November* 1965

sodium deoxycholate were added. After 15 min. 2 ml. of 25 % (w/v) lauryl sulphate were added and the mixture warmed on a water bath at 60° for 10 min. This was followed by addition of solid NaCl to a final concentration M. An equal volume of water-saturated phenol was added followed by centrifugation at 4° for 30 min. The upper aqueous layer was removed and re-extracted with water-saturated phenol for 10 min. in the cold, the mixture being stirred with a magnetic stirrer. The liquid was again centrifuged, the upper layer extracted with ether 4 or 5 times for 10–20 min., traces of ether removed from it and left at 0° for several hours, centrifuged and the supernatant fluid added to an equal volume of 95 % (v/v) ethanol in water. The DNA which was collected on a glass rod was dissolved in 9 ml. dilute saline citrate (0·15 M-NaCl + 0·015 M-sodium citrate) and shaken with an equal volume of chloroform + isoamyl alcohol (24 + 1 by vol.) for 15 min. and centrifuged. This operation was repeated twice and the DNA precipitated by addition of 95 % (v/v) ethanol in water. After centrifugation the DNA was dispersed in half the volume of supernatant 0·15 M-NaCl + 0·015 M-sodium citrate. RNA was removed by incubation with ribonuclease (50 μg./ml.) at 37° for 30 min., followed by centrifugation and further washing with chloroform + isoamyl alcohol. The DNA was re-precipitated with ethanol, dissolved in dilute saline citrate buffer and 0·54 volume isopropyl alcohol added dropwise with stirring. The DNA threads were collected on a glass rod, and placed in 70 % (v/v) ethanol in water and then absolute ethanol was added slowly to a concentration of 95 % (v/v) ethanol. After centrifugation the DNA was finally dissolved in 0·85 % NaCl solution (normal saline).

Genetic markers. The characters of the Azotobacters which were used as genetic markers were the brown melanin pigment, colony characteristics, fermentation reactions and nitrogen-fixing capacity. Ashby's nitrogen-free mannitol agar media in Petri dishes were seeded with a suspension of the donor strain, incubated, and the bacteria harvested 72 hr later by gently scraping-off the gummy growth from the surface of the agar. The organisms were then suspended in normal saline and their DNA isolated by the procedure described above.

Transformation. For transformation a loopful of a 22 hr growth of a receptor strain was suspended in 2 ml. normal saline to provide a concentration of 10^7 organisms/ml. and incubated with DNA of the donor strain at 37° for 30 min. to 1 hr. At the end of this incubation period dilutions of the bacterial suspension were plated on Ashby's mannitol agar and incubated at room temperature (28–30°) for 7–14 days. Prolonged incubation was useful for the development of the melanin pigment. Colonies bearing resemblance to any character of the donor strain which was absent from the recipient strain were transferred to agar slopes for further study.

Pigment formation. The melanin-type pigment was extracted according to the procedure of Dawes (1941) with slight modifications: a bacterial suspension was boiled for 10 min. and the insoluble residue digested for 48 hr with 100 ml. of 1 % pepsin in KCl + HCl buffer (pH 2·0); a little toluene was added and the mixture shaken. The sediment was washed with distilled water, centrifuged and hydrolysed with 4 % (w/v) NaOH, the boiling being continued for 15 min. The brown pigment was estimated with a Unicam absorptiometer at 400 mμ (E_{400}). For qualitative comparisons of amounts of pigment, suspensions of young cultures of similar bacterial concentrations were used directly for spectrophotometry.

Nitrogen-fixing capacity was estimated by a micro-Kjeldahl determination of the total-N of the crop of organisms obtained on Ashby's nitrogen-free mannitol medium.

RESULTS

Transformation of Azotobacter chroococcum with A. vinelandii DNA

When *Azotobacter chroococcum* was transformed with the DNA of *A. vinelandii* 6–10 % of the colonies obtained through dilution plating were found to be different from the parent colonies. Colonies of the original parent strain of *A. chroococcum* were dark coloured, smooth and glistening but never raised. Colonies of the donor strain of *A. vinelandii* were colourless and raised. The 'transformants' showed much lighter shades of brown, some being completely colourless and others raised and very

Table 1. *Characters of transformants obtained by treatment of Azotobacter chroococcum with A. vinelandii DNA*

Organisms	Colony characters	Growth in Nutrient broth	Growth in Peptone water	Utilization of sucrose
Parent strains				
A. chroococcum (strain AZCF)	Deep brown, gummy	−	+ +	+ +
A. vinelandii (strain AZV)	Colourless, very gummy, raised	+ +	±	+ + +
Transformant isolates				
AZ CV 1	White, gummy	−	+	+ +
AZ CV 2	White, gummy	−	+ +	+ +
AZ CV 3	White, gummy	+ +	+	+
AZ CV 4	Faint brown	−	−	+ + +
AZ CV 5	White, gummy	+ +	+ +	+ + +
AZ CV 6	White, gummy	+ +	−	+ +

±, +, + +, + + + indicate doubtful, slight, good and vigorous growth, respectively.

gummy. In several colonies which produced pigment the rate of pigment production was much slower than by the parent strain. Colonies which were faintly brown or white and gummy were used for detailed investigation. The biochemical characteristics of six selected isolates together with a brief description of the features are given in Table 1. All the transformants fermented mannitol, sucrose and xylose like the parent strain, but they differed to some extent in their capacity to grow on peptone or nutrient broth. The AZCF strain of *A. chroococcum* used in this work was unable to grow in nutrient broth, whereas the isolate of *A. vinelandii* grew well in this medium. The transformants CV 3 and CV 5 utilized peptone and nutrient broth whereas the isolate CV 4 did not grow in either of these media. The isolate CV 6 grew well on nutrient broth but not in peptone water.

The pigment content and nitrogen-fixing capacities of these isolates are shown in Table 2. It will be seen that all the transformants shown in this table had very little of the melanin pigment, whereas the receptor strain of *Azotobacter chroococcum* AZCF was quite rich in the pigment. The actual pigment content of the parent strain was much higher than the value shown in the table because the method of extraction used could solubilize only a part of the total pigment, and the pigment which

was tightly bound could not be separated by any method. The pigments of the transformants however were almost completely extracted. The strain of *A. vinelandii* used had a higher capacity for fixing nitrogen than the receptor AZCF strain of *A. chroococcum*. The improvement observed in nitrogen-fixing capacity was quite remarkable and all the transformants studied fixed at least twice as much nitrogen as the receptor AZCF strain of *A. chroococcum*.

Table 2. *Pigment content and nitrogen-fixing capacity of transformant Azotobacter isolates*

Pigment present in 1 mg. dry wt. organism dissolved in 1 ml. 4% (w/v) NaOH.

Organism	Pigment E_{400}	Total N (%)
A. chroococcum	0·10	1·5
A. vinelandii	0·015	3·6
A. chroococcum transformed with *A. vinelandii* DNA		
AZ CV 1	0·02	3·0
AZ CV 2	0·01	3·1
AZ CV 3	0·02	—
AZ CV 4	0·02	3·0
AZ CV 5	0·02	3·6
AZ CV 6	0·02	3·4

Table 3. *Characters of transformants obtained by treatment of Azotobacter vinelandii with A. chroococcum DNA*

Organism	Colony characters	Growth in Nutrient broth	Growth in Peptone water	Utilization of sucrose
Parent strains				
A. chroococcum (strain AZCF)	Deep brown, gummy	−	+ +	+ +
A. vinelandii (strain AZV)	White, very gummy, raised	+ +	±	+ + +
Transformant isolates				
AZ VC 1	Deep brown	−	+	+ + +
AZ VC 2	Deep brown	−	±	+ +
AZ VC 3	Deep brown	−	−	+ +
AZ VC 4	White, raised, slightly wrinkled	−	−	+ + +

±, +, + +, + + + indicate doubtful, slight, good, vigorous growth, respectively.

Transformation of Azotobacter vinelandii with A. chroococcum DNA

Attempts to transform *Azotobacter vinelandii* to *A. chroococcum* have also been successful. Several transformant strains have been isolated which have the property of melanin production as indicated by the formation of brown pigment. The biochemical characteristics, pigment production, and nitrogen-fixing capacities are given in Tables 3 and 4. While the strain of *A. vinelandii* used grows well on nutrient broth but poorly, if at all, in peptone and the converse is true for *A. chroococcum*, the transformants varied considerably in their capacity to utilize these substrates. All of them, however, could metabolize sucrose, mannitol and xylose,

though in varying degrees. The three transformants vc1, vc2 and vc3 increased considerably in pigment production. The nitrogen-fixing capacity was, however, lowered in all the cases and intermediate values were obtained.

Table 4. *Pigment content and nitrogen-fixing capacity of transformant Azotobacter isolates*

Relative amounts of pigment present in 1 mg. dry wt. organism dissolved in 1 ml. 4% (w/v) NaOH.

Strain	Pigment E_{400}	Total N (%)
A. chroococcum	0·10	1·5
A. vinelandii	0·01	3·6
A. vinelandii transformed with *A. chroococcum* DNA		
AZ VC1	0·06	2·4
AZ VC2	0·08	2·7
AZ VC3	0·05	3·4

DISCUSSION

The investigations described here indicate the transformability of *Azotobacter chroococcum* and *A. vinelandii*. The property of the production of brown melanin pigment, alteration in the consistency of the gum produced and the shape of the colony can be acquired by the receptor strain when treated with the DNA of the donor strain. The strains of both the species of *Azotobacter* studied were fairly sensitive to penicillin and streptomycin, and the property of resistance to these antibiotics could not be studied.

Genetic implication of the findings will be presented in a subsequent paper. It is, however, of considerable interest that when *Azotobacter chroococcum* is treated with the DNA of the non-pigment-producing *A. vinelandii* a large number of transformants failed to produce the melanin pigment. Whether this is due to the acquisition of the capacity to elaborate a substance which interferes with the production of melanin, or there was some position effect, cannot be decided at present. The capacity to fix nitrogen can apparently be modified and this observation also merits careful consideration and further biochemical studies.

The significance of interspecific transformation in Azotobacter in speciation cannot be assessed at present. Variations in the shape of the colony, pigment content and gum production of the transformants are, however, indicative of such a possibility since strains of Azotobacter isolated from a given soil are very often found to vary considerably in their morphological features and biochemical characteristics, as has been found in the case of the transformants described here. The significance of genetic transformation in speciation and variability of soil micro-organisms has been discussed elsewhere (Biswas, Sen & Sen, 1964).

REFERENCES

ANAGNOSTOPOULOS, C. & SPIZIZEN, J. (1961). Requirements of transformation in *Bacillus subtilis*. *J. Bact.* **81**, 741.

BISWAS, G. D., SEN, M. & SEN, S. P. (1964). Genetic transformation in soil microorganisms and its probable significance in the variability and speciation of microorganisms. Symposium on 'Frontiers of Plant Sciences'. *Bull. Bot. Soc. Bengal*, **18** (Suppl.), p. 29 (abstract).

COLTER, J. S., BROWN, R. A. & ELLEM, K. A. O. (1962). Observations on the phenol method of isolation of deoxyribonucleic acid. *Biochim. biophys. Acta*, **55**, 31.

DAWES, B. (1941). The melanin content of the skin of *Rana temporaria* under normal conditions and after prolonged light and dark adaptation. A photometric study. *J. exp. Biol.* **18**, 26.

MCCARTY, M. & AVERY, O. T. (1946). Chemical nature of the substance inducing transformation of pneumococcal types. III. Improved method for the isolation of the transforming substance and its application to Pneumonococcus type II, III and VI. *J. exp. Med.* **83**, 97.

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

RAVIN, A. W. (1961). The genetics of transformation. *Advanc. Genet.* **10**, 61.

J. gen. Microbiol. (1965), 41, 7–21
Printed in Great Britain

The Genetics of *Bacillus licheniformis* Penicillinase: a Preliminary Analysis from Studies on Mutation and Inter-strain and Intra-strain Transformations

BY D. A. DUBNAU* AND M. R. POLLOCK
National Institute for Medical Research, Mill Hill, London, N.W. 7

(*Received* 5 *February* 1965)

SUMMARY

A system of transformation in *Bacillus licheniformis* is described, in which a wide variety of markers can be transferred from one strain to another. A number of mutants affecting the regulatory, enzymic and serological aspects of penicillinase production are described, classified and discussed. A control gene for penicillinase synthesis was found to be linked to the structural gene in four wild-type strains of *B. licheniformis*. The electrophoretic properties of the penicillinases produced by the progeny of inter-strain crosses appeared to be affected by the type of cell in which the relevant structural gene was expressed.

INTRODUCTION

This paper presents part of a biochemical, serological and genetic study of bacterial penicillinases currently under way in this laboratory. It is based on a number of inter-strain and intra-strain crosses in *Bacillus licheniformis* which involve penicillinase production, studied by means of transformation with DNA. The phenotypic properties of various penicillinase mutants used and of the transformants obtained are also described and discussed. Since the work was completed Gwinn & Thorne (1964) have reported the transformation of several nutritional markers in a strain of *B. licheniformis*, and Leonard, Mattheis, Mattheis & Housewright (1964) have studied transformation to prototrophy and polyglutamic acid synthesis in this species.

METHODS

Organisms. The parent of all the mutant strains used for transformation studies was *Bacillus licheniformis* 749/1, derived from strain 749 by 'training' it to grow rapidly in 'minimal' medium having NH_4^+ as sole source of N. Strain 749/110 (subsequently referred to as 110) is a streptomycin-resistant and histidine-requiring derivative of 749/1. Strains 749/48 and 749/81 are streptomycin-resistant and methionine-requiring mutants, respectively, also derived from 749/1. These three strains were obtained from Dr P. H. A. Sneath. Strain 246 was received from Dr I. Takahashi (Canadian Department of Agriculture, Ottowa). Strain IRC-1 was

* Present address: Department of Biochemistry, Albert Einstein College of Medicine, Bronx, N.Y., U.S.A.

received from the Institute of Research, Brussels, Belgium. As with strain 749 and its derivatives, strain 6346 was previously referred to as *B. subtilis* (NCTC 6346) (see Pollock, 1965). All of these strains are penicillinase-inducible.

Media. The minimal (glucose, ammonium, salts) medium used was that of Anagnostopoulos & Spizizen (1961). Andrade agar (Kogut, Pollock & Tridgell, 1956) was used as a standard solid nutrient medium and for detecting the production of penicillinase on solid medium. Cephalosporin C (5 μg./ml.) was included in this medium as an inducer when required. Difco Penassay broth was used as a standard liquid nutrient medium. Enzyme preparations for starch electrophoresis and immunological studies were obtained from organisms grown in CHS medium (Collins, 1964). Transformation medium (TM) consisted of the salt mixture used by Anagnostopoulos & Spizizen (1961) + 0·5 % glucose, 0·0075 % Casamino acids, 0·02 % yeast extract, L-histidine (50 μg./ml.), 0·2 % additional sodium citrate and 0·005 ml. each of stock iron solution and oligodynamic mixture (Pollock & Kramer, 1958) per 100 ml. medium. Solid medium contained 2 % Difco agar. L-Histidine was added to solid media to a concentration of 50 μg./ml., and streptomycin to 1 mg./ml.

Mutagenic treatment. Ultraviolet radiation. Two ml. of distilled water containing about 3×10^6 spores/ml. were irradiated in a Petri dish for 25 sec. at a distance of 50 cm. with a 300 watt u.v. lamp (Hanovia, Slough). The survival after this treatment was about 1 %. The irradiated spores were diluted 30 times with Penassay broth and incubated overnight.

Ethylmethane sulphonate (EMS) as mutagen. Spores were suspended in 2 ml. of 0·1 M-sodium phosphate buffer (pH 7·0) containing 0·1 ml. EMS. The suspension was incubated for 20 min. at 35°, washed once with phosphate buffer, diluted 30 times into Penassay broth and incubated overnight.

N-Methyl-N'-nitro-N-nitrosoguanidine (NG) as mutagen. An overnight culture in Penassay broth was centrifuged and resuspended to original volume in 0·2 M-sodium acetate buffer (pH 5·0). To 0·5 ml. of this suspension was added 0·12 ml. of a solution of NG (4 mg./ml.) in acetate buffer. The suspension was incubated, without shaking, for 210 min. at 35° and then diluted 30 times in Penassay broth, followed by overnight growth. This method is essentially that used by A. Garen (personal communication).

Selection of penicillinase mutants. Constitutive mutants were detected by plating about 10^3 colony-forming units in a 2 ml. layer of Andrade agar on a previously poured Andrade plate. The bacteria were then covered with an additional 2 ml. of Andrade agar. After overnight incubation, the plates were flooded with a solution containing about 500,000 units benzylpenicillin. Colonies of penicillinase-constitutive mutants produced pink zones after a few minutes, and were easily picked and purified.

Mutants producing low amounts of enzyme activity were detected by plating 500–1000 constitutive colony-forming units as described above. After overnight incubation at room temperature, the plates were incubated for 4 hr at 50°. The resulting colonies were then 'developed' for penicillinase by the method of Novick (1963), modified by J. F. Collins and M. H. Richmond (personal communication), as follows. A saturated solution of N-phenyl-1-naphthylamine-azo-*o*-carboxybenzene (PNAC) was prepared in dimethylformamide. This was mixed with 10^{-2}

M-phosphate buffer (pH 7·0) to a final dimethylformamide concentration of 30 %, allowed to cool for several hours and then filtered. This preparation was poured on to the Petri dishes to be tested and allowed to soak in for about 1 hr. The excess PNAC was rinsed off with distilled water and 10 ml. of 10^{-2} M-phosphate buffer (pH 7·0) containing 300 mg. benzylpenicillin poured on the dish. Within a few minutes the constitutive parent colonies stained a deep purple. Colonies which remained pale were picked with a Pasteur pipette into Penassay broth containing 100 units sterile penicillinase/ml., to destroy excess penicillin. After overnight incubation, the mutant strains were purified and tested.

Transformation procedure. An entire single colony of a competent strain was picked from a minimal medium plate and inoculated into 3 ml. of transformation medium (TM) in a large culture tube. No attempt was made to disperse the organisms. The tube was then incubated on a reciprocal shaker for 20 hr at 35°. At this time 0·15 ml. was removed and added to 1·35 ml. of TM containing 7×10^{-3} M-NaCl. Deoxyribonucleic acid (DNA) prepared from the donor strain was added to the desired concentration (usually 1 μg./ml.). The suspension was incubated and vigorously aerated through a glass tube in a water bath at 40° for 1–2 hr. During this time little or no change in viable count occurred. Deoxyribonuclease (DNAse) was added to a final concentration of 20 μg./ml. along with 10^{-2} M-$MgSO_4$. After 10 min. the culture was plated as desired in order to select transformant clones. The viable count at this time was about 5×10^8/ml.

'$A \rightarrow B$' signifies a cross in which DNA from A (the donor) is used to transform B (the recipient).

Selection of transformants. Transformants to prototrophy were selected by plating on the appropriate minimal medium. Streptomycin-resistant transformants were selected by plating in a layer of agar on a previously prepared bed of Andrade agar. After a 2-hr delay at 35° a third layer of agar containing streptomycin was added. The final streptomycin concentration attained after uniform diffusion throughout the plate was 1 mg./ml.

Penicillinase-producing transformants were selected as follows. Molten Andrade agar (25 ml.) containing about 3×10^6 colony-forming units was pipetted into each Petri dish. Benzylpenicillin was added with the bacteria, to reach a final concentration of 0·03 units/ml. in half the plates and 0·04 units/ml. in the rest. Both concentrations were used in each experiment since the optimal selection concentration was extremely critical and somewhat variable. A top layer of 5 ml. 1·5 % (w/v) Difco agar was poured when the bottom layer had set. The plates were then incubated at 35° for 18 hr. At this time large clusters of minute satellite colonies were visible, often with a larger transformant colony in the centre. Each plate then received $2\text{–}10 \times 10^4$ units benzylpenicillin in 1 ml. M-sodium phosphate buffer (pH 7·0). After 20–48 hr at 35°, the satellite growth had lysed and the transformant colonies could be easily picked and purified. Reconstruction experiments showed that, with the recipient strain used throughout these experiments, recovery of both inducible and constitutive donor types was 90–100 %. Transformant colonies were picked with a needle and purified on appropriate media before testing.

Penicillinase assay. Penicillinase was assayed by the method of Perret (1954) and expressed as units (μmoles benzylpenicillin hydrolysed/hr at 30°; Pollock &

Torriani, 1953). Benzylpenicillin, methicillin and cephalosporin C were used as substrates at concentrations of 2·4, 2·4 and 3·0 mg./ml., respectively.

When cephalosporin C was used as a substrate, 20 min. were allowed for the hydrolysis product to react with iodine (on the suggestion of J. F. Collins, personal communication) rather than the usual 10 min. used with benzylpenicilloic acid. A correction factor of 2 was applied, since it is reported that the product (presumed to be cephalosporoic acid) reacts with only 4 equivalents of iodine (Fleming, Goldner & Glass, 1963) as compared with 8 in the case of benzylpenicilloic acid. It should, however, be noted that values for 'cephalosporinase' activity thus assayed are about 30 % below those obtained manometrically (Pollock, 1965).

Induction tests: 'induction ratio'. Overnight cultures in Penassay broth were diluted tenfold in the same medium and incubated at 35° with shaking. When an opacity equivalent to a bacterial concentration of 0·1 mg. dry wt. bacteria/ml. (measured by reference to a standard opacity/dry wt. curve) was reached, each culture was divided into two portions, one of which received 2 μg. cephalosporin C/ml. Penicillinase values were measured after further incubation for 1·5 hr and expressed in units/mg. dry wt. bacteria (specific activity). The specific activity of the induced cultures divided by that of the uninduced is referred to as the 'induction ratio'. It should be noted that Induction Ratios are only really valid for comparative purposes under standard conditions, and do not measure the maximum possible extent of induction in any particular case; this would require much longer incubation with inducer.

Preparation of DNA. DNA was prepared by the method of Marmur (1961) and estimated by the method of Burton (1956).

Immunological analysis. Enzyme/antiserum precipitation reactions in agar were detected, and enzyme samples quantitatively titrated, by means of antiserum to wild-type *B. licheniformis* strain 749/c penicillinase, according to methods described elsewhere (Pollock, 1964).

Starch-gel electrophoresis. Horizontal starch-gel electrophoresis was done with penicillinase samples soaked on filter paper, according to the method of Smithies (1955), using gel prepared in 5×10^{-2} M-glycine buffer (pH 8·9). A voltage gradient of about 10 V./cm. was applied at room temperature for 4 hr. The starch blocks were sliced, and penicillinase bands developed on one half by using PNAC and on the other by spraying with 2×10^{-2} M-iodine containing 100,000 units benzylpenicillin/ml. The latter method was more sensitive but more transitory than the first. The resulting patterns were photographed.

Reagents. N-Phenyl-1-naphthylamine-azo-*o*-carboxybenzene was bought from The British Drug Houses Ltd., Poole, England, the ethylmethane sulphonate from Eastman Kodak Co. and the *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine from The Aldrich Chemical Co. Methicillin and cephalosporin C were gifts from Beecham Research Laboratories Ltd. and Glaxo Laboratories, respectively.

RESULTS

Throughout this work derivatives from *Bacillus licheniformis* strain 749 were used as recipients.

Table 1 shows that pre-treatment of DNA with DNAse (20 μg./ml.) for 10 min.

completely prevented transformation. DNA prepared from a histidine auxotroph (strain 110) could transform to methionine prototrophy but did not transform strain 110 to independence of histidine. The same 110 culture was competent since it was transformed to prototrophy by wild-type DNA. It proved possible to transform cells of various mutant strains of *B. licheniformis* 749 routinely at a frequency of 10^{-5} to 10^{-6}. All markers tested have been successfully transformed, including streptomycin resistance, histidine, methionine, adenine and arginine prototrophy, and penicillinase production.

When *Bacillus licheniformis* 749-type cultures were plated on minimal medium, two morphological colony types were apparent. The majority were flat, smooth in texture, sticky and soft. A small and variable minority (0·1–10 %) were smaller, rough, often crinkled, and turned reddish brown after 2–3 days. The predominant colony type will be referred to as 'smooth' and the other as 'rough'. In a typical

Table 1. *Strains, markers and transformation frequencies in Bacillus licheniformis*

Strain from which DNA was isolated	Recipient	Selected marker	No. of transformant-type colonies appearing after treatment with DNA
749/48	749/81	Strept. -res.	1930
749/48 (preparation pre-treated with DNAse)	749/81	Strept. -res.	10
749/48	110	*his*$^+$	1850
110	110	*his*$^+$	10
110	749/81	*met*$^+$	2240
None	749/81	*met*$^+$	30
None	749/81	Strept. -res.	15
None	110	*his*$^+$	0

experiment in which 2 smooth and 5 rough variants from strain 110 were transformed to histidine independence, the smooth variants gave 10 and 17 prototrophs/ml. and the rough gave 2540, 3970, 286, 473 and 1430 prototrophs/ml., respectively. When a mixture of rough and smooth variants was used as recipient, all of the colonies selected for histidine or methionine independence were rough. The viable count plates contained about 80 % smooth colonies. It is apparent that the smooth variants were not competent under the conditions used. When rough variants were streaked on minimal medium, reversion to the smooth state was easily demonstrated. The rate of reversion varied markedly among rough strains. Competent rough variants were maintained on minimal agar and re-streaked from single colonies about once a week on fresh plates. Single colonies from these streaks were used to initiate competent cultures.

The concentrations of the ingredients in transformation medium (TM) were those experimentally determined to be optimal. These components were required only during the period of growth preceding the addition of DNA. Once the organisms had become competent, they could be diluted into Spizizen salts medium or saline containing 10^{-3} M-$MgSO_4$ and then exposed to DNA without loss of competence, as compared with controls treated in the usual way. In saline without $MgSO_4$, the transforming efficiency was much lower.

Competence was found to reach a maximum during the stationary phase of growth and to decline rapidly after about 3 hr. The yield of transformants increased linearly with increasing DNA concentration, reaching a maximum at about 1 μg. DNA/ml.

Characterization of penicillinases

Mutants. Table 2 presents some properties of the penicillinase mutants obtained from the standard wild-type ('magno-inducible') strains used. In addition to 'magno-constitutive' strains, several loss mutants, some producing immunologically cross-reacting material (CRM) were obtained. Other mutants with abnormally low induction ratios, presumably with regulatory lesions in a control gene, may be described as 'semi-constitutive' (raised basal, normal maximum induction) or 'semi-inducible' (normal basal, subnormal maximal induction). Others were strictly non-inducible (induction ratios of unity), each producing its characteristic level of enzyme with or without inducer, covering a range from around normal basal or below ('micro-constitutive') up to 10 % of the maximum ('magno-') constitutive value ('meso-constitutive'). Treatment at 50° for 1 hr caused more than 90 % loss of activity in crude culture supernatant fluids from both these mutants, but had no effect on the parent, wild-type enzyme.

Strain 9 behaved like the inducible wild-type at 35°, but was semi-constitutive at 50°, forming at the higher temperature about 500 units penicillinase/mg. dry wt. bacteria in the absence of inducer and the normal fully inducible complement after induction.

Strain 18 (obtained from a constitutive mutant of 100) represents a particular type of enzyme mutant of which strains 19, 71 and 20 later proved (Pollock, Fleming & Petrie, 1965) to be further examples. In Penassay broth, the total enzyme activity of strain 18 was only about 5–10 % of that produced by the parent strain. When the bacteria were lysed with lysozyme, about 50 % or more of the penicillinase activity was lost, whereas the low activity in the supernatant fluid was unaffected by lysozyme. When a constant amount of supernatant fluid enzyme was titrated with increasing amounts of specific antiserum to *B. licheniformis* 749/c penicillinase, a three- to four-fold stimulation of penicillinase activity was observed. Under similar conditions wild-type enzyme was 50 % inhibited by this antiserum (Pollock, 1964).

Strains 22 and 72 produced no enzyme activity detectable by the methods used; these strains were derived from constitutive strains and produced cross-reacting material (CRM) in the absence of inducer. They were thus double penicillinase mutants, with damage presumably in both the control and the structural genes. Strain 22 was used throughout this work as a recipient in crosses involving selection for penicillinase.

Homologous crosses. The term 'homologous' is used here to describe a cross in which the donor and recipient strains were derivatives of the same wild-type isolate. When the donor and recipient were derived from distinct wild-type strains, the cross is described as 'heterologous'. Strain 749/c3 (a constitutive mutant strain) was transformed to prototrophy by using DNA from the wild-type strain 749. The resulting *his*$^+$ strain (c3/1) was used to transform strain 22. Selection was for penicillinase production and for histidine prototrophy. In this cross 17 penicillinase

Table 2. *Penicillinase properties of strains derived from Bacillus licheniformis strain 749*

Strains Standard	Parent	Mutagen	Penicillinase (units/mg. dry wt. bacteria) Uninduced	Induced	Induction ratio*	Phenotypic description†	Precipitation with specific antiserum to wild-type penicillinase‡
749	749	—	12–24	840–1400	35–117	Magno-inducible (standard wild-type)	+ +
749/1	749	—	12–24	840–1400	35–117	Magno-inducible (trained to minimal medium)	+ +
110	749/1	—	12–24	840–1400	35–117	Magno-inducible (histidine auxotroph: streptomycin-res.)	+ +
Penicillinase mutants							
749/c	749	EMS	3000–5000	3000–5000	1·0	Magno-constitutive (standard constitutive strain)	+ +
749/c2	110	EMS	4530	4750	1·05	Magno-constitutive	n.t.
749/c3	110	EMS	3660	4120	1·02	,,	+ +
749/c4	110	EMS	4190	4320	1·03	,,	n.t.
749/c5	110	EMS	3370	3910	1·16	,,	n.t.
749/c6	110	[illegible]	4440	4510	1·02	,,	n.t.
749/c7	110	EMS	5920	5850	0·99	,,	n.t.
749/c8	110	EMS	6970	7430	1·07	,,	n.t.
18	749/c3	UV	280	270	0·95	Meso-constitutive	+
32	110	NG	208	208	1·0	,,	—
19	749/c3	NG	90	90	1·0	,,	+ +
77	749/c	NG	79	77	0·98	,,	—
71	749/c	NG	50	52	1·04	,,	+ +
20	749/c3	NG	33	24	0·7	,,	+ +
17	749/c3	NG	21	25	1·2	Micro-constitutive	—
28	110	NG	24	23	0·95	,,	—
76	749/c	NG	19	25	1·3	,,	+ +
31	110	NG	9	10	1·1	,,	—
75	749/c	NG	3·9	4·2	1·1	,,	+ +
16	749/c3	NG	3·8	2·6	0·7	,,	+ +
74	749/c	NG	2·4	2·2	0·92	,,	—
25	749/c3	NG	2·8	2·1	0·75	,,	—
26	749/c3	NG	0·80	0·90	1·1	,,	—
72	749/c	NG	< 0·05	< 0·05	—	Negative	+ +
22	749/c3	NG	< 0·05	< 0·05	—	,,	+ +
23	749/c3	NG	4250	n.t.	—	(Thermo-labile enzyme)	+
24	749/c3	NG	1420	n.t.	—	,,	+
c1	749/1	EMS	2865	4130	1·44	Semi-constitutive	n.t.
9	749/1	EMS 35°	19	1250	66	Magno-inducible at 35°	+ +
		50°	400	1400	2·8	Semi-constitutive at 50°	n.t.
27	110	NG	15	130	8·6	Semi-inducible	+ +
29	110	NG	21	88	4·1	,,	+
34	110	NG	18	43	2·9	,,	—
30	110	NG	14	31	2·3	,,	—
11	749/1	EMS	12	18	1·5	,,	—

* Induction ratio = differential rate of enzyme synthesis under standard conditions for maximal induction/differential rate of enzyme synthesis without induction. (see Text)

† See text for description of nomenclature.

‡ Based on plate Ouchterlony test on surface growth (in case of inducible strains, after maximal induction) as described by Pollock (1964).

n.t. = not tested.

transformants were picked, and all were found to be constitutive; control platings of strain 22 alone yielded no revertants.

When strain 110/1, an inducible wild-type *his*+ transformant derived from 110, was used to transform strain 22, 89 out of 101 penicillinase transformants tested were inducible; the remaining 12 % were constitutive. When a DNA concentration 20 times lower was used, 11 out of 15 transformants (73 %) were inducible. These structural and regulatory mutant sites in strain 22 are therefore closely linked by transformation. Two constitutive and 5 inducible transformants from this cross were assayed for penicillinase production, with and without induction. The values of enzyme produced in these transformants were not significantly different from those of the constitutive parent strain of 22 and of the inducible donor strains.

Some crosses were performed with strain 9 (the temperature-sensitive control mutant) as donor and strain 22 as recipient. Transformants were tested on Andrade plates, with and without inducer, at 35° and at 50°. Of 15 transformants tested 6 were constitutive and 9 showed the donor temperature-sensitive character. It was concluded that the regulatory mutation of strain 9 is also linked to the structural gene.

Table 3. *Linkage of structural and control loci for penicillinase in Bacillus licheniformis*

The recipient in all crosses was *B. licheniformis* strain 22 (see Table 2).

Donor strain	Transformants tested (no.)	Inducible (%)	Frequency of histidine and penicillinase transformation*
749/1	101	88	100
IRC-1	37	76	370–610
6346	185	52	46–73
246	45	31	32–43

* These values are percentages of the average transformation frequency obtained in the homologous cross.

Heterologous crosses. A series of crosses was undertaken by using DNA from a number of magno-penicillinase-inducible wild-type *Bacillus licheniformis* strains: 6346, IRC-1-s′ (an EMS-induced mutant of IRC-1, resistant to 1 mg. streptomycin/ml.), 246. In these crosses 20,000 rather than 100,000 units benzylpenicillin/plate were used on the second day of selection, because of the lower resistance of 246 and 6346 to saturating concentrations of benzylpenicillin. Separate experiments showed that the use of 20,000 rather than 100,000 units in homologous crosses with strain 749 would not have affected the earlier results obtained. This permits comparison of results derived from the homologous and heterologous crosses.

Table 3 shows the numbers of inducible and constitutive transformants and the relative transformation frequencies obtained for histidine and penicillinase in the various crosses. Throughout this work the frequency of histidine and penicillinase transformation never differed by more than 50 % in a given cross. The last column in Table 3 therefore presents combined data for both these markers. (Neither the strain 22 penicillinase structural marker nor the *his*− marker has ever been observed to revert.) All three heterologous donors could co-transform strain 22 to inducibility

and enzyme function, thus demonstrating linkage of the structural and regulatory loci. The degree of linkage varied from about 80 to 30%. Table 3 shows that the frequency of linked transformation of the structural and regulatory markers varied directly with the frequency of transformation of the histidine and penicillinase markers between heterologous strains.

Properties of penicillinase produced by the progeny of heterologous crosses

An attempt was made to characterize the enzyme produced by transformants from heterologous crosses containing a 749 genome + genetic material from a foreign strain carrying the structural and regulatory determinants for penicillinase. The specific benzylpenicillinase activities of strains 6346 and 246 (which appear to be indistinguishable, judged by all tests so far made) are about sixfold lower than that of strain 749. Indeed, the purified 749/c penicillinase has a molecular activity on benzylpenicillin nearly 6 times that of the purified enzyme from strain 6346/c (a magno-constitutive mutant from strain 6346; Pollock, 1965). On Andrade agar plates ('developed' by the addition of about 2×10^5 units benzylpenicillin, after induction with 5 μg. cephalosporin C/ml.), this difference was clearly recognizable from the zone size of acid production. The penicillinase types of the progeny of a number of heterologous crosses involving 6346 and 246 donors were tested in this way.

Of 185 strain 6346 → strain 22 'hybrids' examined in the first batch, 182 had maximal values of benzylpenicillinase activity similar to that produced by strain 6346. About half of these were constitutive (Table 3). The remaining 3 transformants, however, produced penicillinase at the strain 749 value, and all were constitutive. Repeated control tests never revealed any reversions of strain 22 back to the magno-constitutive phenotype from which it was derived.

Among 145 further strain 6346 → strain 22 hybrids, another 3 transformants (all constitutive) formed penicillinase at the strain 749 value. Finally, out of 45 hybrid strains obtained by transforming strain 22 with DNA from strain 246, 2 produced 749-type enzyme, and both were likewise constitutive. Thus, out of a total of 475 hybrid transformants, 8 (all constitutive) produced recipient-type enzyme, indicating that repair or suppression of the structural gene mutation in strain 22 had been achieved by means of transformation with heterologous DNA.

The validity of the rapid plate test for distinguishing between 6346-type and 749-type penicillinases was confirmed in several cases by measuring the ratios of activity in hydrolysing cephalosporin C and methicillin relative to benzylpenicillin, as described in Methods. (These differ markedly and characteristically in the wild types; Pollock, 1965.) The results for cephalosporin C are shown in Table 4. The classification of a given transformant penicillinase as 6346-like or 749-like on the basis of the plate test was confirmed in all cases by its relative activity on cephalosporin C and in two cases (T21 and T25) on methicillin.

Finally, the apparent identity of the transformant 6346-type penicillinase with the normal wild-type 6346/c enzyme was confirmed in two of these inter-strain hybrids by comparative titrations with anti-749/c penicillinase antiserum (Fig. 1). Wild-type 6346/c and 749/c penicillinases have been shown to differ by 100% in the extent of maximal stimulation of their methicillinase activities by this antiserum, and by approximately sixfold in their equivalence points, when this is expressed

ห้องสมุด กรมวิทยาศาสตร์

in terms of the ratio of the minimum amounts of antiserum causing maximal stimulation of the activities of the two types of enzyme respectively (Pollock, 1964). Figure 1 shows that transformants T21 and T25 gave titration curves indistinguishable from those given by normal 6346/c enzyme. In an analogous experiment with benzylpenicillin as substrate, transformants T13 and T33 both gave, with the same antiserum, the 'biphasic' titration curves characteristic of the 6346-type penicillinase (Pollock, 1964).

Table 4. *Bacillus licheniformis: relative activities of wild-type and inter-strain (6346 → 22) transformant penicillinases on different substrates*

Type strains (parental)	Phenotype inferred from benzylpenicillinase plate test	Activity of penicillinase in hydrolysing cephalosporin C (as % activity on benzylpenicillin)
6346/c	6346 constitutive	10·3
749/c3	749 constitutive	0·76
Inter-strain transformants tested		
T17	6346 inducible	10·4
T18	6346 inducible	9·6
T20	6346 constitutive	10·3
T21	6346 constitutive	9·0
T22	6346 constitutive	10·5
T23	6346 constitutive	10·0
T16	749 constitutive	0·73
T25	6346 constitutive	9·7
T27	6346 constitutive	9·5
T28	6346 constitutive	10·0
T30	6346 constitutive	9·3
T31	6346 inducible	9·4
T33	6346 constitutive	9·0
V-3	749 constitutive	0·78
V-4	749 constitutive	0·83

In spite of the fact that all the strain 6346 → strain 22 transformants tested produced a type of penicillinase with enzymic and serological properties indistinguishable from one or other of the two wild-types, electrophoretic mobilities of some of these transformant enzyme samples appeared to be significantly different from both. On electrophoresis through starch gel (see Methods), samples of wild-type 6346/c penicillinase always moved more rapidly than the 749/c enzyme, though both types showed multiple banding (Pollock, 1965). Culture supernatant fluids from ten of the strain 6346 → strain 22 transformants which were enzymically 6346-like and from both wild-types were dialysed and concentrated 15-fold by freeze-drying, and mobilities compared by this method. The penicillinase, in preparations from all ten transformants, migrated at speeds intermediate between those of 6346/c and 749/c. All these produced an enzyme-type enzymically similar to that of 6346/c: typical results are presented in Fig. 2. In electrophoretic runs of artificial mixtures of preparations from transformants and 6346/c wild-type strains, each 'brand' of

penicillinase retained its characteristic mobility unaffected by the presence of the other. Two 749-like transformant preparations from strain 6346 → strain 22 crosses migrated at the same speed as the 749/c preparation.

To determine whether transformation of strain 22 to produce active penicillinase led to elimination of the ability to form the mutant penicillinase protein (cross-reacting material: CRM) normally produced by strain 22, culture supernatant fluids from strain 6346 → strain 22 transformants were examined for the presence of material interfering with the normal penicillinase/antipenicillinase reaction. Interference by the enzymically inactive strain 22 mutant-penicillinase CRM itself was

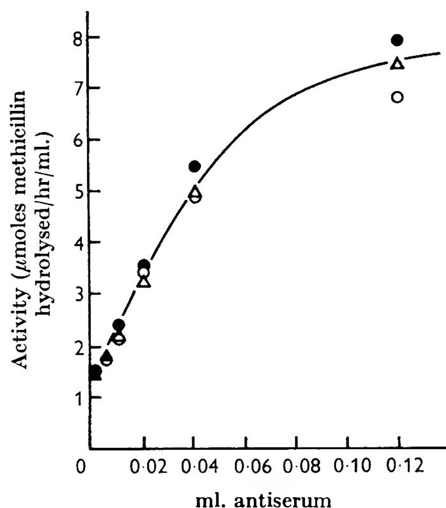


Fig. 1

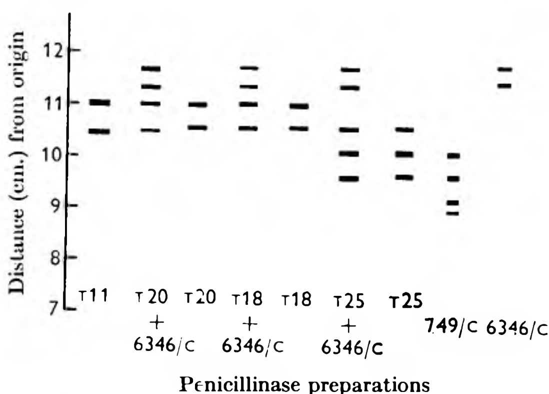


Fig. 2

Fig. 1. Immunological titration curves of penicillinase from 6346/c (wild-type) and 6346→ 22 transformants. Increasing quantities of anti-749/c penicillinase antiserum were added to a constant amount (150 units) of enzyme from untreated culture supernatants, and the resulting enzyme activities determined with methicillin as substrate. —●— 6346/c control; —△— T21; —○— T25.

Fig. 2. Tracings of patterns of enzyme reaction bands after starch gel electrophoresis of inter-strain transformant (T11, T18, T20 and T25) and parental-type (749/c and 6346/c) penicillinases (singly and together). Migration is toward the anode. See text for origin and relationship of enzyme types.

shown by measuring the stimulating effect of increasing quantities of antiserum to 749/c penicillinase on the rate of methicillin hydrolysis by normal (strain 6346) and transformant (strain T25) enzyme, as follows. 100 units of normal enzyme contained in 0·45 ml. supernatant fluid from cultures of strains 6346/c and T25, grown to a population density equiv. to 1·2 mg. dry wt. bacteria/ml., were titrated (*a*) alone and (*b*) after addition of an equivalent quantity (0·5 ml. of culture supernatant fluid from mutant 22 grown to an opacity equiv. to 1·1 mg. dry wt. bacteria/ml.) of the CRM penicillinase. The titration curves obtained (Fig. 3) show (*a*) significant (up to 50 %) inhibition, by an equivalent amount of strain 22 CRM penicillinase, of the reaction between antiserum and both wild-type and transformant enzymes, indicating nearly equal competition between CRM-22 and wild-type enzyme for combination with limiting quantities of antibody; (*b*) no significant titration differences between

normal 6346-type enzyme and that formed by T 25 (as previously indicated in Fig. 1). Similar results were obtained with another strain 6346 → strain 22 transformant, T21. If either of these two transformants had formed significant quantities of 22 CRM penicillinase as well as normal 6346-type enzyme, their enzyme titration curves with antiserum would have been different from that of the 6346/c enzyme alone.

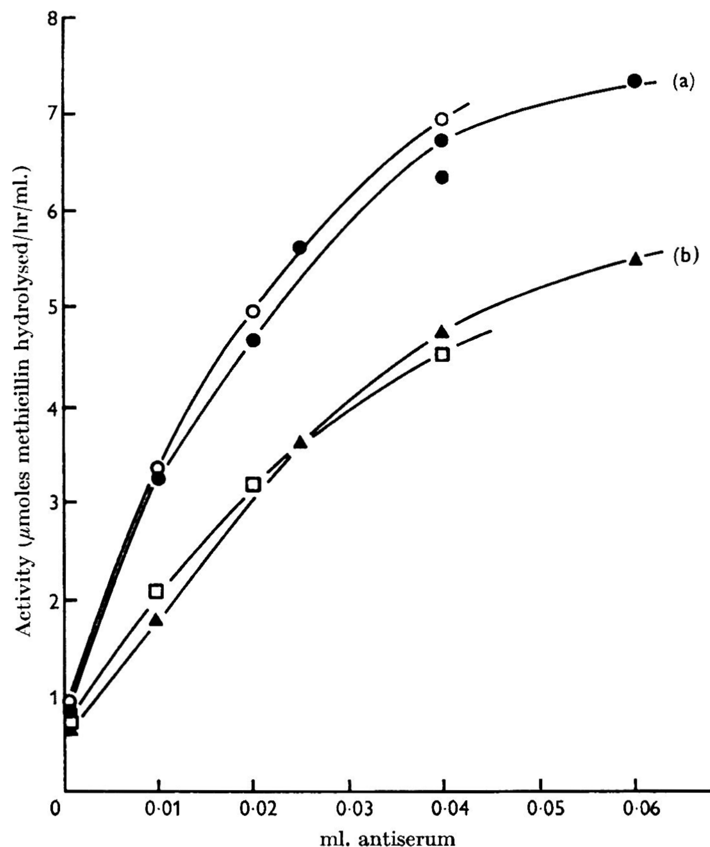


Fig. 3. Interference in the penicillinase/antipenicillinase reaction through addition of enzymically inactive CRM penicillinase from mutant 22. Titration curves showing comparative stimulating effects of increasing quantities of anti-749/c penicillinase antiserum on rates of methicillin hydrolysis by 100 units of wild-type (6346/c) and transformant (T25) enzyme (*a*) alone, and (*b*) after addition of equivalent amount of 22 CRM penicillinase. 6346/c alone —●—●—, T25 alone —○—○—, 6346/c+22 CRM —▲—▲—, T 25 + 22 CRM —□—□—.

DISCUSSION

The various penicillinase mutants listed in Table 2 can be provisionally grouped into two main categories.

'*Control*' *mutants*, which produce penicillinase qualitatively indistinguishable from the 'magno-inducible' wild-type, but at a significantly different rate or with an altered induction response. These comprise: (i) strains 749/c to 749/c8 inclusive (see Table 2), which form maximal amounts of enzyme in the absence of inducer

and conform to the original definition of 'magno-constitutive' (Pollock, 1957); (ii) strains 27, 29, 30 and 11 (all 'semi-inducible'), which retain some response to induction but form less enzyme than the wild-type, and strain C1 ('semi-constitutive'), which has a significantly increased basal (uninduced) value; both these subgroups have, of course, subnormal induction ratios; (iii) 'micro-constitutives' and 'meso-constitutives', which show no induction response but form enzyme at rates well below the maximum; some of these (77, 17, 74, 25 and 26) arise from strains already mutated to magno-constitutivity, whereas others (28, 31 and 32) stem directly from the original inducible strain 110; (iv) Strain 9, a 'facultative' constitutive, inducible at 35° but semi-constitutive at 50°, having properties consistent with the presence of a thermo-labile repressor, and very similar to the *Escherichia coli* β-galactosidase mutant described by Horiuchi & Novick (1961).

Structural gene mutants, which form enzyme with properties clearly differentiated from those of the wild-type—having either markedly decreased specific activity (as indicated by the presence of a normal serological precipitation reaction, combined with greatly decreased enzyme activity: strains 18, 19, 71, 20, 76, 75, 16, 72 and 22) or decreased thermostability (strains 23 and 24). Strains 18, 19, 71 and 20 show altered serological behaviour, wild-type activity being partially or completely restored after interaction with specific antiserum. One explanation is that this is due to partial restoration of the original wild-type conformation by combination with antiserum, it being supposed that the mutated enzyme in solution adopts an altered conformation which is enzymically inefficient (see Pollock *et al.*, 1965).

Novick (1963) reported on a genetic analysis by transduction of the penicillinase of *Staphylococcus aureus*, and presented evidence indicating that the penicillinase gene of that organism was carried on a plasmid. In *Bacillus licheniformis*, however, there is no suggestion that the penicillinase genes are extra-chromosomal; the structural gene is stable and, unlike the situation in *S. aureus*, there is no large class of absolute negative, irreversible, loss mutants.

The data in Table 4 show that the frequency of co-transformation of the structural and control genes in three heterologous crosses was directly proportional to the frequency of histidine and penicillinase transformation. It seems reasonable to interpret these results in terms of the degree of genetic homology between the strains, since homology over a large stretch of chromosome may be required to integrate a more extended region of donor DNA. B. W. Catlin (private communication) has obtained a similar result in transformational crosses between strains of *Neisseria catarrhalis*; the frequency of co-transformation of resistance to streptomycin and kanamycin was higher in homologous than in heterologous strains. This concept is borne out in the present case by what is known about the phenotype of the four strains of *Bacillus licheniformis* involved. The penicillinase of *B. licheniformis* IRC-1 is more closely related in substrate 'profile' and antiserum titration values to that of strain 749 than are the penicillinases produced by strains 246 or 6346. Furthermore, several phages, isolated from soil, form plaques on either strain 749 or strain IRC-1, but not on strain 6346 or 246 (D. A. Dubnau, unpublished observations).

It seems highly probable that the structural penicillinase loci in the wild-type strains 749 and 6346 are strictly allelic. Apart from *a priori* considerations based on the similarity of the two strains and the two types of enzyme, the results of

interstrain (6346 → 22) crosses indicate that (*a*) insertion of the 6346 type of enzyme into a 749-type recipient (22) involves the exclusion of the normal cross-reacting material (CRM) product of the mutant 22 structural gene, and (*b*) in a few, but significant number of, instances apparently complete repair of the defective 749 penicillinase structural gene in strain 22 can be achieved by means of transformation with DNA from the heterologous wild-type strain 6346. The 749 and 6346 types of penicillinase have been shown (Pollock, 1965) to be closely related proteins with similar molecular weights, differing considerably in their enzymic properties but with only marginal distinctions physico-chemically and serologically. Analyses of their overall amino-acid compositions do not show significant differences, and it is not therefore yet certain that their primary amino-acid sequences are dissimilar. It is, however, more likely that they do differ by a number of residues.* If this be so, the most probable explanation of the repair, by strain 6346, of the defect in strain 22 is the occurrence of an intra-cistronic recombination involving the restricted insertion into the 749-type (22) genome of that portion of the 6346 penicillinase structural gene which is identical with, or very similar to, the homologous region on the 749 genome containing the mutational defect expressed in strain 22.

The fact that 100 % (8 out of 8) of these particular transformants are constitutive (i.e. retain the control mutation of the recipient) indicates that crossing-over must have occurred between the control and structural mutated loci in strain 22. If a region of dissimilarity within the 6346 and 749 genes exists between the position of the mutation in strain 22 and the proximal end of the control gene, a strong selection for constitutivity among the 749-like transformants would be expected.

The overwhelming majority of strain 6346 → strain 22 transformants produced a donor (6346)-type penicillinase. This was to be expected, and it is probable that in most of these transformations the 749-type recipient has accepted the 'foreign' heterologous gene, with little or no modification. However, in some instances it is possible that a 6346-type enzyme may also result from intra-cistronic recombination. The relatively high (about 50 %) incidence of constitutives amongst such transformants suggests that, since close linkage between control and structural penicillinase genes has already been demonstrated in homologous crosses, a proportion of recombinations between the control and structural mutated loci in strain 22 may have occurred within the structural gene. If so, they must involve insertion not only of the area defective in strain 22 but of the fragment of the strain 6346 structural penicillinase gene which carries all or most of the sequences differing in the two types. The distinctive electrophoretic behaviour of all of the 6346-like transformants is also consistent with this possibility, although three inducible hybrid transformants tested showed this same behaviour. The fact that differently treated preparations of exo-enzyme from the same wild-type strain can also differ significantly in their mobilities as shown by this technique (e.g. as between a supernatant fluid concentrate and a fully purified sample; Pollock, 1965) suggests that these differences do not involve the primary amino acid sequence and may arise from the influence of a 749-type cytoplasmic environment acting on a 6346-type of penicillinase molecule.

* See Note page 21

We are grateful to Miss Joan Fleming, Mr D. J. McGillicuddy, Miss S. Petrie and Mr P. Thompson for their able technical assistance, and to Dr A. H. Campbell (Glaxo Research Laboratories) for his gift of cephalosporin C and to Dr G. N. Rolinson (Beecham Research Laboratories) for gifts of methicillin. The work was supported by a United States Public Health Service Postdoctoral Fellowship held by one of us (D.A.D.).

REFERENCES

ANAGNOSTOPOULOS, C. & SPIZIZEN, J. (1961). Requirements for transformation in *Bacillus subtilis*. *J. Bact.* **81**, 741.

BURTON, K. (1956). A study of the conditions and mechanism of the diphenylamine reaction for the colorimetric estimation of deoxyribonucleic acid. *Biochem. J.* **62**, 315.

COLLINS, J. F. (1964). The distribution and formation of penicillinase in a bacterial population of *Bacillus licheniformis*. *J. gen. Microbiol.* **34**, 363.

FLEMING, P. C., GOLDNER, M. & GLASS, D. G. (1963). Observations on the nature, distribution and significance of cephalosporinase. *Lancet*. ii, 1399.

GWINN, D. D. & THORNE, C. B. (1964). Transformation of *Bacillus licheniformis*. *J. Bact.* **87**, 519.

HORIUCHI, T. & NOVICK, A. (1961). A thermolabile repression system. *Cold Spr. Harb. Symp. quant. Biol.* **26**, 247.

KOGUT, M., POLLOCK, M. R. & TRIDGELL, E. J. (1956). Purification of penicillin-induced penicillinase of *Bacillus cereus* NRRL 569: a comparison of its properties with those of a similarly purified penicillinase produced spontaneously by a constitutive mutant strain. *Biochem. J.* **62**, 391.

LEONARD, C. G., MATTHEIS, D. K., MATTHEIS, M. J. & HOUSEWRIGHT, R. D. (1964). Transformation to prototrophy and polyglutamic acid synthesis in *Bacillus licheniformis*. *J. Bact.* **88**, 220.

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

NOVICK, R. P. (1963). Analysis by transduction of mutations affecting penicillinase formation in *Staphylococcus aureus*. *J. gen. Microbiol.* **33**, 121.

PERRET, C. J. (1954). Iodometric assay of penicillinase. *Nature, Lond.* **174**, 1012.

POLLOCK, M. R. (1957). Penicillin-induced resistance to penicillin in cultures of *Bacillus cereus*. In *Drug Resistance in Micro-organisms*, p. 78, *Ciba Foundation Symposium*. Ed. by G. E. W. Wolstenholme & C. M. O'Connor.

POLLOCK, M. R. (1964). Stimulating and inhibiting antibodies for bacterial penicillinase. *Immunology*, **7**, 707.

POLLOCK, M. R. (1965). Purification and properties of penicillinases from two strains of *Bacillus licheniformis*: a chemical, physico-chemical and physiological comparison. *Biochem. J.* **94**, 666.

POLLOCK, M. R. & KRAMER, M. (1958). Intermediates in the biosynthesis of bacterial penicillinase. *Biochem. J.* **70**, 665.

POLLOCK, M. R. & TORRIANI, A. M. (1953). Purification et caractéristiques physico-chimiques de la pénicillinase de *Bacillus cereus*. *C.r. hebd. Séanc. Acad. Sci., Paris*, **237**, 276.

POLLOCK, M. R., FLEMING J. & PETRIE, S. (1965). The effects of specific antibodies on the biological activities of wild-type bacterial penicillinases and their mutationally altered analogues. *Second Meeting of European Federation of Biochemical Societies, Vienna, April 1965. Report of Colloquium on 'Antibodies to Biologically Active Molecules'*. (In the press).

SMITHIES, O. (1955). Zone electrophoresis in starch gels: group variations in the serum proteins of normal human adults. *Biochem. J.* **61**, 629.

Note added in proof. At least one difference in the amino-acid sequences has now been unequivocally demonstrated by R. P. Ambler (private communication).

J. gen. Microbiol. (1965), 41, 23–27
Printed in Great Britain

Utilization of Sodium Acetate by Shigella and Escherichia

By I. D. COSTIN
State Inspection for Hygiene, Enterobacteria Laboratory, Timişoara, Rumania

(*Received* 16 *February* 1965)

SUMMARY

The chemically defined medium with sodium acetate as sole carbon source (sodium acetate agar) devised by Trabulsi & Ewing (1962) was used to examine 414 strains of Shigella and 446 strains of Escherichia. None of the 414 Shigella cultures (belonging to the subgroups, A, B, C, D) utilized acetate and did not develop on sodium acetate agar, whereas about 95% of 283 anaerogenic and non-motile and all 163 aerogenic Escherichia isolates studied, utilized sodium acetate and grew on sodium acetate agar. It is concluded that the utilization of acetate on sodium acetate agar has a definite value for the differentiation of strains of the genera *Shigella* and *Escherichia*.

INTRODUCTION

Between the Shigella and Escherichia groups, as now defined (Ewing & Edwards, 1962; Edwards & Ewing, 1962; Carpenter, 1963*a*) there is a wide spectrum of relationships, expressed also through an appreciable genetic homology (Luria & Burrows, 1957; Stenzel, 1961; Falkow, Schneider, Baron & Formal, 1963; Stenzel, 1963; Stenzel & Meier, 1964) as well as through biochemical and antigenic similarities (Ewing, Hucks & Taylor, 1952; Ewing, 1953; Ewing, Tatum, Davis & Reavis, 1956; Ewing, Reavis & Davis, 1958; Edwards & Ewing, 1962). As the procedures for the determination of genetic compatibilities are not included among those used in most diagnostic laboratories, and pathogenic properties are not internationally accepted as taxonomic criteria for the groups of the family Enterobacteriaceae (Carpenter, 1963*b*), the positive and differential diagnosis of Shigella and Escherichia (as of all other groups of the family) is based on biochemical and antigenic tests; for group determination biochemical characters are used predominantly. With Shigella and Escherichia, of the 41 serotypes accepted in the Shigella group, only 5 do not exhibit relationships with the O (1–145) antigens of the Escherichia group; 14 Shigella serotypes have identical O antigens with that of some Escherichia types, and the remaining 24 serotypes of Shigella present different degrees of antigenic relationships of the type a-a, b or a, b-a, c with the members of the Escherichia group (Edwards & Ewing, 1962). It is evident that biochemical tests have particular value in the examination of isolates which might belong to one of these groups. There is no difficulty in the recognition of 'typical' Escherichia strains (prompt lactose fermentation, motility, aerogenesis) which are most frequently encountered, but the identification of slow lactose-fermenting (24 hr or more) non-motile and anaerogenic strains which conform to Shigella and Escherichia definitions after the usual biochemical screening tests is sometimes difficult.

Up to September 1962 we used for the separation of the lactose non-fermenting, non-motile and anaerogenic strains which for the rest conformed to the Shigella and Escherichia groups, the tests for lysine and ornithine decarboxylation, Christensen's citrate, sodium mucate and the decomposition of several carbohydrates. In September 1962 we were informed, through the courtesy of Dr W. H. Ewing (Atlanta, Georgia, U.S.A., who kindly supplied the manuscript of a then unpublished paper) about a chemically defined medium containing sodium acetate as sole carbon source, which Trabulsi & Ewing (1962) had found valuable in the differentiation of Shigella and Escherichia cultures. From that date we used this test in our routine biochemical tests for the examination of Enterobacteriaceae. The present paper reports the results obtained with this test of sodium acetate utilization in the differentiation of Shigella and Escherichia strains.

METHODS

Organisms. Between September 1962 and January 1965, 860 isolates which were found to belong to Shigella and Escherichia were tested for their ability to utilize acetate. With some few exceptions, all the strains studied had been freshly isolated

Table 1. *Shigella serotypes tested on sodium acetate agar*

	Species	Serotype	No. of isolates tested
A	*S. dysenteriae*	1	2
		2	24
		3	1
B	*S. flexneri*	1a	2
		1b	26
		2a	62
		2b	3
		3a	2
		3b	58
		4	16
		5	11
		6 (Boyd 88)	16
		6 (Manchester)	24
		var. X	3
		var. Y	5
C	*S. boydii*	1	28
		4	1
D	*S. sonnei*	6	2
		Form I and II	128
	Total		414

from stools of diarrhoeal and non-diarrhoeal persons, who were being examined for diagnostic or prophylactic purposes. The identification of these isolates was made biochemically and serologically (not all cultures) according to internationally accepted procedures (*Report*, 1958; Edwards & Ewing, 1962). Other technical details about the biochemical methods were given elsewhere (Costin, 1965). The Shigella strains belonged to the subgroups B, D, C, A (in decreasing numerical frequency; see Table 1).

Medium. The sodium acetate agar medium (SAA) was prepared according to Trabulsi & Ewing (1962): distilled water, 100 ml.; NaCl, 0·5 g.; $MgSO_4.7H_2O$, 0·02 g.; $NH_4H_2PO_4$, 0·1 g.; K_2HPO_4, 0·1 g.; sodium acetate 0·2 g.; agar (washed 3 days), 2·0 g.; bromthymol blue (1/500) aqueous solution), 4 ml.

The ingredients were dissolved by boiling, the agar added, and the solution adjusted to pH 7–7·1. The medium was dispensed in 120 × 12 mm. tubes in about 3 ml. amounts, the tubes closed with rubber stoppers, autoclaved for 15 min. at 121° and cooled in a sloped position to form a butt of about 10 mm. The tubes were inoculated over the surface of the slopes with saline suspensions from fresh (20 hr) agar cultures and incubated at 37° for 7 days. Readings were made daily. Since it was noticed that correct results were obtained only when there was some syneresis liquid in the bottom of the tubes, only such tubes were used. In all instances, in which non-lactose-fermenting, anaerogenic and non-motile strains were examined, other significant biochemical tests (lysine and ornithine decarboxylation, Christensen's citrate, sodium mucate, carbohydrates) were also done; beginning in 1963, the β-galactosidase test (Le Minor & Ben Hamida, 1962) was also used.

RESULTS

Table 2 summarizes the results obtained; none of the 414 Shigella isolates used acetate as sole carbon source in the presence of inorganic nitrogen, and did not develop on the sodium acetate agar (SAA) during the 7 days of observation.

Table 2. *Growth of some Shigella, Escherichia and Salmonella cultures on sodium acetate medium*

Group (serotype)	No. of strains tested	No. of positive strains in			%	
		1 day	2 days	3–7 days	+	−
Shigella (A, B, C, D)	414	.	.	.	.	100
E. coli (lactose non-fermenting, non-motile, anaerogenic, serologically undetermined)	169	134	22	4	94·7	5·3
E. coli (belonging to Alcalescens-Dispar)	114	103	5	1	95·6	4·4
E. coli (lactose non-fermenting/24 hr/aerogenic)	68	65	3	.	100	.
E. coli (prompt lactose-fermenting)	57	57	.	.	100	.
E. coli 124:72 (B_{17})	37	22	13	2	100	.
E. coli 112ac:66 (B_{11})	1	1	.	.	.	.
Salmonella typhi	10	.	.	.	.	100
S. gallinarum and *S. pullorum*	602	.	.	.	.	100
Other Salmonella (B-group)	34	34	.	.	100	.

From the 169 strains of lactose non-fermenting (< 24 hr), non-motile and anaerogenic *Escherichia coli*, which did not belong to alkalescens-dispar, 124:72 and 112ac:66 serotypes, and which were not further identified serologically, 94·7 % developed and made alkaline the SAA medium, mostly after 24 hr incubation.

A similar proportion of positive results (95·6 %) was obtained with 114 Escherichia strains, which were biochemically similar to the above collection but which

belonged to 0 (1–8) A–D antigens (mostly to 0_1). In the case of the strains from the above two collections which did not grow on SAA (5·3 and 4·4%, respectively) the diagnosis of Escherichia was established on the ground of positive results in one or more of the significant biochemical tests (lysine, ornithine, mucate, Christensen's citrate) and confirmed serologically by lack of significant reaction with the Shigella antisera. We encountered no strain which was positive on SAA and negative in all the other above-mentioned significant biochemical tests. In no instance, therefore, was the diagnosis of Escherichia established only on a positive result in the SAA test. Further, the SAA test was positive with all the 68 motile and aerogenic, slow lactose-fermenting Escherichia strains tested and also with all the other 57 prompt lactose-fermenting Escherichia strains originating from diarrhoeal cases, which belonged to certain determined 0-antigen groups and which were described previously (Costin, 1964).

Thirty-seven *Escherichia coli* 124:72 strains from those so far tested utilized acetate (see also Costin & Olinici, 1965); similarly one strain of *E. coli* 112ac:66.

From the results obtained with the sodium acetate agar test in other groups of Enterobacteriaceae, only those of some Salmonella strains are included in Table 1 for comparison; the strains of *Salmonella gallinarum-pullorum* were all of avian origin (see Costin *et al.* 1964).

DISCUSSION

The fact that none of the 414 Shigella strains examined developed on the sodium acetate agar (SAA) medium, seems to indicate that the inability to use acetate as sole carbon source in the presence of inorganic nitrogen in a chemically defined medium is a constant feature of members of the Shigella group. Though many of the accepted Shigella serotypes were not represented in our material, yet the agreement of the results presented here with those reported by Trabulsi & Ewing (1962) is strong support for the conclusion that a culture which is able to develop on SAA medium can be excluded from the Shigella group, as at present defined. The high proportion of positive results (growth on SAA medium) obtained with the non-motile, anaerogenic and lactose non-fermenting *Escherichia coli* strains, which are easily mistaken for Shigella strains, especially in view of the antigenic relationships mentioned in the Introduction, shows the usefulness of the SAA medium for differentiating these Escherichia variants from true Shigellas. Such *E. coli* variants (belonging or not to the specified A–D antigenic groups) are not rare in the material encountered in routine work. Thus, in the period September 1962 to January 1965, we encountered 283 such strains, representing about 4% of all strains of lactose-negative (24 hr) Enterobacteriaceae isolated by us from faeces mostly from non-diarrhoeal subjects. Since about 5% of the anaerogenic Escherichia isolates gave negative results with SAA, it is evident that the acetate utilization test cannot be recommended as a sole differentiating test between Shigella and Escherichia. The results of the work reported here show that the acetate utilization in the SAA medium of Trabulsi & Ewing (1962) is useful for the differentiation of the members of the Shigella and Escherichia groups. Therefore, it may be said that any taxonomical study of strains called 'intermediate' between Shigella and Escherichia is incomplete (Ewing & Edwards, 1962), if there is no mention of results obtained with the sodium acetate utilization test.

The writer wishes to acknowledge his indebtedness to Dr W. H. Ewing, Atlanta, Georgia, for the courtesy of having supplied the manuscript on SAA medium before its publication.

REFERENCES

Carpenter, K. P. (1963*a*). Report of the Subcommittee on Taxonomy of the Enterobacteriaceae. *Int. Bull. bact. Nomencl. Taxon*, **13**, 69.

Carpenter, K. P. (1963*b*). Minutes of Enterobacteriaceae Subcommittee meeting, Montreal, August 1962. *Int. Bull. bact. Nomencl. Taxon*, **13**, 139.

Costin, I. D. (1964). Maladies diarrhéiques chez enfants et adultes associées à *Escherichia coli* n'appartenant pas aux sérotypes dits entéropathogènes. *Budapest, 4th Congress Microbiology.*

Costin, I. D. (1965). Bemerkungen zur Praxis der biochemischen Identifizierung der Darmbakterien in der Routinearbeit. *Zentbl. Bakt ParasitKde I.* Ref. **198**, 289.

Costin, I. D. & Olinici, N. (1965). Studiul unor tulpini de *Escherichia coli* serotip 124:72 (B_{17}), izolate de la copii şi adulţi cu enterite. *Microbiologia Parazit. Epidem.* **10**, 49.

Costin, I. D., Petrica, L., Gariou, M., Onica, P., Pelle, A. & Dinu, N. (1964). Correlation between origin and biochemical behaviour of *Salmonella gallinarum* and *Salmonella pullorum* cultures. *Zentbl. Bakt. ParasitKde I. Orig.* **194**, 342.

Edwards, P. R. & Ewing, W. H. (1962). *Identification of Enterobacteriaceae*, 2nd edition. Minneapolis: Burgess Publishing Comp.

Ewing, W. H. (1953). Serological relationships between Shigella and coliform cultures. *J. Bact.* **66**, 333.

Ewing, W. H. & Edwards, P. R. (1962). The *Principal Divisions and Groups of Enterobacteriaceae and their Differentiation.* Atlanta, Georgia, U.S.A.: Communicable Diseases Center Publication.

Ewing, W. H., Hucks, M. C. & Taylor, M. W. (1952). Interrelationships of certain Shigella and Escherichia cultures. *J. Bact.* **63**, 319.

Ewing, W. H., Reavis, R. W. & Davis, B. R. (1958). Provisional Shigella serotypes. *Can. J. Microbiol.* **4**, 89.

Ewing, W. H., Tatum, H. W. Davis, B. R. & Reavis, R. W. (1956). *Studies on the Serology of the Escherichia coli Group.* Atlanta, Georgia, U.S.A.: Communicable Diseases Centre Publication.

Falkow, S., Schneider, H., Baron, L. S. & Formal, S. B. (1963). Virulence of Escherichia-Shigella genetic hybrids for the guinea pig. *J. Bact.* **86**, 1251.

Le Minor, L. & Ben Hamida, F. (1962). Avantages de la recherche de la β-galactosidase sur celle de la fermentation du lactose en milieu complexe, dans le diagnostic bactériologique en particulier des Enterobacteriaceae. *Ann. Inst. Pasteur*, **102**, 267.

Luria, S. E. & Burrous, J. W. (1957). Hybridization between *Escherichia coli* and Shigella. *J. Bact.* **74**, 461.

Report of the Enterobacteriaceae Subcommittee of the Nomenclature Committee of the International Association of Microbiological Societies, 1958. *Int. Bull. bact. Nomencl. Taxon* **8**, 25.

Stenzel, W. (1961). *Escherichia coli*-Shigella-Hybriden und ihre Schleimhautpathogenitat. *Z. Hyg. InfektKrankh.* **147**, 521.

Stenzel, W. (1963). Untersuchungen über die Hybridisierung zwischen *E. coli* und Shigella in vivo. *Arch. Hyg. Bakt.* **147**, 444.

Stenzel, W. & Meier, R. (1964). Experimentall-genetische Untersuchungen zur Frage einer Motilitätsübertragung auf unbewegliche Typen der Coli-Shigella-Gruppe. *Arch. Hyg. Bakt.* **148**, 475.

Trabulsi, L. R. & Ewing, W. H. (1962). Sodium acetate medium for the differentiation of Shigella and Escherichia cultures. *Publ. Hlth Lab.* **20**, 137.

J. gen. Microbiol. (1965), 41, 29–36
With 3 plates
Printed in Great Britain

The Morphology of Proteus Bacteriophages

BY O. W. PROZESKY, H. C. DE KLERK AND J. N. COETZEE
Department of Microbiology, University of Pretoria, Pretoria, South Africa

(*Received* 1 *March* 1965)

SUMMARY

The morphology of 28 bacteriophages active on members of the Proteus–Providence group of organisms was examined by a negative staining technique. Examples of the 'T-even', T3 and P22 morphologies were encountered as well as phages with non-contractile cross-striated tails resembling a group of coliphages. Others resemble staphylococcal and pseudomonas phages. Two phages (9, 7479) with octahedral capsids were encountered. A temperate *Proteus mirabilis* phage (12/57) and a temperate *P. morganii* phage (10041/2815) differ from phage P22 in that they possess collars. A *P. rettgeri* phage (7480b) is unique in that it possesses a head with a regular hexagonal outline, a collar and a non-contractile tail while a Providence phage (9266) is believed to be the largest phage active on Gram-negative bacteria.

INTRODUCTION

The morphology of Proteus bacteriophages was first examined by Ruska (1943, 1950). He examined shadow-cast preparations and recorded the dimensions of tadpole-shaped phages. Coetzee (1958) examined shadow-cast preparations of phages active on *Proteus vulgaris* and *P. mirabilis*. They were also tadpole shaped. In a paper on defective lysogeny Taubeneck (1963) published photographs of shadow-cast preparations of a tadpole-shaped phage derived from a lysogenic strain of *P. mirabilis*. Other illustrations in the paper show tail cores surrounded by contractile sheaths adsorbed to *P. mirabilis* and *P. vulgaris* strains. These tails were liberated by defective lysogenic strains and no head structures were visible.

In the family Enterobacteriaceae bacteria of the Proteus and Providence groups share the distinction that they alone possess a phenylalanine deaminase (Shaw & Clarke, 1955). They display many intra- and inter-group differences (Ewing, 1958) and their classification is uncertain. Rauss (1962) would allocate them tribal rank in the family with genera *Proteus*, *Morganella*, *Rettgerella* and *Providencia*. *Topley & Wilson's Principles* (1964) recognize 'vulgaris, mirabilis, morganii and rettgeri' species in a genus *Proteus* of the tribe Enterobacterieae. They do not commit themselves on the position of the Providence group. Kauffmann (1951) has combined 'mirabilis' and 'vulgaris' strains in a species 'hauseri'. Falkow, Ryman & Washington (1962) suggest that *P. morganii* is distinct genetically, possessing a guanine + cytosine molar content of 50 %, compared with other Proteus and Providence strains, where content is about 40 %. We decided to study the structure of phages active on the above organisms as an approach to the taxonomy of both the phages and their hosts.

METHODS

Bacteriophages and host organisms. Phages were isolated from sewage by the enrichment technique of Adams (1959) or were derived from lysogenic strains, spontaneously or by ultraviolet induction. All but 4 of the phages have been used in other investigations and are listed in Table 1 together with the method of isolation, indicator host and reference to the literature. Sewage phages 50, 67b and 7480b productively lyse *Proteus morganii* strain M50, *P. vulgaris* strain 67 and *P. rettgeri* NCTC7480 respectively. Phage 13 *vir* is present in plaques which appear spontaneously on cultures of *P. mirabilis* strain 13. Organisms of this strain cannot be freed of the phage by suspension in high-titre phage antiserum, and this phage is therefore regarded as a virulent mutant of a temperate phage of strain 13. The temperate phage has not yet been demonstrated. The general phage techniques were those of Adams (1959).

Table 1. *Sources of bacteriophages*

Phages	Isolation	Indicator	Reference
Proteus hauseri			
9	Sewage	*P. vulgaris* 9	Coetzee, 1958
15	Sewage	*P. vulgaris* 15	
65	Sewage	*P. vulgaris* 65	
67b	Sewage	*P. vulgaris* 67	See Methods
78	Sewage	*P. vulgaris* 78	Coetzee, 1958
12	Sewage	*P. mirabilis* 12	
14	Sewage	*P. mirabilis* 14	
57	Sewage	*P. mirabilis* 57	
34/13	*P. mirabilis* 34	*P. mirabilis* 13	Coetzee & Sacks, 1959, 1960*a*, *b*
12/57	*P. mirabilis* 12	*P. mirabilis* 57	
$\frac{24}{25}$/14	*P. mirabilis* 24, 25	*P. mirabilis* 14	
13 *vir*	*P. mirabilis* 13	*P. mirabilis* 13	See Methods
P. morganii			
50	Sewage	*P. morganii* M50	See Methods
5845	Sewage	NCTC 5845	Coetzee, 1963*a*
10041/2815	NCTC 10041	NCTC 2815	
47	M47	*P. morganii* M235	
Providence			
9248	Sewage	NCTC 9248	Coetzee, 1963*b*
9266	Sewage	NCTC 9266	
9211/9295	NCTC 9211	NCTC 9295	
9213/9211a, b	NCTC 9213	NCTC 9211	
9000/9402	NCTC 9000	NCTC 9402	
P. rettgeri			
8893	Sewage	NCTC 8893	Coetzee, 1963*c*
7480	Sewage	NCTC 7480	
7480b	Sewage	NCTC 7480	See Methods
7479	Sewage	NCTC 7479	Coetzee, 1963*c*
7478/325	NCTC 7478	R325	
7476/322	NCTC 7476	R322	

Media. The nutrient agar was that of Adams (1959) and the broth was that of Coetzee & Sacks (1960*c*).

Electron microscopy. High-titre phage lysates of sewage phage were prepared by infecting young aerated broth cultures from single plaques. Temperate phages were

prepared by the modification (Adams, 1959) of the double-agar-layer method of Hershey, Kalmanson & Bronfenbrenner (1943). The lysates were purified and concentrated by differential centrifugation (de Klerk, Coetzee & Fourie, 1965). The purified phages (plaque-forming titres about 1×10^{11}/ml.) were suspended in 0·1 M-ammonium acetate (pH 7·2). The negative staining method of Brenner & Horne (1959) was used. Phage suspensions in neutral potassium phosphotungstate were mounted on carbon support films by the spreading technique (Bradley, 1962) and examined with a Philips EM 200 electron microscope. In the absence of spontaneous contraction of tail sheaths, hydrogen peroxide (3 %, v/v) and ethyl alcohol (10 %, v/v) were added to phage suspensions to cause possible sheath contraction (Kellenberger & Arber, 1955).

RESULTS

Dimensions of the bacteriophages examined are listed in Table 2.

Table 2. *Dimensions (in Å) of Proteus bacteriophages*

Phage	Head*	Tail		Core	Overall
		Length	Width	width	length
Proteus hauseri					
15	518	148	222	—	666
34/13	630	167	204	—	797
65	518	148	222	—	666
12/57	589	185	204	—	774
9	826	1837	89	—	2663
13 *vir*	600	1019	167	60	1619
57	619	882	178	56	1501
67b	589	981	174	74	1570
78	611	889	163	60	1500
12	593	2163	115	—	2756
14	660	2045	115	—	2705
$\frac{24}{25}$/14	715 × 604	1715	108	—	2430
P. morganii					
50	970 × 670	1252	159	52	2222
5845	1022 × 763	1252	156	74	2274
10041/2815	544	182	167	—	726
47	556	1481	85	—	2037
Providence					
9248	611	185	—	—	796
9266	1193 × 822	2152	248	96	3345
9213/9211a	626	1696	137	—	2322
9213/9211b	608	152	204	—	760
9211/9295	533	148	196	—	681
9000/9402	682	2304	148	—	2986
P. rettgeri					
8893	926 × 741	1296	178	74	2222
7480	634	2185	126	—	2819
7480b	367	330	63	—	697
7479	737	1852	85	—	2589
7478/325	604	1741	115	—	2345
7476/322	533	1600	77	—	2133

* Dimensions between apices are given, or apex to tail-joint and width. Figures are the mean of 8 to 12 measurements.

Phages active on Proteus hauseri strains

Phages 15, 34/13, 65. (Pl. 1, figs. 1, 2.) These are similar in morphology. Phages 65 and 34/13 are presented. The heads have hexagonal outlines and attached to one of the angles by means of a short neck is a base-plate with a number of pins. These phages resemble salmonella phage P22 (Anderson, 1960) and pseudomonas phage 12B (Bradley, 1963*a*) but phages 15 and 65 are slightly smaller.

Phage 12/57. (Pl. 1, fig. 3.) This phage resembles those just mentioned but possesses a delicate collar about 200 Å wide, around the short neck. No tail fibres were seen.

Phage 9. (Pl. 1, fig. 4.) The head of this phage is large, hexagonal in outline and octahedral in shape. The tail is thin, cross-striated, non-contractile and ends in a point with tail fibres. It resembles pseudomonas phage 24 (Bradley, 1963*a*), phage 1C (Bradley, 1962) and phages F1 (Bradley, 1963*b*) and WAK/2 (Bradley, 1964).

Phages 13 vir, 57, 67b, 78. (Pl. 1, figs. 5, 6, 7.) These phages have a similar morphology and 13 *vir*, 57 and 78 are shown. The head is regular hexagonal in plan view. The tail consists of a core surrounded by a contractile sheath to which is attached an inconspicuous base-plate, probably with 6 prongs. Cross-striations are visible on some of the sheaths with a periodicity of about 40 Å. These phages resemble Vi phage 1 (Bradley & Kay, 1960) although their heads are smaller.

Phages 12, 14. (Pl. 1, fig. 8.) These phages are similar and correspond to the group of 'smaller phages without contractile tails' of Bradley (1963*b*). Phage 12 is shown. Their heads are polygonal and often hexagonal in outline. The tails are non-contractile and markedly cross-striated with a periodicity of about 40 Å. A small bundle of fibres is present at the tail tip.

Phage $\frac{24}{25}$*/14.* (Pl. 1, fig. 9.) This phage has a polyhedral head slightly longer than wide which closely resembles that of phage ZG/3A (Bradley, 1964). It has a non-contractile tail with cross-striations about 30 Å apart. No tail fibres have been observed.

Phages active on strains of Proteus morganii

Phages 50, 5845. (Pl. 1, fig. 10; Pl. 2, figs. 11, 12.) These phages are similar in shape. The dimensions and appearance of the heads suggest that they may be bipyramidal hexagonal prisms and the tails consist of cores surrounded by cross-striated contractile sheaths. A base-plate with pins moves with the sheath. Tail fibres are present and in some photographs they form a fibrous network which extends to the collar (Bradley, 1963*b*). The collars have a width of 360 Å and a periodicity of the sheaths of this group is about 40 Å. In Pl. 2, fig. 11, is a separate rosette-shaped structure which may be either a base-plate or a collar. These phages resemble phage T4, which is reported to possess a bipyramidal hexagonal head (Bradley, 1963*b*).

Phage 10041/2815. (Pl. 2, fig. 13.) This phage is similar to but slightly smaller than *P. hauseri* phage 12/57. The collar above the base-plate can be clearly seen on one of the phages in the figure. No tail fibres have been observed.

Phage 47. (Pl. 2, figs. 15, 18.) This phage has a head with a hexagonal outline. The tail is non-contractile and cross-striated. It has no neck or collar, tail fibres have not been identified and it resembles the SBL group of typhoid phages (Bradley, 1963*b*).

Phages active on Providence strains

Phage 9248. (Pl. 2, fig. 14.) The head of the phage is polyhedral and hexagonal in plan view. It has a wedge-shaped tail attached to one corner and resembles coliphage T3. It also resembles brucella phages (Brinley-Morgan, Kay & Bradley, 1960; McDuff, Jones & Wilson, 1962) and a group of pseudomonas phages (Bradley, 1963*a*).

Phage 9266. (Pl. 2, figs. 16, 17.) This large phage has a head which is hexagonal in outline and resembles those of the T-even phages. The tail core is surrounded by a contractile sheath with cross-striations. No base-plate is evident. Attached to the tail sheath and visible in the contracted state (Pl. 2, fig. 16) are 6 short spikes and a tangled mass of fibres. It has a neck but no collar.

Phage 9213/9211a. (Pl. 2, fig. 19.) The phage has a head with a regular hexagonal outline in certain views. The tail is non-contractile, has cross-striations and ends in a rosette-shaped structure. It resembles staphylococcal phages 187, 52 (Bradley, 1963*a*) and pseudomonas phages Pc, Pz (Bradley & Kay, 1960).

Phages 9213/9211b, 9211/9295. (Pl. 3, figs. 20, 21). These phages resemble the *P. hauseri* groups 34/13, 15, 65 and 12/57 and the *P. morganii* phage 10041/2815. On occasions a rod 30–40 Å wide which projects about 150 Å beyond the base-plate was seen, but could not be consistently demonstrated. No tail fibres were seen.

Phage 9000/9402. (Pl. 3, fig. 23.) The outline of the head is a regular hexagon. It has a long non-contractile tail with cross-striations and some fibres at the tip. This phage is slightly longer than the *P. hauseri* phages 12, 14, but smaller than the C_1, F_1 group of coliphages (Bradley, 1963*b*).

Phages active on strains of Proteus rettgeri

Phage 8893. (Pl. 3, fig. 26.) The structure is similar to *P. morganii* phages 50, 5845.

Phage 7480. (Pl. 3, fig. 22.) This phage is similar to Providence phage 9000/9402.

Phage 7480b. (Pl. 3, fig. 25.) The head has a regular hexagonal outline. A short tail is attached to one corner by a neck which is surrounded by a collar. The tail could not be made to contract by the methods used and cross-striations were not seen. Despite repeated efforts the detailed structure of the tail-tip could not be resolved. The collar is 100 Å wide.

Phage 7479. (Pl. 3, fig. 24.) This phage resembles *P. hauseri* phage 9 although the octahedral head is slightly smaller.

Phage 7478/325. (Pl. 3, fig. 27.) This phage resembles *P. hauseri* phage 12, 14, *P. morganii* phage 47, Providence phage 9000/9402 and *P. rettgeri* phage 7480. The dimensions are similar to those of T5 (Bradley & Kay, 1960).

Phage 7476/322. (Pl. 3, fig. 28.) The phage head is hexagonal in outline. The tail is thin, cross-striated and measures about 30 Å less in width than tails of the SBL group (Bradley & Kay, 1960).

Many of the capsids have an uneven rough appearance suggestive of arrays of capsomeres (Bradley, 1964) but neither their shape nor their packing arrangements could be determined. Apart from those whose heads are obviously octahedral, the head shapes of the phages could not be determined. We have indicated those which may have bipyramidal shapes and the remainder are possibly icosahedra.

DISCUSSION

Undescribed varieties of phage have been found. The *Proteus mirabilis* transducing phage 12/57 differs from its salmonella counterpart P22 in that it has a delicate collar. The same applies to *P. morganii* phage 10041/2815. Phage 7480b is unique in that it has a collar, a non-contractile tail and a head with a regular hexagonal outline. Phage ZG/3A described by Bradley (1964) is similar, but has a bipyramidal prismatic head. The overall dimensions of phage 9266 are larger than those of phage P1 (Anderson, 1960) (which is described as the largest phage yet examined (Hayes, 1965)) though some staphylococcal phages have longer tails (Bradley & Kay, 1960). Phage 13 *vir* has a unique morphology for a phage derived from a lysogenic bacterium. The nearest to it are phages P1, P2 (Anderson, 1960), which are much larger, lack tail pins and have less conspicuous tail fibres. The original lysate of phage 9213/9211 (Coetzee, 1963*b*) was thought to be pure, but it has yielded two morphological types. There is a possibility that one of the phages may be derived from the indicator strain NCTC 9211 although this strain has not been proved lysogenic.

It is not known how the P22 group of phages infect their hosts (Anderson, 1960). The finding here of phages which resemble P22 but have collars may eventually cast light on the process. Tail fibres were never observed in these phages. The nature of the structure which projects beyond the base plates of phages 9213/9211b and 9211/9295 is not known. It could not always be demonstrated and may be nucleic acid.

Providence phage 9248 resembles coliphage T3. Its head shape could not be established but it may be octahedral like that of phage T3 (Bradley, 1963*b*). Two other phages (9, 7479) of this series possess octahedral capsids. Caspar & Klug (1962) have cast doubt on the octahedral shapes of phage capsids but Bradley (1963*b*; 1964) has clearly demonstrated such capsids among coliphages.

Good correlation between the morphological and serological types of phages have been recorded (Bradley, 1963*a*; de Klerk, Coetzee & Theron, 1963; de Klerk *et al.* 1965). Phages 15, 65, which are morphologically identical, are related serologically but the identical structural types 57, 78 differ serologically and in other aspects (Coetzee, 1958). The bacteriophages studied show considerable morphological variation and identical types grow on representatives of most of the groups of organisms. This lack of morphological specificity is a great problem in bacteriophage taxonomy (Dawson, Smillie & Norris, 1962; Bradley, 1963*b*). Results also diminish the hope of finding a particular morphological type with a predilection for one or other of these groups of organisms like that of the RNA and filamentous phages for male strains of *Escherichia coli* (Bradley, 1964).

One of us (J.N.C.) is in receipt of a grant from the South African Council for Scientific and Industrial Research.

REFERENCES

ADAMS, M. H. (1959). *Bacteriophages*. New York: Interscience Publishers Inc.

ANDERSON, T. F. (1960). On the fine structure of the temperate bacteriophages P1, P2 and P22. *Proc. Eur. Reg. Conf. Electron Microscopy, Delft*, 2, 1008.

BRADLEY, D. E. (1962). A study of the negative staining process. *J. gen. Microbiol.* **29**, 503.

BRADLEY, D. E. (1963*a*). The structure of some *Staphylococcus* and *Pseudomonas* phages. *J. Ultrastruct. Res.* **8**, 552.

BRADLEY, D. E. (1963*b*). The structure of coliphages. *J. gen. Microbiol.* **31**, 435.

BRADLEY, D. E. (1964). The structure of some bacteriophages associated with male strains of *Escherichia coli. J. gen. Microbiol.* **35**, 471.

BRADLEY, D. E. & KAY, D. (1960). The fine structure of bacteriophages. *J. gen. Microbiol.* **23**, 553.

BRENNER, S. & HORNE, R. W. (1959). A negative staining method for high resolution electron microscopy of viruses. *Biochim. biophys. Acta*, **34**, 103.

BRINLEY-MORGAN, W. J., KAY, D. & BRADLEY, D. E. (1960). *Brucella* bacteriophage. *Nature, Lond.* **188**, 74.

CASPAR, D. L. D. & KLUG, A. (1962). Physical principles in the construction of regular viruses. *Cold Spr. Harb. Symp. quant. Biol.* **27**, 1.

COETZEE, J. N. (1958). The characterization of a series of *Proteus* bacteriophages. *S. Afr. J. Lab. clin. Med.* **4**, 147.

COETZEE, J. N. (1963*a*). Host-range of *Proteus morganii* bacteriophages. *Nature, Lond.* **199**, 827.

COETZEE, J. N. (1963*b*). Lysogeny in *Providence* strains and the host-range of *Providence* bacteriophages. *Nature, Lond.* **197**, 515.

COETZEE, J. N. (1963*c*). Lysogeny in *Proteus rettgeri* and the host-range of *P. rettgeri* and *P. hauseri* bacteriophages. *J. gen. Microbiol.* **31**, 219.

COETZEE, J. N. & SACKS, T. G. (1959). Lysogeny in the genus *Proteus. Nature, Lond.* **184**, 1340.

COETZEE, J. N. & SACKS, T. G. (1960*a*). Lysogeny in the genus *Proteus.* I. Incidence of lysogenic strains. *S. Afr. J. Lab. clin. Med.* **6**, 123.

COETZEE, J. N. & SACKS, T. G. (1960*b*). Transduction of streptomycin resistance in *Proteus mirabilis. J. gen. Microbiol.* **23**, 445.

COETZEE, J. N. & SACKS, T. G. (1960*c*). Morphological variants of *Proteus hauseri. J. gen. Microbiol.* **23**, 209.

DAWSON, I. M., SMILLIE, E. & NORRIS, J. R. (1962). The morphology of *Bacillus cereus* bacteriophages. *J. gen. Microbiol.* **28**, 517.

EWING, W. H. (1958). The nomenclature and taxonomy of the *Proteus* and *Providence* groups. *Int. Bull. bact. Nomencl.* **8**, 17.

FALKOW, S., RYMAN, I. R. & WASHINGTON, O. (1962). Deoxyribonucleic acid base composition of Proteus and Providence organisms. *J. Bact.* **83**, 1318.

HAYES, W. (1965). *The Genetics of Bacteria and their Viruses.* Oxford: Blackwell Scientific Publications.

HERSHEY, A. D., KALMANSON, G. R. & BRONFENBRENNER, J. (1943). Quantitative methods in the study of the phage-antiphage reaction. *J. Immunol.* **46**, 267.

KAUFFMANN, F. (1951). *Enterobacteriaceae.* Copenhagen: Einar Munksgaard.

KELLENBERGER, E. & ARBER, W. (1955). Die Struktur des Schwanzes der Phagen T2 und T4 und der Mechanismus der irreversiblen Adsorption. *Z. Naturf.* **10***b*, 698.

DE KLERK, H. C., COETZEE, J. N. & FOURIE, J. T. (1965). The fine structure of *Lactobacillus* bacteriophages. *J. gen. Microbiol.* **38**, 35.

DE KLERK, H. C., COETZEE, J. N. & THERON, J. J. (1963). The characterization of a series of *Lactobacillus* bacteriophages. *J. gen. Microbiol.* **32**, 61.

MCDUFF, C. R., JONES, L. M. & WILSON, J. B. (1962). Characteristics of brucellaphage. *J. Bact.* **83**, 324.

RAUSS, K. (1962). A proposal for the nomenclature and classification of the Proteus and Providence groups. *Int. Bull. bact. Nomencl.* **12**, 53.

RUSKA, H. (1943). Ergebnisse der Bakteriophagenforschung und ihre Deutung nach morphologischen Befunden. *Erg. Hyg.* **25**, 437.

RUSKA, H. (1950). Die *Elektronenmikroskopie in der Virusforschung.* In *Handbuch der Virusforschung*, 2. Ergänzungsband, p. 221. R. Doerr & C. Hallauer. Wien: Springer-Verlag.

SHAW, C. & CLARKE, P. H. (1955). Biochemical classification of Proteus and Providence cultures. *J. gen. Microbiol.* **13**, 155.

TAUBENECK, U. (1963). Über die Produktion biologisch aktiver Phagen-Schwänze durch einen defekt lysogenen *Proteus mirabilis*-Stamm. *Z. Naturf.* **18*b***, 989.

Topley and Wilson's Principles of Bacteriology and Immunity, (1964) 5th ed. Ed. by G. S. Wilson and A. A. Miles. London: Edward Arnold.

EXPLANATION OF PLATES

The magnification in all Figs. is ×270,000. All phages in ammonium acetate and phosphotungstate. Figs. 1–9. Phages active on *Proteus hauseri* strains. Figs. 10–13, 15, 18: phages active on *P. morganii* strains. Figs. 14, 16, 17, 19–21, 23: phages active on Providence strains. Figs. 22, 24–28: phages active on *P. rettgeri* strains.

PLATE 1

Fig. 1. Phage 65.

Fig. 2. Phage 34/13.

Fig. 3. Phage 12/57.

Fig. 4. Phage 9.

Fig. 5. Phage 13 *vir*.

Fig. 6. Phage 78.

Fig. 7. Phage 57.

Fig. 8. Phage 12.

Fig. 9. Phage $\frac{24}{25}$/14.

Fig. 10. Phage 50.

PLATE 2

Figs. 11, 12. Phage 5845.

Fig. 13. Phage 10041/2815.

Fig. 14. Phage 9248.

Fig. 15. Phage 47.

Figs. 16, 17. Phage 9266.

Fig. 18. Phage 47.

Fig. 19. Phage 9213/9211 a.

PLATE 3

Fig. 20. Phage 9213/9211 b.

Fig. 21. Phage 9211/9295.

Fig. 22. Phage 7480.

Fig. 23. Phage 9000/9402.

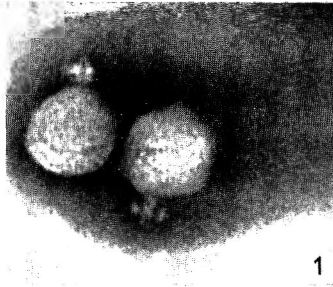
Fig. 24. Phage 7479.

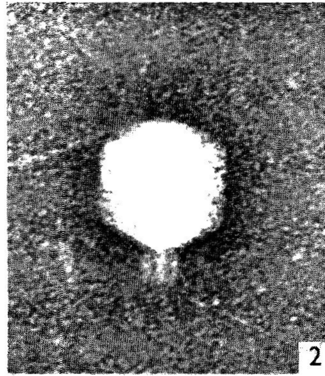
Fig. 25. Phage 7480 b.

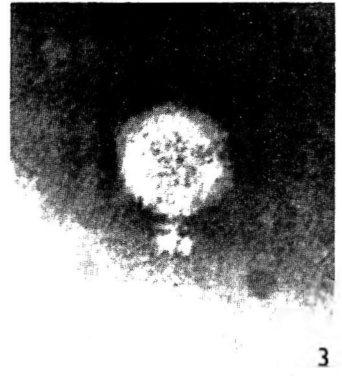
Fig. 26. Phage 8893.

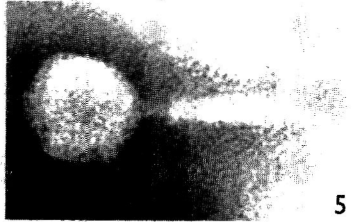
Fig. 27. Phage 7478/325.

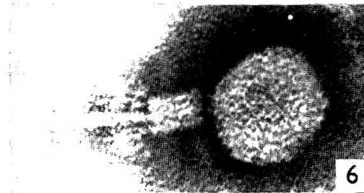
Fig. 28. Phage 7476/322.

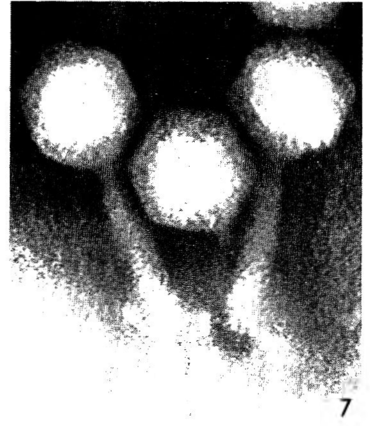


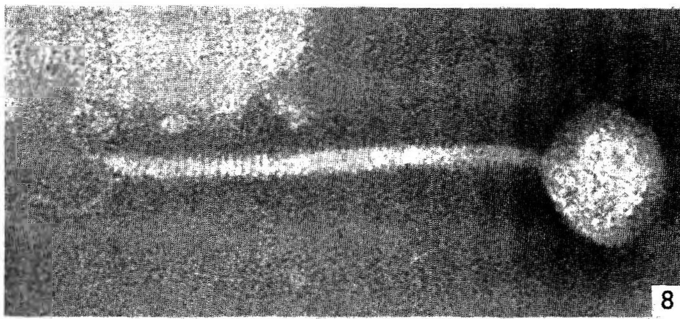


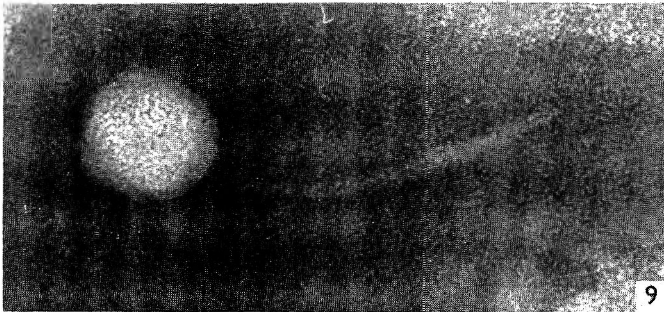


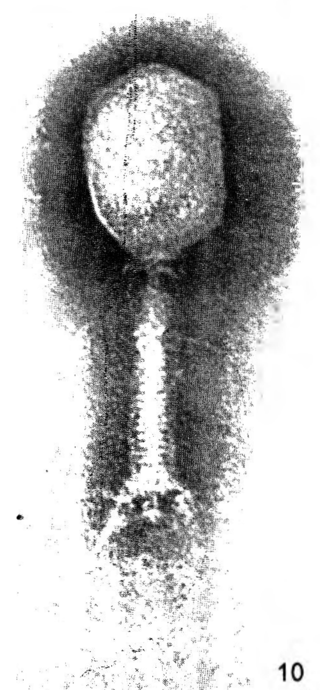








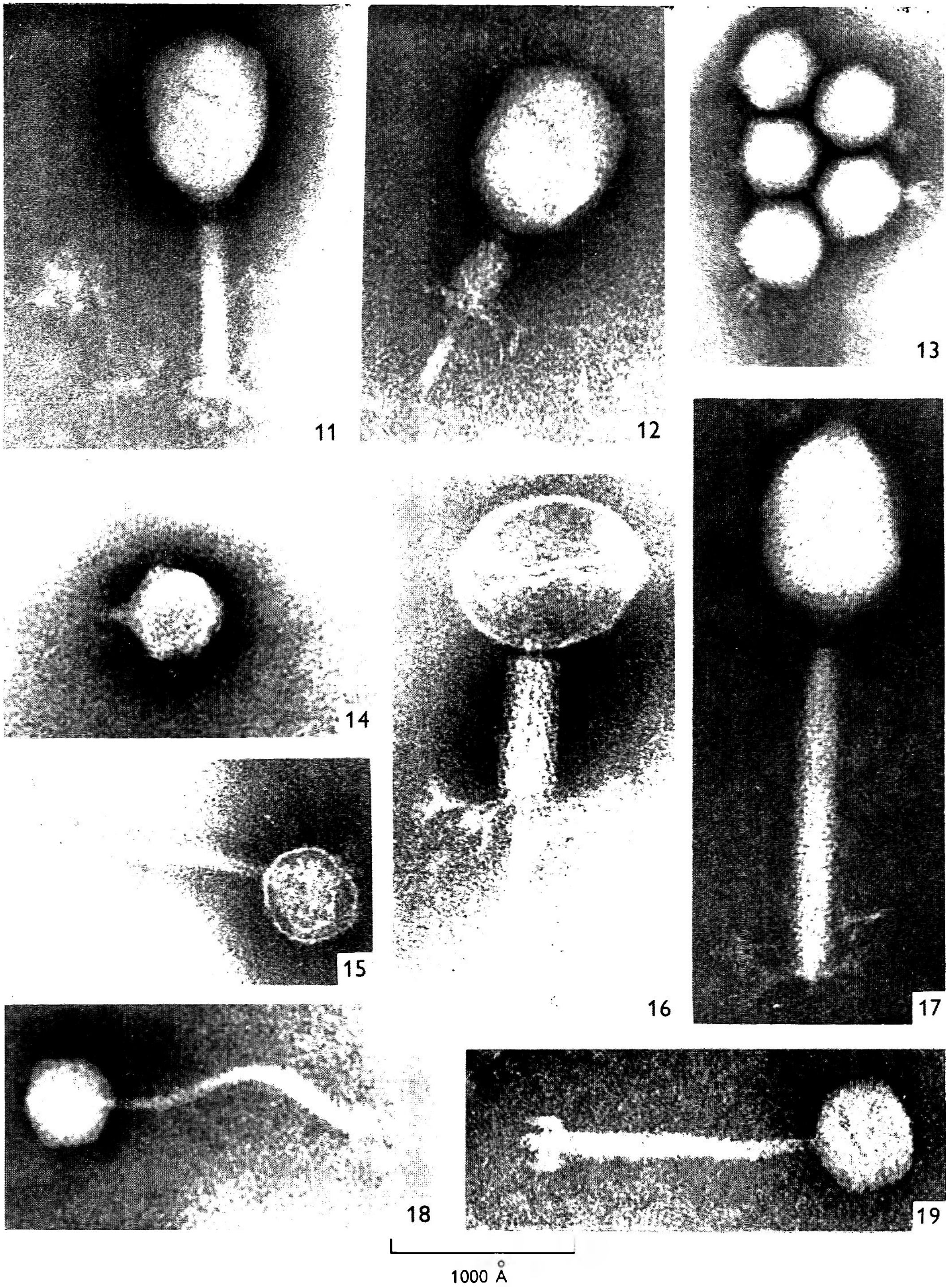




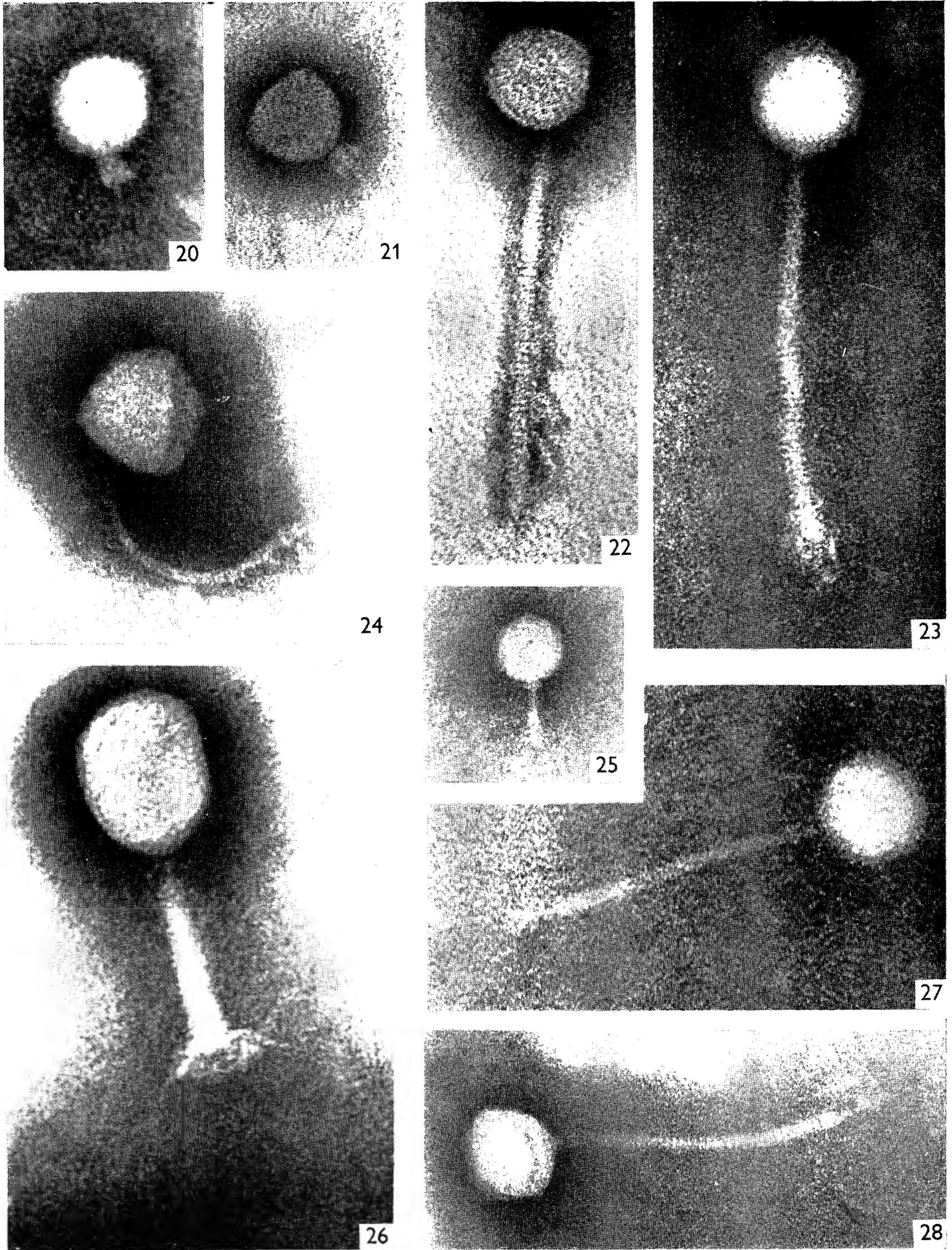
1000 Å

O. W. PROZESKY, H. C. DE KLERK AND J. N. COETZEE

(*Facing p.* 36)



O. W. PROZESKY, H. C. DE KLERK AND J. N. COETZEE



O. W. PROZESKY, H. C. DE KLERK AND J. N. COETZEE

J. gen. Microbiol. (1965), **41**, 37–45
Printed in Great Britain

RNA Metabolism of *Rhodopseudomonas spheroides* during Preferential Photopigment Synthesis

BY T. G. LESSIE*
Microbiology Unit, Department of Biochemistry, University of Oxford

(*Received* 4 *January* 1965)

SUMMARY

During anaerobic growth of *Rhodopseudomonas spheroides* the specific RNA content (μg. RNA/μg. protein) of cultures was directly proportional to the light intensity in which they were grown. Cultures subjected to a step-up in light intensity preferentially synthesized RNA until their specific RNA content increased to the value characteristic of growth at the higher light intensity. Conversely following a step-down in light intensity the rate of RNA synthesis fell below the rate of protein synthesis, and cellular RNA was diluted out to the value characteristic of growth at the lower light intensity. Adjustment of the differential rate of RNA synthesis in response to a change in light intensity was thus opposite to that of the differential rate of photopigment synthesis. Although a decrease in light intensity drastically decreased RNA synthesis some RNA continued to be formed. The RNA synthesized, like that in cultures maintained at a constant light intensity, consisted of soluble and ribosomal RNA and unstable RNA with sedimentation behaviour like m-RNA. Aerobic cultures of *R. spheroides* subjected to a marked decrease in O_2 tension synthesized photopigments *de novo* without appreciably increasing their net content of RNA, but continued to synthesize small amounts of all the usual classes of RNA.

INTRODUCTION

Rhodopseudomonas spheroides when grown aerobically is devoid of bacteriochlorophyll and contains only traces of carotenoids, but it synthesizes large amounts of these photopigments when grown anaerobically in the light or when incubated semi-aerobically in the dark (Cohen-Bazire, Sistrom & Stanier, 1957). The extent of pigment formation is inversely proportional to light intensity or to the O_2 tension under semi-aerobic conditions. Formation of bacteriochlorophyll is strictly dependent upon protein synthesis (Sistrom, 1963; Bull & Lascelles, 1963). This is in part explained by the fact that certain enzymic activities associated with bacteriochlorophyll formation increase in parallel with the ability to form photopigments (Lascelles, 1959; Gibson, Neuberger & Tait, 1963). However, the obligatory requirement for protein synthesis in already pigmented cultures, which presumably contain all the enzymes necessary for bacteriochlorophyll synthesis, suggests that bacteriochlorophyll formation must proceed with joint synthesis of some protein component of the photosynthetic apparatus. Evidence that proliferation of intracellular membranes is concomitant with development of photopigments in the Athiorhodoaceae

* Present address: Department of Biological Sciences, Purdue University, Lafayette, Indiana, U.S.A.

supports this view (Cohen-Bazire & Kunisawa, 1963). The integral relationship between photopigment synthesis and protein synthesis suggests that control of bacteriochlorophyll formation may be at the genetic level. The role of RNA in mediating synthesis of specific proteins is well known. To gain information about the relationship of protein synthesis to bacteriochlorophyll formation I examined the RNA metabolism of *R. spheroides* under conditions where the organisms were increasing their specific photopigment content.

METHODS

Organisms. The strains of *Rhodopseudomonas spheroides* used were strain GA, and strain M-25 which requires uracil for growth. These bacteria and the methods used to grow them were described previously (Lessie, 1965).

Semi-aerobic incubation of bacteria. Cultures were grown aerobically to a population equivalent to about 100 μg. protein/ml. The organisms were centrifuged and suspended to ten times their original concentration in fresh medium, and 60 ml. volumes of the suspension were shaken at 34° in ⊥-tubes (see Lascelles, 1959).

Determination of cellular nucleic acids, protein, and bacteriochlorophyll. RNA and DNA were extracted from organisms with hot trichloroacetic acid (TCA). RNA was estimated colorimetrically by reaction with orcinol (Schneider, 1945). DNA was estimated by reaction with indole (Ceriotti, 1952). TCA-insoluble protein was digested in NaOH and estimated by the Folin phenol method (Lowry *et al.* 1953). The details of these procedures were given elsewhere (Lessie, 1965). Bacteriochlorophyll was extracted from bacteria with methanol, and estimated by measuring the extinction of the methanolic extracts at 775 mμ (see Cohen-Bazire *et al.* 1957).

Incorporation of ^{14}C-uracil into bacteria. Uptake of ^{14}C-uracil into cellular RNA was measured as follows. Culture samples were diluted 1/2 to 1/10 in cold trichloroacetic acid (TCA) to give a concentration of between 20 and 100 μg. protein/ml. in 5 % TCA. Part of the TCA-treated sample (containing about 100 μg. protein) was filtered through Millipore membranes (0·65 μ pore size) and the organisms washed with cold 5 % TCA. The membranes were dried, cemented to planchettes, and their radioactivities determined by using a thin window Geiger tube. At the concentration of bacteria used it was unnecessary to correct for self-absorption. In a few experiments ^{14}C-labelled RNA was extracted from the bacteria with hot TCA. The results were identical to those obtained using the Millipore technique.

Preparation and characterization of ribosomes and RNA. Bacteria were disrupted in a French press to obtain crude extracts containing ribosomes and soluble RNA. Ribosomal RNA was obtained by deproteinizing the crude extracts with sodium lauryl sulphate and phenol. The ribosomes and RNA were characterized by sucrose gradient centrifugation using an SW-39 rotor in a Spinco Model L ultracentrifuge, The detailed procedures followed to obtain and characterize *Rhodopseudomonas spheroides* ribosomes and RNA have already been reported (Lessie, 1965).

2-^{14}C-uracil was obtained from the Radiochemical Centre Amersham, Buckinghamshire. Millipore filters were obtained from the Millipore Filter Co., Bedford, Mass., U.S.A.

RESULTS

Effect of light on the differential rate of RNA synthesis. Cultures of *Rhodopseudomonas spheroides* subjected to an increase in light intensity temporarily stopped forming photopigments and synthesized RNA preferentially. After the specific RNA content (μg. RNA/μg. protein) increased to the value value characteristic of growth at the higher light intensity increase of RNA proceeded in step with increase of protein. For example a culture of *R. spheroides* strain GA subjected to a step-up

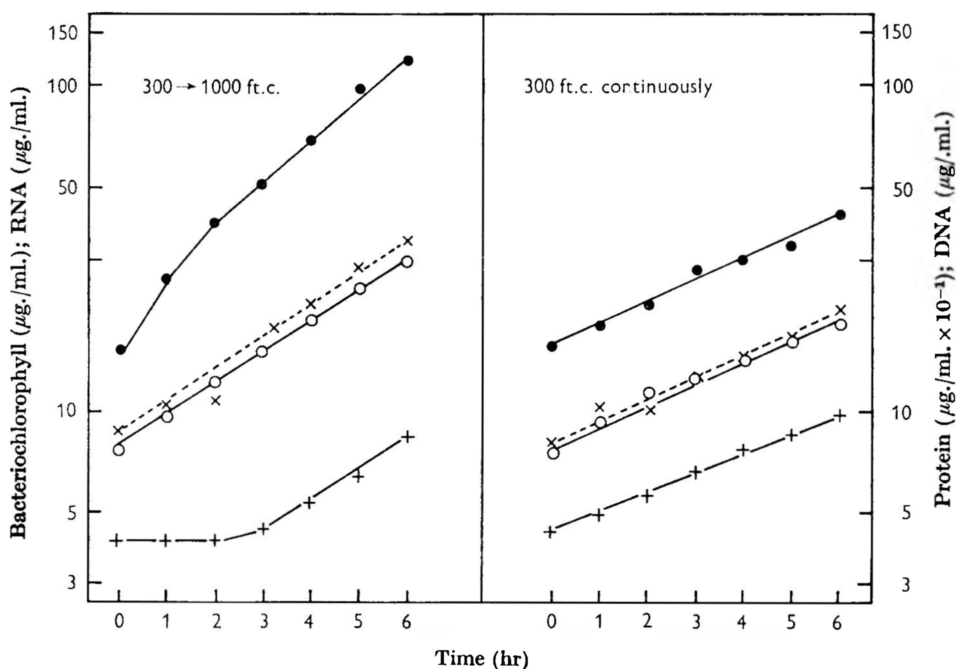


Fig. 1. Increase of specific RNA content following an increase in light intensity. *Rhodopseudomonas spheroides* GA was grown in 400 ml. SG medium at a light intensity of 300 ft.c. The culture was bubbled with a mixture of 5% (v/v) CO_2 and 95% (v/v) N_2. When the culture reached a concentration equivalent to about 50 μg. protein/ml. it was divided into two portions. One portion was incubated as before in light at 300 ft.c.; the other portion was incubated in light at 1000 ft.c. At the indicated times duplicate samples were taken for determination of bacteriochlorophyll (+—+), protein (○—○), RNA (●—●), DNA (×—×).

in light intensity from 300 to 1000 foot-candles (ft.c.) synthesized RNA preferentially until its specific RNA content was increased about twofold (from 0·13 to 0·25, see Fig. 1). The amount of DNA/protein remained constant with a specific DNA content of about 0·1.

Conversely, a decrease in light intensity caused the rate of RNA synthesis to decrease below the rate of protein synthesis; as a consequence the cellular RNA was diluted. After the specific RNA content decreased to the value characteristic of growth at the lower light intensity, balanced synthesis of RNA was resumed. Accordingly, a culture subjected to a step-down in light intensity formed bacteriochlorophyll without significantly increasing its net content of RNA. For example,

following a decrease in light intensity from 1000 to 200 ft.c. the specific bacteriochlorophyll content of a culture of *Rhodosopeudomonas spheroides* strain GA more than doubled while net increase of RNA was arrested (see Fig. 2). The specific DNA content increased slightly following the step-down in light intensity and then readjusted to its initial value.

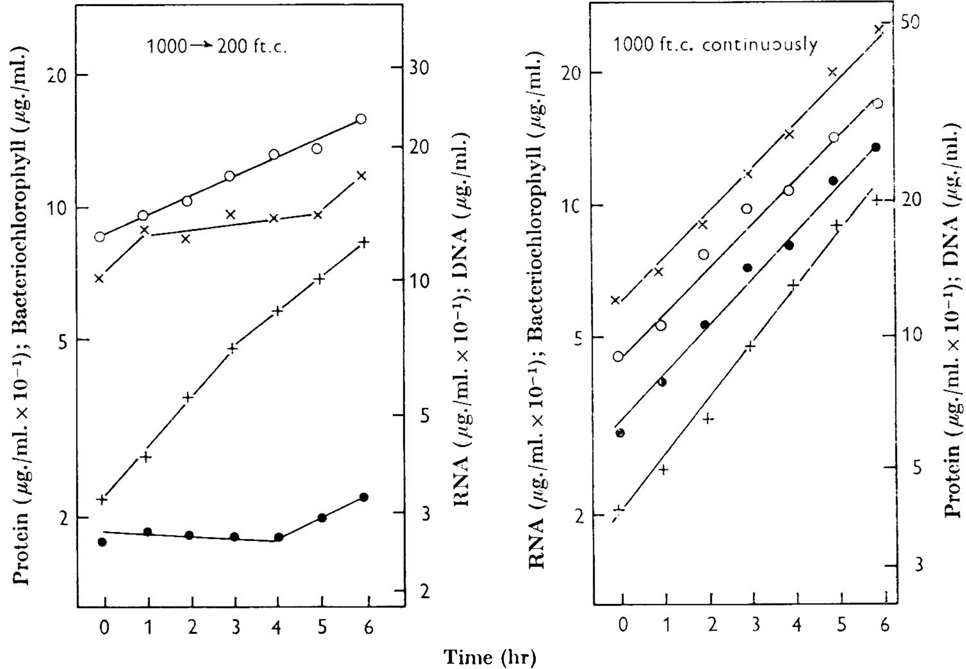


Fig. 2. Decrease of specific RNA content following a decrease in light intensity. *Rhodopseudomonas spheroides* was grown in 400 ml. SG medium in light at an intensity of 1000 ft.c. The culture was bubbled with a mixture of 5 % (v/v) CO_2 and 95 % (v/v) N_2. When the culture reached a concentration equivalent to about 50 μg. protein/ml. it was divided into two portions. One portion was maintained in 1000 ft.c.; the other was placed in light at 200 ft.c. Duplicate samples were taken at specified times for determination of bacteriochlorophyll (+—+), protein (O——O), RNA (●–●), DNA (×—×).

RNA synthesis following a decrease in light intensity. RNA synthesis was not abolished by a decrease in light intensity. The bacteria incorporated radioactive uracil into their RNA at the same time as they preferentially synthesized bacteriochlorophyll. Figure 3 shows the kinetics of entry of ^{14}C-uracil into the cold-TCA insoluble fraction of a culture of *Rhodopseudomonas spheroides* strain M-25 following a step-down in light intensity from 1200 to 120 ft.c. Incorporation which occurred after the initial rapid uptake amounted to about 10 % of that in cultures maintained at 1200 ft.c. Following a decrease in light intensity from 1200 to 400 ft.c. the rate of uracil incorporation was about 20 % the value at 1200 ft.c.

In order to ascertain that under the above conditions uracil was incorporated into RNA and to determine which classes of RNA were being synthesized the following experiments was performed. Cultures of *Rhodopseudomonas spheroides* M-25 were subjected to a step-down in light intensity from 1200 to 120 ft.c., and then given a

2 min. pulse of ^{14}C-uracil which was 'chased' for different times with excess ^{12}C-uracil. Phenol purified RNA was prepared from the bacteria, and characterized by sucrose gradient centrifugation. Figure 4 shows the sucrose gradient profiles of RNA from bacteria which were given a 2 min. pulse of ^{14}C-uracil, and then incubated for 30 sec. or 2, 10, or 20 min. with an excess of unlabelled uracil. The sedimentation patterns were similar when the bacteria were subjected to a step-down in light

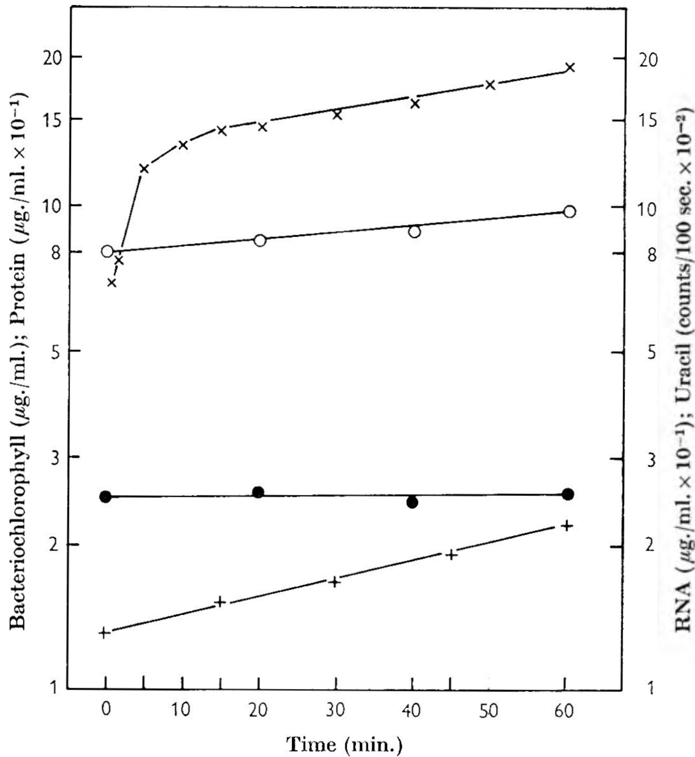


Fig. 3. Uracil incorporation into RNA during preferential bacteriochlorophyll formation under anaerobic conditions. *Rhodopseudomonas spheroides* M-25 was grown in 1200 ft.c. in 200 ml. SG medium containing 5 μg. uracil/ml. The culture was bubbled with a mixture of 5 % (v/v) CO_2 and 95 % (v/v) N_2. When the cells reached a concentration equivalent to about 100 μg. protein/ml. they were centrifuged and suspended in 200 ml. SG medium containing 5 μg. uracil/ml. and a total of 20 microcuries 2-^{14}C-uracil (3730 counts/100 sec./μg. uracil). The suspension was incubated in light at an intensity of 120 ft.c., and gassed as before. At the indicated times samples were removed for determination of bacteriochlorophyll (+—+), RNA (●—●), protein (○—○), radioactive uracil in cold-TCA washed cells from 1 ml. culture (×—×).

intensity from 1200 to 400 ft.c. or maintained at 1200 ft.c. It should be noted that *R. spheroides* is devoid of the usual 23S ribosomal RNA (Lessie, 1965). The first RNA to be labelled was unstable and sedimented differently from the bulk ribosomal and soluble RNAs. The sedimentation behaviour of this heterogeneous fraction of RNA was similar to that of m-RNA. Within 10 min. most of the label passed into the stable ribosomal and soluble RNA fractions.

As expected from the kinetics of labelling of phenol-purified RNA, label was found to enter rapidly into the ribonucleoprotein particles of bacteria subjected to a

decrease in light intensity. Figure 5 shows the kinetics of appearance of ^{14}-uracil in the 30S and 50S ribosomes of bacteria which were subjected to a step-down in light intensity from 1200 to 120 ft. c. Similar incorporation of label into ribosomes were obtained with bacteria transferred from 1200 to 400 ft.c. or bacteria maintained at 1200 ft.c. In all three instances the 30S ribosomes were labelled more rapidly than were the 50S ribosomes.

RNA synthesis in semi-aerobic cultures. Suspensions of aerobically grown *Rhodopseudomonas spheroides* subjected to a marked decrease in O_2 tension formed photopigments without significantly increasing their net content of RNA. The bacteria did, however, continue to incorporate ^{14}C-labelled uracil into cellular RNA (see

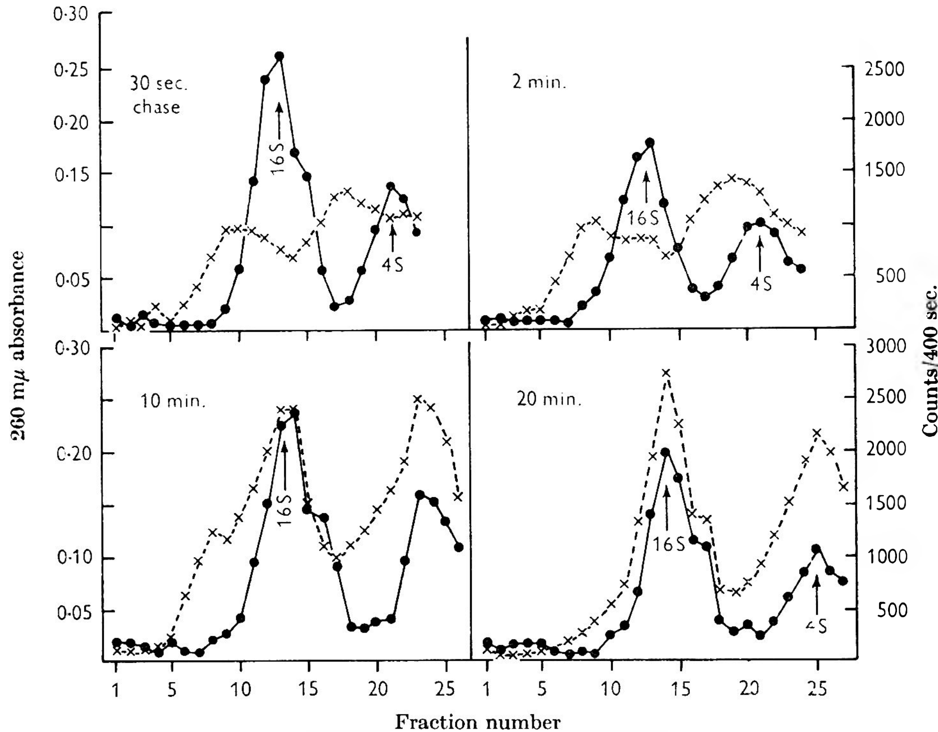


Fig. 4. RNA synthesized following a decrease in light intensity. *Rhodopseudomonas spheroides* was grown in 1200 ft.c. and transferred to 120 ft.c. as described in Fig. 3, except that transfer was to medium containing one μg. uracil/ml. and no ^{14}C-uracil. After 3 min. 20 μc. 2-^{14}C-uracil were added in one ml. (zero time). Two min. later 3·6 ml. 10^{-3} M-^{12}C-uracil were added (final uracil conc. 20 μg./ml.). At 30 sec. and at 20 min. 100 ml. volumes of culture were poured onto 50 g. crushed ice containing 0·75 ml. 2×10^{-1} M-NaN_3. The bacteria were centrifuged, washed twice with cold SG medium containing 10^{-3} M-NaN_3, and then frozen. An identical culture was given a two min. pulse of ^{14}C-uracil, but the bacteria were sampled, washed, and frozen after 2 min. and after 10 min. chase periods in the presence of excess ^{12}C-uracil. Crude ribosome extracts and phenol-purified RNA in 5×10^{-3} M-tris buffer (pH 7·4) and 10^{-4} M-$MgCl_2$ were prepared from thawed pulse-labelled bacteria combined with unlabelled bacteria from a 300 ml. culture (100 μg. protein/ml.) grown in 1200 ft.c. 0·2 ml. portions of the phenol-purified RNA preparations were centrifuged at 38,000 rev./min. for 5 hr. through 5 ml. 5–15% sucrose gradients containing 5×10^{-3} M-tris buffer (pH 7·4) and 10^{-4}M-$MgCl_2$. The bottoms of the tubes were punctured, and 5 drop fractions were collected. Extinction at 260 mμ (●—●) and radioactivities (x --- x) of each sample were determined.

Fig. 6). Pulse experiments with ^{14}C-uracil indicated that, during preferential photopigment synthesis and during the period of adaptation to form photopigments, label was incorporated into ribosomal and soluble RNAs after flowing through unstable RNA like that in Fig. 4.

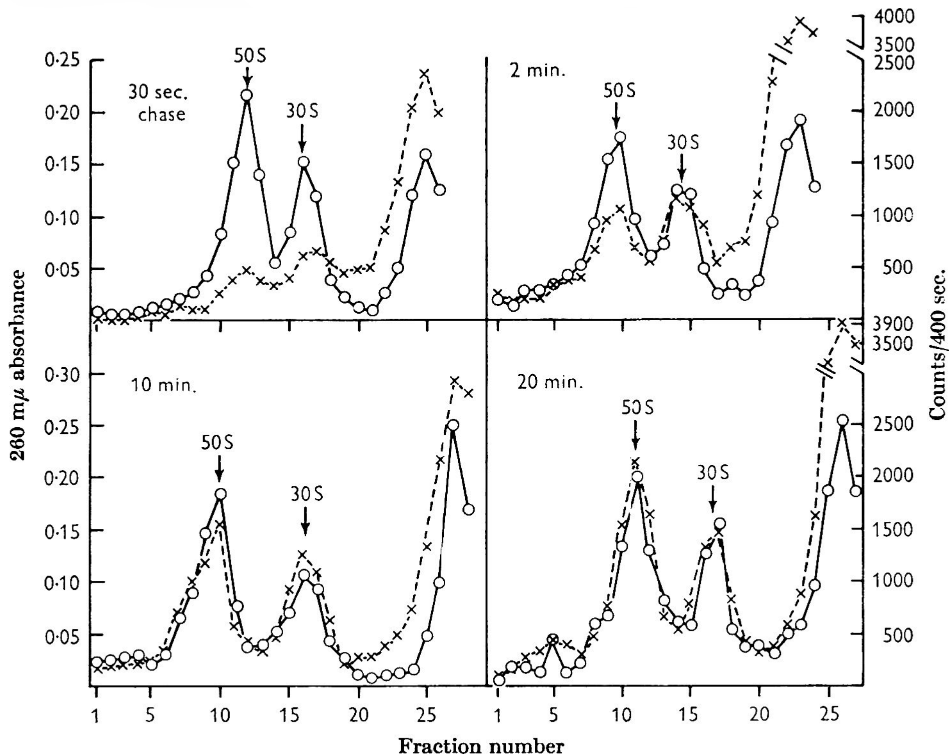


Fig. 5. Synthesis of ribosomes following a decrease in light intensity. 0·2 ml. samples of the crude ribosome extracts obtained from the samples described in Fig. 4 were centrifuged at 38,000 rev./min. for 2·5 hr through 5–10 % sucrose gradients containing 5×10^{-3} M-tris buffer (pH 7·4) and 10^{-4} M-$MgCl_2$. Samples were collected and their radioactivities (× --- ×) and 260 mμ extinctions (O—O) were determined.

Uracil requirement for bacteriochlorophyll synthesis. Aerobically grown *Rhodopseudomonas spheroides* incubated semi-aerobically without uracil formed no photopigments, nor did anaerobically grown bacteria deprived of uracil and subjected to a stepdown in light intensity.

DISCUSSION

The present results indicate that while preferentially synthesizing photopigment *Rhodopseudomonas spheroides* synthesizes all the usual classes of RNA, although more slowly than during balanced growth. There appears to be no striking change in nucleic acid metabolism (such as a greatly increased output of m-RNA) signalling a preferential rise in baeteriochlorophyll formation. However, the observation that *R. spheroides* strain M-25 did not form bacteriochlorophyll when its RNA synthesis was restricted by deprivation of uracil suggests that RNA synthesis is necessary for bacteriochlorophyll formation. These data are consistent with a role of RNA

synthesis in bacteriochlorophyll formation as already implied by the results of Sistrom (1963) and Bull & Lascelles (1963) which established that bacteriochlorophyll formation is coupled to protein synthesis. It is possible that the primary control of photopigment synthesis operates at the genetic level by regulating synthesis of specific informational RNA required to promote synthesis of some protein integrally involved in bacteriochlorophyll synthesis. Our results indicate that if such a control exists detection of the informational RNA involved would depend upon more sensitive technique than sucrose gradient resolution of the entire bacterial complement of RNA.

It has been shown that *Rhodopseudomonas spheroides* is devoid of the usual 23S ribosomal RNA (Lessie, 1965). The experiments reported here show that *R. spheroides* contains unstable RNA which sediments more rapidly than does 16S

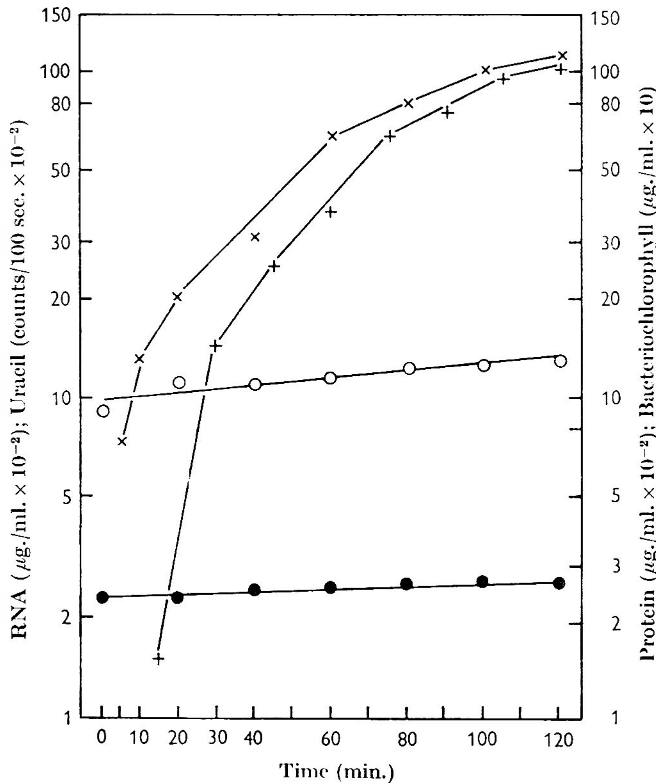


Fig. 6. Uracil incorporation into RNA during semi-aerobic incubation. *Rhodopseudomonas spheroides* was grown aerobically to a concentration equivalent to about 100 μg. protein/ml., centrifuged, and suspended to about 1 mg. protein/ml. in SG medium containing 20 μg. uracil/ml. Three 65 ml. volumes of the suspension were distributed into separate ⊥-tubes. 20 microcuries 2-^{14}C-uracil in one ml. were added to one of the tubes (2210 counts/100 sec./μg. uracil). All three tubes were shaken gently at 34° C. At 15 min. intervals 3 ml. samples for bacteriochlorophyll determination were taken alternatively from the two tubes without ^{14}C-uracil. At the indicated times 0·5 ml. samples of the culture with ^{14}C-uracil were pipetted into 4·5 ml. cold 5 % TCA. 4 ml. of the TCA-treated sample was assayed for RNA and for protein; the remainder was used to determine radioactivity in the cold-TCA insoluble cell fraction. ●—●, RNA; ○—○, protein; +—+, bacteriochlorophyll; ×—×, radioactive uracil in cold TCA-washed bacteria from one ml. culture.

ribosomal RNA. Two observations make it unlikely that this RNA represents a small pool of 23S ribosomal RNA. First, examination of the kinetics of incorporation of labelled uracil into RNA indicated that the RNA under discussion was labelled more rapidly than was 16S RNA. Secondly, under the same conditions 30S ribosomes were labelled more rapidly that 50S ribosomes. Since 16S RNA is presumed to comprise the RNA of 30S ribosomes, it is unlikely that 16S RNA would be labelled more slowly than RNA of 50S ribosomes. I presume that the RNA in question was not derived from 50S ribosomes.

I am grateful to Dr June Lascelles for her help and advice during this work. The author is a post-doctoral fellow of the United States Public Health Service. The work was aided by a grant to the Department from the Rockefeller Foundation.

REFERENCES

Bull, M. J. & Lascelles, J. (1963). The association of protein synthesis with formation of pigments in some photosynthetic bacteria. *Biochem. J.* **87**, 15.

Ceriotti, G. (1952). A microchemical determination of deoxyribonucleic acid. *J. biol. Chem.* **198**, 297.

Cohen-Bazire, G. & Kunisawa, R. (1963). The fine structure of *Rhodospirillum rubrum*. *J. cell. Biol.* **16**, 401.

Cohen-Bazire, G., Sistrom, W. R. & Stanier, R. Y. (1957). Kinetic studies of pigment synthesis by non-sulphur purple bacteria. *J. cell. comp. Physiol.* **49**, 25.

Gibson, K. D., Neuberger, A. & Tait, G. H. (1963). Studies on the biosynthesis of porphyrin and bacteriochlorophyll by *Rhodopseudomonas spheroides* 4. S-adenosylmethionine magnesium protoporphyrin methyl transferase. *Biochem. J.* **88**, 325.

Lascelles, J. (1959). Adaptation to form bacteriochlorophyll in *Rhodopseudomonas spheroides*: changes in activity of enzymes concerned in pyrrole synthesis. *Biochem. J.* **72**, 508.

Lessie, T. G. (1965). The atypical ribosomal RNA complement of *Rhodopseudomonas spheroides*. *J. gen. Microbiol.* **39**, 311.

Lowry, O. H., Rosebrough, N. J., Farn, A. L. & Randall, R. J. (1951). Protein measurement with the Folin phenol reagent. *J. biol. Chem.* **193**, 265.

Schneider, W. C. (1945). Phosphorous compounds in animal tissues. 1. Extraction and estimation of desoxypentose nucleic acid and of pentose nucleic acid. *J. biol. Chem.* **161**, 293.

Sistrom, W. R. (1963). Observations on the relationship between formation of photopigments and the synthesis of protein in *Rhodopseudomonas spheroides*. *J. gen. Microbiol.* **28**, 599.

J. gen. Microbiol. (1965), **41**, 47–55
Printed in Great Britain

Complementation of Non-flagellate Salmonella Mutants

By T. M. JOYS* and B. A. D. STOCKER

Guinness–Lister Research Unit, Lister Institute of Preventive Medicine, Chelsea Bridge Road, London, S.W. 1

(*Received* 4 *March* 1965)

SUMMARY

Selection for resistance to phage χ led to the isolation from *Salmonella typhimurium* strain LT2 of fifteen non-flagellate (*fla*⁻) mutants due to spontaneous mutation at sites such that *H1* could be co-transduced with *fla*⁺. Treatment with phage P22 grown on LT2 *fla*⁺ evoked swarms, i.e. motile (*fla*⁺) transductant clones, from all fifteen *fla*⁻ mutants and from a *fla*⁻ strain of *S. paratyphi* B; and also evoked trails, i.e. *fla*⁺ abortive transductants, from all these mutants except four. When the *fla*⁻ strains were crossed with each other by transduction all pairs yielded *fla*⁺ clones, which indicated that none of the mutated sites was identical with, or overlapped, any other. In most pairs the appearance of trails showed that abortive transductants, of constitution *fla1*⁻*fla2*⁺/*fla1*⁺*fla2*⁻, were flagellate, as a result of complementation. The mutants fell into five groups, such that mutants of any one group complemented mutants of all other groups; these groups perhaps correspond to five *fla* genes. Some pairs of the ten strains of group A complemented, perhaps through intragenic complementation.

INTRODUCTION

In Salmonella *fla*⁻ mutations which cause absence of flagella map at sites distinct from the loci *H1* and *H2*, which control the serological character of, respectively, the phase-1 and phase-2 flagellar antigens (Lederberg & Edwards, 1953). Many *fla*⁻ mutants, when treated with phage P22 lysates of *fla*⁺ strains differing from them in respect of their *H1* allele, yield a proportion of swarms (*fla*⁺ transductant clones) with the phase-1 flagellar antigen of the donor strain. It is inferred that the *fla* sites concerned are closely linked to *H1*, so that the *fla*⁺ and *H1* genes of the donor can be simultaneously transduced by a single phage particle. However, even these *fla*⁻ sites map outside *H1*, which is believed to be the structural gene for the phase-1 flagellar protein (flagellin) constituting phase-1 flagella (McDonough, 1965). Moreover in diphasic strains *fla*⁻ mutants form neither phase-1 nor phase-2 flagella though the *H2* locus, believed to be the structural gene for phase-2 flagellin, is remote from *H1* on the chromosome (Smith & Stocker, 1962). The *fla*⁺ genes therefore play some role in the formation of flagella other than in the determination of the structure of flagellin, their only known component. Most complementation studies in micro-organisms have been made with auxotrophic or non-fermenting mutants, and have concerned complementation of mutated forms of genes which

* Present address: Department of Bacteriology, University of Oregon Medical School, Portland, Oregon, 97201, U.S.A.

determine the structure of enzymically active proteins. The availability of many *fla*⁻ mutants permits a study of complementation in genes which determine a different sort of character. It is easy to test whether, in cells which are diploid for the *H 1* region, pairs of non-identical *fla*⁻ mutants complement each other to produce the wild-type (*fla*⁺) phenotype. This can be done by looking for 'trails', the linear groups of micro-colonies which mark the path through a semi-solid medium of bacteria of a non-motile strain made motile by abortive transduction (Stocker, Zinder & Lederberg, 1953; Stocker, 1956; Lederberg, 1956). Two reports (Iino, 1958; Iino & Enomoto, 1962) recorded complementation of some but not all pairs of *fla*⁻ mutants, the results defining several complementation groups of which three at least are linked to *H1*. An investigation of mutation of the *H1* allele, *H1-i*, resulting in altered forms of antigen *i* of *S. typhimurium* strain LT2 (Joys, 1961; Joys & Stocker, 1963) necessitated the isolation, in each of several such mutants, of one or more *fla*⁻ mutants at sites co-transducible with *H1*. We here report the behaviour in complementation tests of eleven *fla*⁻ mutants isolated for this purpose, of four other LT2 *fla*⁻ mutants isolated in this laboratory, and of one *fla*⁻ strain of *S. paratyphi* B. The LT2 *fla*⁻ strains were obtained as spontaneous mutants by selection with phage χ, which attacks various Salmonella species only when they are motile (Sertic & Boulgakov, 1936; Meynell, 1961).

METHODS

Bacterial strains. The parent *Salmonella typhimurium* LT2 *fla*⁺ stocks used comprised: LT2 *athA-4 pro-401* (*ath*, *pro*, *ade*, *met*, *try* indicate loci mutation at which results in auxotrophy for, respectively, adenine + thiamine, proline, adenine (or other purine), methionine, tryptophan); seven mutants, indicated M5, M6, M7, etc., in which the wild-type phase 1 antigen *i* of *S. typhimurium* had been changed by mutation to *iM5*, *iM6*, *iM7*, etc. (Joys, 1961; Joys & Stocker, 1963), all obtained in LT2 *adeC-7 proA-46*; lines SD7 and SD14, derived from LT2 *metA-22 tryB-2* by transductional replacement of, respectively, *H1-i*, and successively of this gene and of gene *H2-1, 2*, by the corresponding alleles, *H1-b* and *H2-e,n,x*, of *S. abony* strain SW803 (Spicer & Datta, 1959). The non-flagellated strain of *S. paratyphi* B, SW543, was originally isolated as such; its *fla*⁻ site is closely linked to *H1* (Stocker *et al.* 1953).

Isolation of non-flagellate mutants. Phage χ was propagated and titrated on *Salmonella typhimurium* strain Q1 (Boyd & Bidwell, 1957). A *fla*⁺ parent strain was first passaged through semi-solid medium (Stocker *et al.* 1953) to ensure good motility, then streaked out. A portion of a broth culture, inoculated with a discrete colony in the desired antigenic phase and incubated at 37° until just turbid, was spread on the surface of a deep tryptic beef-digest nutrient agar plate. Drops of a suspension of phage χ (about 10^6 plaque-forming units/ml.) were applied and allowed to dry, and the plate incubated at 23°. This procedure was necessary to get sufficient lysis since *S. typhimurium* strain LT2 is only moderately sensitive to this phage (Joys, 1961; Meynell, 1961). Discrete colonies which appeared in the area of lysis were picked. Clones which were non-motile in broth culture were tested by slide agglutination, to ensure they were *fla*⁻ rather than *mot*⁻ (flagellate but not-motile) mutants, then tested for rate of reversion to *fla*⁺ by streaking about 10^{10}

bacteria on semi-solid medium. Mutants which gave swarms after overnight incubation at 37° were discarded. The remaining, stable *fla*$^-$ mutants were tested to see whether they gave any swarms with the phase-1 flagellar antigen of the donor when treated with a phage P22 lysate of a strain with a different phase-1 antigen. Mutants which met this test were retained and assigned *fla* mutation numbers starting from *fla-50*.

Transduction methods. Phage P22 (Zinder & Lederberg, 1952) was propagated by the soft-agar layer method on strains to be used as donors; lysates were titrated on strain Q1 (Boyd & Bidwell, 1957). For transduction an overnight shaken broth culture (about 10^9 bacteria/ml.) of a *fla*$^-$ strain to be used as recipient was mixed with a phage P22 lysate of the donor strain (generally another *fla*$^-$ mutant) at a multiplicity of about 10; after 20 min. at 37°, 10^{-1}, 10^{-2}, 10^{-3}, 10^{-4} dilutions were made in broth and one standard drop (about 0·02 ml.) of each dilution was streaked on each of three plates of semi-solid motility medium, poured 1 cm. deep in 5 cm. diameter Petri dishes for the easier observation of trails. The plates were incubated at 37°. The production of swarms of spreading growth, and their absence from control plates inoculated with untreated recipient bacteria, then indicated the production of *fla*$^+$ recombinants. In *fla*$^-$ → *fla*$^-$ crosses the presence of trails, best seen with inocula dilute enough to yield few or no swarms, and their absence from control plates, indicated complementation of the two *fla*$^-$ mutants. To test for linkage of the *fla* site of a mutant to *H1*, the same method was used except that for *fla*$^-$ strains with the unexpressed phase-1 antigen *i* (or *iM5*, *iM6*, etc.) the donor strain used was SD7, that is, LT2 with the phase-1 antigen *b*. Drops of the transduction mixture were streaked on semi-solid medium without serum and on semi-solid medium containing sufficient anti-*i* serum to prevent the spread of *i* swarms, and anti-*1,2* serum to prevent the spread of any phase-2 swarms. For tests on *fla*$^-$ mutants with the unexpressed phase-1 antigen *b*, the donor had the phase-1 antigen *iM10*, and anti-*b* serum was used instead of anti-*i* serum. It was necessary to ensure that the recipient strain was in latent phase 1 (see Stocker *et al.* 1953). The appearance of swarms on the serum-containing plates indicated co-transduction of *H1* with *fla*$^+$ from the donor; this was confirmed by slide agglutination after streaking out. In tests on some of the *fla*$^-$ mutants comparison of the lowest dilutions which yielded swarms on the semi-solid plates with serum or without serum gave an approximate estimate of the proportion of *fla*$^+$ transductants with the phase-1 antigen of the donor, i.e. of the rate of co-transduction of *H1* with *fla*$^+$.

RESULTS

The properties of the fifteen LT2 *fla*$^-$ mutants isolated by selection with χ phage and of the *fla*$^-$ strain of *Salmonella paratyphi* B and the characters of the motile parent strains are recorded in Table 1. Seven of the *fla*$^-$ mutants on occasion yielded motile revertants; but not at a frequency high enough to interfere with the transduction experiments. The rate of co-transduction of *H1* with the *fla* loci, i.e. the estimated proportion of swarms manifesting the phase-1 antigen of the donor strain, ranged, in those mutants in which it was determined, from about 0·5 to $< 0{\cdot}01$ $> 0{\cdot}001$. All sixteen *fla*$^-$ strains on treatment with a lysate of a wild-type donor gave swarms, i.e. *fla*$^+$ transductants (Table 2). Twelve of them also gave abundant

Table 1. *Properties and origin of non-flagellate mutants*

fla⁻ mutant					*fla*⁺ parent				
Mutant no.	Reversion	Proportion donor-type swarms	Trails (fla^+ donor)	Complementation group	Strain no.	$H1$	$H2$	Other characters	
fla 50	−	0·1	++	B	M 5	*i*M 5			
fla 59	+	n.d.	++	B	M 5	*i*M 5			
fla 52	−	0·3	−	A	M 6	*i*M 6			
fla 60	+	n.d.	++	E	M 6	*i*M 6			
fla 61	−	n.d.	−	A	M 6	*i*M 6			
fla 53	+	< 0·01	−	C	M 7	*i*M 7	1, 2	*adeC*-7	*proA*-46
fla 54	−	0·01	++	C	M 9	*i*M 9			
fla 56	−	0·2	++	A	M 10	*i*M 10			
fla 57	−	0·1	++	A	M 10	*i*M 10			
fla 55	−	0·5	−	A	M 11	*i*M 11			
fla 58	−	0·1	++	D	M 12	*i*M 12			
fla 68	+	< 0·01	++	A	SL 482	*i*	1, 2	*athA*-4	*pro*-401
fla 65	−	n.d.	++	A	SD 7	*b*	1, 2	*metA*-22	*tryB*-2
fla 66	+	n.d.	++	A	SD 14	*b*	*e*, *n*, *x*	*metA*-22	*tryB*-2
fla 67	+	n.d.	++	A	SD 14	*b*	*e*, *n*, *x*	*metA*-22	*tryB*-2
fla 69	+	0·5	++	A	*	*b*	—	*S. paratyphi* B	

Proportion donor-type swarms: proportion of swarms (*fla*⁺ transductants) with phase-1 antigen of donor—i.e. antigen *b* of strain SD 7 for all *fla*⁻ mutants with *H1-i*, or a mutant form of it; or antigen *i* M 10 of strain M 10, for *fla*⁻ mutants with *H1-b*.

n.d.: not determined.

Trails: ++, trails from one drop of mixture of *fla*⁻ mutant with wild-type (*fla*⁺) lysate; −, no trails from undiluted mixture.

* *fla* 69 is the *fla* no. assigned to the naturally occurring non-flagellate *S. paratyphi* B strain SW 543 (Stocker, Zinder & Lederberg, 1953). All the other *fla*⁻ strains were spontaneous mutants in LT2 lines, selected by use of phage.

Table 2. *Complementation and recombination between pairs of fla⁻ mutants*

Recip. *fla* no.	w.t. (*fla*+)	Donor *fla*⁻ mutant no: 66	69	65	67	68	56	52	55	61	57	50	59	53	54	58	60	Group
66	t, s	(−, −)	−, s	−, s	−, s	−, s	−, s	−, s	−, s	−, s	−, s	t, s	t, s	t, s	t, s	t, s	t, s	A
69	t, s	−, s	(−, −)	−, s	−, s	−, s	−, s	−, s	−, s	−, s	−, s	t, s	t, s	t, s	t, s	t, s	t, s	A
65	t, s	−, s	−, s	(−, −)	−, s	−, s	−, s	−, s	−, s	−, s	t, s	t, s	t, s	t, s	t, s	t, s	t, s	A
67	t, s	−, s	−, s	−, s	(−, −)	−, s	−, s	−, s	−, s	−, s	t, s	t, s	t, s	t, s	t, s	t, s	t, s	A
68	t, s	−, s	−, s	−, s	−, s	(−, −)	−, s	−, s	−, s	−, s	t, s	t, s	t, s	t, s	t, s	t, s	t, s	A
56	t, s	−, s	−, s	−, s	−, s	−, s	(−, −)	t, s	t, s	t, s	t, s	t, s	t, s	t, s	t, s	t, s	t, s	A
52*	−, s	−, s	−, s	−, s	−, s	−, s	−, s	(−, −)	−, s	−, s	−, s	−, s	−, s	−, s	−, s	−, s	−, s	A
55*	−, s	−, s	−, s	−, s	−, s	−, s	−, s	−, s	(−, −)	−, s	−, s	−, s	−, s	−, s	−, s	−, s	−, s	A
61*	−, s	−, s	−, s	−, s	−, s	−, s	−, s	−, s	−, s	(−, −)	−, s	−, s	−, s	−, s	−, s	−, s	−, s	A
57	t, s	−, s	−, s	t, s	t, s	t, s	t, s	t, s	t, s	t, s	(−, −)	t, s	t, s	t, s	t, s	t, s	t, s	A
50	t, s	t, s	t, s	t, s	t, s	t, s	t, s	t, s	t, s	t, s	t, s	(−, −)	−, s	t, s	t, s	t, s	t, s	B
59	t, s	t, s	t, s	t, s	t, s	t, s	t, s	t, s	t, s	t, s	t, s	−, s	(−, −)	t, s	t, s	t, s	t, s	B
53*	−, s	−, s	−, s	−, s	−, s	−, s	−, s	−, s	−, s	−, s	−, s	−, s	−, s	(−, −)	−, s	−, s	−, s	C
54	t, s	t, s	t, s	t, s	t, s	t, s	t, s	t, s	t, s	t, s	t, s	t, s	t, s	−, s	(−, −)	t, s	t, s	C
58	t, s	t, s	t, s	t, s	t, s	t, s	t, s	t, s	t, s	t, s	t, s	t, s	t, s	t, s	t, s	(−, −)	t, s	D
60	t, s	t, s	t, s	t, s	t, s	t, s	t, s	t, s	t, s	t, s	t, s	t, s	t, s	t, s	t, s	t, s	(−, −)	E
Group		A										B		C		D	E	

t, s: trails and swarms.
−, s: no trails, swarms.
−, −: no trails, no swarms.
( ): selfed cross.
* : *fla* mutants not forming trails even with *fla*+ donor.
Group: complementation group.

trails, i.e. trails even from an inoculum of a 10^{-2} dilution of the transductant mixture (Table 2). Four mutants (M6 *fla 52*, M6 *fla 61*, M7 *fla 53*, M11 *fla 55*) gave no trails even from inocula of undiluted transduction mixture. We considered the possibility that these four mutants might not form trails because they carried not only *fla*$^-$ genes but also modifier genes, not co-transducible with the *fla–H1* region, resulting in poor motility even when a *fla*$^+$ gene was introduced. A comparison of the rate of spread of *fla*$^+$ transductional swarms derived from the *fla*$^-$ mutants which did not form trails, with the rate of spread of swarms from two trail-forming mutants (M5 *fla 50*, M10 *fla 57*), did not support this hypothesis. Swarms from two of the non-trail-forming mutants (M6 *fla 52*, M11 *fla 55*) spread more slowly than did the control swarms, but those from the other two non-trail-forming mutants spread faster than the control swarms. We do not know why these four strains did not give trails. Evidently no inference about complementation can be drawn from a test with a non-trail-forming recipient strain.

Each of the sixteen *fla*$^-$ strains was tested with phage P22 lysates of every one of the *fla*$^-$ strains (Table 2). Every combination except the 'selfed' crosses (e.g. M10 *fla 58* treated with a lysate of M10 *fla 58*) gave swarms. It therefore appears that none of the sixteen *fla*$^-$ mutations coincides with, or overlaps, the site of any other. The selfed combinations did not produce trails, as was to be expected. The four mutants which gave no trails on treatment with wild-type lysate also did not give trails on treatment with any other lysate; but each of them when used as a donor evoked trails from several other *fla*$^-$ strains. Otherwise every pair of *fla*$^-$ mutants gave the same result (swarms and trails = complementation; or swarms but no trails = no complementation) when tested in the two possible directions.

The complementation results divide the mutants into several complementation or cistronic groups: (i) *fla-60* complements all *fla* mutants except itself, and constitutes group E; (ii) *fla-58* behaves similarly and forms group D; (iii) *fla-50* and *fla-59* do not complement each other but complement all other mutants, and form group B; (iv) *fla-54* and the non-trail-forming *fla-53* behave similarly and form group C. The ten remaining mutants, which complement mutants of groups E, D, B and C, form group A, with a complex pattern of complementation with each other which may be represented:

Group A i	66 and 69
Group A ii	57
Group A iii	65, 67 and 68
Group A iv	56
Group A v	52*, 55* and 61*

where overlapping of the bars representing groups of mutants indicate that they fail to complement—or are deficient in the same 'unit of function', and non-trail-forming mutants are indicated by asterisks. Mutants *fla-66* and *fla-69* (the *S. paratyphi* B o strain) do not complement any of the ten mutants of group A and we term them group A i. *fla-57* complements all A strains except those of group A i and we assign it to a group A ii. Mutants *fla-65*, *fla-67* and *fla-68* complement *fla-57* of group A ii, but no other group A mutant; these three mutants thus form group A iii. Group A iv comprises mutant *fla-56*, which complements group A ii and also the three

remaining strains of the A group, *fla-52**, *fla-55** and *fla-61**. These last three strains behave alike in that when tested as donors they complement the A ii and A iv mutants but no others of the A group. As none of the three produces trails they cannot be tested for ability to complement each other; we tentatively group them as group A v.

DISCUSSION

In 12 of the 16 *fla* strains examined the production of trails, i.e. motile abortive fla^+ transductants, showed that the fla^- lesion was recessive to fla^+. Our experiments on the rate of spread of fla^+ derivatives of the four non-trail-forming fla^- mutants gave inconclusive results; despite this we think the most plausible explanation of their character is that they carry unstable modifiers, at loci not co-transducible with *H1*, causing poor development of flagella, so that abortive fla^+ transductants are insufficiently motile to travel through semi-solid medium. If the chromosome fragments involved in transduction by phage P22 are indeed of constant composition (Ozeki, 1959) the ability of lysates of the non-trail-forming strains to evoke trails from other fla^- mutants (Table 2) shows that these four fla^- mutations are not dominant to their fla^+ alleles. However, recent experiments (Pearce & Stocker, 1965; Roth & Hartman, 1965) show some heterogeneity in composition of transduced chromosome fragments, and the hypothesis of dominant fla^- mutation is thus not entirely disproved by our observation.

Drs Iino and Enomoto in a communication submitted to this Journal while our paper was in proof describe five complimentation groups amongs their *H1*-linked *fla* mutants of *Salmonella typhimurium* and *S. abortus-equi*, and show by crosses with representatives of our groups that four of the five correspond to our groups, A, B, C and D; our group E was not represented amongst their mutants, nor their group J amongst ours. Our five complementation groups may represent five distinct *fla* genes; or, less probably, reflect to some extent intra-genic complementation. If all the group A mutants are mutated in a *fla*A gene, their *fla* sites should map adjacent to each other; but in our tentative map (Joys & Stocker, 1963) the locus *H1* separates *fla-57* from *fla-52* and *fla-55*, though all these three mutants fall in complementation group A. However, subsequent experiments (Mrs Ursula Pearce & Stocker, to be published) suggest that *fla-52* and *fla-55* were incorrectly mapped.

Some *fla* mutations map at a locus between *gal* and *try*, that is, far from *H1* (Smith & Stocker, 1962; Iino & Lederberg, 1964). The production of flagella therefore requires the functioning of at least two genes, and, if each complementation group represents a distinct gene, of at least six genes, distinct from *H1* and *H2*, the structural genes for the alternative forms of flagellin, the only known structural component of Salmonella flagella. Several possible roles for such genes can be envisaged, and most of them have been discussed by Iino & Lederberg (1964). If flagella contain a second structural component, so far undetected and serologically inactive, the gene or genes for its production would presumably have the properties of *fla* genes. The synthesis of flagellin or of flagella may involve special structures: for instance, a polymerization enzyme or a special sort of ribosome ('flagellosome', Iino & Lederberg, 1964); the genes determining their protein or RNA components would then have the properties of *fla* genes. It is also possible that one or more *fla* loci are concerned in regulating the production of flagellin, probably by regulating

the production of $H1$ and $H2$ messenger RNA. The existence of some such regulatory system is suggested by the effect of some environments in suppressing production of new flagella (e.g. Quadling & Stocker, 1962) and by the absence of cytoplasmic flagellin in most *fla*⁻ mutants (Iino & Enomoto, 1962). By analogy with a class of dominant *lac*⁻ mutant (Wilson *et al.* 1964) a *fla*⁻ mutation at a gene whose function was the production of a repressor of synthesis of flagellin would be expected to be dominant to its *fla*⁺ allele. There is at present no good evidence for the existence of dominant *fla*⁻ mutants. However, recessive mutations at regulator loci resulting in failure to produce the regulated protein have been reported in other systems (Garen & Echols, 1962; Englesberg *et al.* 1964). A *fla* gene of this sort might produce an 'internal inducer' for production of flagellin (Iino & Lederberg, 1964).

The work forms part of the London University Ph.D. Thesis of T. M. Joys. We thank Drs T. Iino and M. Enomoto for exchange of information, and for their courteous agreement to adopt our nomeclature for *fla* complementation groups A-E, even though this involves an interchange of the designations B and C used in an earlier review paper in Japanese (Iino, 1964).

REFERENCES

BOYD, J. S. K. & BIDWELL, D. E. (1957). The type A phages of *Salmonella typhimurium*: identification by a standard cross-immunity test. *J. gen. Microbiol.* **16**, 217.

ENGLESBERG, E., IRR, J. & POWER, J. (1964). Evidence for a new type of regulatory gene in the L-arabinose gene enzyme complex. *Bact. Proc.* p. 19.

GAREN, A. & ECHOLS, H. (1962). Genetic control of induction of alkaline phosphatase synthesis in *E. coli*. *Proc. natn. Acad. Sci., U.S.A.* **48**, 1938.

IINO, T. (1958). Cistron test of motility genes in Salmonella. *Natn. Inst. Genetics, Japan, Annu. Rep.* **9**, 96.

IINO, T. (1964). Genetical studies of Salmonella flagella. *Jap. J. Genet.* **39**, 313 (text in Japanese, English summary and tables).

IINO, T. & ENOMOTO, M. (1962). Further studies on non-flagellate mutants of Salmonella. *Natn. Inst. Genetics Japan, Annu. Rep.* **13**, 73.

IINO, T. & LEDERBERG, J. (1964). Genetics of Salmonella. In *The World Problem of Salmonellosis*, Ed. by E. van Oye, *Monographiae biol.* **13**, 111.

JOYS, T. M. (1961). *Mutation of flagellar antigen i of Salmonella typhimurium.* Ph.D. Thesis, University of London.

JOYS, T. M. & STOCKER, B. A. D. (1963). Mutation and recombination of flagellar antigen *i* of *Salmonella typhimurium*. *Nature, Lond.* **197**, 413.

LEDERBERG, J. (1956). Linear inheritance in transductional clones. *Genetics*, **41**, 845.

LEDERBERG, J. & EDWARDS, P. R. (1953). Serotypic recombination in Salmonella. *J. Immunol.* **71**, 232.

McDONOUGH, M. W. (1965). Amino acid composition of antigenically distinct Salmonella flagellar proteins. *J. molec. Biol.* **12**, 342. *Proc. 6th Int. Congr. Biochem.* p. 169.

MEYNELL, E. W. (1961). A phage, $\phi\chi$, which attacks motile bacteria. *J. gen. Microbiol.* **25**, 253.

OZEKI, H. (1959). Chromosome fragments participating in transduction in *Salmonella typhimurium*. *Genetics*, **44**, 457.

PEARCE, U. & STOCKER, B. A. D. (1965). Variation in composition of chromosome fragments transduced by phage P22. *Virology* (submitted).

QUADLING, C. & STOCKER, B. A. D. (1962). An environmentally induced transition from the flagellated to the non-flagellated state in *Salmonella typhimurium*: the fate of parental flagella at cell division. *J. gen. Microbiol.* **28**, 257.

ROTH, J. R. & HARTMAN, P. E. (1965). Variation in P22 transducing particles. *Virology* (submitted).

SERTIC, V. & BOULGAKOV, N. A. (1936). Bactériophages spécifiques pour des variétés bactériennes flagellées. *C.r. hebd. Séanc. Soc. Biol., Paris*, **123**, 887.

SMITH, S. M. & STOCKER, B. A. D. (1962). Colicinogeny and recombination. *Br. med. Bull.* **18**, 46.

SPICER, C. C. & DATTA, N. (1959). Reversion of transduced antigenic characters in *Salmonella typhimurium*. *J. gen. Microbiol.* **20**, 136.

STOCKER, B. A. D. (1956). Abortive transduction of motility in Salmonella: a non-replicated gene transmitted through many generations to a single descendant. *J. gen. Microbiol.* **15**, 575.

STOCKER, B. A. D., ZINDER, N. D. & LEDERBERG, J. (1953). Transduction of flagellar characters in Salmonella. *J. gen. Microbiol.* **9**, 410.

WILSON, C., PERRIN, D., COHN, M., JACOB, F. & MONOD, J. (1964). Non-inducible mutants of the regulator gene in the 'lactose' system of *Escherichia coli*. *J. molec. Biol.* **8**, 582.

ZINDER, N. D. & LEDERBERG, J. (1952). Genetic exchange in Salmonella *J. Bact.* **64**, 679.

J. gen. Microbiol. (1965), **41**, 57–65
Printed in Great Britain

Inheritance of the O Antigens of Salmonella Groups B and D

By P. HELENA MÄKELÄ

Guinness-Lister Research Unit, Lister Institute of Preventive Medicine, Chelsea Bridge Road, London, S.W. 1 and Department of Serology and Bacteriology, University of Helsinki, Helsinki, Finland

(*Received* 9 *March* 1965)

SUMMARY

The inheritance of the O antigens of Salmonella was studied by $Hfr \times F^-$ crosses of strains of groups B (antigens 4,5,12) and D (antigens 9,12). The partial antigens 4 and 9 behaved as if determined by allelic loci, in the sense that all recombinants had either antigen 4 or antigen 9; never both or neither of them. The *O–4/9* locus thus defined was rather closely linked to *his*, the locus for histidine biosynthesis. The gene *O–5* determining the presence or absence of partial antigen 5 (an O-acetyl group on a galactose unit) in group B was very probably introduced into a number of 9,12 recombinants. No antigen 5 appeared. The *O–5* locus was also found to be linked to *O–4/9* and *his* but the linkage was less close than that between *O–4/9* and *his*. It is suggested that the loci *O–4/9* and possibly *O–5* are part of a large *O*-locus concerned with the synthesis of the specific side-chains of the somatic lipopolysaccharide, which perhaps includes the *rou-B* locus of Subbaiah & Stocker (1964).

INTRODUCTION

The specificity of the various somatic or O antigens of enteric bacteria, e.g. Salmonella, resides in a lipopolysaccharide (LPS) component of the cell wall. More precisely, the structures responsible for the specificity are relatively short repeating sequences, 'repeat units', of monosaccharides which constitute the 0-specific part of polysaccharide side-chains (Staub, 1964; Robbins & Uchida, 1962). The innermost portions of the side-chains and the backbone to which these are attached form a 'core' probably common to all Salmonella species, irrespective of their antigenic character (Kauffmann, Krüger, Lüderitz & Westphal, 1961; Lüderitz, Beckmann & Westphal, 1964). The main features of this structure are apparent from the diagram of Fig. 1.

A number of different mutations are known that result in a defective lipopolysaccharide, all bacteriologically recognized as rough (R) forms and apparently possessing a more or less complete core but not the specific parts of the side-chains. Of special interest among them are *rou-B* mutants whose lipopolysaccharides have the antigenic type called R_{II} (Subbaiah & Stocker, 1964: Beckmann, Subbaiah & Stocker, 1964; Beckman, Lüderitz & Westphal, 1964). These seem to have the complete core, with *N*-acetylglucosamine as the terminal monosaccharide unit (Osborn *et al.* 1964). Different enzymic defects can result in this same phenotype: defective rhamnose synthesis in one case, unknown defects in many others (Nikaido,

Nikaido, Subbaiah & Stocker, 1964). Since the *rou-B* mutants are by definition genetically linked to *his*, the locus of histidine biosynthesis, it is likely that they occur in a perhaps large region of the chromosome which is concerned with the synthesis of the O-specific part of the side-chains. This region could reasonably be called an *O* locus. Earlier, a locus controlling the specificity of the O antigen in *Escherichia coli* was mapped near *his* (Ørskov & Ørskov, 1962); this could well be the same locus.

```
                                                              Lipid
                                                                :
(Repeat unit comprising  ) → GNac → Glc → Gal → Glc → (Hept, KDO, P)
(3 to 5 monosaccharides  )                |          |
                          n               └── Gal. ──┘
 ____________________________________________________   ____________
                     Sidechain                             Backbone
 _________________________   ___________________________________________
 Specific part of the         R_II (rough) LPS = the complete core
      sidechain
```

GNac = N-acetyl-D-glucosamine; Glc = D-glucose; Gal = D-galactose; Hept = L-glycero-D-mannoheptose; KDO = 2-keto-3-deoxyoctonic acid; P = phosphate; LPS = lipopolysaccharide.

Fig. 1. A schematic representation of the somatic lipopolysaccharide of Salmonella (mainly according to Osborn *et al.* 1964). The structure of some O repeat units is shown in Fig. 3.

Serologically, each specific O antigen is a composite of various partial antigens (Kauffmann, 1954). These partial antigens represent different aspects of the same lipopolysaccharide molecule (Furth & Landsteiner, 1929; Staub & Pon, 1956; Lüderitz, O'Neill & Westphal, 1960), and possibly of the same repeating unit (Robbins & Uchida, 1962; Uchida, Robbins & Luria, 1963). The antibodies used for identifying the partial antigens recognize a structure definitely larger than a monosaccharide, and probably of the order of a tri- or tetra-saccharide. However, the non-reducing mono- and di-saccharides at the ends of side-chains, and/or their branches, play a prominent role in the specificity (Staub, Tinelli, Lüderitz & Westphal, 1959; Staub, 1964).

The present work was started in the hope of eventually clarifying the genetic determination of the specific side-chains of the somatic lipopolysaccharide. *Salmonella* species seemed to offer several advantages over *Escherichia coli*, even though the work was started with the latter (Ørskov & Ørskov, 1962). The Salmonella antigens have been more exhaustively classified both serologically (Kauffmann, 1954) and chemically (Kauffmann, Lüderitz, Stierlin & Westphal, 1960; Uchida *et al.* 1963; Staub, 1964). The usual absence of capsular antigens in Salmonella organisms makes them technically easier to analyse. The recent development of an effective $Hfr^- \rightarrow F^-$ conjugation system permitting inter-species and inter-O-group crosses in Salmonella was a necessary prerequisite for this genetic analysis (Mäkelä, 1963). Two questions seemed to be of special interest: (1) whether one would find allelic genes concerned with the determination of the alternative specificities of polysaccharide structures; (2) to what extent the partial antigens would be independently determined, e.g. whether it would be possible to synthesize new combinations thereof?

METHODS

Bacterial strains. Those used came from the author's collection in Helsinki (SH and SW strains) or from the collection of the Guinness–Lister Research Unit at the Lister Institute, Chelsea Bridge Road, London, S.W. (SL strains).

Group B (4, 5, 12) bacteria:

SW 1444. *Salmonella abony Hfr* met^- aro^- *str-r H1-b H2-e,n,x*, which is identical with sw 1391 of Mäkelä (1963).

SL 803. *S. typhimurium LT-2* F^- *hisD37*$^-$ *athA* 2^- *str-r H1-i H2-1,2.*

SH 142. *S. typhimurium LT-2* F^- his^- phe^- *H1-i H2-1,2.*

Group D (9, 12) bacteria:

SH 673. *Salmonella enteritidis F* 59^+ try^- *H1-g,m.* The *F'* episome, *F 59*, converts bacteria carrying it into donors of the same type as SW 1444 (Mäkelä & Ziegler unpublished).

SH 674. *S. enteritidis* F^- his^- try^- *H1-g,m.*

The location of markers and origins and directions of chromosome transfer of the donor (*Hfr* and *F59*$^+$) strains, as well as abbreviations used are given in Fig. 2. F^- or = female recipient strain.

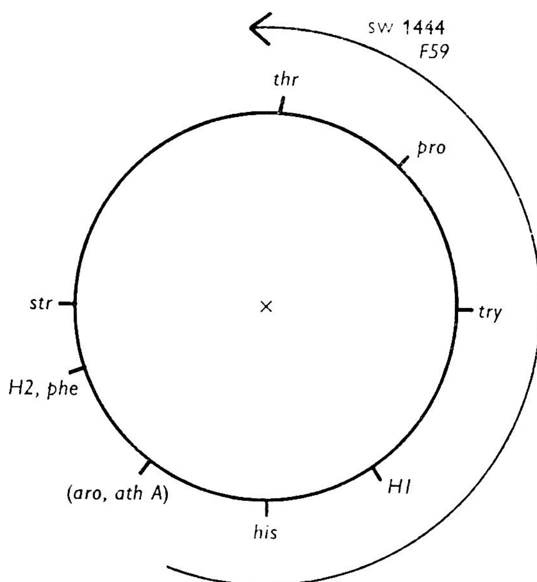


Fig. 2. A chromosome map of Salmonella showing the approximate location of markers and the point of origin (point of the arrow) and direction of transfer of the donor strains (according to Smith & Stocker, 1962 and personal communication; Mäkelä, 1963; Mäkelä & Ziegler, unpublished). *thr*, threonine requirement; *pro*, proline requirement; *try*, tryptophan requirement; *his*, histidine requirement; *aro*, phenylalanine and tyrosine requirement; *ath*, adenine and thiamine requirement; *phe*, phenylalanine requirement; *str*, streptomycin resistance; *H1*, phase-1 flagellar antigen; *H2*, phase-2 flagellar antigen.

Culture media. Broth: Difco nutrient broth +0·5% NaCl. Nutrient agar: Difco nutrient broth +1·6% agar. Motility medium: Difco nutrient broth+0·5% agar +8% gelatin (Stocker, Zinder & Lederberg, 1953). Defined medium: Davis

minimal medium (Lederberg, 1950): 1·6 % agar, 0·2 % glucose, amino acids and/or adenine, 20 μg./ml.; thiamine, 0·002 μg./ml.; streptomycin, 1000 ug./ml. when indicated.

Crosses were made between exponentially growing cultures of *Hfr* or *F 59*$^+$ donor and F^- recipient mixed so as to give about 5×10^7 donor bacteria/ml. and 5×10^8 recipient bacteria. This mating mixture was kept undisturbed at 37° for 2 hr, then plated without washing on selective defined medium. Recombinant colonies which appeared after 2 days were streaked on nutrient agar, from which single colonies were picked and tested for their growth requirements and antigenic characters.

H antigens were determined by slide agglutination in rabbit anti-H sera diluted 1/100. Before testing, the strains (or recombinants) were passed through motility medium to enhance their motility.

O antigens were determined by slide agglutination in appropriate antisera at predetermined dilutions. Single factor sera anti-4 and anti-9 were a gift from the Serological Standards Laboratory, Central Public Health Laboratory, Colindale, London, N.W. 9. Anti-5 factor serum was prepared by absorbing an anti-4,5,12 serum with strain SL 681 of *Salmonella typhimurium*, an LT2 mutant which lacks antigen 5. An unabsorbed serum against *S. paratyphi A* (O antigens 2, 12), also from the Standards Laboratory, was used as an anti-12 reagent.

RESULTS

Cross 1 was between a 4,5,12 *Salmonella abony* donor and a 9,12 *S. enteritidis* recipient; the outcome is seen in Table 1. In bacterial crosses most recombinants resemble the recipient parent more than the donor parent, having inherited only a

Table 1. *Cross 1. 4,5,12 donor Salmonella abony Hfr* SW *1444* (*met*$^-$ *aro*$^-$ *str-r H1-b H 2-e,n,x*); *9,12 recipient S. enteritidis F*$^-$ SH *674* (*try*$^-$ *his*$^-$ *str-s H 1-g, m*)

Selected marker from: *Hfr* donor	*F*$^-$ recipient	Recombinants no.	%	O serotype	Markers tested (in their proposed map order): *try*	*H 1*	*his*	*O–4/9*	*O–5*	*aro*	*H 2*	*str*
his$^+$	*aro*$^+$	21	43	9,12	R	R	**D**	R	—*	**R**	R	R
		21	43	4,12	R	R	**D**	D	R	**R**	R	R
		7	14	4,5,12	R	R	**D**	D	D	**R**	R	R
		49										
try$^+$	*aro*$^+$	10	100	9, 12	**D**	R	R	R	R	**R**	R	R

R represents the recipient (F^-) allele, D the donor (*Hfr*) allele. *—indicates no reaction in anti-5 immune serum, which perhaps is not an adequate test for the presence of *0–5* in these recombinants (see discussion, p. 63). The alleles used in selection in bold face. For abbreviations see Fig. 2.

small part of the donor chromosome. However, when the donor *his*$^+$ allele was selected, 57 % of the recombinants had the 4-specificity of the donor instead of the 9 of the recipient. All the recombinants tested showed strong and typical O-agglutination in either anti-4 or anti-9 serum, and none had both of these specificities. Thus the partial antigens 4 and 9 behaved as if determined by alleles of one locus, which might be called *O–4/9*, approximately 60 % linked to *his*.

The specificities 4 and 5 were separated in several cases. Indeed there were more 4,12 than 4,5,12 recombinants although the donor was 4,5,12. None of the recombinants with the partial antigen 9 had 5-specificity. If the order of the loci concerned is:—*his—O–4/9—O–5*—such recombinants are not to be expected since the incorporation of *his* and *O–5* from the donor without that of *O–4* requires a quadruple crossover. They are, however, to be expected with his^+ but without *O–4* in the reversed cross, with a 9, 12 his^+ strain as donor to a 4, 5, 12 his^+ *str-r* recipient (see below).

When the donor try^+ allele was selected instead of his^-, all recombinants had completely recipient-like O antigens. They also all had the recipient his^-. Both his^+ and try^+ were simultaneously selected from the donor in the hope of obtaining the integration of the whole *try—his* segment in many recombinants. If *O–4/9* or *O–5*

Table 2. *Cross 2. 9,12 donor Salmonella enteritidis F 59*$^+$ *SH 673 (try*$^-$ *str-s H 1-g, m); 4,5,12 recipient S. typhimurium F*$^-$ *SL 803 (his*$^-$ *ath*$^-$ *str-r H 1-i, H 2-1,2)*

Selected marker from				Markers tested (in their proposed map order)						
F 59$^+$ donor	*F*$^-$ recipient	No. of recombinants	O serotype	*try*	*H 1*	*his*	*O–4/9*	*O–5*	*ath*	*str*
his$^+$	***str-r***	9	4, 5, 12	R	R	**D**	R	R	R	**R**
		8 } 10	9, 12	R	R	**D**	D	—*	R	**R**
		1		R	D	**D**	D	—	R	**R**
		1		D	R	**D**	D	—	R	**R**
		1 } 2	4, 12	D	R	**D**	R	D	R	**R**
		1		R	R	**D**	R	D	R	**R**
		21								

R represents the recipient (F^-) allele, D the donor ($F'+$) allele. *—indicates no reaction in anti-5 immune serum, which perhaps is not an adequate test for the presence of *O–5* in these recombinants (see discussion, p. 63). The alleles used in selection are in bold face. For abbreviations see Fig. 2.

were located between *try* and *his* then all or nearly all such recombinants would be expected to have the donor O alleles. However, the integration of the whole segment did not occur, as indicated by the absence of the donor phase-1 flagellar antigen *b* among the recombinants; the gene *H1* determining the specificity of this antigen is known to be located between *his* and *try*. The reason for this unexpected behaviour remains unknown. A high degree of chromosomal non-homology in the region might be an explanation. The *H1* genes (determining the specificities *b* for the donor, *g,m* for the recipient) could replace each other in transduction tests.

In crosses 2 and 3 the 'reverse' cross was made, crossing a 9,12 (*Salmonella enteritidis*) *F'* donor to two *S. typhimurium* (4,5,12) his^- *str-r* F^- recipients. The results are shown in Tables 2 and 3. The donor allele his^+ was selected in both cases. Again in all recombinants either antigen 4 of the recipient or antigen 9 of the donor was fully expressed. The donor allele *O–9* was 50% linked to *his*.

The partial antigens 4 and 5 were again separated, for in cross 2 of the recombinants with antigen 4 of the recipient two failed to agglutinate with anti-5 serum. These two 4,12 recombinants constitute a minority out of 17 recombinants with antigen 4. They represent quadruple cross-overs according to the map order proposed above, which on other evidence is believed to be correct (see discussion, p. 64.)

But according to this order a majority of the 10 recombinants with antigen 9 would have inherited the gene O–5^+ of the recipient. No expression of it could, however, be detected by agglutination in anti-5 factor serum. In all crosses, all recombinants were agglutinated by the anti-12 serum, as were the parent strains.

Table 3. *Cross 3. 9,12 donor Salmonella enteritidis F 59⁺ SH 673 (try⁻ H 1-g, m); 4,5,12 recipient S. typhimurium F⁻ SH 142 (his⁻ phe⁻ H 1-i H 2-1, 2)*

Selected marker from: $F\,59^+$ donor	Selected marker from: F^- recipient	No. of recombinants	O serotype	Markers tested (in their proposed map order): *try*	*H 1*	*his*	*O–4/9*	*O–5*	*phe*
his⁺	*try*⁺	6	4, 5, 12	**R**	R	**D**	R	R	R
		6 } 7	9, 12	**R**	R	**D**	D	—*	R
		1 }		**R**	R	**D**	D	—	D
		13							

R represents the recipient (F^-) allele, D the donor (F') allele. *— indicates no reaction in anti-5 immune serum, which perhaps is not an adequate test for the presence of O–5 in these recombinants (see discussion, p. 63). The alleles used in selections are in bold face. For abbreviations see Fig. 2.

DISCUSSION

Let us first examine what is known of the chemical structure of the O antigens 4,(5),12 and 9,12 with which the experiments were concerned. According to recent results the repeat units of the respective lipopolysaccharides are very much alike. Figure 3 shows their proposed structure, where the only known difference is in a terminal di-deoxyhexose attached to a mannose unit (Westphal & Lüderitz, 1960; Staub, 1964); in group B this di-deoxyhexose is abequose, i.e. 3,6-di-deoxy-D-galactose, in group D tyvelose, i.e .3,6-di-deoxy-D-mannose. These sugars are the most important determinants of the partial antigens 4 or 9.

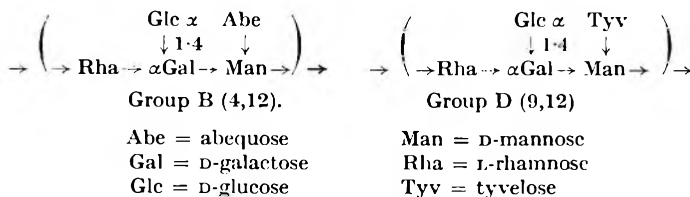


Fig. 3. The proposed structure of the O repeat unit of the lipopolysaccharide of group B (4,12) and group D (9,12) Salmonella strains (Staub. 1964).

The partial antigen 5, when present, is determined by an O-acetyl group on the galactose moiety (Kotelko, Staub & Tinelli, 1961). Other aspects of the same repeat unit represent the antigen factor 12, but do not require further attention here, as the experimental data give no information about it.

In crosses between group B (4,5,12) and group D (9,12) bacteria, irrespective of which was the donor or recipient, the partial antigens 4 and 9 replaced each other as if determined by allelic genes.

Both the di-deoxyhexoses mainly concerned with these specificities are very uncommon, being found only in these specific antigens (and certain other O antigens

of some other Gram-negative genera). Therefore it is improbable that either parent was able to synthesize both of them. In fact an enzymic conversion of a common precursor, cytidine-diphospho-glucose (synthesized by both group B and D bacteria), to cytidine-diphospho-abequose by extracts of *Salmonella typhimurium* (group B), or to cytidine-diphospho-tyvelose by extracts of *S. enteritidis* (group D) was recently demonstrated (Nikaido & Jokura, 1961; Drs H. and K. Nikaido, personal communication). Thus a donor-type (in respect of O antigen) recombinant must have inherited from the donor parent the genes for all the enzymes leading to the synthesis of the donor-type di-deoxyhexose, and probably also a gene for a transferase needed to carry this sugar from its cytidine-diphosphate precursor compound to its place in the lipopolysaccharide. The number of different enzymes involved is not known but must be more than one; each of these enzymes must be specified by a separate structural gene. Because all recombinants had either donor-like or recipient-like antigens, i.e. 4 or 9, all these postulated genes must be located in one cluster so as to be inherited together. This cluster, the *O–4/9* locus, is situated near *his*, the locus for histidine biosynthesis.

In naturally occurring Salmonella strains the partial antigen 5 often appears with 4,12, but never with 9,12 (or other O antigens) (Kauffmann, 1954). The corresponding gene *O–5*$^+$, mutation of which leads to the loss of 5-specificity, transforming 4,5,12 bacteria into 4, 12, is situated close to *his* (Iseki & Sakai, 1952; Smith & Stocker, 1962). This finding is corroborated by the present study, too, if it be assumed that group D Salmonella lack this gene or, less probably, have a negative allele of it. In some of the crosses reported above a majority of the 9,12 recombinants must have inherited the *O–5*$^+$ gene of the 4,5,12 parent, but no expression of it could be detected by anti-5 factor serum. However, this is not conclusive proof of the absence of acetylated galactose in these recombinants, because anti-5 serum might require for reaction a larger antigenic grouping than just the acetylated galactose. Even so, one would expect strong cross-reactions between the acetylated-galactose forms of groups B and D, because the units on each side of the galactose are common to both groups: no such cross-reactivity was found. A more definite test would be the use as immunogens of O9 recombinants in which the presence of the gene *O–5*$^+$ has been ascertained by genetic means, i.e. by backcrossing to a 4,12 recipient. Another possibility is that the acetyl group is transferred on to lipopolysaccharide by an enzyme whose specificity is such that only the abequose-containing repeat unit can serve as acceptor. The absence of a variable subfactor in group D corresponding to the variable antigen 5 in group B makes the latter possibility more attractive. Other possibilities are not excluded, e.g. a repression of the activity of the gene *O–5*$^+$ by the (short) piece of *Salmonella enteritidis* genetic material incorporated in these recombinants, but are perhaps less likely. Experiments are under way to test these alternatives.

The present results indicate a linkage between *O–4/9* and *his* of the order of 50–60 %. This is a real linkage in this sort of cross and indicates quite close proximity of the loci. The crosses were made between different sero-groups (different species according to many classifications), and pertain to a chromosomal region with a high degree of known non-homology. Both these facts tend to diminish linkage; e.g. *try*, a proximal locus about 1/4 of the map length removed from *his* and expected to be 50 % linked to it (Hayes, 1964; Smith & Stocker, 1962) shows

only 3 % linkage. Another factor tending to diminish linkage between the *O* loci and *his* in these crosses is the fact that the *O* loci are probably further from the 'point of origin' of the donors than the selected locus *his*. *O–5* shows even less linkage to *his*. The extent of this linkage cannot, however, be accurately estimated because it is not possible to test for the presence of $O\text{–}5^+$ in all recombinant classes. In cross 1 this linkage is 14 % or slightly more. Again, *O–5* is probably further from the point of origin than both *his* and *0–4/9* (see below).

From the data presented it is not possible to determine on which side of *his* the *O* loci lie, indeed not even whether *O–4/9* and *O–5* are on the same side or not. From other crosses, between group C (antigens 6, 7 and 6, 8) and group B Salmonella (Mäkelä, unpublished), it has become apparent that *O–4*, which seems allelic to *O–7* and *O–8* as well as to *O–9*, is between *his* and *str* and not between *his* and *try*, i.e. the order is *try—H1—his—O–4/7/8/9—O–5—ath—H2—str* (see Fig. 2). This is map order used in the tables above and fits all the data. It also fits the map position of *O–5* inferred by Smith & Stocker (1962).

It is possible that many of the *rou-B* mutants linked to *his* in *Salmonella typhimurium* (Subbaiah & Stocker, 1964) have occurred with in the compound O locus now described. Evidence to this effect has been obtained from crosses of some *rou-B* mutants of *S. typhimurium* with group C (6,7) smooth bacteria (Mäkelä, unpublished). If the correct repeat unit of the O-specific side-chain is not formed, the result would be a rough bacterium with the core of the lipopolysaccharide only, as observed. Whether the partial antigen 12 is determined at the same locus could not yet be ascertained. Even so, this 'locus' would be a quite large region, containing information for the synthesis of perhaps all the smooth-specific sugars (those present only in the O-specific part of the lipopolysaccharide), and for the transfer of these and other monosaccharides (or perhaps oligosaccharide units) on to the lipopolysaccharide being synthesized. The $O\text{–}5^+$ gene determining the acetylation of galactose units in the side-chain of 4,5,12 bacteria in the present study was only 25 % linked to *O–4* in a cross where *O–5* was distal to *O–4*. It might perhaps still be a part of the general O-locus if there were exceptionally much breakage of the chromosome in this cross. Other, unrelated genes present between *O–5* and *O–4* are not known. One ought to test for this possibility, e.g. in respect of the *metG* (methionine requirement) and *tre* (trehalose fermentation) loci reported to be very close to *his* (Sanderson & Demerec, 1965; Subbaiah, 1964).

This study was supported in part by the Finnish Medical Research Council, and by grants from the Sigrid Juselius Foundation and from the International Federation of University Women; this aid is gratefully acknowledged.

REFERENCES

Beckmann, I., Lüderitz, O. & Westphal, O. (1964). Zur Immunchemie der somatischen Antigene von Enterobacteriaceae. IX. Serologische Typisierung von Salmonella-R-Antigenen. *Biochem. Z.* **339**, 401.

Beckmann, I., Subbaiah, T. V. & Stocker, B. A. D. (1964). Rough mutants of *Salmonella typhimurium*. 2. Serological and chemical investigations. *Nature, Lond.* **201**, 1299.

Furth, J. & Landsteiner, K. (1929). Studies on the precipitable substances of bacilli of the Salmonella group. *J. exp. Med.* **49**, 727.

HAYES, W. (1964). *The Genetics of Bacteria and their Viruses*. Oxford: Blackwell.

ISEKI, S. & SAKAI, T. (1952). A method of differentiation of bacterial variant which lost partial antigen. *Proc. Japan Acad.* **28**, 450.

KAUFFMANN, F. (1954). *Enterobacteriaceae*. Copenhagen: Ejnar Munksgaard.

KAUFFMANN, F., KRÜGER, L., LÜDERITZ, O. & WESTPHAL, O. (1961). Zur Immunchemie der O-Antigene von Enterobacteriaceae. VI. Vergleich der Zuckerbausteine von Polysacchariden aus Salmonella S-und R-Formen. *Zentbl. Bakt.* (1. Abt. Orig.), **182**, 57.

KAUFFMANN, F., LÜDERITZ, O., STIERLIN, H. & WESTPHAL, O. (1960). Zur Immunchemie der O-Antigene von Enterobacteriaceae. I. Analyse der Zuckerbausteine von Salmonella-O-Antigen. *Zentbl. Bakt.* (1. Abt. Orig.), **178**, 442.

KOTELKO, K., STAUB, A. M. & TINELLI, R. (1961). Étude immunochimique sur les Salmonella. VIII. Rôle des groupements O acetyls dans la spécificité du facteur 0:5. *Ann. Inst. Pasteur*, **100**, 618.

LEDERBERG, J. (1950). Isolation and characterization of biochemical mutants of bacteria. *Meth. med. Res.* **3**, 5.

LÜDERITZ, O., BECKMANN, I. & WESTPHAL, O. (1964). Zur Immunchemie der somatischen Antigene von Enterobacteriaceae. X. R-spezifische Strukturen in Salmonella-O-Antigenen. *Biochem. Z.* **339**, 416.

LÜDERITZ, O., O'NEILL, G. & WESTPHAL, O. (1960). Zur Immunchemie der O-Antigene von Enterobacteriaceae. V. Die Antigenfaktoren in isolierten Salmonella-O-Antigenen. *Biochem. Z.* **333**, 136.

MÄKELÄ, P. H. (1963). *Hfr* males in *Salmonella abony*. *Genetics*, **48**, 423.

NIKAIDO, H. & JOKURA, K. (1961). Isolation of cytidine diphosphate 3, 6-dideoxyhexoses from Salmonella. *Biochem. biophys. Res. Commun.* **6**, 304.

NIKAIDO, H., NIKAIDO, K., SUBBAIAH, T. V. & STOCKER, B. A. D. (1964). Rough mutants of *Salmonella typhimurium*. 3. Enzymatic synthesis of nucleotide-sugar compounds, *Nature, Lond.* **201**, 1301.

ØRSKOV, F. & ØRSKOV, I. (1962). Behaviour of *E. coli* antigens in sexual recombination. *Acta path. microbiol. scand.* **55**, 99.

OSBORN, M. J., ROSEN, S. M., ROTHFIELD, L., ZELEZNIK, L. D. & HORECKER, B. L. (1964). Lipopolysaccharide of the Gram-negative cell wall. *Science*, **145**, 783.

ROBBINS, P. W. & UCHIDA, T. (1962). Studies on the chemical basis of the phage conversion of O-antigens in the E-group Salmonellae. *Biochemistry (Chicago)*, **1**, 323.

SANDERSON, K. E. & DEMEREC, M. (1965). The linkage map of *Salmonella typhimurium*. *Genetics* (in the Press).

SMITH, S. M. & STOCKER, B. A. D. (1962). Colicinogeny and recombination. *Br. med. Bull.* **18**, 46.

STAUB, A. M. (1964). The role of the polysaccharide moiety in determining the specificity and immunological activity of the O-antigen complex of Salmonellae. In *Bacterial Endotoxins*, Ed. by M. Landy & W. Braun. p. 38. New Brunswick, N.J.: Rutgers University Press.

STAUB, A. M. & PON, G. (1956). Étude immunochimique sur les Salmonella. 1. Polyosides extraits de *S. typhi*, *S. paratyphi B.* et *S. typhimurium*. *Ann. Inst. Pasteur*, **90**, 441.

STAUB, A. M., TINELLI, R., LÜDERITZ, O. & WESTPHAL, O. (1959). Étude immunochimique sur les Salmonella. V. Rôle de quelques sucres, et en particulier des 3,6-didesoxyhexoses, dans la spécificité des antigens O du tableau de Kauffmann-White. *Ann. Inst. Pasteur*, **96**, 303.

STOCKER, B. A. D., ZINDER, N. & LEDERBERG, J. (1953). Transduction of flagellar characters in Salmonella. *J. gen. Microbiol.* **9**, 410.

SUBBAIAH, T. V. (1964). *Ph. D. Thesis, University of London.*

SUBBAIAH, T. V. & STOCKER, B. A. D. (1964). Rough mutants of *Salmonella typhimurium*. 1. Genetics. *Nature, Lond.* **201**, 1298.

UCHIDA, T., ROBBINS, P. W. & LURIA, S. E. (1963). Analysis of the serologic determinant groups of the Salmonella E-group O-antigens. *Biochemistry (Chicago)*, **2**, 663.

WESTPHAL, O. & LÜDERITZ, O. (1960). 3,6-Didesoxy-hexosen-Chemie und Biologie. *Angew. Chem.* **72**, 881.

J. gen. Microbiol. (1965), **41**, 67–75
Printed in Great Britain

Effects of Nutrients on Self-Inhibition of Germination of Conidia of *Glomerella cingulata*

By B. T. LINGAPPA and YAMUNA LINGAPPA
College of the Holy Cross, Worcester, Massachusetts, U.S.A.

(*Received* 22 *March* 1965)

SUMMARY

The percentage germination of suspensions of washed conidia of *Glomerella cingulata* decreased with increasing concentrations of the conidia in distilled water. With 30 conidia/mm.^{2} greater than 90% germination was obtained, whereas the conidia from the same batch gave less than 3% germination when there were 3000 conidia/mm.^{2}. This inhibition of germination of crowded conidia was nullified by adequate amounts of peptone; but aeration by shaking had no influence, so that oxygen lack was ruled out as a factor in the inhibition. Various adsorbents, ion exchange resins, vitamins and germination stimulating chemicals were ineffective in inducing germination of crowded conidia. Czapek–Dox mineral solution, Hoagland's mineral solution and Czapek–Dox glucose solution gave respectively 10, 30 and 40% germination of crowded conidia. The germination of conidia was improved by repeated washing with distilled water. Addition of various mineral salts and trace elements (Mg, Fe, Mn, Bo, Zn, Mo, Cu) were relatively ineffective or toxic. Glucose, or mannitol did not induce germination. Peptone and phenylalanine were most effective in promoting the germination of crowded conidia, but only in massive doses. Bimodal response of germination of conidia to added nutrients was noticed. Since the conidia germinated, when present at low concentrations in redistilled water without added nutrients, the conidia could not be considered as nutritionally deficient. The beneficial effect of nutrients in promoting the germination of crowded conidia may be due to inactivation of inhibitory metabolites from the conidia.

INTRODUCTION

Conidia of *Glomerella cingulata* (Stoneman) Spaulding and Schrenk have been used extensively for bioassay and fungicide studies. The results of germination of conidia of this fungus reported from time to time have shown many inconsistencies. Hawkins (1913) found no difficulty in germinating the conidia in distilled water, whereas Goldsworthy & Green (1938) found that the leached conidia required an exogenous supply of 'accessory' substances for germination. Germination of cleaned conidia of *G. cingulata* was obtained by Lin (1945) only after addition of suitable sources of carbon, nitrogen, phosphorous, magnesium and sulphur Richardson & Thorn (1962) and Lingappa & Lingappa (1964) reported that washed conidia of *G. cingulata* germinated in distilled water without the addition of nutrients, and that the germination of conidia was inversely proportional to the concentration of conidia. This inhibitory effect of concentration of conidia on germination may be called self-inhibition of germination. Washed conidia of *G. cingulata* germinated readily in distilled water when their number was less than 100/mm.^{2}. On the other hand, we had repeatedly observed that the germination of conidia was inhibited

when dispersed in concentrations above 3000/mm.[2] in highly nutritive laboratory media such as full strength potato glucose agar. Experiments devised to examine these inconsistencies in the germination behaviour of conidia of *G. cingulata* are reported in this paper.

METHODS

A culture of *Glomerella cingulata* was derived from Dr C. Leben's isolate no. 22 which Dr Leben had found in 1954 to be pathogenic to Jonathan apples. It was originally isolated from apples by Dr J. O. Andes as isolate G. (personal communication from Dr C. Leben, Ohio Agriculture Experiment Station, Wooster, Ohio). A single-spore isolate from this culture which sporulated better in our laboratory conditions was selected and designated G. 21. Potato glucose agar (PDA; Johnson, Curl, Bond & Fribourg, 1960) or potato sucrose agar which contained 8·5 % (w/v) sucrose in place of 2 % (w/v) glucose in PDA or the respective liquid media without agar, were used to grow this fungus. Liquid cultures were shaken on a rotary shaker at 150 rev/min. at 25° in a temperature-controlled. chamber Both liquid and solid cultures yielded abundant conidia.

Germination studies were done under aseptic conditions to avoid contamination. Conidia were dislodged into water from slopes of culture by using an inoculation needle, and quickly washed twice in redistilled water by centrifugation. Cleaned conidia were resuspended in water to read 1 unit at 400 mμ. in a spectrophotometer (Bausch & Lomb, Spectronic 20). This stock suspension was serially diluted 1:1 through 10 steps. The stock and the first two dilutions were discarded and 1·5 ml. of the dilutions 3 to 10 were pipetted into separate disposable 35 × 10 mm. Petri dishes (Falcone Plastics, Los Angeles, California) in duplicate. The contents of the Petri dishes were finally made up to 2 ml. by adding supplements and/or water. Conidia so dispersed settled rapidly to the bottom of the Petri dish as a single layer covered by a 2 mm. layer of water. These were incubated at 25° for 8 hr. The conidia were killed and stained by adding 0·1 ml. rose bengal/dish. The staining solution was prepared by dissolving 1 g. rose bengal in 100 ml. of 5 % (w/v) aqueous solution of phenol containing 0·01 g. $CaCl_2$ (Waksman, 1932). This solution was filtered and diluted 1/3 with aqueous phenol. From each treatment the germinated conidia in eight separate fields or of 500 or more conidia were counted randomly. All conidia in the microscope fields (0·102 mm.[2] area) were counted, and the number of conidia/mm.[2] *versus* % germination calculated. The dosage (amount of nutrients or number of conidia) was plotted on log scale and the response (% germination) on probit scale. In this work conidia were considered to be germinated when they produced discernible germ tubes longer than the minor diameter of the conidium.

For nutritional work bacteriological grade substances, Bacto-Peptone (Difco Laboratories, Detroit, Michigan) and reagent grade chemicals were used. Redistilled water from a Pyrex glass still was used throughout.

Czapek–Dox mineral solution contained (g.): $NaNO_3$, 3; K_2HPO_4, 1; $MgSO_4.7H_2O$, 0·5; KCl, 0·5; $FeSO_4.7H_2O$, 0·01; in 1 l. distilled water; (Johnson *et al.* 1960).

Modified Hoagland's solution contained (g.): $Ca(NO_3)_2.4H_2O$, 1·18; $MgSO_4.7H_2O$, 0·493; KNO_3, 0·506; KH_2PO_4, 0·136; ferric tartrate, 0·01; H_3BO_3, 0·00286; $MnCl_2.4H_2O$, 0·00362; $ZnSO_4.7H_2O$, 0·00022; MoO_3, 0·00014; $CuSO_4.5H_2O$, 0·00008; in 1 l. distilled water (Thimann & Edmondson, 1949).

RESULTS

Eight dilutions of washed conidia were uniformly distributed in duplicate Petri dishes to give average conidial concentrations from 30 to 3000 conidia/mm.^{2} in distilled water. The data obtained from these experiments clearly indicated that the washed conidia gave 90 % or better germination when present at concentrations of 30/mm.^{2}, whereas the conidia from the same lot gave less than 3 % germination when the concentration was 3000 conidia/mm.^{2} in water (Fig. 1). Different batches of conidia from cultures grown under different conditions gave 80% or less germination in water. Examination of water controls in Fig. 2, 3 and 5 showed, however, that 50 % conidia germinated in water at concentrations of about 100/mm.^{2} The germination of conidia was directly proportional to the concentration of conidia in water (Fig. 1): an instance of 'self-inhibition'.

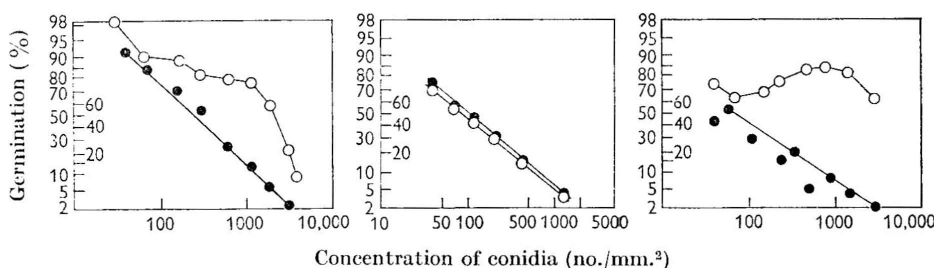


Fig. 1. Germination of conidia (probit scale) of *Glomerella cingulata*, in different densities (log scale), in 1·25 mg./ml. peptone solution (○—○) and in water (●—●).

Fig. 2. Germination of conidia (probit scale) of *Glomerella cingulata*, in different densities (log scale), in 30 mg./ml. sucrose solution (○—○) and in water (●—●).

Fig. 3. Germination of conidia (probit scale) of *Glomerella cingulata*, in different densities (log scale) in 12·5 mg./ml. L-phenylalanine (○—○) and in water (●—●).

The dependence of germination on the concentration of conidia might be due to decreased availability of oxygen or of nutrients to individual conidia, or to decreased ability of the conidia to utilize these in presence of toxic metabolites in or from the conidia. Decreased availability of oxygen did not appear to be responsible for this effect. Suspensions of conidia of different concentrations were shaken, with or without peptone, on a rotary shaker at 50, 100 and 200 rev./min. They did not show increased germination over the static controls. Germination was increased markedly by adding peptone (Fig. 4).

Influence of various nutrients

More conidia germinated in potato extracts or in peptone solutions than in water. Therefore components of various laboratory media were separately added to suspensions of crowded conidia. About 3000 conidia/mm.^{2} were enough to give negligible germination in water and complete germination in peptone solution (30 mg./ml.), but were not too concentrated to count. This concentration was obtained by serial dilution no. 3 and will be referred to as crowded conidia. Addition of D-glucose, sucrose or D-mannitol 30 mg./ml. showed no significant effect on

germination (Fig. 2, 5). Figure 1 shows that high % germination was readily obtained without addition of nutrients when the conidia were less crowded. On the other hand % germination of crowded conidia was directly proportional to the amount of peptone added (Fig. 4), and that even in presence of peptone increasing the concentration of conidia resulted in progressively less germination (Fig. 1). The amount of peptone required to annul inhibition by crowding and to promote

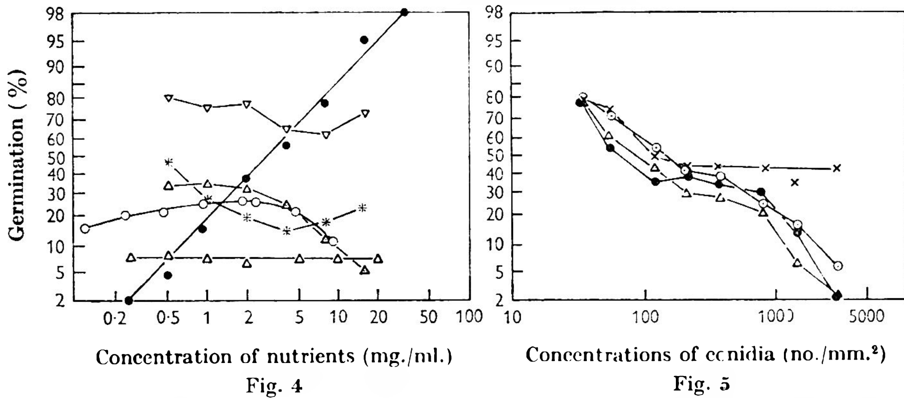


Fig. 4. Germination of conidia (probit scale) of *Glomerella cingulata*, in increasing amounts (log scale), of peptone (●—●), L-phenylalanine (▽—▽), L-asparagine (△—△) DL-alanine (*—*), Hoagland's salts (○—○), and Czapek-Dox salts (△—△); conidia were 3000/mm.².

Fig. 5. Germination of conidia (probit scale) of *Glomerella cingulata*, in different concentrations (log scale), in Czapek-Dox solution (⊙—⊙), in 30 mg./ml. glucose solution (●—●), in Czapek-Dox with 30 mg./ml. glucose (×—×) and in water (△—△).

Table 1. *Effect of concentration of peptone on crowded conidia (3000/mm.²) of Glomerella cingulata*

μg. peptone/μg. conidia (oven dry wt.)	Germination (%)
0·428	0·9
0·855	4·0
1·710	15·3
3·422	31·8
6·844	56·5
13·687	77·5
27·374	94·7
54·747	99·0

the emergence of germ tubes was nearly 30 to 50 times the weight of the conidia (Table 1). These results suggested that only a very minor component of peptone was involved in annulling the inhibition or that some component of the peptone might be serving a non-nutritional role such as binding toxic metabolites of the conidia.

Casein hydrolysate (vitamin free) was ineffective from 1 to 20 mg./ml. Richardson & Thorn (1962) found, of 19 amino acids tested that only arginine, asparagine, aspartic acid, glutamic acid, glutathione, leucine, phenylalanine, tryptophan and tyrosine were effective in bringing about germination of crowded conidia. The

concentration of conidia, duration and manner of incubation and criterion of beneficial effect on germination used by these workers were different. Therefore we tested those amino acids and found that L-glutamic acid and glutathione gave a maximum of 10% germination between 0·01 and 1 mg./ml., and less germination at higher concentrations (1·6 mg./ml.). L-asparagine gave 20–35% germination in concentrations from 0·1 to 1·6 mg./ml. The effect of asparagine above 0·5 mg./ml. is shown in Fig. 4; higher concentrations were toxic. L-phenylalanine and DL-alanine showed a bimodal response, inducing high germination at 0·5 mg./ml. and again at 16 mg./ml. (Fig. 4). Phenylalanine was like peptone in its effect on increasing concentrations of conidia (Fig. 3). Although phenylalanine induced germination, like peptone, the germ tubes formed ended in appressoria and did not grow any further. In peptone the germ tubes were robust, branched and grew profusely.

Table 2. *Effect of various substances on germination of crowded conidia (3000/mm.²) of Glomerella cingulata*

Substances	24 hr of incubation, germination (%)	2nd incubation 8 hr with peptone*, germination
Silica gel (E. Merck A.G. Darmstadt, Germany)	0	+
Cellulose powder (Macherey, Nagel & Co. Düren, Germany)	0	+
Aluminum oxide (Merck)	0	+
Cation exchange resin (Rexyn 101 (Na), (Fisher Scientific Co., New York, N.Y.)	0	+
Anion exchange resin (Rexyn CG (Cl), (Fisher Scientific Co.)	0	+
Charcoal, Bacteriological (Oxo Ltd., London)	1	+
Thiourea (Baker & Adams, Allied Chemicals, New York, N.Y.)	1	+
Adenosine (Nutritional Biochemicals Corp. Cleveland, Ohio)	5	+
L-cysteine HCl (Nut. Biochem Corp.)	0	0
L-cystine (Nut. Biochem. Corp.)	2	+
Menadione (K & K Labs. Plainview, N.Y.)	0	0
Coumarin (Aldrich Chem. Co., Milwaukee, Wisc.)	2	+
† Biotin (Nut. Biochem. Corp.)	2	...
† Thiamine HCl (Eastman)	2	...
† Riboflavin (Merck)	2	...
Water control	1	+
3% (w/v) Peptone (Difco)	98	...

* Nearly all spores germinated = +, no spores germinated = 0, results not available = ...
† Germination was similar in all concentrations, viz. 0·005, 0·05, 0·2 μg./ml.

Other agents tested included various adsorbents, vitamins and chemicals used by various investigators for the activation of dormant biological forms. Adsorbents were added 1 mg./ml. to conidial suspension and other substances were in 10^{-4} M final concentration unless otherwise mentioned. These preparations were incubated for 24 hr, instead of the usual 8 hr, and the conidia counted without killing and staining. Peptone was then added to the Petri dishes and incubation continued for 8 hr. The data shown in Table 2 indicated that the adsorbents, various activating chemicals, and vitamins tested were non-toxic but ineffective in inducing the germination of crowded conidia. Re-incubation with peptone indicated that menadione and 2,4-dinitrophenol were toxic at the concentrations used.

Effect of mineral salts

Conidia were dislodged from slopes of culture as before and the suspensions quickly centrifuged. The deposited conidia were resuspended in sterile distilled water and a sample suitably diluted and put for germination. The remaining spores were repeatedly washed with glass redistilled water, taking samples for germination after 5, 10 and 20 washings. The results shown in Table 3 indicate great improvement in the germination of conidia when washed 5 times or more, as compared with unwashed conidia. When Czapek–Dox mineral solution was used for washing, primarily to prevent osmotic damage to spores, a high % germination was obtained without any addition of carbon source. Corresponding series in peptone 5 mg./ml. solutions gave uniformly 90 % or higher germination at all spore densities, irrespective of the medium or number of washings.

Table 3. *Effect of washing on germination of crowded conidia (3000/mm.²) of Glomerella cingulata in water*

Unwashed		Washed 5 ×		Washed 10 ×		Washed 20 ×		Washed 10 × in Czapek–Dox solution	
Conidia/ mm.²	Germ. (%)	Conidia/ mm.²	Germ. (%)	Conidia/ mm.²	Germ. (%)	Conidia/ mm.²	Germ. (%)	Conidia/ mm.²	Germ. (%)
1800	5	1750	43	1300	37	1176	53	1400	92
80	56	138	80	140	90	223	91	157	84
22	33	88	91	29	82	38	81	48	90

These experiments indicated that either a soluble inhibitor was leached away by repeated washing of the conidia or that these were accumulating stimulatory 'trace' minerals from the 'distilled' water. The later suggestion appeared possible because washing with Czapek–Dox (C–D) mineral solution gave a high stimulation of germination. This was examined by adding C–D mineral solution into crowded conidial suspensions. The results showed (Fig. 4) that the amount of C–D salts added did not make much difference, about 10 % of crowded conidia germinated throughout the range of 0·25 mg. to 20·04 mg. salts/ml. water (normal strength was 5·01 mg. salts/ml. water). It was also evident that the pattern of germination at different concentrations of conidia were unaffected by the addition of C–D minerals (Fig. 5). The addition of C–D mineral salts did not show an effect on lower concentrations of conidia. Singly or in admixture these minerals, with or without glucose, were ineffective in inducing germination of conidia (Lin, 1945). But nearly 50 % conidia germinated in concentration from 1000 to 3000 conidia/mm.² when glucose C–D salts were added (Fig. 5). To examine whether other minerals might increase germination, Hoagland's mineral solution as modified by Thimann & Edmondson (1949) was used; it contained iron, manganese, boron, zinc, molybdenum and copper in traces. This Hoagland solution gave consistently higher germination than the corresponding C–D solution (Fig. 4). At concentrations of 1000–3000 conidia/mm.² 15–20 % conidia germinated in diluted Hoagland's solution (0·6 mg. salt/ml.). Normal strength Hoagland's solution (2·332 mg./ml.) promoted about 25 % germination of crowded conidia (Fig. 4); higher concentrations were toxic.

Trace element components of Hoagland solution were separately added in various concentrations to crowded conidial suspensions. But they did not show any stimulation of germination; concentrations greater than (μg./ml.) 0·004, $CuSO_4 . 5H_2O$; 0·07, MoO_3; 0·011, $ZnSO_4 . 7H_2O$; 1·43, H_3Bo; 1·81, $MnCl_4 . 4H_2O$; 9·84, $MgSO_4 . 7H_2O$ were toxic. Only Mo and Mn slightly stimulated germination (10 %) at concentrations 1/10 of the preceding sentence. Ferric tartrate gave increasing germination (18 %) up to 8 μg./ml.; the corresponding germination in distilled water (control) was 1 %.

DISCUSSION

Decreased germination due to crowding of spores had been called self-inhibition or auto-inhibition; this phenomenon is known for many fungi (Cochrane, 1958). Various studies have shown that germination of uredospores of rust fungi is prevented by the concentration of uredospores and this has been ascribed to substances which diffuse from the spores (Allen, 1957). Trimethylethylene (Forsyth, 1955), phenolic compounds, coumarins and glycosides (van Sumere, van Sumere-De Preter, Vining & Ledingham, 1957), and glutamic and aspartic acids (Wilson, 1958) from the uredospores have been considered as possible self-inhibitors. The results of Bell (1960) suggested the existence of unidentified inhibitory substances other than glutamic and aspartic acids in the uredospores.

Conidia of *Glomerella cingulata* also exhibit strong self-inhibition of germination in water (Fig. 1). Washed conidia were capable of germination in increasing numbers with decreasing concentration in redistilled water; when conidia were less than 100/mm.^{2} a large percentage of them germinated readily in water (Fig. 1). Therefore it is most likely that self-inhibition of germination of conidia might not be due to lack of nutrients. Increased concentration of conidia in water might lead to decreased supply of oxygen and of trace elements that may still be contaminating the twice distilled water, or might lead to increased concentration of self-inhibitory emanations from conidia. Improved germination of repeatedly washed conidia might be due to leaching of toxic metabolites of conidia or to accumulation of trace elements by the conidia from successive batches of water.

In as much as oxygen was ruled out as a factor in limiting germination of crowded conidia there were two questions to be investigated, viz. whether the spores depended upon an exogenous supply of some nutrients or whether self-inhibitory metabolites were responsible for the inhibitory effect of crowding. Inhibitory effect of crowded conidia was not overcome by addition of various growth stimulating and dormancy-breaking chemicals (Table 2). They included menadione, coumarin and 2,4-dinitrophenol which remove self-inhibition of uredospores (cf. Farkas & Ledingham, 1959). These results might indicate that the mechanisms involved in the self-inhibition of germination of conidia of *Glomerella cingulata* might be different from those of the uredospores.

Inhibition of germination of crowded conidia (3000/mm.^{2}) was effectively nullified by peptone (Fig. 4). Peptone required was as much as 30 to 50 times the weight of conidia to bring about germination (Table 1). The ascospores of *Neurospora tetrasperma* utilize only endogenous reserves during germination (Lingappa & Sussman, 1959) and the amount utilized was less than 10 % of the spore weight and this gives an idea as to the amounts of substrates that may be required for germination of

spores. It is inconceivable that peptone might be required in such large quantities as a nutrient source to effect germination of conidia. Therefore, it appeared to us that peptone might be antagonizing or inactivating some toxic spore metabolites. The beneficial effects of phenylalanine decreased and again increased with increasing concentration (Fig. 4) and its effect on different densities of conidia was like peptone (Fig. 3). Nearly 80% of crowded conidia germinated in both peptone and in phenylalanine solutions of 1·25% concentrations. In phenylalanine the germ tubes failed to grow and ended in appressoria. Germination was good in alanine (45% at 0·5 mg./ml.) and growth of germ tubes was normal but the germination of conidia decreased to 14% with 4 mg. alanine/ml. These effects of amino acids indicate that the beneficial effects of peptone might be most likely due to mixtures of amino acids and peptides rather than to some trace or minor constituents of peptone functioning as germination stimulator. The requirement of large amounts of peptone as well as the pure amino acid phenylalanine further indicated the possible non-nutritive role of these substances in inducing germination of crowded conidia of *Glomerella cingulata*.

Glucose and C–D salts appeared to establish a state of equilibrium between germination and germination inhibition over a wide range of spore densities. Peptone, on the other hand, gave a bimodal response curve; the slope of the curve between 100 and 1000 conidia/mm.^{2} was different from those of lower as well as higher densities (Fig. 1). According to Dr J. G. Horsfall (private communication) permeation of alanine moiety might be necessary for germination. The phenyl group in phenylalanine would almost surely increase permeation of the alanine group and increase germination. The second approximation is—(*a*) that alanine and phenylalanine are toxic in the concentrations used, and (*b*) that they also prevent the action of the inhibitor (after they permeate). The deleterious and beneficial effects could generate the polymodal curve. Dosage response curve of the fungicide tetramethylthiuram disulphide (TMTD) on *Macrosporium sarciniforme* showed that toxicity of TMTD increased with increasing concentration of the fungicide but higher concentrations were less toxic and at much higher concentrations toxicity reappeared again (Dimond, Horsfall, Heuberger & Stoddard, 1941). Horsfall & Dimond (1963) considered that such bimodal response curves were generated by the action of two factors acting at different rates or in opposite directions. Petersen (1959) encountered such a non-linear bimodal response of infection of wheat plants to inoculum density of wheat rust uredospores. The observation of Petersen was especially interesting because of the role of a self-inhibitor in germination of uredospores. An unusual phenomenon was observed by Albert and co-workers (cf. Hewitt & Nicholas, 1963) in connexion with the toxicity of oxine to *Staphylococcus aureus*; toxicity of oxine decreased with increase in concentration so much so that a saturated oxine was non-toxic. The 'concentration quenching' was due to oxine or to formation of non-toxic 3:1 oxine:iron complexes which result when oxine was in excess whereas unsaturated 1:1 and 2:1 oxine:iron complexes were toxic. Bimodal response was clearly evident in the alleviation of self-inhibition of germination of conidia of *Glomerella cingulata* by nutrients (Fig. 1, 3, 4, 5). The inhibition of germination of conidia might not be primarily due to nutritional deficiencies but to inhibitory substances in the conidia which may be functioning as antimetabolite(s). The self-inhibitory antimetabolite(s) may be excluded, complexed, or otherwise

inactivated by large amounts of added nutrients. Failure of added trace elements to improve germination of crowded conidia further lent support to the view that the beneficial effect of repeated washing of conidia was due to leaching away of self-inhibitory substance(s) from the conidia and not to the exogenous supply of trace elements.

Financial support provided by U.S. National Science Foundation Grant GB-645 is gratefully acknowledged. We are indebted to Dr J. G. Horsfall and to Dr K. V. Thimann for valuable criticism and advice.

REFERENCES

ALLEN, P. J. (1957). Properties of a volatile fraction from uredospores of *Puccinia graminis* var. *tritici* affecting their germination and development. I. Biological activity. *Plant Physiol.* **32**, 385.

BELL, A. A. (1960). Partial purification of self-inhibitors of germination from uredospores of *Uromyces phaseoli* var. *typica*. (Abstract). *Phytopathology*, **50**, 629.

COCHRANE, V. W. (1958). *Physiology of Fungi.* New York: John Wiley and Sons, Inc.

DIMOND, A. E., HORSFALL, J. G., HEUBERGER, J. W. & STODDARD, E. M. (1941). Role of the dosage-response curve in the evaluation of fungicides. *Conn. Agric. Expl. Station Bull.* **451**, 635.

FARKAS, G. L. & LEDINGHAM, G. A. (1959). The relation of self-inhibition of germination to the oxidative metabolism of stem rust uredospores. *Can. J. Microbiol.* **5**, 141.

FORSYTH, F. R. (1955). The nature of the inhibiting substance emitted by germinating uredospores of *Puccinia graminis* var. *tritici*. *Can. J. Bot.* **33**, 363.

GOLDSWORTHY, M. C. & GREEN, E. L. (1938). Effect of low concentrations of copper on germination and growth of conidia of *Sclerotinia fructicola* and *Glomerella cingulata*. *J. agric. Res.* **56**, 489.

HAWKINS, L. A. (1913). The influence of calcium, magnesium and potassium nitrates upon the toxicity of certain heavy metals toward fungus spores. *Physiol. Res.* **1**, 57.

HEWITT, E. J. & NICHOLAS, D. J. D. (1963). Cations and anions: Inhibitions and interactions in metabolism and in enzyme activity. In *Metabolic Inhibitions*, vol. 2. Ed. by R. M. Hochster & J. H. Quastel. New York and London: Academic Press.

HORSFALL, J. G. & DIMOND, A. E. (1963). A perspective on inoculum potential. *J. Indian Bot. Soc.* **38**, 161.

JOHNSON, L. F., CURL, E. A., BOND, J. H. & FRIBOURG, H. A. (1960). *Methods for Studying Soil Microflora-Plant Disease Relationships.* Minneapolis, Minn: Burgess Publishing Company.

LIN, C. K. (1945). Nutrient requirements in the germination of the conidia of *Glomerella cingulata*. *Am. J. Botany*, **32**, 296.

LINGAPPA, B. T. & LINGAPPA, Y. (1964). Self-inhibitory products of fungi. *Bact. Proc.* p. 35.

LINGAPPA, B. T. & SUSSMAN, A. S. (1959). Endogenous substrates of dormant, activated, and germinating ascospores of *Neurospora tetrasperma*. *Plant Physiol.* **34**, 466.

PETERSEN, L. J. (1959). Relations between inoculum density and infection of wheat by uredospores of *puccinia graminis* var. *tritici*. *Phytopathology*, **49**, 607.

RICHARDSON, L. T. & THORN, G. D. (1962). Stimulation of spore germination and growth of *Glomerella cingulata* by copper and other heavy metal ions. *Phytopathology*, **52**, 865.

THIMANN, K. V. & EDMONDSON, Y. H. (1949). The biogenesis of the anthocyanins. I. General nutritional conditions leading to anthocyanin formation. *Arch. Biochem.* **22**, 33.

VAN SUMERE, C. F., VAN SUMERE-DE PRETER, C., VINING, L. C. & LEDINGHAM, G. A. (1957). Coumarins and phenolic acids in the uredospores of wheat stem rust. *Can. J. Microbiol.* **3**, 847.

WAKSMAN, S. A. (1932). *Principles of Soil Microbiology.* 2nd ed. Baltimore, Md.: The Williams and Wilkins Company.

WILSON, E. M. (1958). Aspartic and glutamic acid as self-inhibitors of uredospore germination. *Phytopathology*, **48**, 595.

J. gen. Microbiol. (1965), **41**, 77–84
Printed in Great Britain

Differentiation of Porcine Mycoplasma Strains

By Z. DINTER, D. DANIELSSON and K. BAKOS
Institutes of Virology and Bacteriology, Uppsala University, Uppsala, and State Veterinary Medical Institute, Stockholm, Sweden

(*Received* 23 *March* 1965)

SUMMARY

Porcine Mycoplasma strains, previously called 'swine enzootic pneumonia strains' are antigenically closely related to *Mycoplasma hyorhinis*. Other porcine Mycoplasma strains which are culturally and antigenically distinct from *M. hyorhinis* are more heterogeneous and fall into four serotypes. *M. granularum*, however, is antigenically unrelated to these strains.

INTRODUCTION

Different types of Mycoplasma have been isolated from pigs with enzootic pneumonia or atrophic rhinitis in Sweden (Bakos & Dinter, 1963). The majority of isolates were shown to be culturally closely related to a cytopathogenic agent cultivated by Wesslén & Lannek (1954) from pigs with a disease called swine enzootic pneumonia (SEP). These isolates were hence labelled SEP strains, although no evidence was presented that such strains cause a transmissible pneumonia in pigs (Goodwin & Whittlestone, 1964). A minor group of isolates, designated B strains, were culturally distinct from the SEP strains.

In this study the SEP and B strains have been compared with each other and with reference strains of *Mycoplasma hyorhinis* (Switzer, 1955) and *M. granularum* (Switzer, 1964)—the two hitherto described species of Mycoplasma occurring in pigs. The results show that porcine Mycoplasma strains are a heterogeneous group.

METHODS

Mycoplasma strains. Two strains of *Mycoplasma hyorhinis*, kindly supplied by Professor A. Freundt, University of Aarhus, Denmark, and Dr W. P. Switzer, Iowa State University of Science and Technology, Ames, Iowa, U.S.A., and one strain of *M. granularum*, kindly supplied by the latter, were investigated. The *M. hyorhinis* strains were labelled F and S7, respectively. Other strains used in this study belonged to our collection of isolates from pigs. The SEP strains labelled SEP/200, SEP/sk and SEP/G as well as six B strains labelled B1 to B6 were used. The strains SEP/G and B6 were isolated from pig material sent by Dr H.-J. Neumann, Kiel, Germany. Stock cultures of the strains were prepared in PPLO broth, distributed in tubes in amounts of 5 ml. per tube and stored at −60° before use.

PPLO media. PPLO broth medium consisted of beef heart infusion with addition of 0·5 % (w/v) yeast extract (Difco), 0·1 % (w/v) glucose, 20 % (v/v) unheated horse serum, penicillin (100 i.u./ml.) and 1 % (v/v) of a 5 % (w/v) solution of thallium acetate. PPLO agar medium was composed of the same ingredients with addition

of 1·2 % (w/v) Special Agar-Noble (Difco). Penicillin and thallium acetate were omitted when satellite growth was required.

Viable cell count. Serial tenfold dilutions of a Mycoplasma suspension were made in PPLO broth and inoculated in amounts of 0·1 ml. on plates with PPLO agar. The plates were incubated at 37° and the number of colonies counted on day 6 after inoculation.

Satellite growth. The surface of PPLO agar was first inoculated with Mycoplasma and then, along the diameter of the plates, with staphylococci according to the method of Morton, Smith & Leberman (1949). The staphylococcus strain KC3H, kindly supplied by Dr J. I. Terpstra, Rotterdam, Holland, was used for this purpose.

Egg inoculation. Suspensions of Mycoplasma were inoculated into the yolk sac of chick embryos aged 5–7 days as previously described (Dinter, Wesslén & Lannek, 1957). Pools of amniotic and allantoic fluids were used for further passages in eggs.

Cell cultures. Primary cultures of calf cells were prepared from trypsinized kidneys of newborn calves and grown at 37° in tubes. Growth medium was Hanks's salt solution with 0·5 % (w/v) lactalbumin hydrolysate (H+Lah) enriched with 10 % (v/v) newborn calf serum. The maintenance medium consisted of H+Lah+5 % (v/v) unheated horse serum. Penicillin (100 i.u./ml.) was added to both media.

Titration of infectivity in cell cultures. Serial tenfold dilutions of a Mycoplasma suspension were made in maintenance medium and inoculated in 0·1 ml. amounts in each of 4 cell culture tubes. Four additional, non-inoculated cultures served as controls of the specificity of cell degeneration induced by Mycoplasma. All cultures were incubated at 37° in upright position and in a volume of maintenance medium sufficient to cover the entire cell sheet. The results were read on day 6 or 7 after inoculation. The titres were calculated according to Kärber and are expressed as $\log_{10}$ units of TCD50/0·1 ml.

Preparation of antigens for gel diffusion. The strains were transferred into PPLO broth by inoculating 200 ml. of this medium with 10 ml. of stock culture. A dense outgrowth was obtained after incubation for 2 or 3 days at 37°. The organisms were concentrated by sedimentation for 30 min. at 35,000g and 4° and then washed 3 times in isotonic saline. The washed sediments were resuspended in 1 ml. of distilled water per 100 ml. of the original volume and the suspensions frozen and thawed 5 times before their use as antigens. A similar technique has previously been applied by Taylor-Robinson, Somerson, Turner & Chanock (1963) for the preparation of antigens from human Mycoplasma strains. The antigen stocks were kept at −20°.

Preparation of antisera. Antisera were prepared in rabbits with PPLO broth cultures as antigens. The Mycoplasma strains were sedimented and washed as described above. The washed sediments were resuspended in 5 ml. of isotonic saline per 100 ml. of the original volume. The suspensions were then mixed thoroughly with an equal amount of Freund's complete adjuvant (Difco). Each rabbit was given 2 ml. of the antigen–adjuvant mixture intramuscularly into each leg. After 5 or 6 weeks the rabbits were injected intravenously with 1 ml. of a suspension without adjuvant prepared as described above. Serum samples were taken 1 week after the second injection of antigen and were stored at −20°. They were used unheated.

Absorption of antisera. Antisera used for gel diffusion were absorbed with the PPLO broth medium in order to remove antibodies to the ingredients of this medium. PPLO broth was concentrated 10 times by pressure dialysis in a collodion bag (Membranfilter AG, Göttingen, Germany). Equal amounts of concentrate and antiserum were mixed and the mixture kept for 16 hr at 4°. The mixture was then centrifuged for 30 min. at 4500*g* and 4° and the supernatant concentrated to the original volume by pressure dialysis as mentioned above.

Gel diffusion. The micro modification of the Ouchterlony method (Wadsworth, 1957) was used. Tests were performed on glass plates with a 0·4 mm. thick layer of agar gel consisting of 1% (w/v) Special Agar-Noble (Difco) in phosphate-buffered saline (PBS), pH 7·2. The wells were 3 mm. in diameter and made in 3 mm. thick plexiglass templates. The apertures connecting the wells with the gel were 2 mm. wide. Templates with 3, 5 or 7 wells at a distance of 5 or 6 mm. between the edges were used. When the agar had congealed the wells were filled once with reactants. The plates were kept in a humidified atmosphere at room temperature for 2 days. The templates were then removed and the precipitation patterns recorded. The plates were washed for a few days in PBS and then dried and stained with amido black as recommended by Wadsworth (1957).

Growth inhibition. The technique was that of Clyde (1964) as applied by Taylor-Robinson, Canchola, Fox & Chanock (1964) for the differentiation of human Mycoplasma strains. The strains were cultivated in PPLO broth for 2 or 3 days at 37° and the cultures then spread on PPLO agar in amounts of 0·1 ml. containing 10^5 to 10^6 colony-forming organisms. The PPLO agar was dried for 1 hr at 37° prior to inoculation. Filter-paper discs were soaked with 0·025 ml. of undiluted antiserum by means of wire loops invented by Takátsy (1955) and manufactured by Cooke Engineering Co., Alexandria, Virginia, U.S.A. The discs were then placed at appropriate distances from one another on the surface of PPLO agar. The results were read after incubation for 3 days at 37°. Zones of growth inhibition were measured as the distance between the margin of the disc and the margin of inhibition.

RESULTS

Cultural characteristics of SEP *strains and Mycoplasma hyorhinis*

The occurrence of different Mycoplasma strains in pigs was evident at the time of isolation (Bakos & Dinter, 1963). The SEP strains were easier to isolate in tissue cultures (Wesslén & Lannek, 1954) or in the yolk sac of chick embryos (Dinter *et al.* 1957) than on PPLO growth media. Similar results were obtained when isolating *Mycoplasma hyorhinis* (L'Ecuyer, Switzer & Roberts, 1961).

A property common to SEP strains was that their growth on PPLO agar was promoted by the simultaneous cultivation of staphylococci. This phenomenon was most pronounced with SEP strains not adapted to cell-free media—the number and size of the colonies formed by SEP strains increased closer to the streak of staphylococci (Bakos & Dinter, 1963). The strains, however, were easily adapted to cell-free media after a few passages in PPLO broth.

The adapted strains showed an extensive growth on PPLO agar. The colonies were embedded in the agar and had a granular appearance with the larger colonies showing dark centres. Smears from colonies stained with Giemsa stain (Kebo AB,

Stockholm) showed particles with an approximate size of 0·2 μ. A similar colonial structure was observed after cultivation of reference strains of *Mycoplasma hyorhinis*. When the method of satellite growth was applied, the colonies were enlarged adjacent to the staphylococci.

Three-day-old cultures in PPLO broth were titrated in cultures of calf kidney cells. The SEP strains and *Mycoplasma hyorhinis* produced cytopathic changes of the same type as described for the strains of the cytopathogenic agent isolated by Wesslén & Lannek (1954). The titres of infectivity varied between $10^{6\cdot25}$ and $10^{7\cdot75}$ TCD50/0·1 ml. The SEP strains and *M. hyorhinis* proved to be lethal for chick embryos but the mortality rates were irregular as described by Dinter *et al.* (1957) for the cytopathogenic agent of Wesslén & Lannek (1954).

Table 1. *Differentiation of porcine Mycoplasma strains by some cultural properties*

Organism and strain	Promoted by satellite growth	Cytopathogenic for calf kidney cells	Lethal for chick embryos
M. hyorhinis F	+	+*	(+)†
M. hyorhinis S7	+	+	(+)
SEP/200	+	+	(+)
SEP/sk	+	+	(+)
SEP/G	+	+	(+)
M. granularum	−	(+)‡	−
B1	−	(+)	−
B2	−	(+)	−
B3	−	+	+§
B4	−	(+)	−
B5	−	(+)	−
B6	−	(+)	−

* Degeneration and cell death.
† Irregular rates of mortality.
‡ Various degrees of cytoplasmic granulation.
§ Regular rates of mortality.

Cultural characteristics of B *strains and Mycoplasma granularum.* Five out of six B strains were isolated directly on PPLO agar. One strain, B6, had to be enriched in PPLO broth prior to cultivation on PPLO agar. The growth of B strains and *Mycoplasma granularum* was not stimulated by satellite cultivation but was dependent upon the presence of serum. The viable cell count gave 2 to 7×10^7 colony-forming units per 0·1 ml. of 2-day-old PPLO broth cultures. The colonies were embedded in the agar. Most colonies were of the 'fried egg' type, although the colonies of one strain, B6, differed from those of the other strains by having a structure similar to that described for *M. pneumoniae* (Channock, Hayflick & Barile, 1962). The size of the organisms in the colonies of B strains was approximately 0·2 μ.

One of the B strains, B3, was shown to be strongly cytopathogenic for cultures of calf kidney cells and regularly lethal for chick embryos. Acid production was observed in cell cultures inoculated with this strain. The other B strains and *Mycoplasma granularum* have been passaged 3 times in eggs but proved to be non-lethal for chick embryos. Cell cultures inoculated with these strains showed various degrees of a cytoplasmic granulation.

On the basis of some cultural properties indicated in Table 1, the porcine Mycoplasma strains can be differentiated into two groups, one represented by *Mycoplasma hyorhinis* and the other by *M. granularum.*

Differentiation by gel diffusion. In preliminary experiments, unabsorbed antisera and antisera absorbed with the PPLO broth medium were tested with this medium as antigen. Some unabsorbed sera gave one precipitation line with PPLO broth but most unabsorbed sera as well as all absorbed sera did not react with this medium. The results of cross-testing eleven absorbed antisera against suspensions of 12 strains are shown in Table 2. According to these results a close relationship exists between *Mycoplasma hyorhinis* and the SEP strains. The precipitation lines showed reactions of identity. SEP strains are thus identified as strains of *M. hyorhinis.* No cross-reactions were found between the group of strains represented by *M. hyorhinis* and the B strains including *M. granularum.*

Table 2. *Differentiation of porcine Mycoplasma strains by gel diffusion*

Organism and strain	Antiserum against: *M. hyorhinis* F	*M. hyorhinis* S7	SEP 200	SEP sk	SEP G	*M. granularum*	B 1	B 2	B 3	B 4	B 6
	Number of precipitation lines										
M. hyorhinis F	3	4	3	2	5	0	0	0	0	0	0
M. hyorhinis S7	3	4	3	2	5	0	0	0	0	0	0
SEP/200	3	4	3	2	5	0	0	0	0	0	0
SEP/sk	3	4	3	2	5	0	0	0	0	0	0
SEP/G	3	4	3	2	5	0	0	0	0	0	0
M. granularum	0	0	0	0	0	4	0	0	0	1	0
B1	0	0	0	0	0	0	5	5	0	0	1
B2	0	0	0	0	0	0	5	5	0	0	1
B3	0	0	0	0	0	0	0	0	8	0	0
B4	0	0	0	0	0	1	0	0	0	4	0
B5	0	0	0	0	0	0	5	5	0	0	1
B6	0	0	0	0	0	0	1	1	0	0	4

Within the group comprising the B strains and *Mycoplasma granularum*, a reciprocal relationship was established between the strains B1 and B2. Since the antigen of the B5 strain was precipitated by antisera to strains B1 and B2, these three strains were considered to belong to the same serotype. Reciprocal cross-reactions of low order were noted between *M. granularum* and strain B4 as well as between strain B6 and the strains B1, B2 and B5. With heterologous antisera one precipitation line was formed by these strains whereas homologous antisera gave with them four or five lines (Table 2). No cross-reactions were observed between strain B3 and the other strains although B3 produced at least 8 lines when tested against homologous antiserum. According to these results the strains of the present study fall into six serotypes (Table 3).

Differentiation by growth inhibition. Antisera used in gel diffusion tests were also examined in growth inhibition tests. The results are summarized in Table 4. Antisera to SEP strains and strains of *Mycoplasma hyorhinis* inhibited the growth of the strain F of *M. hyorhinis* but had no inhibitory effect on the S7 strain of *M.*

hyorhinis. All SEP strains were inhibited by homologous antisera and two of them also by certain heterologous antisera. The reciprocal relationship between SEP strains and strains of *M. hyorhinis* as shown by gel diffusion could thus not be demonstrated by growth inhibition. Concerning the B strains and *M. granularum*, the specific reactions seen in gel diffusion tests were also observed in growth inhibition tests (Table 4). A close relationship was established between strains B1, B2, and B5. The other three B strains and *M. granularum* were inhibited only by homologous antisera. Cross-reactions of low order seen between some strains in gel diffusion tests were not observed in growth inhibition tests.

Table 3. *Serotypes of porcine Mycoplasma strains as shown by gel diffusion*

Serotype	Organism and strain
1	*M. hyorhinis*: F, S7; SEP: 200, sk, G
2	*M. granularum*
3	B3
4	B4
5	B1, B2, B5
6	B6

Table 4. *Differentiation of porcine Mycoplasma strains by growth inhibition*

	Antiserum against										
	M. hyorhinis		SEP			*M. granularum*	B				
Strain	F	S7	200	sk	G		1	2	3	4	6
	Zone of inhibition in millimeters										
M. hyorhinis F	5	3	4	3	4·5	–	–	–	–	–	–
M. hyorhinis S7	–	–	–	–	–	–	–	–	–	–	–
SEP/200	2·5	1·5	3	–	2·5	–	–	–	–	–	–
SEP/sk	–	–	–	2	–	–	–	–	–	–	–
SEP/G	2	–	2	–	2	–	–	–	–	–	–
M. granularum	–	–	–	–	–	5	–	–	–	–	–
B1	–	–	–	–	–	–	5	3	–	–	–
B2	–	–	–	–	–	–	5	3	–	–	–
B3	–	–	–	–	–	–	–	–	3·5	–	–
B4	–	–	–	–	–	–	–	–	–	4	–
B5	–	–	–	–	–	–	5	3	–	–	–
B6	–	–	–	–	–	–	–	–	–	–	5

– = no inhibition.

DISCUSSION

The porcine Mycoplasma strains isolated in this country could be divided at isolation into two groups. One group comprised isolates which originally grew better in cultures of animal cells and the yolk sac of chick embryos than on PPLO media unless satellite growth was employed. Isolates with such properties were encountered in more than 60% of pigs suffering from enzootic pneumonia or atrophic rhinitis (Bakos & Dinter, 1963). These isolates were provisionally called 'SEP strains'.

The present study was carried out using SEP strains well-adapted to growth on

PPLO media but these strains retained the properties of being cytopathogenic, irregularly lethal for chick embryos and promoted by satellite growth. It is interesting that they were shown to be closely related to *Mycoplasma hyorhinis*, originally isolated from pigs with atrophic rhinitis (Switzer, 1955) but considered to be a secondary invader (Switzer, 1964).

The strains of the other group were provisionally called B strains. The growth of these strains was dependent upon the presence of serum in the medium but was not stimulated by animal or bacterial cells as shown for SEP strains and *Mycoplasma hyorhinis*. *M. granularum* showed similar properties to the B strains. The existence of two different species, *M. granularum* and *M. hyorhinis*, was first established when porcine Mycoplasma strains were compared by indirect haemagglutination (Ross & Switzer, 1963).

In the present study serological differentiation was carried out by gel diffusion and growth inhibition. These tests were found reliable for differentiation of human Mycoplasma strains (Taylor-Robinson *et al.* 1963, 1964). The gel diffusion tests showed that strains of *Mycoplasma hyorhinis* and SEP strains are antigenically identical. The growth inhibition test, on the other hand, proved to be unsatisfactory when applied for identification of *M. hyorhinis*. Antisera effective against one strain were less effective or ineffective against the other; one strain was not inhibited even by homologous antiserum. Klieneberger-Nobel (1962) and Huijsmans-Evers & Ruys (1956) made similar observations when working with other Mycoplasma strains but no explanation is available for this phenomenon.

Both tests, the gel diffusion and growth inhibition, demonstrated that the B strains, including *Mycoplasma granularum*, are distinct from strains of *M. hyorhinis* but show great heterogeneity within the group. Only three out of six B strains belong to a single serotype whereas the other three B strains and *M. granularum* each represent separate serotypes. Although some heterologous strains showed cross-reactions in gel diffusion tests, the homologous reactions were sufficiently complex to identify the strains as separate serotypes. Similar observations were made with other Mycoplasma strains by Lemcke (1965).

Finally, a classification of porcine Mycoplasma strains must consider many more isolates than the small number studied here. Of special interest are the results of investigations carried out by the Cambridge group in this field. Goodwin & Whittlestone (1964) cultivated a visible pleomorphic organism, the J agent, from pigs with enzootic pneumonia, which was shown to induce a transmissible pneumonia in pigs. This agent is probably a Mycoplasma.

REFERENCES

Bakos, K. & Dinter, Z. (1963). Das SEP-Agens. *Proc. 17th World Vet. Congr.* **1**, 549.

Chanock, R. M., Hayflick, L. & Barile, M. F. (1962). Growth on artificial medium of an agent associated with atypical pneumonia and its identification as a PPLO. *Proc. natn. Acad. Sci., U.S.A.* **48**, 41.

Clyde, W. A., Jun. (1964). Mycoplasma species identification based upon growth inhibition by specific antisera. *J. Immunol.* **92**, 958.

Dinter, Z., Wesslén, T. & Lannek, N. (1957). Die Kultivierung des SEP-Agens im Dottersack des Bruteies. *Zbl. VetMed.* **4**, 581.

Goodwin, R. F. W. & Whittlestone, P. (1964). Production of enzootic pneumonia in pigs with a microorganism grown in media free from living cells. *Vet. Rec.* **76**, 611.

HUIJSMANS-EVERS, A. G. M. & RUYS, A. C. (1956). Microorganisms of the pleuropneumonia group (Family of Mycoplasmataceae) in man. II. Serological identification and discussion of pathogenicity. *Antonie van Leeuwenhoek*, **22**, 377.

KLIENEBERGER-NOBEL, E. (1962). *Pleuropneumonia-Like Organisms (PPLO) Mycoplasmataceae*. London and New York: Academic Press.

L'ECUYER, C., SWITZER, W. P. & ROBERTS, E. D. (1961). Microbiologic survey of pneumonic and normal swine lungs. *Am. J. vet. Res.* **22**, 1020.

LEMCKE, R. M. (1965). A serological comparison of various species of Mycoplasma by an agar gel double-diffusion technique. *J. gen. Microbiol.* **38**, 91.

MORTON, H. E., SMITH, P. F. & LEBERMAN, P. R. (1949). Symbiotic growth of pleuropneumonia-like organisms with bacterial colonies. *Proc. Soc. exp. Biol. Med.* **72**, 328.

ROSS, R. F. & SWITZER, W. P. (1963). Comparison of isolates of *Mycoplasma hyorhinis* by indirect hemagglutination. *Am. J. vet. Res.* **24**, 622.

SWITZER, W. P. (1955). Studies on infectious atrophic rhinitis. IV. Characterization of a pleuropneumonia-like organism isolated from the nasal cavities of swine. *Am. J. vet. Res.* **16**, 540.

SWITZER, W. P. (1964). Mycoplasmosis. In *Diseases of Swine*, 2nd ed, p. 498. Ed. by H. W. Dunne. Ames, Iowa, U.S.A.: The Iowa State University Press.

TAKÁTSY, GY. (1955). The use of spiral loops in serological and virological micro-methods. *Acta microbiol. Acad. sci. hung.* **3**, 191.

TAYLOR-ROBINSON, D., CANCHOLA, J., FOX, H. & CHANOCK, R. M. (1964). A newly identified oral mycoplasma (*M. orale*) and its relationship to other human mycoplasmas. *Am. J. Hyg.* **80**, 135.

TAYLOR-ROBINSON, D., SOMERSON, N. L., TURNER, H. C. & CHANOCK, R. M. (1963). Serological relationships among human mycoplasmas as shown by complement-fixation and gel diffusion. *J. Bact.* **85**, 1261.

WADSWORTH, C. (1957). A slide microtechnique for the analysis of immune precipitates in gel. *Int. Archs Allergy appl. Immun.* **10**, 355.

WESSLÉN, T. & LANNEK, N. (1954). The isolation and cultivation in tissue culture of a cytopathogenic agent from pigs with enzootic pneumonia (so-called virus pneumonia). *Nord. VetMed.* **6**, 481.

J. gen. Microbiol. (1965), 41, 85–91
With 1 *plate*
Printed in Great Britain

DNA Base Composition, Flagellation and Taxonomy of the Genus *Rhizobium*

By J. DE LEY
Laboratory for Microbiology, Faculty of Sciences, State University, Ghent, Belgium

AND A. RASSEL
Laboratory for Electron Microscopy, Station for Agricultural Chemistry and Physics, Ministry of Agriculture, Gembloux, Belgium

(*Received* 23 *March* 1965)

SUMMARY

There is a correlation between the base composition of pure DNA from 35 strains of Rhizobium and their type of flagellation. There are two groups of Rhizobia. The peritrichously flagellated organisms, which usually grow fast, have a low % (guanine + cytosine) composition in the range 58·6–63·1 %; these organisms occur in all cross-inoculation groups investigated. From a comparison with literature data it appears that this group is constituted by two subgroups: *Rhizobium leguminosarum* and *R. meliloti*. The subpolarly flagellated, slow-growing strains have a somewhat higher (guanine + cytosine) content, mostly in the range 62·8–65·5 %; these organisms appear to be specialized mainly for the lupin, soybean, cowpea, Lotus, Wistaria and Robinia groups, It is proposed that only one genetic species is involved, to be called *Rhizobium japonicum*. The rhizobia would thus contain only three genetic species. The relationship between the high and low (guanine + cytosine) groups is stressed by their closely related %(G + C) values, the near-identity of the average compositional distribution of the DNA molecules and because the peritrichous organisms not infrequently show a subpolar flagellum.

INTRODUCTION

It is well established that the knowledge of the base composition (expressed as molar percentage of guanine + cytosine) of pure DNA will considerably improve and clarify the present bacterial classification.

The present paper is a contribution towards an improved classification of the rhizobia along these lines. The present classification (*Bergey's Manual*, 1957) of these bacteria is still based on the cross-inoculation groups proposed by Fred, Baldwin & McCoy (1932). Many authors have expressed dissatisfaction with this system (see Discussion) and one can expect that a future classification will look different. During the course of our work it was noticed that there is a distinct correlation between the average molar (guanine + cytosine) content (%(G + C)) and the type

of flagellation. The conclusion which emerged from it agrees remarkably well with the principle of the proposal recently made by Graham (1964), although in the latter case an entirely different approach was made, namely by Adansonian analysis.

METHODS

Organisms used. All strains with a number of two or three symbols were obtained through the courtesy of Prof. Ch. Bonnier, Chair of Biochemistry and Microbiology, Institut Agronomique de l'Etat, Gembloux, Belgium. All other strains were obtained through the courtesy of Dr L. W. Erdman, U.S.D.A., Beltsville, Md., U.S.A. Upon receipt, and later during the experiments proper, all strains were checked for bacteriological purity.

Growth conditions. Mass cultures were prepared by growing the organisms for 2–3 days at 25° in Roux flasks with 150 ml. of a modified medium of Ashby (1907) containing (in %, w/v) 1 mannitol, 0·02 NaCl, 0·05 K_2HPO_4, 0·02 $MgSO_4.7H_2O$, 0·01 $CaSO_4.2H_2O$, 0·01 $CaCO_3$, 0·1 yeast extract, 2·5 agar. The organisms were harvested; they were washed by repeated suspending in 0·15 M-NaCl, 0·1 M-EDTA buffer pH 8 and centrifuging for 30 min. at 18,000*g*. Care was taken to remove as much as possible of the polysaccharides.

Preparation and thermal denaturation of pure DNA. The centrifuged cell paste was quickly frozen by immersion of the container in a bath at −30° and lyophilized overnight. It was found that this procedure resulted in an improved cell breakage and release of DNA in the preparation proper. DNA was purified according to the procedure of Marmur (1961). The molecular weights of the samples were determined in the Spinco model E analytical ultracentrifuge and found to be in the range $10–18 \times 10^6$. Thermal denaturation was determined by automatic registration as previously described (De Ley & Van Muylem, 1963). T_m, the mid-point of the absorbance–temperature curve, was determined and the average molar (guanine + cytosine) content was calculated with the formula $0{\cdot}41[\%(G+C)] = T_m - 69{\cdot}3$ (Marmur & Doty, 1962). We have previously checked this formula by plotting T_m against direct estimations of %(G+C) (De Ley & Schell, 1963) and found it to be quite satisfactory. All results in Table 1 were calculated with this formula. If the calculations of Colwell & Mandel (1964) were followed, the %(G+C) would be 4–4·4 % lower. The standard deviation σ of the compositional distribution around the average %(G+C) was calculated from the same absorbance–temperature curve (Doty, Marmur & Sueoka, 1959).

Flagella staining. The organisms were grown on agar slants with the modified Ashby medium, containing 1 ml. of distilled water in the bottom (Rhodes, 1958). After several days at room temperature a loopful of the liquid was suspended gently in water; 35 % formalin was added to make a final concentration of 5–10 %. The organisms were centrifuged for 5 min. at 3000 r.p.m. and repeatedly washed by suspending in water and centrifuging at the same speed. A drop of the final suspension (about 10^7–10^8 cells per ml.) was applied on a perfectly clean microscope slide and dried (Leifson, 1960). The staining procedure of Rhodes (1958) was followed.

Electron microscopy. The organisms were grown in liquid medium in Kohl flasks and incubated at 20°. Every 48 hr a sample was taken in the following way: 200-

mesh grids covered with a 200 Å thick Formvar film were floated on the surface for 30 min. They were then deposited for 30–120 sec. on a 2% phosphotungstic acid solution, pH 5–7 (Brenner & Horne, 1959; Valentine & Horne, 1962). They were washed for 15 sec. in distilled water and dried in the air in Petri dishes. A Philips EM 200 electron microscope was used. Photographs were taken on Ilford 9BII and enlargements made on Gevarto 8.

RESULTS

The results are compiled in Table 1.

Table 1. *Base composition, expressed as* %$(G+C)$, *'melting points'* T_m *values, standard deviation* σ *of the compositional distribution about the mean* %$(G+C)$ *of purified DNA, and type of flagellation of several Rhizobium strains*

Strain no.	Host plant	T_m in °C	%(G+C)	σ	Cross-inoculation group
		Peritrichous			
6.2	*Lupinus luteus*	93·6	59·1	1·18	Lupin
3H0t19	*Pisum arvense*	93·85	59·7	0·44	Pea
14.1	*Vigna sinensis*	93·9	59·9	0·12	Cowpea
5.62	*Trifolium repens*	94·1	60·4	0·0	Clover
316ml	*Strophostyles pauciflora*	94·15	60·5	0·06	Strophostyles
9.5	*Phaseolus vulgaris*	94·2	60·6	0·0	Bean
3D1x3	*Trifolium dubium*	94·2	60·6	0·49	Clover
3E0b5	*Lotus americanus*	94·2	60·6	0·0	Lotus
5.0	*Trifolium pratense*	94·4	61·1	0·25	Clover
3F6g2	*Caragana arborescens*	94·5	61·3	0·75	Caragana
316c10(a)	*Phaseolus vulgaris*	94·55	61·4	0·06	Bean
3c2nl	*Lupinus densiflorus*	94·7	61·8	0·25	Lupin
10.0	*Arachis hypogea*	94·7	61·8	0·0	Cowpea
11.1	*Vicia sativa*	94·8	62·1	0·38	Pea
9.14	*Phaseolus vulgaris*	94·9	62·3	0·63	Bean
3D0a30	*Medicago sativa*	94·9	62·3	0·0	Alfalfa
3F3cl	*Wistaria frutescens*	95·0	62·5	0·88	Wistaria
1.5	*Medicago sativa*	95·0	62·5	0·60	Alfalfa
4.1	*Pisum sativum*	95·0	62·5	0·12	Pea
3F4b7	*Robinia pseudoacacia*	95·2	63·1	0·50	Robinia
		Subpolar			
3I2b1	*Erythrina indica*	94·6	61·6	0·0	Cowpea
314a8	*Pueraria thunbergiana*	95·1	62·8	0·87	Cowpea
3.9	*Soja hispida*	95·2	63·1	0·63	Soybean
3c2k4	*Lupinus* sp.	95·3	63·3	0·36	Lupin
10.1	*Arachis hypogea*	95·3	63·3	0·5	Cowpea
316n10	*Vigna sinensis*	95·3	63·3	0·37	Cowpea
3.15	*Soja hispida*	95·4	63·5	0·38	Soybean
3c3a1	*Ulex europaeus*	95·5	63·8	0·48	Cowpea
3.23	*Soja hispida*	95·5	63·8	0·12	Soybean
3.14	*S. hispida*	95·5	63·8	0·38	Soybean
3I1B59	*S. hispida*	95·6	64·0	0·96	Soybean
3.1	*S. hispida*	95·6	64·0	0·36	Soybean
3F3d	*Wistaria speciosa*	95·7	64·3	0·62	Wistaria
3.2	*Soja hispida*	95·9	64·8	0·0	Soybean
1B0a2	*Albizzia julibrissin*	96·2	65·5	1·0	Cowpea

Flagellation. The strains from the Beltsville collection were selected because their type of flagellation is known (Leifson & Erdman, 1958). Several of them were

rechecked and the type of flagellation was confirmed. The strains of the Gembloux collection were studied both by electron microscopy and by ordinary staining. The latter procedure yields less reliable results because the flagella are easily detached. This appears to happen during the drying of the drop containing the live bacteria. The use of formalin improves the results. Electron microscopy is preferable.

There are two types of strains: peritrichously (Fig. 1) and subpolarly (Fig. 2) flagellated. This confirms previous observations (Hansen, 1919; Löhnis & Hansen, 1921; Shunk, 1921; Leifson & Erdman, 1958). Several peritrichously flagellated strains, such as 5.62 from *Trifolium repens* (Fig. 3), 5.0 from *Trifolium dubium* and 11.1 from *Vicia sativa*, are surrounded by fimbriae. We are not aware that these structures have previously been observed in rhizobia.

The peritrichous organisms bear 2–5 flagella, dispersed all over the cell. Quite often one of these flagella is subpolarly implanted. There appears to be a difference between the subpolar and the peritrichous flagella of the same cell since the latter are very easily detached. In several strains only careful inspection reveals the peritrichous individuals amongst an excess of apparently subpolarly flagellated cells, which frequently still have short stumps of broken-off peritrichous flagella. This phenomenon may give rise to erroneous conclusions, e.g. such as the one by Krassilnikov (1959) that all rhizobia have polar flagella. It is also possible that peritrichous strains indeed contain a number of subpolar individuals, a situation which exists, for example, in several acetic acid bacteria (Asai, Iizuka & Komagata, 1964).

DNA base composition. All strains have a T_m in the narrow range 93·6°–96·2° and a %(G+C) between 59·1 and 65·5. The peritrichous organisms have the lowest %(G+C), ranging from 59·1 to 63·1; nearly all the subpolarly flagellated strains are in the upper range from 62·8 to 65·5 (except strain 3I2B1, which has 61·6 %). Both groups are not sharply separated; there is overlapping in the range 61·6–63·1 %(G+C). The compositional distribution σ of the DNA molecules for both groups is nearly identical: these values range from 0 to 1·2 with an average of 0·3 for the peritrichous strains and of 0·46 for the subpolar ones.

DISCUSSION

The classification within the genus Rhizobium. In the first generally accepted system Fred *et al.* (1932) proposed 16 cross-inoculation groups. Six of them were elevated to species level: *R. meliloti* (alfalfa), *R. trifolii* (clover), *R. leguminosarum* (pea), *R. phaseoli* (bean), *R. lupini* (lupin), and *R. japonicum* (soybean); the other groups did not receive species status. This is the origin of the species differentiation in *Bergey's Manual* (1957) and in Prévot's *Traité* (1961). Krassilnikov (1959) added another four species to them. Later the number of cross-inoculation groups was increased to 22 (Allen & Allen, 1947). However, innumerable exceptions show that a better system of classification is required (Wilson, 1939, 1944; Johnson & Allen, 1952; Bonnier, 1958; Lange, 1961; Manil, 1963; Graham, 1964).

A new system of classification. Graham (1964) proposed, after Adansonian analysis, that there would be only three species of rhizobia: the fast-growing *Rhizobium meliloti* and *R. leguminosarum* (a pool of *R. leguminosarum*, *R. trifolii* and *R. phaseoli* from *Bergey's Manual*) and the slow-growing *Phytomyxa japonicum* (a

renamed pool of *R. lupini*, *R. japonicum* and organisms from the cowpea group). Our results likewise advocate a renewal in the classification of the rhizobia and they are in agreement with the basic aspects of Graham's proposal. The following facts are new: (1) there is a distinct correlation between the DNA base composition and the type of flagellation: the peritrichous organisms have a low %(G+C) and the subpolarly flagellated ones have a high %(G+C); (2) within each group the %(G+C) range is very narrow, indicating that the organisms are genetically closely related; (3) the %(G+C) of both groups are close together and even overlap, an argument in favour of their close genetic relatedness.

The basis for the new classification may be summarized as follows:

(*a*) *The fast-growing, peritrichous, low (G+C) group.* This group coincides with *Rhizobium leguminosarum* and *R. meliloti* of Graham (1964). This finer distinction between species cannot be detected by the method of DNA base composition, because all strains have %(G+C) which are very close together. *R. leguminosarum* has a %(G+C) from 59·1 to 63·1. Both strains of *R. meliloti* are in the vicinity of 62·4 %. Organisms of the leguminosarum type have been isolated from almost all host plants and they thus belong to almost all cross-inoculation groups. Our results on the peritrichous flagellation of the alfalfa, clover, pea and bean groups are in perfect agreement with previous reports by Hansen (1919), Löhnis & Hansen (1921), Shunk (1921), Müller & Stapp (1925) and Leifson & Erdman (1958). Several of the other smaller groups are likewise peritrichously flagellated (Leifson & Erdman, 1958; Leifson, 1960). Table 1 shows this also to be the case for bacteria isolated from the Lotus, Strophostyles, Robinia, Caragana and Wistaria groups.

(*b*) *The slow-growing, subpolarly flagellated, high (G+C) group.* This group coincides with *Phytomyxa japonicum* from Graham (1964). Their base composition is in the range 62·8–65·5, except for an aberrant strain 312B1 which has 61·6 %. Organisms of this type have been mainly isolated from plants of the lupin, soybean and cowpea groups; a few subpolar strains from *Wistaria*, *Lotus* and *Robinia* are known. Both the results from the present paper and from Graham (1964) favour the view that only one genetic group is involved. Whether it is a species of *Rhizobium*, to be called *R. japonicum*, or a separate genus, to be called *Phytomyxa japonicum*, can only be decided unbiasedly after DNA-hybridization experiments.

(*c*) *Arguments for the close genetic relatedness of both groups.* (i) Their %(G+C) are very near and even overlap. The compositional distribution σ of their DNA molecules is small and of the same order.

(ii) Similarities in morphology and root-nodule-forming properties.

(iii) Frequently peritrichous strains bear a more firmly attached subpolar flagellum.

(iv) The Adansonian similarity is of the order of 80 % (Graham, 1964).

(v) R. Balassa (Balassa, 1963) demonstrated exchange of genetic material by transformation between strains of the alfalfa, lupin and soybean groups.

(vi) Plants of the lupin, soya, cowpea and some of the minor groups are a host to both peritrichous and subpolar strains. This is exemplified in the present paper by strains 6.2, 3c2nl and 3c2k4 from Lupinus, 10·0 and 10·1 from Arachis, 14.1 and 316n10 from Vigna, 3F3cl and 3F3d from Wistaria. Similar situations have been encountered by Wilson (1917), Fred & Davenport (1918), Löhnis & Hansen (1921), Shunk (1921), Müller & Stapp (1925) and Leifson & Erdman (1958). The peritrichous

organisms can grow in a wide variety of leguminous plants, whereas the subpolar ones are much more narrowly specialized and limited to plants of the lupin, soybean, cowpea and some of the minor groups (Lotus, Wistaria, Robinia).

One of us (J. D. L.) is indebted to the Fonds voor Collectief Fundamenteel Wetenschappelijk Onderzoek for a research and personnel grant as well as to his senior assistant Dr K. Kersters for assistance with the flagella staining. Both authors are indebted to Prof. Ch. Bonnier and Dr L. W. Erdman for providing the strains.

REFERENCES

Allen, O. N. & Allen, E. K. (1947). A survey of the nodulation among leguminous plants. *Proc. Soil Sci. Soc. Am.* **12**, 203.

Asai, T., Iizuka, H. & Komagata, K. (1964). The flagellation and taxonomy of genera *Gluconobacter* and *Acetobacter* with reference to the existence of intermediate strains. *J. gen. appl. Microbiol.* **10**, 95.

Ashby, S. F. (1907). Some observations on the assimilation of atmospheric nitrogen by a free living soil organism—*Azotobacter chroococcum* of Beyerinck. *J. agric. Sci., Camb.* **2**, 35.

Balassa, G. (1963). Genetic transformation of Rhizobium: a review of the work of R. Balassa. *Bact. Rev.* **27**, 228.

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

Bonnier, Ch. (1958). La spécificité dans la symbiose Rhizobium–légumineuses. *Antonie van Leeuwenhoek*, **24**, 1.

Brenner, S. & Horne, R. W. (1959). A negative staining method for high resolution electron microscopy of viruses. *Biochim. biophys. Acta*, **34**, 103.

Colwell, R. R. & Mandel, M. (1964). Adansonian analysis and composition of some Gram-negative bacteria. *J. Bact.* **87**, 1412.

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.

Fred, E. B., Baldwin, I. L. & McCoy, E. (1932). Root nodule bacteria and leguminous plants. *Univ. Wisc. Stud. Sci.* no. 5.

Fred, E. B. & Davenport, A. (1918). Influence of reaction on nitrogen-assimilating bacteria. *J. agric. Res.* **14**, 317.

Graham, P. H. (1964). The application of computer techniques to the taxonomy of the root-nodule bacteria of legumes. *J. gen. Microbiol.* **35**, 511.

Hansen, R. (1919). Note on the flagellation of the nodule organism of the Leguminosae. *Science*, **50**, 568.

Johnson, M. D. & Allen, O. N. (1952). Nodulation study with special reference to strains isolated from Sesbania species *Antonie van Leeuwenhoek*, **18**, 13.

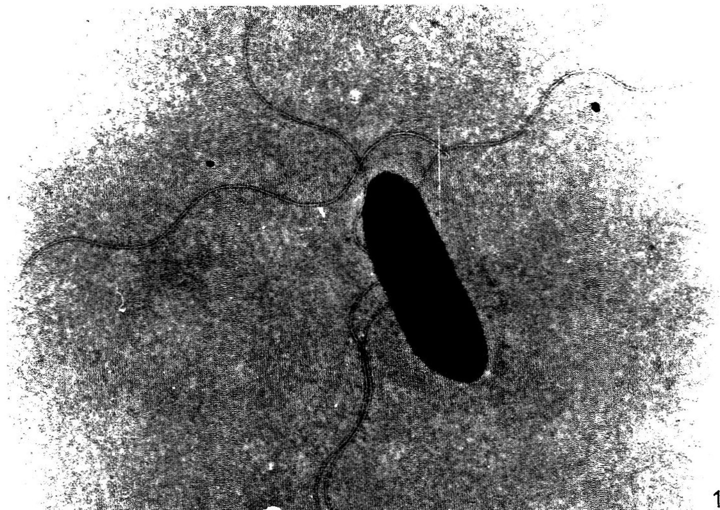
Krassilnikov, N. A. (1959). *Diagnostik der Bakterien und Actinomyceten.* Jena: VEB. Gustaf Fischer Verlag.

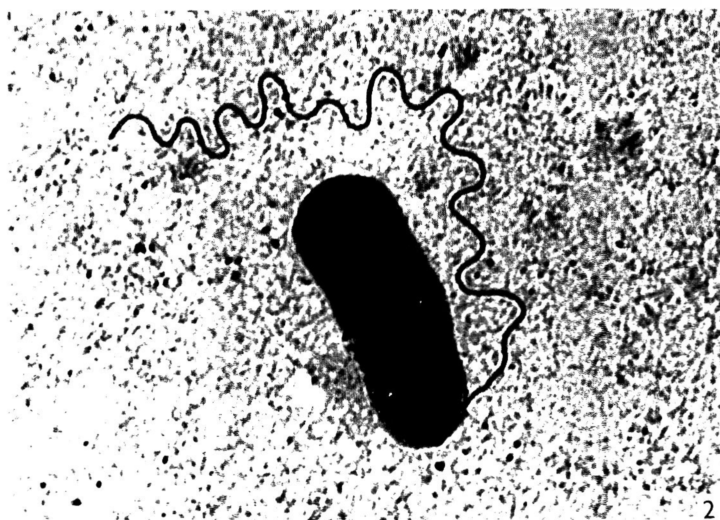
Lange, R. T. (1961). Nodule bacteria associated with the indigenous leguminosae of South-Western Australia. *J. gen. Microbiol.* **26**, 351.

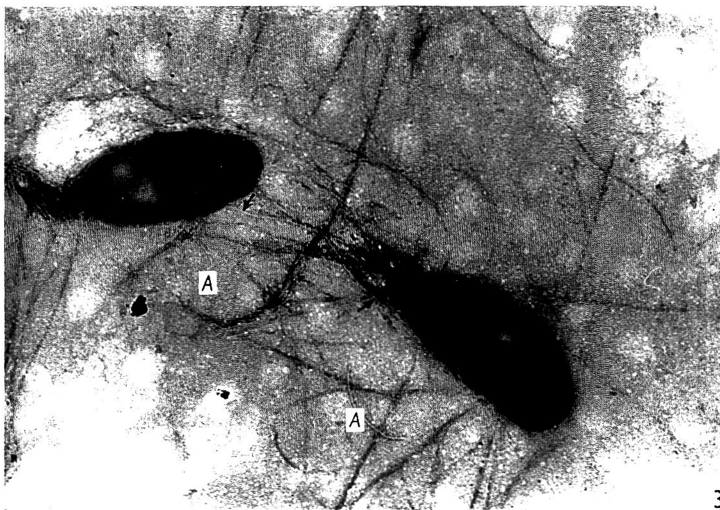
Leifson, E. (1960). *Atlas of Bacterial Flagellation.* New York: Academic Press.

Leifson, E. & Erdman, L. W. (1958). Flagellar characteristics of Rhizobium species. *Antonie van Leeuwenhoek*, **24**, 97.

Löhnis, F. & Hansen, R. (1921). Nodule bacteria of leguminous plants. *J. agric. Res.* **20**, 543.


1


2


A
A
3

MANIL, P. (1963). Les Rhizobium et la fixation symbiotique de l'azote. *Ann. Inst. Pasteur*, **104**, 19.

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.

MÜLLER, A. & STAPP, C. (1925). Beiträge zur Biologie der Leguminosenknöllchenbakterien mit besonderer Berücksichtigung ihrer Artverschiedenheit. *Arb. Biol. Reichsanst. Land-. u. Forstw.* **14**, 455.

PRÉVOT, A. R. (1961). *Traité de Systématique Bactérienne.* Paris: Dunod.

RHODES, M. E. (1958). The cytology of Pseudomonas species as revealed by a silver-plating staining method. *J. gen. Microbiol.* **18**, 639.

SHUNK, I. V. (1921). Notes on the flagellation of the nodule bacteria of Leguminosae. *J. Bact.* **6**, 239.

VALENTINE, R. C. & HORNE, R. W. (1962). An assessment of negative staining techniques for revealing ultrastructure. In *The Interpretation of Ultrastructure.* Ed. by R. J. C. Harris. *Symp. int. Soc. Cell Biol.* **1**, 263.

WILSON, J. K. (1917). Physiological studies of the *Bacillus radicicola* of the soybean and of the factors influencing nodule production. *Bull. Cornell Univ. agric. Exp. Stn*, no. 386, p. 369.

WILSON, J. K. (1939). Symbiotic promiscuity in the leguminosae. *Trans. Third Comm. Intern. Soc. Soil Sci* A, p. 49.

WILSON, J. K. (1944). Over five hundred reasons for abandoning the cross-inoculation groups of the legumes. *Soil Sci.* **58**, 61.

EXPLANATION OF PLATE

Fig. 1. Electron micrograph of a peritrichously flagellated cell of a Rhizobium, strain 10.0 from *Arachis hypogea* (22,200 ×).

Fig. 2. Electron micrograph of a subpolarly flagellated cell of a Rhizobium, strain 3.15 from *Glycine hispida* (39,500 ×).

Fig. 3. Fimbriated cells from a Rhizobium, strain 5.62 from *Trifolium repens*. Fragments of flagella (*A*) may be seen (30,000 ×).

J. gen. Microbiol. (1965), **41**, 93–98
With 2 plates
Printed in Great Britain

Chromosome Behaviour at Meiosis in *Saccharomyces cerevisiae*

By HIDEO TAMAKI
Doshisha Women's College Kyoyo, Japan

(*Received* 24 *March* 1965)

SUMMARY

The behaviour of the chromosomes of diploid strains of *Saccharomyces cerevisiae* was studied in organisms which had been treated with snail digestive juice after fixation, and then stained with the HCl–Giemsa stain. Coagulation of chromatin was observed after late prophase-I in some strains and for this reason the chromosome number of these strains could not be determined. One diploid strain (H 102 × H 9) showed no chromatin coagulation, and was shown to have eighteen bivalents at metaphase-I.

INTRODUCTION

Since the investigations of van Leeuwenhoek, cytological observations of nuclear behaviour in yeast have been made by many cytologists, but the details of the division process and the chromosome number have not yet been clarified in mitosis or in meiosis. Drs R. K. Mortimer and D. C. Hawthorne have genetic evidence indicating that *Saccharomyces cerevisiae* has a minimum of fifteen linkage groups (personal communication). In the present study an attempt has been made to determine the chromosome number of *S. cerevisiae* by direct cytological methods, and to study the movement of the chromosome at meiosis in diploid strains.

METHODS

Organisms. The following strains of *Saccharomyces cerevisiae* were used in the present work: H 9, mating type a, histidine requirer (hi_2); H 11, mating type α, histidine and uracil requirer (hi_2, ur_1); H 102 mating type α, adenine and tryptophan requirer (ad_6, tr_5); H 104, mating type a, adenine, arginine and tryptophan requirer (ad_6, ar_4, tr_5); H 203, mating type α; H 204, mating type a.

Diploid strains were obtained from the following crosses: H 11 × H 104; H 203 × H 204; H 102 × H 9. These crosses were made by the minimal plate mating technique of Takahashi, Saito & Ikeda (1958) or the mass mating technique of Lindegren & Lindegren (1943). The genotype of each diploid strain was confirmed by tetrad analysis.

Culture media. The complete culture medium had the following composition (g./l.); peptone, 10; Difco yeast extract, 10; glucose, 20. Spore-forming cultures of yeast were maintained in the sodium acetate medium of Fowell (1952) or the potassium acetate medium of McClary, Nulty & Miller (1959). Complete medium agar and sodium acetate or potassium acetate sporulation medium agar contained Bacto-agar 20 g./l.

Cytological procedures

Organisms were prepared for cytological observation by the following procedure.

1. When grown in liquid complete medium at 28° for 48 hr they were transferred to liquid sporulation medium; when they were grown on solid complete medium at 28° for 48 hr they were transferred to solid sporulation medium.

2. Organisms were collected either by centrifugation of liquid cultures, or by scraping growth from the surface of solid media with a platinum needle; they were fixed in Flemming's strong fluid for 24 hr in a cold dark room. The fixed materials were washed five times with distilled water by repeated centrifugations, and material which showed a darker appearance was bleached in 3 % H_2O_2 solution and washed twice as before.

3. The next step was treatment with snail digestive juice at 28° for 30 min., washing off the residue by three successive centrifugations in distilled water. This facilitated the subsequent smearing of the fixed cell materials. The juice was got from snails which had not been fed for 2 days, and it was sterilized by antibiotics without dilution.

4. The materials were hydrolyzed with N-HCl at 60° for 16 min., washed three times as before, and the final water suspension spread on clean cover glasses and dried at room temperature.

5. The material which adhered to the cover glass was stained with diluted Giemsa solution (1 vol. Giemsa stock solution diluted with 30 vol. 0·02 M-phosphate buffer, pH 6·8) at 28° for 7 min. and washed with the same buffer solution. The stained material was then mounted and squashed.

In addition to the Giemsa stain, the Feulgen stain and methyl green (1 %) were also used. The results obtained were the same, i.e. the same bodies (chromatin in meiosis) were well stained. The Giemsa stain was preferred for further study, since it gave somewhat better staining.

RESULTS

Nuclear behaviour in meiosis of Saccharomyces cerevisiae diploid strain H 11 × H 104

The *Saccharomyces cerevisiae* diploid strain H 11 × H 104, was grown in liquid sporulation medium with aeration at 28° and samples prepared every 24 hr as described in Methods. Various shapes were observed in each sample; for convenience, these have been divided into seven stages as shown Pl. 1, figs. A–G. The frequencies of appearance of each stage after different times of incubation are shown in Table 1. Stage A cells and possibly some of the stage E cells will sporulate; B, C and D are intermediate stages in sporulation. Stage F shows tetrad formed at completion of sporulation.

The coagulation of chromatin observed in this strain in liquid and solid sporulation cultures, was also observed in the diploid strain H 203 × H 204. Because of this high chromatin coagulation, it was not possible to determine the chromasome number from these figures.

Meiosis of Saccharomyces cerevisiae diploid strain H *102* × H *9*

As shown in Table 1, after *Saccharomyces cerevisiae* strain H 11 × H 104 had been kept for 24 hr on sporulation medium, metaphase I was observed frequently, but no tetrads. After 48 hr, however, all the stages of meiosis were observed. Accordingly, the *S. cerevisiae* diploid strain H 102 × H 9 was incubated on a solid complete medium at 28° for 48 hr, then transplanted to solid sporulation medium and fixed after incubation for 48 hr. Plate 2, figs. A–C, shows three stages of the prophase of meiosis I in strain H 102 × H 9. In the case of strains H 11 × 104 or H 203 × H 204,

Table 1. *Frequency of meiotic stages at spore formation in Saccharomyces cerevisiae cross* H *11* × H *104*

		Times after culture on spore forming media (hr)					
		0	24	48	72	96	120
Stage A	Observed	56*	36	7	0	0	0
	%	25·34	10·06	1·93	0	0	0
Stage B	Observed	0	26	15	2	0	0
	%	0	7·26	4·14	0·74	0	0
Stage C	Observed	0	43	33	31	0	0
	%	0	12·01	9·12	11·40	0	0
Stage D	Observed	0	114	62	80	47	0
	%	0	31·84	17·13	29·41	20·17	0
Stage E	Observed	132†	31	109	33	78	81
	%	59·73	8·66	30·11	12·13	33·48	36·99
Stage F	Observed	0	0	31	29	32	57
	%	0	0	8·56	10·66	13·73	26·02
Stage G	Observed	33	108	105	97	76	81
	%	14·93	30·17	29·01	35·66	32·62	36·99
Total	Observed	221	358	362	272	233	219
	%	100	100	100	100	100	100

Stages A–G correspond with those of Pl. 1, figs. A–G, respectively.
* Mitotic figure. † Stage of Pl. 1, Fig. H.

some of the chromatin showed coagulation as well as heteropycnosis in late prophase. But in the case of strain H 102 × H 9, no coagulation into chromatin bodies was observed. Plate 2, fig. C respresents diakinesis and Pl. 2, fig. J is a diagrammatic sketch of the chromosomes of Pl. 2, fig. C. Plate 2, fig. D shows metaphase I with eighteen bivalents; one of twelve cells examined which showed essentially the same number of bivalents; fig. K is a sketch of fig. D. Plate 2, fig. E may be taken as anaphase I, since the chromosomes appear to be separated into two poles. The next expected stage should be a diad stage, but no such stage was seen. Plate 2, fig. F shows what may be a stage of metaphase II: 36 chromosomes were easily counted (see sketch, fig. L). Plate 2, fig. G shows anaphase II and 58 chromosomes were counted. The number of chromosomes in anaphase II ought to be 72, according to the number found at metaphase I (Pl. 2, fig. D), but some of the chromosomes might have been pushed out of the cell by the squash method used. The following stage should be the distribution of these chromosomes of four daughter nuclei (Pl. 2, fig. H), with subsequent formation of a discrete tetrad (Pl. 2, fig. I).

The strains used in this study seem to be normal diploids. Polyploicy would be expected to be detected through irregular segregation of genetic markers (Roman, Phillips & Sands 1955, Mortimer, 1958). The results of tetrad analysis of 32 asci showed decreased spore viability (Table 2). The decreased viability suggests poly-polyploidy or polysomy, but the segregation ratios are consistent only with this strain being diploid. More extensive analysis with markers for each linkage group should be undertaken to settle this point.

Table 2. *Segregation of three markers among ascospores of Saccharomyces cerevisiae cross* H *102* × H *9 diploid strain. Only asci containing four spores were dissected*

Ascus	Segregational ratio AD:ad	HI:hi	TR:tr
	4 viable spores		
8	2:2	2:2	2:2
12	2:2	2:2	2:2
26	2:2	2:2	2:2
	3 viable spores		
3	2:1	1:2	2:1
5	1:2	1:2	2:1
10	2:1	1:2	1:2
14	1:2	2:1	1:2
19	1:2	1:2	1:2
	2 viable spores		
1	2:0	1:1	1:1
9	1:1	1:1	1:1
13	2:0	2:0	1:1
16	1:1	0:2	2:0
18	1:1	1:1	2:0
21	0:2	1:1	1:1
23	2:0	2:0	1:1
24	1:1	1:1	0:2
	1 viable spore		
2	1:0	0:1	0:1
4	1:0	1:0	1:0
6	1:0	0:1	0:1
7	0:1	0:1	0:1
11	1:0	1:0	0:1
15	0:1	1:0	1:0
17	1:0	0:1	0:1
20	1:0	0:1	0:1
22	0:1	1:0	1:0
25	0:1	1:0	0:1
	0 viable spore		
27	0:0	0:0	0:0
28	0:0	0:0	0:0
29	0:0	0:0	0:0
30	0:0	0:0	0:0
31	0:0	0:0	0:0
32	0:0	0:0	0:0
Total	29:24	26:27	25:28
	$\chi^2 = 0{\cdot}472$	$\chi^2 = 0{\cdot}008$	$\chi^2 = 0{\cdot}170$
	$0{\cdot}5 < P < 0{\cdot}75$	$0{\cdot}95 < P < 0{\cdot}99$	$0{\cdot}75 < P < 0{\cdot}9$

DISCUSSION

Many observations have been made by several investigators about the chromosome number in yeast, but the numbers reported hitherto have varied considerably and no definite conclusion has been reached. For example, Swellengrabel (1905). Fuhrmann (1906), Badian (1937), Shinoto & Yuasa (1941), and DeLamater (1950) reported that the diploid chromosome number in a presumably diploid yeast was four. Levan (1947) reported at least ten chromosomes in the mycelial type of a brewery yeast cell after camphor treatment. Leitz (1951) found three chromosomes in the haploid and six in the diploid stages of *Zygosaccharomyces priorianus*. Subramaniam (1946) presented evidence for what he considered to be two chromosomes in a brewery yeast, but subsequently, Prahlada Rao & Subramaniam (1952) found four, six and eight chromosomes in this organism, and concluded that these variations were due to 'endopolyploidy'. After examining many diploid, triploid and tetraploid cells of a strain of Saccharomyces, McClary, Williams, Lindegren & Ogur (1957) came to the conclusion that the haploid chromosome number is four.

All these demonstrations were made by using certain squash methods or preparations which did not yield satisfactory mitotic and meiotic figures. Also some of the difficulties may be attributed to unusual behaviour of the strains used by many of these investigators; i.e. some strains showed very high incidence of chromatin coagulation throughout the mitotic or meiotic cycles similar to that shown by our strain *S. cerevisiae* H 11 × H 104. It is not known whether this coagulation results from endopolyploidy, as postulated by Subramaniam, or from other causes.

The success of the present studies is the result of the use of snail digestive juice, which facilitated the spreading of cells and chromosomes into one plane. For this reason, the normal orientation of the chromosomes during the different stages of meiosis was disturbed. A similar attempt was made by Yuasa (1960) with a lytic enzyme preparation from Streptomyces, and which was also capable of digesting yeast cell walls. However, the enzyme used by Yuasa was not helpful in discerning individual chromosomes. The present observation is well supported by recent genetic studies of Drs R. K. Mortimer and D. C. Hawthorne (personal communication). They have obtained evidence for at least fifteen linkage groups based on independently segregating centromeres and, in addition, have identified three more groups of genes that are not yet associated with any of the established linkage groups.

The support of the Research Grant of Doshisha Women's College for this project is gratefully acknowledged. The author wishes to thank Drs E. Balbinder and R. K. Mortimer for valuable help in the preparation of the manuscript.

REFERENCES

Badian, J. (1937). Sur la cytologie des levures. *Bull. intern. acad. polon. sci., Classa sci. math. nat. Bi.* (1–5 B 1). 61.

DeLamater, E. D. (1950). The nuclear cytology of the vegetative diplophase of *Saccharomyces cerevisiae*. *J. Bact.* **60**, 321.

Fowell, R. R. (1952). Sodium acetate agar as a sporulation medium for yeasts. *Nature, Lond.* **170**, 578.

Fuhrmann, F. (1906). Die Kernteilung von *Saccharomyces ellipsoideus*. *Zbl. Bakt., II*, **15**, 769.

LEITZ, K. (1951). Beitrag zur Hefecytologie. *Arch. Mikrobiol.*, **16**, 275.
LEVAN, A. (1947). Studies on the camphor reaction of yeast. *Hereditas*, **33**, 431.
LINDEGREN, C. C. & LINDEGREN, G. (1943). A new method for hybridizing yeast. *Proc. Nat. Acad. Sci., Wash.* **29**, 306.
McCLARY, D. O., NULTY, W. L. & MILLER, G. R. (1959). Effect of potassium versus sodium in the sporulation of *Saccharomyces*. *J. Bact.* **78**, 362.
McCLARY, D. O., WILLIAMS, M. A., LINDEGREN, C. C. & OGUR, M. (1957). Chromosome counts in a polyploid series of *Saccharomyces*. *J. Bact.* **73**, 360.
MORTIMER, R. K. (1958). Radiobiological and genetic studies on a polyploid series (haploid to hexaploid) of *Saccharomyces cerevisiae*. *Radiation Research* **9**, 312.
PRAHLADA RAO, L. S. & SUBRAMANIAM, M. K. (1952). Somatic chromosomal reduction in yeast. *Arch. Mikrobol.*, **17**, 160.
ROMAN, H., PHILLIPS, M. M. & SANDS, S. M. (1955). Studies of polyploid *Saccharomyces*. I. Tetraploid segregation. *Genetics*, **40**, 546.
SHINOTO, Y. & YUASA, A. (1941). Karyological studies in *Saccharomyces cerevisiae*. *Cytologia*, **11**, 464.
SUBRAMANIAM, M. K. (1946). Studies on the cytology of yeasts. I. Mitosis in *Saccharomyces cerevisiae*. *Proc. Natn. Inst. Sci. India*, **12**, 143.
SWELLENGREBEL, M. (1905). Sur la division nucleaire de la levure pressee. *Ann. Inst. Pasteur*, **19**, 505.
TAKAHASHI, T., SAITO, M. & IKEDA, Y. (1958). Heterothallic behaviour of a homothallic strain *Saccharomyces* yeast. *Genetics*, **43**, 249.
YUASA, A. (1960). The spindle of the yeast-cell. *Bot. Mag. Tokyo*, **73**, 474.

EXPLANATION OF PLATES

PLATE 1

Figs. A–H. Meiosis in *Saccharomyces cerevisiae*, cross H 11 × H 104. Magnification × 3600

Figs. A–C. These stages are considered to correspond to the prophase of meiosis.

Fig. A. The resting nucleus (Fig. H) has started to divide.

Fig. B. Middle prophase; note clarified chromosome and chromatin.

Fig. C. Late prophase; some of the chromatin already coagulated and showing heteropycnosis.

Fig. D. Probably metaphase I, but showing considerable coagulation of chromatin; the number of chromatin masses was not constant from cell to cell.

Fig. E. Advanced stage of meiosis I; the chromatin coagulation has become more pronounced and one or more masses of chromatin are visible.

Fig. F. Tetrad.

Fig. G. Cells with diffuse nuclei; these cells were observed at almost equal frequencies in materials fixed after different times of growth on sporulating media (see Table 1). Accordingly, it seems reasonable to assume that these cells have no relation to spore formation.

Fig. H. Cell with resting nucleus.

PLATE 2

Figs. A–L. Meiosis in *Saccharomyces cerevisiae* cross H 102 × H 9. All magnifications × 3600

Figs. A–C. Stages of prophase of meiosis I.

Fig. C. Represents diakinesis.

Fig. D. Metaphase I with eighteen bivalents.

Fig. E. Anaphase I.

Fig. F. Metaphase II.

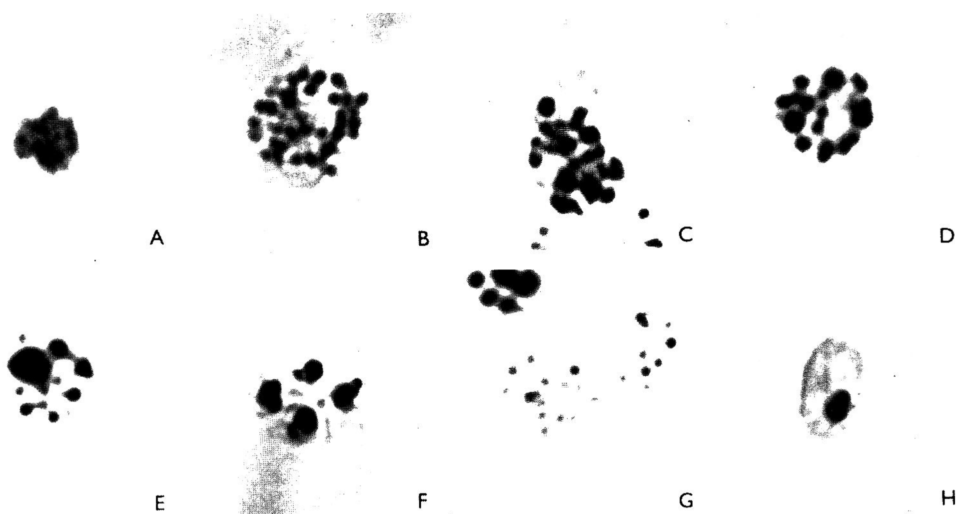
Fig. G. Anaphase II.

Fig. H. Telophase II.

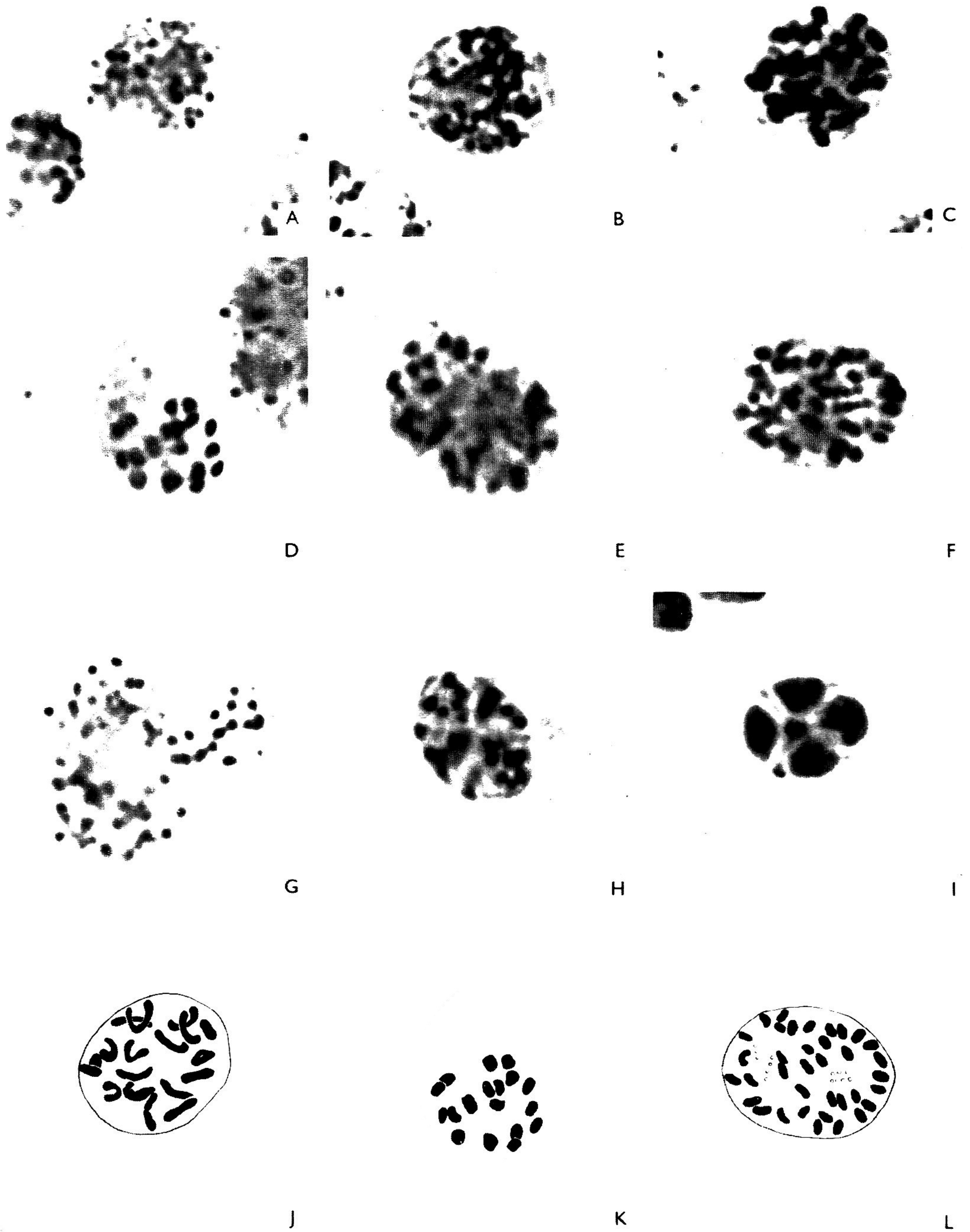
Fig. I. Tetrad.

Figs. J, K and L are diagrammatic representations of photographs C, D and F.

White rings of Fig. L represents unstained granules.



H. TAMAKI (*Facing p.* 98)



H. TAMAKI

J. gen. Microbiol. (1965), **41**, 99–107
Printed in Great Britain

Metal-Complexing Agents as Metal Buffers in Media for the Growth of *Aspergillus niger*

By A. QADEER CHOUDHARY and S. J. PIRT
Microbiology Department, Queen Elizabeth College, University of London, W. 8

(*Received* 8 *April* 1965)

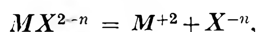
SUMMARY

The influence of metal-complexing agents on the mycelial growth rate, conidial germination and morphology of *Aspergillus niger* in shake-flask cultures was studied. The agents tested were: ethylenediaminetetra-acetic acid (EDTA), diaminocyclohexane-*N*,*N*-tetra-acetic acid (CDTA), diethylenetriaminepenta-acetic acid (DTPA), and nitrilotriacetic acid (NTA), which form soluble complexes, and ferrocyanide, which forms insoluble complexes. The agents were added singly to culture media in concentrations up to 9·4 mM. The sum of the concentrations of the complex-forming metals (Fe, Cu, Zn and Mg) added to the medium was 1·04 mM. At pH 6·5 mycelial growth rate was almost unaffected by any of the agents when they were added after the germination of conidia. At pH 3·5 ferrocyanide at 84 μM increased the mycelial doubling time by 30 %, but the other agents had little effect on the mycelial growth rate at pH 3·5. EDTA, CDTA and DTPA at 1·04 mM inhibited germination of conidia at pH 6·5; lowering the pH partially relieved this inhibition. Ferrocyanide at 9·4 mM was without effect on germination at pH 6·5, but at pH 3·5 it strongly inhibited germination at 84 μM. The morphology of the organism was markedly affected by EDTA, CDTA, DTPA and ferrocyanide but not by NTA. In cultures without complexing agents or with NTA the organism grew in the form of large pellets with a filamentous periphery; such pellets formed a gelatinous conglomerate in the culture. With either EDTA, CDTA or DTPA at 1·04 mM or ferrocyanide at 84 μM, the organism grew in the form of small pellets with a smooth exterior; such pellets remained discrete and dispersed in the medium. These physiological effects are attributed to changes in the metal ion concentrations in the medium brought about by the complexing agents.

INTRODUCTION

The trace-metal concentrations in culture media may be controlled in either of two ways. The commoner method is to purify the medium so as to remove certain metal ions and then add known amounts of the corresponding metal salts. This method is exacting, laborious and subject to failure by recontamination of the medium from dust and the walls of vessels. A newer and as yet little used method is to add metal-chelating agents to the medium so as to decrease the concentration of free metal ions to the desired values. This method has the advantage that the metal complex acts as a 'metal buffer' which reversibly dissociates so as to replace free metal ions as they are consumed by a growing organism, or to combine with metal ions added to the system. For wider aspects of the subject the symposium in

Federation Proceedings (1961) should be consulted. Many metal-complexing agents are available but their physiological effects have been little investigated and need to be better known if metal buffers are to be used more widely. The object of the present work was to study the effect of some metal-complexing or chelating agents on the growth of *Aspergillus niger*. The accumulation of citric acid in the medium when this mould is grown is a process known to be sensitive to the concentrations of trace metals. The theory of metal-complexing agents, which is analogous to that of hydrogen-ion buffers, has been treated, for example, by Chaberek, Bersworth & Martell (1955). The dissociation of a simple metal complex, MX, is represented by



$$MX^{2-n} = M^{+2} + X^{-n},$$

where M^{+2} is the metal ion and X^{-n} is the anion of the chelating agent of valency n. The 'stability constant' K is given by

$$K = \frac{[MX^{2-n}]}{[M^{+2}][X^{-n}]},$$

where the brackets indicate molar concentrations. The stability constant is used as a measure of the degree of chelation. Values of the stability constants for the metals and chelating agents used in this work are given in Table 1.

Table 1. *Log K values (stability constants) of metal chelates**

Metal	DHEG	NTA	EDTA	CDTA†	DTPA
Fe^{3+}	?	15·87	25·1	31·0	28·6
Cu^{2+}	8·15	12·68	18·8	21·3	21·1
Zn^{2+}	5·36	10·45	16·5	18·67	18·3
Fe^{2+}	4·31	8·84	14·33	?	16·5
Mn^{2+}	3·15	7·44	14·04	16·78	?
Mg^{2+}	1·15	7·00	8·69	10·32	?

* All values from Bjerrum, Sillèn & Schwarzenbach (1957), except † from Simons, Swidler & Benedict (1963).
? = Not known.

The removal of iron and other biologically important metals from molasses by the formation of complexes with ferrocyanide ions was suggested by Mezzadroli (1938) in order to make this cheap source of carbohydrate suitable for citric acid production by *Aspergillus niger*. Since then, potassium ferrocyanide has been extensively used for the treatment of molasses to be used as an ingredient in media for citric acid production. Ferrocyanide ions form highly insoluble precipitates with the ions of many heavy metals. The order of increasing solubility of metallic ferrocyanides of importance in this study is as follows: Fe^{3+}, Cu^{2+}, Zn^{2+}, Mn^{2+}, Fe^{2+}, Mg^{2+} (*American Cyanamid*, 1953). It has proved useful to compare the biological actions of the soluble chelate–metal complexes with those of the insoluble ferrocyanides. In this paper the effects on the growth of *Aspergillus niger* are considered.

METHODS

Strains of organism. The strain of *Aspergillus niger*, Wis 72-4, kindly supplied by Professor M. J. Johnson (Biochemistry Department, University of Wisconsin), was used. Some work was done with the Kew strain IMI-31821(2) derived from Wis 72-4, and supplied by the Mycological Institutue, Ferrylane, Kew, Surrey, U.K.

Media. The compositions of the media used are given in Table 2. All reagents were of analytical grade. Glass-distilled water was used for the preparation of solutions unless otherwise stated. All media, unless otherwise stated, were autoclaved at 121° for 15 min. Media were divided into several parts, in graduated bottles, for sterilization: the parts were: for medium M1: (1) glucose 50%, w/v; (2) KH_2PO_4; (3) NH_4NO_3; (4) $FeCl_3$; (5) $MgSO_4 + CuSO_4 + ZnSO_4$. The salt solutions were ten times concentrated. Medium M3 was sterilized in three parts: (1) glucose 50%, w/v; (2) $(NH_4)_2SO_4$ ten times concentrated; (3) inorganic salt solutions containing citrate buffer, double strength. Decrease of volume, because of evaporation in the autoclave, was made up in the graduated bottles before mixing all the solutions aseptically. The pH value of a medium was adjusted with sterile N-NaOH or N-HCl after mixing all solutions.

Table 2. *Composition of media used for growth of Aspergillus niger*

Constituents	M1	M2	M3	M4*
	g./l.			
Glucose	150	150	150	91
Agar	20	0	0	30
NH_4NO_3	2·5	2·5	0	0·45
$(NH_4)_2SO_4$	0	0	4·7	0
KH_2PO_4	1·0	2·5	2·5	0
$MgSO_4.7H_2O$	0·25	0·25	0·25	0·06
Na citrate buffer (pH 6·4)†	0	0	Present	0
	mg./l.			
Trace metals‡				
Fe^{3+}	2·20	1·3	1·3	0
Cu^{2+}	0·48	0·06	0·06	0
Zn^{2+}	3·80	0·25	0·25	0
	pH value			
	4·5	3·5	6·5	4·5

* Also contained beer (English bitter), 60 ml./l.
† 0·054 mM citrate, pH adjusted with NaOH.
‡ Fe as $FeCl_3$; Cu as $CuSO_4$; Zn as $ZnSO_4$.

Inoculum preparation. Stock cultures were maintained as freeze-dried conidial suspensions. The mould was transferred from the stock culture to surface cultures in medical flat bottles plugged with cotton-wool. The bottles were of 8 oz. capacity and contained 15 ml. agar medium. Medium M1 was used for strain 72-4 and medium M4 for the Kew strain. The bottles were incubated at 30° for 5–7 days. To wash off the spores the cultures were wetted with 5 ml. of 0·05% aqueous solution of 'Monoxal O.T.' (di-octyl ester of sodium sulphosuccinic acid). The agar was washed twice with sterile distilled water and the combined washings were made to 50 ml. and shaken with glass beads to break up clumps of conidia. The number of conidia was determined with a Thoma counting chamber. The final stock conidial suspension was adjusted to contain 50 million conidia/ml.; 1 ml. of this suspension was used to inoculate 25 ml. medium.

Shake-flask culture. For shake-flask culture 25 ml. medium including 1 ml. conidial suspension was held in a 250 ml. conical flask plugged with cotton-wool. The flasks were shaken on a rotary shaker (throw 25 mm., 180 cyc./min.) and incubated at 25°. In the kinetic experiments on growth a series of shake-flasks were set up and duplicate flasks removed at intervals for analysis.

Stirred-fermenter culture. Aspergillus niger, strain 72-4, was also grown in a 2 l. fermenter of the type described by Elsworth, Meakin, Pirt & Capell (1956). The vortex aeration system (Callow & Pirt, 1961) was used. The impeller was a vaned disc of 63 mm. diameter and vane depth 10 mm.; the stirrer speed was 1120 rev./min. Polyglycol P2000 (R. W. Greef and Co., London) was added at a concentration of 0·2 ml./l. to prevent foaming.

Metal-complexing agents. The chelating agents were dissolved and brought to pH 7–8 by adding NaOH. Potassium ferrocyanide was used as source of ferrocyanide ions; the solution was sterilized by membrane filtration.

Mycelial dry weight was determined by filtering off mycelium in a tared sintered glass crucible (porosity 2), washing the mycelial mat thoroughly with water and drying to constant weight at 100–105°.

RESULTS

Effects of chelating agents and ferrocyanide on mycelial growth rates of Aspergillus niger

To determine the mycelial growth rate, mycelial dry weights in shake-flask cultures were determined at intervals after the conidia had germinated, that is about 24 hr after inoculation. On plotting the logarithms of dry weights against time, a straight line was obtained. An example of such a plot is shown in Fig. 1. This straight line relation shows that growth of the mycelium followed the exponential law, $dx/dt = \mu x$, where x is the mycelial dry weight, t is time and μ is a constant called the 'specific growth rate'. The doubling time of the mycelium (t_d) is given by the formula $t_d = (\log_e 2)/\mu$. The growth rates of the mycelium under different conditions are expressed in terms of the specific growth rate or the doubling time. To examine the effects of chelating agents on mycelial growth rates, these

Table 3. *Effects of chelating agents added after germination on the mycelial doubling times of Aspergillus niger strain 72-4*

Concn. (mM)	Chelating agent			
	CDTA	DTPA	EDTA	NTA
Initial pH 6·5; medium M3, doubling time (hr)				
0·00	6·9	6·9	6·9	6·9
1·04	7·5	7·4	7·4	7·2
3·13	7·5	7·5	7·5	7·5
9·40	8·1	8·0	7·3	7·2
Initial pH 3·5; medium M2, doubling time (hr)				
0·00	7·7	7·7	7·7	7·7
1·04	8·0	7·7	7·7	7·7
3·13	8·0	8·1	8·0	7·9
9·40	8·3	8·3	8·0	7·9

agents were added to shake-flask cultures after the conidia had germinated, and the doubling times determined (Table 3). The coefficients of variation of the doubling times were 6 % in medium M3 and 7 % in medium M2. The pH value decreased from 6·5 to 3·5 in medium M3 and from pH 3·5 to 1·8 in medium M2 over 48 hr. The exponential growth phase in both media generally came to an end about 55 hr after inoculation.

The growth rates were only slightly decreased by increasing the molarity of the chelating agent up to nine times the summed molarity of Mg+Fe+Cu+Zn. The addition of the chelating agents did not affect the maximum mycelial dry weight attained.

Addition of ferrocyanide to the medium gave rise to a blue colour which may be attributed to formation of Prussian blue, a ferric ferrocyanide complex. The mycelial growth rates obtained when potassium ferrocyanide was added after the conidia had germinated are given in Table 4. The pH value of the medium had a marked effect on the action of ferrocyanide: at pH 6·5 the growth rate was hardly affected by ferrocyanide to 9·4 mM, whereas, at pH 3·5, 84 μM ferrocyanide appreciably decreased the growth rate.

Table 4. *Effect of ferrocyanide on the mycelial doubling time of Aspergillus niger strain 72-4*

Ferrocyanide (mM)	Doubling time (hr)
Initial pH 6·5; medium M3	
0·00	6·9*
1·04	7·2
3·13	7·6
9·40	7·2
Initial pH 3·5; medium M2	
0·00	7·7†
84 μM	10·0

* Standard deviation 0·052 hr.
† Standard deviation 0·071 hr.

Effect of chelating agents and ferrocyanide on germination of conidia

Some of the chelating agents tested markedly inhibited the germination of conidia. The effects of the agents on germination were determined by adding them to the medium before inoculation with conidia. Inhibition of germination showed as an increase in the lag before the exponential increase of mycelial weight occurred (Fig. 2). The lag increased progressively with increase in concentration of EDTA. When EDTA at 9·4 mM was present before the conidia germinated it had a more marked inhibitory effect on mycelial growth than when added after germination. At the lower concentrations, however, the only effect of EDTA was to inhibit germination and the subsequent mycelial growth rate was unaffected. Table 5 shows the effects of CDTA, DTPA, EDTA and NTA on the germination period and subsequent growth rate of *Aspergillus niger* strain 72-4; similar results were obtained with the Kew strain. The most powerful inhibitor of germination was CDTA; at

3·13 mM and pH 6·5 it completely inhibited germination. In contrast, NTA was without effect on germination up to 9·4 mM at pH 3·5 and 6·5. The effect of decreasing the pH value of the medium from 6·5 to 3·5 was to alleviate the inhibition (except with NTA). Since decreasing the pH value would decrease the degree of metal chelation the pH effect indicates that the inhibition was due to metal chelation.

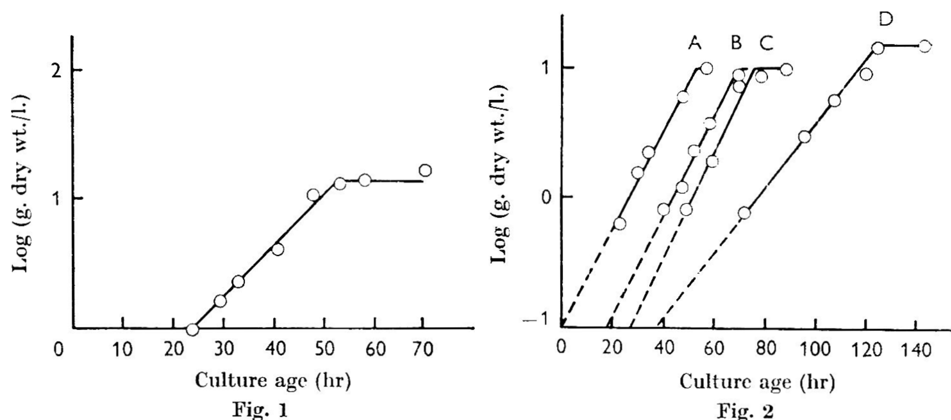


Fig. 1. Mycelial growth curve for *Aspergillus niger* strain 72-4 in medium 3 in shake-flasks.

Fig. 2. Increase in the lag (germination period) of *Aspergillus niger* strain 72-4 brought about by EDTA in medium M3 in shake-flask cultures. A, No EDTA; B, 1·04 mM-EDTA; C, 3·13 mM-EDTA; D, 9·4 mM-EDTA.

Table 5. *Effect of chelating agents on germination period and subsequent mycelial doubling time of Aspergillus niger strain 72-4*

Chelating agent concn. (mM)	CDTA: Increase in germination period (hr)	CDTA: Doubling time (hr)	DTPA: Increase in germination period (hr)	DTPA: Doubling time (hr)	EDTA: Increase in germination period (hr)	EDTA: Doubling time (hr)	NTA: Increase in germination period (hr)	NTA: Doubling time (hr)
				Initial pH 6·5; medium M3				
0·00	0	6·9	0	6·9	0	6·9	0	6·9
1·04	35	8·5	18	7·5	18	7·2	0	7·5
3·13	No germination	—	21	7·2	27	7·2	0	7·2
9·40		—	40	9·3	38	11·6	0	7·5
				Initial pH 3·5; medium M2				
0·00	0	7·7	0	7·7	0	7·7	0	7·7
1·04	24	9·2	5	8·4	7·5	9·2	0	7·6
3·13	31	13·0	12	8·5	21·0	9·2	0	8·0
9·40	No germination	—	23	8·7	33·0	16·2	0	8·4

Standard deviations of doubling time in control media M3 and M2 were 0·052 and 0·071 hr. respectively.

Ferrocyanide up to 9·4 mM was without effect on germination in medium M3 at pH 6·5; but in medium M2 at pH 3·5 it was a powerful inhibitor of germination; concentrations above 28 μM prevented germination.

Effect of increasing EDTA and metal concentrations in parallel

An experiment was done to see whether inhibition of germination and of the subsequent mycelial growth by EDTA could be antagonized by adding more metal ions. The EDTA concentration was increased up to 9·4 mM and extra amounts of the Mg, Fe, Cu and Zn sources were added to keep the EDTA: metal ratio constant. The lag period brought about by inhibition of germination, and the mycelial growth rates in the medium after inoculation with conidia are given in Table 6. These results show that the inhibition of germination and of mycelial growth was not antagonized by adding extra Mg, Fe, Cu and Zn salts. The failure to prevent the inhibition may be attributed to chelation of some trace metal other than one of those added, or to some effect of the chelating agent other than to deprive the organism of metal ions.

Table 6. *Effect of increasing concentrations of EDTA and of metal ions (Mg + Fe + Cu + Zn) on the lag (germination) period and growth rate of strains of Aspergillus niger*

		Strain 72-4		Kew strain	
EDTA concn. (mM)	Metal concentration relative to concentration in medium M3	Increase in lag period (hr)	Doubling time (hr)	Increase in lag period (hr)	Doubling time (hr)
0·00	1	0	6·9	0	6·3
1·04	1	19	7·3	21·0	8·2
3·13	3	26	8·0	24·0	8·6
9·40	9	37	12·1	39·0	17·3

Effects on mould morphology. Ferrocyanide and EDTA, CDTA and DTPA affected the morphology of the mould growth in shake-flask cultures. In medium M2 without complexing agents the mould grew as a viscous conglomerate of pellets (average diameter 2 mm.) with a filamentous periphery. The filamentous exteriors of the pellets interlocked to form a gelatinous conglomerate. In the presence of 1·04 mM-EDTA, CDTA, DTPA or 84 μM-ferrocyanide, the mould formed smaller pellets (average diameter, 1·3 mm. with EDTA; 0·7 mm. with ferrocyanide) with smooth exteriors. Such pellets remained dispersed in the medium as granules. In contrast, addition of NTA did not affect the mould morphology.

The highly viscous conglomerate was obviously much less readily agitated and aerated than the smooth pellet suspension. Considerable significance is attached to this morphological alteration in the citric acid fermentation, the smaller smooth pellets being considered desirable (Clark, 1962). It was also observed that citrate added as a pH buffer in medium M3 caused the formation of smooth separate pellets, like those obtained with EDTA, except that the citrate pellets were slightly larger. Addition of EDTA, CDTA, DTPA or ferrocyanide to medium M3 decreased the pellet size to that usually obtained with the complexing agent.

The effect of EDTA, CDTA and DTPA on morphology was modified when a

conidial rather than a mycelial inoculum was used. When a conidial inoculum was used, smooth pellets were obtained only when the concentration of chelating agent was 1·04 mM, not at 3·13 or 9·40 mM.

Stirred fermenter culture. The mould was grown in medium M2 in the stirred fermenter with excess of available oxygen and with growth limited by the oxygen supply. Under both these conditions the organism grew in the form of a uniformly filamentous and highly viscous mass; it did not form pellets, as was the case in shake-flasks.

The growth rate was decreased in the stirred fermenter, the doubling time in medium M2 being 13 hr as compared with 7·7 hr in shake-flasks with the same medium. Addition of ferrocyanide (126 μM) slightly decreased the growth rate but did not affect the morphology of the mould growth. It is concluded that vigorous stirring can counteract the tendency of the organism to grow in the pellet form.

DISCUSSION

Exponential growth of filamentous moulds in mass culture is now becoming generally recognized; it has been previously reported for Neurospora (Zalokar, 1959), Penicillium (Pirt & Callow, 1960) and Gibberella (Borrow *et al.* 1964). *Aspergillus niger* is now shown to be another mould which will grow exponentially under mass-culture conditions. The achievement of exponential growth greatly facilitates growth studies in that the mycelium doubling time can be used for the quantitative comparison of growth rates.

The effects of the metal-complexing agents on growth of two *Aspergillus niger* strains have been analysed in terms of growth rate, germination period and morphological changes. The nature of the effects on growth produced by the complexing agents were qualitatively similar but differed quantitatively, and were also considerably dependent on the pH value of the medium. EDTA, CDTA and DTPA at concentrations up to nine times the summed molarity of the complexed metals (Mg, Fe, Cu, Zn) had little effect on growth rate when added after germination, either at pH 6·5 or pH 3·5. Ferrocyanide up to 9·4 mM had no effect on the growth rate at pH 6·5, but at pH 3·5 it strongly inhibited growth. The conidia were much more sensitive than was the mycelium to the complexing agents. EDTA, CDTA and DTPA at low concentrations inhibited germination at pH 6·5 and 3·5; ferrocyanide at comparable concentrations did not affect the germination rate at pH 6·5, but, at pH 3·5, strongly inhibited germination at a concentration about equal to that of the Fe ions. Inhibition of the germination of conidia by *Aspergillus niger* by ferrocyanide at low pH has been previously observed by Martin (1955).

The form of the mass of growth in shake-flask cultures was also highly sensitive to the metal-complexing agents: EDTA, CDTA, DTPA and ferrocyanide induced the formation of small smooth granular easily dispersed pellets of mycelium, in contrast to the viscous conglomerate of larger pellets with filamentous peripheries formed in the absence of metal-complexing agents. On a molecular concentration basis, ferrocyanide was most effective in producing this change of form of growth.

The effects of the agents may be attributed to their metal-chelating power. This conclusion is supported by the following evidence. (1) The only known action of physiological importance which the agents have in common is to complex with

metal ions. (2) The antagonism of the effect of EDTA, CDTA or DTPA on conidial germination caused by decreasing the pH value of the medium can be accounted for by the decreased degree of metal chelation at the lower pH values. The lack of physiological action by NTA may be attributed to two possible mechanisms. One is that it has the lowest complexing power of the agents tested. The other is that it is trivalent and therefore will form a neutral complex with the Fe^{3+} ion. Such a neutral complex would be expected to be lipid soluble and therefore able to diffuse through the plasma membrane into the cell and thus make the iron available. This point suggests that the iron concentration may be largely responsible for the effects observed with the agents. Other evidence in favour of the iron concentration being critical is that the chelating agents and ferrocyanide complex iron before Cu, Zn or Mg, and that the effective concentration of ferrocyanide was about the same as the concentration of added Fe (23·3 μM).

The anomalous results in vigorously stirred cultures remain unexplained. Here the high shearing effect, and the presence of stainless steel parts in the vessel which possibly contributed traces of metal, especially at the low pH value, are new factors to be taken into account.

REFERENCES

American Cyanamid Co. (1953). *The Chemistry of the Ferrocyanides.* 30 Rockfeller Plaza, New York 20.

BJERRUM, J., SILLÉN, L. G. & SCHWARZENBACH, G. (1957). *Stability Constants, Part I. Organic Ligands.* London: The Chemical Society.

BORROW, A., BROWN, S., JEFFERYS, E. G., KESSELL, R. H. J., LLOYD, E. C., LLOYD, P. B., ROTHWELL, A., ROTHWELL, B. & SWAIT, J. C. (1964). The kinetics of metabolism of *Gibberella fujikuroi* in stirred culture. *Can. J. Microbiol.* **10**, 407.

CALLOW, D. S. & PIRT, S. J. (1961). Developments in apparatus for the culture of micro-organisms: design features of a laboratory two-stage continuous culture apparatus. *J. appl. Bact.* **24**, 12.

CHABEREK, S., BERSWORTH, F. C. & MARTELL, A. E. (1955). Chelating agents as metal buffers in biological systems. I. Principles and use of ethylenediamine tetracetic acid. *Arch. Biochem. Biophys.* **55**, 321.

CLARK, D. S. (1962). Submerged citric acid fermentation of ferrocyanide-treated beet molasses: morphology of pellets of *Aspergillus niger*. *Can. J. Microbiol.* **8**, 133.

ELSWORTH, R., MEAKIN, L. R. P., PIRT, S. J. & CAPELL, G. H. (1956). A two-litre scale continuous culture apparatus for micro-organisms. *J. appl. Bact.* **19**, 264.

Federation Proceedings (1961). *Biological Aspects of Metal-binding. Fed. Proc.* **20**,, No. 3, Part 2, Suppl. no. 10.

MARTIN, S. M. (1955). Effect of ferrocyanide on growth and acid production of *Aspergillus niger*. *Can. J. Microbiol.* **1**, 644.

MEZZADROLI, G. (1938). French patent 833, 631. Quoted from Perlman, D. & Sik, C. J. (1960). *Prog. ind. Microbiol.* **2**, 167.

PIRT, S. J. & CALLOW, D. S. (1960). Studies of the growth of *Penicillium chrysogenum* in continuous flow culture with reference to penicillin production. *J. appl. Bact.* **23**, 87.

SIMONS, J. N., SWIDLER, R. & BENEDICT, H. M. (1963). Kinetic studies of re-greening of iron-deficient soy beans. *Plant Physiol.* **38**, 667.

ZALOKAR, M. (1959). Enzyme activity and cell differentiation in Neurospora. *Am. J. Bot.* **46**, 555.

J. gen. Microbiol. (1965), 41, 109-118
Printed in Great Britain

The Effect of Calcium Nutrition on the Production of Diffusible Antigens by *Rhizobium trifolii*

By BEVERLEY A. HUMPHREY and J. M. VINCENT
Microbiology Laboratory, School of Agriculture, University of Sydney, Australia

(*Received* 9 *April* 1965)

SUMMARY

Rhizobium trifolii grown in a defined medium deficient in calcium yielded additional diffusible antigens as compared with organisms receiving calcium or strontium. The two kinds of organisms were indistinguishable in respect of their somatic agglutinogens. The same additional precipitinogens were released from Ca-adequate bacteria by mechanical disintegration, freezing, drying, lysozyme or chloroform. Their occurrence in the untreated Ca-deficient bacteria is attributed to autolysis of these more fragile bacteria. The precipitinogen ('a' band) that was common to the Ca-adequate and Ca-deficient bacteria was not shared by a second strain of *R. trifolii* nor by 2 strains of *R. meliloti*. This 'a' antigen was heat stable and appeared to be a component of the cell wall, from which it could be separated in diffusible form by formamide extraction of whole bacteria, trypsin digestion of broken bacteria and by mechanical disintegration of prepared cell walls. Neither of the additional antigens ('b', 'c') was related to agglutination; both were found in Ca-deficient or mechanically broken bacteria of the other strain of *R. trifolii*; 'c' only was similarly shared with *R. meliloti*. The 'b' band antigen appeared to be an intracellular component and was heat labile. Like 'a', 'c' was heat stable and associated with cell wall, but evidently was not ordinarily exposed, although readily released by lysis or mechanical breakage. The extracellular polysaccharide was neither antigenic nor haptenic, but the preparation obtained from Ca-deficient and mechanically broken Ca-adequate organisms was persistently associated with a co-precipitating antigenic component (chiefly 'a' band).

INTRODUCTION

Previous studies of a strain of *Rhizobium trifolii* (Vincent & Colburn, 1961; Humphrey & Vincent, 1962) showed that calcium deficiency produced morphological abnormality, and that the swollen spherical shape of the Ca-deficient bacteria could be attributed to shortage of Ca in the cell walls, with consequent weakening of structure. There appeared, however, to be no other readily detected change in the gross chemical structure of the cell walls. It seemed worth while, therefore, to use serological analysis to examine whether there are detectable differences in the organization of cell wall components. The work to be described was based partly on the agglutination of whole bacteria, but also used gel-diffusion methods. The latter have recently been applied to Rhizobium by Dudman (1964); Dr Dudman generously supplied us with details of his experience in advance of publication.

METHODS

Organism and cultural conditions. Rhizobium trifolii strain SU 297/31 was grown in a defined liquid medium (Vincent, 1962) having mM total divalent cation, made up of either 0·5 mM-Ca^{2+} + 0·5 mM-Mg^{2+} (Ca-adequate) or mM-Mg^{2+} (Ca-deficient). Cultures were shaken and incubated at 25° and harvested after 72 hr (early stationary phase).

Preparation of antisera. Antisera were prepared by a method similar to that of Dudman (1964) except that whole bacteria in a 72 hr liquid culture were used throughout. The rabbits were injected first intramuscularly with 2 ml. of an emulsion containing equal volumes of Freund's complete adjuvant (Difco) and the bacterial culture. This culture thus included a considerable amount of extracellular polysaccharide (Humphrey & Vincent, 1959) as well as any other soluble substances arising from metabolism or autolysis. One month later, 2 ml. of the same culture, which had been stored deep frozen, were injected intravenously without adjuvant. The animals were bled after one more week, by which time the agglutination titre, against the dialysis residue organisms of whole culture, was greater than 1600. Two animals were used for each form of culture, Ca-adequate and Ca-deficient.

Agglutination and absorption by intact organisms. Intact-organism suspensions, either in saline (0·85 %, w/v, NaCl) after centrifuging down from whole culture, or the dialysis residue organisms from whole cultures, were used for somatic agglutination and antibody absorption by the usual methods (e.g. Vincent, 1941); three successive absorption cycles were required to remove all agglutinins (negative at 1/50).

Gel diffusion. The Petri plate method of double diffusion (Dudman, 1964) was used. Oxoid Ionagar no. 2 (0·75 %, w/v, in 0·85 % NaCl + 0·25 % sodium azide added when the plates were poured) was used to give a 4 mm. depth of layer. Plastic Petri dishes were preferred since they had a flatter and more uniform base than had glass ones. Wells cut in the agar were 4 mm. in diameter and were usually spaced 8 mm. apart. Antisera were used undiluted. Bacteria centrifuged down from whole cultures at 27,000*g* were resuspended in 0·85 % NaCl at a concentration equiv. 150 mg. dry wt. organism/ml. Maximum development of the precipitation lines was seen after incubation at 25° in a moist atmosphere for 3–6 days.

Direct absorption of antibody or antigen could be demonstrated by mixing both reactants in a well in the agar. A suspension of bacteria containing equiv. 10–20 mg. dry wt. bacteria/ml. saline was able to absorb precipitins from antiserum at a 1 : 1 by vol. ratio of reactants. Non-absorption, or excess of antibody or antigen, was then shown by formation of precipitation lines between the absorption well and a well containing antigen or antibody, as appropriate.

Cell walls. These were prepared as previously (Humphrey & Vincent, 1962), but with omission of lyophilization of the bacteria before extracting the β-hydroxybutyrate polymer with chloroform. The sequence used in the present work, starting with the debris obtained by breaking the bacteria in a Mickle disintegrator with ballotini was: (i) to extract by shaking on a wrist-action shaker for 2 hr with an equal volume of chloroform and then to remove the chloroform by centrifuging it to a clear bottom layer; (ii) to digest the wall material with trypsin; (iii) finally to wash the walls with M-NaCl and then water, and to separate any whole bacteria by differential centrifugation.

Ultraviolet (u.v.) absorption spectra. These were made on a Unicam SP 700 automatic recording spectrophotometer (Cambridge Instrument Co., Cambridge, England). The extinction at 260 mμ (E_{260}) was taken as an index of the leakage of cytoplasmic components from the bacteria.

RESULTS

Agglutination reactions with intact Rhizobium trifolii organisms

There was no distinction between Ca-adequate and Ca-deficient *Rhizobium trifolii*, either by somatic agglutination or by cross-absorption. It was concluded therefore that both kinds of organism had the same agglutinogens, namely, antigens at or near the surface which involved the whole organisms in their reaction with antibody.

Gel-diffusion precipitin tests with whole Rhizobium trifolii organism

Homologous reactions. These are shown as the upper patterns of Fig. 1. Ca-adequate *Rhizobium trifolii* (positions i and iv) slowly formed a band of two precipitation lines near the antigen well when tested against the homologous antisera.

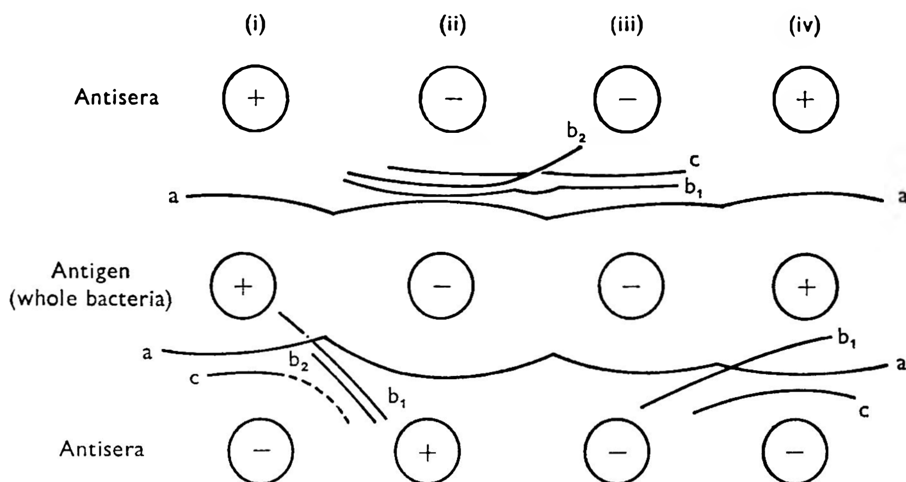


Fig. 1. Schematic representation of gel-diffusion patterns with Ca-adequate and Ca-deficient organisms of *Rhizobium trifolii*. Centre row of wells with suspensions of whole organisms of *R. trifolii* (Ca-adequate, +; Ca-deficient, −). Top row of duplicate antisera arranged to give homologous patterns; bottom row, heterologous and test of unique antigens. ×2.

These lines, lying close together and slightly curved with the concave side towards the antigen well, will be referred to as the 'a' band. Ca-deficient organisms (positions ii and iii) reacted differently with their homologous antisera in that they developed, in addition to the 'a' band, at least two fast-moving and quickly developing bands nearer the antiserum well, and concave towards it. These will be designated 'b' and 'c' (see Fig. 1). The 'b' band was made up of one ('b_1', position iii) or two ('b_1', 'b_2', position ii) thin sharp lines, and was the first to appear. The diagonal between the Ca-deficient organisms (position iii) against antiserum to

Ca-deficient organisms (position ii) resulted from the additional 'b' line ('b_2') antibody in the latter. The 'c' band constituted a weaker line nearest the antiserum well and was not further resolved. Both 'b' and 'c' bands disappeared when the antiserum was diluted more than twofold, but the 'a' band was still seen at a 32-fold dilution.

Heterologous and cross reactions for unique antigens. Antisera to Ca-adequate organisms produced only the 'a' band with Ca-deficient organisms (Fig. 1, lower positions ii, iii), but the reciprocal heterologous reaction sometimes showed the 'c' band as well (e.g. lower patterns i, iv). The crossing lines formed between antisera to Ca-deficient organisms and the diagonally opposite Ca-deficient organisms (i/ii; iv/iii) showed the 'b' band unique to this situation. In agreement with the difference in the two 'Ca-deficient' antisera as revealed with the homologous reaction, i/ii gave two lines ('b_1', 'b_2') whereas iv/iii gave only 'b_1'.

The detailed difference between the two antisera to Ca-deficient organisms in respect of the 'b' band antibodies is of the kind likely to be encountered between different animals receiving the same course of injections (Crowle, 1960).

The occurrence of a weak 'c' band in the reaction between about half the batches of Ca-adequate organisms and antisera to Ca-deficient bacteria needs to be considered in the light of further observations (below) that the 'c' antigen diffused slowly from isolated cell walls. It seemed that some batches of Ca-adequate organisms liberated more of this near-surface antigen than others. The absence of the corresponding antibody in the antisera to Ca-adequate organisms indicates that either the particular immunizing suspensions were relatively intact, or that the substance released from some of the Ca-adequate organisms was an incomplete antigen (complex hapten). The absence of any 'b' band between Ca-adequate bacteria and the antisera to Ca-defieient cells showed that autolysis as such was not involved. *Rhizobium trifolii* grown on an entirely different medium (yeast-extract mannitol agar) exhibited the same gel-diffusion pattern against these antisera as did those organisms grown on a defined Ca-adequate medium. Organisms grown with strontium instead of calcium, and which therefore showed normal morphology (Vincent & Humphrey, 1963), gave a 'Ca-adequate' diffusion pattern.

Absorption directly in the well showed the identity of the antigens responsible for the 'a' bands of both Ca-adequate and Ca-deficient organisms. Ca-deficient bacteria (with 'a', 'b' and 'c') were able to absorb completely the antisera to Ca-adequate organisms (anti-'a'). On the other hand Ca-adequate organisms absorbed anti-'a' and anti-'c', but not anti-'b', from 'Ca-deficient' antisera.

Fragility of Ca-deficient Rhizobium trifolii

Ca-deficient *Rhizobium trifolii* organisms appeared to be more fragile than the normal bacteria in that about 50% more E_{260}-absorbing compounds accumulated in the culture fluid and twice as much was released when the harvested bacteria were suspended overnight in distilled water or saline, or were frozen and thawed in distilled water. This was confirmed by the greater mass of debris seen in electron micrographs of suspension of Ca-deficient organisms as compared with that in suspensions of Ca-adequate bacteria.

Extracellular antigens

A crude polysaccharide-containing fraction was prepared from culture fluids (bacteria removed at 27,000g), by evaporation to small bulk in a rotary vacuum distillation apparatus at 40°, dialysis to remove salts and mannitol and the dialysis residue evaporated to dryness at 40° or freeze-dried. The end-product, dissolved in saline, was used for gel diffusion. This method is similar to that described by Dudman (1964) to prepare his extracellular antigens. Such a fraction from Ca-adequate cultures showed the 'a' band only, whereas the 'a', 'b' and 'c' bands appeared in the extracellular antigens from Ca-deficient cultures. Like Dudman, we found that all precipitation lines in the extracellular preparations were readily shown with suspensions of washed whole bacteria containing the same weight of dry substance/ml.

More highly purified polysaccharide fractions were prepared by precipitation from the supernatant fluids of centrifuged cultures, either by adding cetyltrimethylammonium bromide or ethanol, followed by reprecipitation with ethanol and deproteinization by the Sevag method (Sevag, Lackman & Smollens, 1935). The polysaccharide fraction prepared in any of these ways from Ca-adequate cultures gave no gel-diffusion lines. On the other hand, polysaccharide from Ca-deficient cultures, even when precipitated twice from ethanol and deproteinized, showed the 'a' band and occasionally faint 'b' and 'c' bands. The possibility that Ca, bonded with the polysaccharide fraction of Ca-adequate bacteria, might thereby restrict the diffusion or functioning of constituent antigens was not supported by the fact that the addition of EDTA to the polysaccharide solution did not produce any precipitation bands. Moreover, polysaccharide prepared from a mechanically disintegrated whole culture of Ca-adequate bacteria showed a definite 'a' band even after repeated precipitation and deproteinization.

If the polysaccharide were to represent a partial antigen unable to precipitate with antibody after separation from a larger or cell-bound component, it might still be expected to inhibit reactions involving the whole antigen. To test this, antisera against Ca-deficient and Ca-adequate bacteria were mixed in the agar wells with an equal volume of concentrated polysaccharide solution, and tested against Ca-adequate and Ca-deficient bacteria. In no case did the polysaccharide from Ca-adequate bacteria inhibit the reaction between antisera and bacteria, suggesting that the lack of precipitation bands from this preparation did indeed indicate lack of antigenic activity. As expected, the polysaccharide fraction from Ca-deficient bacteria known to contain the 'a' antigen absorbed the corresponding 'a' antibody from both antisera, whilst leaving the 'b' and 'c' antibodies of 'Ca-deficient' antisera free to react. These results lend no support to any suggestion that the extracellular polysaccharide itself is even haptenic.

Gel diffusion with disintegrated Rhizobium trifolii organisms

Ca-adequate *Rhizobium trifolii* organisms which had been shaken with ballotini in a Mickle disintegrator were practically all broken in 5 min., as indicated by the stained appearance, viable counts, and the u.v.-absorption of the supernatant fluid. This broken unfractionated material showed all of the gel-precipitation bands of unbroken Ca-deficient organisms. When broken organisms were centrifuged at

27,000*g*, the debris, consisting of cell walls, β-hydroxybutyrate polymer (Humphrey & Vincent, 1962) and a few unbroken bacteria, showed the 'a' and 'c' lines. The supernatant fluid (cell contents and finest debris), when concentrated by lyophilization and resuspended in saline, produced a strong 'b' line, in addition to lines 'a' and 'c'. Continuation of disintegration for 120 min. did not further change the gel-diffusion pattern. It was also found that Ca-adequate organisms liberated some of the 'b' and 'c' antigens when frozen, and particularly when freeze-dried. Curiously these antigens were the first to disappear from very old (22–40 day) Ca-deficient cultures.

Heat stability of precipitinogens. A suspension of disintegrated bacteria containing antigens of all three bands was heated in sealed ampoules at 96°. The antigens of the 'a' and 'c' bands were stable for the full period of 2 hr; 'b' disappeared within 30 min.

Chloroform-extracted residue. The residue of the debris from breaking with the Mickle disintegrator showed mainly the 'a' band after chloroform extraction. The extract itself after evaporation to dryness yielded a thin plastic film which, when taken up with saline, gave faint 'a', 'b' and 'c' bands, probably due to some bacteria and cell walls caught up in the sticky mass.

Chloroform extraction of whole bacteria, under conditions similar to those used for extracting the debris, did not decrease their agglutinability. Gel diffusion with the same extracted bacteria now showed the pattern typical of 'Ca-deficient' and mechanically disrupted bacteria.

Trypsin digestion. The residue after disintegration and extraction with chloroform was treated with trypsin to remove adhering cytoplasm, and centrifuged at 27,000 *g*. The supernatant fluid, dialysed, lyophilized and resuspended in saline, showed the 'a' band. Concentrated washings from the debris, first with M-NaCl and then repeatedly with water, showed traces of all three bands, particularly the 'a' band.

Antigenic properties of cell walls. The bacterial residues retained their agglutinability through all stages, up to and including the cell-wall preparation itself. In all cases the titre remained high (> 800), but the nature of the agglutination changed from the fine granular deposit, characteristic of the whole bacteria and unextracted broken bacteria, to an abundant loose flocculent precipitate in the cases of the trypsin-digested residue and the final cell-wall preparation. In gel diffusion, the 'a' band antigens were associated with the residue up to the stage of trypsin digestion; they were then readily shown in the supernatant fluid but not in the residue and final cell-wall preparation, both of which were still agglutinable. Long diffusion with most cell-wall preparations, shown under the electron microscope to be relatively clean, still gave a faint 'c' line, and occasionally a close almost non-diffusing area of precipitation around the antigen well. When the cell walls of such preparations and cell walls which showed no diffusion pattern at all were suspended in saline and treated in the Mickle disintegrator for 1 hr, the diffusion pattern then showed definite 'a' and 'c' lines. With the appropriate parallel arrangement of wells, it was found that the 'a' band which developed from disintegrated cell walls curved in to join up with the closer 'non-diffusing' area of precipitation seen around the wells which contained intact cell walls.

Rhizobium trifolii was grown in a medium relatively deficient in mannitol (0·2 %,

w/v, as compared with 1 %, w/v) so as to accumulate less polymer (10–12 % of dry wt. bacteria as compared with 40–50 %) and permit the preparation of cell walls without chloroform extraction, since this might destroy a lipopolysaccharide layer in the walls. However, no difference was detected in agglutinability or diffusion pattern between cell walls prepared in this way and those which had had the usual treatment with chloroform.

Other methods of breakdown and extraction

Lysozyme. Lysozyme even without EDTA, liberated the 'b' and 'c' antigens from Ca-adequate bacteria. This might mean that the small degree of lysis found under these conditions (17 % as compared with 61 % with EDTA; Vincent & Humphrey, 1963) was enough to liberate a reacting concentration of intracellular antigen.

Formamide extractions. The conditions were those of Bjorklund (1953), with lyophilized Ca-adequate whole bacteria in 25 parts by wt. formamide at 5° for 30 min. shaken in a Mickle disintegrator without ballotini. The extract gave a heavy flocculent precipitate on adding 2 vol. acetone; on resolution and diffusion this material produced a heavy double 'a' band. The extracted bacteria showed a new complexity of diffusible antigens. In particular, the 'a' antigens diffusing from extracted bacteria gave a pattern of three lines.

Intra- and interspecific cross-reactions

Cross-reactions between *Rhizobium trifolii* strain SU 297/31 and *R. trifolii* strain TA 1 were studied by agglutination and gel diffusion. Somatic cross-agglutination was negative at 1/50 dilution of antiserum but some cross-precipitation was observed with mechanically broken organisms. Antigens responsible for 'b' and 'c' lines appeared to be common to both strains, but the 'a' band was found only with each homologous antiserum. When disintegrated bacteria of two strains of *R. meliloti* (SU 47, U 45) were tested by gel diffusion against the antisera against both strains of *R. trifolii*, a faint but definite line was observed in the 'c' position near the antiserum well.

DISCUSSION

Whereas the agglutination reaction, as ordinarily observed, depends on a firm structural relationship between an exposed antigen (agglutinogen) and the bacterium, precipitation reactions are able to reveal only soluble antigen (precipitinogen). Gel diffusion is likely not to detect agglutinogens (except so far as these might become detached, probably as incomplete antigens), and is less readily interpreted in spatial terms in that it need not be restricted to surface antigens. In this connexion, the structural integrity of the bacteria used for the preparation of antisera and in testing is obviously of paramount importance. Various means of disintegration can be used to reveal additional diffusible antigens (mechanical, sonic, chemical, enzymic) and the results can be interpreted accordingly. However, care must be used to avoid uncontrolled autolysis (Lennox, 1960). The age of a culture, its aeration and the nature of the culture medium are factors which must be controlled and their effects examined.

In the present work, the Ca-adequate *Rhizobium trifolii* organisms, well aerated

and harvested early in the stationary phase appeared to be relatively unautolysed. The 'a' band, shown in the gel-diffusion test, can therefore be regarded as due to soluble precipitinogen(s) located at or near the surface. The consistent difference between antisera against the Ca-adequate and Ca-deficient bacteria is reassuring, in that it shows that there was little, if any, liberation of deep-seated antigens in the animal being immunized.

The 'b' and 'c' antigens regularly revealed in Ca-deficient bacteria, and also liberated from mechanically broken Ca-adequate bacteria, do not represent new antigens in the strict sense. Rather they are an expression of leakage from the more fragile Ca-deficient bacteria of internally located antigenically competent macromolecules. The work of Niwa, Yamadeya & Kuwajima (1964), with Bordetella (also Gram-negative), is significant in this connexion. These workers found that leakage of RNA was increased by a variety of agents which might be expected to remove Ca from the cell walls (including oxalic acid, EDTA, phosphoric and arsenic acids); on the other hand Ca, but not Ba, decreased the leakage. No details were given, however, concerning the initial Ca status of the organisms. Although the lipoprotein–lipopolysaccharide outer layer of the wall of Gram-negative bacteria is considered to be the dominant antigenic surface, most parts of the cell have been found, in various organisms, to be antigenic. Antigenicity has been observed in the cytoplasmic membrane of *Bacillus megaterium* (Vennes & Gerhardt, 1956), which contains lipoprotein and some RNA; in a protein and nucleic acid–polysaccharide complex from Rhizobium (Lorkiewicz, Kwas & Szwed-Nabialek, 1963); and in intracellular enzymes of Neurospora (Roberts & Pateman, 1964). That deep-seated antigens should be shared between varieties of *Rhizobium trifolii* and between Rhizobium species is not perhaps unexpected; compare, for example, intracellular group-specific glycerol teichoic acids of Lactobacillus species (Sharpe, Davison & Baddiley, 1964), and the intracellular protein that constitutes the group D specific antigen of Streptococcus (Jones & Shattock, 1960).

The qualitative identity of the Ca-adequate and Ca-deficient *Rhizobium trifolii* organisms in the cross-absorption agglutination test shows that the additional precipitinogens shown with Ca-deficient organisms were unrelated to agglutinogens. Reciprocally this was confirmed by the fact that 'b' and 'c'-band antigens were shared between the two strains of *R. trifolii* and the 'c' band with *R. meliloti*, in both cases without cross-agglutination. There might, however, be some relationship in the case of the 'a' band precipitinogens in that: (i) they were common to the cross-agglutinating Ca-adequate and Ca-deficient organisms; (ii) they were not shared with the non-cross-agglutinating strain and species; (iii) like the somatic agglutinogens, they were heat stable; (iv) they occurred at the surface and were closely associated with the cell wall. It seems sound to conclude that the 'a'-band antigens are part of the cell-wall structure which may, when thus anchored on the organisms, be responsible for agglutination. The fact that the 'a'-band disappeared from the gel-diffusion pattern of the residue after trypsin digestion but appeared in the supernatant fluid, indicates the release of an antigenic complex by the breakage of peptide links. The fact that formamide extracted the 'a' antigen from Rhizobium organisms may further indicate its lipopolysaccharide nature. The 'a' antigen may be a complex of related or unrelated chemical entities; indeed the band we observed was made of at least two closely associated lines. The fact that relatively violent

reagents (e.g. phenol, Davies, 1956; formamide, Krause & McCarty, 1961) have been used to isolate specific antigenic substances from bacterial walls makes it likely that the walls would retain non-diffusible antigens in spite of the rigours of cell-wall preparation, and that these might be available for agglutination but not immediately for diffusion. In our case, mechanical disintegration released a diffusible fraction which reacted in the 'a' band. The greater diffusibility of these disintegrated walls seems likely to reflect the release of antigenic components in a smaller and more mobile form. The antigen of the 'c' band, although evidently playing no role in agglutination, appeared to be relatively superficial, as judged by the ease with which it was liberated from Ca-adequate organisms and from isolated walls. Its heat stability as compared with heat-labile 'b'-band antigens might reflect association with the wall rather than with cytoplasm.

Our present work, like earlier results (Kleczkowski & Kleczkowski, 1952; Humphrey & Vincent, 1959) indicates that extracellular polysaccharide of Ca-adequate *Rhizobium trifolii* is neither antigenic nor haptenic (except so far as it may react with antibody already developed against polysaccharide of type III Pneumococcus; Schluchterer & Stacey, 1945). The persistent diffusible antigens of 'purified' polysaccharide of Ca-deficient *Rhizobium trifolii* organisms seem to represent a coprecipitating fragment released from the fragile organism. This conclusion is supported by the fact that a similar persistent antigen occurred in polysaccharide from mechanically disrupted Ca-adequate organisms. Our experience, like that of Dudman (1964) with *R. meliloti*, in which diffusing antigens were better demonstrated in washed whole organisms than in concentrated extracellular preparations, supports the idea that such reactions as have been observed with the water-soluble polysaccharides of rhizobia were due to contaminating cellular components. The extracellular polysaccharide appears to be a diffuse exudate without a morphological role. Glucuronic acid, known to be a major component of the extracellular polysaccharide could not be detected in the cell walls of *R. trifolii* (Humphrey & Vincent, 1962). Pappagianis, Putman & Kobayashi (1961) attributed to somatic contamination some antigens which occur in polysaccharide preparations from culture filtrates of *Coccidioides immitis*. The same considerations would contraindicate the preparation of polysaccharides by cetyltrimethylammonium bromide precipitation from whole cultures in view of this agent's known lytic action (Salton, 1957).

Our thanks are due to Dr D. G. Drummond and Mr R. G. Wright of the Electron Microscope Unit, Sydney University; and to Dr G. F. Humphrey of the C.S.I.R.O. Division of Fisheries and Oceanography for the ultraviolet spectra. The work reported in this paper was supported by the Reserve Bank of Australia (Rural Credits Division), the Wheat Industry Research Funds and the Research Committee, University of Sydney.

REFERENCES

BJORKLUND, B. (1953). Immunological studies of rabbit bone marrow with the aid of gel diffusion and serological techniques. *Internat. Arch. Allergy*, 4, 379.

CROWLE, A. J. (1960). Interpretation of immunodiffusion tests. *Ann. Rev. Microbiol.* 14, 161.

DAVIES, D. A. L. (1956). A specific polysaccharide of *Pasteurella pestis*. *Biochem. J.* **63**, 105.

DUDMAN, W. F. (1964). Immune diffusion analysis of the extracellular soluble antigens of two strains of *Rhizobium meliloti*. *J. Bact.* **88**, 782.

HUMPHREY, B. A. & VINCENT, J. M. (1959). Extracellular polysaccharides of *Rhizobium*. *J. gen. Microbiol.* **21**, 477.

HUMPHREY, B. A. & VINCENT, J. M. (1962). Calcium in cell walls of *Rhizobium trifolii*. *J. gen. Microbiol.* **29**, 557.

JONES, D. & SHATTOCK, P. M. F. (1960). The location of the group antigen of group D *Streptococcus*. *J. gen. Microbiol.* **23**, 335.

KLECZKOWSKI, J. & KLECZKOWSKI, A. (1952). The effect of specific polysaccharide from the host bacteria and of ribonuclease on the multiplication of *Rhizobium* phages. *J. gen. Microbiol.* **7**, 340.

KRAUSE, R. M. & MCCARTY, M. (1961). Studies on the chemical structure of the streptococcal cell wall. I. The identification of a mucopeptide in the cell walls of groups A and A variant streptococci. *J. exp. Med.* **114**, 127.

LENNOX, E. S. (1960). Antigenic analysis of cell structure. In *The Bacteria*, vol. 1, ed. I. C. Gunsalus & R. Y. Stanier. New York: Academic Press.

LORKIEWICZ, Z., KWAS, S. & SZWED-NABIALEK, G. (1963). Antigenic properties of nucleic acids in *Rhizobium*. *Acta Microbiol. Polon.* **12**, 113.

NIWA, M., YAMADEYA, Y. & KUWAJIMA, Y. (1964). Leakage of cell components of *Bordetella pertussis*. *J. Bact.* **88**, 809.

PAPPAGIANIS, D., PUTMAN, E. W. & KOBAYASHI, G. S. (1961). Polysaccharide of *Coccidioides immitis*. *J. Bact.* **82**, 714.

ROBERTS, D. B. & PATEMAN, J. A. (1964). Immunological studies of amination deficient strains of *Neurospora crassa*. *J. gen. Microbiol.* **34**, 295.

SALTON, M. J. R. (1957). The action of lytic agents on the surface structures of the bacterial cell. *2nd International Congress on Surface Activity* (*London*), p. 245. London: Butterworths.

SCHLUCHTERER, E. & STACEY, M. (1945). The capsular polysaccharide of *Rhizobium radicicolum*. *J. chem. Soc.* p. 776.

SEVAG, M. G., LACKMAN, D. B. & SMOLLENS, J. (1935). The isolation of the components of streptococcal nucleoproteins in serologically active form. *J. biol. Chem.* **124**, 425.

SHARPE, M. E., DAVISON, A. L. & BADDILEY, J. (1964). Teichoic acids and group antigens in *Lactobacilli*. *J. gen. Microbiol.* **34**, 333.

VENNES, J. W. & GERHARDT, P. (1956). Immunological comparison of isolated surface membranes of *Bacillus megaterium*. *Science*, **124**, 535.

VINCENT, J. M. (1941). Serological studies of the root-nodule bacteria. I. Strains of *Rhizobium meliloti*. *Proc. Linn. Soc. N.S.W.* **66**, 145.

VINCENT, J. M. (1962). Influence of calcium and magnesium on the growth of *Rhizobium*. *J. gen. Microbiol.* **28**, 653.

VINCENT, J. M. & COLBURN, J. R. (1961). Cytological abnormalities in *Rhizobium trifolii* due to deficiency of calcium or magnesium. *Aust. J. Sci.* **23**, 269.

VINCENT, J. M. & HUMPHREY, B. A. (1963). Partition of divalent cations between bacterial wall and cell contents. *Nature, Lond.* **199**, 149.

J. gen. Microbiol. (1965), 41, 119–126
Printed in Great Britain

An Induced Enzyme in X-irradiated *Escherichia coli*: Comparison with Lethal Effects

BY J. L. MOORE

Experimental Radiopathology Research Unit, Medical Research Council, Hammersmith Hospital, Ducane Road, London, W. 12

(*Received* 13 *April* 1965)

SUMMARY

The effect of X-rays on the synthesis of induced β-D-galactosidase was examined with *Escherichia coli* strain B and three of its mutants, i.e. the sensitive strain BS and two resistant strains B/r and B/H. With the parent strain *E. coli* B the extent of radiation-induced lethal damage depended on conditions of culture after irradiation, whereas radiation-induced inhibition of β-D-galactosidase was unaffected by the composition of the culture medium. The sensitivity of radiation damage to induction of β-D-galactosidase was about the same for *E. coli* strains B, B/r and B/H, the dose required to give 37% survival being 42 ± 6 krad; in *E. coli* BS the sensitivity to radiation was three times as great. Enzyme induction was one-half to one-third as radiosensitive as viability for the strains B/r, B/H and BS but with strain B it was one-tenth as sensitive when Oxoid Nutrient Medium was used. Oxygen enhancement ratios for lethal effects were always smaller than those for inhibition of enzyme synthesis.

INTRODUCTION

Many biochemical effects of ionizing radiation are difficult to observe unless very large doses are used, as compared with those used to study loss of reproductive ability. It has been found, however, that the ability of bacteria to synthesize induced enzymes may be inhibited by doses of X-rays comparable with those used to establish survival curves (Billen & Lichstein, 1952; Pauly, 1959, 1963; Dewey, 1962; Pollard & Vogler, 1961). There is evidence that ionizing radiations interfere with many synthetic pathways in bacteria; the synthesis of proteins as well as nucleic acids has been shown to be affected (Gros, 1960; Pauly, 1963). Swenson & Setlow (1964) found that ultraviolet inactivation of β-D-galactosidase M-RNA was more sensitive than general protein synthesis. Kameyama & Novelli (1962) found that if the bacteria were ultraviolet-irradiated before induction β-D-galactosidase synthesis was more sensitive than general protein synthesis. If it be accepted that the synthesis of each protein depends on a specific expression of the genetic code, then the study of X-ray effects on the synthesis of a particular enzyme may be expected to throw light on the pathways leading to that synthesis. Pauly (1959) investigated the inhibition by X-rays of the synthesis of lysine decarboxylase in 'Bacterium cadaveris'. The exclusion of oxygen from the irradiated material decreased the effects of the X-rays by a factor of four. Dewey (1962) reported that the ability of a strain of Pseudomonas to synthesize histidine-oxidizing enzymes

was partly protected against radiation by glycerol. Pollard & Vogler (1961) observed that the doses required to inhibit induction of β-D-galactosidase in *Escherichia coli* were similar to those reported by Pauly for inhibition of lysine decarboxylase induction in 'B. cadaveris'. The lethal effects of ionizing radiation on bacteria may be modified by a variety of treatments, and in particular *E. coli* strain B and some of its mutants are very susceptible to the influence of cultural conditions after irradiation (Alper & Gillies, 1958*a*, 1960*b*; Gillies & Alper, 1959). The inhibition of enzyme synthesis presented an end-point other than lethality for testing the modifying effects of some of these conditions, and also for comparing the response of *E. coli* B with that of mutants which differed greatly from it in sensitivity to radiation in respect of survival.

METHODS

Organisms

The strains of bacteria used were *Escherichia coli* B, B/r, BS and B/H. *E. coli* B/H is a resistant mutant of *E. coli* B isolated in this laboratory. Cultures were grown in Oxoid Nutrient Broth and aerated by gentle shaking in an incubator at 37° for $3\frac{1}{2}$ hr and then harvested while in logarithmic growth. Suspensions were prepared for irradiation by centrifuging down and washing the organisms three times in 0·066 M-phosphate buffer (pH = 7·0). Suspensions containing 10^9 bacteria/ml. were used for measuring inhibition of enzyme induction and about 5×10^5 bacteria/ml. for measuring survival.

Enzymes

For induction of β-D-galactosidase 5 ml. of double-strength nutrient medium were placed in a conical flask with 3 ml. sterile distilled water; 1 ml. of irradiated bacterial suspension was added, the flask placed in a water bath and gently shaken at 37°. Two minutes later 1 ml. of 1% (w/v) lactose solution was added to the flask and a sample immediately removed. Further samples were taken at intervals of 15 min. over the next 75 min. The samples were placed in test tubes containing chloramphenicol to give a final concentration in excess of 100 μg./ml., to arrest induction of enzyme. Two drops of toluene and two drops of 1% (w/v) sodium deoxycholate were added to the samples which were shaken vigorously for 1 min. and then placed in an incubator at 37° for a further 15 min. This last procedure ruptured the cell wall and membrane so that the enzyme was released into the surrounding medium.

The amount of β-D-galactosidase was estimated by a modification of the method of Lederberg (1950); with *o*-nitrophenyl β-D-galactoside (ONPG) as substrate the *o*-nitrophenol (ONP) is split off, being measured in a spectrophotometer (Unicam) at 420 mμ. Samples of enzyme were placed in Unicam glass cuvettes with buffer (pH = 7·0) and the substrate ONPG at a final concentration of 0·0028 M. The cuvettes were placed in a cell carrier fitted with a thermostatic device to keep the contents at 28°. (The cuvette carrier was designed by A. Lowe and modified by W. Siddiqi and J. Woo Sam in this Unit so that four 1 cm. cuvettes can be kept at controlled temperatures). Measurements of ONP were taken every few minutes until the increase in concentration was linear with time.

Enzyme activity was measured in millimoles-*o*-nitrophenol liberated/min./ml. of initial culture.

Survival curves were determined by suitably diluting samples removed from the irradiation vessel, plating on Nutrient Agar, the plates being incubated overnight at 37°. The nutrient media used were Oxoid Nutrient Agar (CM 55), Oxoid Nutrient Broth (CM 67), Difco Nutrient Agar and Difco Nutrient Broth (Difco Laboratory Detroit, U.S.A.). These were prepared from commercial preparations of dehydrated granules. In some experiments these media were supplemented with 1 % (w/v) lactose.

Irradiation and dosimetry

Radiation was from an X-ray unit operating without added filtration at either 250 kV., 12mA. or 250 kV., 15 mA. Dose rates were 1000 rad. or 2000 rad./min. as determined by ferrous dosimetry. The irradiation vessel used was designed by Alper (1955) to enable irradiation to be carried out under controlled gas conditions, and to allow removal of samples without altering the dose rate. Nitrogen containing less than 10 p.p.m. oxygen was bubbled through the suspensions to obtain anoxic conditions.

RESULTS

A typical curve for the induction of β-D-galactosidase after adding lactose to a culture of *Escherichia coli* strain B showed a lag of 15 min. before measurable amounts of enzyme were detected, followed by a linear increase in concentration until 60 min. after adding the inducer, when the concentration per bacterium became constant. It was assumed that by this time all the bacteria were fully induced and any further increase in enzyme concentration is due to growth with an increase in the number of bacteria present. When induction of β-D-galactosidase in irradiated organisms is compared with that in unirradiated organisms the slope of the linear portion of the induction curve is used to establish the surviving fraction for enzyme induction after a given dose of X-rays. Results of Pauly (1963) indicate a somewhat greater effect of X-rays on the synthesis of a specific enzyme (lysine decarboxylase in 'B. cadaveris') than on total protein synthesis. In the experiment here with β-D-galactosidase the decreasing rate of enzyme synthesis may therefore be due partly to a lessening in total synthesis since this must also be affected by X-rays. During induction in these experiments a certain amount of cell lysis occurs and this makes it difficult to correct changes in enzyme concentration for changes in turbidity that occur during induction of β-D-galactosidase. 'Surviving activity' is defined by the ratio of the slopes pertaining to irradiated and non-irradiated bacteria. By plotting the logarithm of the surviving activity against dose a curve is derived which is comparable with survival curves as conventionally plotted.

Figure 1 shows survival curves after plating *Escherichia coli* B on media with and without added lactose. Of the strains used, this is the most readily affected in its radiation response by slight alterations in post-irradiation conditions of growth (Alper & Gillies, 1958*a*, 1960*b*). The results obtained with the four strains of *E. coli* used are shown in Table 1 and from this the following points can be made: (*a*) There was no difference in the effect of aerobic or anoxic irradiation on synthesis of β-D-galactosidase whether Difco or Oxoid Nutrient Broth was used for induction after irradiation. As shown by Alper & Gillies (1958*a*, *b*), however, colony forming ability was markedly influenced by plating on comparable solid media, the influence

being much greater after anoxic irradiation (Fig. 2). (*b*) With every strain of *E. coli* the oxygen enhancement ratio was higher for damage to enzyme induction than damage to colony forming ability. (*c*) When Oxoid Nutrient Broth was used after irradiation, the inhibition of enzyme induction was nearly as great in *E. coli* B as in

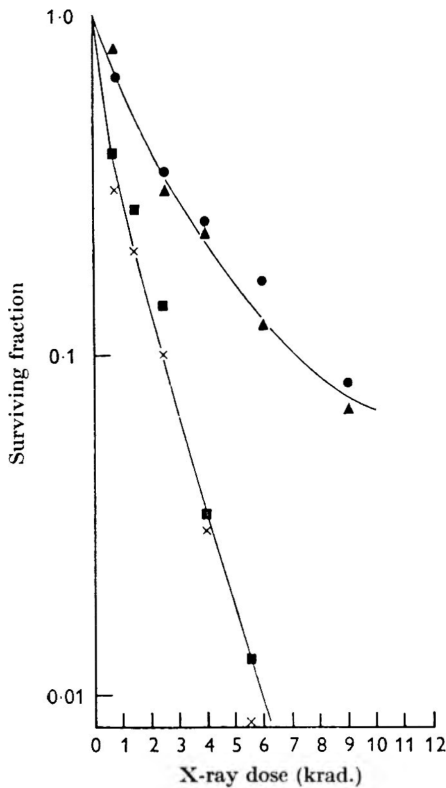


Fig. 1

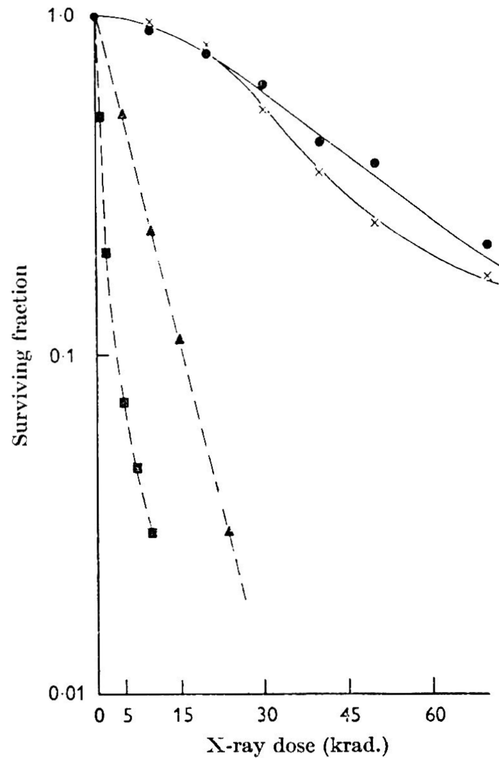


Fig. 2

Fig. 1. The effect of X-irradiation on survival of colony forming ability in *Escherichia coli* strain B after exposure in buffer solution to X-rays under oxygenation or anoxia, and subsequently incubated on Oxoid Nutrient Agar with or without added lactose. ■, Irradiated aerobicially and incubated without lactose; ●, irradiated anaerobically and incubated without lactose; ×, irradiated aerobically and incubated with lactose; ▲, irradiated anaerobically and incubated with lactose.

Fig. 2. Inhibition by X-rays of induction of β-D-galactosidase and survival of colony formation in *Escherichia coli* strain B after exposure in buffer solution to X-rays in the absence of oxygen. Lactose was used as inducer for β-D-galactosidase. ●, Induction of β-D-galactosidase in Difco Nutrient Broth; ×, induction of β-D-galaetosidase in Oxoid Nutrient Broth; ▲, survival of colony forming ability on Difco Nutrient Agar; ■, survival of colony forming ability on Oxoid Nutrient Agar.

E. coli B/r, the ratio of doses to give the same effect being only 1·3 both for aerobic and anaerobic irradiations. This may be contrasted with their relative sensitivities to radiation-induced killing when this medium was used, the ratio of doses to give the same effect then being 7 for aerobic irradiation and 9 for anaerobic irradiation. The results for anaerobic irradiation are shown in Fig. 3. (*d*) Inhibition of enzyme induction in *E. coli* BS was more sensitive than in the other three strains so that

only about one-third of the dose was required to diminish enzyme induction to a similar degree. Survival and inhibition of enzyme induction for *E. coli* BS are shown graphically in Fig. 4.

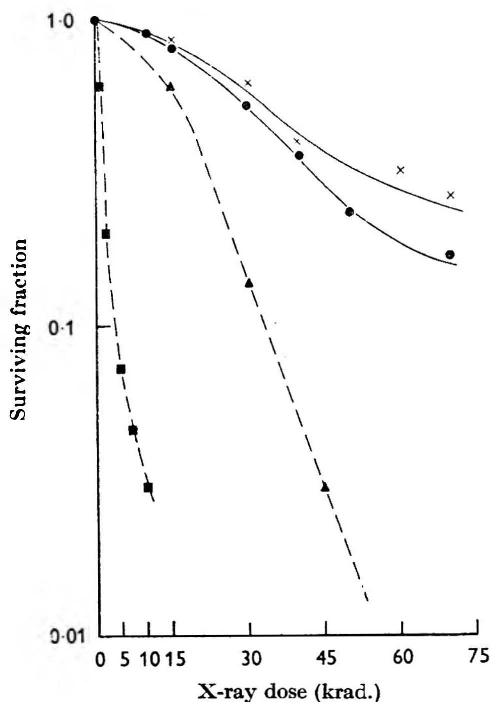


Fig. 3

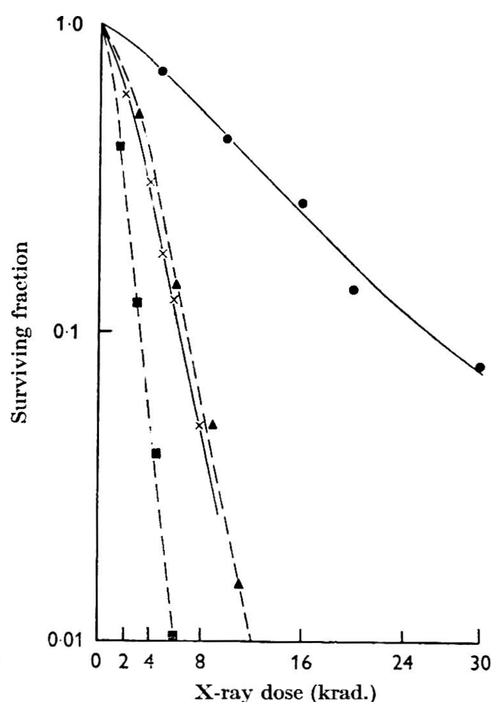


Fig. 4

Fig. 3. Inhibition by X-rays of the induction of β-D-galactosidase and survival of colony forming ability in *Escherichia coli* strain B and *E. coli* strain B/r; exposure to X-rays was carried out in buffer solution and in the absence of oxygen. Oxoid Nutrient Agar was used for determining survival of colony formation and Oxoid Nutrient Broth for the survival of the induction of β-D-galactosidase. Inducer was lactose. ×, Induction of β-D-galactosidase in *E. coli* B/r; ●, induction of β-D-galactosidase in *E. coli.* B; ▲, survival of colony formation in *E. coli* B/r; ■, survival of colony formation in *E. coli* B.

Fig. 4. Inhibition of the induction of β-D-galactosidase and survival of colony formation in *Escherichia coli* strain BS. A bacterial suspension in buffer solution was irradiated in the absence and presence of oxygen. Induction of β-D-galactosidase by lactose and survival of colony-forming ability was carried out with Oxoid Nutrient Broth and Oxoid Nutrient Agar respectively. ●, Induction of β-D-galactosidase after anaerobic irradiation; ×, induction of β-D-galactosidase after aerobic irradiation; ▲, survival of colony formation after anaerobic irradiation; ■, survival of colony formation after aerobic irradiation.

DISCUSSION

These results show that the two types of radiation damage studied were differently affected by post-irradiation cultural conditions, by strain differences and by the presence of oxygen. Oxygen enhancement ratios for killing varied between 2·1 and 4·0 with the different strains used; with *E. coli* B variations from 1·8 to 3·5 have been observed (Alper & Gillies, 1958*a*; Gillies & Alper, 1959; Alper & Gillies, 1960*a*; Alper, 1961; Hodgkins & Alper, 1963). The oxygen enhancement ratio for inhibition of induction was always higher than for killing and never less than 3·5. Inhibition

of enzyme induction showed many similarities for the four strains used, which suggests that this is a more specific type of damage than cell survival. The results of Pauly (1963) indicate a somewhat greater effect of X-rays on the synthesis of a specific enzyme (lysine decarboxylase in 'Bacterium cadaveris') than on protein synthesis as a whole. Since the total synthesis must encompass that of many enzymes it seems likely that there is variation in the effects of radiation on specific enzyme systems. In previous work of my own, for example (Moore unpublished) I found that the inhibition of the synthesis of malic decarboxylase in *Lactobacillus arabinosus* to 37 % required ten times as much ionizing radiation as did β-D-galactosidase in *E. coli* ML 30. Thus the relationship between dose of ionizing radiation and effect on the synthesis of a specific enzyme should provide information about the radiation sensitivity of the sum of all the steps leading to the formation of that enzyme. It is clear that the induction of certain enzymes is particularly sensitive to ionizing radiations.

Table 1. *The effect of aerobic and anaerobic X-irradiation on the inhibition of the synthesis of β-D-galactosidase and survival of colony formation in four strains of Escherichia coli*

Strains of *E. coli*	Inactivation dose*				Oxygen enhancement ratio	
	For survival		For induction			
	Aerobic	Anoxic	Aerobic	Anoxic	For survival	For induction
B	0·8	2·1	9·0	36·0	2·6	4·0
B†	2·8	7·0	12·0	47·0	2·5	5·0
B/r	6·0	19·5	12·5	48·5	3·3	3·9
BS	1·8	3·8	3·2	11·8	2·1	3·7
B/H	4·5	18·0	9·0	38·5	4·0	4·3

Inhibition of enzyme induction and survival of viability was established with Oxoid Nutrient Media for all four strains of *E. coli*.

* The inactivation doses are those required to reduce the surviving fraction of enzyme induction and viability to a value of 0.37.

† For *E. coli* strain B inhibition of enzyme induction and survival of viability was established with Difco Nutrient Media.

Dewey (1962) reported that induction of histidine oxidizing enzymes in a strain of Pseudomonas was half as radiosensitive as survival. Induction of β-D-galactosidase in *E. coli* strains BS, B/r, B/H was one-half to one-third as radiosensitive as survival, but in *E. coli* strain B induction was one-tenth as radiosensitive as survival when Oxoid medium was used. The oxygen enhancement ratios for inhibition of β-D-galactosidase in all strains of *E. coli* used in this present work varied between 3·7 and 4·3 and both Pauly (1963) and Dewey (1962) reported oxygen enhancement ratios of value about 4 for inhibition of other inducible systems.

Jacob & Monod (1961) suggest that induction of β-D-galactosidase requires specific pieces of DNA for its expression. The importance of DNA has been endorsed by the work of Novelli, Kameyama & Eisenstadt (1961). These authors reported that the induction of β-D-galactosidase in a bacterial extract obtained from irradiated *Escherichia coli* was absent until the addition of DNA from unirradiated induced bacteria. This work of Novelli *et al.* (1961) might suggest that the integrity of the enzyme synthesizing system depends only on the presence of undamaged

DNA. However, the present work, and that of others (Dewey, 1962; Pauly, 1963) throws some doubt on the concept that radiation damage to DNA is solely responsible for the inhibition of enzyme synthesis. This doubt arises in part from the magnitude of the oxygen enhancement ratios. From work which is more specificially concerned with damage to DNA the oxygen enhancement ratios have been much smaller. Anderson (1951) reported that a radiation-induced back mutation had a smaller oxygen enhancement ratio than killing. More recently Bridges (1963) and Deering (1963) have reported similar observations. Howard-Flanders, Levin & Theriot (1963) reported an oxygen enhancement ratio of 1·8 for inactivation of T_2 bacteriophage after it had been injected into the host. In these model systems oxygen enhancement ratios are considerably lower than those reported for lethal effects (Alper, 1963), so the high oxygen enhancement ratios seen in experiments with inducible systems may indicate that damage to some part of the induction system other than DNA is responsible.

It is possible to estimate 'target size' for a given end-point from the dose required (D_{37}) to give an average of one damaging event per cell (Lea, 1946). The D_{37} value of 42 ± 6 krad. for inhibition of β-D-galactosidase in three strains of *Escherichia coli* is similar to that reported by Pauly (1963) for lysine decarboxylase. D_{37} values after anaerobic irradiations have been used to calculate the approximate molecular weight of inactivated enzymes (Pollard *et al.* 1955; Pollard & Barrett, 1959). If the doses of radiation to reduce the surviving fraction to 37 % for enzyme induction are used in conjunction with Pollard's formula, the site responsible for induction has a molecular weight of 25 million, similar to that deduced by Pauly (1959) for induction of lysine decarboxylase. Electron microscope studies have suggested that the messenger RNA involved in the synthesis of β-D-galactosidase is 1·5 million (Kioh & Rich, 1964). Therefore the molecular weight determination based on inhibition of enzyme induction under anoxic conditions yields a molecular weight that is too large to be appropriate for the corresponding piece of DNA code. This may be further evidence suggesting that not only damage to DNA is being observed and that it is some other form of damage which is responsible for the high values of oxygen enhancement ratio.

The author wishes to thank Miss T. Alper for advice given during this work and during the preparation of the manuscript.

REFERENCES

ALPER, T. (1955). Bacteriophage as indicator in radiation chemistry. *Radiat. Res.* **2**, 119.

ALPER, T. (1961). Variability of the oxygen effect observed with micro-organisms. Part II. *Escherichia coli* B. *Int. J. Radiat Biol.* **3**, 369.

ALPER, T. (1963). Lethal mutations and cell death. *Physics Med. Biol.* **8**, 365.

ALPER, T. & GILLIES, N. E. (1958*a*). Restoration of *Escherichia coli* strain B after irradiation: its dependence on suboptimal growth conditions. *J. gen. Microbiol.* **18**, 461.

ALPER, T. & GILLIES, N. E. (1958*b*). 'Restoration' of micro-organisms and suboptimal growth conditions. *Radiat. Res.* **9**, 86.

ALPER, T. & GILLIES, N. E. (1960*a*). The influence of metabolic events immediately after irradiation on the difference in sensitivity between *E. coli* B and *E. coli* B/r. 'Immediate and low level effects of ionizing radiations' *Proceedings of Symposium held at Venice, June 22–26, 1959*. Edited by A. A. Buzzati-Traverso, p. 205. London: Taylor & Francis, Ltd.

ALPER, T. & GILLIES, N. E. (1960*b*). The relationship between growth and survival after irradiation of *Escherichia coli* strain B and two resistant mutants. *J. gen. Microbiol.* **22**, 113.

ANDERSON, E. H. (1951). The effect of oxygen on mutation induced by X-rays. *Proc. natn. Acad. Sci., U.S.A.* **37**, 340.

BILLEN, D. & LICHSTEIN, H. C. (1952). The effect of X-radiation on the adaptive formation of formic hydrogenlyase in *Escherichia coli*. *J. Bact.* **63**, 533.

BRIDGES, B. A. (1963). Effect of chemical modifiers on inactivation and mutation by gamma radiation in *Escherichia coli*. *J. gen. Microbiol.* **31**, 405.

DEERING, R. (1963). Mutations and killing of *Escherichia coli* WP-2 by accelerated heavy ions and other radiations. *Radiat. Res.* **19**, 169.

DEWEY, D. L. (1962). X-ray inactivation of inducible enzyme synthesis and the effect of oxygen and glycerol. *Nature, Lond.* **194**, 158.

GILLIES, N. E. & ALPER, T. (1959). Reductions in the lethal effects of radiations on *Escherichia coli* B by treatment with chloramphenicol. *Nature, Lond.* **183**, 237.

GROS, F. (1960). *The Nucleic Acids*, vol. III, p. 409. Ed. E. Chargaff & J. N. Davidson, New York: Academic Press.

HODGKINS, B. & ALPER, T. (1963). Anaerobic growth as a factor influencing radio-sensitivity. *J. gen. Microbiol.* **30**, 307.

HOWARD-FLANDERS, P., LEVIN, J. & THERIOT, L. (1963). Reaction of desoxyribonucleic acid radicals with sulphydryl compounds in X-irradiated bacteriophage systems. *Radiat. Res.* **18**, 593.

JACOB, F. & MONOD, J. (1961). Genetic regulatory mechanisms. *J. molec. Biol.* **3**, 318.

KAMEYAMA, T. & NOVELLI, G. D. (1962). Effect of ultraviolet inactivation and photoactivation on the induced synthesis of β-galactosidase by *Escherichia coli*. *Arch. Biochem. Biophys.* **97**, 529.

KIOH, Y. & RICH, A. (1964). Induced enzyme formed on bacterial polyribosomes. *Proc. natn. Acad. Sci., U.S.A.* **51**, 111.

LEA, D. E. (1946). *Actions of Radiation on Living Cells*. Cambridge University Press.

LEDERBERG, J. (1950). β-D-Galactosidase of *E. coli* K 12. *J. Bact.* **60**, 381.

NOVELLI, G. D., KAMEYAMA, T. & EISENSTADT, J. M. (1961). The effect of ultraviolet light and X-rays on an enzyme forming system. *J. cell. comp. Physiol.* **58**, 225.

PAULY, H. (1959). X-ray sensitivity and target volume of enzyme induction. *Nature, Lond.* **184**, 1570.

PAULY, H. (1963). Synthesis of protein, RNA, DNA and induced enzyme in Bacterium cadaveris after X-irradiation. *Int. J. Radiat. Biol.* **6**, 221.

POLLARD, E. C. & BARRETT, N. (1959). The radiation sensitivity and molecular weight of dry β-galactosidase. *Radiat. Res.* **11**, 781.

POLLARD, E. C., GUILD, W. R., HUTCHINSON, F. & SETLOW, R. B. (1955). The direct action of ionising radiation on enzymes and antigens. *Prog. Biophys. biophys. Chem.* **5**, 72.

POLLARD, E. C. & VOGLER, C. (1961). Radiation action on some metabolic processes in *Escherichia coli*. *Radiat. Res.* **15**, 109.

SWENSON, P. A. & SETLOW, R. B. (1964). β-Galactosidase: inactivation of its messenger RNA by ultraviolet irradiation. *Science*, **146**, 791.

J. gen. Microbiol. (1965), **41**, 127–134
Printed in Great Britain

Nutritional Factors in Relation to Growth and Fat Synthesis in *Mortierella vinacea*

BY C. G. C. CHESTERS AND J. F. PEBERDY
Department of Botany, University of Nottingham,

(*Received* 20 *April* 1965)

SUMMARY

Growth and fat synthesis in *Mortierella vinacea* are influenced by a number of nutritional factors. Monosaccharide carbohydrates provided the best carbon source for growth and fat synthesis. Organic nitrogen substrates were inferior to ammonium phosphate for fat accumulation, although high yields of mycelium were obtained. These media probably enhanced protein synthesis. The balance between carbon and nitrogen in the culture medium influenced the rate at which fat synthesis occurred. High concentrations of carbohydrate (glucose) resulted in large yields of mycelium with a high fat content.

INTRODUCTION

Many fungi are now known which have the ability to synthesize intracellular fat from carbohydrates supplied in the culture medium (Woodbine, 1959). Several detailed investigations have been carried out with species of the genera *Penicillium*, *Aspergillus* and *Fusarium* and these have shown that the synthesis of fats by fungi is controlled by a number of nutritional factors.

Although species of Mortierella are common isolates from soils there have been relatively few studies on the physiology of this group of fungi. The proteolytic activity of a number of species has been demonstrated (Wetter, 1952; Turner, 1956) and fat synthesis in *Mortierella alpina* has been investigated by Galloway (1949) and Woodbine, Gregory & Walker (1951). A closer study of fat synthesis by species of *Mortierella* seemed worthy of attention, and it was thought interesting, therefore, to extend the study on growth and fat synthesis with one of these fungi. *M. vinacea* was selected after preliminary investigations using seven species.

METHODS

The organism. The strain of *Mortierella vinacea* Dixon-Stewart used was kindly supplied by Mrs M. Turner of the Botany Department, University of Nottingham (Un. Nott. no–133 obtained from C.B.S.). Cultures were maintained on slopes of potato glucose agar and were subcultured every 4 weeks. Cultures for the preparation of inocula were grown on malt extract agar in 8 oz. medical flat bottles.

Composition of media, inoculation and incubation. The basal mineral salts medium used in all experiments contained the following (g./100 ml.): KH_2PO_4, 0·04; $MgSO_4.7H_2O$, 0·005; $FeCl_3$, 0·001. To this solution carbon and nitrogen sources were added as required, and in other experiments glucose and $(NH_4)_2HPO_4$ in a

range of concentrations. The medium was adjusted to pH 6·5 before autoclaving. The solutions of KH_2PO_4 and $FeCl_3$ were autoclaved separately and afterwards were mixed with the bulk of the medium contained in cotton-wool-plugged 250 ml. conical flasks. The final volume of the mixed solutions was 100 ml. All solutions were autoclaved at 108° for 30 min.

On cooling, the flasks were inoculated with a spore suspension containing about 1×10^6 spores/ml. Spore suspensions were prepared in sterile distilled water from a 7-day culture of the fungus growing on malt extract agar. Hyphal fragments were removed from the suspension by filtration through sterile glass wool. The spore content of a suspension was determined with a counting chamber; and colony counts were made on potato glucose agar. One ml. of spore suspension was added to each of the culture flasks which were then placed on a shaker in a constant temperature room at 20°.

In some experiments the cultures were harvested after 9 days; in others the cultures were harvested at intervals over an incubation period of 15 or more days. A set of three or five flasks was withdrawn at each sampling time. The mycelium developed as numerous small discrete pellets which were filtered off on weighed papers, dried, re-weighed and, together with samples of culture filtrates, were set aside for analysis.

Estimation of fat. The extraction of fat from dried mycelium was done in a multiple Soxhlet apparatus. Light petroleum (b.p. 40–60°) was used as solvent and the extraction was done for 24 hr. The solvent was then distilled off, and the residual fat weighed after drying at 60°.

Analyses of culture filtrates. Glucose concentration in culture fluids was estimated by the anthrone method (Yemm & Willis, 1954). Spectrophotometric readings were made on a Hilger Spectrophotometer with Ilford orange filter no. 607 and 5 mm. cells.

Nitrogen estimations were made by a modified micro-Kjeldahl method (E. C. Cocking, personal communication). A sample (usually 1 ml.) of diluted culture filtrate was boiled for several hours with 0·2 ml. of 1 % (w/v) solution of selenium dioxide in 50 % (v/v) sulphuric acid. The contents of the tube (about 0·1 ml.) were diluted to 3 ml. with distilled water; 1 ml. of this solution was mixed with 8 ml. distilled water and 1 ml. Nessler's solution. The extinction of the solutions was measured on the spectrophotometer, with Ilford spectrum violet filter no. 601 and 1 cm. cells.

RESULTS

Fat synthesis from different carbon sources

Several different carbon sources were incorporated with $(NH_4)_2HPO_4$ in the basal mineral salts solution. The cultures were harvested after 9 days.

Of the five carbon sources used, maltose gave the best yield of mycelium and a high degree of fat accumulation. Mycelium production on glucose was not so good, although this carbohydrate was as good as maltose for fat synthesis. Whilst supporting good mycelium formation, lactose was not a carbon source readily converted to fat. In contrast both starch and sodium acetate supported poor growth, but the mycelium contained a large proportion of fat. It would appear ,therefore, that carbon sources supporting the production of large amounts of mycelium do not necessarily stimulate fat synthesis. These results are summarized in Table 1.

Fat synthesis with different nitrogenous substrates

The yields of mycelium and fat obtained after 9 days are shown in Table 2.

Like other members of the group, *Mortierella vinacea* utilized various organic nitrogen sources; growth on these substrates was better than with ammonium

Table 1. *Formation of fat by Mortierella vinacea grown on various carbon sources*

Concentration of carbon source 1 g./100 ml. and $(NH_4)_2HPO_4$ 0·05 g./100 ml. Cultures harvested after 9 days. Values given are the means from five flasks with standard deviations.

Carbon source	Mycelium mean dry wt. (g./100 ml.)	Fat (g.)	Fat g./100 g. dry mycelium
Glucose	0·2643 (±0·0248)	0·1005 (±0·0092)	38·02 (±4·01)
Lactose	0·1959 (±0·0201)	0·0298 (±0·0025)	15·22 (±1·47)
Maltose	0·3395 (±0·0316)	0·1169 (±0·0104)	34·44 (±3·28)
Starch	0·0834 (±0·0076)	0·0209 (±0·0022)	25·05 (±2·62)
Sodium acetate	0·0653 (±0·0058)	0·0184 (±0·0020)	28·18 (±2·90)

Table 2. *Formation of fat by Mortierella vinacea grown on various sources of nitrogen*

Medium was made up of basal mineral salts medium + 1 % (w/v) glucose + nitrogen source added to give a final concentration of 1 mg. N/100 ml. medium. Cultures harvested after 9 days. Values are means from five flasks with standard deviations.

Nitrogen source	Mycelium mean dry wt. (g./100 ml.)	Fat (g.)	Fat g./100 g. dry mycelium	Nitrogen utilized (g./100 ml.)	Glucose utilized (g./100 ml.)
Ammonium phosphate	0·2295 (±0·0115)	0·1016 (±0·0055)	34·00 (±2·05)	0·0050	0·94
Aspartic acid	0·3171 (±0·0045)	0·0916 (±0·0042)	29·72 (±1·06)	0·0039	0·96
Arginine	0·3523 (±0·0082)	0·0514 (±0·0088)	14·46 (±2·19)	0·0073	0·70
Lysine	0·3254 (±0·0104)	0·0513 (±0·0175)	15·96 (±1·90)	0·0080	0·97
Asparagine	0·3295 (±0·061[illegible]	0·0633 (±0·0124)	19·01 (±2·10)	0·0076	0·89
Urea	0·3518 (±0·0111)	0·0440 (±0·0013)	12·53 (±0·78)	0·0075	0·81
Control	0·0085) (±0·0009)	—	—	—	0·27

phosphate, and nitrate did not support growth. In contrast fat synthesis was in most cases poor on the media which contained the amino acids and urea; an exception was aspartic acid which gave results similar to the ammonium salt. There was a correlation between the amount of fat synthesized and the amount of nitrogen utilized. Uptake of nitrogen was greatest from the amino acid and urea media

which supported only limited fat production, but was low from the media containing aspartic acid and ammonium phosphate. Glucose utilization was about the same in all media.

Comparative rates of growth and fat synthesis

The fungus was grown on basal salts medium +1 % (w/v) glucose + 0·05 % (w/v) $(NH_4)_2HPO_4$. Three flasks were removed after various periods of incubation. As seen in Table 3, rapid formation of mycelium occurred and the maximum yield was

Table 3. *Rate of fat synthesis in Mortierella vinacea*

Cultures were grown on basal salts medium + 1 % (w/v) glucose + 0·05 % (w/v) $(NH_4)_2HPO_4$. Mean values from three replicate cultures with standard deviations are given. Glucose estimations were made on combined filtrates.

Days	Mycelium mean dry wt. (g.)	Fat (g.)	Fat g./100 g. dry mycelium	Glucose utilized g./100 ml.
2	0·0694 (±0·0055)	0·0022 (±0·0002)	3·17 (±0·25)	0·28
4	0·2536 (±0·0264)	0·0701 (±0·0062)	27·68 (±1·51)	0·60
6	0·2970 (±0·0288)	0·1370 (±0·0110)	39·13 (±2·67)	0·92
8	0·2897 (±0·0240)	0·1116 (±0·0097)	38·58 (±3·11)	0·94
11	0·2806 (±0·0142)	0·1327 (±0·1063)	47·30 (±5·09)	0·95
13	0·2882 (±0·0210)	0·1477 (±0·0091)	51·40 (±3·14)	0·96
15	0·2789 (±0·0246)	0·1348 (±0·0125)	48·27 (±4·55)	1·00
18	0·2887 (±0·0258)	0·1342 (±0·0154)	47·32 (±5·24)	1·00
20	0·2742 (±0·0221)	0·1338 (±0·0061)	46·16 (±1·89)	1·00

obtained at 6 days, the amount of mycelium remaining fairly constant for the following 14 days. Utilization of glucose followed a linear pattern, 30 % (w/w) utilization after 2 days, 60 % after 4 days and 90 % after 6 days; average uptake 0·15 g. glucose/100 ml. medium/day.

The accumulation of fat reached a maximum after 13 days. During the initial stages of growth, fat synthesis proceeded rapidly and this condition was maintained for 6 days, after which the rate of synthesis decreased. At this stage the amount of fat in the mycelium was increasing, but there was no simultaneous increase in weight of mycelium. It is likely that much of the fat formed after the 6th to the 13th day, must have been derived from some other non-fat reserve in the mycelium.

The effect of the C:N ratio of the culture medium on growth and fat synthesis

Media containing glucose and $(NH_4)_2HPO_4$ were prepared in which carbon and nitrogen were present in ratios of 10:1, 25:1, 50:1 and 80:1. The results are shown in Table 4.

On medium 1 (C:N = 10:1) and medium 2 (C:N = 25:1) maximum production

Table 4. *The influence of the C:N ratio in the growth medium on the formation of fat in Mortierella vinacea*

Medium 1. C:N = 10:1, glucose 0·212 g./100 ml. Medium 2. C:N = 25:1, glucose 0·53 g./100 ml. Medium 3. C:N = 50:1, glucose 1·06 g./100 ml. Medium 4. C:N = 80:1, glucose 1·696 g./100 ml.

Concentration of $(NH_4)_2HPO_4$ in all media was 0·05 g./100 ml. Three replicate flasks were harvested at each sampling time. Mean values with standard deviations are given. Glucose analyses were made on combined filtrates.

Days	Mycelium mean dry wt. (g./100 ml.)	Fat (g.)	Fat g./100 g. dry mycelium	Glucose utilized (g./100 ml.)
		Medium 1		
3	0·1208 (±0·0078)	0·0038 (±0·0002)	3·38 (±0·25)	0·172
6	0·1125 (±0·0071)	0·0054 (±0·0002)	4·74 (±0·19)	0·212
9	0·1038 (±0·0025)	0·0101 (±0·0034)	9·76 (±3·30)	0·212
12	0·1003 (±0·0032)	0·0074 (±0·0017)	7·42 (±1·80)	0·212
15	0·0914 (±0·0056)	0·0055 (±0·0006)	5·61 (±0·92)	0·212
		Medium 2		
3	0·0988 (±0·0024)	0·0064 (±0·0009)	6·53 (±0·89)	0·46
6	0·0929 (±0·0075)	0·0112 (±0·0013)	12·22 (±2·20)	0·49
9	0·0905 (±0·0100)	0·0130 (±0·0008)	14·43 (±1·27)	0·49
12	0·0821 (±0·0037)	0·0049 (±0·0002)	5·22 (±0·98)	0·49
15	0·0826 (±0·0120)	0·0044 (±0·0002)	5·07 (±0·41)	0·50
		Medium 3		
3	0·2196 (±0·0052)	0·0468 (±0·0047)	21·56 (±2·70)	0·50
6	0·3122 (±0·0172)	0·0786 (±0·0095)	21·59 (±2·66)	1·02
9	0·3315 (±0·0065)	0·1298 (±0·0146)	39·10 (±3·58)	1·06
12	0·3292 (±0·0017)	0·1359 (±0·0019)	40·99 (±0·83)	1·06
15	0·3289 (±0·0038)	0·1514 (±0·0024)	46·22 (±1·19)	1·06
		Medium 4		
3	0·2338 (±0·0016)	0·0600 (±0·0035)	25·34 (±1·71)	0·292
6	0·3826 (±0·0062)	0·2350 (±0·0172)	60·56 (±3·36)	1·270
9	0·4567 (±0·0082)	0·3033 (±0·0054)	66·47 (±1·62)	1·696
12	0·4749 (±0·0167)	0·2852 (±0·0380)	61·45 (±7·70)	1·696
15	0·4502 (±0·0072)	0·2809 (±0·0028)	62·05 (±1·95)	1·696

of mycelium occurred after 3 days, followed by autolysis. The maximum yield of mycelium on these media was 20–30% (w/w) of that obtained on medium 3 (C:N = 50:1) and medium 4 (C:N = 80:1).

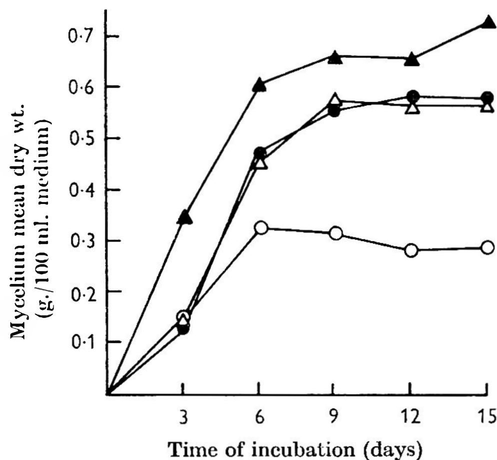


Fig. 1

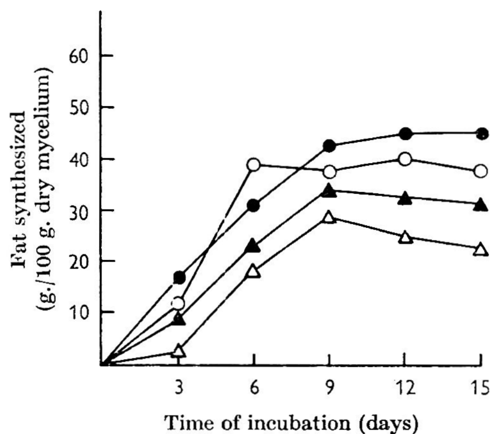


Fig. 2

Fig. 1. Growth of *Mortierella vinacea* on media containing various concentrations of glucose and ammonium phosphate, but maintaining a constant C:N ratio of 40:1 in the medium —○—○—, medium 5, glucose 1 g./100 ml., $(NH_4)_2HPO_4$ 0·05 g./100 ml.; —●—●—, medium 6, glucose 2 g./100 ml., $(NH_4)_2HPO_4$ 0·10 g./100 ml.; —△—△—, medium 7, glucose 5 g./100 ml., $(NH_4)_2HPO_4$ 0·25 g./100 ml.; —▲—▲—, medium 8, glucose 10 g./100 ml., $(NH_4)_2HPO_4$ 0·50 g./100 ml.

Fig. 2. Fat accumulation in the mycelium of *Mortierella vinacea* grown on media containing various concentrations of glucose and ammonium phosphate, but maintaining a constant C:N ratio of 40:1 in the medium. —○—○—, medium 5, glucose 1 g./100 ml., $(NH_4)_2HPO_4$ 0·05 g./100 ml.; —●—●—, medium 6, glucose 2 g./100 ml., $(NH_4)_2HPO_4$ 0·10 g./100 ml.; —△—△—, medium 7, glucose 5 g./100 ml., $(NH_4)_2HPO_4$ 0·25 g./100 ml.; —▲—▲—, medium 8, glucose 10 g./100 ml., $(NH_4)_2HPO_4$ 0·50 g./100 ml.

Table 5. *The influence of the C:N ratio in the growth medium on the efficiency of conversion of glucose to fat by Mortierella vinacea*

Details of media are given in Table 4.

	Fat coefficient*
Medium 1	4·74
Medium 2	2·64
Medium 3	14·34
Medium 4	17·74

* The fat coefficient is defined as the amount of fat produced by the organism per 100 g. carbon source utilized. The coefficients given here are calculated from data obtained at the time of maximum fat accumulation in the mycelium.

An increase in fat accumulation resulted from supplying the fungus with increased carbohydrate. As shown in Table 4, the culture growing on medium 4 produced mycelium which contained over 60% (w/w) fat. This yield is high in comparison with reports for other fungi. The results obtained suggest that the balance

between fat synthesis and protein synthesis was affected by the amounts of carbon and nitrogen available. Consideration of the fat coefficients for each culture at the time of maximum fat synthesis (Table 5) suggests that protein formation may have been occurring to a greater extent in cultures growing on low carbon media.

The influence of concentration of carbon and nitrogen at a constant C:N ratio

Media containing a range of concentrations of glucose and $(NH_4)_2HPO_4$ but maintaining a constant C:N ratio of 40:1 were prepared. Cultures were harvested after various periods of incubation and the results are given in Figs. 1 and 2. A tenfold increase in glucose concentration in medium 8 (10 %, w/v, glucose) over medium 5 (1 % glucose) gave an increased yield of mycelium. This increase was over 100 %. The growth produced on medium 6 (2 % glucose) and medium 7 (5 % glucose) was the same. The highest yields on these media were almost twice that on medium 5. There were noticeable differences in the times at which maximum growth occurred on the different media. On medium 5 maximum growth was produced after 6 days, on media 6 and 7 at 9 days, and on medium 8 mycelium formation was still taking place after 15 days.. These results are shown in Fig. 1.

The corresponding rates of fat synthesis and yields of fat are shown in Fig. 2. Fat synthesis proceeded more rapidly and more fat was formed (per g. mycelium) by the cultures growing on medium 5 (1 % glucose). There were different rates of fat synthesis in the four cultures during the first 3 days, the cultures growing on medium 5 producing most fat. After this initial period the rates of synthesis in the four cultures were about equal.

DISCUSSION

The nature of the carbon and nitrogen sources, together with the relative proportions of each, have a marked effect on growth and fat synthesis in *Mortierella vinacea*. The results given here show also that the formation of fat is an important aspect of the general metabolism of this fungus.

Nutrients which support good growth do not necessarily support the synthesis of large amounts of reserve food products. Some nutrients are used solely for the production of new protoplasm which contains little reserves, others support the formation of abundant reserves of a particular type as well as new protoplasm.

A wide range of carbon sources are utilized by fungi; glucose and maltose give rise to the highest yields of mycelium for most, e.g. *Penicillium lilacinum* (Philip & Walker, 1958), *Aspergillus nidulans* (Naguib, 1959). These fungi produced more fat from glucose than maltose and it was suggested that maltose favoured the accumulation of polysaccharides. In contrast, maltose was better than glucose for growth of *Mortierella vinacea* but the amount of fat synthesized was similar for both sugars.

Synthesis of fat on media containing inorganic forms of nitrogen occurs to varying extent. There are several reports that this form of nitrogen stimulates the formation of more fat than organic nitrogen sources, although mycelium production is better on the latter type of medium. This has been shown by Turpenein (1936) with Geotrichoides and by Gad & El-Nockrashy (1960) with *Aspergillus fumigatus*. Naguib & Saddik (1960) found that *A. nidulans* behaved differently, producing

larger yields of fat on medium containing asparagine than on media containing nitrate or ammonium salts.

The time at which fat formation occurs also varies. Naguib (1959) found that *Aspergillus nidulans* synthesized its fat during the later stages of growth. He found that at this stage increases in the weight of mycelial felts were due mainly to accretion of fat. With the organism used in this study, however, the processes of formation of new protoplasm and fat occurred simultaneously and some of the fat produced was utilized later in some cultures. This observation supports the view of Gyllenberg & Raitio (1952) who found that fat synthesized in the mycelium of a Penicillium species was later used when the fungus was starved of nutrients.

The effects of concentration of carbon and nitrogen in the medium have been investigated with a number of organisms. All behave in a similar manner, producing more fat when the amount of carbon in the medium is increased. The effects of increasing the concentrations of these two nutrients but maintaining a constant C:N ratio have not been studied previously. It is significant to note that whilst an increase in the amount of carbon and nitrogen in the medium was correlated with increased yield of mycelium, this was not so for fat synthesis. The concentration of nutrients in a medium is obviously of as much importance as their relative proportions in the control of metabolic processes in fungi.

We wish to thank Mrs M. Turner for helpful discussion and comments. This work was carried out during the tenure of a D.S.I.R. Research Studentship awarded to one of us (J.F.P.).

REFERENCES

Gad, A. M. & El-Nockrashy, A. S. (1960). Microbiological synthesis of fat. II. Effect of different cultural conditions on the synthesis of fat by *Aspergillus fumigatus*. *J. Chem. U.A.R.* **3**, 57.

Galloway, L. D. (1949). Patent Prov. Spec. 32887.

Gyllenberg, H. & Raitio, A. (1952). Studies on the decomposition of cellular lipids of moulds. *Phys. Plant.* **5**, 367.

Naguib, K. (1959). Growth and metabolism of *Aspergillus nidulans* Eidam in surface culture. *Can. J. Bot.* **37**, 353.

Naguib, K. & Saddik, K. (1960). Growth and metabolism of *Aspergillus nidulans* Eidam on different nitrogen sources in synthetic media conducive to fat formation. *Can. J. Bot.* **38**, 613.

Philip, S. E. & Walker, T. K. (1958). Mycological formation of fat. V. Factors which influence the formation of fat in surface cultures of *Penicillium lilacinum* Thom. *J. Sci. Fd. Agric.* **9**, 223.

Turner, M. (1956). *Mortierella globulifera*. Rostrup. *Trans. Br. mycol. Soc.* **39**, 291.

Turpenein, O. (1936). Studies on microbiological lipides. The formation and chemical composition of lipides of *Geotrichoides* sp. Langeron & Talli:e. *Ann. Acad. Sci. fenn.* A, **46**, 110.

Wetter L. R. (1952). The proteolytic enzymes of micro-organisms. IV. Partial purification of extracellular protease from *Mortierella renispora* Dixion-Stewart. *Can. J. Bot.* **30**, 685.

Woodbine, M. (1959). Microbiological fat: Micro-organisms as potential fat producers. *Prog. ind. Microbiol.* I.

Woodbine, M., Gregory, M. E. & Walker, T. K. (1951). Microbiological synthesis of fat. Preliminary survey of the fat producing moulds. *J. Expl. Bot.* **2**, 204.

Yemm, E. W. & Willis, A. J. (1954). The estimation of carbohydrates in plant extracts by anthrone. *Biochem. J.* **57**, 508.

J. gen. Microbiol. (1965), 41, 135–142
Printed in Great Britain

Isolation of Variant Strains from Foot-and-Mouth Disease Virus Propagated in Cell Cultures Containing Antiviral Sera

By N. St G. HYSLOP
The Animal Virus Research Institute, Pirbright, Woking, Surrey

(*Received* 23 *April* 1965)

SUMMARY

Three strains of foot-and-mouth disease virus of type SAT 1 were propagated serially in cultures of pig kidney cells in the presence of gradually increased concentrations of specific antiserum. All showed an increase in their ability to multiply in the presence of the serum. With two of the strains, complement-fixation tests revealed that propagation under these conditions was associated with modification of antigenic structure, but antigenic differences were detected later than changes in the ability of all strains to multiply in the presence of antiserum. Processes of this nature may possibly result *in vivo* from similar interactions of virus and antibody in host populations.

INTRODUCTION

Within each of the seven principal types of foot-and-mouth disease virus certain strains have been isolated which differ from one another in their host-specificity, virulence and invasiveness. In addition to differences of this nature, dissimilarity in antigenic constitution is frequently detectable by complement-fixation tests and other methods. The accumulated evidence for the existence of field strains possessing antigenic differences sufficiently great to justify their classification as subtypes was reviewed in a previous report (Hyslop, Davie & Carter, 1963); that report also described the practical effect of subtype variants on the apparent efficacy of vaccination campaigns. The influences which lead to the emergence of subtype variants in the field remain obscure but the interplay of infection and immunity in the host population is probably an important factor. The present report records the isolation of variants from three strains of foot-and-mouth disease virus, which were each passaged serially in secondary monolayer cultures of pig kidney cells in the presence of gradually increasing concentrations of antisera specific for the original strains.

METHODS

Foot-and-mouth disease (*FMD*) *virus.* All strains were of type SAT 1. Strain RV. 11/37 was isolated in Southern Rhodesia in 1937, strain SA. 13/61 was isolated in the Transvaal in 1961 and strain Tur. 323/62 was isolated in western Turkey during the epizootic of 1962. Before passage in pig-kidney tissue culture, the 3 strains were each propagated on the tongues of cattle at Pirbright and then were typed by complement-fixation tests.

Titration. Serial threefold dilutions of virus were inoculated intraperitoneally into randomized groups of 4- to 6-day-old albino mice of the Pirbright 'P' strain.

Usually 8 mice were inoculated with each dilution and the 50 % fatality end-points were estimated by the method of Kärber (1931).

Hyperimmune guinea pig sera. Virus strains were usually passaged twice in guinea pigs before being used to produce hyperimmune sera. Groups of guinea pigs, of about 500 g. body weight, from the colony at this Institute, were hyperimmunized by two doses of virus suspension. The first intradermal inoculation into the tarsal pads contained 0·05 % (w/v) saponin; this was followed after 21 days by an intramuscular inoculation (without saponin). The animals were bled out 10 days later.

Hyperimmune cattle serum. Virus of strain Tur. 323/62 passaged in cattle was inoculated into the dermal mucosa of a fully susceptible steer, which developed clinical foot-and-mouth disease. A second inoculation of virus of the same strain was made 76 days later. The animal was bled for serum 2 weeks after the second inoculation.

Propagation of virus in pig-kidney tissue culture. Pig kidney cells, grown as primary monolayers for 6–7 days in Roux flasks containing Earle's saline with 0·5 % lactalbumin hydrolysate and 0·01 % yeast extract (EYL medium) plus 10 % bovine serum, were harvested by means of 0·02 % EDTA + trypsin solution and, after washing, were seeded into 4 oz. flat bottles containing the same medium. After about 2 days of incubation at 37° the secondary monolayers were washed and warm fresh medium, usually containing the required concentration of either inhibitory or normal serum, was added. The culture was inoculated with virus and incubation continued until either cytopathic effect was observed or the medium became acid.

Complement-fixation tests. Guinea pig hyperimmune sera were prepared by using virus isolated at several passage stages during propagation in pig-kidney tissue cultures containing antisera; similar sera were prepared at the corresponding passage stages in which identical amounts of normal serum had been incorporated in the medium. The sera were used in complement-fixation tests (Brooksby, 1952; Bradish & Brooksby, 1960) to determine the cross-fixation ratios of the various virus isolates with one another. The cross fixation ratio is:

$$\frac{\text{Amount of complement fixed in the heterologous serum-virus system}}{\text{Amount of complement fixed in the homologous serum-virus system}}.$$

Calculation of the product of these ratios excludes differences attributable to the relative concentrations of individual reagents. Some degree of antigenic variation was indicated when the product of these ratios was significantly less than unity; and when the product was less than 0·5 strains were considered to be distinct subtypes (Bradish & Brooksby, 1960).

Serum–virus neutralization tests. Cross-neutralization tests in mice, using homologous and heterologous substrain-specific sera, were done by the method of Skinner (1953).

RESULTS

Strain RV. 11/37

Cattle-passaged FMD virus of strain RV. 11/37 was passaged once in mice and a Seitz EK filtrate (infectivity titre $10^{5\cdot4}$ mouse LD 50/ml.) was prepared from triturated mouse carcasses. Equal volumes of the filtrate and of a $10^{-5\cdot0}$ dilution

of guinea pig hyperimmune serum (homologous strain) were mixed and incubated for 30 min. at 26°. Two ml. of the mixture were inoculated into a pig-kidney tissue culture monolayer; the same volume of virus mixed with 0·4 M-phosphate buffer (pH 7·6) was inoculated into a further pig-kidney tissue culture monolayer. After 30 min. incubation, the monolayers were washed and 20 ml. EYL medium added. Cytopathic effect was evident in both cultures after incubation for 24 hr at 37° and the culture fluids were used to inoculate the next cultures of the series. The procedure was repeated 4 times, the serum concentration being increased tenfold at each stage.

After the 5th passage, virus and serum were not mixed before the cultures were inoculated, but the serum was added directly to the culture medium and then 1 ml. of virus suspension was added to 19 ml. of the serum + medium mixture. A further 41 passages of strain RV. 11/37A were made in medium containing guinea pig hyperimmune serum at a dilution of $10^{-2\cdot0}$, followed by four passages in which the dilution was decreased to $10^{-1\cdot7}$. At the 1st, 5th, 31st and 46th passages the amounts of virus inoculated were $10^{5\cdot7}$, $10^{6\cdot5}$, $10^{6\cdot4}$ and $10^{5\cdot3}$ mouse LD 50. Concurrently, substrain RV. 11/37B was passaged in medium containing corresponding dilutions of normal serum for a total of 50 passages.

Table 1. *Decrease of the neutralizing power of 1/1000 antiserum for foot-and-mouth disease virus strain RV. 11/37 propagated serially in pig-kidney tissue culture containing homologous antiserum*

Passage no.	Log. depression of virus titre after 30 min. incubation with antiserum		Log. difference between virus substrains
	Substrain A	Substrain B	
1	2·2	1·9	≤ 0·0
4	1·8	2·6	0·8
10	1·8	2·3	0·5
30	1·5	2·3	0·8
45	0·6	2·6	2·0

Substrain A, passaged in the presence of immune serum; substrain B, passaged in the presence of normal serum.

Sensitivity to incubation with antiserum. The sensitivities of the two substrains to the neutralizing effect of RV. 11/37 guinea pig hyperimmune serum were compared at intervals during the experiment, the first and last isolates being passaged once in serum-free cultures before being used for neutralization tests. Virus suspensions were mixed with equal volumes of a 1/500 dilution of serum in 0·4 M-phosphate buffer (pH 7·6) and, after incubation for 30 min. at 37°, the two mixtures and serum-free controls were titrated in mice. Little change occurred in the sensitivity of substrain RV. 11/37A before the 30th passage but the substrain became much less susceptible to inactivation by serum between the 30th and 45th passages (Table 1).

Complement-fixation tests. Because the virus suspensions harvested from the 50th pig-kidney tissue culture were consistently anticomplementary, both substrains were passaged once on the tarsal pads of guinea pigs to produce antigen suitable for

complement-fixation tests. The cross-fixation product of substrains RV. 11/37 A and RV. 11/37 B was 0·03, indicating that the substrains were antigenically distinct after the 50th passage.

Strain SA. 13/61

A 1/10 suspension of bovine tongue epithelium infected with FMD virus of strain SA. 13/61 was filtered and 2 ml. samples used to inoculate two pig-kidney tissue culture monolayers maintained in 18 ml. EYL medium. To these cultures had been added either (A) pooled serum from cattle recovered from clinical infection with virus of strain SA. 13/61, or (B) normal cattle serum, in volumes sufficient to produce a final dilution of serum of $10^{-5\cdot0}$. Only limited cytopathic effect was noted after incubation for 24 hr but virus from each of the cultures was passaged serially in gradually increasing concentrations of the appropriate sera. At the start of the experiment the strain did not multiply in the presence of immune serum at a dilution of $10^{-4\cdot0}$. Serial passage of the substrains continued until strain SA. 13/61 A had been passaged twice in the presence of convalescent serum concentrations of $10^{-5\cdot0}$, once at each of the concentrations $10^{-4\cdot0}$, $10^{-3\cdot0}$, $10^{-2\cdot0}$, and $10^{-1\cdot7}$ and finally 4 times at a serum concentration of $10^{-1\cdot5}$. Substrain SA. 13/61 B was passaged similarly in the presence of normal serum. Although the presence of increasing amounts of antiserum during serial passage appeared to exert progressively less inhibitory effect on the virus, ten passages were insufficient to cause significant change either in the sensitivity to incubation with 1/1000 antiserum or in the antigenic structure of the substrain A.

Strain Tur. 323/62

The general procedure followed that of the previous experiment but hyper-immune cattle serum was employed instead of convalescent serum. The initial inoculum was a filtrate from a suspension of triturated bovine tongue epithelium infected with strain Tur. 323/62; the infectivity titre was $10^{5\cdot5}$ mouse LD 50/ml.

Substrain Tur. 323/62 A was passaged in pig-kidney tissue culture 3 times in the presence of a serum dilution of $10^{-4\cdot0}$, twice at a dilution of $10^{-3\cdot0}$, 5 times at $10^{-2\cdot5}$, 5 times at $10^{-2\cdot3}$, 3 times at $10^{-1\cdot7}$ and then twice at a serum dilution of $10^{-1\cdot5}$. Substrain Tur. 323/62 B was passaged concurrently in the presence of corresponding dilutions of pooled serum from normal cattle. Well-marked cytopathic effect was observed with both substrains. After the 20th passage the substrains were stored at -20° pending completion of the complement-fixation tests. The stored material was revived by a single passage in serum-free pig-kidney tissue culture; then a further five passages were made in a serum concentration of $10^{-1\cdot5}$ and at each stage an attempt was made to propagate virus of substrain Tur. 323/62 A in a serum concentration of $10^{-1\cdot3}$ (Table 2). The effect is not attributable to a gradual increase in the infectivity titre of the inoculum during passage.

Sensitivity to incubation with antiserum. Virus from the 1st, 10th, 15th and 20th passage levels of substrains Tur. 323/62 A and Tur. 323/62 B were each passaged once in serum-free pig-kidney tissue culture and then used in cross-neutralization tests against homologous and heterologous guinea pig sera. Modification occurred in substrain Tur. 323/62 A between the 15th and 20th passages and this was sufficient to cause a difference greater than tenfold in the neutralization indices of the homologous and heterologous serum + virus pairs (Table 3).

Table 2. *Adaptation of foot-and-mouth disease virus strain Tur. 323/62 A to multiplication in pig-kidney tissue culture in the presence of bovine hyperimmune serum*

Passage no.	Titre of virus inoculated (mouse LD 50/ml.)	Serum in culture medium	Dilution of serum in culture medium										
			$10^{-1.0}$	$10^{-1.3}$	$10^{-1.5}$	$10^{-1.7}$	$10^{-2.0}$	$10^{-2.3}$	$10^{-2.5}$	$10^{-2.7}$	$10^{-3.0}$	$10^{-4.0}$	$10^{-5.0}$
1	$10^{5.5}$	Normal	+ + +	.	.	.	+ + +	.	.	.	+ + +	+ + +	+ + +
1	$10^{5.5}$	Hyperimmune	.	.	.	.	–	.	.	.	–	+	+ +
2	$10^{4.9}$	Hyperimmune	.	.	.	.	.	.	.	.	.	±	.
3	n.a.*	Hyperimmune	.	.	.	.	.	.	.	.	±	+ + +	.
4	$10^{4.6}$	Hyperimmune	.	.	.	.	.	.	.	.	+ +	+ + +	.
5	n.a.	Hyperimmune	.	.	.	.	.	.	.	+	+ +	.	.
6	$10^{4.0}$	Hyperimmune	.	.	.	.	–	+	+	+ +	.	.	.
11	$10^{4.6}$	Hyperimmune	.	.	.	.	–	+	+ + +	.	.	.	.
12	n.a.	Hyperimmune	.	.	.	.	±	+ +	.	.	.	.	.
14	n.a.	Hyperimmune	.	.	.	.	+	+ +	.	.	.	.	.
16	$10^{5.2}$	Hyperimmune	.	.	–	+	+ +	+ + +	.	.	.	.	.
20	$10^{1.4}$	Hyperimmune	.	.	+ +	.	.	.	.	.	.	.	.
21	n.a.	Hyperimmune	–	–	+	.	.	.	.	.	.	.	.
24	n.a.	Hyperimmune	–	±	+ +	+ + +	.	.	.	.	.	.	.
25	$10^{3.9}$	Hyperimmune	–	±	+ +	.	.	.	.	.	.	.	.
25	$10^{3.9}$	Normal	+ + +	.	.	.	+ + +	.	.	.	.	.	.

* n.a. = not available.

– = no reaction; ± = small zones of cellular swelling and degeneration; + = small zones of fenestration with release of cells into medium; + + = large areas of cellular detachment; + + + = almost complete destruction of monolayer.

Complement-fixation tests. Virus suspensions collected at several passage levels were used as antigens for complement-fixation tests with homologous and heterologous antisera; a significant difference in the amount of complement fixed was not observed, irrespective of whether supernatant or whole-culture material was employed. Little change in antigenic structure was detectable during the first 15 passages (Table 4), though some modification may have occurred already in the ability of substrain Tur. 323/62 A to multiply in the presence of hyperimmune cattle serum (Table 2). By the 15th passage, however, antigenic differences were just becoming apparent. After the 20th passage the mean cross-fixation product of the substrains had decreased to 0·4 and they may be regarded as distinct strains. The 1st and 20th passages of substrain Tur. 323/62 B were antigenically similar.

Table 3. *Cross-neutralization indices (log.) of substrains of foot-and-mouth disease virus strain Tur. 323/62 in neutralization tests with homologous and heterologous antisera*

Virus, substrain and passage no.	Serum, substrain and passage no.			
	B1	A1	B20	A20
B1	3·2	3·3	3·4	1·5
A1	3·5	3·3	4·2	1·5
A10	3·5	3·5	3·8	1·6
A15	3·2	3·2	3·4	2·4
A20	2·2	2·1	2·4	3·5
B20	3·5	3·3	3·7	1·9

Substrain A, passaged in the presence of immune serum; substrain B, passaged in the presence of normal serum.

Table 4. *Complement-fixation products of substrains A and B of foot-and-mouth disease virus strain Tur. 323/62 during pig-kidney tissue culture passage in hyperimmune serum and in normal serum, respectively*

Substrain and passage no.	Substrain and passage no.									
	B1	A1	B5	A5	B10	A10	B15	A15	B20	A20
B1	1·00	1·00	1·03	0·96	1·02	.	0·90	.	1·00	0·37
A1	1·00	1·00	.	.	.	.	.	.	.	.
B5	1·03	.	1·00	1·00	.	.	.	.	.	.
A5	0·96	.	1·00	1·00	.	.	.	.	.	.
B10	1·02	.	.	.	1·00	0·96	.	.	.	.
A10	.	.	.	.	0·96	1·00	.	.	.	.
B15	0·90	.	.	.	.	.	1·00	0·80	.	.
A15	.	.	.	.	.	.	0·80	1·00	.	.
B20	1·00	.	.	.	.	.	.	.	1·00	0·40
A20	0·37	.	.	.	.	.	.	.	0·40	1·00

Substrain A, passaged in the presence of immune serum; substrain B, passaged in the presence of normal serum.

DISCUSSION

The availability of a strain-specific antiserum of high titre prompted the use of guinea pig serum for the early experiments with foot-and-mouth disease (FMD) virus strain RV. 11/37 but, in an attempt to relate experiments *in vitro* more

closely to the conditions which might occur *in vivo*, strains SA. 13/61 and Tur. 323/62 were passaged with bovine sera. Because of this and other differences in technique, comparison between strains is rendered difficult. Irrespective of the source of antiserum, however, propagation of FMD virus of type SAT 1 in the presence of subinhibitory concentrations of homologous antibody was associated with gradual change in one or more of the characteristics of the strains. Thus, by the 50th passage in the presence of guinea pig hyperimmune serum, a very profound change in both serum sensitivity and antigenic structure had occurred in substrain A of strain RV. 11/37. Similar modification occurred during 20 passages of strain Tur. 323/62 in pig-kidney tissue cultures containing cattle hyperimmune serum. With strain SA. 13/61 the changes were limited to an apparent increase in the ability of the strain to multiply in greater concentrations of antiserum.

By the 5th passage, the substrain Tur. 323/62A was able to multiply in a concentration of hyperimmune serum at least 12-fold greater than at the 1st passage (Table 2). Complement-fixation tests did not reveal any antigenic modification until the 10th passage, when the difference in cross-fixation product was of doubtful significance. Although 20 passages apparently modified the antigenic constitution of the strain sufficiently for it to be considered distinct from the original strain, it cannot be concluded that as many as 20 passages would necessarily be required to produce such an effect on this or on other strains. The rate of change of substrain Tur. 323/62A was greatest between the 16th and 20th passages, when the antiserum dilution was $10^{-1.7}$ or less (Tables 2 and 3); it might well have been possible to propagate the virus in lower dilutions of serum at a somewhat earlier stage and thus to have accelerated the rate of antigenic variation. However, because the animal providing the antiserum had been inoculated twice with virulent virus, the neutralizing titre of its serum was $\geqslant 1/4096$ and this precluded the use of low dilutions of serum in the early passages. There was no evidence that passage in pig-kidney tissue culture containing normal serum decreased the sensitivity of the strain to immune serum or caused modification of antigenic structure.

The data from these experiments do not reveal the mechanism by which the variants arose. Because field strains of virus were used to initiate each passage series, the new substrains may have been isolated either as a result of mutation or by selection from an originally heterogeneous population. The alternatives are not mutually exclusive but, partly because of the progressive nature of the changes observed, it appears less likely that selection from a mixed population was the predominant factor in the isolation of variants. In this context it is noteworthy that strain Tur. 323/62 and other strains isolated at about the same time, during the epizootic in the Middle East, have all shown evidence of remarkable stability and antigenic similarity; and strain RV. 11/37 has been maintained for more than 25 years at Pirbright without exhibiting any obvious 'drift' in its properties. Stability of the degree exhibited by these strains appears inconsistent with very heterogeneous populations and it is likely that mutants begin to form an important fraction of the population only when adverse factors tend to suppress the original strain.

Selection may greatly alter the frequency of one-step mutants during relatively few passages and it is relevant to recall that the mutation rate for marker systems such as thermal inactivation may be as high as $10^{-3.8}$ to $10^{-5.6}$ for some strains of

foot-and-mouth disease virus (Pringle, 1964). Furthermore, Pringle (1965) has indicated that the differences between strains which are revealed by complement-fixation tests are a manifestation of a complex genetic characteristic. Consequently, it is probable that the variation in subtype characters observed in the present experiments occurred principally as a result of cumulative selection of a number of independent mutations during serial passage in the strongly selective system employed. Moreover, recent experiments of a similar nature (to be reported elsewhere) indicate that virus of plaque-purified strains may also develop, during relatively few passages, a marked increase in ability to grow in the presence of antiserum prepared against virus from the same clone. Changes in antigenic structure, resembling those reported above, were detected subsequently during experimental passage of foot-and-mouth disease virus in partly immunized cattle (Hyslop & Fagg, 1965).

The author wishes to thank Mr R. H. Fagg, who supervised most of the complement-fixation tests, and to acknowledge the technical assistance of Mr R. L. G. King and Mr K. C. Cranston.

REFERENCES

Bradish, C. J. & Brooksby, J. B. (1960). Complement-fixation studies of the specificity of the interactions between components of the virus system of foot-and-mouth disease and its antibodies. *J. gen. Microbiol.* **22**, 405.

Brooksby, J. B. (1952). The technique of complement fixation in foot-and-mouth disease research. *A.R.C. Rep. Ser.* no. 12.

Hyslop, N. St G., Davie, J. & Carter, S. P. (1963). Antigenic differences between strains of foot-and-mouth disease virus of type SAT 1. *J. Hyg., Camb.* **61**, 217.

Hyslop, N. St G. & Fagg, R. H. (1965). Isolation of variants during passage of a strain of foot-and-mouth disease virus in partly immunized cattle. *J. Hyg., Camb.* (in the Press).

Kärber, G. (1931). Beitrag zur kollektiven Behandlung pharmakologischer Reihenversuche. *Arch. exp. Path. Pharmak.* **162**, 480.

Pringle, C. R. (1964). Genetic aspects of the thermal inactivation properties of foot-and-mouth disease virus strains. *Bull. Off. int. Epiz.* **61**, 619.

Pringle, C. R. (1965). Evidence of genetic recombination in foot-and-mouth disease virus. *Virology*, **25**, 48.

Skinner, H. H. (1953). One week old white mice as test animals in foot-and-mouth disease research. *Proc. XV int. vet. Congr., Stockholm*, 1953, p. 208.

J. gen. Microbiol. (1965), **41**, 143–150
Printed in Great Britain

Effect of Dilution Rate and Growth-Limiting Substrate on the Metabolic Activity of *Torula utilis* Cultures

By D. W. TEMPEST and D. HERBERT
Microbiological Research Establishment, Porton Down, Wiltshire

(*Received* 27 May 1965)

SUMMARY

Torula utilis was grown in a chemostat, at several dilution rates, in media containing growth-limiting concentrations of glucose, xylose and ethanol; it was similarly grown in an NH_4^+-limited medium (carbon source, glucose). The 'steady state' rates of oxygen uptake of cultures, and Q_{O_2} values for washed suspensions of organisms separated from the cultures and incubated with each substrate, were determined at each dilution rate. Differences in oxidation rates indicated quantitative changes in the 'constitutive' enzyme content of organisms, which varied according to the growth rate and/or the nature of the growth-limiting substrate. The significance of these changes in terms of metabolic regulation and economy of enzyme synthesis is discussed.

INTRODUCTION

With the introduction and development of techniques for the continuous cultivation of micro-organisms (Monod, 1950; Novick & Szilard, 1950; Herbert, Elsworth & Telling, 1956), it is possible to study the influence of specific environmental changes on the chemical composition and metabolic activity of micro-organisms under conditions where the physical and chemical environment is rigidly controlled. Much data have accumulated on the variations in chemical composition and in macromolecular organization of organisms which occur when specific changes in environment are made; for example in intracellular polysaccharide content (Holme, 1957), in RNA content (Neidhardt & Magasanik, 1960; Dean, 1962; Rosset, Monier & Julien, 1964), ribosomal composition and content (Ecker & Schaechter, 1963; Sykes & Tempest, 1965; Tempest, Hunter & Sykes, 1965; Tempest & Hunter, 1965) and in cell wall composition and content (Collins, 1964; Sud & Schaechter, 1964). The present paper reports results of experiments designed to determine whether quantitative changes in some 'constitutive' enzyme systems can occur.

In C-limited cultures the carbon-containing substrate is present in the environment at a concentration which is insufficient to saturate the oxidative enzymes of the organisms (see Herbert *et al.* 1956). It follows, therefore, that the changes in substrate oxidation rate which accompany changes in 'steady state' growth rate need not necessarily require similar changes in cellular oxidative enzyme concentration but could result solely from changes in the degree of subsaturation of a constant amount of enzymic material. A measure of cellular oxidative enzyme concentration can be obtained from the rates of oxidation of excess substrate by washed suspensions of organisms prepared from chemostat-grown cultures. We report here the rates of oxidation of glucose, xylose and ethanol by *Torula utilis* organisms grown at various dilution rates in glucose-, xylose-, ethanol- and NH_4^+-limited environments.

METHODS

Organism. Torula utilis (NCYC 321) maintained by fortnightly subculture on a medium containing (% w/v): 0·3 yeast extract, 0·5 peptone, 1 glucose, 0·2 potassium dihydrogen phosphate, 2 agar; pH 5·5.

Media. The carbon-limited media contained: $(NH_4)_2SO_4$, 0·15 M; KH_2PO_4, 5×10^{-2} M; citric acid, 4×10^{-3} M; biotin, 1×10^{-7} M; $MgCl_2$, $1{\cdot}25 \times 10^{-3}$ M; traces of Ca^{2+}, Fe^{3+}, Zn^{2+}, Mn^{2+}, Cu^{2+}, Co^{2+}, MoO_4^{2-}; the carbon source was added to a final concentration of 1% (w/v). To obtain NH_4^+-limited conditions $(NH_4)_2SO_4$ was reduced to ($1{\cdot}8 \times 10^{-2}$ M) and glucose was increased to 5% (w/v).

Apparatus. A chemostat of 0·5 l. working volume, with automatic pH control, which permitted the growth of organisms to a high concentration, was used. Temperature was automatically controlled at 30°, and the pH value at 5·5.

Dry weights. Duplicate samples of culture (5 ml.) were centrifuged (3000*g* for 10 min.) in weighed 100 mm. × 12 mm. Pyrex tubes; the organisms were washed in distilled water by centrifugation and dried at 105° for 16 hr. Results for duplicate samples were within 5%, and generally within 2%, of each other.

Protein. The method of Stickland (1951) was used; a standard was provided by a solution of bovine plasma albumin (Armour Laboratories Ltd.).

Determination of culture Q_{O_2} values. These are reported as μl. oxygen uptake/mg. protein/hr and were calculated from the rates of air flow through the chemostat oxygen content of the effluent air (determined with a Beckman Model E-2 oxygen analyser), culture dry weight of organisms and their protein contents. It was necessary to base oxygen uptake rates on culture protein content because of the gross variations in polysaccharide content and hence of the dry weight of organisms grown in the NH_4^+-limited medium.

Determination of Q_{O_2} values of washed suspensions. These were determined by the conventional manometric technique. Each manometer vessel contained substrate (0·1 ml. 1M; in the side-arm) and approximately 2 mg. dry weight organisms, suspended in 1·9 ml. 0·06 M-phosphate buffer (pH 5·5) in the main compartment; 0·2 ml. 3 N-NaOH, and a pleated square of filter paper, was placed in the centre well. Temperature was adjusted to 30° and the oxygen uptake followed for 60 min. after mixing the contents of the side-bulb and main compartment. The oxygen uptake rates were linear over the period of observation; endogenous respiration rates (simultaneously measured) were subtracted from the substrate oxidation rates.

Experimental procedure. Organisms from an overnight culture grown on the maintenance medium were inoculated into the selected medium contained in the chemostat. After growth had ceased, a regulated flow of fresh medium to the culture was started. The medium flow rate was initially adjusted to 0·1 vol./hr and the culture left to equilibrate for 2–3 days before the start of each experiment. After this period a sample (50 ml.) of culture was removed from the chemostat, cooled to 4° and used for dry-weight determination (2 × 5 ml.) for metabolic studies (10 ml.) and for chemical analysis of organisms (30 ml. washed in distilled water and lyophilized). The flow rate of medium was then changed to a new value, the culture left to equilibrate for 2 days, and the above procedure repeated. The flow rate was progressively increased until the 'critical' dilution rate was reached; the flow rate

was then progressively decreased in similar increments to a suitable minimum value (usually 0·05 vol./hr). Each experiment took 3–4 weeks to complete, and successively employed glucose-, xylose-, ethanol-, and NH_4^--limited conditions.

RESULTS

Effect of growth rate on respiration rate of Torula utilis cultures

When growth is limited by the supply of carbon source the rate of oxygen uptake will depend on the dilution rate. The relationship between respiration rate and growth rate in cultures of *Torula utilis* growing in either a xylose-limited or an ethanol-limited medium was linear (Fig. 1). At zero growth rate neither plot passes through the origin but extrapolates back to a finite Q_{O_2} value which probably represents the endogenous respiration rate (see similar result with *Aerobacter aerogenes* culture; Herbert, 1958). Under nitrogen-limited conditions with excess carbon source, a similar result was obtained (Fig. 2) but here the extrapolated Q_{O_2} value was much higher since it included not only the endogenous respiration but also the oxidation of glucose in the absence of utilizable nitrogen. The rates of endogenous metabolism were dependent on growth rate with both C- and N-limited organisms (Fig. 3) though in different ways. The increase in endogenous metabolism at low growth rates in N-limited organisms correlated with a gross increase in intracellular glycogen-like material (Herbert, 1961).

Effect of growth rate on the metabolic activity of Torula utilis organisms

To grow at any specific rate the organisms must oxidize the carbon source at a corresponding minimum rate: in C-limited cultures however the capacity to oxidize the substrate may be much greater than the rate required to sustain growth. This was apparent with the glucose oxidizing system of *Torula utilis* organisms grown at a low dilution rate in a glucose-limited medium (compare Figs. 2 and 4). In Fig. 4 it is apparent that the oxidative ability of glucose-limited organisms varied little with growth rate and that differences in substrate dissimilation rates, in the culture, resulted from changes in the degree of saturation of enzymes on changing the substrate input rate. In contrast, the xylose oxidizing system of xylose-limited organisms varied markedly with growth rate (Fig. 5) in a manner which paralleled the changes in culture respiration rate (Fig. 1). The enzyme system oxidizing ethanol in ethanol-limited organisms responded in a different manner to changes in dilution rate (Fig. 5) in that it varied only slightly between dilution rates of 0·05 and 0·35 vol./hr but then markedly at higher growth rates.

In chemostat cultures a simple relationship between specific growth rate and the concentration of growth-limiting substrate has been demonstrated (Monod, 1942, Herbert *et al.* 1956); it seems likely therefore, that changes in the oxidative capacity, of C-limited organisms are due primarily to changes in intracellular substrate concentration which influence mechanisms controlling enzyme synthesis. In N-limited cultures, where the carbon-containing substrate concentration is independent of growth rate, a direct relationship between substrate oxidizing capacity and dilution rate would not be expected. However it is apparent (Fig. 6) that the glucose oxidizing ability of N-limited *Torula utilis* organisms (carbon source, glucose) did vary markedly with growth rate, and to a greater extent than that of glucose-limited

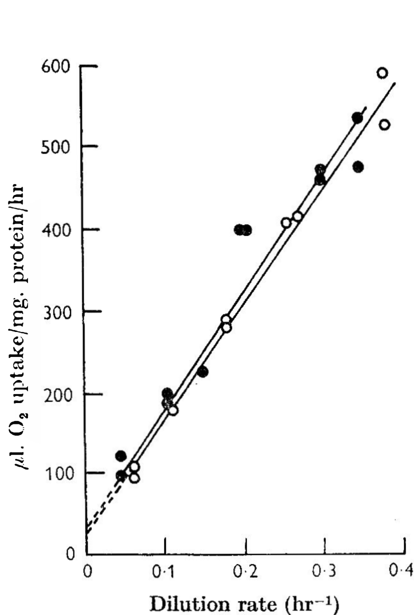


Fig. 1

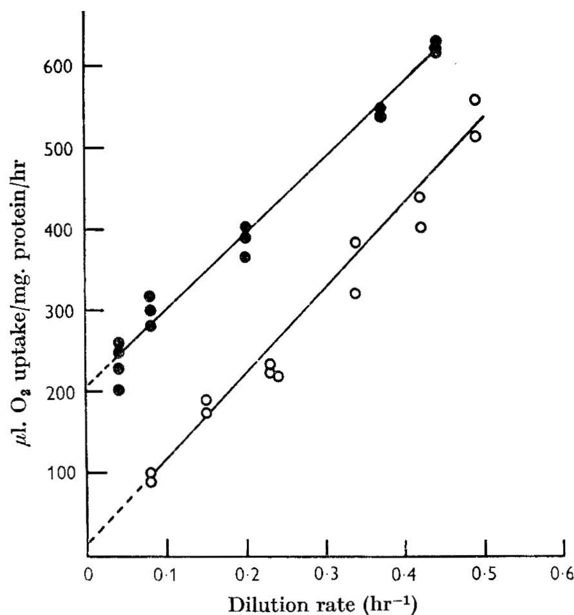


Fig. 2

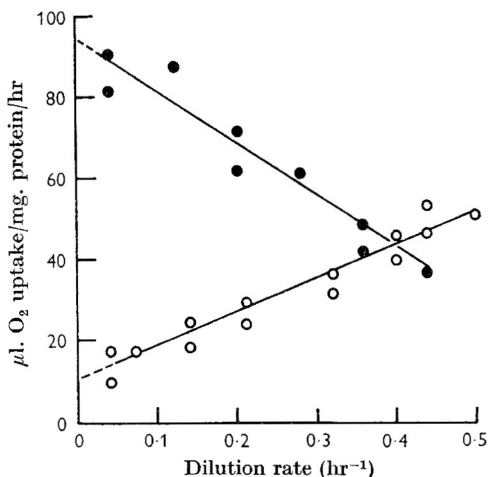


Fig. 3

Fig. 1. Rates of oxygen uptake (μl./mg. protein/hr) by *Torula utilis* organisms growing in xylose-limited (●) and ethanol-limited (○) media at several dilution rates.

Fig. 2. Rates of oxygen uptake (μl./mg. protein/hr) by *T. utilis* organisms growing in a glucose medium NH_4^+-limited (●) and C-limited (○).

Fig. 3. Rates of endogenous respiration (μl./mg. protein/hr) of NH-limited (●) and C-limited (○) *T. utilis* organisms grown at several dilution rates, washed free from the culture medium and suspended in 0·06 M-phosphate buffer (pH 5·5).

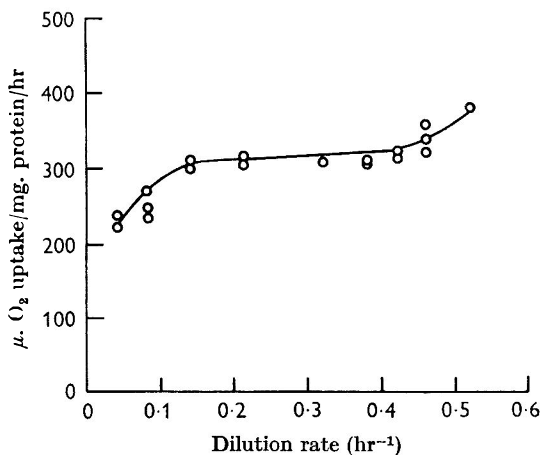


Fig. 4

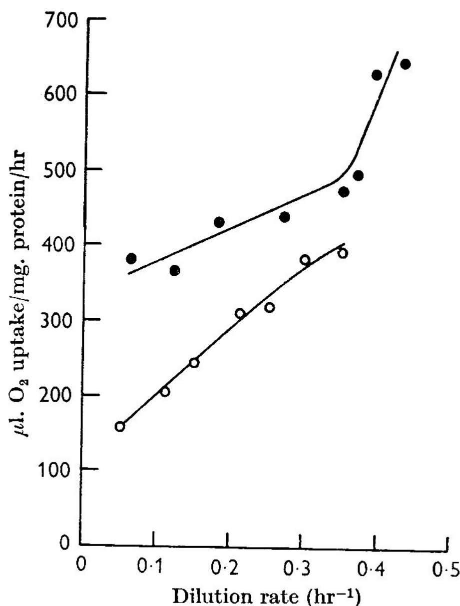


Fig. 5

Fig. 4. Rates of oxidation (μl./mg. protein/hr) of 50 mM-glucose by washed suspensions of C (glucose)-limited *T. utilis* organisms grown at several dilution rates; endogenous values subtracted.

Fig. 5. Rates of oxidation (μl./mg. protein/hr) of 50 mM-ethanol by C (ethanol)-limited (●), and 50 mM-xylose by C (xylose)-limited (○) *T. utilis* organisms, grown at several dilution rates; endogenous values subtracted.

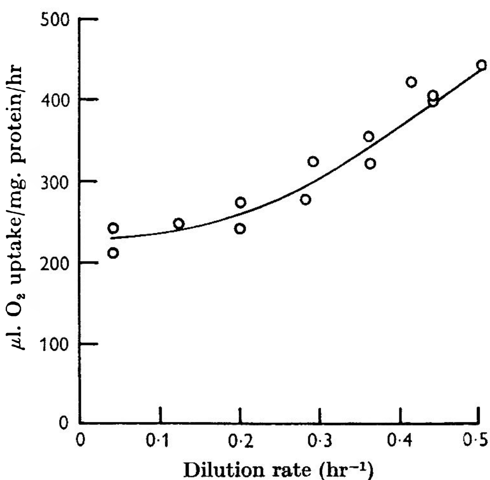


Fig. 6

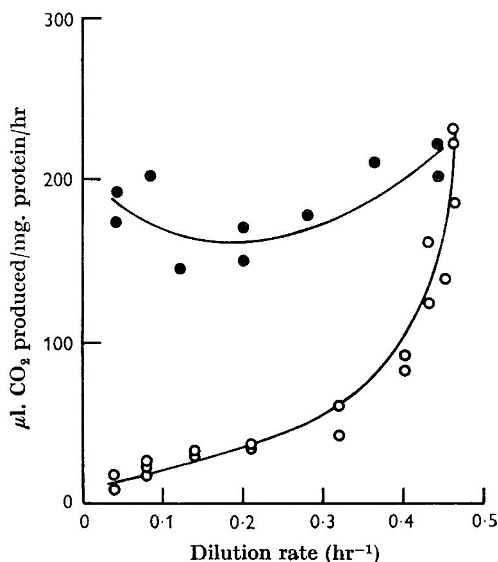


Fig. 7

Fig. 6. Rates of oxidation (μl./mg. protein/hr) of 50 mM-glucose by washed suspensions of NH_4^+-limited *T. utilis* organisms grown at several dilution rates; endogenous values subtracted.

Fig. 7. Rates of fermentation (μl. CO_2 produced/mg. protein/hr) of 50 mM-glucose by NH_4^+-limited (●) and C (glucose)-limited (○) *T. utilis* organisms grown at several dilution rates; endogenous values subtracted.

organisms (Fig. 4). Changes in the ability of glucose-grown organisms to dissimilate glucose anaerobically accompanied changes in their oxidative capacity. Here (Fig. 7) fermentative capacity correlated with the concentration of glucose in the culture, indicating an inductive synthesis of the enzyme(s).

Changes in the ability of Torula utilis organisms to oxidize compounds other than the growth substrate

The rate at which washed suspensions of organisms oxidized a substrate varied not only when organisms were grown at different rates in a medium containing that substrate, but also when they were grown in media containing other compounds.

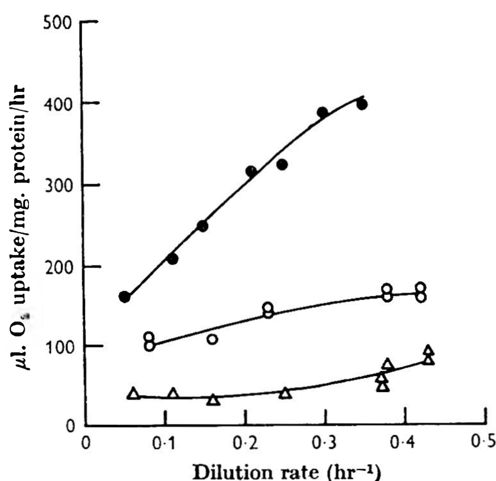


Fig. 8

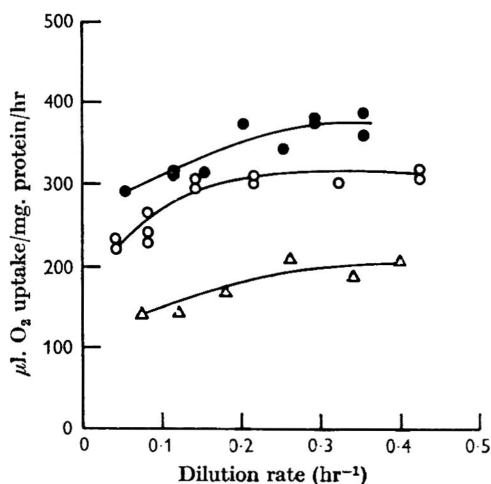


Fig. 9

Fig. 8. Rates of oxidation (μl./mg. protein/hr) of 50 mM xylose by washed suspensions of *T. utilis* organisms grown on an xylose-limited medium (●), a glucose-limited medium (○) and an ethanol-limited medium (△) at several dilution rates; endogenous values subtracted.

Fig. 9. Rates of oxidation (μl./mg. protein/hr) of 50 mM glucose by washed suspensions of *T. utilis* organisms grown on a xylose-limited (●), a glucose-limited (○) and an ethanol-limited (△) medium at several dilution rates; endogenous values subtracted.

Comparison of the rates of xylose oxidation by xylose-, glucose- and ethanol-limited organisms showed (Fig. 8) that at each dilution rate xylose-grown organisms had a greater xylose oxidizing capacity than glucose-grown organisms, and that the latter were able to oxidize this substrate more readily than were ethanol-grown organisms. In contrast to this observation it was found (Fig. 9) that glucose was oxidized more rapidly by xylose-grown organisms than by glucose-grown organisms, though again ethanol-grown organisms had a much lower glucose oxidizing capacity.

DISCUSSION

Although oxidation of a substrate is a multi-enzyme process, the overall rate must be a function of a single rate-limiting reaction in the chain of reactions which starts with the permeation of substrate into the organism and terminates with its oxidation

and assimilation. It seems reasonable to assume that in our experiments such a rate-limiting step was not located on that part of the oxidation pathway which was common to all three substrates (e.g. the tricarboxylic acid cycle or the electron transport system), otherwise a common pattern of change in oxidative capacity with dilution rate would have resulted and this was not observed. Data derived from studies on isolated enzymes (see Gorini & Maas, 1957) would have provided definite results, assuming that the specific rate-limiting reactions were known, but as a first approach the results obtained with washed suspensions of organisms are uggestive. Comparing the Q_{O_2} values of the growing cultures, which represent their 'oxidative demands' (Figs. 1, 2), with corresponding Q_{O_2} values of washed suspensions of organisms (Figs. 4–6), which represent their 'oxidative capacities', it is evident that in most cases a decrease in growth rate caused a decrease in both values, indicating an economy in enzyme synthesis. This economy was not apparent in the glucose-limited culture (Fig. 4) but was observed in the corresponding N-limited culture (Fig. 6), where glucose was present in excess of requirement in the environment. The reasons for this difference are not obvious, but may reflect the differing metabolic priorities when protein synthesis is limited by the energy supply as opposed to the supply of a structural element.

The oxidative enzyme systems of *Torula utilis* investigated here are 'constitutively' produced and yet there appeared to have been considerable variations in their rates of synthesis which suggest that the mechanisms controlling their biosynthesis were quantitatively rather than qualitatively different from those which controlled 'adaptive' enzyme synthesis. The differing abilities of glucose-, xylose- and ethanol-limited organisms to oxidize xylose (Fig. 8) emphasize the quantitative nature of the mechanisms which control 'constitutive' enzyme synthesis; they also reflect the degrees of similarity between the three oxidative pathways. In this connexion the enhanced rate of glucose oxidation by xylose-limited organisms (Fig. 9) may have resulted from glucose being preferentially oxidized via the 'pentose phosphate cycle'. Blumenthal, Lewis & Weinhouse (1954) showed that this pathway and the 'glycolytic' pathway of glucose catabolism were operative in *T. utilis*; growth on xylose causes, presumably, increased synthesis of enzymes of the pentose phosphate cycle.

It is possible that the observed changes in oxidative capacity of organisms resulted from variations in the rate of substrate permeation into the organisms, though this seems unlikely, since substrate oxidation, by washed suspensions of organisms, occurred without a lag and was linear for at least an hour. Thus it seems that growth rate and growth-limiting substrate affect not only the composition of organisms (Herbert, 1961; Neidhardt, 1963) but also their metabolic activity.

REFERENCES

BLUMENTHAL, H. J., LEWIS, K. F. & WEINHOUSE, S. (1954). An estimation of pathways of glucose catabolism in yeast. *J. Am. chem. Soc.* **76**, 6093.

COLLINS, F. M. (1964). The effect of growth rate on the composition of *Salmonella enteritidis* cell walls. *Aust. J. exp. Biol. med. Sci.* **42**, 255.

DEAN, A. C. R. (1962). Nucleic acid and protein content of *Bact. lactis aerogenes*. I. Equilibrium and non-equilibrium conditions. *Proc. Roy. Soc.* B, **155**, 580.

ECKER, R. E. & SCHAECHTER, M. (1963). Ribosome content and the growth of *Salmonella typhimurium*. *Biochim. biophys. Acta*, **76**, 275.

GORINI, L. & MAAS, W. K. (1957). The potential for the formation of a biosynthetic enzyme in *Escherichia coli*. *Biochim. Biophys. Acta*, **25**, 208.

HERBERT, D. (1958). Some principles of continuous culture. In *Recent Progress in Microbiology, VII Int. Congr. Microbiol.* p. 381.

HERBERT, D. (1961). The chemical composition of micro-organisms as a function of their environment. In *Microbial Reaction to Environment. Symp. Soc. gen. Microbiol.*, **11**, 391.

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

HOLME, T. (1957). Continuous culture studies on glycogen synthesis in *Escherichia coli* B. *Acta chem. scand.* **11**, 763.

MONOD, J. (1942). *Recherches sur la croissance des cultures bactériennes.* Paris: Hermann & Cie.

MONOD, J. (1950). La technique de culture continué; theorie et applications. *Ann. Inst. Pasteur*, **79**, 390.

NEIDHARDT, F. C. (1963). Effect of environment on the composition of bacterial cells. *Annu. Rev. Microbiol.* **17**, 61.

NEIDHART, F. C. & MAGASANIK, B. (1960). Studies on the role of ribonucleic acid in the growth of bacteria. *Biochim. biophys. Acta*, **42**, 99.

NOVICK, A. & SZILARD, L. (1950). Description of the chemostat. *Science*, **112**, 715.

ROSSET, P., MONIER, R. & JULIEN, J. (1964). RNA composition of *Escherichia coli* as a function of growth rate. *Biochem. biophys. Res. Commun.* **15**, 329.

STICKLAND, L. H. (1951). The determination of small quantities of bacteria by means of the biuret reaction. *J. gen. Microbiol.* **5**, 698.

SUD, I. J. & SCHAECHTER, M. (1964). Dependence of the content of cell envelopes on the growth rate of *Bacillus megatherium*. *J. Bact.* **88**, 1612.

SYKES, J. & TEMPEST, D. W. (1965). The effect of magnesium and of carbon limitation on the macromolecular organisation and metabolic activity of *Pseudomonas* sp., strain C-1 B. *Biochim. biophys. Acta*, **103**, 93.

TEMPEST, D. W. & HUNTER, J. R. (1965). Influence of temperature on the macromolecular composition of C (glycerol)-limited and Mg^{2+}-limited *Aerobacter aerogenes*, growing in a chemostat. *J. gen. Microbiol.* (Vol. 41, in the Press).

TEMPEST, D. W., HUNTER, J. R. & SYKES, J. (1965). Magnesium-limited growth of *Aerobacter aerogenes* in a chemostat. *J. gen. Microbiol.* **39**, 355.

[*The Editors of the* Journal of General Microbiology *accept no responsibility for the Reports of the Proceedings of the Society. Abstracts of papers are published as received from authors.*]

THE SOCIETY FOR GENERAL MICROBIOLOGY

The Society for General Microbiology held its forty-second General Meeting at Queen Elizabeth College, Campden Hill Road, London, W. 8 *on Thursday and Friday* 7 *and* 8 *January* 1965. *The following communications were made:*

ORIGINAL PAPERS

Mass Culture of Organisms in a Fermenter. By R. K. B. Robinson and H. K. King (*Biochemistry Department, University of Liverpool*)

Culture of micro-organisms for examination of minor chemical constituents or for preparation of enzymes often requires larger masses of cells than can be obtained readily through ordinary laboratory culture methods. One solution is the continuous culture machine; an alternative is the pilot fermenter. The latter was developed in Chain's laboratory primarily for studies on antibiotic production by the aerated deep culture method (Chain, Paladino, Ugolini, Callow & Van der Sluis (1954), *Rend. Ist sup. Sanit. Roma* (English ed.), 17, 62). We have modified this design (including provision for illumination for photosynthetic organisms) and examined its utility for mass culture, using an instrument with four vessels of 10 l. working capacity each (manufactured by Taylor Rustless Fittings Ltd., of Leeds). Maximum yield in terms of cell dry weight per given volume of medium was obtainable only with careful control, involving addition at appropriate times of antifoaming agents, of acids or alkalis to control pH, and possibly additional nutrients. For most purposes, however, organisms in the logarithmic phase are required, so our investigations have centred on obtaining satisfactory yields of log-phase organisms with the minimum of control during fermentation. Typical non-exacting heterotrophic bacteria gave 2–4 g. dry wt./l. of culture medium at 4–8 hr from start of growth on synthetic media of simple composition, using a very heavy inoculum. Growth of *Saccharomyces cerevisae* and of *Aspergillus fumigatus* was slower, but yields of 6 and 12 g./l., respectively, were obtained. *Euglena gracilis* on a complex medium containing sucrose as carbon source gave yields of 4·6 g./l. in the dark and of 6·2 g./l. under illumination. *Chlorella pyrenoidosa* gave 3 g./l. on glucose-containing medium with illumination. Two obligate autotrophs were grown: *Thiobacillus thioparus* yielded 0·2 g./l., and the blue-green alga *Anabaena variabilis* (Kutzing) gave approx. 0·5 g./l. with CO_2 as sole carbon source.

Oxygen Uptake in Shake-flask Penicillin Fermentations. By C. T. Calam (*Imperial Chemical Industries Ltd., Pharmaceuticals Division, Alderley Park, Macclesfield, Cheshire*)

Measurements of O_2 uptake were made in 500 ml. conical flasks on a rotary shaker (250 r.p.m.). The medium (100 ml.) contained lactose, corn-steep solids, chalk and precursor, and was inoculated with a penicillin-producing strain of *Penicillin chrysogenum*. The flasks were plugged with cotton wool. Analysed on the first to sixth day, the atmosphere in the flasks contained 18–19 % of O_2 and 2–3 % of CO_2. The use of tight plugs produced little change. Respiration was measured by closing the flasks with rubber bungs and analysing the air inside. O_2 absorption was linear for about 3 hr when the concentration had fallen to approx. 10 %, it then continued at a slower rate, almost ceasing after 7 hr when the concentration fell to 4 %. CO_2 production was slightly less than oxygen consumption. The rate of O_2 uptake under normal conditions was approximately 1·65 ml. O_2/l./min. or 4 mM O_2/l./hr, which was insufficient to decompose all the lactose in the medium.

Tests with a membrane electrode dipping in the culture gave oxygen tensions of 15–40 % saturation. On stopping the shaker, dissolved O_2 rapidly disappeared but reappeared when shaking was restarted.

In the past opinions have differed about the O_2 supply in shaken cultures. The view has been expressed that shaken flask cultures must be semi-anaerobic, but recently Schultz ((1964), *Appl. Microbiol.* **12**, 305) has concluded from physico-chemical data that while aeration may be limited in large flasks it should be adequate in smaller ones. The present experiments suggest that although the O_2 transfer rate is lower than it is in stirred fermenters, and transfer from gas to liquid is poor, the cultures are by no means starved of O_2 by poor diffusion into the flasks.

The Effect of Increased Oxygen Partial Pressures on the Growth of *Pseudomonas aeruginosa* in a Closed Aeration System. By T. R. Owen and E. C. Hill (*Department of Microbiology, University College, Cardiff*)

A laboratory-scale fermentor with a closed aeration and a gas circulating system was evolved. Metabolically produced CO_2 was removed during circulation and O_2 was allowed to enter through a one-way valve to compensate for the reduction in pressure caused by CO_2 removal. This ensured that the partial pressure of O_2 in the system remained constant.

Batch cultures of *Pseudomonas aeruginosa* were grown in this fermentor on a glucose–mineral-salts medium with aeration at varied O_2 and N_2 partial pressures (air, 45, 75 and 100 % O_2 mixtures). Growth curves measured by dry-weight determinations were plotted for each O_2 partial pressure. It was found that when higher O_2 partial pressures than in air were used for aeration, and when nutrients were not growth-limiting, then the logarithmic phase of growth was extended for a period of time longer than when air was used. When O_2 eventually became the growth-rate limiting factor, growth ceased to be logarithmic, became linear and proportional to the quantity of O_2 available, and hence to the partial pressure of O_2 used for aeration.

Thus, increased growth rates and over-all yields of organisms were obtained at the higher O_2 tensions.

It is also of note that this closed aeration system has advantages for the cultivation of pathogenic organisms, for the growth of organisms on volatile substrates, and for studying the effect of constant gas partial pressures.

Simultaneous Estimation of the Uptake of Oxygen and Carbon Dioxide (as $^{14}CO_2$) in *Ferrobacillus ferrooxidans*. By M. C. Allan and J. Meyrath (*University of Strathclyde, Glasgow, C. 1*)

The available methods for estimating the simultaneous uptake of O_2 and CO_2 (e.g. Warburg, O. (1924), *Biochem. Z.* **152**, 51; Schatz, A. (1952), *J. gen. Microbiol.* **6**, 322; Lialikova, N. N. (1958), *Mikrobiologija* (Eng. transl.), **27**, 546; Hagihara, B. (1961), *Biochim. Biophys. Acta*, **46**, 134) were found to be unsuitable for kinetic investigations, where many readings have to be taken, or where exact comparison between variants is desired.

The method described here is based on the estimation of the change of partial pressure of CO_2 using $^{14}CO_2$ as tracer, and determining the change in radioactivity by means of a small Geiger tube inserted into the top of the culture flask. The amount of CO_2 taken up can be obtained from a calibration curve. Simultaneously with the change in CO_2 content the total decrease in volume is measured volumetrically. After subtraction of the volume of CO_2 taken up, and having made a correction for thermobarometric changes (obtained, for example, from a control system), the volume of O_2 taken up can be calculated. The volumetric method has been chosen in preference to the manometric method for reasons of ease and simplicity.

Any $^{14}CO_2$ assimilated by the bacteria is not counted by the Geiger tube since the weak β-rays are not able to penetrate the aqueous phase; therefore, no shielding of the gas phase from the liquid phase is necessary. The major technical difficulty is the prevention of

water vapour condensing on the end window counter. This is overcome by keeping the flask in a constant-temperature water bath which is colder than the ambient temperature, and by keeping the neck of the flask (holding the Geiger tube) above the water level. The error introduced by having a small portion of the system exposed to a temperature different from that of the constant temperature bath is negligible.

The functioning of the apparatus and the procedure is explained by taking *Ferrobacillus ferrooxidans* as example.

Problems in Obtaining Mycelial Preparations of Moulds for Respiratory Studies.

By A. F. McIntosh and J. Meyrath (*University of Strathclyde, Glasgow, C.* 1)

Although specific respiration activity in growing cultures can be examined more suitably by recent techniques (Shu, P. (1953), *Agric. Fd Chem.* **1**, 1119; Chain, E. B. & Gualandi, G. (1954), *Rend. Ist sup. Sanit.* (English ed.), **17**, 5; Allan, M. C. & Meyrath, J. (1965), *J. gen. Microbiol.* (preceding abstract)), the classical method of examining resting cells in manometric or volumetric systems allows ready investigation of the repiratory mechanism of whole cells. Details of the preparatory treatment of cells is often unrecorded although awareness of the importance of such treatment has been shown (Hromatka, O. & Ebner, H. (1949), *Enzymologia*, **13**, 369; Wikén, T. & Somm, H. (1952), *Experientia*, 8, 140). Filamentous mycelium of *Aspergillus oryzae* produced in deep culture from early stages of growth is sensitive to several environmental factors likely to be operating while it is being prepared for use as resting mycelium. Washing with water or phosphate-buffer to free the mycelium from accompanying substrate leads to a decrease in specific respiration activity. This decrease is more pronounced when the washing procedure is intensified and is still more pronounced when at the same time the mycelium comes into contact with adsorbing filters. Washing by centrifuging, technically difficult with mycelium, has a less-pronounced effect on respiration than has filtering with strongly adsorbing filters. The mechanical effect of pressing has only little influence. The mycelium suspension can be stored in culture substrate at low temperature for a limited time without loss of respiration activity; storage in water or phosphate buffer leads to a more rapid decrease in specific rate of respiration. If factors influencing respiration in such ways are known, interpretation of results is thereby aided.

The Role of Metal Complexing Agents in the Production of Citric Acid by *Aspergillus niger*.

By M. A. Qadeer Choudhary and S. J. Pirt (*Microbiology Department, Queen Elizabeth College, London, W.* 8)

Ethylenediamine tetracetic acid (EDTA), cyclohexanediamine tetracetic acid (CDTA), diethylenetriamine pentacetic acid (DTPA), nitrilotriacetic acid (NTA), added during vegetative growth, at a concentration up to 9·4 mM had no effect on the specific growth rate or final dry weight, in media of initial pH 3·5 or 6·5. The chelating agents, except NTA or *N*-N^1-di (β-hydroxyethyl) glycine (DHEG), inhibited germination of spores at pH 6·5, less so at pH 3·5.

Ferrocyanide forms an insoluble complex with iron and other metal ions. Ferrocyanide up to 9·4 mM in medium of initial pH 6·5 did not affect specific growth rate, or final dry weight, or germination rate; in medium of initial pH 3·5, specific growth-rate of mycelium, final dry weight and spore germination rate were all decreased.

The mould morphology in shake-flasks was modified to the form of small separate smooth pellets by EDTA, CDTA, DTPA and ferrocyanide. In control cultures and with NTA or DHEG the mould was in the form of a viscous conglomerate of large filamentous pellets.

Citric acid accumulation was increased by EDTA, CDTA and DTPA but not by NTA or DHEG. Ferrocyanide, added 24 hr after inoculation, stimulated citric acid accumulation more than the chelating agents and had no effect when added 48 hr after inoculation, unlike the chelating agents. The ferrocyanide precipitate is necessary during the citric

acid producing phase. To account for these findings it is postulated that the chelating agents and the ferric-ferrocyanide precipitate, act as metal buffers especially for iron. The absence of activity by NTA and DHEG may be attributed to their lower chelating power or to the fact that they form neutral lipid-soluble ferric complexes, to which the cell may be permeable.

Melanin Synthesis in Wild-type and Mutant Strains of *Aspergillus nidulans*. By A. T. BULL (*Department of Microbiology, Queen Elizabeth College, W.* 8) and B. M. FAULKNER (*Department of Botany, Bedford College, N.W.* 1)

Wild-type strains of *Aspergillus nidulans* produce large amounts of a melanoid pigment. A series of 8-azaguanine-induced and spontaneous nuclear mutants have been isolated (Bull, A. T. & Faulkner, B. M. (1964), *Nature, Lond.* **203**, 506) in which melanogenesis is blocked to a greater or lesser extent. Certain of these mutants appear to be unique in their accumulation of pink and purple pigments which have been identified as the melanin intermediates dopachrome (indole-5,6-quinone 2-carboxylic acid) and melanochrome (indole-5,6-quinone, or, more probably, a small oligomer of the latter). *A. nidulans* melanin is the first fungal melanin to be characterized as an 'indole-type' (Nicolaus, R. A., pers. comm.; see also *Tetrahedron* (1964), **20**, 1163). Tyrosine, but not DOPA has been detected chromatographically in mycelial extracts and may, therefore, be the precursor for melanin synthesis.

Preliminary investigations have been made of the tyrosinase activity in this fungus. The enzyme from wild-type strains exhibits typical tyrosinase kinetics and activity, e.g. 'cresolase' and 'catecholase' activities; a 'cresolase' induction period which can be shortened by the addition of an *o*-dihydric phenol to the reaction mixture; differential inhibition of the two activities by copper-complexing agents. Of particular interest is the tyrosinase activity of a colourless, asexual mutant. Here, 'catecholase' activity is retained whilst the 'cresolase' entity has been eliminated. This loss of 'cresolase' activity is not due to (*a*) the inability to synthesize an essential co-factor, or, (*b*) the presence of an endogenous inhibitor. These and other observations are consistent with the hypothesis that tyrosinase is a single protein having two distinct active centres.

The isolation of both sexual and asexual pigment mutants indicates melanogenesis and sexual development are not closely associated in this fungus.

The Kinetics of Potassium Uptake by *Staphylococcus pyogenes*. By K. A. Wright and H. V. Wyatt (*Department of Biological Sciences, Bradford Institute of Technology*)

The uptake of K^+ by *Staphylococcus pyogenes* is reduced in the presence of Rb^+ (Wyatt (1963), *Exptl Cell Res.* **30**, 62). We have examined the uptake of K^+ by *Staphylococcus*, and the effect of Rb^+ and Cs^+ on this uptake, and analysed the results according to Michaelis–Menten kinetics for enzymic reactions (Conway & Duggan (1958), *Biochem. J.* **69**, 265; Rothstein (1959). *Bact. Rev.* **23**. 175). 5 hr cultures grown in low (K^+) broth were, after centrifugation, resuspended in new media of graded $^{42}K^+$ concentrations. Samples were removed at 5 min. intervals and after rapid filtering the filtrate was analysed for $^{42}K^+$. The velocities of uptake were calculated, and because these were not linear with time the initial velocity was used (5 min.) and converted to μM K^+ uptake/100 mg. dry wt./hr.

A Lineweaver–Burk plot was made, which showed that K^+ uptake by *Staphylococcus* could be analysed by Michaelis–Menten kinetics. With K^+ alone, K_m was 10^{-4}M: Rb^+ increased this to $1{\cdot}85 \times 10^{-4}$M but had little effect on V_m. Caesium affected neither the K_m nor V_m. These results suggest that Rb^+ competes with K^+ for uptake, but Cs^+ does not. This is in agreement with growth studies; Rb^+ can replace K^+, but Cs^+ cannot. The value for K_m compares with that given for yeast, 3×10^{-4}M, at a similar pH (Rothstein (1959), *Bact. Rev.* **23**, 175). K_m values were reproducible in different experiments, but V_m values varied.

The Metabolism of Glucose by 'Bacteroides' Strains. By D. H. SHRIMPTON and B. J. H. STEVENS (*Low Temperature Research Station, Cambridge*)

Microbial metabolism within the paired caeca of the fowl is essentially fermentative (Beattie, J. and Shrimpton, D. H. (1958), *Quart. J. exp. Physiol.* **43**, 399) and is partly characterized by the presence of volatile fatty acids (VFA) (Shrimpton, D. H. (1963), *J. appl. Bact.* **26**, i). Obligate Gram-negative anaerobes may be present at $> 10^8$ organisms per g. fresh weight (Barnes, E. M. & Goldberg, H. S. (1962), *J. appl. Bact.* **25**, 94). Predominant amongst this flora are 'bacteroides' strains which differ from previously described organisms within the family Bacteroidaceae and have now been characterized by Goldberg, H. S., Barnes, E. M. & Charles, A. B. ((1964), *J. Bact.* **87**, 737). The carbohydrate metabolism of these isolates is being studied. The production of VFA by a dozen isolates from glucose broth has been examined by vapour phase chromatography by the technique of Shelley, R. N., Salwin, H. & Horwitz, W. ((1963), *J. Ass. off. agric. Chem.* **46**, 486) and they have been found to produce formic, acetic and propionic acids in simple ratios which differ between strains.

The metabolism of washed suspensions of one of the isolates (61/42) is being studied, following broadly the experimental techniques developed for studying anaerobes isolated from the rumen. All experiments were carried out in the presence of sodium sulphide (Sijpesteijn, A. K. & Elsden, S. R. (1952), *Biochem. J.* **52**, 41) at 0·02 % as hydrate (Doetsch, R. N., Robinson, R. Q., Brown, R. E. & Shaw, J. C. (1953), *J. Dairy Sci.* **36**, 825), using ammonium chloride as the source of nitrogen with cysteine hydrochloride and additions of calcium and magnesium (Bryant, M. P., Robinson, I. M. & Chu, H. (1959), *J. Dairy Sci.* **42**, 1831) and B vitamins.

Under these conditions, and averaging from separate experiments, 10^9 cells metabolized 1 μM of glucose to 0·3 μM CO_2, 0·4 μM lactic acid, 0·5 μM acetic acid and 0·5 μM propionic acid. If metabolism followed a similar course in the caecum, the total amount of VFA could be of the order of 0·4 % of the contents by weight. Values between 0·2 and 1·0 % have been reported (Shrimpton, D. H. (1963), *J. appl. Bact.*, **26**, i; Hill, K. J., Annison, E. F. & Noakes, D. E. (1964), Private communication).

Assimilation of Organic Compounds by a Strictly Chemoautotrophic Thiobacillus. By D. P. KELLY (*Microbiology Department, Queen Elizabeth College, London, W.* 8)

Thiobacillus strain C (Kelly, D. P. & Syrett, P. J. (1964), *J. gen. Microbiol.* **34**, 307) cannot grow on organic nutrient media or on mineral media containing acetate but lacking thiosulphate. Acetate-2-^{14}C added to cultures growing logarithmically in mineral media (Kelly, D. P. & Syrett, P. J. (1964), *ibid.*) was incorporated by the bacteria at a constant differential rate during subsequent growth. Fractionation of the bacteria by the method of Syrett, Bocks & Merrett ((1964), *J. exp. Bot.* **15**, 35) after 1 hr or 3 hr exposure to ^{14}C-acetate showed the approximate distribution of the incorporated ^{14}C among the fractions to be: hot-water soluble (34 %), ethanol soluble (35 %), nucleic acid + polysaccharide (16 %) and protein (14 %). Less than 1 % was phenol extractable, suggesting little poly-β-hydroxybutyrate accumulation. At least 11 labelled compounds were demonstrated chromatographically in the water extract (Wilson & Calvin (1955), *J. Amer. Chem. Soc.* **77**, 5948). Major constituents were a substance running at the front of both solvents (possibly β-hydroxybutyrate), glutamate, compounds remaining near the origin, and a compound running at the phenol front.

Sodium monofluoroacetate (5–50 μM) immediately stopped logarithmic growth, but did not affect thiosulphate oxidation or CO_2-fixation by washed cells. Growth inhibition could be due to blockage of a tricarboxylic acid cycle by fluorocitrate formed metabolically from fluoroacetate; but if glutamate, rather than TCA cycle intermediates, is a primary product of acetate and fluoroacetate incorporation, inhibition might be by a different mechanism.

This demonstration of acetate assimilation is believed to be the first report of metabolic

transformation of exogenously supplied organic material by a 'strict chemoautotroph' and may be comparable with light-dependent acetate assimilation by the strict photo-autotroph *Chlorobium* (Hoare & Gibson (1964). *Biochem. J.* **91**, 546).

Azurin Production by Bacteria. By I. W. SUTHERLAND (*Bacteriology Department, Edinburgh University*)

A blue protein of relatively low molecular weight was extracted from cells of *Pseudomonas aeruginosa* and studied by Horio ((1956), *J. Biochem.* (*Tokyo*), **45**, 195, 267). Subsequently, a similar protein was isolated from species of *Bordetella* (Sutherland, I. W. & Wilkinson, J. F. (1963), *J. gen. Microbiol.* **30**, 105). This substance, to which the name azurin was given, contained one copper atom per molecule. It has now been confirmed that this type of protein is present in all species of *Bordetella* and *Alacaligines* and in some *Pseudomonas*. It was not detected in certain other *Pseudomonas* nor in representative strains of other bacterial species tested. Examination of cells of one strain each of *P. aeruginosa*, *B. bronchiseptica* and *A. denitrificans* showed that azurin was almost absent from cells grown in medium containing less than 0·1 μg. copper/ml. However, cells grown in such medium had apparently normal respiratory activities. The azurin content of cells increased with the copper content of the medium up to a concentration of about 5 μg./ml. Increase of copper content above this level did not lead to increase in the azurin content of cells. At the copper levels used, there was no appreciable difference in the azurin contents of the three bacterial species tested.

Azurin is produced during aerobic and anaerobic growth of *Pseudomonas aeruginosa*. Although growth was poorer under anaerobic conditions, the azurin content was similar to that of cells grown aerobically in the presence of similar amounts of copper. It is concluded that the primary function of azurin is not the detoxification of copper. It is probably involved in the electron transport system as was suggested by Horio ((1956), *J. Biochem.* (*Tokyo*), **45**, 267) for *Pseudomonas* azurin. However, the growth of bacteria under conditions where almost no azurin is produced would indicate that it is probably not an essential cell component. It may thus form part of an alternative pathway for electron transport.

The Influence of Incubation Temperature on the Viability of *Escherichia coli* Damaged by Phenol, X-Rays and Radio-Mimetic Agents. By N. D. HARRIS and M. WHITEFIELD (*The School of Pharmacy, Chelsea College of Science and Technology, London, S.W.* 3)

Various temperatures have been reported as optimal for bacteria damaged by ionizing and ultraviolet radiations and by chemicals, and a temperature of 30–32° has been recommended for sterility testing (*Wld Hlth Org. techn. Rep. Ser.*, **1960**, 200). It was thought useful, therefore, to compare the temperature responses of several strains of *Escherichia coli* damaged by phenol and X-rays. Radio-mimetic agents were included also to see whether the responses of bacteria damaged by them were similar to those observed after X-irradiation, since such a similarity would indicate a possible identity of damage produced by the two agencies.

Three strains of *E. coli* were damaged by treatment with phenol, X-rays and the radio-mimetic drugs dimethyl Myleran and chlorambucil. They were then exposed for 24 hr to temperatures in the range 8–37° before their viability was finally tested by incubation at 37°. All the strains showed similar patterns of responses. The exposure temperature resulting in the best viability was 15° for X-irradiated organisms and 30° after phenol treatment. Change of temperature had little effect on counts from suspensions treated with radio-mimetic drugs. Incubation of X-irradiated *E. coli* B/r on media containing up to 5 μg./ml. of chloramphenicol resulted in low counts. It is concluded that the beneficial effects of incubation at temperatures below 37° were not due to retardation of protein synthesis, and that the damage caused by X-rays differed from that caused by radio-mimetic agents.

A Temperature Gradient Incubator for Studies of the Effect of Temperature on Bacterial Growth and Revival. By N. D. Harris and I. A. Martin (*The School of Pharmacy, Chelsea College of Science and Technology, London, S.W.* 3)

In studies of the effect of temperature on bacterial growth and revival, damaged cells may be inoculated into molten agar and then spend a variable period cooling to and at laboratory temperature, followed by an unknown heating period in the incubator before reaching equilibrium. Recordings of the temperatures of agar media in Petri dishes in an incubator showed that the time required to reach 37° depended primarily upon the number of dishes stacked together and on the position within the stack. Depending upon the loading conditions plates could take between 40 and 200 min. to reach 36°, the approach to 37° being asymptotic. Plates pre-warmed to 37° before inoculation with phenol-treated *Escherichia coli* gave much lower counts than those inoculated and placed in an incubator set at 37°. Also there was some correlation between the count and the heating rate.

These difficulties have been overcome with a temperature gradient incubator consisting of an insulated aluminium block, each end of which can be heated to any desired temperature. This gives a linear temperature gradient in nutrient agar contained in dishes in a trough in the block, with only minor transient fluctuations during inoculation. Preliminary results showed that the optimal temperature for phenol-treated *Escherichia coli* lay in the range 27–29° and that counts dropped sharply above 30°. As expected, the observed optimum was lower than that indicated (30–32°) by conventional incubation procedures. The apparatus provides a means of examining the variation in response of damaged cells with temperature, with greater accuracy and detail than techniques previously available.

The Repair of Combined Ionizing and Ultraviolet Radiation Damage in *Micrococcus radiodurans*. By B. E. B. Moseley (*Molteno Institute, University of Cambridge*)

Micrococcus radiodurans. a non-sporing bacterium, is unusual in comparison with the majority of bacteria, in that it can survive very large doses of both UV and ionizing radiations. Both survival curves have a shoulder followed by an exponential slope. Setlow & Duggan ((1964), *Biochim. biophys. Acta*, **87**, 664) have shown that this bacterium has a very efficient repair system for UV damage. Moseley & Laser ((1965), *Proc. roy. Soc.* B, in the Press) have presented evidence which shows that it also possesses a repair system for at least two types of X-ray damage.

The question arises of whether the UV and ionizing radiation damage is repaired by the same or by separate enzyme systems. In order to elucidate this point, the viability of *Micrococcus radiodurans* after receiving mixed doses of ionizing and UV radiations has been measured. After pre-irradiation with γ-rays, the ability of the cells to repair UV damage is reduced. A linear relationship exists between the dose of ionizing radiation and the reduction in the ability of the cell to repair UV damage. On giving a dose of γ-rays which is large enough to cause exponential death, subsequent UV radiation also causes exponential death. This means that the final lesion which renders the cell non-viable can be caused either by UV or ionizing radiation. Since UV radiation is absorbed almost specifically in the nucleic acids it follows that the site of ionizing radiation damage in *M. radiodurans* is also in the nucleic acids.

From this and other evidence which will be presented, it can be demonstrated that at least two types of lesion are produced by irradiation and that to some extent UV and ionizing radiations cause some common damage which can be repaired by the same repair system.

SYMPOSIUM: 'THE ROLE OF OXYGEN IN MICROBIAL GROWTH AND FUNCTION'

Introduction. By S. J. Pirt (*Microbiology Department, Queen Elizabeth College, London. W. 8*)

Few, if any, microbes seem to be inert to free oxygen (O_2). It controls the life of microbes either by stimulating or inhibiting their functions. There are diverse microbial enzyme systems for reaction with O_2: cytochrome pathways for transport of electrons or hydrogen atoms to O_2, cytochrome independent pathways, and oxygenases for incorporation of O_2 in carbon substrates. In aerobic electron transport the cytochrome system is intimately connected with energy provision by high-energy phosphates, although the mechanism involved is obscure (Dolin, M. I. (1961), *The Bacteria*, **2**, 319. New York: Academic Press). Ideas about the role of O_2 utilization by cytochrome independent pathways, notably in lactic acid bacteria and clostridia, are more speculative (Dolin, M. I. (1961), *ibid.* 425). Recent work indicates that the synthesis of large groups of enzymes, such as those of the tricarboxylic acid cycle and cytochrome pathway, are subject to induction and repression by O_2 (e.g. Englesberg, E., Levy, J. B. & Gibor, A. (1954), *J. Bact.* **68**, 178). If such is the case, change in O_2 level can cause a major upheaval in cell organization. There seem to be no reports so far of analogous effects in organisms which utilize O_2 by a cytochrome-independent pathway. From the modern concepts of metabolic control we shall perhaps derive a definitive explanation of the Pasteur effect. Growth rate and environmental conditions, such as iron supply, are reported to have a marked effect on the cytochrome system (Smith, L. (1961), *The Bacteria*, **2**, 365. New York: Academic Press), and possibly therefore on reaction with O_2, but these influences have received little attention. Iron nutrition seems especially relevant to O_2 effects. For instance, *Pasteurella pestis* grows well from small inocula under anaerobic conditions but requires haemin for aerobic growth (Herbert, D. (1949), *Brit. J. Exp. Path.* **30**, 509).

The possibility of using substitutes for O_2 is of great interest. H_2O_2, nitrate and organic compounds such as methylene blue may serve as alternative electron acceptors. The extent to which these substances fulfil the role of O_2 is little explored.

The development of aeration methods over the last 15 years has greatly assisted control of aerobiosis. Unfortunately, the academic microbiologist seems little aware of the quantitative basis of aeration, with the result that he may have been unwittingly using anaerobic conditions thinking they were aerobic. The efficiency of aeration is stated in terms of the oxygen solution rate. Another parameter required to define the availability of O_2 is O_2 tension or activity in the medium. The membrane electrode seems at last to provide a convenient means for measuring this quantity.

The control of anaerobiosis with its use of redox potential of the medium is still an obscure process, but should it be divorced from control of aerobiosis, especially in view of the observation of O_2 uptake by 'anaerobes' belonging to the clostridia? In no case does the mechanism of O_2 toxicity for anaerobes, or aerobes, seem to be established.

Another aspect of major importance is the ecological role of O_2. In this respect O_2 must be ranked with such ubiquitous factors as temperature and pH value. Study of O_2 effects here may reveal new effects such as accelerated selection against virulent types in cultures of *Pasteurella pestis* caused by the presence of oxygen (Delwiche, E. A., Fukui, G. M., Andrews, A. W. & Surgalla, M. J. (1959), *J. Bact.* **77**, 355).

Microbial Respiration and Oxygen Tension. By D. Herbert (*Microbiological Research Establishment, Porton, Salisbury, Wilts*)

The respiration of resting cell suspensions of several bacteria and yeasts has been shown to depend on the concentration of dissolved O_2 in the suspending fluid according to the equation

$$q = q_{max.}\left(\frac{c}{K_c + c}\right),$$

where q is the respiratory coefficient, c the concentration of dissolved O_2 and K_c is a 'saturation constant' equal to the O_2 concentration at which $q = q_{max}/2$. The analogy with the Michaelis–Menten equation of enzyme kinetics is obvious, but the K_c of whole bacterial cells is not necessarily to be identified with the Michaelis constant of any single respiratory enzyme.

Early measurements on resting cells were made by manometric methods, but the K_c values of bacteria are so low that a very elaborate technique is required (e.g. Warburg & Kubowitz, 1929, *Biochem. Z.* **214**, 5). More recent measurements have been made by polarographic techniques, e.g. with rotating or vibrating noble-metal electrodes. Longmuir ((1954), *Biochem. J.* **57**, 81) applied this technique to a number of bacteria and yeasts and found K_c values ranging from 0·01 to 0·6 μM, corresponding to half-maximal respiration at O_2 tensions of $4{\cdot}5 \times 10^{-4}$ to 8×10^{-6} atmospheres. There was a strong correlation with cell size, larger organisms having higher K_c values, suggesting an influence of diffusion.

Hitherto such measurements have been made on resting cell suspensions. It is of even greater interest to determine the effect on bacteria of growing them in known concentrations of dissolved O_2. It is difficult to control the dissolved O_2 content of a conventional batch culture, since this depends both on the O_2 supply (usually constant) and the O_2 demand, which increases with cell concentration. The dissolved O_2 concentration therefore decreases continuously during growth and unless aeration is very efficient, the final stages of batch growth will be semi-anaerobic.

The problem is easier if continuous culture techniques are used, since one then has steady-state growth and a constant cell population, so that the O_2 demand is constant; by regulating the O_2 supply, growth can be made to take place at a controlled concentration of dissolved O_2. (The required regulation of O_2 supply can, with some trouble, be effected manually, but the only ultimately satisfactory method is servo-control, using the signal from the O_2 electrode to control the aeration rate and hold it constant.)

When bacteria are grown in this way at different dissolved O_2 levels (Moss & Herbert, unpublished), the most striking effect is on the cytochrome content of the cells, and particularly on the a cytochrome. With *Bacillus megaterium* grown in high dissolved O_2 concentrations, cytochromes a_1 and a_2 are undetectable and only cytochrome b_1 is visible. At lower dissolved O_2 levels both a cytochromes appear and increase to a maximum as the O_2 concentration is further reduced, while cytochrome b_1 remains relatively constant: if the O_2 concentration is reduced still further, all three cytochromes decrease, finally to the very low levels found in anaerobically grown cells. Similar results were found with yeast, in which the cytochrome a/a_3 complex increases as the dissolved O_2 decreases; in this case, however, the a cytochromes are never completely repressed even at high O_2 levels.

Oxygen Tension and Glucose Metabolism of *Klebsiella aerogenes*. By D. E. F. Harrison and S. J. Pirt (*Microbiology Department, Queen Elizabeth College, London, W.* 8)

Klebsiella aerogenes shows a wide range of metabolic products depending on the conditions of growth (Pirt, S. J. & Callow, D. S. (1958), *J. appl. Bact.* **21**, 188). The present work attempts to relate these products to the O_2 tension in a growing culture and to define the critical O_2 tension. Several methods for measuring O_2 tension were investigated in order to find the most suitable for use in continuous culture. A Bishop electrode (EIL, Richmond, Surrey) was first used in conjunction with a 'Lectrona' O_2 analyser (British Oxygen Company Ltd., Wembley, Middlesex). This system had a very high residual current which lowered its sensitivity at low O_2 tensions. When used to measure O_2 tension by means of diffusion currents, however, the Bishop electrode had a much lower residual current. In both these systems the calibration of the electrode drifted considerably when used continually over a period of days. It was found that after about 7 days the drift became considerably slower and so the electrode could be used with greater confidence but with reduced sensitivity. A gasometric determination of dissolved O_2 (Calam, C.T. Imperial Chemical Industries, Alderley Park, Cheshire, private communication) was

investigated which, it was thought, might provide a means of checking electrode calibration, but this method was found too inaccurate over low O_2 tensions. The Mackereth electrode (Mackereth, F. J. H. (1964), *J. Sci. Instrum.* **41**, 38) was found to give a much higher degree of sensitivity and stability than the Bishop electrode, there being no detectable drift in the calibration over a period of 1 month.

A continuous culture apparatus similar to that described by Elsworth, R., Meakin, L. R. P., Pirt, S. J. & Capell, G. H. ((1956), *J. appl. Bact.* **19**, 264) was used. This enabled the temperature, pH, and growth rate of the culture to be kept constant. The O_2 tension was varied by passing different mixtures of air and N_2 through the culture, the total gas flow-rate remaining constant. Experiments were carried out with glucose-limited growth, and in the presence of excess glucose with N_2-limited growth.

The culture demonstrated three different phases in relation to the O_2 tension: (1) at O_2 tensions above 15 mm. Hg. the O_2 uptake and metabolic products were unaffected by changes in O_2 tension, and O_2 tension was proportional to the rate at which O_2 was supplied to the culture; (2) at O_2 tensions between approximately 1 and 15 mm. Hg. the O_2 tension reading showed wide fluctuations with a constant rate of O_2 supply. At growth rates of 0·4 and 0·5 $hr.^{-1}$ and with excess of glucose, these fluctuations were in the form of quite regular oscillations; (3) at O_2 tensions below 1 mm. Hg. the fluctuations ceased.

It cannot be stated at this stage whether the fluctuations in O_2 tension obtained in the second phase were a reflexion of changes in the respiration rate of the organism or were an artifact resulting from small changes in the physical conditions of the culture.

At O_2 tensions above 15 mm. Hg. and with glucose-limited growth, most of the glucose carbon was accounted for in the form of organism dry-weight and CO_2. When growth was N_2-limited, and glucose present in excess, large amounts of pyruvic acid were formed and also small quantities of butanediol, acetoin, alcohol and volatile acids. As O_2 tension was reduced below 15 mm. Hg., pyruvate and organism dry-weight production fell and butanediol, alcohol and volatile acid production increased. Butanediol production began to increase at a higher O_2 tension than alcohol production. Under anaerobic conditions butanediol, alcohol and volatile acid production were at a maximum, organism dry-weight production was at a minimum, and no pyruvate was produced.

Oxygen in Relation to the Growth and Activities of Fungi. By A. Borrow and E. G. Jefferys (*Imperial Chemical Industries Limited, Alderley Park, Macclesfield*)

The work described relates to the growth of *Gibberella fujikuroi* in submerged culture. Under suitable conditions, growth and the uptake of nutrients, proceed at exponentially increasing rates until some restriction is imposed on the system. One such restriction is the availability of O_2, which as well as affecting the rate of various metabolic activities, initiates qualitative changes in the nature of subsequent growth.

The equipment used to date includes the EIL–Bishop electrode, linked to the Polariter P04 polaragraph. Respiratory activities are measured by conventional manometric methods, but analyses of the flowing gases are also made, using Hilger–IRD CO_2 meter and the Servomex O_2 meter. Results obtained in these studies support the hypothesis that the change from exponential growth can, in suitable circumstances, be used as a biological measure of the gas transfer efficiency of submerged culture systems.

Oxygenases. By S. Dagley (*Department of Biochemistry, University of Leeds*)

Many biochemical oxidations are accomplished by the removal of hydrogen atoms or electrons which are transferred from primary substrates through carriers to molecular O_2: sometimes O_2 serves as direct acceptor but in all oxidations of this type O_2 is converted into H_2O or H_2O_2. However, in the case of those enzyme systems to which the name ‘oxygenase’ has been given, oxygen is incorporated into a substrate molecule. A ‘true’ oxygenase incorporates two atoms of atmospheric oxygen into one molecule of substrate,

as in the bacterial oxidation of catechol to *cis-cis*-muconic acid; whereas a 'mixed function' oxygenase, or hydroxylase, incorporates one atom of the oxygen molecule into the substrate and requires an electron donor despite the fact that the substrate is oxidized: the second atom of molecular oxygen is reduced to water. Since oxygenases participate in the metabolism of many compounds by soil bacteria it is evident that they are also responsible for biological fixation of oxygen which was not formerly recognized.

Bacterial attack upon a chemically inert molecule appears often to be initiated by a mixed-function oxygenase: thus, M. J. Coon and colleagues have studied bacterial enzyme systems that require NADH and ferrous ions for the aerobic conversion of octane into octanol and for the ω-oxidation of fatty acids. I. C. Gunsalus, H. E. Conrad and colleagues have shown that mixed function oxygenases are involved in the degradation of camphor; and before aromatic compounds are degraded by bacteria, two hydroxyl groups are introduced into the benzene nucleus by oxygenases of this type. The elucidation of the course of the reaction catalysed by these oxygenases of bacterial origin has been difficult because they are so labile, and studies have not yet been made as complete as those of S. Kaufman for the mammalian system that converts phenylalanine into tyrosine and for which dihydrobiopterin has been implicated in addition to NADPH. However, O. Hayaishi and his group have shown that the bacterial hydroxylation of kynurenic acid to give 7,8-dihydroxykynurenic acid proceeds through the 7,8-dihydrodiol, and also that anthranilic acid is oxidized to catechol by another cell-free system, possibly with the intermediate formation of an epoxide but not of 3,5-cyclohexadiene-1,2-diol. These recent findings will be described.

Once a dihydric phenol has been formed, the benzene nucleus is opened by an oxygenase that may cleave the ring between hydroxyl groups (as in pyrocatechase), or in the 2,3 position (metapyrocatechase, recently crystallized by Nozaki, Kagamiyama & Hayaishi), or between one hydroxyl group and a side chain (homogentisicase). The metabolic significance of oxygenases of this type will be discussed with reference to enzymic reactions, some of them elucidated recently, which convert the products of ring-fission into compounds of the Krebs cycle. Knowledge of the type of ring-fission catalysed also may aid the difficult task of taxonomic analysis of the aerobic pseudomonads (Stanier, R. Y. & Palleroni, N. J., personal communication).

Induction and Repression of Enzymes by Oxygen. By J. W. T. WIMPENNY (*Biochemistry Department, Oxford*) and D. E. HUGHES (*Microbiology Department, Cardiff*)

Facultative bacteria can grow in a wide range of O_2 tensions from that at normal atmospheric pressure to complete anaerobiosis. The question to be discussed is whether, when growing anaerobically, a typical facultative organism such as *Escherichia coli* is enzymically and structurally similar to an obligate anaerobe. Alternatively, is it essentially an aerobic organism in which O_2 has, either directly or indirectly, induced new enzymes and repressed those connected with aerobic metabolism?

Much of the experimental work to be discussed has been carried out by Dr Wimpenny and Dr Gray at Dartmouth Medical School (Gray, C. T. (1964), *Biochem. J.* **90**, 23). This has involved the estimation of the enzymic and chemical constitution and the location of enzymes in cells of *Escherichia coli* grown at different O_2 tensions. It was found that under anaerobic conditions there was a considerable reduction but not complete suppression of enzymes associated with electron transport to O_2 as final electron acceptor. At the same time, new enzymes such as a bound hydrogenase and a soluble, low redox, c type cytochrome appeared (Gray, C. T., Wimpenny, J. W. T., Hughes, D. E. & Ranlett, M. (1963), *Biochem. Biophys. Acta*, **67**, 157).

The anaerobically grown facultative organism therefore contrasted sharply with obligate anaerobes especially in that the composition of the membrane was more like that of an obligate aerobe than that of a typical obligate anaerobe. There was no marked change in structure or chemical constitution in any cell component measured.

It would appear that the ability of facultative organisms to grow anaerobically involves the production of enzymes concerned in the reduction of alternate substrates to O_2, while at the same time the membrane-bound electron transport system of aerobic cells remains, although it is quantitatively less significant.

Oxygen and the Photosynthetic Apparatus of Athiorhodaceae. By JUNE LASCELLES (*Microbiology Unit, Department of Biochemistry, University of Oxford*)

Many photosynthetic bacteria of the Athiorhodaceae family are facultative aerobes, since they grow either anaerobically in the light or aerobically in the dark. The synthesis of the photosynthetic pigments, bacteriochlorophyll and carotenoids, is considerably influenced by the growth conditions; organisms grown photosynthetically contain about 100 times more of these pigments than those grown aerobically. The effect of the environment on the pigment system is clearly established as repression by O_2 rather than as induction by light since (1) introduction of O_2 into cultures growing under continuous illumination immediately stops pigment synthesis (Cohen-Bazire, G., Sistrom, W. R. & Stanier, R. Y. (1957), *J. Cell. comp. Physiol.* **49**, 25), and (2) pigment formation occurs in the dark provided that the O_2 concentration is low (Lascelles, J. (1959), *Biochem. J.* **72**, 508.

Oxygen also influences other components of the photosynthetic apparatus. Aerobically grown organisms have a lower concentration of cytochromes and ubiquinone than do those grown anaerobically in the light and they exhibit little or no ribulosediphosphate carboxylase activity; they also contain less phospholipid. The adaptation of suspensions of Athiorhodaceae from the non-pigmented to the pigmented state which occurs in the dark under low aeration is accompanied by increases in the various components that are found in high concentration in photosynthetically grown organisms. It can therefore be concluded that O_2 represses the synthesis of other components of the photosynthetic apparatus besides the pigments.

The 100-fold difference in bacteriochlorophyll content of *Rhodopseudomonas spheroides* that can be achieved by varying the growth conditions is reflected in the concentration of enzymes concerned in the synthesis of this pigment from succinyl CoA and glycine. The enzymes responsible for the first steps in the pathway, δ-aminolaevulinate synthase and ALA dehydratase, are five to ten times higher in pigmented cells. Their synthesis is repressed by O_2, though not completely since they are also needed to form haems which are present in cells grown either aerobically or photosynthetically. The ability to methylate magnesium protoporphyrin monomethylester is found only in photosynthetically grown organisms (Gibson, K., Neuberger, A. & Tait, G. (1963), *Biochem. J.* **88**, 325). This suggests that O_2 causes co-ordinate repression of all the enzymes of the pathway to bacteriochlorophyll though confirmation of this requires further knowledge of the individual steps in the biosynthesis.

Control of bacteriochlorophyll synthesis by O_2 is not achieved solely by repression of the biosynthetic enzymes. The immediate effect of O_2 on pigment synthesis could occur by inhibition of the action of enzymes concerned in the initial stages of the pathway. Another possibility is that O_2 favours oxidation of succinate via the tricarboxylic acid cycle, thereby reducing the availability of succinyl CoA for diversion towards tetrapyrrole synthesis.

The function of ribulosediphosphate carboxylase in the Athiorhodaceae cannot be to render CO_2 available as a bulk carbon source since these organisms use organic substrates for this purpose. The primary function of the carboxylase may be to convert CO_2 to phosphoglycerate, which, under photosynthetic conditions, could act as a 'sink' for excess reducing power generated by oxidation of the organic substrate. Repression of the enzyme by O_2, an alternative outlet for reoxidation of NADH, might therefore be analogous to the repression of nitrate reductase by O_2 in organisms capable of dissimilatory nitrate reduction.

The development of the photosynthetic apparatus in Athiorhodaceae involves the co-ordinated synthesis of the individual units, which include the pigments, components

of the electron transport chain and ancillary enzymes. Electron microscopy has shown that modifications also occur in the cytoplasmic membrane, resulting in the appearance of structures termed chromatophores. The repression by O_2 of the photosynthetic apparatus and of the associated structures may be compared with the repression by glucose of the respiratory apparatus in yeast and other micro-organisms. The mechanism of O_2 repression is not known.

Inhibitory Effects of Oxygen. By P. N. Hobson (*Rowett Research Institute, Bucksburn, Aberdeen*)

Bacteria have for long been grouped into aerobes, facultative anaerobes, and anaerobes, but there are many gradations in these broad categories. The initiation of growth from small inocula of anaerobic bacteria and protozoa is inhibited by oxygen and by a high E_h in the culture medium, but it is not easy to determine exactly what are the conditions in a medium, especially at the cell surface, from measurements of oxidation-reduction potentials or O_2 content of the gaseous phase of the medium. The measurable conditions, such as E_h, under which growth can be initiated also vary with composition of the culture medium. The conditions for continuation of growth of anaerobes, once rapid growth has started, can be different from those needed to initiate growth, and there is evidence that some anaerobes can utilize O_2 in growing cultures as well as in non-growing suspensions. The effects of O_2 on non-growing anaerobic and facultative anaerobic bacteria vary with the physical state of the cells. Some of these varying effects will be discussed and examples given of deleterious effects of O_2 on growing and resting bacteria under different conditions. There is evidence that O_2 in excess is toxic to all micro-organisms, even those classed as strict aerobes, and this may be due to a non-specific oxidation of thiol groups in the cells. The reasons for the greater sensitivity of anaerobic bacteria to O_2 cannot so far be definitely stated. The 'catalase theory' of oxygen-inhibition of anaerobes cannot be generally applicable, but some of the recent evidence for the presence in some anaerobes of enzyme systems which are inactivated by O_2, or H_2O_2 formed by the metabolic activities of the enzymes, will be discussed. These enzyme systems have been found mainly in Clostridia, but there exist many bacteria such as the photosynthetic anaerobic bacteria, sulphate-reducing bacteria and carbohydrate-fermenting rumen bacteria which have more exacting requirements for anaerobic growth conditions than the Clostridia. The presence of easily autoxidizable cytochrome-linked electron transport systems in these bacteria may be the cause of their need of highly reduced conditions for growth.

CORRIGENDA

In J. F. M. Hoeniger (1965). *J. gen. Microbiol.* **40**, 29–42

Page 34, Results, Fig. 2:

For 'Percentage of bacteria having the indicated lengths of flagella'

Read 'Percentage of flagella having the indicated lengths'

In C. A. Schindler (1965). *J. gen. Microbiol.* **40**, 199–205

Page 200:

Line 4 *For* Nossil *Read* Nossal

Line 18 *For* tent-butanol *Read* tert-butanol

Line 43 *For* 540 μ *Read* 540 mμ

Starting with Volume 42 in 1966, the subscription price for *The Journal of General Microbiology* will be £5 (U.S.A. $17.50) a volume including postage. Single parts will be £2. 5*s*. (U.S.A. $7.50) postage extra.

Four volumes are issued yearly.