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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.

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

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(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.

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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*).

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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: *c.* = circa or approximately; 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.

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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 Vitimins. 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 references 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.

ADDENDUM

An earlier paper by Postgate & Hunter (1962) contained several errors. We publish the following table of corrections and apologize to anyone who may have been misled.

Reference	*Correction*
Page 236, line 13	*For* $\pm 0{\cdot}01/hr^{-1}$ *read* $\pm 0{\cdot}01\ hr^{-1}$
Page 237, line 22	*For* Millipore *read* membrene
Page 240, sketch	*For* 26·7 *read* 96·7
Page 245, last line	*For* $\times 10^{+5}$ M *read* $\times 10^{-5}$ M
Page 246, table 2	*Insert* no *after* or *in last column*
Page 246, table 2	*For* Fig. 9 *read* Fig. 7 (in note marked †)
Page 248, line 36	*For* uracil *read* uridine
Page 253, table 4 Page 254, line 10	*For* 0·31 mM-Na_2SO_4 *read* 0·031 mM-Na_2SO_4
Page 255, Fig. 12	*Label ordinate* viable/total, %
Page 256, Fig. 13*a*	*Third point in D = 0·44 curve should have symbol* ◒
Figs. 2–5, 11, 13–16	*For* % viable total *read* viable/total, %

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The Effect of Enzyme Treatments on *Brucella abortus* Cell Walls

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(*Received* 1 *April* 1963)

SUMMARY

Cell walls of *Brucella abortus* which were treated with trypsin, 'Pronase' (a protease from *Streptomyces griseus*), or lysozyme were more serologically reactive than cell walls treated with ribonuclease, pepsin or lipase or those with no enzymic treatment. Ribonuclease decreased reactivity of cell wall suspensions. Electron microscopy revealed the presence of round 'plates' following treatment with certain of these enzymes. 'Plates' were also shown to be embedded in fragments of the cell wall following lysozyme treatment. Still smaller round bodies were also seen to be present. These particles are believed to be a sub-unit of *B. abortus* cell walls and are correlated with serologic reactivity.

INTRODUCTION

In 1959, Markenson, Sulitzeanu & Olitzki described the immunizing activity of insoluble fractions of Brucella organisms which had been sonically disintegrated and concluded that these insoluble fractions were most likely cell-wall fragments. Foster & Ribi (1960, 1962) showed that most of the immunizing activity of the brucellas was in the cell-wall fraction. Markenson, Sulitzeanu & Olitzki (1962) showed that the cell walls were better antigens than the intact bacteria, and that the method of preparing the cell walls appeared to influence their activity, in that the protection index given by cell wall prepared by trypsinization was higher than that shown by cell wall prepared by sonic disintegration only. The purpose of the present work was to evaluate the antigenicity of certain preparations made from *Brucella abortus* and to study, in particular, some effects of enzymes on the antigenicity of Brucella cell walls.

METHODS

The culture used throughout was *Brucella abortus* strain 19 taken from the culture collection of the School of Veterinary Medicine, University of Georgia. Cell walls and cytoplasm of Brucella were prepared according to the method of Foster, Cowan & Maag (1962) or Foster & Ribi (1962). Endotoxin was prepared by the aqueous extraction of ether-treated organisms (Foster & Ribi, 1962). Acetone-sterilized organisms were prepared by adding 2 vol. of cold acetone to 1 vol. of washed brucellas in cold saline. The brucellas were adjusted to a concentration equivalent to about 50 mg. dry weight/ml. suspension. The suspension was re-

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frigerated at 0–6° overnight and dried on a Buchner funnel by washing with cold acetone. Cytoplasm, endotoxin, cell walls and ether-treated whole organisms were lyophilized.

Enzymic treatment of cell walls. The enzymic treatment of cell walls was as follows. Samples of cell wall (equiv. 5 mg. dry wt.) in 0·15 M-phosphate buffer (5 ml.) were treated with the following enzyme preparations (2·5 mg. at the pH values indicated): ribonuclease (5 × cryst., Nutritional Biochemicals, Cleveland, 18, Ohio, U.S.A.), pH 7·6; pepsin (5 × cryst., Nutritional Biochemicals), pH 2·5; lipase (Nutritional Biochemicals), pH 7·0; lysozyme (Nutritional Biochemicals), pH 7·2; trypsin (trypsin 1/250, Difco Laboratories), pH 8·2; Pronase (*Streptomyces griseus* protease, California Corporation for Biochemical Research, 3625 Mediford Street, Los Angeles 63, Cal., U.S.A.), pH 8·6. Controls with the same reagents as the test, but without the corresponding enzymes, were included and handled in the same way as the test suspensions. These were incubated for 3 hr at 37° in a water bath. Two ml. samples of the test and control suspensions were removed for part of the tests and the remainder centrifuged at 12,000 *g* and 0° for 20 min. The sediment was reconstituted to the original volume with 0·15 M-NaCl. The supernatant fluid and sediment from pepsin-treated cell walls were neutralized with 0·1 N-NaOH.

Preparation of antisera. Before injection, non-living antigens were adjusted to a concentration of 1 mg./ml. in saline and tested for sterility by inoculation on tryptose and blood agar plates. Serologically negative rabbits were then given an intravenous injection of 2·0 mg. antigen. Antisera against viable brucellas were produced by inoculation of a suspension containing about 10^9 cells. These brucellas were grown on a chemically defined medium to avoid production of antibodies against culture medium ingredients. Seven days after inoculation all rabbits were bled by heart puncture, the sera collected and stored at 5° without preservative.

Serological analysis. The serological reactivity of all the preparations was measured by the capillary flocculation test (Larson, 1951) with antigen dilutions with constant antibody. A parallel set of controls was routinely set up using the same antigen dilutions + 0·15 M-NaCl instead of serum. The tubes were incubated at 25° and read at the end of 24 and 48 hr. The end-point adopted was the highest dilution in which a 'floc' visible with a × 10 hand lens had settled to the lower meniscus in the tube. The 'floc' was readily distinguished from the more compactly sedimented control. Control dilutions showed visible flocculation only twice and then only in dilutions less than 1/10. Titres are expressed as the reciprocal of the dilution and are based on the dry weights of the various fractions; thus 1 mg./ml. was considered to be undiluted. In interpreting the results, it should be remembered that the antigens used in these tests were in different physical states (Shepard, Ribi & Larson, 1954; Ribi, Milner & Perrine, 1959).

Tube agglutination titres were determined in twofold dilutions of antisera in a final quantity of 0·5 ml./tube to which 0·5 ml. of standard ARS Brucella antigen (obtained through courtesy of Dr C. A. Manthei, National Animal Disease Laboratories, U.S., A.R.S., Ames, Iowa, U.S.A.) was added. An antigen + saline control was included with each test. The tubes were then incubated in a water bath at 37° for 48 hr before final readings were made. Total agglutination resulting in a completely clear supernatant fluid was scored as 4+, and all titrations were read at this end-point.

Electron micrographs were made with an RCA EMU-2C electron microscope. Preparations were mounted on 200-mesh copper screens covered with a thin collodion film and shadowed with chromium or platinum + palladium alloy.

RESULTS

Serological activity of various Brucella abortus fractions

The results of capillary flocculation tests with various Brucella fractions are given in Table 1. These results show that Brucella cell walls and endotoxin were the most antigenically active of the various fractions. Viable organisms showed little flocculation while ether and acetone-sterilized organisms were more reactive.

The data given in Table 2 were obtained with tube agglutination by using antisera for the various preparations and phenolized Brucella organisms of various species.

Table 1. *Reciprocal flocculation titres to fractions of Brucella abortus strain 19*

Preparation (equiv. 1 mg. dry wt./ml. saline)	Antiserum to: Viable organisms	Acetone-sterilized whole organisms	Ether-treated whole organisms	Cell walls	Endotoxin	Cytoplasm
	Reciprocal flocculation titre					
Viable organisms	20	20	5	5	5	5
Acetone-sterilized whole organisms	40	160	20	20	80	10
Ether-treated whole organisms	40	40	40	40	40	40
Cell walls	80–160	320	40	80	80	40
Endotoxin	640–1280	160	40	40	160	40
Cytoplasm	10	20	5	5	40	40

Table 2. *Serological response in rabbits following the intravenous injection of Brucella abortus fractions*

B. abortus 19 antigens	Antiserum to: *B. abortus* 19	*B. suis* 1776	*B. melitensis* 2500
	Reciprocal of serum titres in tube agglutination		
Viable organisms	640	640	320
Cell walls	640	640	640
Endotoxin	1280	640	320
Cytoplasm	320	160	80
Acetone-sterilized whole organisms	640	640	640
Ether-treated whole organisms	320	320	320
Cell walls + ribonuclease	320	320	160
Cell walls + pepsin	320	640	320
Cell walls + lipase	640	640	320
Cell walls + trypsin	640	640	320
Cell walls + Pronase	1280	1280	1280
Cell walls + lysozyme	1280	1280	1280

These titres showed little difference in antigenicity of viable and killed organisms. They also suggest that the distribution of A and M antigens in the cell walls did not differ from their distribution in whole organisms. Possibly there is a difference in anti-A and anti-M antibodies produced by inoculation of cytoplasm and endotoxin because the titre of *Brucella melitensis* was one-fourth that of *B. abortus*. There was no evidence of a differential sensitivity of A and M antigens to the enzymes used.

Effect of enzymes on cell walls. Initial evidence for enzymic effect on Brucella cell walls was a decrease in opacity following Pronase and lysozyme treatment. Lysozyme increased flocculation titres of cell-wall suspensions two- to fourfold and ribonuclease decreased the titre twofold; pepsin, lipase, Pronase or trypsin had no detectable effect.

Table 3. *Reciprocal flocculation titres of enzyme-treated cell-wall sediments and supernatant fluids to Brucella abortus strain 19 antiserum prepared with viable organisms; cell walls treated with enzymes and samples of sediment and supernatant fluids after centrifugation tested for flocculation against antiserum to whole viable B. abortus 19*

	Antiserum to whole viable *B. abortus* 19			
	Test		Control	
Enzyme-treated cell-wall preparations (equiv. 1 mg. preparation/ml.)	Sediment	Supernatant fluid	Sediment	Supernatant fluid
	Reciprocal flocculation titre			
Cell walls + ribonuclease	40	10	160	neg
Cell walls + pepsin	80	20	80	10
Cell walls + lipase	80	20	160	neg
Cell walls + trypsin	80	40	160	neg
Cell walls + Pronase	160	80	160	neg
Cell walls + lysozyme	320	160	160	neg

These results led to an examination of the relative reactivity of the sediments and supernatant fluids of the cell-wall suspension after enzyme treatment. The data given in Table 3 show that Pronase and lysozyme released significant amounts of antigen into the supernatant fluid; trypsin apparently released more antigen than lipase, pepsin or ribonuclease. Ribonuclease decreased the flocculation titre of the sediment and released little antigen into the supernatant fluid. Buffer released little soluble antigen from cell walls.

The results given in Table 4 furnish additional evidence that lysozyme increased the flocculation titre of the cell walls. Decreased activity of pepsin-treated cell walls also was seen with endotoxin or cell-wall antiserum. However, the low pH value used during enzyme treatment may have caused the decreased reactivity. The antiserum for cytoplasm reacted more strongly with untreated cell-wall preparations than with enzyme-treated preparations. This might have been due to the effect of the enzymes on cytoplasm which may have contaminated the cell walls.

Morphological study of enzyme-treated Brucella cell walls. From a study of the

electron micrographs (Pls. 1 and 2, figs. 1–7) it is apparent that the treatment of cell walls with enzymes resulted in not only a loss of Brucella cell-wall rigidity but also a loss of cell-wall integrity. Salton (1960) concluded that the enzymes ribonuclease, trypsin and lipase could be used without destroying rigidity of, or apparently degrading, the wall structure. From a comparison of Pl. 1, fig. 1, which is a control specimen, with Pl. 1, figs. 3–5, it is apparent that there was a degradation of *Brucella abortus* cell walls with all of these enzymes, as well as with pepsin (Pl. 1, fig. 2) and Pronase (Pl. 2, fig. 7).

Table 4. *Reciprocal flocculation titres of Brucella abortus strain* 19 *fractions to B. abortus* 19 *fraction antisera*

	Antiserum to			
Cell-wall preparation (equiv. 1 mg. dry wt./ml.)	Viable organisms	Cell walls	Endotoxin	Cytoplasm
	Reciprocal flocculation titre			
Viable organisms	20	5	5	5
Cell walls	80–160	80	80	40
Endotoxin	640–1280	40	160	40
Cytoplasm	10	5	40	40
Cell walls + ribonuclease	80	40	40	20
Cell walls + pepsin	80	40	20	10
Cell walls + lipase	80	80	40	20
Cell walls + trypsin	80	80	40	20
Cell walls + Pronase	160	80	80	20
Cell walls + lysozyme	320	80	80	20

Salton (1960) also showed that after treatment with lysozyme, the cell walls of *Rhodospirillum rubrum* lost rigidity and became spherical. This he attributed to the release by lysozyme of alanine, glutamic acid, diaminopimelic acid, and glucosamine. Plate 2, figs. 6*a*, *b*, are electron micrographs of Brucella cell walls treated with lysozyme. In comparing these figures with Pl. 1, fig. 1, it seems that there is not so much a loss of cell rigidity as there is a loss in cell-wall integrity. The small round 'plates' seen in Pl. 1, fig. 2, and Pl. 2, fig. 6*b*, in particular, which vary in size from less than 0·1 μ to about 0·3 μ, are too small to be intact cell walls which, owing to lysozyme and Pronase, have lost their rigidity and assumed a spherical shape. The flattening might be an artifact. Although there is certainly a decrease in the size of the intact cell wall treated with any of the enzymes, this decrease is not sufficient to account for the extremely small size of the 'plates'. If the supernatant fluid, separated from the lysozyme-treated cell walls at 3800 *g* for 1 hr at 0°, is centrifuged for successive 30 min. periods at 12,000 *g* and 90,000 *g* at 0°, fragments are sedimented which contain 'plates'. Further centrifugation at 140,000 *g* and 0° sediments smaller fragments (Pl. 2, fig. 6*b*) which still contain 'plates'. In these pictures some 'plates' seem to be held in membranous material. The appearance is that of a defined structural component, a 'plate', in the cell wall. There also are still smaller particles (Pl. 2, fig. 6*a*) visible in disintegrating cell walls, and in the background material. These are similar to those seen in *Escherichia coli* by Weidel, Frank & Martin (1960). The particles released by lysozyme and sedimented from

a supernatant fluid as above react to high titre (greater than 1/5120) with *Brucella abortus* antiserum. It appears that enzymic treatment of Brucella cell walls has brought about a degradation of the structure and hence a loss of cell-wall integrity.

DISCUSSION

The results of the present work confirm earlier reports (Markenson *et al.* 1959, 1962; Foster & Ribi, 1960, 1962) that potent antigenic substances are to be found in the cell walls of *Brucella abortus*. The apparent increase in serological reactivity obtained when Brucella cell walls were treated with trypsin, Pronase or lysozyme is of significance in view of the recent findings of Olitzki & Sulitzeanu (1958) and Markenson *et al.* (1962). The former workers treated Brucella cell walls with trypsin and intact organisms with lysozyme. They observed that lysozyme treatment enhanced the antigenicity of their preparations and concluded that the enzyme caused more efficient release of antigen by breaking up cell walls; they also found that pepsin inactivated some antigens in the cell wall. The results of the present study are compatible with their findings (Tables 3 and 4). Markenson *et al.* (1962) found that trypsin digestion of ethanol-treated brucellas yielded cell walls which were more immunogenic than those obtained by sonic disintegration only, and sonic disintegration of trypsin cell wall further enhanced immunogenicity. The results in the present work are less convincing, but cannot be directly compared since the former study was made *in vivo*.

It is interesting to note that viable brucellas gave low flocculation titres (Tables 1 and 4) with the antisera tested. Viable brucellas probably release less antigen than brucellas which have been altered by acetone (Olitzki & Sulitzeanu, 1958; present paper, Table 1) or ether (Foster & Ribi, 1962).

Electron micrographs show that maximum disintegration of cell walls follows treatment with lysozyme and Pronase. Lesser degrees of dispersal occurred with other enzymes, but in all cases round 'plates' were released. Increased flocculation titres were correlated to some extent with degrees of dispersion, and the 'plates' may account for the flocculating activity of the supernatant fluids shown in Table 3. Markenson *et al.* (1959, 1962) described similar particles of cell walls which they considered to be cell-wall fragments; we concur in this opinion. It seems possible that the 'plates' and smaller round particles observed in the present work are normally occurring structural sub-units. Pl. 2, figs. 6*a*, *b*, supports this idea. If so the cell-wall structure may be different from that seen in *Escherichia coli* (Weidel *et al.* 1960). However, their cell walls were treated with base which would probably release some cell-wall material from *Brucella abortus* (Olitzki & Sulitzeanu, 1958; Olitzki, 1960; Foster & Ribi, 1962). The *E. coli* cell walls were also treated with sodium dodecyl sulphate which is known to affect the state of aggregation of Brucella antigens (Miles & Pirie, 1939) and these differences in treatment make direct comparison impossible. The possibility cannot be overlooked that the 'plates' might be derived from cell membrane which adhered to the cell wall. The indiscriminate effects of the several enzymes in producing 'plates' might indicate action on a membrane. Finally, the increase of flocculation titres by an increase in dispersion of the cell wall poses the following questions: is this increase due simply to an increase in surface area exposed, or is it due to exposure of a new and

different surface? That the inner surface of the cell wall may differ from that of the outside has been shown by electron micrography of the cell wall of a Spirillum (Houwink, 1953) and *E. coli* (Weidel *et al.* 1960).

We wish to thank Dr T. A. Maag of this department for assistance in preparation of the final enlargements. This investigation was supported, in part, by a research grant (E-1247) from the National Institute of Allergy and Infectious Diseases, and by a training grant (2E-197) U.S. Public Health Service. Part of this work is taken from a thesis presented by the senior author in partial fulfilment of the requirements for the degree of Master of Science, University of Georgia, 1962. It was presented, in part, at the Annual Meeting for the Society for Microbiology, Cleveland, Ohio, 6 May 1963.

REFERENCES

FOSTER, J. W., COWAN, R. M. & MAAG, T. A. (1962). Rupture of bacteria by explosive decompression. *J. Bact.* **83**, 330.

FOSTER, J. W. & RIBI, E. (1960). The role of the cell wall of *Brucella abortus* in immunity. *Bact. Proc.* 1960.

FOSTER, J. W. & RIBI, E. (1962). The role of the cell wall of *Brucella abortus* in immunity. *J. Bact.* **84**, 258.

HOUWINK, A. L. (1953). A macromolecular mono-layer in the cell wall of *Spirillum* sp. *Biochim. biophys. acta*, **10**, 360.

LARSON, C. L. (1951). Studies on thermostable antigens extracted from *Bacterium tularense* and from tissues of animals dead of tularemia. *J. Immunol.* **66**, 249.

MARKENSON, J., SULITZEANU, D. & OLITZKI, A. (1959). Immunizing properties of insoluble cell material derived from Brucella. *Nature, Lond.* **183**, 1693.

MARKENSON, J., SULITZEANU. D. & OLITZKI, A. (1962). Immunogenic activity of Brucella cell wall. *Brit. J. exp. Path.* **39**, 219.

MILES, A. A. & PIRIE, N. W. (1939). The properties of antigenic preparations from *Brucella melitensis*. II. Serological properties of the antigens. *Brit. J. exp. Path.* **20**. 109.

OLITZKI, A. L. (1960). The antigenic relationship between phenol extracted bacterial deoxyribonucleic acid and other soluble antigens of brucellae studied with the aid of the agar-gel precipitation technique. *Brit. J. exp. Path.* **41**, 623.

OLITZKI, A. L. & SULITZEANU, D. (1958). Studies on the antigenic structure of *Brucella suis* with the aid of agar-gel precipitation technique. *Brit. J. exp. Path.* **39**, 219.

RIBI, E., MILNER, K. C. & PERRINE, T. D. (1959). Endotoxic and antigenic fractions from the cell wall of *Salmonella enteritidis*. *J. Immunol.* **82**, 75.

SALTON, M. R. S. (1960). *Microbial Cell Walls. Ciba Lectures in Microbial Biochemistry.* New York: John Wiley and Sons Inc.

SHEPARD, C. S., RIBI. E. & LARSON, C. L. (1954). Electron microscopically revealed structural elements of *Bacterium tularensis* and their *in vitro* and *in vivo* role in immunologic reactions. *J. Immunol.* **75**. 7.

WEIDEL, W., FRANK, H. & MARTIN, H. H. (1960). The rigid layer of the cell wall of *Escherichia coli* strain B. *J. gen. Microbiol.* **22**, 185.

EXPLANATION OF PLATES

PLATE 1

Fig. 1. Control: *Brucella abortus* strain 19 cell walls untreated with enzyme.

Fig. 2. *B. abortus* cell walls treated with pepsin.

Fig. 3. *B. abortus* cell walls treated with lipase.

Fig. 4. *B. abortus* cell walls treated with ribonuclease.

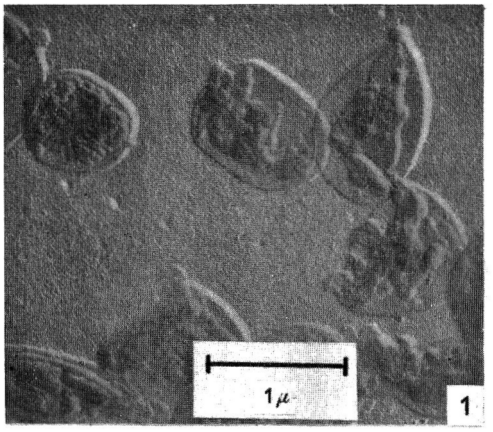
Fig. 5. *B. abortus* cell walls treated with trypsin.

PLATE 2

Fig. 6*a*. *B. abortus* cell walls treated with lysozyme.

Fig. 6*b*. Particles from lysozyme-treated cell walls, not sedimented at 90,000 *g* in 2 hr. but sedimented at 140,000 *g*.

Fig. 7. *B. abortus* cell walls treated with Pronase.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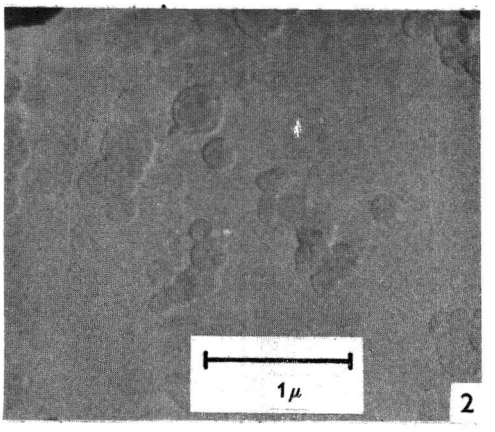
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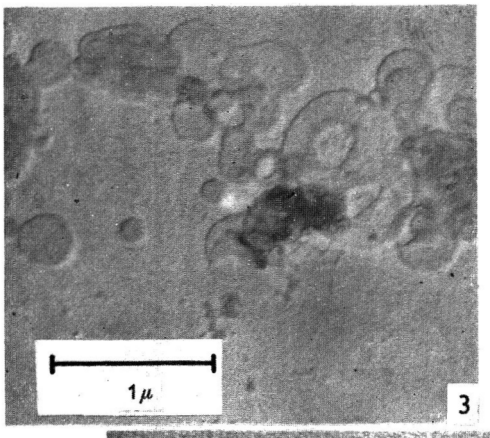
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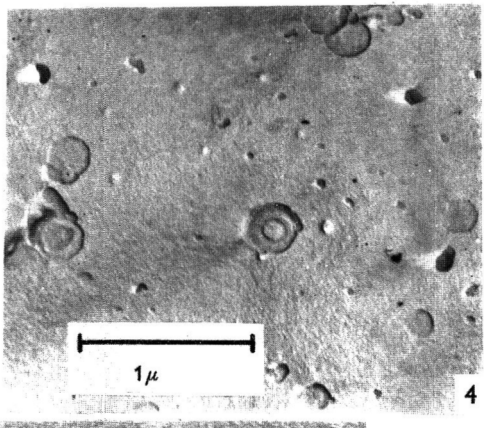
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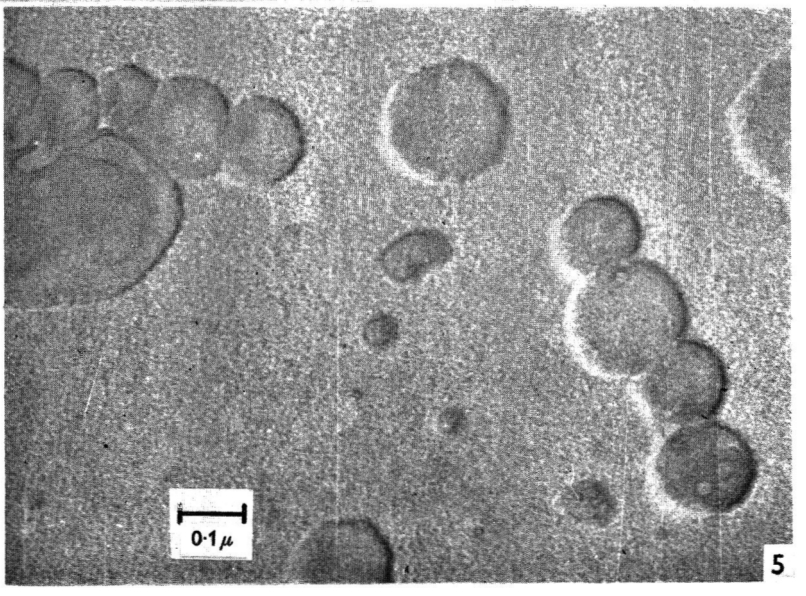
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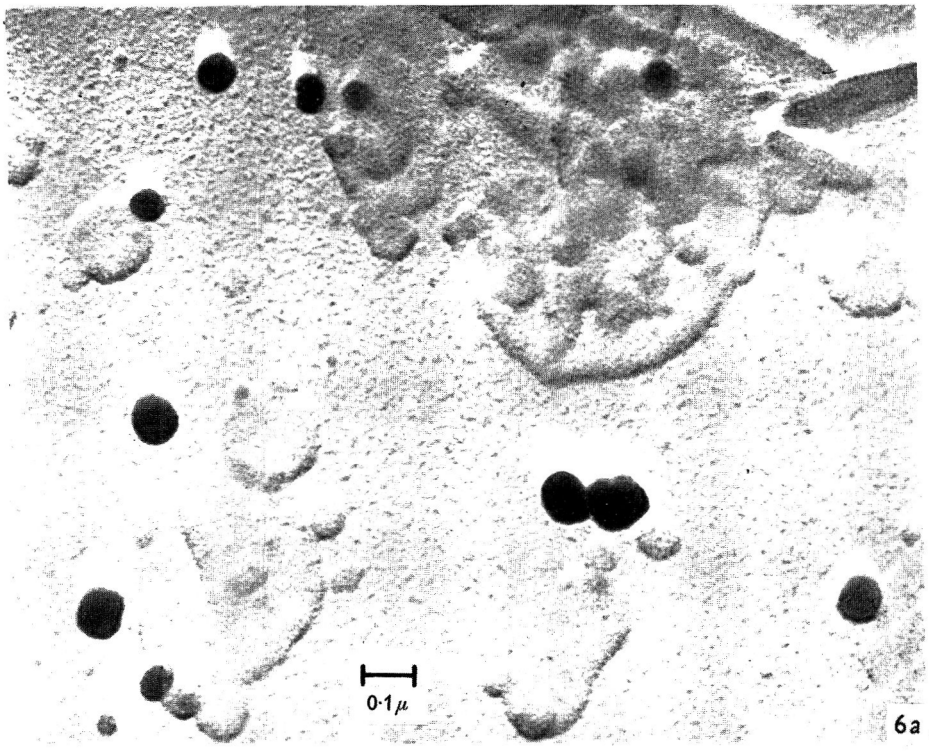
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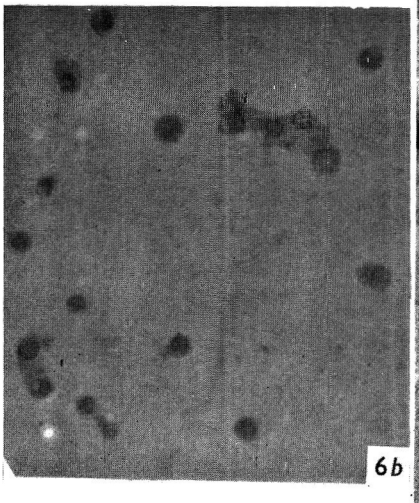
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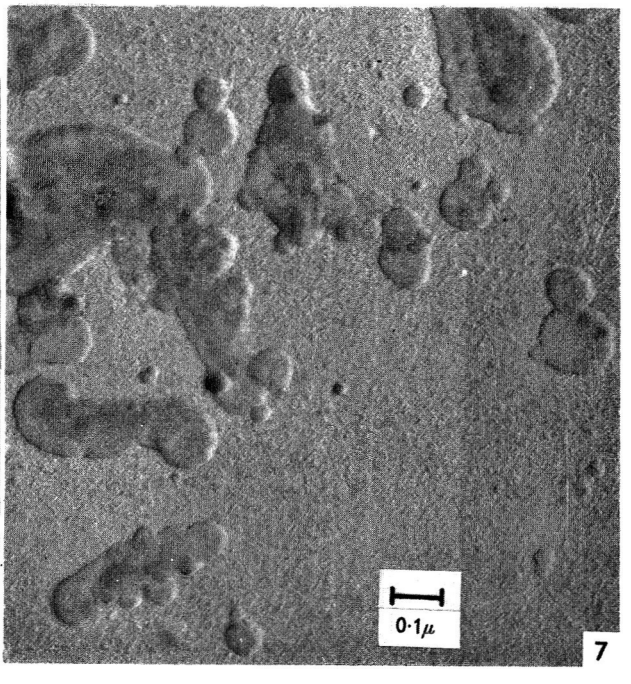
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Kinetic Studies of the Oxidative Assimilation of Acetate by a Non-photosynthetic Strain of *Euglena gracilis*

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SUMMARY

Kinetic studies of the effects of unlabelled acetate on $^{14}CO_2$ production by a non-photosynthetic strain of ^{14}C-labelled Euglena indicate the existence of a 'labile reserve', containing 1·5–2·0 μg.-atoms C/million organisms, which is an early product of acetate assimilation and a substrate of endogenous metabolism. When this Euglena assimilated radioactive acetate, radioactivity in an ethanol-soluble form (Soluble I) increased rapidly at first, then levelled off at a value corresponding to about 0·2 μg.-atom C/million organisms. Ethanol-insoluble radioactivity appeared slowly at first, then at an increasing rate which became linear at about 30–60 min. after addition of acetate. Kinetic analyses suggest that Soluble I is the precursor of the insoluble material. A second ethanol-soluble component began to become labelled some time after the first hour, and reached maximum radioactivity, corresponding to about 0·5 μg.-atom C/million organisms, 2–3 hr after addition of tracer acetate. The chemical composition of the ethanol-soluble and ethanol-insoluble components and their relationship to the 'labile reserve' are discussed.

INTRODUCTION

During oxidative assimilation of acetate by a non-photosynthetic strain of *Euglena gracilis* var. *bacillaris*, 42 % of the acetate-carbon appeared as CO_2, while the remaining 58 % was assimilated. Carbon and oxygen balance studies indicated

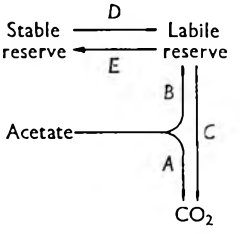

Fig. 1. Diagram of the postulated role of the 'labile reserve' in acetate metabolism of Euglena. *A* is the oxidation pathway for acetate; *B* is the pathway of acetate assimilation; *C* is the pathway of CO_2 production from intracellular reserves; *D* and *E* are exchange reactions between the 'labile reserve' and other reserve substances. (After Danforth & Wilson, 1961.)

that the major assimilatory products have the empirical composition of carbohydrate (Wilson & Danforth, 1958; Danforth, 1961). Oxidation of cellular reserves continues during the oxidative assimilation of acetate (Wilson & Danforth, 1958). Tracer studies with radioactive carbon led Danforth & Wilson (1961) to suggest

that the most recently assimilated acetate-carbon forms a 'labile reserve', which is also the immediate substrate for the reserve oxidation process. This 'labile reserve' was believed to undergo a slow exchange with a much larger 'stable reserve' (Fig. 1). The data presented at that time were not adequate to estimate the capacity of the 'labile reserve', nor to indicate whether the 'labile reserves' consisted of a homogeneous pool or of several compartments. The present study is directed toward further investigation of these problems.

METHODS

General methods. The non-photosynthetic strain of *Euglena gracilis* var. *bacillaris* used, the general methods of growth and handling of the euglenas, and the respirometric tracer techniques were as described in previous publications (Danforth, 1953, 1961; Danforth & Wilson, 1957, 1961; Wilson & Danforth, 1958).

Buetow (1961) showed that the respiratory rate of Euglena decreases with increasing centrifugation during harvesting. The harvesting procedure used routinely in our laboratory regularly yields euglenas with respiratory rates of 20 and 30 μl. O_2/million euglenas/hr., well below the 50–60 μl./million euglenas/hr found by Buetow after minimal centrifugation. We have seen no indication that this decreased metabolic rate alters the patterns of carbon distribution which are the subject of the present work, but this problem clearly deserves further study.

The 'labile reserve'. Labelled euglenas were grown in 1 l. Erlenmeyer flasks containing 500 ml. of medium composed of (w/v): Bacto-Tryptone (Difco), 0·3%; sodium acetate $3H_2O$, 0·3%. The acetate was uniformly labelled with 80 μC. ^{14}C per flask. Cultures were inoculated with 5 ml. of a heavy suspension of euglenas, and incubated for 5 days at 26°.

Experimental euglenas were incubated in special 50 ml. Erlenmeyer flasks (equipped with centre wells) containing 13 ml. of 20 mM-sodium acetate (non-radioactive) in 9 mM phosphate buffer (pH 7·0). Control euglenas were treated similarly, except that acetate was omitted from the medium. The centre wells in the flasks contained 1·0 ml. 5 M-NaOH plus filter paper. Incubation was at about 26°, with shaking at 120 cycles/min. Samples were removed at intervals, centrifuged, washed, and resuspended in fresh medium. Samples (1·5 ml.) of these fresh suspensions were transferred to Warburg flasks whose centre wells contained 0·1 ml. 5 M-NaOH (without filter paper), and whose side arms contained 0·5 ml. M-H_2SO_4. After incubation with shaking for the desired intervals, the side arms were tipped, thus killing the euglenas and acidifying the medium. The flasks were shaken for a further 2 hr to ensure complete transfer of CO_2 to the centre wells. The centre-well contents were withdrawn, and the centre wells rinsed twice, first with 0·3 ml., then with 0·4 ml. distilled water. The centre-well contents and rinses were pooled, transferred to planchets, dried under an infrared lamp, and counted for radioactivity. A drop of ethanol was added to each planchet before drying to facilitate uniform spreading of the sample. Similar samples were transferred at the beginning of the experiment to Warburg flasks for measurement of respiration. Filter paper was added to the centre wells of these flasks, H_2SO_4 being omitted from the side arms. Oxygen consumption of experimental and control euglena suspensions was followed for the entire experimental period.

Kinetics of acetate assimilation. Euglenas were grown at about 26° in a medium consisting of (w/v) Bacto Tryptone, 0·3%; sodium acetate $3H_2O$, 0·5%. The euglenas were incubated at 26°, with shaking at 120 cycles/min., in stoppered 50 or 120 ml. Erlenmeyer flasks equipped with centre wells. The main compartments of the flasks contained: acetate-^{14}C, 10 mM; phosphate buffer (pH 7·0), 10 mM; 1·5–4·8 million euglenas/ml. Uniformly labelled acetate was used in some experiments, methyl-labelled acetate in others. Centre wells contained 0·3 ml. 5 M-NaOH and a square of filter paper. At intervals, 0·5 ml. samples were withdrawn from the main compartments and pipetted into centrifuge tubes containing 0·8 ml. 95% (v/v) ethanol in water, giving a final ethanol concentration of 60% (v/v). The samples were heated to 65° for 30 min., then centrifuged, and the supernatant fluid decanted into a second tube. The precipitate was washed by recentrifugation from the same volume of 60% ethanol, and resuspended in 1·3 ml. 60% ethanol. Samples of the original supernatant fluid and of the resuspended precipitate were transferred to planchets and dried under an infrared lamp. Before counting, the dry samples were flooded twice with 0·5 ml. glacial acetic acid, drying each time, to drive off residual acetate. (In the initial experiments, samples of the ethanol wash were prepared in similar fashion. Since no radioactivity was found in these washes, they were discarded in later experiments.) An initial sample was prepared at zero time by pipetting 0·5 ml. of the reaction mixture into 0·8 ml. 80% (v/v) ethanol containing 0·1 M-NaOH; this sample was dried and counted directly to determine the specific activity of the acetate initially present.

For measurements of respiration, two Warburg flasks were prepared in each experiment, one containing the same reaction mixture as in the experimental flask (except that the acetate was non-radioactive), and one in which acetate was omitted.

Assimilation of acetate carbon into ethanol-soluble and ethanol-insoluble fractions was determined by counting the radioactivity of the appropriate planchets. Total acetate consumption could be estimated by using the stoichiometric carbon–oxygen balance equations of Wilson & Danforth (1958; Danforth, 1961), either from the respiration or independently from the amount of acetate-carbon assimilated. In some experiments, utilization was determined directly by counting the radioactivity remaining in the extracellular medium. All three methods gave equivalent results, within the expected precision of the methods; estimation from respiration was most convenient and was generally used.

RESULTS

Capacity of the 'labile reserve'

If it be assumed that the 'labile reserve' consists of a homogeneous pool of fixed capacity, V, it can be shown (see Theoretical Appendix) that during the oxidation of non-labelled acetate by Euglena uniformly labelled with ^{14}C:

$$\ln \frac{(C+E)R-D}{B} = \frac{-(C+E)t}{V}, \tag{5}$$

where R is the specific activity of the carbon in the 'labile reserve', t is the time after addition of unlabelled acetate, and B, C, D and E are the rates of the corresponding

reactions shown in Fig. 1. These rates can be evaluated from the experimental data (see Theoretical Appendix). Plotting $\ln[\{(C+E)R-D\}/B]$ against $(C+E)t$ should give a straight line through the origin, with slope equal to $-1/V$.

Figures 2 and 3 show an example of the experimental evaluation of V by this method; Table 1 presents the numerical results of this experiment. For this experiment, the line of best fit (by eye) yielded a value for V of 1·70 μg.-atoms C/million

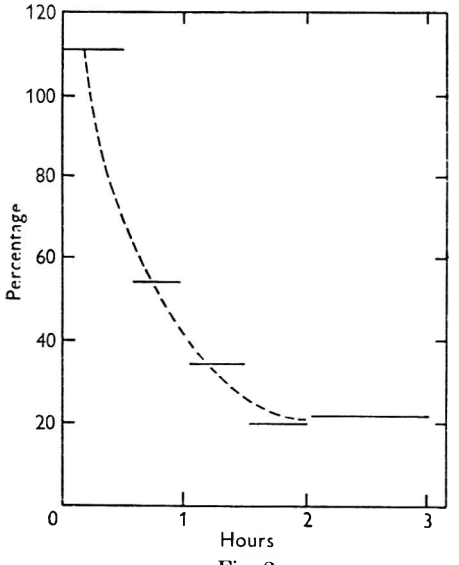

Fig. 2

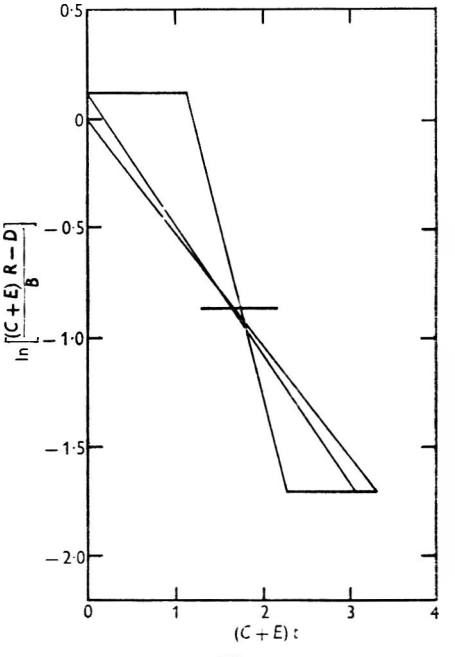

Fig. 3

Fig. 2. Effect of unlabelled acetate on the production of $^{14}CO_2$ by ^{14}C-labelled euglenas. Rate of $^{14}CO_2$ production is expressed as percentage of the rate in the absence of acetate. Each horizontal line is the average rate over a single period of measurement.

Fig. 3. Same data as in Fig. 2, plotted to provide an estimate of the capacity of the 'labile reserve'.

Table 1. *Numerical values of constants in equation (5), and the resulting estimates of* V, *for the experiment shown in Figs. 2 and 3*

Constants A–E are the rates of corresponding reactions in Fig. 1, estimated as described in the Theoretical Appendix. V is the capacity of the 'labile reserve', estimated graphically from Fig. 3.

Pathway or fraction	Value (μg.-atoms C/10^6 euglenas/hr)
A	1·28
B	1·76
C	0·18
D	0·44
E	1·01
	(μg.-atoms C/10^6 euglenas)
V (best)	1·70
V (maximum)	1·95
V (minimum)	0·58

euglenas. A second experiment gave an estimated V of 1·55 μg.-atoms C/million euglenas by the same procedure. Extreme estimates of V from the same two experiments ranged from 0·58 to 1·95 μg.-atoms C/million euglenas. Despite the uncertainties of the calculation, it seems likely that the actual amount of material making up the 'labile reserve' falls somewhere within this range.

A simpler, partially independent estimate of the capacity of the 'labile reserve' may be obtained directly from the data presented in Fig. 2 and Table 1. The rate of $^{14}CO_2$ production at about 40–50 min. is halfway between the initial and final rates. It may be assumed that this degree of 'dilution' occurs when the amount of unlabelled C assimilated is slightly more than one-half the capacity of the labile reserve. From Table 1, the assimilation at this time is about 1·0–1·3 μg.-atoms C/million euglenas, again suggesting that V is in the neighbourhood of 2 μg.-atoms C/million euglenas.

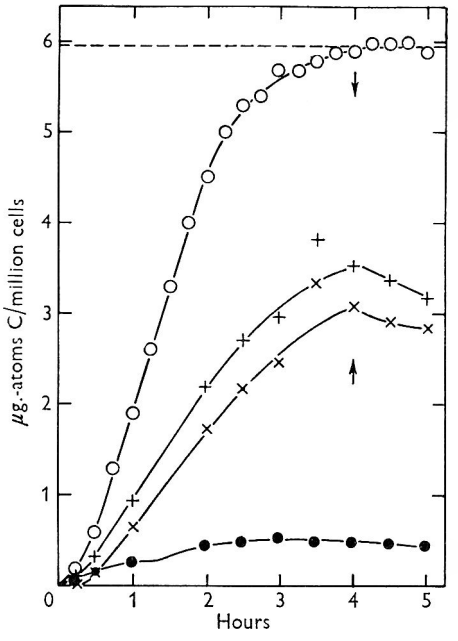

Fig. 4

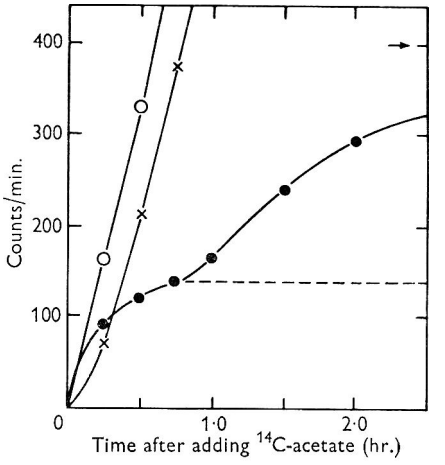

Fig. 5

Fig. 4. Time course of metabolism of ^{14}C-acetate. ○, Total acetate consumption; +, total assimilation; ×, assimilation into ethanol-insoluble material; ●, assimilation into ethanol-soluble material. Arrows indicate the time of exhaustion of acetate; dotted line indicates the amount of acetate initially present.

Fig. 5. Early stages of assimilation of ^{14}C-acetate. ○, Total assimilation; ×, assimilation into ethanol-insoluble material; ●, assimilation into ethanol-soluble material. Dotted line shows estimated maximum ^{14}C content of Soluble I. Arrow shows ultimate maximum value reached by ethanol-soluble fraction.

Kinetics of assimilation of radioactive acetate

Study of the assimilation of radioactive acetate by non-radioactive euglenas provides an independent means for studying the metabolic processes concerned in the supposed 'labile reserve'; Fig. 4 shows the results of a typical experiment.

As concluded from previous experiments (Wilson & Danforth, 1958; Danforth, 1961) acetate consumption (after a very brief lag) occurred at a constant rate until the acetate was nearly exhausted from the medium, and the fraction of this acetate assimilated was essentially constant during the entire course of the experiment. Assimilatory products soluble in 60% ethanol accumulated rapidly at first, but soon reached a relatively constant 'plateau' of about 0·5 μg.-atom C/million euglenas. Ethanol-insoluble products, on the other hand, appeared slowly at first, then at an increasing rate, and finally increased linearly until the acetate was exhausted.

The general appearance of the curves in Fig. 4 suggests that the ethanol-soluble products were precursors of the ethanol-insoluble products. Closer examination, however, suggests that the situation was more complex. Fig. 5 shows the early stages of an experiment similar to that shown in Fig. 4, plotted on a larger scale.

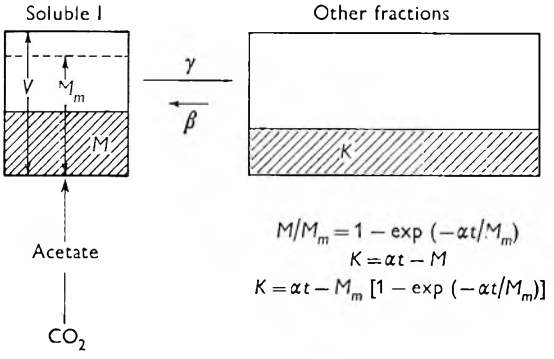

Fig. 6. Model used in kinetic analysis of acetate assimilation. See text for definitions of symbols.

In this case, it is apparent that the ethanol-soluble products accumulate in two stages, one beginning immediately and reaching a plateau after about the first hour, the second beginning after the first hour, and reaching a plateau only after 2 or more hours. A similar two-stage accumulation of ethanol-soluble products was observed in every experiment suitable for such analysis.

Of these two subdivisions of the soluble products, only the first to appear (hereafter designated Soluble I) has the characteristics expected of a precursor of the insoluble material. Examination of Fig. 5 shows that the rate of accumulation of insoluble radioactivity is roughly proportional to the radioactivity of Soluble I, and reaches a constant maximum at about the time Soluble I reaches a plateau, as would be expected if Soluble I were the immediate precursor of the insoluble material. The second stage of accumulation of soluble material ('Soluble II'), on the other hand, begins to appear only after the insoluble radioactivity is accumulating at maximum rate, a relationship incompatible with the hypothesis that Soluble II is a precursor of the insoluble products. Instead, the time relations suggest that Soluble II is derived either from Soluble I or, more probably, from the insoluble material.

The assumption that Soluble I is the precursor of both the insoluble products

and of Soluble II can be tested more critically by making use of the model shown in Fig. 6, and the equations derived from this model:

$$M/M_m = 1 - \exp(-\alpha t/M_m),$$
$$K = \alpha t - M,$$

where M is the amount of ^{14}C in Soluble I, K is the sum of the amounts of ^{14}C in Soluble II and the insoluble fraction, t is time, and α and M_m are constants subject to experimental evaluation (see Theoretical Appendix).

Figure 7 compares the experimental course of appearance of radioactivity with the expected results calculated from the above equations; the experiment is that shown in Fig. 5. The agreement between theory and experiment is fairly good,

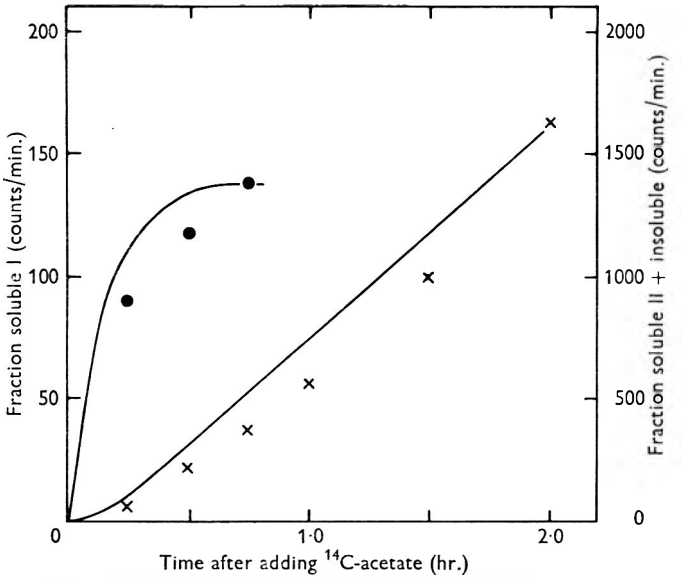

Fig. 7. Comparison of the actual time course of acetate assimilation with that predicted by the model shown in Fig. 6. ● and left ordinate, Soluble I; × and right ordinate, Soluble II plus insoluble. Points are experimental data, lines are calculated.

except that the experimental values consistently lag behind the theoretical by about 10–15 min. This systematic deviation can probably be accounted for by the fact that α, the rate of total assimilation, was assumed to be constant over the entire time period. In actual fact, the rate of acetate consumption during the first few minutes of such experiments was consistently found to be slower than the later steady-state rate (see Fig. 4). The amount of this delay, 10–20 min., is of the right order to explain the lag in the experimental values. It would seem, therefore, that the model shown in Fig. 6, in which Soluble I is considered to be a single homogeneous pool, the first product of acetate assimilation, and the precursor of all other assimilation products, gives a reasonable approximation of the actual events.

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Amounts of the ethanol-soluble products

Table 2 presents the maximum (plateau) values of ^{14}C found in the two subdivisions of the ethanol-soluble fraction in several experiments of the type just described. The total carbon content of these pools may be somewhat greater than these values because of the possibility that back reactions occur, which feed a certain amount of non-radioactive carbon into the pools (e.g. reaction β in Fig. 6). The magnitude of such possible back reactions is unknown, but is probably not large for intermediates on the direct pathway of acetate assimilation. Any extensive recycling of carbon between intermediates and products of assimilation would be expected to produce randomization between the methyl and carboxyl carbons of acetate; in fact, the fates of the two carbons are markedly different (Danforth, 1961). In *Polytomella coeca*, whose acetate metabolism resembles that of Euglena in many respects (Danforth, 1961), this lack of randomization is apparent even in long-term growth experiments (Bevington, Bourne & Turton, 1953; Barker & Bourne, 1955). Similarly, recycling should produce apparent changes in oxidation–assimilation ratios, and in the relative fates of methyl and carboxyl carbons, during the course of acetate utilization; no such changes have been detected (Danforth, 1961). It seems probable, therefore, that acetate assimilation is largely a one-way process, and that back reactions do not result in any gross underestimate of the pool sizes presented in Table 2.

Table 2. *Maximum values of ^{14}C found in the ethanol-soluble fractions*

Data from four experiments of the type shown in Fig. 4 were interpreted graphically by the method shown in Fig. 5.

	Ethanol-soluble fraction		
	Soluble I	Soluble II	Total soluble
	Maximum values of ^{14}C (μg.-atom C/10^6 euglenas)		
Expt. no.			
M1	0·22	0·54	0·76
M3	0·17	0·32	0·49
M4	0·23	0·30	0·53
M6*b*	0·14	0·13	0·27
Mean	0·19	0·32	0·51

DISCUSSION

Nature of the assimilation products. It has been shown (Marzullo & Danforth, 1964) that nearly 90% of the acetate carbon found in ethanol-insoluble products can be recovered in the glucose units of the reserve polysaccharide, paramylon, which occurs as granular inclusions within the Euglena organisms. The nature of the ethanol-soluble subfractions is relatively unknown. If the scheme shown in Fig. 6 be correct, Soluble I should consist largely of intermediates in the conversion of acetate to carbohydrates, presumably Krebs cycle acids, the phosphorylated intermediates of glucose metabolism and oligosaccharides. In partial support of this theory, Lynch & Calvin (1952, 1953) found that, of the acetate methyl-carbon incorporated into ethanol-soluble materials during incubation for 12 min. in the dark, nearly 50% was in the form of phosphorylated intermediates, 4% in the form of 'dextrins', and about 25% in the form of amino acids related to the Krebs cycle.

Even less is known about the composition of Soluble II; investigations of the components of both soluble subfractions are under way at present.

Relation of the 'labile reserves' to ethanol fractions. Of the three carbon pools inferred from the kinetics of assimilation of radioactive acetate, only Soluble I shows kinetic characteristics similar to those postulated for the 'labile reserve', rising to a maximum during the first hour or so after addition of acetate, and remaining at a constant level thereafter. There is, however, a great discrepancy between the estimated size of Soluble I and that of the 'labile reserve'. The capacity of the 'labile reserve' was estimated to be in the neighbourhood of 1·5–2 μg.-atoms C/million euglenas, while that of Soluble I is only about one-seventh as great, about 0·2 μg.-atom C/million euglenas. Even allowing for the considerable uncertainties involved in both estimates, this quantitative difference seems to preclude the possibility that the 'labile reserve' is identical with Soluble I. Indeed, the 'labile reserve' seems to be about three times as large as Soluble I and Soluble II together, which strongly suggests that the 'labile reserve' includes insoluble as well as soluble components. Since the insoluble materials are so largely paramylon, this probably indicates that paramylon contributes to the 'labile reserve'.

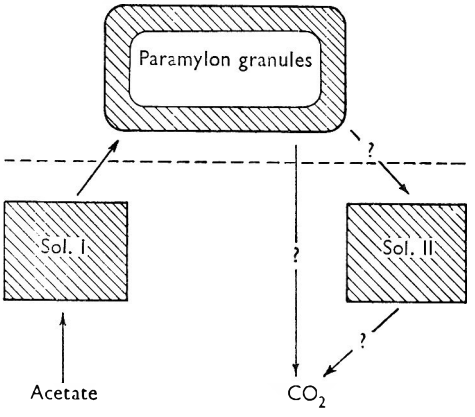

Fig. 8. Hypothetical explanation of the relationships among the products of acetate assimilation. Cross-hatched portions are believed to constitute the 'labile reserve'. Materials above the dotted line are considered to be ethanol-insoluble; materials below the line, ethanol-soluble.

To account for these relationships, it is suggested that the 'labile reserve' consists in part of the surface layers of some or all of the paramylon granules. It would be expected that newly synthesized paramylon would be deposited on the surfaces of the granules, and that these surface layers would be the first portion of the granules to be attacked by degradative enzymes. Thus the finding that newly assimilated carbon is the first to be oxidized by the endogenous pathway can be accounted for. It was this finding which led originally to the concept of a 'labile reserve'. The intermediates in the conversion of acetate to paramylon (presumably included in Soluble I) would also contribute to the 'labile reserve' (Fig. 8). The lag which is sometimes apparent in the dilution of endogenous CO_2 production by exogenous acetate (Fig. 2) suggests that the 'labile reserve' contains more than one component. The place of Soluble II in this scheme is not clear. Soluble II might

consist of intermediates in the oxidation of acetate via the endogenous pathway, or of by-products derived from paramylon by side reactions. The very late appearance of radioactivity in Soluble II seems to favour the latter possibility.

THEORETICAL APPENDIX

Kinetics of the 'labile reserve'. Assume the model system presented in Fig. 1, and further assume that the 'labile reserve' is a homogeneously mixed pool of fixed capacity, V. When non-radioactive acetate is supplied to fully labelled euglenas, the amount of ^{14}C in the 'labile reserve' will decrease according to the relationship

$$\frac{dX}{dt} = D - \frac{X}{V}(C+E), \tag{1}$$

where X = amount of ^{14}C in the 'labile reserve', and C, D, and E the rates of the corresponding reactions in Fig. 1. Letting $X/V = R$ = specific activity of carbon in the 'labile reserve',

$$\frac{dR}{dt} = \frac{D-(X/V)(C+E)}{V}. \tag{2}$$

Integrating equation (2) and evaluating the constant of integration for the values ($t = 0$, $R = 1{\cdot}0$), gives

$$-\ln[(C+E)R-D] = \frac{(C+E)}{V} - \ln(C+E-D). \tag{3}$$

But, if V is constant,

$$B+D = C+E, \quad \text{or} \quad C+E-D = B. \tag{4}$$

Substituting from (4) into (3),

$$-\ln[(C+E)R-D] = \frac{(C+E)t}{V} - \ln B,$$

$$\ln\left[\frac{(C+E)R-D}{B}\right] = -\frac{(C+E)t}{V}. \tag{5}$$

From Fig. 1 it may be seen that B is the rate of conversion of acetate carbon to reserves, which may be determined from the respiration by using the carbon:oxygen balance equations of Wilson & Danforth (1958; Danforth, 1961). C, the rate of CO_2-production from reserves, is equal to the endogenous oxygen consumption, since the endogenous respiratory quotient is 1·0 (Danforth & Wilson, 1961).

Since the 'labile reserve' is considered to be the direct precursor of the endogenous CO_2, the specific activity (R), of the 'labile reserve' (relative to that of control cells without acetate) equals

$$\frac{\text{Rate of } ^{14}CO_2 \text{ production by experimental euglenas}}{\text{Rate of } ^{14}CO_2 \text{ production by control euglenas}},$$

and is experimentally determined (Fig. 2).

Solving equation (5) for R gives

$$R = \frac{B\exp[-(C+E)t/V]+D}{C+E}, \tag{6}$$

or, substituting $B+D = C+E$ from equation (4),

$$R = \frac{B \exp[-(C+E)\, t/V]+D}{B+D}. \tag{6a}$$

As t increases, the exponential term in equations (6) and (6a) approaches zero, and R approaches a steady-state value

$$R \text{ (steady state)} = \frac{D}{B+D}. \tag{7}$$

Since B is known, D may be calculated from the steady-state value of R. E may then be estimated from equation (4), using values of B, C and D derived as described above.

Kinetics of the ethanol fractions. Assume the relationship indicated in Fig. 6 between Soluble I and the other assimilatory products, and further assume that Soluble I is a homogeneously mixed pool of fixed capacity W. If uniformly labelled acetate-^{14}C is supplied to initially unlabelled euglenas,

$$\frac{\mathrm{d}(M/W)}{\mathrm{d}t} = \frac{\alpha-\gamma(M/W)}{W}, \tag{8}$$

where M = amount of ^{14}C in Soluble I, and α and γ are the rates of the corresponding reactions in Fig. 6. Integrating, and evaluating the constant of integration for ($t = 0$, $M = 0$) gives

$$\frac{M}{W} = \frac{\alpha}{\gamma}\,[1-\exp(-\gamma t/W)]. \tag{9}$$

Neither γ or W are subject to experimental evaluation. But, as t becomes large, M approaches a constant maximum value M_m, and $\exp(-\gamma t/W)$ approaches zero, giving

$$\frac{M_m}{W} = \frac{\alpha}{\gamma}, \quad W = \frac{\gamma}{\alpha} M_m. \tag{10}$$

Substituting this expression for W in equation (9) gives

$$\frac{M}{M_m} = 1-\exp(-\alpha t/M_m), \tag{11}$$

in which all quantities can be experimentally evaluated.

Also, from the initial assumptions,

$$\frac{\mathrm{d}K}{\mathrm{d}t} = \frac{\gamma M}{W} = \frac{\alpha M}{M_m}, \tag{12}$$

where K is the amount of label in all fractions other than Soluble I. Integrating (12), and evaluating the constant of integration for ($t = 0$, $K = 0$), gives

$$K = \alpha t - M_m[1-\exp(-\alpha t/M_m)] = \alpha t - M. \tag{13}$$

The total assimilation after 2 hr. was used to evaluate α, assuming the assimilatory rate to be constant over this period. M_m was estimated graphically as illustrated in Fig. 5, while K, M, and t are derived directly from the experimental data.

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REFERENCES

BARKER, S. A. & BOURNE, E. J. (1955). In *Biochemistry and Physiology of Protozoa*. Ed. by S. H. Hutner and A. Lwoff, vol. II, p. 45. New York: Academic Press Inc.

BEVINGTON, J. C., BOURNE, E. J. & TURTON, C. N. (1953). Chemical degradation of ^{14}C-glucose and its application to ^{14}C-starch from *Polytomella coeca*. *Chem. & Ind.* p. 1390.

BUETOW, D. E. (1961). Variation of the respiration of protozoan cells with length of centrifuging. *Analyt. Biochem.* **2**, 242.

DANFORTH, W. (1953). Oxidative metabolism of Euglena. *Arch. Biochem.* **46**, 164.

DANFORTH, W. F. (1961). Oxidative assimilation of acetate by Euglena. Carbon balance and effects of ethanol. *J. Protozool.* **8**, 152.

DANFORTH, W. F. & WILSON, B. W. (1957). Adaptive changes in the acetate metabolism of Euglena. *J. Protozool.* **4**, 52.

DANFORTH, W. F. & WILSON, B. W. (1961). The endogenous metabolism of *Euglena gracilis*. *J. gen. Microbiol.* **24**, 95.

LYNCH, V. H. & CALVIN, M. (1952). Carbon dioxide fixation by microorganisms. *J. Bact.* **63**, 525.

LYNCH, V. H. & CALVIN, M. (1953). CO_2 fixation by Euglena. *Ann. N.Y. Acad. Sci.* **56**, 890.

MARZULLO, G. & DANFORTH, W. F. (1964). Composition of ethanol-insoluble assimilatory products of oxidative assimilation of acetate by *Euglena gracilis*. *J. gen. Microbiol.* **34**, 21.

WILSON, B. W. & DANFORTH, W. F. (1958). The extent of acetate and ethanol oxidation by *Euglena gracilis*. *J. gen. Microbiol.* **18**, 535.

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Composition of Ethanol-Insoluble Assimilatory Products of Oxidative Assimilation of Acetate by *Euglena gracilis*

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SUMMARY

After incubation for 6 hr. with uniformly labelled ^{14}C-acetate, about 90% of the acetate carbon assimilated by non-photosynthetic *Euglena gracilis* var. *bacillaris* was in the form of cellular materials insoluble in 60% (v/v) ethanol in water. Within this fraction about 80% of the assimilated carbon was in the form of glucose units of the polysaccharide paramylon, about 9% was in a fraction containing proteins, nucleic acids and probably other materials, and about 2% was in lipid. The ratio of assimilated carbon to total carbon in the paramylon was 3 to 5 times that in the protein + nucleic acid or lipid fractions.

INTRODUCTION

Previous studies (Wilson & Danforth, 1958; Danforth, 1961) showed that when a non-photosynthetic strain of *Euglena gracilis* var. *bacillaris* was incubated with acetate as a sole carbon and energy source, 42% of the acetate carbon was oxidized to CO_2, while 58% was assimilated. Carbon:oxygen balance studies indicated that the assimilatory product(s) have the empirical composition of carbohydrate. When euglenas which have been permitted to utilize ^{14}C-acetate are extracted with 60% (v/v) ethanol in water, radioactive assimilatory products are found in both the ethanol-soluble and ethanol-insoluble fractions (Marzullo & Danforth, 1964). The time course of labelling is quite different, however; the labelling of the soluble fraction increases rapidly at first, but soon steadies to a relatively constant value, while the rate of labelling of the insoluble is slow at first, increasing to a more rapid rate which is maintained while acetate assimilation continues. Kinetic analysis strongly suggests that some, but not all, of the soluble products serve as precursors for the insoluble material. The present work was directed toward identification of the ethanol-insoluble assimilation products.

METHODS

Organisms of a non-photosynthetic strain of *Euglena gracilis* var. *bacillaris* were grown, harvested, and washed by procedures described in preceding publications (Danforth & Wilson, 1957, 1961). Incubation mixtures were prepared in Erlenmeyer flasks by suspending the washed euglenas in media composed of 0·035 M-sodium acetate uniformly labelled with ^{14}C and 0·002 M-sodium phosphate buffer (pH 7·0). The flasks were stoppered with cotton plugs and incubated at 25–27° for 6 hr. The rate of oxygen consumption and the release of ^{14}C-CO_2 were measured

in Warburg flasks containing the same mixtures and incubated at the same temperature, as described previously (Marzullo & Danforth, 1964). A similar flask from which acetate was omitted was used to estimate the rate of endogenous respiration.

Figure 1 summarizes the following fractionation procedure. At the end of the incubation period, the euglenas were separated from the medium by centrifugation, washed three times with water, and resuspended in 60% (v/v) ethanol in water. This suspension was held at 65° for 30 min., then centrifuged. The pellet was washed twice by resuspension in 3 vol. of 60% (v/v) ethanol in water and recentrifugation. The supernatant fluid and washes were pooled and filtered by suction through a membrane filter; 0·5 ml. samples of this 'ethanol-soluble fraction' (ES) were dried on planchets and their radioactivity counted.

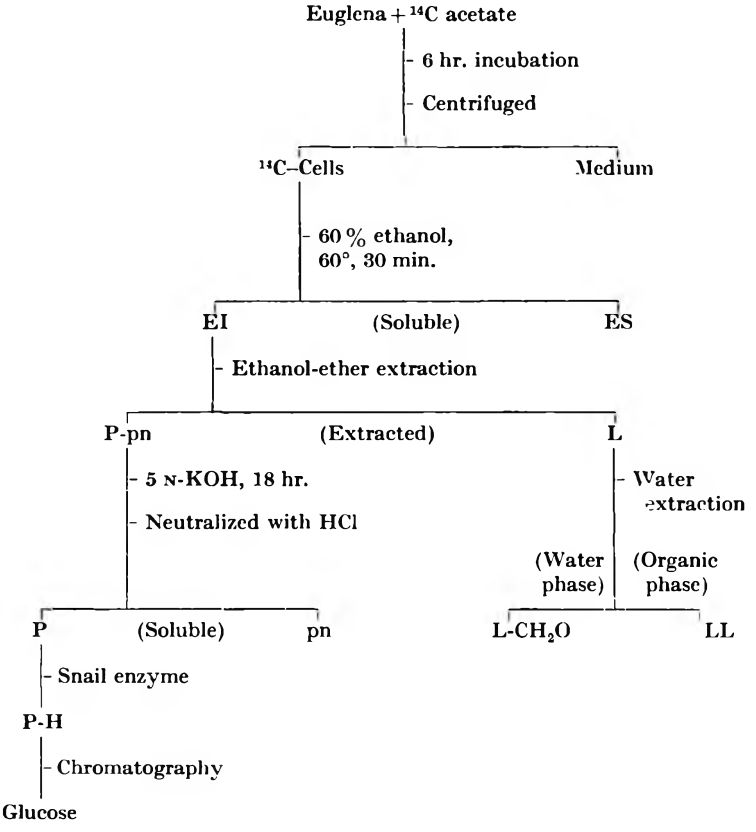

Fig. 1. Fractionation scheme for the separation of products of acetate assimilation.

The washed 'ethanol-insoluble fraction' (EI) was resuspended in 8 ml. 60% (v/v) ethanol in water, and samples counted for radioactivity. The remainder was dried to constant weight in a desiccator over KOH.

The dried EI pellet was ground to a powder; 4 ml. of a mixture of 3 vol. ethanol + 1 vol. ether was added to the powder and the suspension was stirred for 30 min. and centrifuged. The pellet was extracted three more times, and the ethanol + ether extracts combined to form a 'lipid fraction' (L). This L fraction, however,

was found to contain carbohydrate, and was therefore re-extracted by shaking with 1·5 vol. water in a separatory funnel. The aqueous phase was re-extracted with 0·5 vol. fresh ether. The two organic fractions were combined (labelled 'LL') and the aqueous fraction labelled 'L-CH_2O'. Total carbohydrate and total radioactivity were determined on samples of both fractions, and the dry weight of the LL fraction determined.

The residue after ethanol + ether extraction (labelled 'P-pn') was dried over KOH and weighed. Determinations of total carbohydrate, total nitrogen and radioactivity were made on samples of this fraction. Another sample of the P-pn fraction was placed in a 15 ml. conical centrifuge tube, and 2 ml. 5 N-KOH added. The mixture was incubated at 55° for 18 hr. A drop of 0·01 % (w/v) phenolphthalein indicator was added; the tube was placed in an ice bath and neutralized by dropwise addition of N-HCl with careful stirring. More HCl was added to bring to pH 2·0 as tested with 'pHydrion' (Micro Essential Laboratory, Brooklyn 10, New York, U.S.A.) pH paper. Acidification of the clear KOH hydrolysate resulted in the formation of a flocculant gel-like precipitate. The precipitate was centrifuged down, washed six times with 0·01 N-HCl, and labelled 'P' (for paramylon). The washes were combined with the first supernatant fluid and labelled 'pn' (protein, nucleic acid). Determinations of total carbohydrate and reducing sugar were performed on the P fraction, and total carbohydrate, total nitrogen, and radioactivity were determined for the pn fraction.

A sample of the P fraction was incubated at 37° for 18 hr in 0·05 M-sodium acetate buffer with an enzyme preparation extracted from snail intestines, known to contain β-glucosidase activity (L. Lerner & A. H. Roush, personal communication). At the end of this time, the gel-like precipitate had dissolved. The enzymic digest was labelled 'P-H' (paramylon hydrolysate), and total carbohydrate and reducing sugar were determined on it.

The P-H fraction was then concentrated at room temperature under vacuum, and samples of the concentrate were chromatographed on Whatman no. 1 filter paper, with ethyl acetate + acetic acid + water (9 + 2 + 2, by vol.) as a descending solvent. Authentic samples of glucose and ribose were used as markers. Reducing sugars were detected by spraying with a mixture of equal volumes of 0·1 M-silver nitrate and 5 N-ammonium hydroxide, and heated at 110° for 10 min.

Autoradiographs of chromatograms were made with Kodak Blue Brand X-ray film. Discs of Whatman no. 1 paper, which had been allowed to absorb amounts of P-H equivalent of 1, 2, 4, 6 and 10 % of the amount chromatographed, were included in the autoradiographic procedure.

Total carbohydrate was determined by the anthrone method (Mokrasch, 1954; Trevelyan & Harrison, 1952), reducing sugar by Nelson's (1944) method, and total nitrogen by Kjeldahl digestion followed by nesslerization (Johnson, 1941) with commercially prepared Nessler's reagent (Paragon C. and C. Co., Inc., New York 58, New York, U.S.A.).

Total carbon contents of the various fractions were estimated from dry weights on the assumption that carbon accounts for 40 % of the weight of carbohydrate (polyglucose), 77 % of the weight of lipid (stearo-oleopalmatin) and 50 % of the weight of a mixture of protein (55 % C) and nucleic acid (36 % C).

RESULTS

Results of individual fractionation steps

Table 1 summarizes the results of the extraction procedure for lipids. It may be seen that the original ethanol + ether extract (L) contained a considerable amount of carbohydrate. Re-extraction of the ethanol + ether fraction with water removed this carbohydrate (L-CH_2O). Since carbohydrate accounted for most of the weight of the L-CH_2O extract, this material was combined with the paramylon fraction (P), while the purified ethanol + ether fraction (LL) served as the basis for determination of the ^{14}C assimilated as lipid.

Table 1. *Characteristics of the lipid fraction at various stages of purification*

	Fraction			%
	L	L-CH_2O	LL	recovery
Weight (mg.)	16·0	(4·0)	12·0	—
Total counts/min.	20,400	14,200	6,050	99
Carbohydrate (mg., as glucose)	3·32	3·34	Nil	101

L = crude lipid fraction; L-CH_2O = water extract of L; LL = remainder after water extraction; the value in parentheses was estimated by difference.

Table 2. *Distribution of dry weight and carbohydrate during subfractionation of the paramylon + protein + nucleic acid fraction*

	Fraction		
	P-pn	P	pn
Weight (mg.)	113·4	73·4	(40·0)
Carbohydrate (mg. as glucose)	76·0	75·0	0·71

P-pn = paramylon + protein + nucleic acid fraction; P = paramylon fraction; pn = protein + nucleic acid fraction; value in parentheses estimated by difference.

Table 3. *Distribution of carbohydrate and nitrogen during subfractionation of the paramylon + protein + nucleic acid fraction*

	Fraction		
	P-pn	P	pn
Carbohydrate (mg. as glucose)	48·6	47	0·85
Nitrogen (mg.)	1·97	Nil	1·93

P-pn = paramylon + protein + nucleic acid fraction; P = paramylon fraction; pn = protein + nucleic acid fraction.

The rather unorthodox procedure used for separating paramylon (P) from proteins and nucleic acids (pn) was developed empirically, based on characteristics of paramylon described by Clarke & Stone (1960). The residue (P-pn) after ethanol + ether extraction was digested with strong base to hydrolyse proteins and nucleic acids. The paramylon dissolved during this step, but was precipitated by acidifying to pH 2·0. Tables 2 and 3 show analyses of the precipitates (P) and supernates (pn) after this step. All the nitrogen of the original material was found in the pn fraction.

Most of the carbohydrate was found in the P fraction; the small amount of carbohydrate in the pn fraction can easily be accounted for by the pentose portions of the nucleic acids. The carbohydrate content accounted for the total weight of the P fraction, indicating that this material was essentially pure carbohydrate. The nitrogen content of pn was somewhat low for protein or nucleic acid, indicating that other materials may also have been present in this fraction.

Table 4 shows the results of digestion of the P fraction with snail enzyme. The total carbohydrate content of the fraction was unchanged during digestion, while reducing sugar, as glucose, increased from a negligible value to equal the total carbohydrate, indicating complete digestion of the polysaccharide to monosaccharides. Paper chromatograms of the digest showed a single reducing spot which corresponded to that for an authentic glucose sample.

Table 4. *Results of hydrolysis of the paramylon fraction by snail enzyme*

	Carbohydrate (mg. as glucose)	Reducing sugar (mg. as glucose)	Reducing sugar / Carbohydrate
Paramylon (P)	8·00	0·004	0·0005
Hydrolysed paramylon (P-H)	7·80	7·90	1·01
	8·20	8·20	1·00

Table 5. *Gross* ^{14}C *balance after* 6 *hr. incubation of Euglena with* ^{14}C*-acetate*

	Counts/min.	μg-atoms ^{14}C
Total suspension	1,250,000	9,800
Medium	800,000	6,200
Acetate- C utilized		
Suspension *minus* medium	450,000	3,540
Calculated from respiration	—	3,820
CO_2		
Measured	198,000	1,550
^{14}C utilized *minus* ^{14}C assimilated*	189,000	1,480
Acetate-C appearing as CO_2 (% of acetate-C utilized)	38·4	

* The amount of ^{14}C assimilated is the sum of ES and EI (Table 6).

Gross carbon balance

Table 5 shows the over-all carbon balance for one experiment in which euglenas were incubated with uniformly labelled ^{14}C-acetate. It can be seen that the total acetate utilization determined by loss of radioactivity from the medium agrees well with that estimated from the oxygen consumption, and that two methods of estimating CO_2 production from acetate are also in good agreement. The % acetate-carbon converted to CO_2 is very similar to that found in other studies (Wilson & Danforth, 1958; Danforth, 1961).

Table 6 shows the distribution of ^{14}C among the various fractions in the same experiment. When expressed as % total carbon assimilated, the results of a second experiment were very similar. About 8% of the carbon was found in the ethanol-soluble material, and the remaining 92% in the ethanol-insoluble material. Most of the insoluble radioactivity (75–80% of all the carbon assimilated) was in the paramylon fraction, and much smaller amounts in the protein+nucleic acid and

lipid fractions (about 10% and 2%, respectively). In the last column in Table 6, and also in Fig. 2, the ^{14}C content of each fraction is expressed as % of total carbon in that fraction.

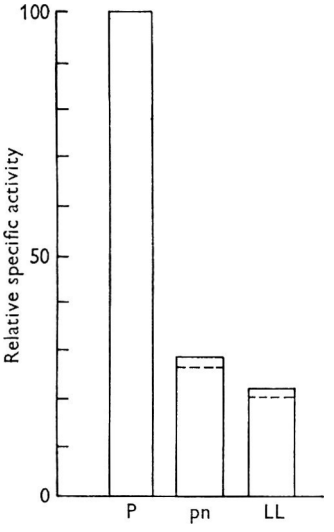

Fig. 2. Relative rates of carbon 'turnover' during the assimilation of acetate carbon by Euglena. 'Relative specific activity' of each fraction is the specific activity (^{14}C/total C) of the fraction expressed as % specific activity of the paramylon fraction in the same experiment. P, Paramylon fraction; pn, protein-nucleic acid fraction; LL, lipid fraction. Bars represent the results of one experiment, and dotted lines the results of a second.

Table 6. *Distribution of ^{14}C among various Euglena fractions after incubation for 6 hr. with ^{14}C-acetate*

Fraction	Counts/min.	μg.-atoms ^{14}C	% of assimilated ^{14}C*	^{14}C Total C (%)
Ethanol-soluble (ES)	20,900	104	8·0	—
Ethanol-insoluble (EI)	240,000	1,880	92	—
Lipid (LL)	6,050	47	2·3	6·1
Paramylon + protein – nucleic acid (P-pn)	234,000	1,830	90	—
Protein + nucleic acid (pn)	23,000	180	8·8	5·0
Paramylon (P)	210,000	1,650	81	23·2

* (^{14}C in fraction)/(total assimilated ^{14}C) × 100.

Nature of the labelled material in the paramylon fraction

After enzymic digestion, the labelled P-H fraction was chromatographed, and the glucose spot, detected by silver nitrate spray, cut out. An amount of labelled P-H fraction identical with that chromatographed was absorbed on a bit of filter paper the same size and shape as the glucose spot. This 'dummy spot' was used to estimate the total radioactivity added to the chromatogram, corrected for self-absorption of the filter paper. Table 7 shows the results of two experiments of this sort. It is apparent that essentially all (94–111%) of the radioactivity originally applied to the chromatogram is found in the glucose spot. The degree of error in

the counting method, however, is too great to preclude the possibility that some other labelled compound might be present in significant amounts. Therefore, autoradiographs of similar chromatograms were prepared. A 'dummy spot', containing ^{14}C-labelled P-H in an amount equivalent to 2% of that applied to the chromatogram, was included in the autoradiographic procedure. The resulting radiogram showed a spot corresponding to glucose, and a much weaker spot corresponding to the 'dummy', but no other spots. Therefore, it may be concluded that no component other than glucose accounted for as much as 2% of the radiocarbon in the paramylon fraction.

Table 7. *Radioactivity of the glucose spot in chromatograms of the hydrolysed paramylon (P-H) fraction*

The glucose spot was cut from the chromatogram; both sides of the spot were counted. A 'dummy spot' contained an amount of P-H equal to the amount chromatographed, absorbed in a piece of filter paper the same size and shape as the glucose spot. Two 'dummy spots' were used in Expt. I.

	Glucose spot			'Dummy spot'			
	Side 1	Side 2	Mean	Side 1	Side 2	Mean	
	Counts/min.						Recovery (%)
Expt. I	70·2	58·0	64·1	69·0 68·0	69·0 66·0	68·0	94
Expt. II	97·3	88·0	92·7	82·5	84·2	83·4	111

DISCUSSION

Wilson & Danforth (1958) concluded, on the basis of carbon balance studies, that nearly all the acetate-carbon assimilated by the euglenas used, under conditions similar to those of the present experiments, was converted to carbohydrate. The present results provide more direct support for this conclusion. About 75–80% of the acetate-carbon assimilated was found in the form of glucose units of the reserve polysaccharide paramylon. Since some of the 8% of the ^{14}C found in the ethanol-soluble fraction is probably in the form of phosphorylated sugars, it seems likely that 80–90% of the acetate-carbon assimilated is converted to carbohydrate.

Under conditions of uniform growth, with acetate as sole carbon source, one would expect all cellular constituents to be synthesized at rates proportional to their abundance in the organisms. The results shown in Table 6 and Fig. 2 show that this is not the case in the present experiments. This almost certainly indicates that preferential net synthesis of carbohydrate is occurring, rather than synthesis of entire new euglenas. The alternative hypothesis, that the high rate of carbohydrate formation is compensated by a correspondingly high rate of carbohydrate degradation, can be ruled out; the required rate of degradation of intracellular carbohydrate would produce CO_2 at a rate practically equal to the total rate of CO_2 production in the presence of acetate. Experimentally, no more than one-third of the CO_2 formed under these conditions is derived from intracellular material (Danforth, 1961; Danforth & Wilson, 1961). Since the experimental media were deficient in nitrogen and vitamins, and since other experimental conditions (shaking, crowding, absorption of CO_2) were probably unfavourable for growth, the preferential synthesis

of carbohydrate may have been simply characteristic of non-growing organisms. Nitrogen deficiency (Witsch, 1948; Myers & Cramer, 1948) or previous starvation (Cramer & Myers, 1949) of Chlorella results in diversion of substrate assimilation toward carbohydrate synthesis; similar phenomena are known in a variety of other organisms.

On the other hand, respirometric studies (Wilson, 1963) on the closely related flagellate Astasia indicate that the % of acetate-carbon assimilated (42%) under growth conditions is identical with that found in Euglena (Wilson & Danforth, 1958) under conditions resembling those of the present experiments. While the data under growth conditions do not permit construction of a complete carbon balance, it seems unlikely that the % assimilation should remain unchanged if the assimilation products differed greatly under the two sets of conditions. A possible alternative explanation for the preferential synthesis of carbohydrate was suggested by Danforth & Wilson (1961). On the basis of quite different considerations, they suggested that assimilation of rapidly utilized substrates such as acetate might at all times be directed primarily toward paramylon synthesis, and that energy and carbon for growth are derived secondarily, from breakdown of paramylon. At present, however, there is no direct evidence bearing on this question. Although assimilation of acetate-carbon appears to be channelled almost exclusively into carbohydrate synthesis, it cannot be assumed that this is true for all substrates, since the carbon balance for ethanol metabolism is not consistent with synthesis of carbohydrate as the sole assimilation product (Wilson & Danforth, 1958).

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REFERENCES

CLARKE, A. E. & STONE, B. A. (1960). Structure of the paramylon from *Euglena gracilis*. *Biochim. biophys. Acta*, **44**, 161.

CRAMER, M. & MYERS, J. (1949). Effects of starvation on the metabolism of Chlorella. *Plant Physiol.* **24**, 255.

DANFORTH, W. F. (1961). Oxidative assimilation of acetate by Euglena. Carbon balance and effects of ethanol. *J. Protozool.* **8**, 52.

DANFORTH, W. F. & WILSON, B. W. (1957). Adaptive changes in the acetate metabolism of Euglena. *J. Protozool.* **4**, 52.

DANFORTH, W. F. & WILSON, B. W. (1961). The endogenous metabolism of *Euglena gracilis*. *J. gen. Microbiol.* **24**, 95.

JOHNSON, M. J. (1941). Isolation and properties of a pure yeast polypeptidase. *J. biol. Chem.* **137**, 575.

MARZULLO, G. & DANFORTH, W. F. (1964). Kinetic studies of the oxidative assimilaton of acetate by a non-photosynthetic strain of *Euglena gracilis*. *J. gen. Microbiol.* **34**, 9.

MOKRASCH, L. C. (1954). Analysis of the hexose phosphate and sugar mixture with the anthrone reagent. *J. biol. Chem.* **208**, 55.

MYERS, J. & CRAMER, M. (1948). Metabolic conditions in Chlorella. *J. gen. Physiol.* **32**, 103.

NELSON, N. (1944). A photometric adaptation of the Somogyi method for the determination of glucose. *J. biol. Chem.* **153**, 375.

TREVELYAN, W. E. & HARRISON, J. S. (1952). Fractionation and micro-determination of all carbohydrate. *Biochem. J.* **50**, 298.

WILSON, B. W. (1963). *Controlled growth and the regulation of the metabolism of the flagellates Astasia and Euglena.* Thesis, University of California, Los Angeles.

WILSON, B. W. & DANFORTH, W. F. (1958). The extent of acetate and ethanol oxidation by *Euglena gracilis*. *J. gen. Microbiol.* **18**, 535.

WITSCH, H. VON (1948). Physiologischer Zustand und Wachstumsintensität bei Chlorella. *Arch. Mikrobiol.* **14**, 128.

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Effects of Amino Acids on the Utilization of Tryptophan and Indole for Growth by a Mutant of *Neurospora crassa*

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SUMMARY

Fourteen of twenty-one naturally occurring amino acids inhibited competitively the utilization of tryptophan for growth by an indole- or tryptophan-requiring strain of *Neurospora crassa*. At less than inhibitory ratios of amino acid to tryptophan, certain amino acids enhanced the growth of this mutant. Growth on indole was neither enhanced nor inhibited by amino acids other than tryptophan. The uptake of tryptophan from the medium was inhibited by phenylalanine. The effects of amino acid on the growth of this strain with tryptophan can be explained by an inhibition of tryptophan uptake from the medium.

INTRODUCTION

Various naturally occurring amino acids can inhibit the growth of certain amino acid-requiring mutants of *Neurospora crassa*. A mutant which grew on isoleucine + valine was inhibited by phenylalanine, norleucine or norvaline (Bonner, Tatum & Beadle, 1943); the growth of a lysine-requiring mutant was competitively inhibited by arginine (Doermann, 1944), and arginine-deficient mutants were competitively inhibited by lysine (Srb, 1953). Lein, Mitchell & Houlahan (1948) noted that three types of Neurospora mutants—histidine-, tryptophan- and methionine-requiring—did not grow on a complete medium. The inhibition of histidine-requiring mutants by the complete medium was later shown to be due to certain mixtures of amino acids (Haas, Mitchell, Ames & Mitchell, 1952); leucine, isoleucine, valine, methionine, glycine, tyrosine, phenylalanine, tryptophan or histamine were inhibitory in combination with lysine or arginine. Mathieson & Catcheside (1955) showed that the two combinations, arginine + methionine and arginine + tryptophan, were competitive with histidine; they also concluded that amino acids inhibit the growth of histidine-requiring mutants by preventing histidine uptake into the mycelium. Norvaline, tyrosine or α-aminobutyric acid inhibited the growth of a mutant which grew maximally on methionine + threonine (Teas, Horowitz & Fling, 1948).

Haddox (1952) reported that the growth of a tyrosine-requiring mutant was inhibited by leucine and that a mutant which required phenylalanine or tyrosine was inhibited by leucine or tryptophan. The growth of another tyrosine-requiring mutant (T-145) on tyrosine was inhibited by 18 of 23 amino acids tested (DeBusk &

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Wagner, 1953). A mutant which grew on any one of the intermediates between indole and nicotinic acid was inhibited by 12 of 21 amino acids that were tested in combination with tryptophan, kynurenine or 3-hydroxykynurenine (Shanmugasundaram & Sarma, 1954; Panicker, Shanmugasundaram & Acharya, 1962). Soboren & Nyc (1961) reported that 4 of 17 naturally occurring amino acids inhibited growth of the tryptophan-requiring strain C-83. Brockman, DeBusk & Wagner (1959) showed that the utilization of phenylalanine for growth by a phenylalanine- or phenylpyruvate-requiring mutant (E-5212) was competitively inhibited by any one of 14 naturally occurring amino acids. The utilization of phenylpyruvate, the α-keto analogue of phenylalanine, for growth by this strain was not inhibited by amino acids but was competitively inhibited by other α-keto acids. Therefore α-amino acids may be antagonists to amino acid utilization for growth in this mutant, and α-keto acids may inhibit keto acid utilization. The importance of the group at the α-position in determining inhibition specificity was further demonstrated by the observation that α-hydroxyisovalerate (the α-hydroxy analogue of valine) did not completely inhibit either phenylalanine or phenylpyruvate utilization for growth (Brockman, 1960).

The effects of amino acids on the growth of two aromatic amino acid-requiring mutants (T-145 and E-5212) of Neurospora were studied by DeBusk & Wagner (1953), Brockman *et al.* (1959) and Brockman (1960). The present report extends the observations to the indole- or tryptophan-requiring strain FS-108. This was done with the following objectives: (*a*) to determine whether the same family of inhibitory amino acids would be found with strain FS-108 as was found with strains E-5212 and T-145; (*b*) to see whether the utilization of indole for growth by strain FS-108 would be unaffected by amino acids as keto acid utilization was in strains E-5212 and T-145; (*c*) to investigate the phenomenon of sparing of the tryptophan requirement by low concentrations of certain amino acids that had been observed in an early experiment with strain FS-108; (*d*) to determine the relation, if any, between the phenomena of amino acid sparing and inhibition. A preliminary report has been given (Brockman & DeBusk, 1960).

METHODS

The mutant strain FS-108 of *Neurospora crassa* was recovered by the author from a filtration-concentration experiment (Woodward, De Zeeuw & Srb, 1954) following ultraviolet irradiation of the St Lawrence wild-type strain ST-74A. Its growth requirement is satisfied only by indole or tryptophan, and the gene governing this requirement is located at the *tryp*-1 locus of linkage group IIIR. Vegetative cultures were maintained on slopes of complete medium + tryptophan. Since the growth of strain FS-108 is inhibited by the amino acids in complete media, a 'weak' complete medium consisting of 2% glycerol, 1·5% agar, 0·05% liver fraction 'L' (Nutritional Biochemicals Corp., Cleveland 28, Ohio), 0·05% yeast extract and 0·05% malt extract (Difco Laboratories, Detroit 1, Michigan) in Fries minimal medium was used.

Growth assays were done in 125 ml. Erlenmeyer flasks containing 25 ml. liquid Fries minimal medium (Beadle & Tatum, 1945). The flasks were inoculated with one to two drops of just visibly turbid conidial suspension and incubated for 3 days

at about 27° unless noted otherwise. The mycelial growth was harvested from each flask with a glass hook and excess medium removed by pressing the mycelial pad on a paper towel. These pads were dried overnight at 60° before weighing. Each weight value quoted is an average from duplicate flasks recorded to the nearest milligram; pads weighing less than 5 mg. were recorded as zero. Tryptophan was assayed colorimetrically by the method of Nason, Kaplan & Colowick (1951).

RESULTS

Many metabolites, including the known intermediates of aromatic amino acid biosynthesis, were tested for growth-promoting activity for the *Neurospora crassa* mutant strain FS-108; and only indole or tryptophan was active. Characteristic growth response curves (Fig. 1) showed that, although the mutant responded better to indole than to tryptophan at low concentrations, the maximum growth achieved in 3 days was nearly identical. The inhibition by indole at concentrations greater than 4 μmole/25 ml. is not unique for strain FS-108, since indole inhibition has also been observed in wild-type strains (Cushing, Schwartz & Bennet, 1949), adenine-requiring mutants (Lein *et al.* 1948) and tryptophan- or indole-requiring mutants (Newmeyer & Tatum, 1953) of *N. crassa.*

Table 1. *Competitive inhibition of tryptophan utilization for growth in the Neurospora mutant strain* FS-108

At each μmole value of L-tryptophan, the concentration of inhibitory amino acid was varied to give the desired ratio of inhibitory amino acid to tryptophan. The dry weight values were determined by the methods described in Fig. 1.

		μmole L-tryptophan/flask					
Inhibitory amino acid	Ratio of inhibitory amino acid : L-tryptophan	1	2	4	8	16	32
		Growth (mg. dry wt.)					
None	—	14	25	43	55	51	42
L-Phenylalanine	10	42	52	70	68	65	61
	20	0	0	0	0	0	0
L-Leucine	20	39	45	51	49	51	51
	30	26	38	33	27	27	18
	40	0	0	0	0	0	0
L-Valine	130	16	32	30	22	12	—
	140	11	10	25	14	5	—
	150	0	0	0	0	0	—

Fourteen of 21 naturally occurring amino acids inhibited the growth of strain FS-108 on L-tryptophan. The inhibition by each of these 14 amino acids was competitive in nature; the inhibition was dependent on the ratio of inhibitory amino acid to tryptophan, rather than on the absolute concentration of inhibitory amino acid. The results with three representative inhibitory amino acids are given in Table 1. The ratio of inhibitory amino acid to tryptophan which resulted in a complete inhibition of growth for 3 days has been defined as the inhibition index for that particular amino acid. The inhibition indices of all the amino acids tested are summarized in Table 2. Growth of the wild-type strain ST-74A was not inhibited

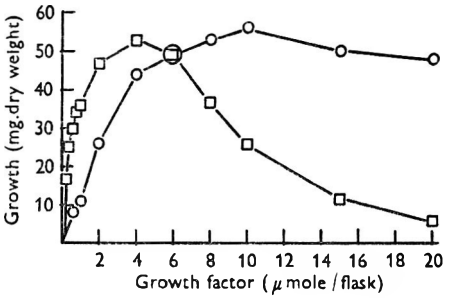

Fig. 1

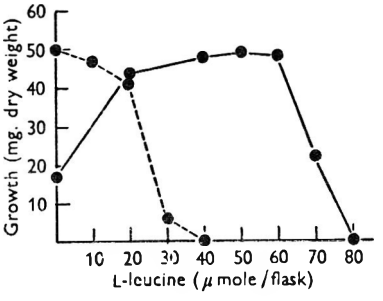

Fig. 2

Fig. 1. Growth response of the tryptophan- or indole-requiring mutant of *Neurospora crassa* strain FS-108. The dry wt. of the mycelium was determined from duplicate 125 ml. Erlenmeyer flasks containing 25 ml. Fries minimal medium following 3 days static incubation at about 27°. □—□ indole; ○—○ L-tryptophan.

Fig. 2. Additive nature of two inhibitory amino acids on the growth of Neurospora strain FS-108. ●—● 2 μmole L-tryptophan/flask; ●---● 2 μmole L-tryptophan + 20 μmole L-phenylalanine/flask, a ratio of phenylalanine:tryptophan which enhances the growth over that given by tryptophan alone.

Table 2. *Amino acid inhibition indices for the Neurospora mutant strain* FS-108

The inhibition index is defined as the ratio of inhibitory amino acid to L-tryptophan which results in a complete inhibition of growth for 3 days. Those amino acids which were not completely inhibitory of growth at a ratio of 500:1 were classified as non-inhibitory.

Inhibition index	Amino acid	
10–20	L-Phenylalanine	.
30–40	L-Leucine	.
50–60	L-Methionine	DL-Norvaline
60–70	L-Tyrosine	DL-α-Aminobutyric acid
80–90	DL-Norleucine	.
120–130	L-Alanine	.
140–150	L-Valine	.
300–325	Glycine	DL-Threonine
325–350	DL-Serine	DL-Isoleucine
350–375	DL-Asparagine	.
Non-inhibitory	L-Lysine	L-Glutamic acid
	L-Arginine	L-Aspartic acid
	L-Proline	L-Glutamine
	L-Histidine	.

by concentrations of the individual amino acids equal to those tested for strain FS-108.

From Table 1 it is evident that less than inhibitory concentrations of certain inhibitory amino acids, such as phenylalanine, enhanced the growth of strain FS-108 over that on tryptophan alone. To test further this stimulatory action of the amino acids on the tryptophan requirement of strain FS-108, two concentrations of each of the amino acids were superimposed on 1 μmole of tryptophan (Table 3); the amino acids are tabulated in the order of increasing inhibition index. By referring to the column in which 10 μmole of each amino acid was tested, it is evident that,

in general, as the inhibition index increased the degree of stimulation decreased. Amino acids with inhibition indices greater than 300 enhanced growth very little, and little or no stimulation was observed with 10 μmole of the non-inhibitory amino acids. Some of the amino acids which were not stimulatory at the 10 μmole concentration did spare the tryptophan requirement when tested at one-half the concentration of the inhibition index. None of the amino acids supported growth of strain FS-108 in the absence of tryptophan.

Table 3. *Stimulation of the growth of Neurospora strain FS-108 on tryptophan by certain amino acids*

One μmole L-tryptophan, which induced 10 mg. dry wt. growth in 3 days, was added to all growth flasks. The number of μmole of inhibitory amino acid equivalent to one-half the inhibition index was calculated from Table 2, and in the case of the non-inhibitory amino acids 250 μmole was tested.

		μmole of amino acid tested	
		10	equivalent to ½ the inhibition index
Amino acid tested	Inhibition index	Growth (mg. dry wt.)	
None	—	10	10
L-Phenylalanine	10–20	41	15
L-Leucine	30–40	32	39
L-Methionine	50–60	33	44
DL-Norvaline	50–60	27	36
L-Tyrosine	60–70	32	45
DL-α-Aminobutyric acid	60–70	25	36
DL-Norleucine	80–90	17	30
L-Alanine	120–130	23	42
L-Valine	140–150	24	37
Glycine	300–325	13	19
DL-Threonine	300–325	16	23
DL-Serine	325–350	17	26
DL-Isoleucine	325–350	17	19
DL-Asparagine	350–375	15	44
L-Lysine	Non-inhibitory	13	15
L-Arginine		15	41
L-Proline		11	20
L-Glutamic acid		10	42
L-Aspartic acid		10	44
L-Histidine		13	15
L-Glutamine		13	48

Bergmann, Sicher & Volcani (1952) reported that *p*-aminobenzoic acid (PABA) inhibition of a tryptophan-requiring *Escherichia coli* strain was competitively annulled by tryptophan, and that phenylalanine and tyrosine, in addition to sparing the tryptophan requirement, annulled the PABA inhibition of tryptophan utilization. In relation to the sparing of the tryptophan requirement in strain FS-108, it was of interest to determine whether inhibition of the tryptophan utilization by a specific amino acid would be annulled by stimulatory concentrations of a second amino acid. This possibility was tested, as shown in Fig. 2. If phenylalanine had annulled the leucine inhibition of tryptophan utilization, more leucine would have been required for complete inhibition of growth in the presence of phenylalanine + tryptophan than in the presence of tryptophan alone. On the contrary, the inhibition

index was less when the initial growth was enhanced by a sparing concentration of phenylalanine, and, furthermore, the results suggest that phenylalanine and leucine are strictly additive in their inhibitory action on tryptophan utilization for growth of strain FS-108.

Since certain amino acids had a marked influence on tryptophan utilization for growth by strain FS-108, all 21 amino acids were retested for their ability to inhibit or spare indole utilization. Either 250 or 500 μmole of each of the 21 amino acids in combination with 1 μmole indole did not generally result in a significantly different growth value from that given on indole alone. Five hundred μmole of glycine, DL-serine, L-histidine, DL-norvaline or DL-α-aminobutyric acid induced a 50 %

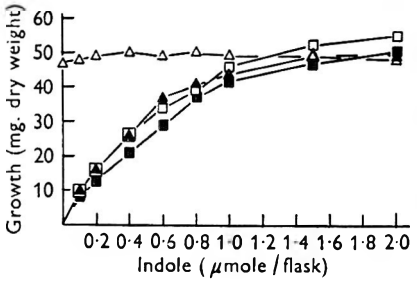

Fig. 3

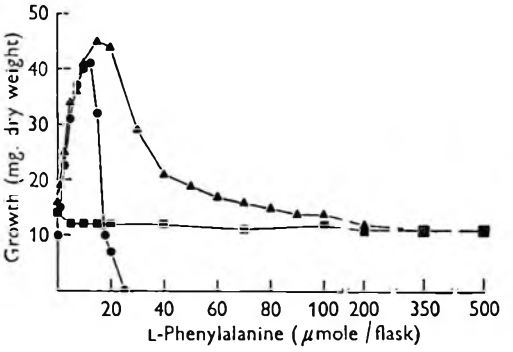

Fig. 4

Fig. 3. Indole annulment of phenylalanine inhibition of growth in the Neurospora strain FS-108. □—□ indole only; ■—■ indole + 500 μmole L-phenylalanine/flask; △—△ indole + 5 μmole L-tryptophan/flask; ▲—▲ indole + 5 μmole L-tryptophan and 500 μmole L-phenylalanine/flask.

Fig. 4. Effect of L-phenylalanine on the growth of Neurospora strain FS-108 under three different nutritional conditions. ■—■ 0·2 μmole indole/flask; ●—● 1 μmole L-tryptophan/flask; ▲—▲ 1 μmole L-tryptophan + 0·2 μmole indole/flask.

growth inhibition, but further tests showed that the antagonisms were not competitive. Various keto acids, vitamins, organic acids, purines and pyrimidines were also tested in combination with indole, but to date no naturally occurring metabolite has been found which will competitively inhibit utilization of indole for growth.

It was further found that indole annulled non-competitively all the amino acid inhibitions of tryptophan utilization. Indole relief of phenylalanine inhibition of tryptophan utilization was studied in detail (Fig. 3). When various concentrations of indole were added to flasks containing 5 μmole tryptophan + 500 μmole phenylalanine, the resulting growth curve was always nearly identical with the control curves of growth on indole alone or on indole + phenylalanine. These results suggest that only indole, and not tryptophan, is utilized for growth by strain FS-108 when indole annuls the phenylalanine inhibition of tryptophan utilization. Other compounds involved in tryptophan metabolism, including 3-hydroxyanthranilic acid, kynurenine and nicotinic acid, were tested for annulment of amino acid inhibition of tryptophan utilization, but only indole was effective.

It is clear from the data so far presented that the growth response of strain FS-108 in the presence of an amino acid other than tryptophan was markedly dependent

on the growth requirement that was supplied: either tryptophan, indole, or indole + tryptophan. To show better the growth responses of strain FS-108 to an inhibitory amino acid, various concentrations of phenylalanine were superimposed on the three possible growth supplements of strain FS-108 (Fig. 4). Growth on indole was neither enhanced nor depressed at any concentration of phenylalanine, except for an initial small decrease. In contrast, growth on tryptophan was both enhanced and inhibited by phenylalanine over a 30 μmole concentration range. In the presence of tryptophan + indole, the growth requirement was again markedly spared, and growth was slowly decreased to 12 mg. dry wt. at very high concentrations of phenylalanine. From Fig. 4 it cannot be determined whether this 12 mg. dry wt. value was equivalent to the growth given on indole or tryptophan, but other experiments (as in Fig. 3) showed that growth was always decreased to a value which would have been obtained on indole alone.

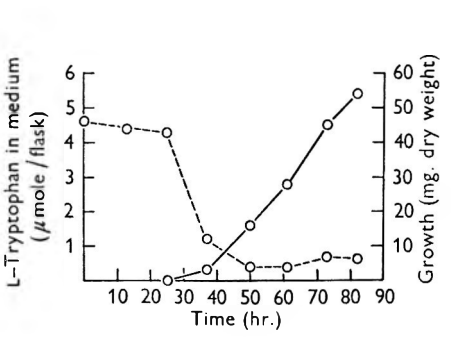

Fig. 5

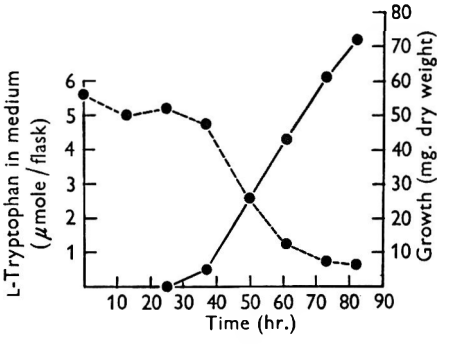

Fig. 6

Fig. 5. Relation between tryptophan uptake and growth in Neurospora strain FS-108 in the absence of inhibitory amino acid. Flasks containing 25 ml. of Fries minimal medium + 5 μmole L-tryptophan were inoculated with $7{\cdot}45 \times 10^4$ conidia/flask. At each indicated time, the tryptophan remaining in the culture fluid was determined colorimetrically by the method of Nason *et al.* (1951) following centrifugation of the conidia and/or mycelium; dry wt. of mycelium was also recorded. ○---○ L-tryptophan; ○—○ growth.

Fig. 6. Relation between tryptophan uptake and growth of Neurospora strain FS-108 in the presence of a sparing concentration of the inhibitory amino acid, L-phenylalanine. Experimental conditions were the same as in Fig. 5 except that 50 μmole of L-phenylalanine were added to each flask. ●---● L-tryptophan; ●—● growth.

The disappearance of tryptophan from the medium during a growth period of 3·5 days was measured colorimetrically by the method of Nason *et al.* (1951). Flasks containing tryptophan, tryptophan + a sparing concentration of phenylalanine, and tryptophan + an inhibitory concentration of phenylalanine, were inoculated with $7{\cdot}45 \times 10^4$ conidia/flask. About every 12 hr the contents of duplicate flasks of each type were centrifuged and a sample of the culture fluid assayed for tryptophan; growth (dry wt.) was measured simultaneously. Tryptophan uptake from the medium and the resulting growth in the absence of inhibitory amino acid during incubation for 3·5 days are given in Fig. 5. Fig. 6 shows the results when a sparing concentration of phenylalanine was added. Tryptophan was taken up at a very rapid rate between 24 and 36 hr. in the absence of inhibitor (Fig. 5), but a sparing concentration of phenylalanine decreased this rate of uptake significantly (Fig. 6). No growth or tryptophan uptake from the medium occurred

during the 3·5 days when an inhibitory concentration of phenylalanine was used (250 μmole phenylalanine + 5 μmole tryptophan). A comparison of Figs. 5 and 6 leads to the surprising conclusion that the growth of strain FS-108 was enhanced by the sparing concentration of phenylalanine even though the uptake of its requirement, tryptophan, was inhibited.

These results led to the formulation of the following hypothesis. In the absence of inhibitory amino acids strain FS-108 transports tryptophan into the organism at a rate greater than that necessary for optimum growth. This accumulation of tryptophan might result in an intracellular imbalance of amino acids (Soboren & Nyc, 1961) unfavourable for growth, or the accumulated tryptophan might be degraded before it could be utilized for growth (Haskins & Mitchell, 1949). Inhibitory amino acids are antagonists to the transport of tryptophan into the cell, and a certain ratio of inhibitory amino acid to tryptophan produces an optimum rate of tryptophan uptake; this ratio is the one which enhances growth most. As the ratio of inhibitory amino acid to tryptophan is further increased, the decreased rate of tryptophan uptake becomes limiting for growth, and complete growth inhibition eventually occurs. According to this hypothesis, the enhancement of growth by sparing concentrations of an inhibitory amino acid is due solely to its regulatory effect on the rate of tryptophan uptake and is not the result of other, unspecified, intracellular functions.

Table 4. *Fractional addition of tryptophan to Neurospora strain* FS-108

Growth in mg. dry wt. was measured 72 hr after the flasks were inoculated. The types of L-tryptophan addition were (*a*) 1 μmole or 5 μmole added at the time of inoculation; (*b*) 0·2 μmole added at the time of inoculation and 12, 24, 36 and 48 hr later for a total of 1 μmole, or 1 μmole added at inoculation time and at the same subsequent times for a total of 5 μmole.

	Type of tryptophan addition	
	(*a*)	(*b*)
μmole L-tryptophan	Growth (mg. dry wt.)	
1	11	23
5	40	57

To test this explanation of sparing, it was necessary to duplicate the action of sparing concentrations of an amino acid, i.e. to decrease the rate of tryptophan uptake in the absence of an inhibitory amino acid. This can be attempted most easily by adding small concentrations of tryptophan to an inoculated growth flask periodically throughout the 3-day incubation period. Table 4 shows the results of such an experiment. It is clear that addition of a total amount of 1 or 5 μmole tryptophan at zero time gave less growth than when those total amounts were added fractionally during the incubation period. Growth was not enhanced to the extent previously shown by sparing concentrations of certain amino acids, but growth could probably be further increased by adding the tryptophan more frequently in even smaller fractions.

DISCUSSION

The ability of certain naturally occurring amino acids to inhibit competitively utilization of the amino acid required for growth and the relative effectiveness for inhibition are similar in three aromatic amino acid-requiring mutants of *Neurospora crassa*: tyrosine-deficient strain T-145 (DeBusk & Wagner, 1953), phenylalanine-deficient strain E-5212 (Brockman *et al.* 1959), and tryptophan-deficient strain FS-108 (this report). In most previous publications, only a limited number of amino acids were reported as inhibitory for a given amino acid-requiring strain (see Introduction). With few exceptions, however, all of the common amino acids were not tested, or they were not tested at sufficiently high ratios of amino acid to required amino acid. Consequently, distinct relations between groups of inhibitory amino acids and classes of amino acid-deficient mutants are not readily apparent from a consideration of the previous literature. It does seem possible now to venture some predictions. Arginine and lysine are non-inhibitory in all aromatic amino acid-requiring mutants of Neurospora studied by the author and others, while arginine-deficient mutants are inhibited by lysine and lysine-requiring mutants by arginine. It might be predicted, then, that glutamic acid-requiring mutants would not be inhibited by any of the amino acids which inhibit aromatic amino acid-requiring mutants; they might, however, be inhibited by some of the amino acids which do not inhibit aromatic amino acid-requiring strains. On the other hand, leucine-requiring mutants might be characterized by the same classes of inhibitory and non-inhibitory amino acids as have been found for aromatic amino acid-requiring mutants. These predictions are based on the hypothesis that the amino acids which inhibit growth of aromatic amino acid-requiring mutants exert their antagonistic action at a common site. The additive effect of phenylalanine and leucine in the inhibition of tryptophan utilization for growth of strain FS-108 supports this hypothesis. It would be of interest to screen for amino acid inhibition of representative amino acid-requiring mutants of Neurospora so that a complete picture could be formulated. Mutants which will grow on more than one amino acid would be especially useful. Neurospora mutants deficient in aminating ability (*am* mutants) do grow on any one of a number of amino acids. Fincham (1950) has shown that four amino acids which are non-inhibitory or weak inhibitors for strain FS-108—glycine, histidine, serine, and threonine—inhibited *am* mutants when they were grown on glutamate but not when they were grown on alanine.

All the results indicate that the inhibitory amino acids prevent tryptophan utilization for growth by inhibiting the entry of tryptophan into the organism rather than at some intracellular site of tryptophan utilization. This is shown directly by the present experiments on tryptophan uptake and is supported by the fact that the amino acids are not inhibitory to the wild-type strain or to strain FS-108 supplemented with indole. The utilization of endogenous tryptophan (that synthesized by wild-type from simple precursors or by strain FS-108 from indole) for growth is not inhibited, but the utilization of exogenous tryptophan for growth by strain FS-108 is inhibited competitively. Mathieson & Catcheside (1955) concluded that the uptake of histidine by histidine-requiring strains is inhibited by certain amino acids. Similarly, casein hydrolysate inhibited tryptophan uptake by the Neurospora mutant strain 39401 (Panicker *et al.* 1962). The data are consistent with the supposition

that there is one site with specificity for the aromatic amino acids and those amino acids which inhibit aromatic amino acid utilization.

The amino acids which are non-inhibitory to aromatic amino acid-requiring mutants can enter the organism at another site or sites. Similarly, there can be other sites specific for indole and for certain α-keto acids.

The stimulation of growth of amino acid-requiring strains of Neurospora by various amino acids has been reported by other authors (Shanmugasundaram & Sarma, 1954; Mathieson & Catcheside, 1955; Newmeyer & Tatum, 1953; Soboren & Nyc, 1961). The present report shows that the ratio of test amino acid to required amino acid determines whether the test amino acids stimulate (spare the tryptophan requirement) or inhibit the growth of strain FS-108 on tryptophan. An explanation of the sparing phenomenon which depends on a competition between the required amino acid and inhibitory amino acids for an uptake site has been advanced above. It was proposed that the ratio which enhances growth over that obtained with tryptophan alone is one which allows the required amino acid to enter the growing mycelium at a rate which is optimal for growth. Generally it is assumed that a metabolite which has no growth-promoting activity for a given mutant, but which spares that mutant's requirement, does so because it is an essential metabolic product of the compound which the mutant requires. The large number of naturally occurring amino acids which can spare the tryptophan requirement of strain FS-108, the inability of these same amino acids to spare the indole requirement, and the fact that an amino acid can either spare or inhibit depending on the ratio of amino acid to tryptophan, support the proposed hypothesis.

This report is prepared in part from a dissertation presented to the Graduate School of Florida State University in partial fulfilment of the requirements for the degree of Doctor of Philosophy. The author expresses his appreciation to Dr A. G. DeBusk for his constant interest and encouragement during this work.

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REFERENCES

BEADLE, G. W. & TATUM, E. L. (1945). Neurospora II. Methods of producing and detecting mutations concerned with nutritional requirements. *Amer. J. Bot.* **32**, 678.

BERGMANN, E. D., SICHER, S. & VOLCANI, B. E. (1952). Sparing effects in the biosynthesis of the aromatic amino-acids in *Escherichia coli*. *Biochim. biophys. Acta*, **9**, 220.

BONNER, D., TATUM, E. L. & BEADLE, G. W. (1943). The genetic control of biochemical reactions in Neurospora: A mutant strain requiring isoleucine and valine. *Arch. Biochem.* **3**, 71.

BROCKMAN, H. E. (1960). *Inhibition analysis of aromatic amino acid-requiring mutants of Neurospora crassa.* Dissertation, Florida State University, Tallahassee, Florida.

BROCKMAN, H. E. & DEBUSK, A. G. (1960). Relationships between sparing and inhibition in a tryptophan-deficient mutant of *Neurospora crassa*. *Ass. Southeastern Biologists Bull.* **7**, 2.

BROCKMAN, H. E., DEBUSK, A. G. & WAGNER, R. P. (1959). Mutant-associated antagonisms in *Neurospora crassa*. *Arch. Biochem.* **84**, 455.

CUSHING, J. E., SCHWARTZ, M. & BENNETT, R. (1949). Altered responses of *Neurospora crassa* to inhibiting concentrations of indole. *J. Bact.* **58**, 433.

DeBusk, A. G. & Wagner, R. P. (1953). *p*-Hydroxyphenylpyruvic acid function in *Neurospora crassa*. *J. Amer. chem. Soc.* **75**, 5131.

Doermann, A. H. (1944). A lysineless mutant of Neurospora and its inhibition by arginine. *Arch. Biochem.* **5**, 373.

Fincham, J. R. S. (1950). Mutant strains of Neurospora deficient in anmating ability. *J. biol. Chem.* **182**, 61.

Haas, F., Mitchell, M. B., Ames, B. N. & Mitchell, H. K. (1952). A series of histidineless mutants of *Neurospora crassa*. *Genetics*, **37**, 217.

Haddox, C. H. (1952). The accumulation of α-phenylglycine by mutants of *Neurospora crassa* stimulated by phenylalanine and tyrosine. *Proc. nat. Acad. Sci., Wash.* **38**, 482.

Haskins, F. A. & Mitchell, H. K. (1949). Evidence for a tryptophan cycle in *Neurospora*. *Proc. nat. Acad. Sci., Wash.* **35**, 500.

Lein, J., Mitchell, H. K. & Houlahan, M. B. (1948). A method for selection of biochemical mutants of Neurospora. *Proc. nat. Acad. Sci., Wash.* **34**, 435.

Mathieson, M. J. & Catcheside, D. G. (1955). Inhibition of histidine uptake in *Neurospora crassa*. *J. gen. Microbiol.* **13**, 72.

Nason, A., Kaplan, N. O. & Colowick, S. P. (1951). Changes in enzymatic constitution in zinc-deficient Neurospora. *J. biol. Chem.* **188**, 397.

Newmeyer, D. & Tatum, E. L. (1953). Gene expression in Neurospora mutants requiring nicotinic acid or tryptophan. *Amer. J. Bot.* **40**, 393.

Panicker, R. K., Shanmugasundaram, E. R. B. & Acharya, U. S. (1962). The effect of amino acid on the uptake and utilization of tryptophan and other precursors of nicotinic acid by *Neurospora crassa*. *J. gen. Microbiol.* **28**, 409.

Shanmugasundaram, E. R. B. & Sarma, P. S. (1954). Tryptophan utilization by *Neurospora crassa* and the influence of pure amino acids. *Current Sci. India*, **23**, 224.

Soboren, J. & Nyc, J. F. (1961). Amino acid interactions in *Neurospora crassa*. *J. Bact.* **82**, 20.

Srb, A. M. (1953). Shifts in heterocaryotic dominance relations effected by modifier genes in Neurospora. *Genetics*, **38**, 694.

Teas, H. J., Horowitz, N. H. & Fling, M. (1948). Homoserine as a precursor of threonine and methionine in Neurospora. *J. biol. Chem.* **172**, 651.

Woodward, V. W., de Zeeuw, J. R. & Srb, A. M. (1954). The separation and isolation of particular biochemical mutants of Neurospora by differential germination of conidia, followed by filtration and selective plating. *Proc. nat. Acad. Sci., Wash.* **40**, 192.

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The Occurrence of Muramic Acid in Wax D Preparations of Mycobacteria

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SUMMARY

Acid hydrolysates of wax D preparations from human and bovine strains of *Mycobacterium tuberculosis* were examined by paper chromatography. The amino acids detected in human wax D preparations were alanine, glutamic acid and *meso*-α,ϵ-diaminopimelic acid. In general, amino acids were not found in bovine wax D preparations. Muramic acid was found in all wax D preparations from human strains of mycobacteria but was not detected in wax D preparations from bovine strains. The presence of this typical cell-wall component, as well as the foregoing amino acids, suggests a close relationship between wax D and the cell-wall mucocomplex. The role of muramic acid in the structure of wax D of human strains of mycobacteria is discussed.

INTRODUCTION

The chemical analogy between the water-soluble moiety of wax D and the cell wall of mycobacteria has been suggested (White, Bernstock, Johns & Lederer, 1958; Asselineau & Lederer, 1960; Lederer, 1961*a*, *b*). The present work was undertaken to determine whether the wax D fraction of mycobacteria represents part of their cell-wall mucocomplex. Following the classical studies on the extraction of lipids from tubercle bacilli by Anderson (1927, 1929) and Anderson & Roberts (1930*a*, *b*), Aebi, Asselineau & Lederer (1953) isolated wax D of tubercle bacilli from the chloroform-soluble waxes. Wax D prepared from bovine or saprophytic strains of mycobacteria consists in general of mycolic acids esterified to a polysaccharide containing arabinose, galactose and mannose, but without a peptide (Lederer, 1960; Jollès, Samour & Lederer, 1962). Asselineau, Buc, Jollès & Lederer (1958) and Jollès *et al.* (1962) showed that a water-soluble peptido-polysaccharide of Wax D preparations from human strains of mycobacteria contained a heptapeptide composed of three amino acids (alanine, glutamic acid, α,ϵ-diaminopimelic acid), hexosamines (glucosamine and a smaller amount of galactosamine), and sugars (arabinose, galactose, mannose). Muramic acid was not found although it was 'not excluded that a little muramic acid may be present'.

Muramic acid is an important specific component of the bacterial cell-wall mucopeptide. Salton (1960) showed that the function of muramic acid is to link peptides to other sugars or sugar residues through an amide bond at the carboxyl group of muramic acid. Since wax D preparations from human strains of mycobacteria possess a peptide, hexosamines and a polysaccharide, the demonstration of muramic acid would be of interest in establishing a relation between the wax D

of mycobacteria and their cell walls. The main purpose of the present work was to determine whether muramic acid was present in wax D preparations from mycobacteria.

METHODS

Strains of mycobacteria used. Strain 'C' *Mycobacterium tuberculosis* var. *hominis* was obtained from the Central Veterinary Laboratories, Weybridge. The strains used in the preparation of the wax fractions were *M. tuberculosis* strains Brévannes, Canetti, Test, BCG and Marmorek.

Chemical fractions of mycobacteria. All chemical fractions of mycobacteria were prepared by Professor E. Lederer (Laboratoire de Chimie biologique, Paris) as follows. Mycobacteria were cultivated for 4 weeks on Sauton medium, filtered off, washed with distilled water and extracted several times at room temperature with 5 vol. of a mixture of ethanol + ether (1 + 1, by vol.). After 2 weeks the organisms were filtered off and re-extracted several times with chloroform. The pooled chloroform extracts were extracted with boiling acetone, the insoluble fraction remaining being crude wax D. This was purified by refluxing 2 g. crude wax with 50 ml. acetone for 3 hr., the solvent was filtered off while still hot; after six such extractions the acetone dissolved no more substance. Preparations thus obtained are called purified wax D. Such preparations were obtained from three human strains and two bovine strains of mycobacteria.

Determination of the number of bacilli in wax D preparations and in known weights of organisms. Amounts of dried bacilli of *M. tuberculosis* strain 'C' corresponding to the weights used for hydrolysis were prepared as a suspension in 0·5% Tween 80 and spread evenly over a 1·5 cm. square marked out on a microscope slide. Each film was dried and stained by the Ziehl–Neelsen method. The bacilli in twenty random fields (area 0·0132 sq. mm.) were counted and the number of bacilli per weight of whole organisms hydrolysed was calculated. The same procedure was carried out with 1 mg. samples of the wax D preparations but the suspensions were prepared in chloroform.

Hydrolysis of wax D preparations and whole organisms. Samples were hydrolysed in sealed tubes with 1 ml. 6 N-HCl at 105° for 18 hr. The hydrolysates were filtered to remove insoluble humins, evaporated to dryness over P_2O_5 and KOH in an Edwards centrifugal freeze-drying apparatus and finally resuspended in 0·25 ml. distilled water.

Chromatography. Amino acids were separated by two-dimensional chromatography on Whatman No. 1 paper (10 × 10 in.) in Smith 10 in. Universal chromatanks (Shandon Scientific Co. Ltd., London). Two solvent systems were used: (*a*) butan-1-ol + acetic acid + water (120 + 30 + 50, by vol.) ascending, followed by water-saturated phenol in NH_3 atmosphere ascending (Smith, 1960); (*b*) the phenol + water solvent ascending followed by lutidine + water (65 + 35, by vol.) either descending or ascending (Cummins & Harris, 1956). Each solvent was run for 17 hr. The chromatograms were dried and the spots located by dipping in ninhydrin in acetone (0·1%, w/v).

Stereoisomers of α,ε-diaminopimelic acid (DAP). These were identified by chromatography on Whatman No. 1 paper (45 × 15 cm.) with methanol + water + 10 N-HCl + pyridine (80 + 17·5 + 2·5 + 10, by vol.) ascending. The spots were located by

dipping in ninhydrin in acetone (0·1 %, w/v) and heating at 100° for 2 min. (Rhuland, Work, Denman & Hoare, 1955; Hoare & Work, 1957). DAP spots were characteristically olive-green at first, fading to a permanent yellow colour, in contrast to the purple colour of the other amino acids.

Hexosamines. These were identified by one-dimensional descending chromatography on Whatman No. 3 MM paper (57 × 23 cm.) or Schleicher 2043b paper. The solvent used was a modification of the one described by Bourillon & Michon (1959), butan-1-ol + pyridine + water containing 0·4 % glacial acetic acid (60 + 35 + 25, by vol.), and was allowed to descend for 36 hr, after which the chromatograms were dried and the hexosamines located with ninhydrin or with the modified Elson & Morgan reaction of Partridge (1948). With the latter method, glucosamine and galactosamine appeared as pink spots and muramic acid as a salmon-pink spot.

RESULTS

The amino acids and hexosamines detected in the wax fractions are shown in Table 1. The hydrolysates of waxes from human strains of mycobacteria contained alanine, glutamic acid and α,ϵ-diaminopimelic acid as major components, with traces of aspartic acid, serine or glycine in some cases. Amino acids were not detected in the hydrolysates prepared from wax fractions of bovine strains of mycobacteria, with the exception of specimen WL 44, the wax D fraction with a high melting point of strain Marmorek, which showed weak spots for alanine, glutamic acid, aspartic acid and glycine. Chromatograms of whole bacilli of *Mycobacterium tuberculosis* strain 'C' revealed thirteen amino acids, with an increase in the intensity of spots proportional to the weight of bacilli hydrolysed. DAP was not present in the same proportion as either alanine or glutamic acid because the former is present only in the cell wall, while the other amino acids are also cytoplasmic components. In all cases where DAP was detected the chromatographic spot corresponded with that of the DD- or *meso*- forms, but was assumed to be *meso*-DAP after the findings of Work (1951). Bacterial counts showed that 85×10^5 whole bacilli were required to give detectable DAP on a chromatogram.

Glucosamine, galactosamine and muramic acid were located on the one-dimensional chromatograms by Partridge's method. Muramic acid was found in eleven wax D preparations from human strains of mycobacteria. Hexosamines were not found in the wax D fractions of bovine strains with the exception of WL 44, the wax D preparation from strain Marmorek, in which glucosamine and galactosamine were detected. During the preparation of this paper it was learned that Professor E. Lederer and Dr P. Jollès (personal communication) had also found muramic acid in wax D preparations by column chromatography.

Since most wax D preparations are contaminated with small amounts of whole mycobacteria, the possibility that this impurity accounted for the presence of muramic acid was considered. This seemed unlikely since the experiments with whole *M. tuberculosis* organisms showed that 26×10^9 bacilli had to be hydrolysed to yield a faint spot for muramic acid on a chromatogram, and at this concentration strong spots for valine, leucine, isoleucine, threonine and lysine were also apparent. In the human mycobacteria wax D preparations muramic acid was detected without the spots for these latter amino acids. Secondly, bacterial counts showed that too

Table 1. *Amino acid and hexosamine composition of wax D preparations from mycobacteria*

Ref. no. of wax sample*	Source and nature of wax D preparation	Wt. wax hydrolysed (mg.)	No. bacilli/mg. substance	Amino acids†							Hexosamines		
				Alanine	Glutamic	DL-DAP	Aspartic	Serine	Glycine	Muramic acid	Glucosamine	Galactosamine
	Human strains of mycobacteria											
W. 6	Test	5·0	34×10^3	+	+	+	±	−	±	±	+	−
				+	+	+						
WL 8	Canetti	3·5	17×10^3	+	+	+	±	±	±	±	±	±
				+	+	+						
WL 13	Brévannes intermediate fraction	2·1	—	+	+	+	±	±	±	+	±	±
				+	+	+						
WL 14	Canetti	10·0	68×10^3	+	+	+	−	−	+	+	+	−
				+	+	+						
WL 16	Test m.p. 217–221°	8·7	17×10^3	+	+	+	−	−	−	+	+	−
				+	+	+						
WL 19	Test m.p. 220°	8·0	51×10^3	+	+	+	−	−	+	+	+	−
				+	+	+						
WL 20	Test	6·4	34×10^3	+	+	+	+	−	−	+	+	−
				+	+	+						
WL 45	Test; chromatographed	2·4	—	+	+	+	+	−	+	+	+	−
				+	+	+						
WL 52	Canetti	7·1	136×10^3	+	+	+	±	±	±	±	+	+
				+	+	+						
WL 53	Brévannes; chromatographed	3·6	—	+	+	+	+	−	±	+	+	+
				+	+	+						
WLT	Lipopolysaccharide strain Test	10·9	None seen	+	+	+	±	±	±	+	+	+
				+	+	+						
	Bovine strains of mycobacteria											
WL 7	B.C.G.	6·0	—	−	−	−	−	−	−	−	−	−
WL 18	Marmorek; m.p. 47–49°	5·3	170×10^3	−	−	−	−	−	−	−	−	−
WL 21	BCG; m.p. 160 165°	5·0	110×10^3	−	−	−	−	−	−	−	−	−
WL 44	Marmorek; m.p. 212°	5·0	—	+	+	−	±	−	±	−	±	±
WL 60	Marmorek	2·0	68×10^3	−	−	−	−	−	−	−	−	−

* White/Lederer reference numbers; these conform with biological results published elsewhere (White *et al.* 1958).

† Valine, leucine, proline, arginine, not detected.

few bacilli were present in the wax D fractions to yield a detectable spot for muramic acid, and although the contamination of bovine wax D preparations with bacilli was as great as that for human wax D preparations, neither amino acids nor hexosamines were detected.

DISCUSSION

The presence of muramic acid in wax D preparations from human mycobacterial strains as an integral component suggests a close relation with the cell wall of the organism. Analyses of mycobacterial cell walls (Cummins & Harris, 1958) have shown the presence of amino acids (alanine, glutamic acid, *meso*-DAP), hexosamines (glucosamine, muramic acid) and sugars (arabinose, galactose). Thus the demonstration of muramic acid together with alanine, glutamic acid and *meso*-DAP in wax D preparations supports the hypothesis that these waxes are integral components of the cell-wall mucocomplex. Further support for this hypothesis comes from the finding (Jollès *et al.* 1962; White, Jollès, Samour & Lederer, 1964) that the molecular proportions of these amino acids in wax D preparations are within the ranges described for the cell walls of mycobacteria (Belknap, Camien & Dunn, 1961).

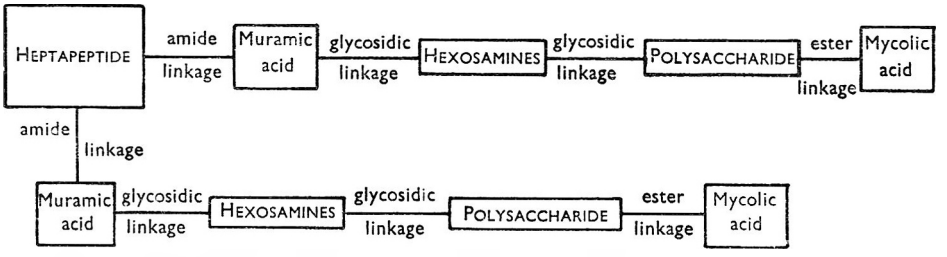

Fig. 1. Incorporation of muramic acid into the hypothetical structure of wax D of human strains of mycobacteria.

Asselineau *et al.* (1958) proposed a structure for the wax D of human strains of *Mycobacterium tuberculosis* in which hexosamines were established as the intermediates between the heptapeptide and the polysaccharide. Jollès, Cros & Lederer (1960) postulated that the heptapeptide and polysaccharide were linked by a bond between a carboxyl group of *meso*-DAP and galactosamine, and a glycosidic linkage between galactosamine and arabinose. Asselineau *et al.* (1958) showed that there were no free amino groups in the heptapeptide. However the structure of Jollès *et al.* (1962) incorporating galactosamine as the intermediate hexosamine, would presumably leave two free amino groups. It is possible that the role of muramic acid as a structural component of wax D could be to link the heptapeptide to sugars or sugar residues as it does in the cell wall. If there are no free amino groups it is conceivable that muramic acid is linked to each molecule of *meso*-DAP and a possible structure for wax D of human strains of *M. tuberculosis* might be as shown in Fig. 1.

The relationship of the wax D of bovine mycobacterial strains to the cell-wall mucocomplex requires further explanation. Recently it has been suggested that the cell-wall mucocomplex may be composed of a mixture of polymers of relatively

high and different molecular weights (Rogers, 1963). The wax D of mycobacteria may also consist of a mixture of different sized polymers. On the basis of the structure proposed above the polymer building-blocks would be: (*A*) muramic acid + heptapeptide, (*B*) *n*-acetylgalactosamine + *n*-acetylglucosamine, and (*C*) polysaccharide + mycolic acid. On the basis of the results of the composition of the waxes, wax D of human strains of *M. tuberculosis* would contain the building-blocks *A*, *B* and *C* linked together. The high melting point fraction of the bovine wax D preparation, WL 44, may contain polymer building-blocks *B* and *C* linked together and the wax D of bovine strains of *M. tuberculosis* could be polymer building-block *C*.

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REFERENCES

AEBI, A., ASSELINEAU, J. & LEDERER, E. (1953). Sur les lipides de la souche humaine 'Brévannes' de *Mycobacterium tuberculosis*. *Bull. Soc. Chim. biol., Paris*, **35**, 661.

ANDERSON, R. J. (1927). The separation of lipoid fractions from tubercle bacilli. *J. biol. Chem.* **74**, 525.

ANDERSON, R. J. (1929). The chemistry of the lipoids of tubercle bacilli: IV. Concerning the so-called tubercle bacilli wax. Analysis of the purified wax. *J. biol. Chem.* **83**, 505.

ANDERSON, R. J. & ROBERTS, E. G. (1930*a*). Chemistry of the lipoids of tubercle bacilli: separation of lipoid fractions from avian tubercle bacilli. *J. biol. Chem.* **85**, 509.

ANDERSON, R. J. & ROBERTS, E. G. (1930*b*). Chemistry of the lipoids of tubercle bacilli: separation of lipoid fractions from bovine tubercle bacilli. *J. biol. Chem.* **85**, 529.

ASSELINEAU, J., BUC, H., JOLLÈS, P. & LEDERER, E. (1958). Sur la structure chimique d'une fraction peptido-glycolipidique (cire D) isolée de *Mycobacterium tuberculosis* var. *hominis*. *Bull. Soc. Chim. biol., Paris*, **40**, 1953.

ASSELINEAU, J. & LEDERER, E. (1960). Chemistry and metabolism of bacterial lipides. *Lipide Metabolism*. Ed. by K. Bloch, pp. 356–358. New York: Wiley.

BELKNAP, D. R., CAMIEN, M. N. & DUNN, M. S. (1961). Amino acid composition of mycobacterial cell walls. *Proc. Soc. exp. Biol., N.Y.* **107**, 289.

BOURILLON, R. & MICHON, J. (1959). Résolution et détermination quantitative par chromatographie sur papier des glucides liés aux protéines. *Bull. Soc. Chim. biol., Paris*, **41**, 267.

CUMMINS, C. S. & HARRIS, H. (1956). The chemical composition of the cell wall in some Gram positive bacteria and its possible value as a taxonomic character. *J. gen. Microbiol.* **14**, 583.

CUMMINS, C. S. & HARRIS, H. (1958). Studies on the cell wall composition and taxonomy of actinomycetales and related groups. *J. gen. Microbiol.* **18**, 173.

HOARE, D. S. & WORK, E. (1957). The stereoisomers of α,ε-diaminopimelic acid. *Biochem. J.* **65**, 441.

JOLLÈS, P., NGUYEN-TRUNG-LUONG-CROS, H. & LEDERER, E. (1960). Sur la structure chimique de la partie peptidique de la cire D d'une souche humaine de *M. tuberculosis*. *Biochim. biophys. Acta*, **43**, 559.

JOLLÈS, P., SAMOUR, D. & LEDERER, E. (1962). Analytical studies on Wax D, a macromolecular peptidoglycolipid fraction from human strains of *Mycobacterium tuberculosis*. *Arch. Biochem.* Suppl. 1, 283.

LEDERER, E. (1960). Lipides des mycobactéries et tuberculose. *Farmaco*, **15**, 44.

LEDERER, E. (1961*a*). Chemistry and biochemistry of some biologically active lipids. *Pure appl. Chem.* **2**, 587.

LEDERER, E. (1961*b*). Glycolipids of acid-fast bacteria. *Advanc. Carbohyd. Chem.* **16**, 207.

PARTRIDGE, S. M. (1948). Filter-paper partition chromatography of sugars. I. General description and application to the qualitative analysis of sugars in apple juice, egg-white and foetal blood of sheep. *Biochem. J.* **42**, 238.

RHULAND, L. E., WORK, E., DENMAN, R. F. & HOARE, D. S. (1955). The behaviour of the isomers of DAP on paper chromatograms. *J. Amer. chem. Soc.* **77**, 4844.

ROGERS, H. J. (1963). The surface structures of bacteria. *Symp. Biochem. Soc.* **22**, 55.

SALTON, M. R. J. (1960). Microbial Cell Walls. *Ciba Lectures in Microbial Biochemistry.* New York: Wiley.

SMITH, I. (1960). *Chromatographic and Electrophoretic Techniques*, vol. I. London: Heinemann.

WHITE, R. G., BERNSTOCK, L., JOHNS, R. G. S. & LEDERER, E. (1958). The influence of components of *M. tuberculosis* and other mycobacteria upon antibody production to ovalbumin. *Immunology*, **1**, 54.

WHITE, R. G., JOLLÈS, P., SAMOUR, D. & LEDERER, E. (1964). Correlation of adjuvant activity and chemical structure of wax D fractions of mycobacteria. *Immunology*, **7**, (in the Press).

WORK, E. (1951). The isolation of α,ϵ-diaminopimelie acid from *Corynebacterium diphtheriae* and *Mycobacterium tuberculosis*. *Biochem. J.* **49**, 17.

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Some Relations between Chemical Structure and Antifungal Effects of Griseofulvin Analogues

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SUMMARY

Inhibitory effects of more than 300 analogues of griseofulvin against six dermatophytic fungi and eight plant pathogenic fungi were determined in an investigation of relations between chemical structure and biological activity. The influence of chemical structure on physical properties of the analogues appeared dominant, but optimum requirements for *in vitro* performance were somewhat different from those required *in vivo* for effective control of plant pathogenic or dermatophytic fungi.

INTRODUCTION

Griseofulvin is firmly established in many countries as an oral antibiotic for treatment of fungal diseases in medical (Barnett, 1960; Williams, 1960; Sternberg, 1960) and in veterinary practice (Uvarov, 1961). The status of griseofulvin in crop protection was reviewed by Rhodes (1962) who concluded that a satisfactory solution to the economics of its utilization might lead to establishment of griseofulvin as a specific plant protectant against the numerous flower-blights and spur-blights induced by *Sclerotinia* species. The relationship between *in vitro* action against *Botrytis allii* and systemic antifungal action against *Alternaria solani* following upon root absorption by tomato plants was examined for griseofulvin and eighteen related compounds by Crowdy, Grove & McCloskey (1959). They found, in a homologous series of 2′-alkyloxy-analogues of griseofulvin, that *in vitro* activity increased to a maximum in the 2′-*n*-propoxy and 2′-*n*-butoxy analogues, but that further ascent of this homologous series resulted in greatly diminished activity. Systemic antifungal properties were observed in the 2′-ethoxy analogue of griseofulvin but higher members of the series were inactive. Rhodes (1962) questioned the need, implicit in this test, for translocation between so distant tissues; application of analogues to the roots takes little account of the potential benefits that might be gained from local systemic action conferred by ability to penetrate cuticle and to move for relatively short distances through leaf tissues.

Following successful clinical use of griseofulvin, a more extensive series of griseofulvin analogues, prepared for an investigation of relations between structure and biological activity, was described (Arkley, Attenburrow, Gregory & Walker, 1963; Gregory, Holton, Robinson & Walker, 1962; Walker, Warburton & Webb, 1962; Stephenson, Walker, Warburton & Webb, 1962; Page & Staniforth, 1962; Arkley, Gregory & Walker, 1963; Goodall, Gregory & Walker, 1963; Page & Staniforth, 1963). We now report some biological results with these compounds against six dermatophytic and eight plant pathogenic fungi *in vitro* together with observations on antifungal action following upon foliage application of selected

analogues *in vivo* against *Botrytis cinerea* on tomatoes, *Erysiphe graminis* on barley seedlings, *E. cichoracearum* on cucumber seedlings, *Oidium chrysanthemi* on chrysanthemums and *Venturia inaequalis* on apple root stocks.

METHODS

Laboratory tests

(i) *Test for 'curling' of hyphae.* All analogues were compared with griseofulvin in the *Botrytis allii* test (Brian, Curtis & Hemming, 1946) for 'curling' and 'stunting' of hyphae, and some of the more active substances were also tested in a similar manner against *B. cinerea.*

(ii) *Test for inhibition of radial growth.* Serial dilutions of the analogues in modified Sabouraud's maltose agar (4% maltose; 1% 'Oxoid' peptone; 2·4% 'Oxoid' malt extract; 2% agar adjusted to pH 7·0; sterilized at 120° for 20 min.) were compared against griseofulvin for inhibition of radial growth of dermatophytic and plant pathogenic fungi by adaptation of a bio-assay for griseofulvin described by Grutter, Gaughran, Swartz & Kamp (1959).

The test micro-organisms were: dermatophytic fungi, *Trichophyton rubrum* (Castellani) Ota; *T. interdigitale* Priestley; *T. mentagrophytes* (Robin) Blanchard; *T. persicolor* Sabouraud; *Microsporum canis* Bodin; *Epidermophyton floccosum* (Herz), Langeron & Milochevitch; plant pathogenic fungi, *Alternaria tenuis* Lees ex Corda; *Aspergillus niger* Van Tiegh.; *Botrytis cinerea* Fr.; *Cercospora melonis* Cooke; *Fusarium nivale* Auct.; *Glomerella cingulata* (Stonem), Spauld.; *Sclerotinia fructigena* Aderk & Ruhl.; *Thielaviopsis basicola* (Berk & Br), Ferraris.

A measured part of a solution of the analogue (1 mg./ml.) in acetone + ethanol (1 + 1 by vol.) was diluted into sterile distilled water to give twice the highest concentration of analogue required in the final agar test plates; dilution of this solution with distilled water afforded a range of twofold serial dilutions. Double strength agar medium (6 ml.) was mixed with an equal volume of each serial dilution and the completed agar test-media, containing analogue 20 to 0·31 μg./ml., were poured into Petri dishes and allowed to set. The three controls consisted of agar containing no solvent and the two highest concentrations of solvent.

Inocula of the test fungi were prepared from cultures on slopes of modified Sabouraud maltose agar medium. The confluent surface growth in each culture was flooded with sterile distilled water and scraped with a wire loop to give a suspension that was homogenized before mixing with molten (47°) agar medium (modified Sabouraud maltose) in Petri dishes and cooled to solidify. Petri dish cultures of *Aspergillus niger* were stored overnight at 4°, but the rest were incubated at 26° for 16, 24 or 48 hr (depending upon the rate of growth of individual organisms) before use. Plugs (7 mm. diameter) cut with a cork-borer from each fungal culture were transferred to the surfaces of the agar test media containing serial dilutions of the analogue to be evaluated. Six dermatophytes, five slow-growing plant pathogens or three fast-growing plant pathogens were distributed in this manner on the surface of each agar test medium and incubated at 26°.

The inhibitory effect of any given concentration of analogue was determined by measuring the radial growth of each test fungus from the seeded plugs at the end of incubation for 3 days (plant pathogens) or 6 days (dermatophytes). When the

analogue concentration prevented radial growth, the extent of inhibition was graded on a scale of 0–3 according to the density of growth within the agar plug inoculum. In each test, a corresponding series of agar media containing serial dilutions of griseofulvin instead of analogue was prepared and inoculated with the same test fungi to compare directly the inhibitory effects of analogue and griseofulvin on fungal growth.

(iii) *Test for growth inhibition in static liquid cultures.* The analogues were compared with griseofulvin by conventional tube dilution assay against liquid cultures of *Candida albicans* (medium: 4% glucose, 1% 'Oxoid' peptone, 2·1% 'Oxoid' malt extract; adjusted to pH 7·0 and sterilized at 120° for 20 min.) and *Bacillus subtilis* (medium: 0·5% NaCl, 1% 'Evans' peptone, 1% 'Lab. Lemco'; adjusted to pH 8·4 and boiled for 30 min. before filtration through kieselguhr, re-adjusted to pH 7·2 with HCl and sterilized at 120° for 20 min.). Complete inhibition of growth in this test may be identified by absence of a white sediment after incubation of *C. albicans* for 24 hr at 26° or by absence of a surface pellicle of *B. subtilis* after incubation for 24 hr at 37°.

Greenhouse tests

(i) *Botrytis cinerea on tomatoes.* The greenhouse test described by Crosse, McWilliam, Rhodes & Dunn (1960) for assessment of fungicidal activity against *Phytophthora infestans* on tomatoes was adapted to evaluation of griseofulvin analogues against *Botrytis cinerea.* Stonor's M.P. tomato plants, grown for about 4 weeks under fluorescent lights (80 W. spaced at 4 in. centres), were ready for use when the sixth compound leaf was clearly visible. In each experiment, forty-nine plants were arranged in a 7 × 7 Latin square. Suspensions or solutions of the analogues were prepared by addition of 'Sorbester PQ 12' (mixed sorbitan monolaurate and polyoxyethylene condensate) to a solution of the analogue in methylethylketone and then dilution with water gave the required concentration of analogue in 0·5% solvent + 0·08% of wetting-out agent. Treatments were applied by means of a B.E.N. model R.F. 4 spray-gun (B.E.N. Patents Ltd. High Wycombe, Bucks.), operated at a constant pressure of 10 lb./sq.in., to both surfaces of the second, third and fourth leaves above the cotyledons, so as to wet the leaflets completely but without run-off.

Inoculum was prepared by suspending in Weindling's medium the conidia from a freely-sporulating culture of *Botrytis cinerea* (isolated from *Vitis vinifera* and maintained on modified Sabouraud maltose agar medium) and diluting with Weindling medium to a concentration of 100,000 spores/ml. This suspension of spores was applied with a B.E.N. model 5 no. 1 spray-gun, operated at a pressure of 10 lb./sq.in., to the ventral surfaces only of the three treated leaves on each plant, with use of no more suspension than was necessary to give a uniform cover of small but discrete droplets of fluid. The inoculated plants were incubated in a saturated atmosphere, provided by a polythene humidity chamber cooled by an external spray of cold water, until discrete lesions developed after 72 hr. The aggregate of lesions on the three inoculated leaves of each plant was taken as a measure of the incidence of disease. The mean number of lesions on seven treated plants compared with the mean obtained from corresponding leaves of control plants gave an estimate of the antifungal protection afforded by treatment.

Effectiveness of treatments against the aggressive form of the disease was

determined by extension of the incubation period to 7 days and then by grading of leaves according to percentage of leaf area destroyed; comparison of mean grades from control and treated plants gave an estimate of protection afforded by treatment.

Local systemic action of the analogues was assessed from the antifungal protection obtained when treatment sprays were confined to the dorsal surfaces only of the leaves, but the pathogenic inoculum was applied to the ventral surfaces in the usual manner.

(ii) *Erysiphe graminis on barley seedlings.* Barley seedlings (variety 'Proctor') were grown, 10 to 15 seedlings in each 3 in. clay pot, to a height of about 5 in. and then decreased in number to leave ten uniform seedlings in each pot. Growing conditions were similar to those already described for raising tomato plants. Each treatment was sprayed from a B.E.N. (model R.F. 4) spray gun, operating at a constant pressure of 10 lb./sq.in., to the foliage of seedlings in three replicate pots and allowed to dry before the pots were transferred to an enclosed section (10 ft. × 22 ft.) of another greenhouse which served as an inoculation chamber. This contained two square tables spaced about 4 ft. apart; one supported the inoculum provided by many boxes of strongly growing barley plants heavily infected with *E. graminis*; on the other, experimental plants were randomly arranged with their foliage orientated in a horizontal position so as to expose a large leaf area to spore deposition.

Inoculation was effected by blowing air from a 'Microsol' mist generator for about 20 sec. across the table of infected plants in the opposite direction from the experimental seedlings, so that a spore cloud moved up the side of the inoculation chamber into the roof. After a further short blast of air, now directed towards the roof, the evenly dispersed spores were allowed to settle in still air for a period of 18 hr. The potted seedlings were then removed to a third greenhouse, free from cereal mildew, and the discrete lesions which appeared 7 days later on the primary leaves were counted to determine the number of lesions on the ten seedlings in each pot. The number of lesions of three treated pots was compared with the corresponding mean from untreated seedlings to give an estimate of the antifungal protection achieved by treatment.

(iii) *Erysiphe cichoracearum on cucumber.* Cucumber seedlings (variety 'Improved Telegraph') grown under conditions similar to those used for the tomato and barley plants were ready for test when the first true leaf was about half expanded. Plants were arranged in a 6 × 6 Latin square and the treatments were applied as foliage sprays at 7-day intervals. Twenty cucumber plants, heavily infected with *E. cichoracearum*, were evenly arranged around the Latin square so as to provide continuous spore inoculum for the test plants. The antifungal effects of treatment were estimated by comparison of the mean number of discrete mildew colonies per plant in each treatment with the corresponding mean obtained from the control plants which had been sprayed with 0·5% methylethylketone + 0·08% 'Sorbester PQ12' only.

(iv) *Oidium chrysanthemi on chrysanthemums.* Pot-grown chrysanthemums (varieties 'Sterling' and 'Mayford Perfection'), lightly infected with mildew, were available for test under glasshouse conditions when about 3 ft. high with crown buds just visible. Each treatment was given to three plants of each variety

selected at random, and the treatments were applied as foliage sprays at fortnightly intervals from the middle of October onwards. The course of the disease was followed by estimations made at intervals from the middle of November until early January. Each leaf was scored according to the area covered by the fungus, and the effect of treatment was estimated by comparing the mean score of the treated plants with the corresponding mean of untreated control plants.

(v) *Venturia inaequalis on apples.* Griseofulvin analogues were tested against *V. inaequalis* on apple root stocks (Malling Merton 106) grown under glass in 10 in. clay pots. Daylight was supplemented as necessary, so as to provide illumination for 16 hr each day throughout the year. The rootstocks were pruned hard to encourage four or five buds to break and develop into shoots about 6 in. in length.

Treatments were sprayed at a constant pressure of 10 lb./sq.in. from a B.E.N. spray gun (model R.F. 4) on the dorsal surfaces of the five youngest leaves of each shoot so as to wet the surfaces completely but without run-off: there were four replicates.

An inoculum containing 300,000 conidia/ml. was prepared by pipetting 10 ml. sterile distilled water into 14-day filter-paper culture tubes (Kirkham, 1956), shaking vigorously, filtering the resultant suspensions through butter muslin to remove fragments of mycelium and finally diluting as necessary. This spore suspension was sprayed from a B.E.N. spray gun (Model S no. 1), operating at a constant pressure of 10 lb./sq.in., onto the leaves, so as to give a uniform cover of small, discrete droplets.

Immediately after inoculation, each rootstock was enveloped in a saturated atmosphere provided by a large polythene bag that was thoroughly moistened on the inside. Each bag was tied firmly around the rim of the pot, and the polythene was held clear of the foliage by four internal supporting canes. The foliage was maintained wet for 48 hr by keeping the plants at 15–20° in subdued light. At the end of this infection period, the polythene bags were removed, and the plants returned to the greenhouse. Lesions appeared after 10–14 days depending on the temperature.

The decrease in disease due to treatment was estimated by comparing the mean lesion count/leaf for each treatment against the corresponding value for control plants. In some experiments the infection of controls was so severe that the lesions coalesced, making it necessary to grade leaves according to the percentage area of damaged lamina: mean grade/leaf was then substituted for mean lesion count/leaf in estimating the effects of treatments.

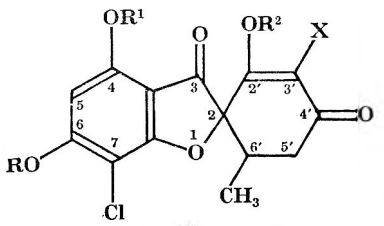
RESULTS

Analogues of griseofulvin which proved to be more active than griseofulvin against one or more of the test fungi *in vitro* are listed in Table I*a*, I*b* and I*c*: none was active against the test bacterium or yeast. It is clear that the antifungal spectrum of griseofulvin can be profoundly altered by changes in chemical structure.

Increase in antifungal action over that of griseofulvin was usually specific to one or a few species. The broadest spectrum of enhanced activity was shown by the 2′-ethoxy-3′-benzyl analogue of griseofulvin (R 10/141), which had greater activity than griseofulvin against all test fungi except *Thielaviopsis basicola.* Increased or decreased length of the 2′-alkyloxy side-chain in this homologous series first made

Table 1*a*. *Analogues of griseofulvin with enhanced* in vitro *activity*

Analogue



(I)

Ratio of griseofulvin concentration to analogue concentration required for specific effects on test fungi

Ratio for 50 % inhibition of radial growth

Reference no.	Analogue	Ratio found in *Botrytis allii* test for 'curling' of hyphae	Dermatophytes: *T. rubrum*	*T. interdigitale*	*T. mentagrophytes*	*T. persicolor*	*M. canis*	*E. floccosum*	Plant pathogenic fungi: *A. tenuis*	*C. meloxis*	*S. fructigena*	*G. cingulata*	*A. niger*	*B. cinerea*	*F. nivale*	*T. basicola*
R 10/130	I; R, $R^2 = CH_3$, $R^1 = nC_4H_9$, X = H	1	—	4	.	.	.	—	.	—	—	.	.	—	—	—
R 10/114	I; R, $R^2 = CH_3$, $R^1 = CH_2.CH{:}CH_2$, X = H	2	—	8	—	.	—	—	.	—	—	.	.	—	—	—
R 10/131	I; R, $R^2 = CH_3$, $R^1 = C_6H_5.CH_2$, X = H	8	.	2	—	.	—	.	.	.	—	.	.	—	—	—
R 10/80	I; R = C_2H_5, R^1, $R^2 = CH_3$, X = H	1	.	4	—	1	.	2	.	.	.	.	.	—	2	—
R 10/106	I; R = nC_3H_7, R^1, $R^2 = CH_3$, X = H	.	.	4	.	.	.	.	.	.	.	.	.	—	—	—
R 10/88	I; R = iso C_3H_7, R^1, $R^2 = CH_3$, X = H	1	.	1	.	1	.	.	.	.	.	.	.	—	—	—
R 10/109	I; R = $CH_2.CH{:}CH_2$, R^1, $R^2 = CH_3$, X = H	1	.	2	.	1	.	1	.	.	.	.	.	—	—	—
R 10/38	I; R, R^1, $R^2 = CH_3$, X = CH_3	2	.	1	.	1	4	.	.	.	.	.	.	—	—	—
R 10/119	I; R, R^1, $R^2 = CH_3$, X = nC_3H_7	2	.	2	.	1	1	.	.	.	.	.	.	—	—	—
R 10/92	I; R, R^1, $R^2 = CH_3$, X = $CH_2.CH{:}CH_2$	2	.	2	.	1	1	.	.	.	.	.	.	—	—	—
R 10/96	I; R, R^1, $R^2 = CH_3$, X = $C_6H_5.CH_2$	2	.	2	.	1	1	.	.	.	.	.	.	—	—	—
R 10/64	I; R, R^1, $R^2 = CH_3$, X = Cl	4	1	16	4	2	1	1	.	4	4	8	.	—	—	—
R 10/20	I; R, R^1, $R^2 = CH_3$, X = Br	4	2	1	2	1	2	2	.	4	2	8	.	—	—	—
R 10/55	I; R, R^1, $R^2 = CH_3$, X = I	8	1	1	.	1	1	2	.	4	2	8	.	—	—	—
R 10/32	I; R, $R^1 = CH_3$, $R^2 = C_2H_5$, X = H	2	.	.	1	.	1	.	2	2	1	4	.	—	—	—
R 10/122	I; R, $R^1 = CH_3$, $R^2 = nC_3H_7$, X = H	4	.	1	1	1	1	1	4	1	2	4	.	—	—	—
S2	I; R, $R^1 = CH_3$, $R^2 = nC_4H_9$, X = H	8	.	8	1	2	.	.	1	1	1	2	.	—	—	—
R 10/18	I; R, $R^1 = CH_3$, $R^2 = nC_6H_{13}$, X = H	.	.	2	1	.	.	.	2	2	1	4	.	—	—	—
R 10/62	I; R, $R^1 = CH_3$, $R^2 = CH_2.CH{:}CH_2$, X = H	4	.	1	1	1	1	1	2	2	1	4	.	—	—	—
R 10/90	I; R, $R^1 = CH_3$, $R^2 = C_6H_5$, X = H	4	.	.	.	.	.	.	2	2	1	4	.	—	—	—
R 10/78	I; R, $R^1 = CH_3$, $R^2 = C_6H_5.CH_2$, X = H	8	.	.	.	.	.	.	2	2	1	4	.	—	—	—
R 10/126	I; R, $R^1 = CH_3$, $R^2 = C_2H_5$, X = Cl	4	1	2	1	.	2	2	2	1	2	4	.	—	—	—
R 10/125	I; R, $R^1 = CH_3$, $R^2 = C_2H_5$, X = Br	4	.	8	2	.	1	1	2	2	1	4	.	—	—	—
R 10/81	I; R, $R^1 = CH_3$, $R^2 = C_2H_5$, X = I	16	.	1	1	.	2	1	2	2	1	4	.	—	—	—
R 10/111	I; R, $R^1 = CH_3$, $R^2 = C_2H_5$, X = C_2H_5	2	.	4	4	4	2	.	2	2	1	4	.	—	—	—
R 10/141	I; R, $R^1 = CH_3$, $R^2 = C_2H_5$, X = $C_6H_5CH_2$	16	2	32	8	8	2	2	2	2	1	4	.	—	—	—
R 10/158	I; R, $R^1 = CH_3$, $R^2 = nC_3H_7$, X = Cl	> 64	1	16	1	.	.	.	.	.	.	.	.	—	—	—
R 10/154	I; R, $R^1 = CH_3$, $R^2 = nC_3H_7$, X = Br	64	.	8	.	.	.	.	.	.	.	.	.	—	—	—
R 10/163	I; R, $R^1 = CH_3$, $R^2 = nC_3H_7$, X = I	512	.	1	.	.	.	.	.	.	.	.	.	—	—	—
R 10/178	I; R, $R^1 = CH_3$, $R^2 = nC_3H_7$, X = nC_3H_7	8	.	1	1	.	.	.	.	.	.	.	.	—	—	—
R 10/162	I; R, $R^1 = CH_3$, $R^2 = nC_3H_7$, X = $C_6H_5.CH_2$	512	.	4	.	.	2	.	.	4	1	4	.	—	—	—
R 10/173	I; R, $R^1 = CH_3$, $R^2 = nC_4H_9$, X = Cl	> 8	.	2	.	.	.	.	.	.	.	2	.	—	—	—
R 10/167	I; R, $R^1 = CH_3$, $R^2 = nC_4H_9$, X = Br	> 512	.	.	.	.	.	.	.	.	.	1	.	—	—	—
R 10/174	I; R, $R^1 = CH_3$, $R^2 = nC_4H_9$, X = I	> 8	.	.	.	.	.	.	.	.	.	.	.	—	—	—
R 10/161	I; R, $R^1 = CH_3$, $R^2 = nC_4H_9$, X = $C_6H_5.CH_2$	1	.	.	.	.	.	.	1	1	.	.	.	—	—	—
R 10/134	I; R, $R^1 = CH_3$, $R^2 = C_6H_5.CH_2$, X = Cl	.	.	.	.	.	.	.	.	.	.	.	.	—	—	—

— = not tested; . = less active than griseofulvin.

Table 1*b*. *2′-Thioether analogues of griseofulvin with enhanced* in vitro *activity*

Reference no.	Analogue (II)	Ratio found in *Botrytis allii* test for 'curling' of hyphae	Ratio of griseofulvin concentration to analogue concentration required for specific effects on test fungi: Ratio for 50 % inhibition of radial growth: Dermatophytes: *T. rubrum*	*T. interdigitale*	*T. mentagrophytes*	*T. persicolor*	*M. canis*	*E. floccosum*	Plant pathogenic fungi: *A. tenuis*	*C. nebulosa*	*S. fructigena*	*G. cingulata*	*A. niger*	*B. cinerea*	*F. nivale*	*T. basicola*
R 10/129	II; R, $R^1 = CH_3$, $R^2 = CH_3$, X = H	4	.	.	.	.	.	.	1	.	.	.	.	.	.	.
R 10/98	II; R, $R^1 = CH_3$, $R^2 = C_2H_5$, X = H	> 8	.	.	.	.	.	.	1	.	.	.	.	.	.	.
R 10/153	II; R, $R^1 = CH_3$, $R^2 = nC_3H_7$, X = H	> 8	.	.	.	.	.	.	1	.	.	.	.	.	.	.
R 10/152	II; R, $R^1 = CH_3$, $R^2 = nC_4H_9$, X = H	> 8	.	.	.	.	.	.	1	.	.	.	.	.	.	.
R 10/128	II; R, $R^1 = CH_3$, $R^2 = CH_2.CH{:}CH_2$, X = H	> 8	1	.	1	.	.	.	1	1	1	1	.	1	.	.
R 10/116	II; R, $R^1 = CH_3$, $R^2 = C_6H_5.CH_2$, X = H	4	.	4	1	.	.	.	1	1	1	1	.	1	.	.
R 10/85	II; R, $R^1 = CH_3$, $R^2 = C_6H_5$, X = H	.	.	2	.	.	.	.	1	1	1	1	.	1	.	.
R 10/184	II; R, $R^1 = CH_3$, $R^2 = CH_3$, X = Cl	1	.	.	.	.	1	.	1	1	1	1	.	1	.	.
R 10/201	II; R, $R^1 = CH_3$, $R^2 = CH_3$, X = $C_6H_5.CH_2$	1	.	2	.	.	.	.	1	1	1	1	.	1	.	.
R 10/185	II; R, $R^1 = CH_3$, $R^2 = C_2H_5$, X = Cl	.	.	.	.	.	.	.	1	1	1	1	.	1	.	.
R 10/197	II; R, $R^1 = CH_3$, $R^2 = C_2H_5$, X = $C_6H_5.CH_2$	.	.	> 4	1	.	> 1	.	1	1	1	1	.	1	.	.
R 10/186	II; R, $R^1 = CH_3$, $R^2 = nC_3H_7$, X = Cl	1	.	.	.	.	.	.	1	1	1	1	.	1	.	.
R 10/225	II; R, $R^1 = CH_3$, $R^2 = nC_3H_7$, X = Br	1	1	.	.	.	.	.	1	1	1	1	.	1	.	.
R 10/206	II; R, $R^1 = CH_3$, $R^2 = nC_3H_7$, X = nC_3H_7	1	1	.	.	.	.	.	1	1	1	1	.	1	.	.
R 10/210	II; R, $R^1 = CH_3$, $R^2 = nC_3H_7$, X = $C_6H_5.CH_2$	1	1	1	1	.	.	.	1	1	1	1	.	1	.	.
R 10/183	II; R, $R^1 = CH_3$, $R^2 = CH_2.CH{:}CH_2$, X = Cl	1	.	1	1	.	.	.	1	1	1	1	.	1	.	.
R 10/203	II; R, $R^1 = CH_3$, $R^2 = CH_2.CH{:}CH_2$, X = $C_6H_5.CH_2$	2	.	.	.	.	.	1	1	1	2	1	.	1	.	.

— = not tested; . = less active than griseofulvin.

Table 1*c*. *Analogues of iso-griseofulvin with enhanced* in vitro *activity*

Reference no.	Analogue (III)	Ratio found in *Botrytis allii* test for 'curling' of hyphae	Ratio of griseofulvin concentration to analogue concentration required for specific effects on test fungi: Ratio for 50% inhibition of radial growth: Dermatophytes: *T. rubrum*	*T. interdigitale*	*T. mentagrophytes*	*T. persicolor*	*M. canis*	*E. floccosum*	Plant pathogenic fungi: *A. tenuis*	*C. melonis*	*S. fructigena*	*G. cingulata*	*A. niger*	*B. cinerea*	*F. nivale*	*T. basicola*
R 10/8	III; R, $R^1 = CH_3$, $R^2 = CH_3$, X = H	.	.	.	.	.	.	.	.	.	1	2	.	.	.	.
R 10/59	III; R, $R^1 = CH_3$, $R^2 = C_2H_5$, X = H	.	.	.	.	.	.	.	.	.	2	1	.	.	.	.
S3	III; R, $R^1 = CH_3$, $R^2 = nC_4H_9$, X = H	2	.	.	.	.	.	.	.	.	1	2	.	1	.	.
R 10/63	III; R, $R^1 = CH_3$, $R^2 = CH_2.CH{:}CH_2$, X = H	.	.	.	.	.	.	.	.	.	1	2	.	.	.	.
R 10/33	III; R, $R^1 = CH_3$, $R^2 = C_6H_5$, X = H	1	.	.	.	.	—	—	2	1	4	.	.	4	2	.
R 10/95	III; R, $R^1 = CH_3$, $R^2 = CH_3$, $X = C_6H_5.CH_2$	4	.	4	> 1	1	—	—	1	2	4	1	2	1	.	.
R 10/36	III; R, $R^2 = C_2H_5$, $R^1 = CH_3$, X = H	.	.	.	.	.	.	.	.	.	.	2	.	.	.	.

— = not tested; . = less active than griseofulvin.

more specific the increase in antifungal action over that of griseofulvin, e.g. the 2′-propoxy-3′-benzyl (R 10/162) and 3′-benzyl (R 10/96) analogues of griseofulvin, and then diminished the antifungal activity against most of the test fungi, e.g. 2′-*n*-butoxy-3′-benzyl analogue of griseofulvin (R 10/161). A similar but not quite so wide range of enhanced antifungal activity was also observed in the 2′-alkyloxy-3′-chloro-, 2′-alkyloxy-3′-bromo- and 2′-alkyloxy-3′-iodo-homologous series of griseofulvin analogues, but the peak activity in each of these series was shown by the 2′-propoxy-3′-halogen analogues of griseofulvin.

Taking the analogues as a whole, it was generally observed that increased activity against the plant pathogens predominated over enhanced action against dermatophytes. Replacements in the 2′-position or substitution in the 3′-position produced the greatest improvement in antifungal action *in vitro*, particularly against *Botrytis cinerea* and *Sclerotinia fructigena*. Replacement of the 2′-methoxy group of griseofulvin by homologous alkyloxy groups had the greatest effect, maximum activity being found in the 2′-propoxy analogue of griseofulvin (R 10/122) with the 2′-*n*-butoxy (S2) and 2′-ethoxy (R 10/32) analogues of griseofulvin next in order of activity. Replacement by allyloxy (R 10/62) or benzyloxy (R 10/78) at the 2′-position in griseofulvin increased activity against *B. cinerea in vitro*, but had no effect (R 10/62) or reduced (R 10/78) activity against *S. fructigena*. Halogen, benzyl or certain alkyl groups substituted at the 3′-position of griseofulvin also resulted in enhanced activity *in vitro* against *B. cinerea* and *S. fructigena*. Enhancement of activity by 3′-halogen substitution increased in the order Cl (R 10/64), Br (R 10/20) to I (R 10/55). Association of the more active substituent groups at the 3′-position with the more active replacement groups at the 2′-position showed a potentiated response against *B. cinerea* and *S. fructigena* in several analogues. The greatest improvement resulted from 2′-propoxy replacement along with 3′-substitution of I (R 10/163), Br (R 10/154) or Cl (R 10/158). Similar but less marked effects were found when 2′-ethoxy replacement was associated with halogen substitution at the 3′-position of griseofulvin.

Some of these 2′, 3′-analogues of griseofulvin showed phenomenal improvement in activity in the 'hyphal curling' test on *Botrytis allii*: the 2′-butoxy-3′-bromo (R 10/167), 2′-propoxy-3′-iodo (R 10/163) and 2′-propoxy-3′-benzyl (R 10/162) analogues of griseofulvin were more than 500 times as active as griseofulvin itself. This exceptionally high activity was also specific, as it was approached neither in the Petri dish tests nor when *B. cinerea* was substituted for *B. allii* in the 'hyphal-curling' test.

Substantially increased activity against most of the plant pathogenic fungi was observed also in the 2′-alkyl-thioether analogues of griseofulvin (Table 1*b*), but in this series there was no potentiated response when any of the 2′-replacements were associated with an activating substituent at the 3′-position. The 2′-ethyl-thioether analogue of griseofulvin (R 10/98) must probably be considered the most active among analogues of this type, with the corresponding 2′-allyl-(R 10/128) and 2′-benzyl-(R 10/116) thioethers next in order of activity and broadness of antifungal spectrum. It is notable that in this series the 2′-*n*-butoxy-thioether (R 10/152) showed improvement only against *Botrytis allii* and *Aspergillus niger*.

Some of the analogues that showed most promise against the plant pathogenic fungi *in vitro* were also tested for inhibiting radial growth of a plant pathogenic

strain of *Stereum purpureum*. The 2′-propoxy-3′-propyl analogue of griseofulvin (R 10/178) was found to be the most active (minimum inhibitory concentration $< 0{\cdot}3$ μg./ml.) of those tested, with the 2′-ethoxy-3′-benzyl analogue of griseofulvin (R 10/141) and the 3′-iodo analogue of griseofulvin (R 10/55) effective at $0{\cdot}6$ μg./ml: griseofulvin required 10–20 μg./ml. for complete inhibition.

Only a few analogues of isogriseofulvin (R 10/8) showed any marked improvement over griseofulvin *in vitro*. The 3′-benzyl analogue of isogriseofulvin (R 10/95) exhibited the broadest spectrum of improved antifungal action, but the improvement shown by most other analogues of this type was fairly specific to *Sclerotinia fructigena* and *Glomerella cingulata* (Table 1*c*). Isogriseofulvin itself was less active

Table 2. *Average performances of some griseofulvin analogues in greenhouse foliage protection tests against* Botrytis cinerea *on tomato*

Ref. no.	Analogue	Approximate activity relative to griseofulvin: Less active	Equal activity	More active
R 10/96	I; R, R^1, $R^2 = CH_3$, X = C_6H_5	*	.	.
R 10/92	I; R, R^1, $R^2 = CH_3$, X = $CH_2.CH{:}CH_2$	*	.	.
R 10/55	I; R, R^1, $R^2 = CH_3$, X = I	.	.	*
R 10/32	I; R, $R^1 = CH_3$, $R^2 = C_2H_5$, X = H	.	*	.
R 10/122	I; R, $R^1 = CH_3$, $R^2 = nC_3H_7$, X = H	.	.	*
S2	I; R, $R^1 = CH_3$, $R^2 = nC_4H_9$, X = H	.	.	*
R 10/18	I; R, $R^1 = CH_3$, $R^2 = nC_6H_{13}$, X = H	*	.	.
R 10/62	I; R, $R^1 = CH_3$, $R^2 = CH_2.CH{:}CH_2$, X = H	.	.	*
R 10/126	I; R, $R^1 = CH_3$, $R^2 = C_2H_5$, X = Cl	.	*	.
R 10/125	I; R, $R^1 = CH_3$, $R^2 = C_2H_5$, X = Br	.	.	*
R 10/81	I; R, $R^1 = CH_3$, $R^2 = C_2H_5$, X = I	.	.	*
R 10/141	I; R, $R^1 = CH_3$, $R^2 = C_2H_5$, X = C_6H_5	.	.	*
R 10/158	I; R, $R^1 = CH_3$, $R^2 = nC_3H_7$, X = Cl	.	.	*
R 10/154	I; R, $R^1 = CH_3$, $R^2 = nC_3H_7$, X = Br	.	.	*
R 10/163	I; R, $R^1 = CH_3$, $R^2 = nC_3H_7$, X = I	.	.	*
R 10/178	I; R, $R^1 = CH_3$, $R^2 = nC_3H_7$, X = nC_3H_7	.	.	*
R 10/162	I; R, $R^1 = CH_3$, $R^2 = nC_3H_7$, X = C_6H_5	.	.	*
R 10/173	I; R, $R^1 = CH_3$, $R^2 = nC_4H_9$, X = Cl	.	.	*
R 10/174	I; R, $R^1 = CH_3$, $R^2 = nC_4H_9$, X = I	.	*	.
R 10/109	I; R = $CH_2.CH{:}CH_2$, R^1, $R^2 = CH_3$, X = H	*	.	.
R 10/129	II; R, $R^1 = CH_3$, $R^2 = CH_3$, X = H	*	.	.
R 10/98	II; R, $R^1 = CH_3$, $R^2 = C_2H_5$, X = H	.	.	*
R 10/153	II; R, $R^1 = CH_3$, $R^2 = nC_3H_7$, X = H	*	.	.
R 10/128	II; R, $R^1 = CH_3$, $R^2 = CH_2.CH{:}CH_2$, X = H	.	.	*
R 10/116	II; R, $R^1 = CH_3$, $R^2 = C_6H_5$, X = H	*	.	.
R 10/85	II; R, $R^1 = CH_3$, $R^2 = C_6H_5.CH_2$, X = H	*	.	.
R 10/95	III; R, R^1, $R^2 = CH_3$, X = C_6H_5	.	.	*

than griseofulvin against all test fungi except *G. cingulata*. Neither griseofulvic acid nor any of its analogues was as effective as griseofulvin in any of the tests. Most replacements at other positions in the griseofulvin molecule diminished antifungal activity to below that of griseofulvin, although a few of the 4- or 6-alkyloxy analogues of griseofulvin (Table 1*a*) showed an improvement limited to a few species.

Selected analogues more active than griseofulvin against *Botrytis cinerea in vitro* were tested at 20 μg./ml. concentration for ability to protect foliage of young

tomato plants against *B. cinerea* in greenhouse tests. Average performances in a considerable number of greenhouse tests are summarized in Table 2. In general, analogues that exhibited enhanced activity *in vitro* also showed to advantage in the greenhouse tests, but it was noted that the most active of the 2′-alkyloxy analogues of griseofulvin were relatively better *in vivo*, whereas the most active of the 2′-alkyloxy-3′-substituted analogues of griseofulvin were relatively worse than had been expected. It was notable that, although the 3′-benzyl analogue of griseofulvin (R 10/96) and the 3′-benzyl analogue of isogriseofulvin (R 10/95) showed a similar enhancement of activity *in vitro*, the former was completely inactive in the greenhouse protection test, but the latter performed much better than might have been expected.

Table 3. *Local systemic protection against* Botrytis cinerea *afforded to ventral leaf surfaces of tomato by griseofulvin analogues applied to dorsal surfaces*

Ref. no.	Analogue	Approximate activity relative to griseofulvin: Less active	Equal activity	More active
R10/92	I; R, R^1, $R^2 = CH_3$, X = $CH_2.CH{:}CH_2$	*	.	.
R10/122	I; R, $R^1 = CH_3$, $R^2 = nC_3H_7$, X = H	.	.	*
S2	I: R, $R^1 = CH_3$, $R^2 = nC_4H_9$, X = H	.	*	.
R10/81	I; R, $R^1 = CH_3$, $R^2 = C_2H_5$, X = I	.	*	.
R10/158	I; R, $R^1 = CH_3$, $R^2 = nC_3H_7$, X = Cl	.	.	*
R10/154	I; R, $R^1 = CH_3$, $R^2 = nC_3H_7$, X = Br	.	.	*
R10/163	I; R, $R^1 = CH_3$, $R^2 = nC_3H_7$, X = I	.	.	*
R10/173	I; R, $R^1 = CH_3$, $R^2 = nC_4H_9$, X = Cl	*	.	.
R10/174	I; R, $R^1 = CH_3$, $R^2 = nC_4H_9$, X = I	.	*	.
R10/129	II; R, $R^1 = CH_3$, $R^2 = CH_3$, X = H	.	*	.
R10/98	II; R, $R^1 = CH_3$, $R^2 = C_2H_5$, X = H	.	*	.
R10/153	II; R, $R^1 = CH_3$, $R^2 = nC_3H_7$, X = H	*	.	.

Similar results were obtained (Table 3) when some of these analogues were tested for local systemic action in experiments that depended upon movement from the dorsal to the ventral leaf surface. In general, the analogues tested were about half as effective in the test for local systemic action as in the normal foliage protection test.

Previous experience had shown that griseofulvin had some activity against apple scab (*Venturia inaequalis*) in greenhouse protection tests on apple root stocks and that a concentration of 200 μg./ml. was about equivalent to LD 60. This test seems to put a premium on systemic action, and it was decided to evaluate in this manner some of the analogues that had proved to have good local systemic action. The analogues listed below were found to be much more effective than griseofulvin in these greenhouse tests against apple scab, 200 μg./ml. of each analogue affording more than 90% protection against the disease: R 10/96—I; R, R^1, $R^2 = CH_3$, X = $C_6H_5.CH_2$; R 10/32—I; R, $R^1 = CH_3$, $R^2 = C_2H_5$, X = H; R 10/141—I; R, $R^1 = CH_3$, $R^2 = C_2H_5$, X = $C_6H_5.CH_2$; R 10/122—I; R, $R^1 = CH_3$, $R^2 = nC_3H_7$, X = H; R 10/158—I; R, $R^1 = CH_3$, $R^2 = nC_3H_7$, X = Cl; R 10/154—I; R, $R^1 = CH_3$ $R^2 = nC_3H_7$, X = Br; R 10/163—I; R, $R^1 = CH_3$, $R^2 = nC_3H_7$, X = I; R 10/162—I; R, $R^1 = CH_3$, $R^2 = nC_3H_7$, X = $C_6H_5.CH_2$.

R 10/129—II; R, $R^1 = CH_3$, $R^2 = CH_3$, X = H; R 10/98—II; R, $R^1 = CH_3$, $R^2 = C_2H_5$, X = H; R 10/153—II; R, $R^1 = CH_3$, $R^2 = nC_3H_7$, X = H; R 10/152—II; R, $R^1 = CH_3$, $R^2 = nC_4H_9$, X = H.

Further investigations with lower concentrations of the same analogues showed that the 2′-ethoxy (R 10/32), the 2′-*n*-propoxy (R 10/122) and the 2′-*n*-propylthioether (R 10/153) analogues of griseofulvin were probably the most active compounds against apple scab, all being superior to the corresponding analogues substituted by halogen or benzyl at the 3′-position.

Griseofulvin is known to be particularly effective against some powdery mildews; as this activity appears to be largely dependent upon systemic action (Rhodes, Crosse, McWilliam, Tootill & Dunn, 1957), we began an investigation into the effectiveness against powdery mildews of the three compounds which had proved to be most effective in the tests against apple scab. All three compounds showed greatly enhanced activity over that of griseofulvin against *Erysiphe graminis* on barley, only 5 μg./ml. of each analogue being required for the LD 95 dose as compared with more than 100 μg./ml. for griseofulvin.

Table 4. *Control of chrysanthemum mildew* (Oidium chrysanthemi) *by 2′-propoxy-griseofulvin* (*R* 10/122) *applied as foliage spray at intervals of* 14 *days from mid-October*

	Percentage reduction of chrysanthemum mildew					
	var. 'Sterling'			var. 'Mayford Perfection'		
	2′-Propoxy-griseofulvin (μg./ml.)		Karathane (μg./ml.)	2′-Propoxy-griseofulvin (μg./ml.)		Karathane (μg./ml.)
Date of disease estimation	100	25	100	100	25	100
15-11-60	61	60	66	26	57	57
28-11-60	100	98	100	46	54	61
19-12-60	98	99	99	75	80	81
4-1-61	100	100	100	85	83	86

Further tests showed that the 2′-propoxy analogue of griseofulvin (R 10/122) was about equal in activity to Dinocap (used as the approximately 25% wettable powder marketed by Rohm and Haas Company under the trade mark 'Karathane') in greenhouse protection tests against powdery mildew of cucumber (*Erysiphe cichoracearum*), both materials giving almost complete control at 80 μg./ml. The dose-response curve of the griseofulvin analogue was, however, much steeper than that of Dinocap, with the result that the analogue compared progressively less favourably as the concentration was diminished. The 2′-propoxy analogue of griseofulvin (R 10/122) proved to be highly effective as a foliage protectant spray against chrysanthemum mildew (*Oidium chrysanthemi*), 25 and 100 μg./ml. applied every 14 days giving virtually complete protection in greenhouse tests (Table 4). This analogue was much less effective against apple mildew (*Podosphaera leucotricha*), 160 μg./ml. giving only partial protection.

DISCUSSION

Rhodes (1962), in a critical review of the status of griseofulvin in crop protection, noted that three distinct processes contribute to the antifungal action of griseofulvin in plants: the effect of griseofulvin on the pathogen; the transportation of the antibiotic within the host; the extent to which chemical degradation occurs in both host and pathogen. These considerations may help to explain some of the results now reported.

In general, analogues which exhibited enhanced activity *in vitro* against plant pathogenic fungi also showed to advantage in greenhouse tests. Maximum *in vitro* activity in the homologous series of alkyloxy replacements at the 2′-position is shown by the 2′-propoxy (R 10/122) and 2′-butoxy (S 2) analogues of griseofulvin; this accords with the relative order of activity in this series of compounds against *Botrytis allii* reported by Crowdy, Grove & McCloskey (1959) and with their suggestion that effects on physical properties may be dominant.

Hyphal walls of the numerous test fungi may be expected to show some diversity in their requirements for the physical properties of an analogue that is to give optimum adsorption by, and penetration of, the hyphae. Restriction of improved activity *in vitro* to a limited number of diverse test organisms may then simply reflect the extent to which these optimum requirements depart from the mean of those against all the test organisms. The small number of analogues which showed improved performance against the dermatophytes may be due to the relative homogeneity of this group and close approximation of their optimum requirements to the physical properties of the 2′-ethoxy-3′-benzyl (R 10/141) analogue of griseofulvin, to which they are highly susceptible.

The most active of the 2′-alkyloxy analogues of griseofulvin performed relatively better *in vivo* but the most active of the 2′-alkyloxy-3′ halogen-substituted analogues of griseofulvin were relatively inferior to expectation in plants, particularly in tests where local systemic action offered a premium. These observations suggest that substitution at the 3′-position, although enhancing *in vitro* antifungal activity, may interfere with processes that affect local systemic action in plants; for example penetration into the leaf or translocation within it. Similarly, optimum requirements for adsorption by hyphae and penetration into dermatophytic fungi must differ from those needed for entry to dermal tissue of the animal host, because Neves (1962) and Munro-Ashman (1962) reported that topical application of the 2′-ethoxy-3′-benzyl (R 10/141) analogue of griseofulvin which showed enhanced activity *in vitro* against dermatophytic fungi did not control *tinea pedis* in man.

These observations on the antifungal activity *in vivo* and *in vitro* of the ring C analogues of griseofulvin accord with the suggestion of Crowdy *et al.* (1959) that for each similar group of griseofulvin analogues there may be an optimum high oil/water partition coefficient, both for increased activity *in vitro* and for systemic antifungal activity. It is notable that Crowdy *et al.* (1959) found the 2′-propoxy analogue of griseofulvin to be non-systemic when tested by root application to tomato plants; in our tests this analogue showed local systemic action and gave good protection when applied as a foliage spray, especially in the test against apple scab where local systemic action was probably at a premium. This suggests a greater divergence between the partition coefficient which gives optimum *in vitro* activity

and that needed for good translocation from roots to shoots, than between the partition coefficients which afford optimum *in vitro* and local systemic action in foliage, respectively.

Physical properties alone can hardly account for the enormous and specific increase in activity observed in the hyphal curling test when halogen or benzyl groups were substituted in the 3′-position. Optimum activity in the 2′-*n*-propoxy analogues of griseofulvin was found with I (R 10/163) or $C_6H_5.CH_2$ (R 10/162) in the 3′-position, but in the 2′-*n*-butoxy analogues of griseofulvin peak activity occurred with bromine substitution in the 3′-position. It is notable that *Botrytis allii* can demethylate griseofulvin at the 2′-position to griseofulvic acid (Boothroyd, Napier & Somerfield, 1961); perhaps the specific and substantial enhancement of activity observed against this organism is due to supplementary effects of the 3′-substituent group on electron distribution within the β-diketone-enol-ether system, diminishing the tendency to de-alkylation of the 2′-position.

The specificity of some of the responses observed suggests that in any antifungal screening it is important where possible to select as test fungi those organisms that are the objectives for control in the field; the selection of fungi for tests because of convenience in the laboratory may result in misleading conclusions. Similarly, correlation of chemical structure with antifungal activity may have little meaning unless the responses of a wide range of fungi are examined.

The 2′-propoxy analogue of griseofulvin, despite its failure to give systemic control of *Alternaria solani* when watered to roots of tomato plants (Crowdy *et al.* 1959), seems to be the best antifungal substance among the 300 or more analogues tested for potential use as plant protectants; in our tests it showed improved activity *in vitro* and *in vivo*, exhibited local systemic action as a foliage spray, gave a good performance against apple scab (*Venturia inaequalis*) and provided excellent control of chrysanthemum mildew (*Oidium chrysanthemi*) in greenhouse tests. The activity of the 2′-propoxy analogue of griseofulvin (R 10/122) against apple scab was, however, much inferior to that of the antibiotic venturicidin (Rhodes *et al.* 1961), and it should be noted that complete control of chrysanthemum mildew has already been obtained by root-watering with griseofulvin itself (Rhodes, 1962). Having regard to the increased cost relative to griseofulvin, the outlook for practical use of analogue R 10/122 must be considered to be unfavourable.

REFERENCES

ARKLEY, V., ATTENBURROW, J., GREGORY, G. I. & WALKER, T. (1963). Griseofulvin analogues. I. Modification of the aromatic ring. *J. chem. Soc.* p. 1260.

ARKLEY, V., GREGORY, G. I. & WALKER, T. (1963). Griseofulvin analogues. VI. Dichlorogriseofulvin and some of its derivatives. *J. chem. Soc.* p. 1603.

BARNETT, S. M. (1960). Treatment of various fungal diseases with griseofulvin discussed. *Wis. med. J.* **59**, 421.

BOOTHROYD, B., NAPIER, E. J. & SOMERFIELD, G. A. (1961). The demethylation of griseofulvin by fungi. *Biochem. J.* **80**, 34.

BRIAN, P. W., CURTIS, P. J. & HEMMING, H. G. (1946). A substance causing abnormal development of fungal hyphae produced by *Penicillium janczewskii* Zal. I. Biological assay, production and isolation of curling factor. *Trans. Brit. mycol. Soc.* **29**, 173.

CROSSE, R., MCWILLIAM, R., RHODES, A. & DUNN, A. T. (1960). Antifungal action of streptomycin-copper chelate against *Phytophthora infestans* on tomato (*Lycopersicon esculentum*). *Ann. appl. Biol.* **48**, 270.

GROWDY, S. H., GROVE, J. F. & MCCLOSKEY, P. (1959). The translocation of antibiotics in higher plants. 4. Systemic antifungal activity and chemical structure in griseofulvin relatives. *Biochem. J.* **72**, 241.

GOODALL, S. R., GREGORY, G. I. & WALKER, T. (1963). Griseofulvin analogues. VII. Replacement in the aromatic ring. *J. chem. Soc.* p. 1610.

GREGORY, G. I., HOLTON, P. J., ROBINSON, H. & WALKER, T. (1962). Griseofulvin analogues. II. Some 3′ alkyl griseofulvic acids and their enol esters. *J. chem. Soc.* p. 1269.

GRUTTER, F. H., GAUGHRAN, E. R. L., SWARTZ, H. E. & KAMP, L. F. (1959). Microbiological assay of griseofulvin using *Neurospora crassa*. *Bact. Proc.* A 46.

KIRKHAM, D. S. (1956). A culture technique for *Venturia* spp. and a turbidimetric method for the estimation of comparative sporulation. *Nature, Lond.* **178**, 550.

MUNRO-ASHMAN, D. (1962). Topical griseofulvin analogue in *tinea pedis*. *Trans. St John's Hosp. derm. Soc., Lond.* **48**, 108.

NEVES, H. (1962). *Tinea pedis*—topical treatment with griseofulvin and an analogue. *Trans. St John's Hosp. derm. Soc., Lond.* **48**, 103.

PAGE, J. E. & STANIFORTH, S. E. (1962). Griseofulvin analogues. V. Infrared absorption. *J. chem. Soc.* p. 1292.

PAGE, J. E. & STANIFORTH, S. E. (1963). Griseofulvin analogues. VIII. Infrared absorption of griseofulvic acid and related compounds. *J. chem. Soc.* p. 1814.

RHODES, A. (1962). Status of griseofulvin in crop protection. In *Antibiotics in agriculture. Proceedings of the University of Nottingham Ninth Easter School in Agricultural Science*, p. 101. London: Butterworth's Scientific Publications.

RHODES, A., CROSSE, R., MCWILLIAM, R., TOOTILL, J. P. R. & DUNN, A. T. (1957). Small-plot trails of griseofulvin as a fungicide. *Ann. appl. Biol.* **45**, 215.

RHODES, A., FANTES, K. H., BOOTHROYD, B., MCGONAGLE, M. P. & CROSSE, R. (1961). Venturicidin: a new antifungal antibiotic of potential use in agriculture. *Nature, Lond.* **192**, 952.

STEPHENSON, L., WALKER, T., WARBURTON, W. K. & WEBB, G. B. (1962). Griseofulvin analogues. IV. The preparation and properties of some chlorides. *J. chem. Soc.* p. 1282.

STERNBERG, T. H. (1960). A brief review of the value of griseofulvin as a therapeutant. *Curr. Ther.* **2**, 158.

UVAROV, O. (1961). Recent advances in the treatment of skin diseases with special reference to griseofulvin. *Vet. Rec.* **73**, 258.

WALKER, T., WARBURTON, W. K. & WEBB, G. B. (1962). Griseofulvin analogues. III. Halogen derivatives of griseofulvin. *J. chem. Soc.* p. 1277.

WILLIAMS, D. I. (1960). The pharmacology, toxicology and clinical uses of griseofulvin are reviewed. *Practitioner*, **184**, 383.

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Studies on the Structure of Vaccinia Virus

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SUMMARY

Vaccinia virus has been examined with the electron microscope by several techniques in conjunction with experimental modifications of structure. Negative staining revealed that over 80% of the virus in highly purified preparations were particles which appeared to have a beaded surface like a mulberry and were termed M forms. The beading was formed by loops of thread-like structures 90 Å wide which were themselves double helices formed from two 30 Å strands coiled to a 120 Å pitch. Twenty per cent or less of the particles appeared as larger more electron-dense bodies with a capsule of complex structure; these have been termed C forms. Experimental interconversion of the two forms showed that both were structurally mature virus. The relationship of structures seen in C forms to those revealed by thin sectioning is considered. Experimental findings and the results of shadowing and replica examinations indicate the presence of a superficial protein layer of antigenic material not revealed by negative staining. The relationship of this layer to the structure of the virus and the nature of the ribbon structure of the M form are discussed.

INTRODUCTION

Electron microscopic examination of shadowed preparations of mature vaccinia virus has shown the virus to be a brick-shaped particle thickened at the centre and at the four corners and possessing a beaded surface. This appearance was well shown by Dawson & McFarlane (1948) who used virus purified by salt flocculation, fixed with osmic acid and shadowed with gold. Examination of thin sections has shown the virus to possess a relatively complex structure with an outer multilayered membrane surrounding a biconcave nucleoid in the concavities of which are situated a pair of lens-shaped lateral bodies which are responsible for the central thickening of the particle (Morgan, Ellison, Rose & Moore, 1954; Peters, 1956; Epstein, 1958*a*, *b*; Dales & Siminovitch, 1961). Recently the negative-staining technique (Brenner & Horne, 1959) has revealed the presence of structures of about 90 Å width which were interpreted as hollow rods by Herzberg, Kleinschmidt, Lang & Reuss (1961), as predominantly spherical structures sometimes running together to form cords by Noyes (1962*a*, *b*), and as long tubular threads by Nagington & Horne (1962). The latter workers showed that in the virus of contagious pustular dermatitis (orf) these threads were wound around the particle in an orderly manner, but little such order is detectable in the case of vaccinia or canary-pox (Herzberg *et al.* 1961). Nagington & Horne also described a second type of particle lacking the thread structure and differing in size from the first type. The present study was

undertaken in order to give a morphological basis to chemical and antigenic studies. Several techniques of examination have been used on highly purified preparations of mature virus. The results suggest that the use of any one technique alone leads to over-simplification in the interpretation of results.

METHODS

Virus. A dermal strain of vaccinia virus was grown on rabbit skin as described by Hoagland, Smadel & Rivers (1940) and purified by density-gradient centrifugation by the method of Zwartouw, Westwood & Appleyard (1962). The final deposit was suspended for electron microscopy in distilled water, to each litre of which one drop of concentrated ammonia was added to assist dispersal of the virus.

Trypsin. Freshly prepared solutions of Armour crystalline trypsin were used in appropriate experiments.

Electron microscopy. Grids coated with carbon or formvar were used throughout. Virus suspensions inactivated by ultraviolet irradiation were either sprayed on to the grids or were applied by means of a pipette, excess fluid being removed with filter paper. Specimens were examined in the Siemens Elmiskop 1 electron microscope using double condenser illumination with 200μ condenser aperture and 50μ objective aperture. 80 kV. accelerating voltage was used for negatively stained specimens and 60 kV. for all others.

Negative staining. Two per cent (w/v) potassium phosphotungstate (PTA) at pH 5–6 was used as originally recommended by Brenner & Horne (1959). Virus was either mixed with the stain in equal volumes and sprayed on to coated grids or was first applied to the grids with a pipette and then stained *in situ*.

Shadowing. Shadowing was carried out by using a device which permitted shadowing through an aperture at various fixed angles (Harris, 1962). Materials used were gold and palladium, carbon and platinum or carbon alone.

Replicas. Self-shadowed replicas were obtained by the evaporation of carbon on to glass slides on which purified virus had been dried. After stripping, the carbon films were floated on to 2% KOH at 60° to dissolve any adherent virus particles.

RESULTS

Observations by using negative staining techniques

Preparations of purified vaccinia virus were shown by negative staining with PTA to contain two types of particle as reported by Nagington & Horne (1962). In fresh preparations between 80 and 90% of the virus consisted of particles which had a beaded appearance like a mulberry; these will be referred to as the 'M' form. The remainder were larger capsulated particles and will be referred to as the 'C' form. The two forms are shown together in Pl. 1, fig. 1.

M form. The M form of the virus (Pl. 1, figs. 3, 4, Pl. 2, fig. 6) possessed a beaded surface and a toothed edge. The central thickening could usually be distinguished and the particle bore an obvious similarity to the usual picture of shadowed virus (Pl. 3, fig. 12). The surface beading was usually completely irregular but occasional particles in suitable orientation revealed a regular arrangement of teeth around the margin of the particle, 40–42 being present at intervals of about 270Å. Despite

their occasional appearance of regularity there is no basic difference between these marginal serrations and the beading over the remainder of the virus surface. Each tooth is formed from a single loop of a long thread as may be seen in Pl. 1, fig. 3. The appearance at A in this figure suggests that the thread is arranged as a coil or zig-zag around the edge of the particle, neighbouring teeth being formed from adjacent coils. However, this appearance is probably illusory since, first, other photographs show the threads to run across the body of the virus in an arrangement more like that of orf (Pl. 1, fig. 5) and, secondly, for reasons discussed below, the thread structure must be entirely superficial, being limited to a depth of no more than 100–150 Å. While this would not preclude a flat zig-zag it would preclude coiling of the order of size suggested by these particles. It seems more likely that neighbouring teeth are formed from neighbouring threads lying parallel to each other and looping around the virus particle and this is in fact seen to be the case in particles viewed on edge.

As noted by previous workers (Nagington & Horne, 1962; Herzberg *et al.* 1961) most threads are about 90 Å wide and frequently appear as double strands suggesting tubes with hollow centres about 30 Å in diameter. The structure, however, appears to be more complex than a simple tube. Owing to the intricacy of their interweaving the threads are seldom seen clearly for more than short distances, though their general course may frequently be traced for many hundred Ångstrom units in length. Over short distances, however, they are often seen with sufficient clarity to suggest that they are helical structures, three types of appearance being distinguishable.

(1) An appearance of frank coiling may sometimes be seen, especially at the edge of a particle (Pl. 2, fig. 6, at A, fig. 7*b* and 7*c*).

(2) Occasionally a chain-stitch appearance may be distinguished (Pl. 1, fig. 5, at B; Pl. 2, fig. 6, at B).

(3) More frequently, each strand of the thread appears as a series of dots at about 60 Å intervals (Pl. 1, fig. 5, at C and C_1; Pl. 2, fig. 6, at C, fig. 7*a*). Occasionally a third row of fainter dots intervenes between the two main series.

The first two appearances directly suggest a helical structure, and the third could be produced by such a structure if each dot represented the point at which the coiled strand turned into the axis of the electron beam, the faint central line of dots being the cross-over points of two interlocking strands (Fig. 1). In Pl. 2, fig. 7*a*, a thread composed of two such series of dots terminates in an open figure of eight in which the terminal coils of the individual strands can be clearly seen. In Pl. 1, fig. 5, at C_1, there is a strong suggestion of the individual strands connecting the dots of the two series in the unusually wide left-hand portion of the thread which appears to pass into a thread of more usual width at the right. It can be seen from this and other photographs that there is, in fact, considerable variation in thread width. It would appear, therefore, that each of the long threads which can be distinguished in this type of particle consists of a double-stranded ribbon coiled in its long axis to form a double helix of about 120 Å pitch. In certain photographs detailed examination of the individual 30 Å strands comprising the ribbons shows a regular arcading or barring at about 17 Å interval. This appearance, which does not seem to be either astigmatic or photographic in origin, suggests that each strand may itself be made up of a filament or filaments of 6–10 Å diameter

coiled to a 17 Å pitch. The appearance must clearly be treated with the greatest caution since it lies near the theoretical limit of resolution of the instrument even under optimal conditions. However, the suggested structure is not illogical if, as seems likely, the thread structure is protein in nature, since the peptide chain complexes of fibrous proteins are probably about 6–10 Å in diameter (Crick & Kendrew, 1957). Three kinds of helical structure might then be involved: (1) a peptide helix; (2) the 30 Å strand made up of a coil of one or more of such helices; (3) the 90 Å thread consisting of a double helix made up from two 30 Å strands.

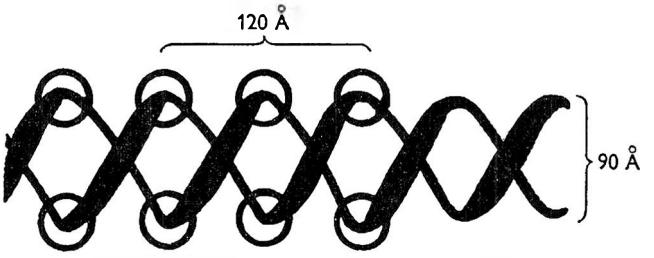


Fig. 1. Suggested structure for the threads of the M form of vaccinia virus. The circles represent the points at which the strands forming the double helix turn into the axis of the electron beam and may show as a series of dots. Compare with Pl. 2, fig. 7*a*.

The arrangement of the threads on the virus particle is difficult to determine. In most particles there appeared to be a complex felt-work of interlacing threads, but occasional particles showed the more orderly arrangements already mentioned, particularly in those regions where the marginal serrations can be clearly seen. It seems likely therefore that the basic arrangement is orderly even if its nature cannot be distinguished.

C form. Of the virus particles in a fresh preparation 20% or less differed from those described above. These C particles were larger than the M form, were more electron dense, did not show the ribbon structure and possessed a capsule (Pl. 1, fig. 1; Pl. 2, fig. 8). The capsule, which was 200–250 Å in thickness, surrounded an inner body of uniform electron density with little or no indication of the central thickening seen in the M form and in shadowed preparations. The outer surface of the capsule of the C form showed as a somewhat ragged membrane which was crenated to form teeth at the same intervals as those on the M form. Its inner surface was smooth, but no definite membrane was distinguished, the capsule being bounded by the outer surface of the smooth inner body. The capsular region was divided into inner and outer portions, the former being subdivided by radially disposed partitions giving an appearance of pallisading (Pl. 1, fig. 1; Pl. 2, fig. 8). The partitions responsible for the pallisading were at about 75–80 Å intervals and could be resolved into double-stranded structures about 45 Å wide, suggestive of tubes with hollow centres (Pl. 2, fig. 8, at A and B; Pl. 3, fig. 10, at A), as suggested by Noyes (1962*b*).

When the side elevation of the C form was studied (Pl. 3, figs. 10, 11) it at once became obvious that the outer zone of the capsule corresponded to the outer multilayered membrane, and the smooth inner body to the nucleoid, as seen in thin section (Pl. 1, fig. 2). The pallisaded zone was seen to be integral with the nucleoid

and followed its contours, while the outer limiting membrane passed over the surface of the lateral body which appeared to lie between this membrane and the pallisaded zone. At each end of the virus particle the nucleoidal structures were within 125 Å of the virus surface; the identity of the pallisaded zones in the side and plan elevations of the virus may be clearly seen from Pl. 3, fig. 10. Except in the region of the lateral bodies, therefore, the nucleoid everywhere approached very close to the virus surface and the superficial structures overlying it were limited to a depth of about 125 Å. The surface of the nucleoid underlying the pallisaded zone presented the appearance of a layer about 45 Å thick surrounding a stippled area comprising the nucleoidal body. This layer was not, however, a continuous membrane, but itself appeared as a series of elongated dots at 45 Å interval (Pl. 2, fig. 8, at A) which seemed to be aligned with the partitions of the pallisaded zone and sometimes appeared to be similarly aligned with rows of dots forming parallel 'tubules' in the stippled area of the nucleoid (Pl. 3, fig. 11, at A). However, the nature of the fine structure in the body of the nucleoid is not easily distinguished and there is no indication that stain penetrates beyond its surface in the untreated virus. Thus the nucleoids in Pl. 3, figs. 10, 11, give no hint of the internal structure suggested in the section shown in Pl. 1, fig. 2. Indications of deeper structure are only seen following chemical treatment of the virus; Peters (1962) has obtained very revealing pictures of such structure following alkali treatment.

Relationship of M forms to C forms. The simplest explanation of the relationship between the two forms is that the larger C form is the complete form of the virus and represents an M particle surrounded by a capsule which masks the thread structure. This explanation was suggested by Nagington & Horne (1962) who interpreted their Type I particle as the inner disc-shaped body described by Morgan *et al.* (1954) and now usually termed the nucleoid, the thread structure possibly representing DNA. There are, however, several objections to this interpretation. In the first place, thin sectioning of the virus pellets obtained by centrifugation of the preparations used in the present work showed that all the virus present was structurally mature. Therefore the M forms, which make up 80 % of fresh preparations, are structurally mature particles. Secondly, the greater electron density of the C forms (Pl. 1, fig. 1) showed that they were more, and not less, penetrable by stain than the M forms. Thirdly, when measurements of the two forms are compared (Table 1), it is seen that, although the C forms were on average larger than the M forms, the difference was not adequate to accommodate a 250 Å capsule. The only explanation which appears to fit the observations is that both forms were structurally mature and the differences between them were due solely to differences in the degree of penetration of the stain; that in the M form the total exclusion of stain from the interior of the virus prevented visualization of the capsule and revealed only superficial structure, while penetration of stain into the C form caused optical obliteration of the superficial M structure, but revealed internal structure down to the surface of the nucleoid, causing at the same time an overall increase in electron density. This view is in agreement with that expressed by Noyes (1962*b*) and Dr D. Peters (personal communication). If this explanation be correct then increasing the penetration of stain into the virus should convert M forms to C forms. The following treatments were found to effect this conversion.

(1) *Trypsin.* Preparations of virus were incubated with 0·01 % crystalline trypsin

for 1 hr. or longer at pH 8. A small proportion of particles was converted into forms intermediate between M and C, in which both the capsule and residual M structure could be seen. Plate 4, fig. 14, shows a particle in which one corner (at A) reveals C structure with a suggestion of pallisading, while the remainder of the particle is clearly M.

(2) *Urea.* Virus treated with trypsin as above and placed on an electron microscope grid was washed and then inverted on a saturated solution of urea for 15 min. at room temperature. The preparation was again washed and then flooded with PTA. After this treatment 82% of the virus in one experiment and 96% in a second was found to be in the C form (Pl. 4, fig. 18). Subsequent experiments showed that urea effected the conversion, without preliminary treatment with trypsin.

(3) *Alkali.* Virus was placed on electron microscope grids, and washed by inversion on distilled water. A series of such preparations were then inverted for 1 hr. at room temperature on glycine buffers ranging from pH 9 to 12·8. After treatment at pH 11, 95%, and at pH 12, 100%, of the virus was found to be in the C form.

Table 1. *Measurements of M and C forms of vaccinia virus in negatively stained preparations*

The table compares measurements from untreated virus with those from virus treated in various ways. Measurements were made on photographs taken at × 10,000 magnification and are about 10% below similar measurements made at × 40,000 and 80,000.

Treatment	Virus form*	Length (range) (mμ)	Breadth (range) (mμ)
Nil (control)	M (40)	254 (235–280)	201 (167–224)
	C (40)	270 (235–339)	218 (180–262)
Trypsin	M (14)	246 (230–260)	187 (180–190)
	C (10)	252 (220–280)	203 (170–230)
Urea	C (23)	280 (245–300)	219 (190–250)
pH 10	M (14)	251 (240–264)	199 (187–211)
	C (14)	277 (246–303)	226 (205–244)
pH 10·5	M (15)	248 (233–261)	196 (182–205)
	C (15)	277 (264–292)	220 (207–250)
pH 11·0	M (15)	239 (219–283)	192 (180–209)
	C (15)	265 (259–280)	212 (201–230)
pH 12·8	M (24)	240 (220–255)	178 (150–190)
	C (21)	273 (250–320)	211 (180–240)
Weighted mean	M (112)	248 (219–283)	192 (150–224)
	C (138)	271 (220–339)	217 (170–262)
Difference:		23	25

* Figures in parentheses denote number of particles measured.

(4) *Drying.* Quantitative discrepancies in the results of repetitive experiments using these and other techniques of conversion led to a recognition of the fact that desiccation of the virus was alone sufficient to cause the conversion. The resulting problems in standardization of the preparative procedure were therefore investigated and the results will be the subject of a separate paper. These investigations showed

that not only did desiccation cause conversion to the C form, but reconstitution of the desiccated virus by soaking in distilled water caused reconversion to the M form.

(5) *Fat solvents.* Similar conversion followed treatment with fat solvents such as ethanol, ether, chloroform, acetone and carbon tetrachloride.

These results confirm that the difference between the two normally occurring forms of virus lies in their relative permeability to stain rather than in some gross structural difference between them, but do not indicate the nature of the barrier to PTA penetration into the M form. The difference may reflect relative states of physiological rather than structural maturity, but there is no evidence for this. While the slightly greater size of the C form might be due to immaturity it seems more likely to be due to swelling as a result of penetration of stain and water. Measurements of the size of particles converted to the C form as a result of chemical treatment showed that conversion was accompanied by swelling, but the converted particles did not differ in size from those in untreated preparations and the mean size of the unconverted M forms was not altered (Table 1). Normally occurring C forms frequently showed some breach in the virus surface which might have permitted the penetration of stain; an experiment showed that mechanical trauma by explosive decompression also converted M forms into C forms. It seems probable therefore that C forms in normal preparations represent virus particles injured or deficient in some way.

From the above findings it follows that, since the thread structure of the M form is revealed only when stain is totally excluded from the virus particle and is obliterated when stain penetrates, this structure must be entirely superficial. Since, further, the pallisaded zone of the capsule is integral with the nucleoid, the thread structure must be limited to the outer zone of the capsule—in a layer only about 125 Å thick. It cannot therefore be regarded as representing the structure of the virus as a whole.

Observations by using shadowing techniques

The appearance of osmic acid-fixed gold-shadowed virus has already been mentioned, but the interpretation of the apparent surface beading has always been complicated by the severity of the gold granulation inseparable from this technique. The surface pattern is better seen in preparations shadowed with platinum and carbon (Bradley, 1960) or better still with carbon alone (W. J. Harris, unpublished); the size of the resulting granulation then lies well below that of the surface beading and the nature of the latter is well indicated (Pl. 3, fig. 12). There is a general similarity to the beading and serration shown by negative staining, and the tooth intervals are similar. However, such structure is clearly shown only by virus which has been flocculated with sodium chloride or pre-treated with trypsin and then fixed with osmic acid before shadowing. When live virus, or virus killed by ultraviolet irradiation, was sprayed on to grids precooled with liquid nitrogen (grid temperature, $-150°$) by a modification of the technique of Williams (1953) and the snapfrozen droplets freeze-dried before shadowing, little indication of surface beading was seen. In fact, when collapse and shrinkage were eliminated, the virus was found to possess a surprisingly smooth surface even when shadowed with gold. On the other hand, if unfixed virus is sprayed at room temperature, the resulting collapse may be extreme and shadowing then reveals the presence of a superficial

wrinkled structure, possibly a membrane, collapsed down on to an underlying beaded surface (Pl. 4, figs. 16, 17). These results confirm the presence of a beaded structure but indicate that it is normally covered by some substance or structure which gives the virus a smooth surface. This substance is not revealed by negative staining but its presence may occasionally be deduced. For instance the M form in Pl. 1, fig. 4, and one of the C forms in Pl. 1, fig. 1, are seen to be surrounded by the remnants of an envelope such as is rarely seen in purified, but is relatively common in unpurified, preparations. The remnants are separated from the virus particle in a manner which suggests that they overlie a layer of some substance which is completely penetrated by the stain. The protein nature of this substance is suggested by the action of trypsin on the purified virus.

Effect of trypsin

In experiments to be reported elsewhere Zwartouw *et al.* (in preparation) showed that suitably controlled treatment with trypsin caused the solution of up to 20% of the virus-nitrogen, including components having virus immunospecificity. We have obtained similar results without the use of proteolytic enzymes, by prolonged incubation at 37° of virus suspensions containing 0·1% sodium azide. In the latter case, although up to 15% of the virus-protein was released into solution, the infectivity of the virus suspension was not detectably diminished. Morphologically, despite chemical and antigenic evidence of protein release, very little difference was detectable between control virus and treated virus, whether these were examined by negative staining or shadowing techniques. In the absence of trypsin there was no detectable difference, but after trypsin treatment there was a softening of the thread detail seen in negatively stained preparations, though evidence of coiling could still be distinguished and no evidence was seen to suggest the digestion of thread structure (Pl. 4, fig. 15). As already mentioned, increased permeability to phosphotungstic acid was induced in some particles with the production of intermediate forms. In shadowed preparations the surface beading appeared to be more conspicuous but the differences were slight and gave rise to the suspicion that the effects of treatment might be due to the disintegration of a non-infective fraction of the virus population, rather than to the release of protein and antigen equally from all the particles present. Counts on treated and untreated samples, carried out by the method of Backus & Williams (1950), did not show any decrease in the total number of apparently intact virus particles present after successive hourly periods during 3 hr trypsin treatment; but it is felt that the method may not be sensitive enough for such results to be conclusive.

With this qualification it may be provisionally concluded that vaccinia virus possesses a superficial coating of protein material, which initially lies under a loose envelope, but which becomes exposed in the mature particle by the shedding of this envelope. The coating of protein material is not removed by physical purification procedures and is therefore strongly adsorbed to the virus surface; its initial location beneath an envelope and its antigenic affinities indicate that it is viral in origin and is not contaminating material derived from the host cells. Finally, its release on incubation without concurrent loss of infectivity suggests that it is not an essential part of the virus structure. These results give morphological form to the early

demonstration by Craigie & Wishart (1936) that soluble antigens may be leached out of the virus elementary bodies. The concept of vaccinia virus structure which emerges from this study is shown diagrammatically in Fig. 2.

Observations by replica technique

The surface appearance revealed by the replica technique (Pl. 2, fig. 9) could not be reconciled with that obtained by either the negative-staining or shadowing techniques. Surface beading was not apparent even after trypsin treatment of the virus. Instead, a complex fine structure was discernible with conspicuous pallisading at the virus margin. In the capsular region the surface configuration indicated a stepped collapse, dividing the capsule into inner and outer portions.

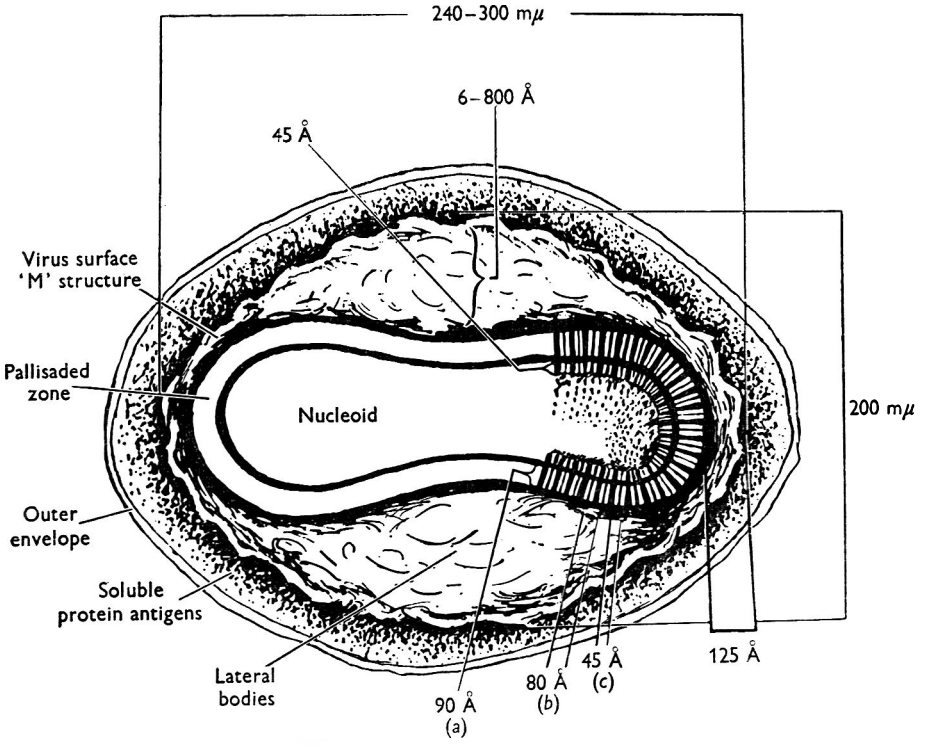


Fig. 2. Diagram of the side elevation of a virus particle showing the various layers identifiable with their suggested structure and principal dimensions.

'Spherical' particles

In all preparations of vaccinia virus and rabbit-pox virus examined a variable but small proportion (less than 10 %) of rounded particles was encountered. These particles were first seen by Sharp, Taylor, Hook & Beard (1946) who interpreted them as being normal particles seen end-on. Our observations and measurements, however, suggest that they are spheroidal bodies of 180–210 mμ diameter, sometimes with an apparent hexagonal outline (Pl. 3, fig. 13) which, by analogy with

adenovirus, might suggest an icosahedral form (Horne, Brenner, Waterson & Wildy, 1959). Their appearance in shadowed and negatively stained preparations indicated that they had a fine structure similar to normal particles.

DISCUSSION

Of the various techniques of examination used in the present work, negative staining undoubtedly contributed more new information than any other, but the interpretation of this information without reference to that supplied by other techniques and to the results of experimental modifications of structure can be misleading. This applies particularly to the relationships of the two predominant morphological forms (C, M) and the significance of the characteristic thread structure of the M form. The latter has been clearly shown to be superficial, probably limited to a surface layer about 125 Å thick, and cannot be taken to represent the structure of the virus as a whole. Furthermore, despite the temptation to interpret double helices in terms of DNA, it is highly unlikely that this structure includes nucleic acid. DNA comprises less than 5% of the dry weight of the virus, of which over 90% consists of protein, and it seems improbable that this genetic DNA material should be spread in a thin layer over the virus surface. It seems more likely that the thread structure is protein, but there is no direct evidence for this. The fact that the M $\rightarrow$ C conversion was brought about by fat solvents suggests that the impermeability of this layer to phosphotungstic acid might be due to the incorporation of lipid in the layer; this might also confer resistance to the action of proteolytic enzymes. Experiments to be reported elsewhere have shown that after ethanol treatment the virus is more susceptible to attack by trypsin. The susceptibility of the layer to desiccation, alkali and urea would also be in keeping with the presence of a lipoprotein structure. If the thread structure is in fact protein or lipoprotein, then its antigenic constitution is of the first importance since it probably represents the true constitution of the virus surface. The present work indicates that the precipitinogens extractable from the purified virus are contained in a structureless outer protein layer which is not essential to infectivity.

REFERENCES

BACKUS, R. C. & WILLIAMS, R. C. (1950). The use of spraying methods and of volatile suspending media in the preparation of specimens for electron microscopy. *J. appl. Phys.* **21**, 11.

BRADLEY, D. E. (1960). Study of background structure in platinum/carbon shadowing deposits. *Brit. J. appl. Phys.* **11**, 506.

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

CRAIGIE, J. & WISHART, F. O. (1936). Studies on the soluble precipitable substances of vaccinia. I. The dissociation of precipitable substances from the elementary bodies of vaccinia. *J. exp. Med.* **64**, 803.

CRICK, F. H. C. & KENDREW, J. C. (1957). X-ray analysis and protein structure. *Advanc. Protein Chem.* **12**, 133.

DALES, S. & SIMINOVITCH, L. (1961). The development of vaccinia virus in Earle's L-strain as examined by electron microscopy. *J. biophys. biochem. Cytol.* **10**, 495.

DAWSON, I. M. & MCFARLANE, A. S. (1948). Structure of an animal virus. *Nature, Lond.* **161**, 464.

EPSTEIN, M. A. (1958*a*). Structural differentiation in the nucleoid of mature vaccinia virus. *Nature, Lond.* **181**, 784.

EPSTEIN, M. A. (1958*b*). An investigation into the purifying effect of a fluorocarbon on vaccinia virus. *Brit. J. exp. Path.* **39**, 436.

HARRIS, W. J. (1962). Device for shadowcasting and making thin films for electron microscopy. *J. sci. Instrum.* **39**, 529.

HERZBERG, K., KLEINSCHMIDT, A., LANG, D. & REUSS, R. (1961). Vaccinevirus und Kanarienpockenvirus elektronenmikroskopisch bei Negativkontrastierung. *Naturwissenschaften*, **48**, 725.

HOAGLAND, C. L., SMADEL, J. E. & RIVERS, T. M. (1940). Constituents of elementary bodies of vaccinia. I. Certain basic analyses and observations on lipid components of the virus. *J. exp. Med.* **71**, 737.

HORNE, R. W., BRENNER, S., WATERSON, A. P. & WILDY, P. (1959). The icosahedral form of an adenovirus. *J. molec. Biol.* **1**, 84.

MORGAN, C., ELLISON, S. A., ROSE, H. M. & MOORE, D. H. (1954). Structure and development of viruses observed in the electron microscope. II. Vaccinia and fowl-pox viruses. *J. exp. Med.* **100**, 301.

NAGINGTON, J. & HORNE, R. W. (1962). Morphological studies on orf and vaccinia viruses. *Virology*, **16**, 248.

NOYES, W. F. (1962*a*). The surface fine structure of vaccinia virus. *Virology*, **17**, 282.

NOYES, W. F. (1962*b*). Further studies on the structure of vaccinia virus. *Virology*, **18**, 511.

PETERS, D. (1956). Morphology of resting vaccinia virus. *Nature, Lond.* **178**, 1453.

PETERS, D. (1962). Substrukturen des Vaccinevirus. 5th int. Congr. Electron Microscopy. *Electron Microscopy*, **2**, S2. New York: Academic Press Inc.

SHARP, D. G., TAYLOR, A. R., HOOK, A. E. & BEARD, J. W. (1946). Rabbit papilloma and vaccinia viruses and T_2 bacteriophage of *E. coli* in 'shadow' electron micrographs. *Proc. Soc. exp. Biol., N.Y.* **61**, 259.

WILLIAMS, R. C. (1953). A method of freeze-drying for electron microscopy. *Exp. Cell Res.* **4**, 188.

ZWARTOUW, H. T., WESTWOOD, J. C. N. & APPLEYARD, G. (1962). Purification of pox viruses by density gradient centrifugation. *J. gen. Microbiol.* **29**, 523.

EXPLANATION OF PLATES

PLATE 1

Fig. 1. Vaccinia virus stained with phosphotungstic acid. M and C forms showing the greater size and electron density of the latter. × 80,000.

Fig. 2. Vaccinia virus. Section of osmic acid-fixed virus stained with lead hydroxide. The appearance of the nucleoid suggests the presence of regular internal structure. × 180,000.

Figs. 3, 4. Vaccinia virus stained with phosphotungstic acid. M forms showing marginal serrations and arrangement of thread structures. Looping of these to form the serrations can be seen at A. Fig. 4 shows, in addition, remnants of the outer envelope. × 170,000.

Fig. 5. Vaccinia virus stained with phosphotungstic acid. One end of an M form showing arrangement and structure of individual threads. The edges of the virus have been printed out and the dotted lines indicate their positions. B, chain-stitch appearance; C and C_1, threads showing as a series of parallel dots. × 550,000.

PLATE 2

Fig. 6. Vaccinia virus stained with phosphotungstic acid. Thread structure of the M form. A, B and C indicate appearances suggestive of coiled structure. × 350,000.

Fig. 7. Vaccinia virus stained with phosphotungstic acid. Individual threads at high magnification showing appearances suggestive of coiled structure. × 680,000.

Fig. 8. Vaccinia virus stained with phosphotungstic acid. C form showing pallisaded capsular structure at A and B. × 250,000.

Fig. 9. Vaccinia virus. Self-shadowed carbon replicas of trypsin-treated virus, showing stepped collapse and pallisading of virus margin, and complex surface patterning. The upper particle shows faint radial striation continuing into the marginal pallisading, but beading appears to be absent. × 125,000.

PLATE 3

Figs. 10, 11. Vaccinia virus stained with phosphotungstic acid. Side elevations of C forms showing nucleoid and lateral bodies. The pallisaded zone of the capsule (seen also in plan at Fig. 10A) is integral with the nucleoid. At Fig. 11A, linear arrangement of the stippling in the nucleoidal area can be seen. × 250,000.

Fig. 12. Vaccinia virus shadowed with carbon after treatment with trypsin and fixation in osmic acid. Surface beading is clearly visible. × 125,000.

Fig. 13. Vaccinia virus stained with phosphotungstic acid. Spheroidal bodies showing M type structure and tendency to hexagonal outline. × 200,000.

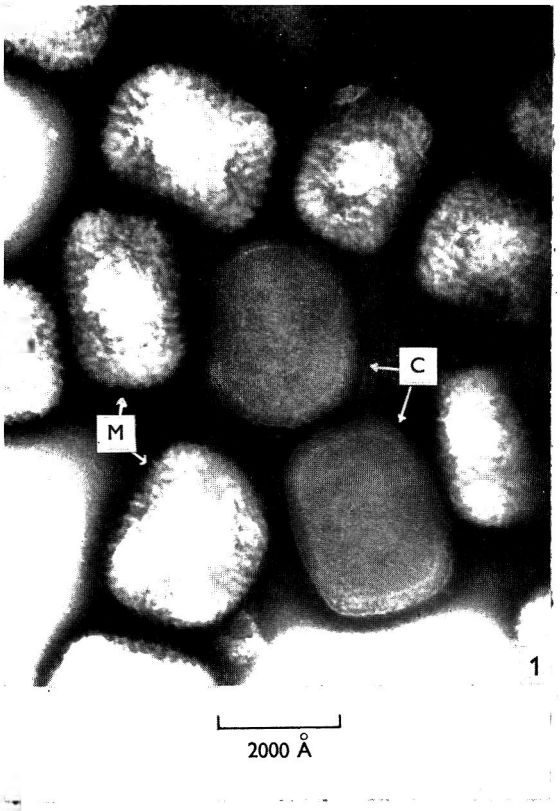
PLATE 4

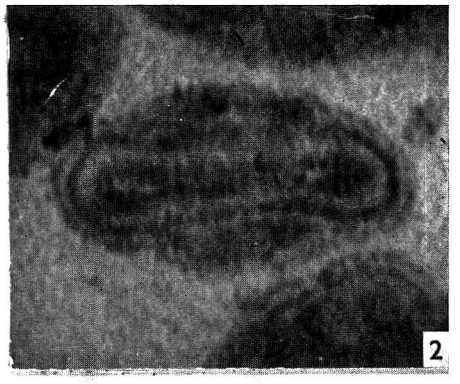
Fig. 14. Vaccinia virus stained with phosphotungstic acid. Intermediate form following trypsin treatment. C structure is visible at A. At B, M structure appears to overlap C structure. × 230,000.

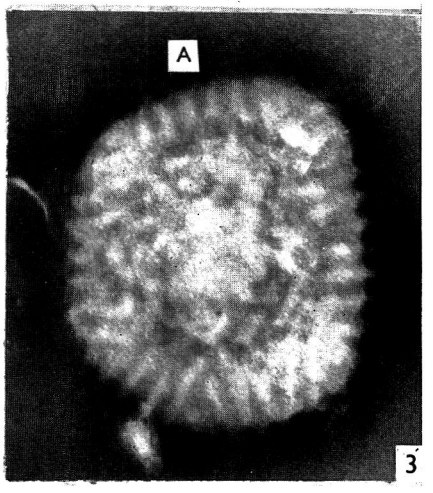
Fig. 15. Vaccinia virus stained with phosphotungstic acid. M form after treatment with trypsin for 2 hr.; no indication of digestion of thread structure.

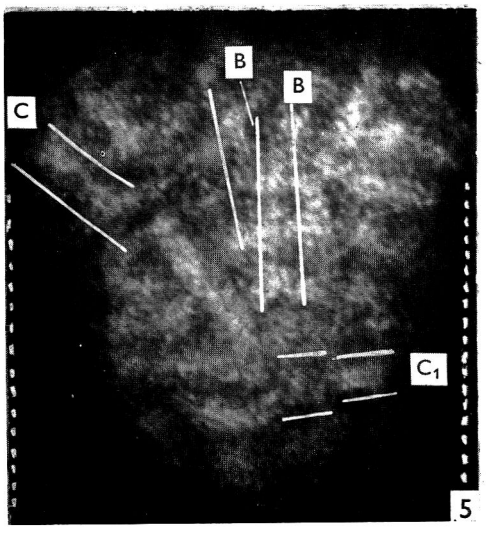
Figs. 16, 17. Vaccinia virus shadowed with carbon and platinum. Unfixed virus sprayed at room temperature. Collapse of the virus has revealed a wrinkled surface structure, resembling a membrane, overlying the beaded structure. × 140,000.

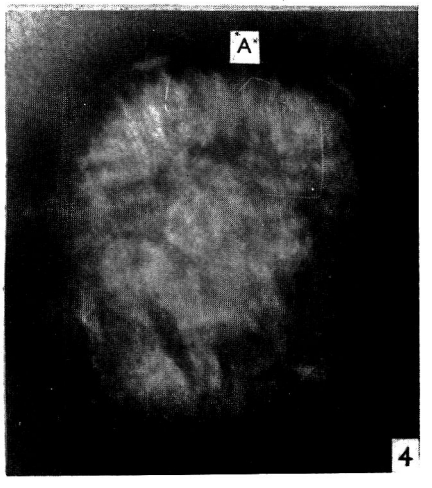
Fig. 18. Vaccinia virus stained with phosphotungstic acid. Group of C forms after treatment of virus on the grid with trypsin followed by saturated urea solution. × 60,000.











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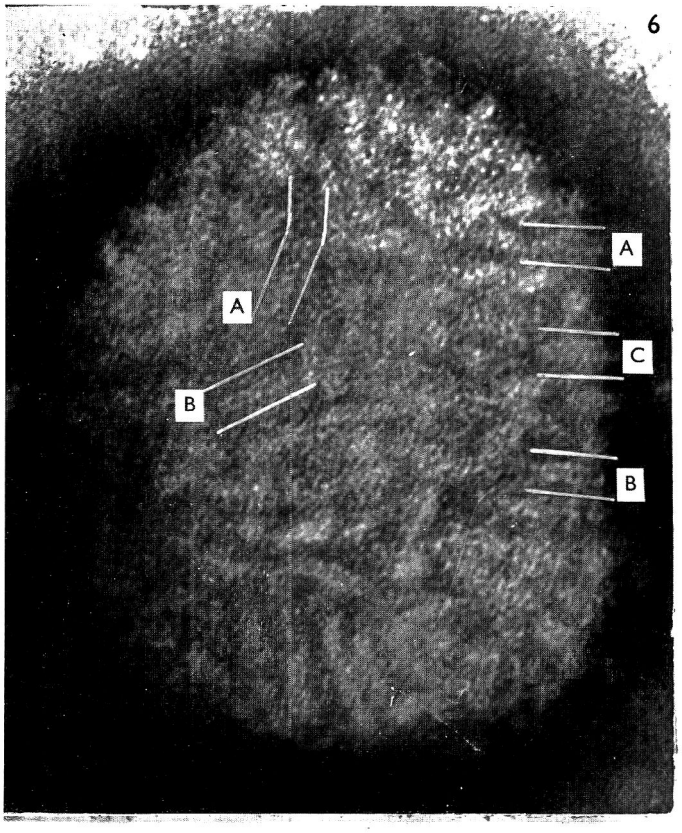
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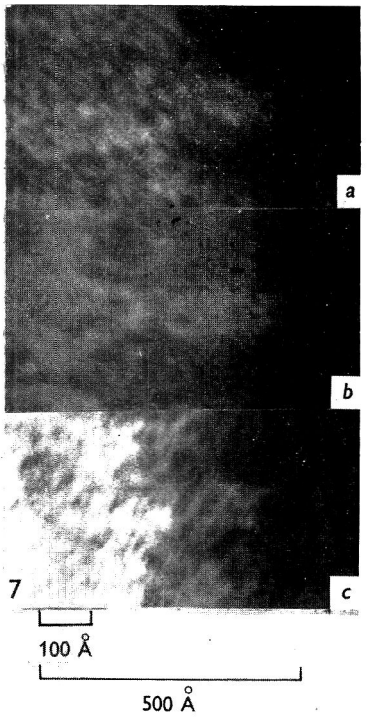
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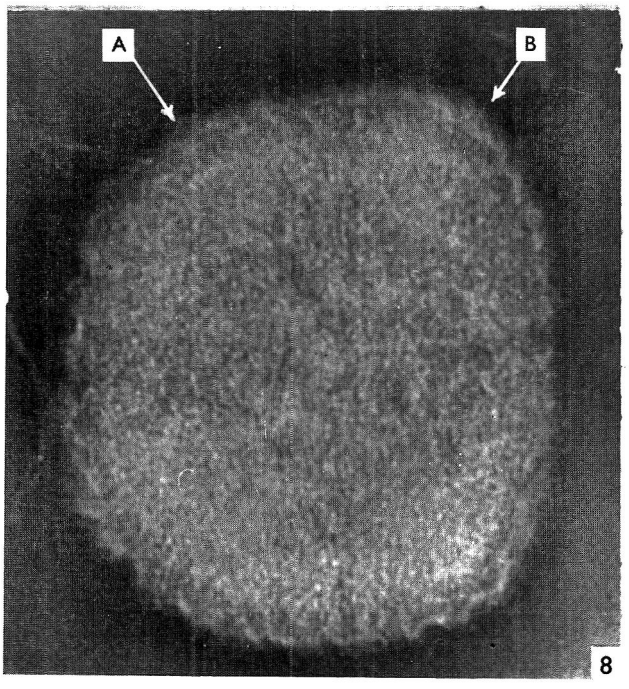
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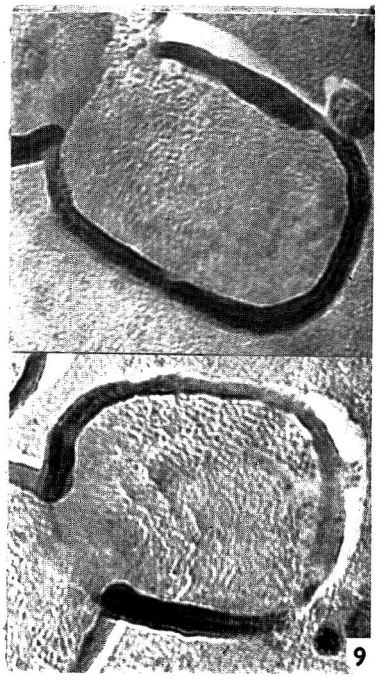
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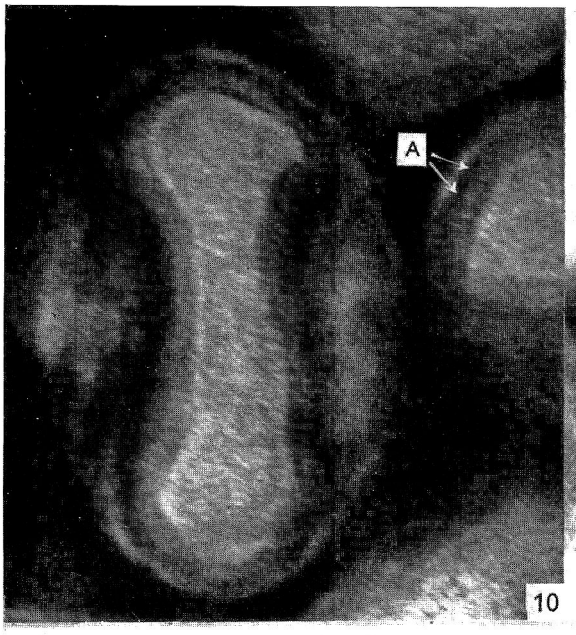

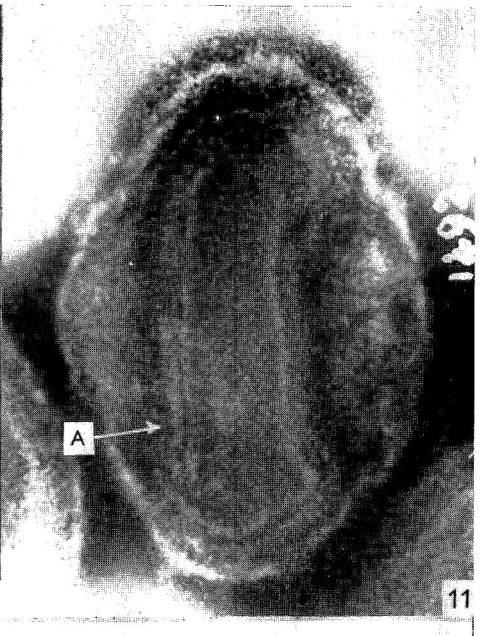

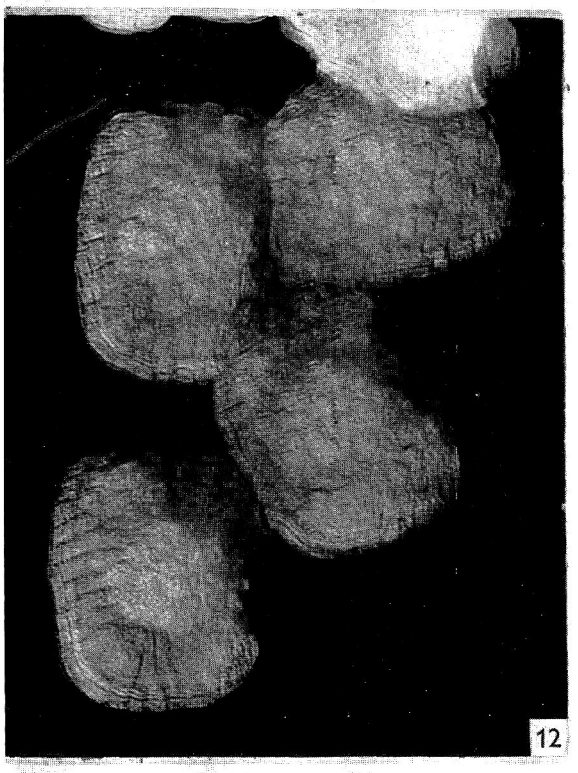

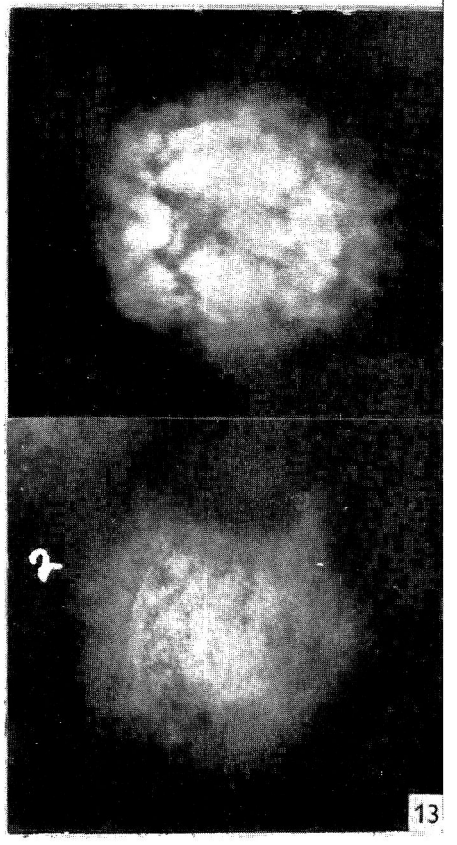

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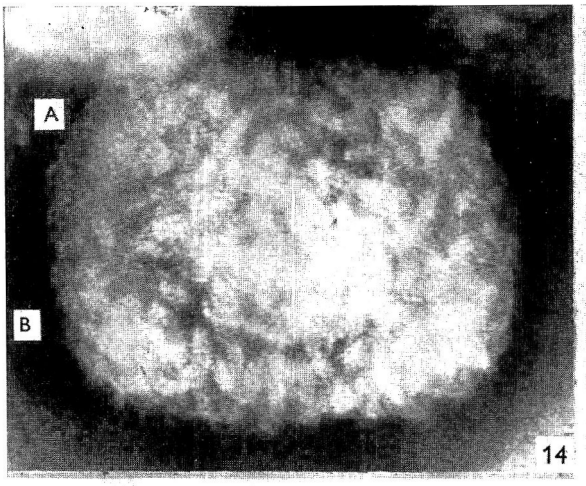
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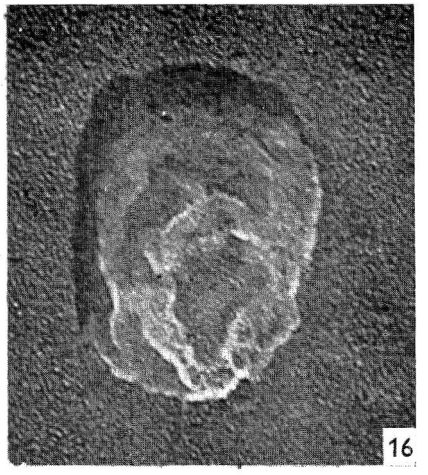
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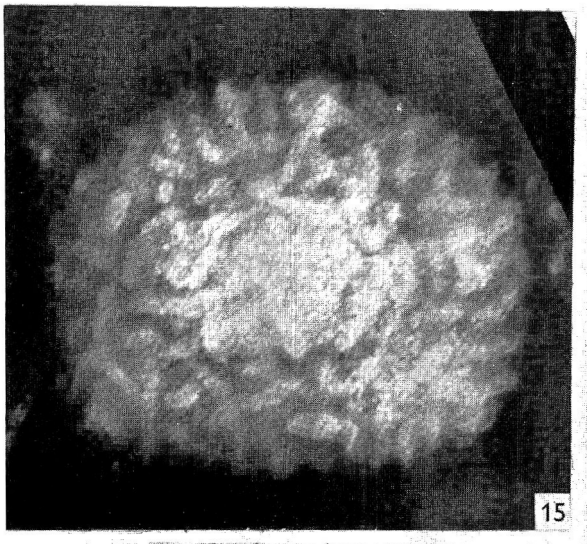
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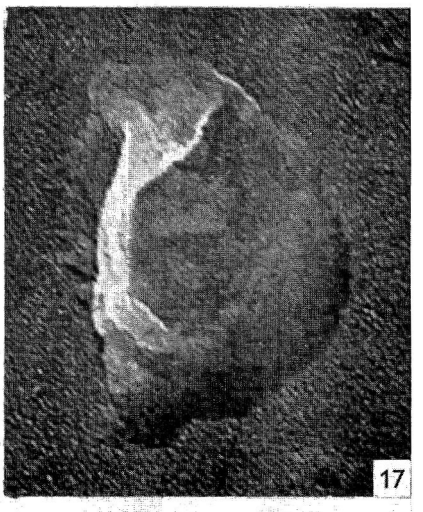
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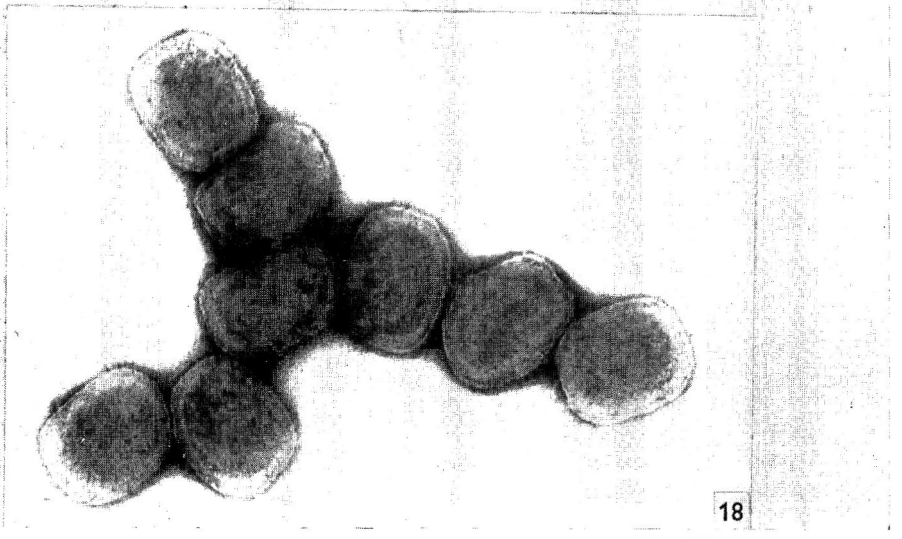
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Growth of a Form 2 Mycobacterium and Various Bacillus Species on Löwenstein-Jensen Medium

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SUMMARY

Vegetative organisms of a Form 2 strain of *Mycobacterium tuberculosis* were inoculated on to Löwenstein–Jensen medium containing malachite green. The multiplication of the organisms was slightly delayed by the presence of the dye, but after a few days growth occurred, even when only a few organisms were used as inoculum. Stimulation of growth of Form 2 mycobacteria by the presence of Form 1 mycobacteria was not observed. Spores of *Bacillus subtilis* and *B. licheniformis* grew on Löwenstein–Jensen medium. Organisms other than Bacillus were usually obtained from Löwenstein–Jensen medium slopes deliberately exposed to airborne contamination, but have never been found in experiments in which *M. tuberculosis* Form 2 organisms were isolated. These results establish the validity of the uninoculated control slopes of Löwenstein–Jensen medium used in all previous experiments in which Form 2 mycobacteria were obtained from Form 1 mycobacteria.

INTRODUCTION

Methods of obtaining rapidly growing spore-forming non-acid-fast rods (Form 2 mycobacteria) from intermittently aerated cultures of *Mycobacterium tuberculosis* (Form 1 mycobacteria) on Löwenstein–Jensen medium were described in previous publications (Csillag, 1961, 1962, 1963*b*). Most of the test cultures were inoculated from old (6 weeks or more) Löwenstein–Jensen medium cultures and were incubated for several months, during which period air was let into the cultures usually once or twice a week and subcultivations were made on to nutrient agar plates. The presence of Form 2 mycobacteria was shown by discoloration of the Löwenstein–Jensen medium and by growth on the nutrient agar. In all of these experiments several uninoculated slopes of the same batch of Löwenstein–Jensen medium were subjected to the same aeration and subcultivation procedures: none showed any evidence of bacterial growth as indicated by discoloration of the medium, the presence of colonies or growth on subculture on nutrient agar. These findings were part of the evidence that Form 2 organisms were derived from Form 1 mycobacteria and were neither medium nor air contaminants. However, Hilson (to be published) has suggested that Form 2 mycobacteria arise from contaminatory Bacillus organisms present in small numbers in the medium or which gain access to the cultures during aeration. His contention is that the contaminants are inhibited by the malachite green in the uninoculated Löwenstein–Jensen medium, but are able to grow in

cultures which contain Form 1 mycobacteria because of destruction of the dye by the Form 1 organisms. The supposition that Form 2 mycobacteria arose from medium contaminants has been shown to be unlikely since they were found to develop in Form 1 cultures on autoclaved medium at a similar rate as on inspissated medium (Csillag, 1962). The possibility that Form 2 organisms arose from airborne contamination has now been further investigated and the results are presented here.

METHODS

Organisms. Mycobacterium tuberculosis. (1) Strain 11413, isolated from the sputum of a British patient with pulmonary tuberculosis (Csillag, 1963*a*), was maintained on Löwenstein–Jensen medium for about 3 years and a 5-week culture was used in the present experiment. (2) A Form 2 strain, isolated from a freeze-dried culture of strain 11413 (Csillag, 1963*b*) was maintained as described earlier (Csillag, 1963*a*), and a 10-week culture on nutrient agar served as the initial culture. *Bacillus licheniformis* (NCTC 1158) and *B. subtilis* (NCTC 2591) were grown on nutrient agar in $\frac{1}{2}$ oz. screw-capped bottles with slightly loosened caps for 3 days at 37°. The caps were then closed and the cultures were kept at room temperature for 2 years (initial cultures).

Media. (1) Löwenstein–Jensen medium (Mackie & McCartney, 1960) without potato starch (Jensen, 1955) and the same medium without malachite green were dispensed in $\frac{1}{2}$ oz. screw-capped bottles. (2) Nutrient agar was prepared by adding 1·4% (w/v) agar to meat-extract + peptone broth (Oxoid No. 2, Oxo Ltd., London) and was used either as slopes in $\frac{1}{2}$ oz. screw-capped bottles or as plates. (3) Hartley's digest nutrient broth (Mackie & McCartney, 1960) was used as the diluent for viable counts. All media were incubated for 3 days at 37° before use, as a test of their sterility. All cultures were incubated at 37° unless otherwise stated.

RESULTS

Growth of vegetative organisms of Form 2 mycobacteria on Löwenstein–Jensen medium. An inoculum containing only a few vegetative organisms of the Form 2 strain of *Mycobacterium tuberculosis* was prepared in the following way. The initial Form 2 culture was plated on nutrient agar; after incubation for 2 days, a suspension, prepared from an isolated colony, was inoculated into nutrient broth and was shaken for 24 hr. Serial ten-fold dilutions of this broth culture were inoculated in 0·02 ml. volumes on two slopes of Löwenstein–Jensen medium + malachite green and on the surface of two nutrient agar plates. The nutrient agar plates were incubated for 1 day, and the colonies then counted. The Löwenstein–Jensen medium cultures were incubated for 4 days, and representative samples of surface growth and of condensation water then subcultivated to further nutrient agar plates which were incubated for 1 day. The 10^{-6} dilution yielded 3 and 9 colonies on the nutrient agar plates. The Löwenstein–Jensen medium slopes inoculated with this dilution turned dark green after incubation for 2 days and colonies were then visible on them; subculture to nutrient agar yielded a heavy growth of Form 2 colonies. The 10^{-7} dilution yielded 1 and 0 colonies on nutrient agar and the Löwenstein–Jensen medium slopes remained unchanged and were sterile on subculture.

The same experiment was repeated with the following modifications. The 10^{-6} dilution only, containing 3–10 organisms capable of yielding colonies on nutrient agar plates, was inoculated on to 6 nutrient agar slopes, on to 6 slopes of Löwenstein–Jensen medium + malachite green and on to 6 slopes of the same medium on which were growing 5-week cultures of Form 1 *Mycobacterium tuberculosis*. On the nutrient agar slopes colonies appeared after incubation for 1 day. The Löwenstein–Jensen medium slopes (without Form 1 growth) became discoloured and colonies appeared on them after incubation for 2–4 days. Brownish discoloration appeared in the Löwenstein–Jensen Form 1 cultures after incubation for 3 days. Nutrient agar plates inoculated from all of the cultures yielded heavy growth of Form 2 mycobacteria.

Growth of spores of various species of Bacillus on Löwenstein–Jensen medium. Inocula containing free endospores of some Bacillus species were prepared in the following way. Smears prepared from 2-year old cultures of *Bacillus subtilis* and *B. licheniformis* showed numerous free endospores and only occasional vegetative forms. A moist sterile loop was placed within the culture bottle but without touching the medium. The bottle was then tapped to dislodge spores on to the loop. Loops, thus inoculated, were spread on 4 slopes of Löwenstein–Jensen medium + malachite green. After incubation for 2 days the medium became yellow and then pink on the following day. Growth was visible after incubation for 2 days and subculture to nutrient agar yielded a heavy growth of Bacillus organisms.

Table 1. *Airborne contaminants obtained on various media*

Twenty slopes of each type of medium were exposed to airborne contamination overnight in the laboratory and resulting growth was identified after subculture to nutrient agar.

	Medium		
Organisms obtained on subculture to nutrient agar	Löwenstein–Jensen + malachite green	Löwenstein–Jensen without malachite green	Nutrient agar
	Number of slopes		
Gram-positive, spore-bearing rods	1	2	1
Other organisms	6	7	9
Sterile	13	11	10

Growth of airborne contaminants on Löwenstein–Jensen medium. Twenty slopes of Löwenstein–Jensen medium + malachite green, 20 slopes of the same medium without malachite green and 20 slopes of nutrient agar were placed in a rack in a random order. The screw-caps were taken off and the media exposed overnight to the air in the same laboratory where Form 2 mycobacteria had been, and were being, isolated from Form 1 strains. The screw-caps were then replaced and the surface of the slopes washed with their own condensation water. After incubation for 2 days, representative samples, taken from the surface of the slopes and from the condensation waters, were plated on nutrient agar and incubated for 1 day. The results obtained on the nutrient agar plates are given in Table 1. About half

of the slopes of each type of medium yielded growth. Gram-positive spore-bearing rods were obtained from 1 Löwenstein–Jensen slope + malachite green, from 2 Löwenstein–Jensen medium slopes without malachite green and from 1 nutrient agar slope. The remainder of the positive cultures yielded various other organisms.

DISCUSSION

The findings reported here may be considered in the light of the suggestion that Form 2 mycobacteria arise from contaminants unable to grow on Löwenstein–Jensen medium + malachite green unless the medium has been conditioned by the previous growth of a Form 1 strain (which destroys the dye). (1) The multiplication of the vegetative organisms of the Form 2 strain investigated was slightly delayed by the presence of malachite green in Löwenstein–Jensen medium but, after a few days, the organisms were capable of multiplication, even when inoculated in small numbers. The presence of the organisms was easily detected by discoloration of the medium and by subcultivation on nutrient agar plates. Further experiments, not reported here, have shown that some Form 2 strains, when inoculated in a vegetative or in a sporulating stage, only yielded visible growth on Löwenstein–Jensen medium + malachite green when the cultures were aerated (by loosening the screw-caps) for about 10 sec. at intervals of 1–2 days. However, even when the presence of these organisms was not detected by visible colony formation or by discoloration of the medium, subcultures on nutrient agar yielded numerous colonies. All of the cultures from which Form 2 organisms were obtained and all of the uninoculated control cultures in previously reported experiments (Csillag, 1961, 1962, 1963*b*) were intermittently aerated and subcultivated on nutrient agar. (2) Stimulation of growth of Form 2 mycobacteria by the presence of Form 1 mycobacteria was not observed. (3) Bacillus spores germinated and formed colonies on Löwenstein–Jensen medium + malachite green and caused discoloration of the medium of a colour different from that produced by Form 2 organisms. Thus, there is evidence from these findings that vegetative Form 2 mycobacteria or Bacillus spores grow on Löwenstein–Jensen medium to an extent that is easily detectable by subculture on nutrient agar, and that conditioning of the medium by growth of Form 1 mycobacteria is not likely to increase the ease with which Form 2 mycobacteria can be isolated. The use of uninoculated medium which is otherwise treated in the same manner as the medium used for Form 2 isolation is therefore established as a valid control.

Further evidence that Form 2 mycobacteria do not arise as air contaminants is provided by the slopes which were deliberately exposed to the air. The contaminants that grew on Löwenstein–Jensen medium and on nutrient agar slopes after exposure to air were not as a rule species of Bacillus. In all the experiments on the production of Form 2 mycobacteria previously reported and in other unreported experiments no organisms other than Form 2 mycobacteria have ever been isolated by subculture from the test Löwenstein–Jensen slopes to nutrient agar. The absence of true contaminants in these experiments is not remarkable since the aeration procedure was carried out in a previously sterilized cabinet and took only a few seconds, whereas in the experiment reported here, only about half of the slopes which were deliberately exposed to the air for as long as 17 hr were contaminated.

In previous publications evidence, independent of the uninoculated controls, was presented that Form 2 mycobacteria were not air contaminants. This evidence can be summarized as follows: (1) A Form 1 culture, inoculated with old organisms yielded Form 2 organisms, whereas the same Form 1 strain, stabilized by frequent rapid passage on Löwenstein–Jensen medium, did not yield Form 2 organisms in the same experiment (Csillag, 1963*b*). (2) The isolation of Form 2 colonies on nutrient agar could be predicted by the previous appearance, within the Löwenstein-Jensen medium test cultures, of their early phases of development, which did not resemble the vegetative form or spores of Bacillus (Csillag, 1961). (3) The bacillary morphology of the organisms and, to some extent the colonial morphology, of Form 2 strains at their first isolation were specific for the Form 1 strain from which they were derived (Csillag, 1961). Form 2 mycobacteria have many characteristics in common with Bacillus, but differ in the production of culturable cocci (*Mycococcus*, Krassilnikov, 1959; Csillag, 1962). A fuller account of the production of these cocci will be reported elsewhere.

I wish to thank Dr D. A. Mitchison for many helpful suggestions and encouragement and Mr M. Clarke for his skilled technical assistance.

REFERENCES

Csillag, A. (1961). Spore formation and 'dimorphism' in the mycobacteria. *J. gen. Microbiol.* **26**, 97.

Csillag, A. (1962). Development of Form 2 mycobacteria on autoclaved Löwenstein–Jensen medium. *Tubercle, Lond.* **43**, 439.

Csillag, A. (1963*a*). Cellular morphology of Form 2 mycobacteria in slide culture. *J. gen. Microbiol.* **30**, 21.

Csillag, A. (1963*b*). Stabilization of various forms of *Mycobacterium tuberculosis*. *Tubercle, Lond.* (in the Press).

Jensen, K. A. (1955). Towards a standardisation of laboratory methods. 2nd Report of the subcommittee of laboratory methods of the International Union against Tuberculosis. *Bull. int. Un. Tuberc.* **25**, 89.

Krassilnikov, N. A. (1959). *Diagnostik der Bakterien und Actinomyceten*, p. 202. Jena: Gustav Fischer.

Mackie and McCartney's Handbook of Bacteriology (1960), 10th ed. Ed. by R. Cruickshank, pp. 192, 212. Edinburgh and London: Livingstone.

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The Nature of the Insensitivity of Gram-Negative Bacteria towards Penicillins

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SUMMARY

The insensitivity of Gram-negative bacteria towards different penicillins has been correlated with the inactivation of these compounds by the penicillinases produced by these bacteria. Penicillins such as phenethicillin, propicillin, methicillin, and cloxacillin were relatively inactive against Gram-negative bacteria, but resistance was not accompanied by inactivation of these compounds. With ampicillin and benzylpenicillin, however, bacterial resistance was associated with destruction of the penicillins. Ampicillin was more stable to the penicillinases produced by certain Gram-negative bacteria than was benzylpenicillin and was correspondingly more active against these organisms. Gram-negative bacteria which produced little or no penicillinase were two to four times more sensitive to ampicillin than to benzylpenicillin, but ampicillin was at least ten times more active than benzylpenicillin against several penicillinase-producing coliform organisms. All strains of bacteria which were resistant to both ampicillin and benzylpenicillin were capable of inactivating both compounds, but the insensitivity of these bacteria was not necessarily due solely to penicillinase production. Strains of *Pseudomonas aeruginosa* and certain strains of *Escherichia coli* and *Proteus mirabilis* were able to grow in high concentrations of these antibiotics, whereas with the strains of *Aerobacter aerogenes*, *Proteus morganii* and *Proteus vulgaris* examined, the resistance appeared to be due solely to inactivation of the penicillins.

INTRODUCTION

The destruction of benzylpenicillin by certain Gram-negative bacteria was reported first by Abraham & Chain (1940) and, later, by Bondi & Dietz (1944), but both groups of workers also observed that the resistance of bacteria to penicillins was not invariably associated with penicillinase formation. Similar findings with ampicillin were reported by Rolinson & Stevens (1961) who showed that certain Gram-negative bacteria were capable of destroying the antibiotic, but that others were capable of growing in high concentrations of it; these results have been confirmed by other workers (Stewart, Coles, Nixon & Holt, 1961; Trafford *et al.* 1962; Ayliffe, 1963). Thus, with ampicillin, as with benzylpenicillin, penicillinase appeared to occupy an indeterminate role in the resistance of Gram-negative bacteria towards penicillins. More recently, the characteristics of the penicillinases elaborated by certain Gram-negative bacteria have been investigated by various workers who have reported striking differences in the activities of these enzymes against various penicillins. For example, Auhagen *et al.* (1962) and Smith (1963) described strains of *Escherichia coli* which destroyed benzylpenicillin but which

had little or no effect on ampicillin, propicillin, methicillin or oxacillin. Ayliffe (1963) showed that ampicillin was more stable than benzylpenicillin to the penicillinase produced by a strain of *E. coli*, but, in addition, reported that ampicillin was inactivated as rapidly, or more rapidly, by penicillinases produced by strains of Proteus and Klebsiella. In these laboratories, Mr F. R. Batchelor (personal communication) has measured marked variation in the reaction of several penicillins to the penicillinases produced by a number of Gram-negative bacteria. It seemed possible that in some cases the differences in the sensitivities of some strains of Gram-negative bacteria towards various penicillins might be correlated with the relative stabilities of these compounds to the bacterial penicillinases. The results reported here were derived from experiments designed to investigate the relationship between the penicillinase activity of Gram-negative bacteria and the sensitivity of these organisms to penicillins, with a view to assessing the significance of these enzymes as factors in the resistance of bacteria to these antibiotics. The experiments comprised tests for antibacterial activity, measurement of the destruction of penicillins by bacteria, and observation of the growth of bacteria in the presence of penicillins. In addition, certain tests were also carried out to distinguish between penicillin inactivation by β-lactamases (penicillinases), which yield penicilloic acids, and deacylases (penicillin amidases) which yield 6-amino-penicillanic acid (6-APA).

METHODS

Measurement of the antibacterial activities of penicillins. Minimal inhibitory concentrations of various penicillins against bacteria were determined by twofold serial dilution in nutrient broth (Oxoid No. 2). The antibiotics were incorporated in graded concentrations in 5 ml. volumes of nutrient broth which were inoculated with 1 drop (0·03 ml.) of an overnight culture of the test organism. Inhibitory concentrations were measured after overnight incubation at 37°. In other experiments, the effect on the antibacterial activity of the penicillins of altering the inoculum size was measured.

Measurement of penicillin destruction. The destruction of penicillins incubated overnight in the antibacterial tests described above was estimated by microbiological assay of residual penicillin activity. Samples were taken from the cultures and were plated in punch-holes in large rectangular plates containing nutrient agar seeded with *Bacillus subtilis* (ATCC 6633). In these assays, standard lines were prepared from standard solutions of penicillins which had been incubated overnight at 37° in nutrient broth to compensate for thermal inactivation of penicillin. The plates containing test and standard samples were incubated overnight at 29° when the diameters of zones of inhibition were measured in the usual way. Tests were also carried out to estimate the extent of penicillin inactivation due to the experimental procedure adopted, and which represented destruction additional to that occurring in the antibacterial tests. Likely factors appeared to be change in pH value of cultures, and the penicillinase activities of test cultures during the microbiological assay procedures.

Measurement of the effect of penicillins on the growth of bacteria. Optically matched tubes containing selected concentrations of penicillins in 10 ml. volumes of nutrient broth were incubated with 0·5 ml. or 1·0 ml. of an overnight culture of test organism.

The solutions were incubated at 37° in a water bath, and growth was measured turbidimetrically over a period of 24 hr by means of an EEL nephelometer. In addition, a replicate set of cultures was prepared in an identical fashion and samples were taken from these at regular intervals and assayed for penicillin content as described above.

Quantitative estimation of penicillins by chromatography. Samples were taken from broth cultures of *Pseudomonas aeruginosa* which had been incubated overnight at 37° with known concentrations of ampicillin or benzylpenicillin and these were assayed as follows. One drop (about 6 μl.) was placed on the origin of filter-paper tapes (1 cm. wide, Whatman No. 1) and developed by descending chromatography in a butanol + pyridine + water (1 + 1 + 1, by vol.) system. Tapes spotted with nutrient broths containing from 2·5 to 100 μg. ampicillin or benzylpenicillin/ml. were treated in a like manner. After 16 hr, the tapes were placed on the surface of large rectangular assay plates containing nutrient agar seeded with *Bacillus subtilis* (ATCC 6633) and incubated for 24 hr at 29°. The penicillin concentrations of the test samples were estimated from standard lines derived from standard samples.

Chromatographic estimation of 6-aminopenicillanic acid. Samples were taken from the antibacterial tests and treated as above for quantitative estimation of penicillin by chromatography. The tapes were sprayed with 5% sodium bicarbonate solution and 5% phenylacetyl chloride in acetone before being plated for microbiological assay (Batchelor, Doyle, Nayler & Rolinson, 1959).

Chromatographic examination for penicilloic acid. Samples from antibacterial tests with ampicillin and benzylpenicillin were spotted on large sheets (Whatman paper No. 1) and developed in the butanol + pyridine + water system as described above. The chromatograms were sprayed with the starch-iodine reagent of Thomas (1961), and the zones which developed compared with those produced by the penicilloic acids which had been prepared by treatment of standard solutions of ampicillin and benzylpenicillin with penicillinase.

RESULTS

Preliminary

Destruction of penicillin due to change in pH values of bacterial cultures was considered to be negligible as a result of experiments which showed that there was little inactivation of standard solutions of benzylpenicillin incubated overnight in nutrient broth buffered at pH 5·0. This was the lowest pH value recorded for a number of overnight cultures of Enterobacteriaceae.

The amount of destruction of ampicillin or benzylpenicillin by bacterial penicillinase during the microbiological assay procedures was estimated by finding the effect of adding, at measured intervals, a culture of a penicillinase-producing strain of *Staphylococcus aureus* to standard solutions of these penicillins contained in punch-holes in an assay plate. There was significant inactivation only when the enzyme system was added within 30 min. of placing the penicillin solution on the assay plate, owing to the very rapid diffusion of the penicillins into the surrounding medium. It seemed improbable therefore that there would be any marked destruction of penicillins during the microbiological assay as a result of the penicillinase produced by the Gram-negative bacteria being examined.

Examination for production of 6-aminopenicillanic acid (6-APA)

Chromatographic examination of broth cultures in which there was marked destruction of penicillin as judged by microbiological assay, showed the presence of large amounts of the cognate penicilloic acid, and little or no 6-APA. The organisms tested included strains of *Escherichia coli*, Proteus, *Pseudomonas aeruginosa* and *Aerobacter aerogenes*. With one strain of *Proteus rettgeri*, however, an appreciable quantity of 6-APA was detected; the penicilloic acids of benzylpenicillin and 6-APA were also observed in cultures of this strain.

The activities of seven penicillins against Gram-negative bacteria

The relative activities of ampicillin, benzylpenicillin, phenoxymethylpenicillin, phenethicillin, propicillin, methicillin, and cloxacillin, against a variety of Gram-negative bacteria are shown in Table 1. From this it can be seen that of these compounds, ampicillin and benzylpenicillin alone showed appreciable activity. In general, ampicillin was two to ten times more active than benzylpenicillin, but certain strains were insensitive to both penicillins.

Table 1. *The relative activities of seven penicillins against certain Gram-negative bacteria*

	Antibiotic						
Organism	Ampicillin	Benzyl-penicillin	Phenoxy-methyl-penicillin	Phene-thicillin	Propi-cillin	Methi-cillin	Cloxa-cillin
	Minimal inhibitory concentration (μg./ml.)						
Escherichia coli 2	2·5	12·5	125	> 500	500	> 500	> 500
E. coli 6	5·0	25	250	500	250	> 500	500
E. coli 37	25	500	> 500	500	250	> 500	500
E. coli 83	500	> 500	> 500	> 500	500	> 500	> 500
Aerobacter aerogenes A	250	125	500	> 500	500	500	500
Klebsiella pneumoniae E	1·25	5·0	125	500	250	.	.
Proteus mirabilis 977	2·5	2·5	50	250	125	125	500
P. mirabilis 889	> 500	> 500	.	.	.	.	.
P. morganii A	125	> 500	> 500	500	500	.	.
Pseudomonas aeruginosa A	> 500	> 500	> 500	> 500	> 500	> 500	> 500
Salmonella typhi	1·25	2·5	50	250	125	> 500	500
S. paratyphi A	1·25	5·0	250	500	250	> 500	> 500
Shigella sonnei	5·0	25	125	500	250	> 500	> 500
S. flexneri	2·5	12·5	125	250	125	500	500

The relative stabilities of seven penicillins to the penicillinases of Gram-negative bacteria

The results of experiments designed to correlate the antibacterial activities of seven penicillins against two strains of *Escherichia coli* and the stabilities of these compounds to the penicillinases produced by the test bacteria are shown in Table 2. In these tests the minimal inhibitory concentrations of the antibiotics were measured in the usual fashion in nutrient broth using an ampicillin-sensitive strain and an

ampicillin-resistant strain of *E. coli*; in addition, the amount of destruction of penicillin which had occurred after overnight incubation was measured by microbiological assay. It will be seen that phenethicillin, propicillin, methicillin and cloxacillin were almost completely unaffected by the penicillinases produced by

Table 2. *The relative stabilities of ampicillin, benzylpenicillin, phenoxymethylpenicillin, phenethicillin, propicillin, methicillin, and cloxacillin to the penicillinases produced by two strains of Escherichia coli*

Minimal inhibitory concentrations of the penicillins against two strains of *Escherichia coli* were determined by serial dilution in nutrient broth; growth and residual penicillin activity after overnight incubation at 37° were determined by visual inspection and microbiological assay respectively.

Penicillin	Organism	Growth and penicillin concentration (μg./ml.) after incubation at 37° for 18 hr.								
Bacteria-free control*		500	250	125	50	25	12·5	5·0	2·5	1·25
Benzylpenicillin	*E. coli* 2	−	−	−	−	−	+	+	+	+
		470	245	96	45	17·2	2·2	2·2	0·9	0·4
	E. coli 83	+	+	+	+	+	+	+	+	+
		0	0	0	0	0	0	0	0	0
Ampicillin	*E. coli* 2	−	−	−	−	−	−	−	+	+
		560	245	120	53	24·0	13·4	5·3	2·4	1·3
	E. coli 83	±	+	+	+	+	+	+	+	+
		400	82	24	1·3	0·7	0	0	0	0
Phenoxymethylpenicillin	*E. coli* 2	±	±	+	+	+	+	+	+	+
		520	240	115	34	13·0	5·8	3·0	1·3	0·6
	E. coli 83	+	+	+	+	+	+	+	+	+
		0	0	0	0	0	0	0	0	0
Phenethicillin	*E. coli* 2	+	+	+	+	+	+	+	+	+
		530	250	132	53	27·0	13·0	4·7	2·6	1·25
	E. coli 83	+	+	+	+	+	+	+	+	+
		550	195	93	41	16·5	8·4	2·6	0·6	0
Propicillin	*E. coli* 2	±	+	+	+	+	+	+	+	+
		490	250	120	45	26·0	12·0	4·4	2·1	0·9
	E. coli 83	±	+	+	+	+	+	+	+	+
		510	250	127	40	23·0	11·0	3·0	1·3	0·4
Methicillin	*E. coli* 2	.	+	+	+	+	+	+	+	+
		.	250	128	54	25·0	13·0	5·1	2·6	1·2
	E. coli 83	.	+	+	+	+	+	+	+	+
		.	250	130	49	25·0	12·5	4·6	1·8	0·9
Cloxacillin	*E. coli* 2	.	+	+	+	+	+	+	+	+
		.	235	128	51	24·0	13·0	4·5	3·1	.
	E. coli 83	.	+	+	+	+	+	+	+	+
		.	245	130	51	25·0	13·0	4·0	0	.

* These concentrations represent the initial concentrations of penicillins prior to incubation with the cultures.
\+ = Growth.
− = No growth.
± = Partial growth.

either strain of *E. coli*, but exerted little or no inhibitory effect on these cultures. In contrast, there was complete destruction of benzylpenicillin and marked destruction of ampicillin by the resistant strain of *E. coli* strain 83, and there was some

obvious destruction of benzylpenicillin by the sensitive strain of *E. coli* 2 at concentrations below the inhibitory value. Ampicillin was, however, unaffected by this strain. Phenoxymethylpenicillin appeared to occupy a mid-way position between these two groups of penicillins, inasmuch as this compound was almost inactive against one strain and was completely inactive against the other; but, nevertheless, there was obvious destruction of this penicillin by the ampicillin-sensitive organism and complete destruction by the second strain.

Table 3. *The activities of ampicillin and benzylpenicillin against Gram-negative bacteria causing minimal destruction of penicillins*

Minimal inhibitory concentrations of the penicillins against five strains of Gram-negative bacteria were determined by serial dilution in nutrient broth; growth and residual penicillin activity after overnight incubation at 37° were determined by visual inspection and microbiological assay respectively.

Organism	Antibiotic	Growth and penicillin concentration (μg./ml.) after incubation at 37° for 18 hr.								
Bacteria-free control*		50	25	12·5	5·0	2·5	1·25	0·5	0·25	0·1
Salmonella typhi	Benzyl-penicillin	.	.	−	−	±	+	+	+	+
		.	.	.	5·0	2·3	1·4	0·7	0·28	0·16
	Ampicillin	.	.	−	−	−	−	±	+	+
		.	.	.	5·0	2·7	1·4	0·53	0·28	0·1
S. paratyphi A	Benzyl-penicillin	.	.	.	−	−	±	+	+	+
		.	.	.	5·0	1·9	1·4	0·7	0·28	0·16
	Ampicillin	.	.	.	.	−	−	±	+	+
		.	.	.	.	2·5	1·8	0·44	0·23	0·12
Proteus mirabilis 4	Benzyl-penicillin	.	.	−	−	−	+	+	+	+
		.	.	12·5	5·0	2·0	1·0	0·52	0·29	0·16
	Ampicillin	.	.	−	−	−	−	−	+	+
		.	.	.	5·0	2·6	1·4	0·53	0·22	0·16
P. mirabilis c977	Benzyl-penicillin	.	.	−	−	+	+	+	+	+
		.	.	10·0	4·2	1·9	1·4	0·44	0·29	0·16
	Ampicillin	.	.	.	−	−	−	+	+	+
		.	.	.	5·0	2·6	1·4	0·53	0·21	0·1
Klebsiella pneumoniae E	Benzyl-penicillin	.	.	−	−	−	±	+	+	+
		.	.	10·5	5·0	2·4	1·4	0·45	0·28	< 0·1
	Ampicillin	.	.	.	−	−	−	−	+	+
		.	.	.	5·6	2·9	1·3	0·6	0·23	< 0·1

* These concentrations represent the initial concentrations of penicillins prior to incubation with the cultures.
\+ = Growth.
− = No growth.
± = Partial growth.

The antibacterial activities and penicillinase stabilities of ampicillin and benzylpenicillin towards Gram-negative bacteria

As a result of numerous experiments to compare the relative antibacterial activities of ampicillin and benzylpenicillin, the Gram-negative bacteria were grouped as follows:

Group 1. *Gram-negative bacteria sensitive to both ampicillin and benzylpenicillin.* The results in Table 3 show the relative activities of ampicillin and benzylpenicillin against two Salmonella species, two strains of *Proteus mirabilis* and one strain of

Klebsiella pneumoniae. These organisms were inhibited by concentrations of 0·5–5·0 μg. penicillins/ml. but, in general, ampicillin was two to four times more active than was benzylpenicillin. Microbiological assay of these samples showed that there had been little or no destruction of either penicillin after incubation with the test organisms at 37° for 16–20 hr.

Group 2. Gram-negative bacteria sensitive to ampicillin but relatively resistant to benzylpenicillin. From Table 4 it can be seen that ampicillin inhibited the growth of three strains of *Escherichia coli* and one strain of each of *Shigella flexneri* and

Table 4. *Penicillinase activities of certain Gram-negative bacteria sensitive to ampicillin but moderately insensitive to benzylpenicillin*

Minimal inhibitory concentrations of the penicillins against five strains of Gram-negative bacteria were determined by serial dilution in nutrient broth; growth and residual penicillin activity after overnight incubation at 37° were determined by visual inspection and microbiological assay respectively.

Organism	Antibiotic	Growth and penicillin concentration (μg./ml.) after incubation at 37° for 18 hr.								
Bacteria-free control*		50	25	12·5	5·0	2·5	1·25	0·5	0·25	0·1
Shigella flexneri	Benzyl penicillin	.	−	±	+	+	+	+	+	+
		.	13·5	13·0	0·44	0·2	0·16	0·12	< 0·1	< 0·1
	Ampicillin	.	.	.	−	−	−	+	+	+
		.	.	.	5·0	2·6	1·7	0·44	0·2	< 0·1
S. sonnei	Benzyl-penicillin	−	±	±	+	+	+	+	+	+
		.	0	0	0	0	0	0	0	0
	Ampicillin	.	.	.	−	−	−	+	+	+
		.	.	.	5·0	2·1	1·05	0·12	< 0·1	< 0·1
Escherichia coli 2	Benzyl-penicillin	.	−	+	+	+	+	+	+	+
		.	17·0	1·9	0·64	0·46	1·8	0·12	< 0·1	< 0·1
	Ampicillin	.	.	.	−	−	±	+	+	+
		.	.	.	5·2	2·6	1·4	0·53	0·14	< 0·1
E. coli 61	Benzyl-penicillin	.	−	−	+	+	+	+	+	+
		.	20·0	6·8	0·34	0	0	0·12	0	0
	Ampicillin	.	.	.	−	−	−	+	+	+
		.	.	.	5·0	2·6	1·4	0·41	0·22	< 0·1
E. coli T341	Benzyl-penicillin	−	−	+	+	+	+	+	+	+
		.	27·0	1·5	0	0	0	0	0	0
	Ampicillin	.	.	−	−	±	+	+	+	+
		.	.	15·0	4·4	2·65	0·63	0·24	0	0

* These concentrations represent the initial concentrations of penicillins prior to incubation with the cultures.
\+ = Growth.
− = No growth.
± = Partial growth.

S. sonnei at concentrations of 1·25–2·5 μg./ml., whereas benzylpenicillin was inhibitory only at 12·5–25 μg./ml., thus showing a tenfold difference in the activities of these penicillins. Moreover, all five strains of bacteria destroyed most or all of the benzylpenicillin at concentrations below the minimal inhibitory concentrations, but there was little or no destruction of ampicillin by these organisms. These results showed the greater stability of ampicillin to the penicillinases produced by these bacteria, and suggested the possibility that the sensitivity of each strain to these

penicillins was related to the stabilities of the compounds to the penicillinase elaborated by each particular strain. This is supported by the results of an experiment shown in Fig. 1, illustrating the growth of one of these strains of bacteria, *E. coli* T341, in the presence of different concentrations of benzylpenicillin, and in Table 5 which shows the rate of destruction of the penicillin by this strain. It can be seen that at the lowest benzylpenicillin concentration (5 μg./ml.) growth was as rapid as in the control tube containing no antibiotic, while the concentration of antibiotic diminished steadily until none could be detected after 7 hr. At the next higher concentration (12·5 μg./ml.), there was little increase in growth for the first

Table 5. *Destruction of benzylpenicillin by cultures of Escherichia coli T341*

Nutrient broth solutions containing graded concentrations of benzylpenicillin were inoculated with *Escherichia coli* T341 and incubated at 37° for 24 hr.; samples were taken at intervals and assayed for penicillin content by microbiological assay.

Incubation time (hr)	Penicillin concentration (μg./ml.)		
0	25	12·5	5·0
1	24	11·5	5·4
2	20	9·2	5·0
3	16	6·3	4·5
4	15	3·0	2·5
5	12	1·3	1·3
6	11	1·3	0·9
7	10	0	0
24	0	0	0

3–4 hr., while the penicillin concentration fell gradually to 3·0 μg./ml.; during the next 3 hr the turbidity of the culture increased steadily and the penicillin concentration fell to zero. At the highest concentration (25 μg./ml.), there was no increase in turbidity over a period of 7½ hr, during which time the penicillin concentration decreased from 25 to 10 μg./ml. After incubation overnight at 37° the turbidities of all these cultures were approximately equivalent to that of the control culture containing no penicillin, and no penicillin was detected for any concentration.

Group 3. *Gram-negative bacteria resistant to both ampicillin and benzylpenicillin.* Examples of bacteria resistant to ampicillin and to benzylpenicillin are shown in Table 6; it can be seen that all strains brought about marked destruction of both penicillins. In some cases, no penicillin could be detected in any of the culture tubes in which there was growth, e.g. *Escherichia coli* 70, *Proteus mirabilis* 889, *Klebsiella pneumoniae* C, *Aerobacter aerogenes* A and G. In other cases there was complete destruction of benzylpenicillin, but only partial destruction of ampicillin, for example, with strains of *P. rettgeri* A and *Ps. aeruginosa* A and C9. Yet again, with some strains there was only partial destruction of either penicillin, e.g. *E. coli* 37 and Paracolon WM29.

Quantitative estimation of penicillinase activity by *Pseudomonas aeruginosa* was made by bioautographic techniques, as it was noted that antibiotic-free cultures of this species displayed marked antibacterial activity against the assay organism, *Bacillus subtilis* ATCC 6633, with the result that microbiological assays showed little

Table 6. *Penicillinase activities of Gram-negative bacteria resistant to ampicillin and benzylpenicillin*

Minimal inhibitory concentrations of the penicillins against ten strains of Gram-negative bacteria were determined by serial dilution in nutrient broth; growth and residual penicillin activity after overnight incubation at 37° were determined by visual inspection and microbiological assay respectively.

Organism	Antibiotic	Growth and penicillin concentration (μg./ml.) after incubation at 37° for 18 hr.							
Bacteria-free control*		500	250	125	50	25	12·5	5·0	2·5	1·25
Escherichia coli 70	Benzyl-penicillin	+	+	+	+	+	+	+	+	+
		0	0	0	0	0	0	0	0	0
	Ampicillin	+	+	+	+	+	+	+	+	+
		0	0	0	0	0	0	0	0	0
Proteus mirabilis 889	Benzyl-penicillin	+	+	+	+	+	+	+	+	+
		0	0	0	0	0	0	0	0	0
	Ampicillin	+	+	+	+	+	+	+	+	+
		0	0	0	0	0	0	0	0	0
Klebsiella pneumoniae C	Benzyl-penicillin	+	+	+	+	+	+	+	+	+
		0	0	0	0	0	0	0	0	0
	Ampicillin	+	+	+	+	+	+	+	+	+
		0	0	0	0	0	0	0	0	0
Aerobacter aerogenes A	Benzyl-penicillin	−	−	+	+	+	+	+	+	+
		170	8	0	0	0	0	0	0	0
	Ampicillin	−	+	+	+	+	+	+	+	+
		90	0	0	0	0	0	0	0	0
A. aerogenes G	Benzyl-penicillin	−	+	+	+	+	+	+	+	+
		55	0	0	0	0	0	0	0	0
	Ampicillin	+	+	+	+	+	+	+	+	+
		0	0	0	0	0	0	0	0	0
Pseudomonas aeruginosa A	Benzyl-penicillin	+	+	+	+	+	+	+	+	+
		0	0	.	.	.	.	.	.	.
	Ampicillin	+	+	+	+	+	+	+	+	+
		530	135	.	.	.	.	.	.	.
Ps. aeruginosa C9	Benzyl-penicillin	+	+	+	+	+	+	+	+	+
		0	0	.	.	.	.	.	.	.
	Ampicillin	+	+	+	+	+	+	+	+	+
		375	150	.	.	.	.	.	.	.
Proteus rettgeri A	Benzyl-penicillin	+	+	+	+	+	+	+	+	+
		0	0	0	0	0	0	0	0	0
	Ampicillin	−	−	−	±	+	+	+	+	+
		520	260	120	40	9·2	0·88	0·2	0	0
E. coli 37	Benzyl-penicillin	+	+	+	+	+	+	+	+	+
		8·5	18·5	12·5	3·2	0·3	< 0·1	0·5	0·4	< 0·1
	Ampicillin	−	−	+	+	+	+	+	+	+
		505	220	105	31	13·0	6·5	1·7	0·6	0·2
Paracolon WM 29	Benzyl-penicillin	+	+	+	+	+	+	+	+	+
		160	72	15	15	0	0	0	0	0
	Ampicillin	+	+	+	+	+	+	+	+	+
		330	135	82	29	9·3	7·1	1·5	0·35	0·1

* These concentrations represent the initial concentrations of penicillins prior to incubation with the cultures.
+ = Growth. − = No growth. ± = Partial growth.

or no destruction of either penicillin by this species. In fact, most strains of *P. aeruginosa* were capable of causing some destruction of both penicillins, ampicillin being more resistant than benzylpenicillin.

It is of interest that of some 30 strains of Gram-negative bacteria tested which were resistant to ampicillin and benzylpenicillin, all were capable of causing some degree of destruction of penicillin. In this series, no organisms which did not produce penicillinase and were inherently penicillin insensitive were isolated. In general, ampicillin was more stable than benzylpenicillin to the penicillinases produced by these Gram-negative bacteria, save that strains of *Aerobacter aerogenes* appeared to destroy ampicillin to a rather greater extent than benzylpenicillin.

Further experiments were designed to attempt to determine to what extent the resistance of these bacteria was due to penicillinase production. A simple type of experiment appeared to be estimation of the effect of decrease in inoculum size on

Table 7. *The effect of alteration of inoculum size on the sensitivity of Gram-negative bacteria to ampicillin and benzylpenicillin*

Organism	Inoculum*	Minimal inhibitory concentrations (μg./ml.) Ampicillin	Benzyl-penicillin
Escherichia coli 70	Neat	> 500	500
	10^{-3}	12·5	25
	10^{-6}	12·5	25
Proteus vulgaris D	Neat	> 500	> 500
	10^{-3}	5·0	12·5
	10^{-6}	1·25	2·5
P. morganii A	Neat	125	> 500
	10^{-3}	25	125
	10^{-6}	2·5	12·5
Aerobacter aerogenes A	Neat	500	125
	10^{-3}	12·5	12·5
	10^{-6}	2·5	5·0
A. aerogenes G	Neat	500	125
	10^{-3}	12·5	12·5
	10^{-6}	2·5	5·0
Pseudomonas aeruginosa A	Neat	> 500	> 500
	10^{-3}	500	> 500
	10^{-6}	500	> 500
E. coli 83	Neat	> 500	> 500
	10^{-3}	> 500	> 500
	10^{-6}	500	> 500
P. mirabilis 889	Neat	500	500
	10^{-3}	500	250
	10^{-6}	250	250

* 1 drop of an overnight culture diluted as specified.

the sensitivity of the organism to penicillin, the rationale being that intrinsically sensitive, penicillinase-producing bacteria would show a marked increase in sensitivity to penicillins with smaller inocula, whereas very small inocula of inherently insensitive organisms would still be capable of proliferating in high

concentrations of penicillin. It can be seen from Table 7 that *Escherichia coli* 70, *Proteus vulgaris* D and *P. morganii* A, and *Aerobacter aerogenes* A and G were very much more sensitive to ampicillin and benzylpenicillin when small inocula were employed, whereas with *Pseudomonas aeruginosa* A, *E. coli* 83, and *P. mirabilis* 889 there was little or no change in sensitivity to ampicillin or benzylpenicillin, even when the inoculum was diminished by a factor of one in a million. From these results it would seem that the former five strains might be examples of intrinsically sensitive, penicillinase-producing bacteria, and that the latter three strains might serve as examples of intrinsically insensitive organisms. These results prompted further experiments designed to confirm these findings by correlating growth with concomitant penicillin destruction.

Table 8. *Destruction of benzylpenicillin by cultures of Aerobacter aerogenes* A

Nutrient broth solutions containing graded concentrations of benzylpenicillin were inoculated with *Aerobacter aerogenes* A and incubated at 37° for 24 hr; samples were taken at intervals and assayed for penicillin content by microbiological assay.

Incubation time (hr)	Penicillin concentration (μg./ml.)		
0	100	25	5·0
1	100	25	4·3
$2\frac{1}{4}$	90	20	2·6
4	37	2·8	1·6
$5\frac{1}{2}$	4·7	2·2	0·7
24	0	0	0

An example of an intrinsically sensitive, but penicillinase-producing organism is illustrated in Fig. 2, which shows the growth of *Aerobacter aerogenes* A in the presence of different concentrations of benzylpenicillin; the rate of destruction of the penicillin by this culture is shown in Table 8. The minimal inhibitory concentration of this culture after overnight incubation was 500 μg. benzylpenicillin/ml., but during the first 6 hr a concentration of 100 μg./ml. completely inhibited growth; during this period the penicillin concentration fell from 100 to 5 μg./ml. Thereafter growth took place so that after 24 hr the turbidity of this culture equalled that of a penicillin-free control culture. These results appear to confirm that the growth of this culture in the presence of benzylpenicillin was related to the rate of destruction of the antibiotic by the organism.

In contrast, an intrinsically insensitive strain of *Escherichia coli* 83 multiplied in the presence of concentrations of up to 500 μg. benzylpenicillin/ml. as rapidly as did an antibiotic-free culture (Fig. 3), although the rate of destruction of penicillin was relatively slow (Table 9), showing that the organism was able to proliferate readily in the presence of high concentrations of penicillin.

Thus, the inferences drawn from the inoculum-dilution experiments regarding the nature of the insensitivity of various Gram-negative bacteria to penicillins appeared to be supported by the growth experiments illustrated above, and would seem to confirm that whereas some penicillinase-producing strains of Gram-negative bacteria are inherently insensitive to penicillins, with other strains resistance is due mainly to destruction of the antibiotic.

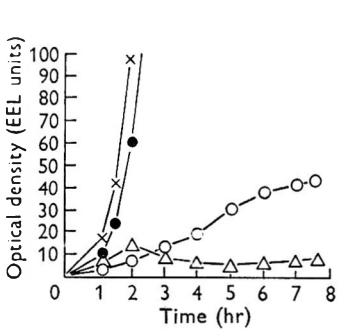

Fig. 1

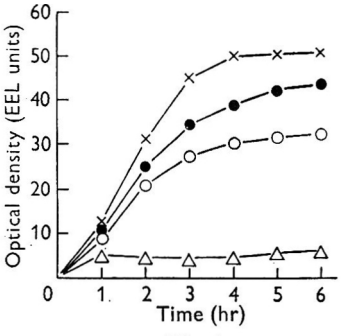

Fig. 2

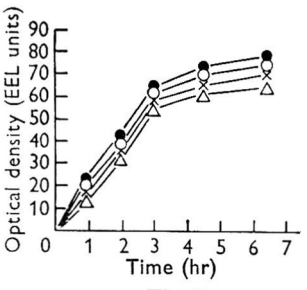

Fig. 3

Fig. 1. Growth of *Escherichia coli* (T341) in the presence of benzylpenicillin. ×, Penicillin-free culture; ●, culture containing penicillin, 5 μg./ml.; ○, culture containing penicillin, 12·5 μg./ml.; △, culture containing penicillin, 25 μg./ml.

Fig. 2. Growth of *Aerobacter aerogenes* A in the presence of benzylpenicillin. ×, Penicillin-free culture; ●, culture containing penicillin, 5 μg./ml.; ○, culture containing penicillin, 25 μg./ml.; △, culture containing penicillin, 100 μg./ml.

Fig. 3. Growth of *Escherichia coli* (83) in the presence of benzylpenicillin. ×, Penicillin-free culture; ●, culture containing penicillin, 25 μg./ml.; ○, culture containing penicillin, 125 μg./ml.; △, culture containing penicillin, 500 μg./ml.

Table 9. *Destruction of benzylpenicillin by cultures of Escherichia coli 83*

Nutrient broth solutions containing graded concentrations of benzylpenicillin were inoculated with *Escherichia coli* 83 and incubated at 37° for 24 hr; samples were taken at intervals and assayed for penicillin content by microbiological assay.

Incubation time (hr)	Penicillin concentration (μg./ml.)		
0	500	125	25
1	490	124	20
3½	465	115	16
5½	425	90	8
24	0	0	0

DISCUSSION

On the basis of the results reported here, the penicillins which display a relatively low order of activity against any of the Gram-negative bacteria examined may be readily distinguished from those penicillins which show a significant effect against certain strains. Compounds in the former group include phenethicillin, propicillin, methicillin and cloxacillin, and it is plain that the insensitivity of the Gram-negative bacteria to these penicillins is in no way associated with penicillinase. In contrast, the sensitivities of Gram-negative bacteria to ampicillin and benzylpenicillin were very varied, and in many cases the reaction of the organism to these drugs appeared to depend upon the ability of the organism to produce a penicillinase capable of destroying the specific penicillin. From the experiments described above, the bacteria tested could be divided into three groups, according to the effect of the specific penicillinase upon ampicillin or benzylpenicillin, e.g.:

(*a*) Strains which produced little or no penicillinase and were sensitive to

ampicillin and to benzylpenicillin, e.g. one Salmonella species, and some strains of *Proteus mirabilis* and Klebsiella.

(*b*) Strains which caused little or no destruction of ampicillin but more marked destruction of benzylpenicillin, and which were, accordingly, sensitive to ampicillin but less sensitive to benzylpenicillin, e.g. strains of *Escherichia coli*, Shigella species.

(*c*) Strains which were capable of destroying both compounds. This group, comprising penicillin-resistant organisms, could be further divided into: (i) intrinsically sensitive, penicillinase-producing, bacteria, e.g. strains of *Aerobacter aerogenes*, *P. vulgaris*, *P. morganii;* (ii) intrinsically insensitive, penicillinase-producing, bacteria, e.g. *Pseudomonas aeruginosa*, strains of *E. coli* and *P. mirabilis*.

It follows from these findings that the main difference in the activities of ampicillin and benzylpenicillin is associated with the increased stability of ampicillin towards the penicillinases produced by certain Gram-negative bacteria. Consequently, while ampicillin is from two to four times more active than benzylpenicillin against Gram-negative bacteria which produce little or no penicillinase, it is at least ten times more active against many strains which do produce penicillinase. The latter would apparently include the large number of strains of *Escherichia coli* which are sensitive to relatively low concentrations of ampicillin, but which require considerably higher concentrations of benzylpenicillin for inhibition. It is worth noting, however, that with these organisms growth is dependent upon destruction of the penicillin in the culture medium, so that benzylpenicillin might seem to be considerably more effective if antibacterial activity were measured over a shorter period of time than is conventional. Strains resistant to both penicillins are usually capable of destroying both compounds, although, again, ampicillin is generally more stable to the action of the penicillinases produced by these organisms. An exception to this finding is that benzylpenicillin appears to be more stable to *Aerobacter aerogenes* than is ampicillin but there is usually extensive destruction of both compounds. These findings are in good agreement with those of various other workers who have reported that ampicillin displays increased stability to the penicillinases produced by Gram-negative bacteria (Auhagen *et al.* 1962; Ayliffe, 1963; Smith, 1963). The last two workers also noted the superior stability of benzylpenicillin to the penicillinases produced by Aerobacter species.

In this series of experiments, all the resistant strains of bacteria produced penicillinase, whereas Trafford *et al.* (1962) reported that a relatively high proportion of bacteria which did not produce penicillinase were resistant to ampicillin. On the other hand, Barber (1962) reported that the majority of coliform bacteria are penicillinase-producing organisms capable of destroying penicillins. It is possible that in some cases at least, divergences in findings may be due to differences in techniques; this is being investigated. Likewise, although Knox & Smith (1962) have described strains of Gram-negative bacteria which are sensitive to ampicillin but with inherent resistance to benzylpenicillin, such strains have not been isolated in the experiments described above.

It thus seems that benzylpenicillin is active against strains of Gram-negative bacteria which produce little or no penicillinase, but is ineffective against penicillinase-producing bacteria. In contrast, ampicillin is relatively stable to the penicillinases produced by certain of the Gram-negative bacteria, and is, as a consequence, substantially more active than benzylpenicillin against many of these

bacteria. With resistant strains of bacteria, however, the situation is much more complex; here insensitivity may be due to penicillinase activity alone, or may be due to inherent resistance, although in the results reported here this inherent resistance was always accompanied by some degree of penicillin destruction.

It is a pleasure to thank Dr M. O. Moss for his assistance in carrying out the chromatographic procedures and Dr G. N. Rolinson for much helpful advice and criticism.

REFERENCES

ABRAHAM, E. P. & CHAIN, E. (1940). An enzyme from bacteria able to destroy penicillin. *Nature, Lond.* **146**, 837.

AUHAGEN, E., GLOXHUBER, C., HECHT, G., KNOTT, TH., OTTEN, H., RAUENBUSCH, E., RISSE, K. H., SCHMID, J., SCHOLTAN, W. & WALTER, A. M. (1962). Ampicillin-Binotal(R), ein Breitspektrum-Penicillin. *Arzneimittel-Forsch.* **12**, 791.

AYLIFFE, G. A. J. (1963). Ampicillin inactivation and sensitivity of coliform bacilli. *J. gen. Microbiol.* **30**, 339.

BARBER, M. (1962). *Resistance of Bacteria to the Penicillins*, p. 86. *Ciba Foundation Study Group*, No. 13. London: J. and A. Churchill, Ltd.

BATCHELOR, F. R., DOYLE, F. P., NAYLER, J. H. C. & ROLINSON, G. N. (1959). Synthesis of penicillin: 6-aminopenicillanic acid in penicillin fermentations. *Nature, Lond.* **183**, 257.

BONDI, A. & DIETZ, C. C. (1944). Production of penicillinase by bacteria. *Proc. Soc. exp. Biol. N.Y.* **56**, 132.

KNOX, R. & SMITH, J. T. (1962). Antibacterial activity, penicillinase stability, and inducing ability of different penicillins. *J. gen. Microbiol.* **28**, 471.

ROLINSON, G. N. & STEVENS, S. (1961). Microbiological studies on a broad-spectrum penicillin, 'Penbritin'. *Brit. med. J.* ii, 191.

SMITH, J. T. (1963). Penicillinase and ampicillin resistance in a strain of *Escherichia coli*. *J. gen. Microbiol.* **30**, 299.

STEWART, G. T., COLES, H. M. T., NIXON, H. H. & HOLT, R. J. (1961). 'Penbritin': an oral penicillin with broad-spectrum activity. *Brit. med. J.* ii, 200.

THOMAS, R. (1961). Colorimetric detection of penicillins and cephalosporins on paper. *Nature, Lond.* **191**, 1161.

TRAFFORD, J. A. P., MACLAREN, D. M., LILLICRAP, D. A., BARNES, R. D. S., HOUSTON, J. C. & KNOX, R. (1962). Ampicillin: a broad-spectrum penicillin. *Lancet*, i, 987.

J. gen. Microbiol. (1964), 34, 99–114
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Effects of Thermal Stress on Viability and Ribonucleic Acid of *Aerobacter aerogenes* in Aqueous Suspension

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SUMMARY

The death-rate of washed *Aerobacter aerogenes* in aqueous suspension at 47° depended on the nature of the growth medium, the composition of the liquid used to wash and resuspend the bacteria, the bacterial growth phase, the bacterial concentration in heated suspensions, the pH value, the oxygen tension and the composition of the diluent in which bacteria were heated. The relative resistance of bacteria in different growth phases differed according to the growth medium and the washing fluid; stationary phase bacteria were not more resistant than exponential phase organisms under all conditions. Starvation increased the thermal resistance of exponential and stationary phase bacteria. High bacterial concentration favoured survival at 47° under most conditions; cell-free filtrate from a heated dense suspension (10^{10} bacteria/ml.) protected a sparser population of fresh bacteria (10^7–10^9/ml.) heated in it. Protective material in filtrate was heat-stable (100°/15 min.) and diffused through cellophan. The optimum pH value for survival at 47° was near pH 6·5. Aerobic conditions favoured survival in distilled water but not in salt solutions or phosphate saline (pH 6·5). The effects of various concentrations of NaCl and KCl on the survival of bacteria at 47° under aerobic conditions were different, K^+ concentrations above 0·1 M being more lethal than equivalent concentrations of Na^+; the lethal effect of heating in mixtures of these salts (total M > 0·1) increased with K^+ concentration. Growth medium, Mg^{2+} (0·01–5 mM) and, to a lesser extent, Mn^{2+} (0·5 mM) or Co^{2+} (5 mM) decreased the death-rate, whereas ethylenediamine tetraacetic acid (mM), or various sugars, increased it. Mg^{2+} but not Mn^{2+} reversed the lethal effect of sugars.

Generally, conditions which accelerated the death-rate of *Aerobacter aerogenes* at 47° also increased the rate of degradation of endogenous RNA. This was accompanied by an increase in the ultraviolet absorption of cold acid-extracts of bacteria and of the suspending fluid. Bacterial protein was degraded to a smaller extent. Depletion of RNA is probably not the primary cause of death at 47° but the effect on bacterial metabolism of a rapid increase in endogenous pool constituents resulting from RNA degradation may contribute to the lethal effect.

INTRODUCTION

The general topic of the heat-inactivation of micro-organisms in aqueous suspension has been reviewed by Rahn (1945) and Wood (1956). Mechanisms proposed for heat destruction of bacteria include coagulation of protein, inactivation of enzymes, disruption of cellular lipids and damage to the genetic apparatus. Chick (1910) proposed protein denaturation as the cause of death during heating. Edwards & Rettger (1937) observed excellent agreement between the maximum growth

temperature of several bacilli and the minimum temperature at which some of their respiratory enzymes were inactivated. However, Rahn & Schroeder (1941) showed that under conditions where 99% of a bacterial population was killed by heat, only 14% of the peroxidase and 20% of the catalase had been inactivated; they concluded that enzyme coagulation was not the cause of death. Califano (1952) also considered that the lethal effect of high temperature on bacteria is not due to protein denaturation. He demonstrated that exposure to heat caused a separation of ribonucleic acid in soluble form from the bacteria into the suspending fluid; the temperature at which the process was initiated depended on the species of bacteria and was related to the temperature of inactivation. Wood (1956) suggested that it is possible to explain the lethal effects of high temperature on various micro-organisms by assuming that heat exerts detrimental effects on the reproductive system of these organisms; but that in yeast, at least a portion of the heat damage is probably due to cytoplasmic injury. The present paper records a study of the effect of comparatively mild thermal stress on the viability and ribonucleic acid of *Aerobacter aerogenes* in aqueous suspension and includes some data obtained with *Serratia marcescens*.

METHODS

Organisms. *Aerobacter aerogenes* (NCTC 418) and a laboratory strain of *Serratia marcescens* were used.

Media and cultural conditions. Organisms were grown at 37° in shaken flasks (2 l.) containing 100 ml. of either the carbon-limiting mannitol + ammonia + salts medium previously described (Strange, Dark & Ness, 1961) or Douglas's digest (TM) broth (Medical Research Council, 1931) seeded with 4 ml. of a fully grown culture (18–20 hr) in the same medium. 'Exponential phase' bacteria were harvested after 3 hr when 40–50% of the maximum growth had occurred. In experiments concerned with the effect of growth phase on thermal resistance, bacteria were grown in a batch culture vessel essentially as described by Elsworth, Meakin, Pirt & Capell (1956) at a continuously maintained pH value of 7·2–7·4 with adequate aeration (Strange *et al.* 1961).

Viability determinations. Viable counts were made using 3 or 5 Douglas's digest broth (TM) agar plates for each determination as previously described (Strange *et al.* 1961). Direct determinations of the % viable bacteria in suspensions were made with dark ground illumination after slide culture on the rich medium (containing glycerol, ammonia, salts, Difco yeast extract, Difco casamino acids, meat digest broth and set with agar) described by Postgate, Crumpton & Hunter (1961).

Heat treatment of bacterial suspensions. Bacteria were separated from the culture by centrifugation, washed once or twice and resuspended to a suitable concentration in diluent. Rapid heating of bacterial suspensions was achieved by dilution (1/10–1/50) in warm diluent held in a temperature-controlled bath at 47° (±0·05°). Washed air or nitrogen was bubbled by means of a Pasteur pipette through diluents before and after adding suspension, to obtain aerobic or anaerobic conditions. The diluents used were: distilled water; phosphate saline which contained NaCl (0·11 M) and appropriate concentrations of $K_2HPO_4 + KH_2PO_4$ (0·02 M-PO_4; pH 6·5); sodium phosphate (0·0375 M) buffers containing appropriate volumes of Na_2HPO_4 (5·3 g./l.) and NaH_2PO_4, $2H_2O$ (5·8 g./l.) solutions to give the required pH value; others are

mentioned in Results. All diluents and diluents+additives were filtered through well-washed sterile Oxoid membrane-filter (grade A.P., Oxo Ltd.) before use.

Materials. Water used for media and phosphate saline was passed twice through a mixed-bed ion-exchange resin (Amberlite MB-1; from British Drug Houses, Ltd.) column before use. Water used for washing bacteria and as a diluent during heating was glass-distilled. Whenever possible, Analytical Reagent grade chemicals were used. Dehydrated firefly tails were obtained from L. Light and Co. Ltd.

Analytical methods. 'Filtrate' from a bacterial suspension (equiv. 0·5–2·5 mg. dry wt./ml.) was obtained by filtration of a heated sample through a well-washed and dried membrane filter. In some experiments, cold acid-soluble substances were then extracted from the bacteria on the filter with chilled 0·25 N-$HClO_4$ (10–15 ml.) and after 30 min. the extract was sucked through under reduced pressure. When assays of nucleic acids were required, bacteria separated from suspension (5 ml.) by centrifugation were extracted with 0·25 N-$HClO_4$ (5 ml.) for 30 min. at 0° and recovered by centrifugation; nucleic acids were then dissolved by two extractions with 0·5 N-$HClO_4$ for 15 min. at 70°. RNA in the combined hot acid extracts was determined with the Bial reaction (Morse & Carter, 1949) with yeast RNA as standard and DNA by Burton's (1956) modification of the diphenylamine reaction, with thymus DNA as standard. Ninhydrin-reacting substances in filtrate from bacterial suspension were determined by the colorimetric method of Yemm & Cocking (1955) with alanine as standard. The ATP-content of cold acid extracts of bacteria was determined by a firefly luminescence technique (Strehler & Totter, 1952) as described previously (Strange & Dark, 1962); before assay, extracts were neutralized with KOH and, after standing for several hr at 0°, precipitated $KClO_4$ was separated by centrifugation. Magnesium was determined colorimetrically with Titan Yellow as described by Gardner (1946) except that preliminary treatment of samples with trichloroacetic acid was omitted; it is generally accepted that this method lacks precision so the results obtained were usually checked by titrating samples with ethylenediamine tetraacetic acid (EDTA) using Eriochrome Black T as indicator (Debney, 1952). Potassium was precipitated with sodium cobaltinitrite and determined colorimetrically (Barry & Rowland, 1953). Bacterial protein and dry weight were determined as previously described (Strange *et al.* 1961). Ultraviolet absorption (u.v.) was measured in a Unicam quartz spectrophotometer, model S.P. 500, with a 1 cm. light path.

RESULTS

Determination of the viability of heat stressed bacteria

Heating bacteria at sublethal temperatures extends the lag phase during subsequent growth on nutrient medium (Hershey, 1939). On TM agar, the colonies produced by heat-treated *Aerobacter aerogenes* usually varied greatly in size among themselves and all were smaller than colonies from untreated bacteria incubated for the same period. Colony counts did not change significantly after incubation of the plates for more than 48 hr. When viability was determined by slide culture, the growth lag period of bacteria was decreased by using a supplemented agar medium (Postgate *et al.* 1961), and the incubation period was extended as long as possible without allowing overgrowth of dead organisms by colonies from organisms

with shorter lag periods. Under these conditions the % viability results were usually close to those obtained by viable counts on TM agar plates (Fig. 2). In experiments reported below, % viability results obtained by only one of the methods are given but all experiments were repeated and results were always confirmed using the other method.

Factors affecting the survival of bacteria at 47°

Preparation of bacterial suspensions. Since certain constituents in culture medium, including Mg^{2+} and sugars (see below), markedly affected the death-rate of bacteria during heating at 47°, bacteria were separated by centrifugation from cultures, then washed and resuspended in diluent before examination of their heat

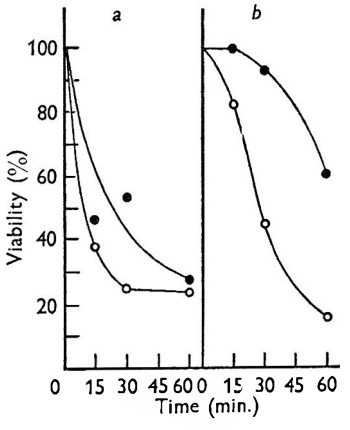

Fig. 1

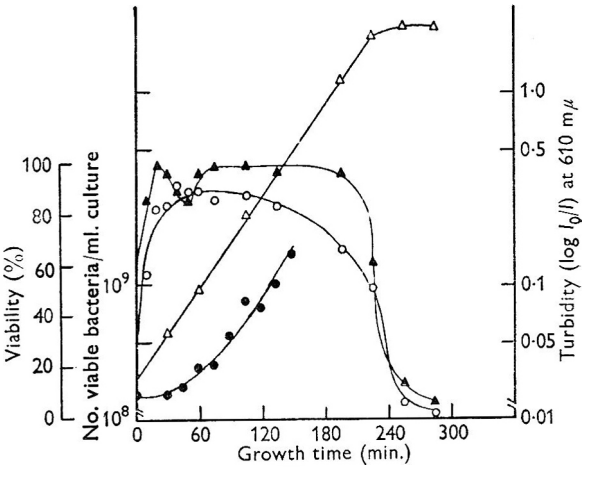

Fig. 2

Fig. 1. Effect of washing procedure on the thermal resistance of exponential phase *Aerobacter aerogenes*. Bacteria washed with water (●) or phosphate saline (○) were resuspended in water (about $2{\cdot}5 \times 10^9$/ml.). Samples of washed suspensions (0·2 ml.) were added to 4·8 ml. each of water (*a*) and phosphate saline (*b*) aerated at 47°. Viabilities of the four suspensions were determined at intervals by plate counts.

Fig. 2. Effect of growth phase on the thermal resistance of *Aerobacter aerogenes*. Bacterial growth in a defined medium was measured turbidimetrically (△) and by viable counts (●). At intervals bacteria centrifuged from the culture were washed and resuspended at about 10^9/ml. in water. Washed suspension (0·1 ml.) was added to phosphate saline (4·9 ml.) at 47° and viability was determined after 0·5 hr. by plate counts (▲) and slide culture (○).

sensitivity. However, the composition of the liquid used to wash bacteria affected their subsequent survival in a given diluent at 47° (Fig. 1). Thus, bacteria washed in distilled water were more resistant than similar bacteria washed in phosphate saline. Further experiments showed that washing bacteria with solutions of NaCl or KCl (0·05–0·15 M) decreased their thermal resistance.

Addition of magnesium ions (0·5 mM) to diluents in which bacteria were heated markedly decreased the death-rate and under these conditions survival was little affected by washing in either distilled water or salt solutions. The possibility that salt solutions displaced magnesium from *Aerobacter aerogenes* was examined as follows. A suspension of bacteria (2×10^{10}/ml.) was washed twice with water by

centrifugation. The resulting pellet was resuspended to the initial concentration in $MgSO_4$ solution (100 μg. Mg^{2+}/ml.) and allowed to stand for 15 min. at 20°. The bacteria were separated by centrifugation and freed from unadsorbed magnesium by washing until the supernatant fluid gave a negative test with the Titan Yellow reagent (Gardner, 1946). Five samples (3 ml.) of the magnesium-treated suspension (about 10^{10} bacteria/ml.) were centrifuged and the pellets resuspended in 5 ml. of water, phosphate saline or 1/4, 1/2 and 3/4 dilutions of phosphate saline and water. After centrifuging, analyses of the supernatant fluids showed the Mg^{2+} concentrations to be 0·2, 7·4, 6·4, 7·2 and 7·5 μg./ml., respectively. Thus, Mg^{2+} was desorbed from *A. aerogenes* by neat or diluted phosphate saline.

Table 1. *Effect of sodium and potassium on magnesium adsorption by Aerobacter aerogenes*

Organisms harvested from defined medium were washed twice with water and resuspended in water (8×10^{10}/ml.). Bacterial pellets centrifuged from 8 samples (1 ml.) were resuspended in water (2·5 ml.) and in 82, 8·2 and 0·82 mM-KCl or NaCl solutions (2·5 ml.). After 15 min. at 20°, 0·82 mM-$MgSO_4$ (2·5 ml.) was added to each suspension. After a further 15 min., supernatant fluid was separated from each suspension and assayed for Mg^{2+}.

	Concentration of Na^+ or K^+ (mM)	Concentration of Mg^{2+} (mM)	Ratio Na^+ or K^+ : Mg^{2+}	Mg^{2+} adsorbed (%)
K^+	0	0·41	0:1	38·5
	0·41		1:1	41·0
	4·1		10:1	34·5
	41·0		100:1	0
Na^+	0		0:1	37·5
	0·41		1:1	33·0
	4·1		10:1	23·0
	41·0		100:1	2·5

Competitive effects of Na^+ or K^+ on Mg^{2+} adsorption by *Aerobacter aerogenes* were demonstrated by exposing samples of a washed suspension to a constant concentration of Mg^{2+} in the presence of increasing concentrations of Na^+ or K^+. The results (Table 1) show that the amount of Mg^{2+} adsorbed remained fairly constant as the Na^+ or K^+ to Mg^{2+} ratio was increased to 10, but at ratios of 10 to 100 there was almost a complete suppression of Mg^{2+} adsorption.

Bacterial growth phase. Several reports (Sherman & Albus, 1923, 1924; Elliker & Frazier, 1938; White, 1951, 1953; Lemcke & White, 1959) indicate that the heat resistance of bacteria varies with growth phase ('age') of the culture and that bacteria in the early exponential phase are the least resistant. It was therefore surprising to find that, when grown in a carbon-limiting defined medium and washed with distilled water, stationary phase *Aerobacter aerogenes* were considerably less heat-resistant than exponential phase organisms (Fig. 2); but when grown in TM broth and tested under similar conditions, stationary phase organisms were the more resistant (Fig. 3). Bacteria separated from defined medium cultures by centrifugation and examined unwashed or washed with phosphate saline, showed least resistance to heating in phosphate saline when in the exponential growth phase. Also, bacterial density during heating was important and, irrespective of preliminary washing with distilled water or salt solutions, stationary phase

populations of above 4×10^9 bacteria/ml. survived as well as or better than similar populations of exponential phase organisms.

Bacterial concentration. As found with *Escherichia coli* by Sherman & Albus (1923) and Watkins & Winslow (1932), the death-rate of a heated suspension of *Aerobacter aerogenes* was lower the higher the initial bacterial concentration (Fig. 4). This phenomenon occurred under aerobic or anaerobic conditions and was probably mainly due to the protective effect of material leaking from heated bacteria. Cell-free filtrate separated from a heated (30 min./47°) suspension containing about 10^{10} bacteria/ml. phosphate saline protected a sparser population of fresh bacteria

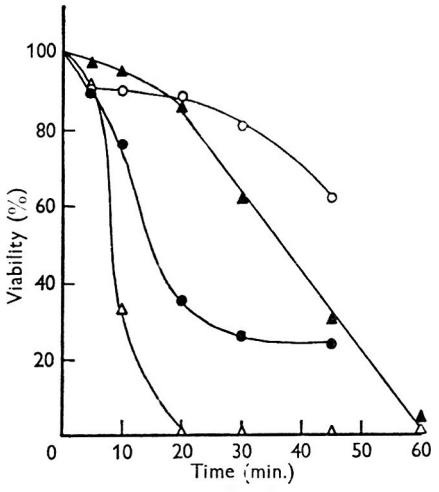

Fig. 3

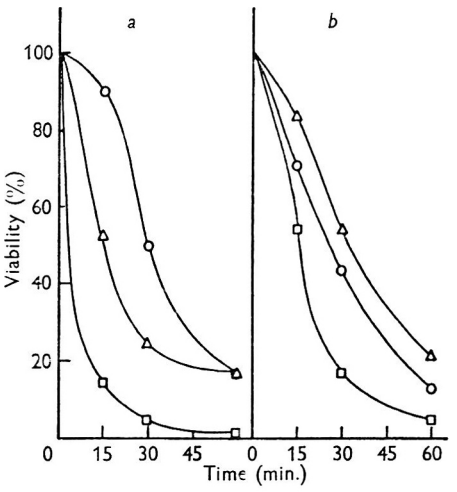

Fig. 4

Fig. 3. Comparison of the thermal resistance of exponential and stationary phase *Aerobacter aerogenes* grown in defined and complex media. Bacteria were prepared and heated as in Fig. 2. Viabilities of heated suspensions were determined at intervals by slide culture. Exponential phase bacteria from defined medium (▲) and TM broth (●); stationary phase bacteria from defined medium (△) and TM broth (○).

Fig. 4. Effect of bacterial concentration on the thermal resistance of *Aerobacter aerogenes*. Exponential phase bacteria from a defined medium were washed and resuspended at 7×10^{10}/ml. in phosphate saline. One series of three serial 1/10 dilutions of washed suspension in phosphate saline at 47° was aerated (*a*), the other had N_2 passed through (*b*). Viabilities by plate counts of suspensions initially containing 7×10^9 (○), 7×10^8 (△) and 7×10^7 (□) viable bacteria/ml.

(about 10^8/ml.) heated in it: viabilities after 10, 20 and 30 min. at 47° were 98, 93 and 86%, respectively, in filtrate, compared with 37, 15 and 12%, respectively, for a similar bacterial suspension heated in phosphate saline alone. Nelson (1943) found that bacteria subjected to heat at partially lethal values were more demanding in their growth requirements than unheated control organisms. It was possible that leakage products which appeared to exert their influence on bacteria during heating may have in fact acted after heating, by supplying substrates to moribund bacteria so that they were able to grow on nutrient agar. This was shown not to be so by adding leakage products to heated bacteria just before slide culture; the viability of the heated suspension was unaffected by added leakage products.

Leakage products from heated bacteria consisted of a complex mixture of substances (see below) and the factor(s) responsible for the protective effect has not been identified. Protective material diffused through cellophan on dialysis against water or phosphate saline (Fig. 5) and its activity was unaffected by heating at 100° for 15 min. Protection by filtrates of heated bacterial suspension was markedly decreased but not entirely eliminated by ethylenediamine tetraacetic acid (2 mM-EDTA).

Diluents in which bacteria were heated. Exponential phase bacteria grown in the defined medium were used for these experiments.

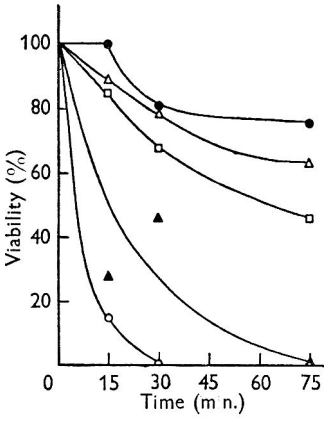

Fig. 5

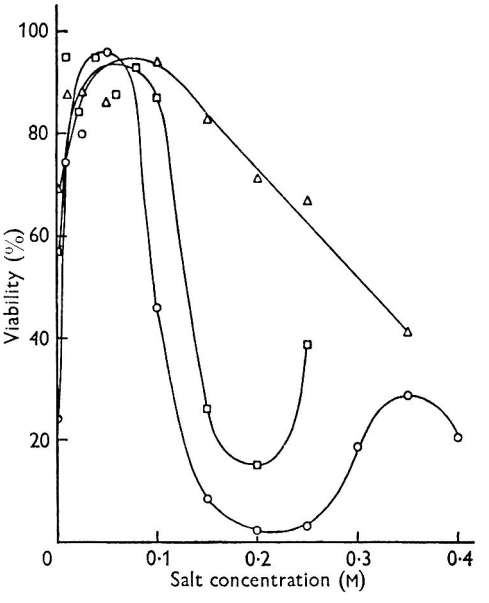

Fig. 6

Fig. 5. Effect of dialysable and non-dialysable leakage products from heated bacteria on the thermal resistance of *Aerobacter aerogenes*. Lyophilized leakage products (60 mg.) from filtrate of an aerated, heated (47°/1 hr.) suspension (equiv. 1·3 g. dry wt. bacteria/125 ml. H_2O) were dialysed against phosphate saline (6 × 5 ml.) at 2°. Diffusates were combined and the sac contents diluted with phosphate saline to 30 ml. Viabilities by plate counts of suspensions (about 10^8 exponential phase bacteria/ml.) at 47° in phosphate saline (○), diffusate + phosphate saline (1 + 1, by vol.) (□), sac contents + phosphate saline (1 + 1, by vol.) (▲), diffusate + sac contents (1 + 1, by vol.) (△) and undialysed leakage products (1 mg./ml. phosphate saline) (●).

Fig. 6. Effect of various concentrations of KCl, NaCl and equimolar NaCl + KCl on the thermal resistance of *Aerobacter aerogenes*. Exponential phase bacteria grown in a defined medium were washed and resuspended at $2·5 \times 10^9$/ml. in H_2O. Washed suspension (0·2 ml.) was added to diluent (4·8 ml.) at 47°. Viability after 1 hr in KCl solutions (plate counts; ○), equimolar NaCl + KCl solutions (plate counts; □) and NaCl solutions (slide culture; △).

(*a*) *pH value of diluent.* As found with *Escherichia coli* by Jordan & Jacobs (1948) and with *Streptococcus faecalis* by White (1963), the death-rate of *Aerobacter aerogenes* subjected to heat was lowest in slightly acid solutions. After 1 hr at 47° in aerated sodium phosphate buffer solutions (0·0375 M), viabilities of suspensions

(5×10^7 bacteria/ml.) at pH values of 4·5 (NaH_2PO_4 alone), 5·6, 5·8, 6·25, 6·6, 6·8, 7·0, 7·3, 7·7 and 9·0 (Na_2HPO_4 alone) were 34, 85, 86, 90, 95, 85, 81, 65, < 1 and < 1 %, respectively.

(*b*) *Oxygen tension of diluent.* The effect of air or nitrogen on the death-rate of bacteria during heating depended on the diluent and on bacterial concentration. In phosphate saline (pH 6·5) the death-rate was accelerated by aeration when suspensions contained up to 10^9 bacteria/ml., but above this bacterial concentration air or nitrogen had less effect (Fig. 4). In distilled water aeration decreased the death-rate (Fig. 11).

(*c*) *Concentration of salt in diluent.* The effects of various concentrations of NaCl, KCl and an equimolar mixture of NaCl and KCl on the thermal resistance of *Aerobacter aerogenes* in aerated suspension at 47° are shown in Fig. 6. At a concentration of 0·1–0·2 M, K^+ or $K^+ + Na^+$ was much more lethal than Na^+ alone. The lethality of $Na^+ + K^+$ (0·1–0·15 M) increased with the relative concentration of K^+ under aerobic conditions: viabilities after 1 hr. at 47° of suspensions (10^8 bacteria/ml.) in aerated 0·15 M-NaCl, 0·1 M-NaCl + 0·05 M-KCl, 0·05 M-NaCl + 0·1 M-KCl and 0·15 M-KCl were 73, 18, 2 and 15 %, respectively. Mg^{2+} and/or anaerobic conditions markedly decreased the death-rate of bacteria during heating in otherwise lethal concentrations of K^+, the concentration of Mg^{2+} required for maximum protection increasing with the K^+ concentration. As a diluent, aerated phosphate saline (pH 6·5; Na:K = 4:1) at 47° was more lethal to bacteria than distilled water or 0·15 M-NaCl, but replacement of the potassium buffer salts with equivalent sodium salts decreased the death-rate to that which occurred in 0·15 M-NaCl.

(*d*) *Trace metal ions in diluent.* The concentration of Mg^{2+} required to provide maximum protection to bacteria during heating depended not only on the other salts present but also on the bacterial density (Fig. 7). Mg^{2+} was taken up by bacteria suspended in water or phosphate saline at 47° (Fig. 8) and, in phosphate saline, absorption rather than adsorption probably accounted for this since desorption of the metal ion occurred from bacteria suspended in phosphate saline at 20° (Fig. 8). The rate of Mg^{2+}-uptake in phosphate saline at 47° was much slower than that which occurred in water at 20° or 47°. Determination of the small amount of Mg^{2+} taken up by bacteria in phosphate saline at 47° (3–4 μg./10^{10} bacteria/ml.) necessitated the use of relatively high bacterial concentrations. Under these conditions, the leakage products available to the bacteria may have affected Mg^{2+}-uptake by supplying energy sources and phosphate. Rothstein, Hayes, Jennings & Hooper (1958) showed that Mg^{2+} can be absorbed by yeast cells provided that glucose is available and that phosphate is absorbed.

Mn^{2+} (0·25–0·5 mM), Ca^{2+} and Co^{2+} (5 mM) also protected bacteria during heating in aerated phosphate saline but to a much smaller extent than Mg^{2+}. Above concentrations of 0·5 mM, Mn^{2+} was toxic at 47°. A mixture of Mg^{2+}, Co^{2+}, Ca^{2+} (each mM) and Mn^{2+} (0·25 mM) provided more protection than Mg^{2+} alone.

(*e*) *Other substances in diluent.* Both TM broth and the defined medium substantially protected bacteria during heating at 47°. Viabilities of bacteria washed with phosphate saline and heated for 30 min. at 47° in phosphate saline, the defined medium, TM broth and distilled water (10^8 bacteria/ml.) were < 1, 81, 71 and 25 %, respectively. The protective effect of TM broth was decreased by EDTA (2 mM); for example, when 10^8 bacteria/ml. were heated at 47° for 10, 20, 30, and 45 min.

viabilities were 84, 56, 38 and 30%, respectively, in TM broth + EDTA compared with 96, 94, 98 and 83%, respectively, in TM broth alone. Addition of EDTA (0·25–2 mM) markedly increased the death-rate of bacteria in distilled water at 47°: after 10 min. viability was < 1% in the presence and 54% in the absence of EDTA. In phosphate saline at 47° EDTA increased the death-rate slightly. 8-Hydroxyquinoline (0·5 mM) somewhat improved the survival of bacteria in phosphate saline but had little effect in distilled water.

Glucose, mannitol, lactose or ribose (10 mM) markedly increased the death-rate of bacteria in phosphate saline at 47°: viabilities after 10, 15 and 30 min. at 47° of

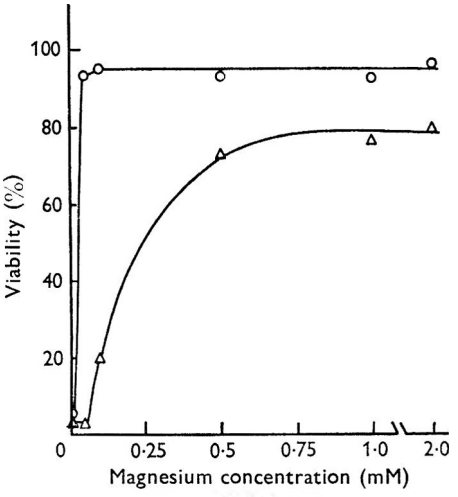

Fig. 7

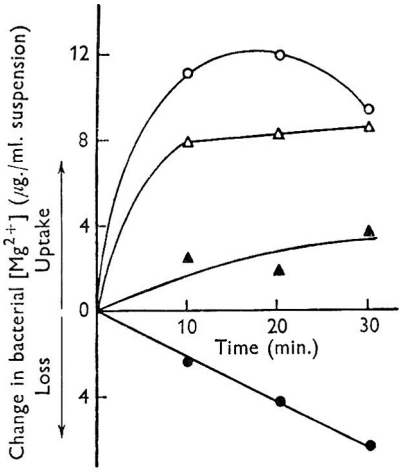

Fig. 8

Fig. 7. Effect of Mg^{2+} on the thermal resistance of *Aerobacter aerogenes* in dilute and dense bacterial suspensions. Exponential phase bacteria grown in a defined medium were washed and resuspended at about 10^{11}/ml. in phosphate saline. Dilutions (1/10 and 1/1000) of washed suspension were made in phosphate saline + $MgSO_4$ (0–2 mM), at 47°. Viability (slide culture) after 30 min. of suspension contained about 10^{10} (△) and 10^8 bacteria/ml. (○).

Fig. 8. Mg^{2+}-uptake by *Aerobacter aerogenes* at 47°. Exponential phase bacteria grown in a defined medium and washed with phosphate saline were: (1) washed and resuspended in H_2O; (2) washed and resuspended in phosphate saline (1 and 2 equiv. 50 mg. bacterial dry wt./ml.). Suspension 1 (5 ml.) added to aerated H_2O + 0·96 mM-Mg^{2+} (20 ml.) at 20° (○) and 47° (△); suspension 2 (5 ml.) added to aerated phosphate saline + 0·96 mM-Mg^{2+} (20 ml.) at 20° (●) and 47° (▲). Supernatant fluids from samples removed at intervals from the four suspensions were assayed for Mg^{2+}.

bacteria washed with phosphate saline were 63, 22 and 1%, respectively, in phosphate saline + ribose (10 mM); 81, 60 and 24%, respectively, in phosphate saline alone. Mg^{2+} (mM) reversed the lethal effect of sugars including glucose (Fig. 9) but Mn^{2+} was ineffective in this respect.

Degradation of RNA and protein in bacterial suspensions at 47°

Catabolism of RNA and protein which occurs in aerated suspensions of *Aerobacter aerogenes* starved at or below the growth temperature (Strange *et al.* 1961; Postgate & Hunter, 1962; Strange, Wade & Ness, 1963) also occurred at 47° but at a faster

rate. For example, the losses of RNA and protein from bacteria washed with and heated in aerated phosphate saline (pH 6·5) for 1 hr. at 47° were 24 and 5%, respectively, of the initial quantities. No significant change in the concentration of DNA was detected during this period (Table 2). Products released into the suspending fluid included u.v.-absorbing substances, ammonia and other ninhydrin-reacting substances, and traces of magnesium and calcium. The absorption maximum of filtrates from heated suspensions was near 255 mμ, indicating the presence of a high proportion of the deaminated base hypoxanthine; the concentration of pentose-reacting substances was low in proportion to the total u.v.-absorption. At least 80% of the total u.v.-absorbing components of the exudate diffused through

Table 2. *Effect of Mg^{2+} on viability and on degradation of RNA and protein in an aerated suspension of Aerobacter aerogenes at 47°*

Exponential phase bacteria harvested from a defined medium and twice washed with phosphate saline were resuspended (equiv. 2·3 mg. bacterial dry wt./ml.) in phosphate saline and phosphate saline + $MgSO_4$ (mM). Suspensions were aerated at 47° and samples removed at intervals for analyses and viability determinations. Results refer to a suspension initially containing equiv. 1 mg. bacterial dry wt./ml.

	Analytical results				
	Time (min.)				
	0	10	20	30	60
	Bacteria in phosphate saline				
Viability (%)	99	88	26	14	11
RNA (μg./ml.)	202	185	186	157	153
$E^{1\,cm.}_{255\,m\mu}$ of filtrate	0	0·46	0·87	1·2	1·5
$E^{1\,cm.}_{260\,m\mu}$ of cold acid bacterial extract	0·36	0·57	0·57	0·53	0·52
Protein (μg./ml.)	620	615	583	583	586
Ninhydrin-reacting substances as alanine in filtrate (μg./ml.)	2·4	6·7	9·8	12·2	16·5
NH_3-N in filtrate (μg./ml.)	0	0·7	1·3	1·7	2·3
DNA (μg./ml.)	24·8	26·0	24·4	24·6	24·8
	Bacteria in phosphate saline + Mg^{2+} (mM)				
Viability (%)	99	95	96	95	82
RNA (μg./ml.)	206	202	198	196	196
$E^{1\,cm.}_{255\,m\mu}$ of filtrate	0	0·15	0·25	0·36	0·62
$E^{1\,cm.}_{260\,m\mu}$ of cold acid bacterial extract	0·32	0·40	0·44	0·45	0·43
Protein (μg./ml.)	605	607	618	630	630
Ninhydrin-reacting substances as alanine in filtrate (μg./ml.)	2·4	5·2	6·5	8·1	10·1
NH_3-N in filtrate (μg./ml.)	0	0·4	0·7	0·9	1·5
DNA (μg./ml.)	24·6	24·4	25·0	24·8	24·8

cellophan during dialysis against phosphate saline or distilled water. Potassium and inorganic phosphate also leaked from bacteria during heating; this was demonstrated by heating bacteria in aerated water when, after 1 hr at 47°, the released material (equiv. about 4% initial bacterial dry weight) contained potassium (10%) and inorganic phosphate (15%). Although u.v.-absorbing fragments from degraded RNA were released rapidly, cold acid-soluble u.v.-absorbing substances present within the bacteria initially increased and then decreased only after a substantial decrease of viability had occurred (Table 2; Fig. 10). ATP was among the

intracellular u.v.-absorbing substances which initially increased during heating: values for the ATP-content of neutralized acid-extracts of samples of water-washed bacteria after 0, 10, 20, 30 and 45 min. at 47° in aerated phosphate saline (3·8 mg. dry wt. bacteria/ml.) were 1·5, 2·7, 2·1, 2·0 and 1·5 μg./mg. bacterial dry wt., respectively; viability of the suspension did not change significantly (99–97 %).

During heating of bacterial suspensions there was an initial period of zero or small viability loss during which RNA degradation proceeded at the maximum observed rate. Under many conditions, the rate of RNA degradation was related to the survival characteristics of a population as is shown in the following examples. The suspensions initially contained $1–4 \times 10^9$ viable exponential phase bacteria/ml. and

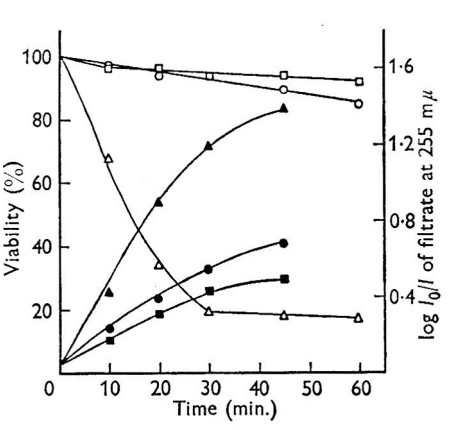

Fig. 9

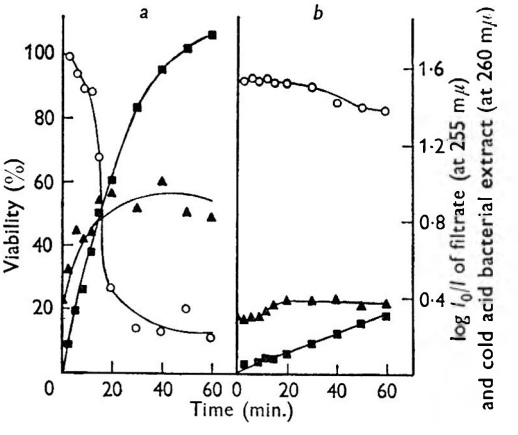

Fig. 10

Fig. 9. Effect of glucose on viability and RNA of *Aerobacter aerogenes* at 47°. Exponential phase bacteria grown in a defined medium were washed and resuspended at $1·4 \times 10^9$/ml. in H_2O. Washed suspension (3 ml.) was added to aerated diluent (27 ml.) at 47°. Viability determinations, open symbols; $E^{1\,cm.}_{255\,m\mu}$ value of filtrate (equiv. 1 mg. dry wt. bacteria/ml.), closed symbols. Diluents: phosphate saline (○, ●); phosphate saline + 10 mM glucose (△, ▲); phosphate saline + 10 mM glucose + 1 mM-$MgSO_4$ (□, ■).

Fig. 10. Effect of thermal stress on viability and RNA of *Aerobacter aerogenes* before and after starvation. Exponential phase bacteria grown in a defined medium were washed and resuspended in phosphate saline (equiv. 6·9 mg. bacterial dry wt./ml.). (*a*) Unstarved bacteria: washed suspension diluted 1/10 in aerated phosphate saline at 47°; (*b*) starved bacteria: washed suspension diluted 1/10 in aerated diluent at 37°; after 19 hr. bacteria (95 % viable) were recovered by centrifugation and diluted in aerated diluent at 47° to give the same no. bacteria/ml. as in (*a*). Viability of slide culture (○); $E^{1\,cm.}_{255\,m\mu}$ value of filtrate (■); $E^{1\,cm.}_{260\,m\mu}$ value of cold acid-extract of bacteria (▲). Absorption values equiv. 1 mg. bacterial dry wt./ml.

quantitative results refer to a suspension initially equiv. 1 mg. dry wt. bacteria/ml. (1) When bacteria washed in water and phosphate saline were heated in aerated phosphate saline for 45 min. at 47°, viabilities were 90 and 14 % and $E^{1\,cm.}_{255\,m\mu}$ values for filtrates were 0·79 and 1·55, respectively. (2) In aerated phosphate saline, Mg^{2+} decreased the death-rate, the loss of RNA and also the loss of protein at 47° (Table 2). (3) In aerated phosphate saline, glucose increased and glucose + Mg^{2+} decreased the death-rate and loss of RNA at 47° (Fig. 9). (4) When water-washed bacteria were heated in aerated 0·1 M-, 0·15 M-, 0·2 M- and 0·3 M-KCl for 1 hr,

viabilities were 93, 77, 35 and 24%, respectively, and $E^{1\,cm.}_{255\,m\mu}$ values for filtrates were 1·1, 1·4, 1·9 and 1·9, respectively. (5) When water-washed bacteria were heated in aerated phosphate saline and phosphate saline + EDTA (mM) for 30 min., viabilities were 90 and 77·5%, respectively, and $E^{1\,cm.}_{255\,m\mu}$ values for filtrates were 0·59 and 0·74, respectively. (6) During heating of phosphate saline-washed bacteria in phosphate saline viabilities after 10, 20 and 30 min. with vigorous aeration were 53, 4 and 2%, respectively, and $E^{1\,cm.}_{255\,m\mu}$ values for filtrates were 0·3, 1·5 and 2·2, respectively; under anaerobic conditions (nitrogen) viabilities were 92, 52 and 26%, respectively, and $E^{1\,cm.}_{255\,m\mu}$ values for filtrates were 0·5, 0·9 and 1·4, respectively.

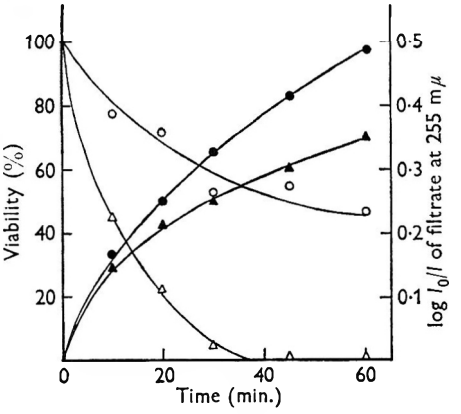

Fig. 11. Effect of air and nitrogen on the thermal resistance of *Aerobacter aerogenes* in H_2O at 47°. Exponential phase bacteria grown in defined medium were washed and resuspended in H_2O (equiv. 5·6 mg. bacterial dry wt./ml.). Samples of washed suspension diluted 1/10 in H_2O at 47° gassed with air (○, ●) and nitrogen (△, ▲). Viability by plate counts, open symbols; $E^{1\,cm.}_{255\,m\mu}$ values of filtrate (equiv. 1 mg. dry wt. bacteria/ml.), closed symbols.

The close relationship thus shown to exist between the loss of viability and the loss of RNA suggested that the lethal effect of heating at 47° on bacteria might be due to loss of, or irreversible damage to, RNA. If this were true, then decrease of the RNA-content of bacteria before heating should decrease or have no effect on their thermal resistance. This was investigated as follows. The death-rate and RNA degradation was measured in a sample of a washed suspension of bacteria (about 10^9/ml.) in phosphate saline at 47° with aeration; a second sample of suspension was held at 37° with aeration for 19 hr., the bacteria separated by centrifugation and then treated like those in the first sample. Despite the fact that the starved bacteria had lost 38% of their RNA, they were still 95% viable and their thermal resistance was much greater than that of unstarved bacteria (Fig. 10). During heating, both increase in endogenous u.v.-absorbing substances and leakage was much less in starved than in unstarved bacteria (Fig. 10).

An exception to the rule that an increased death-rate was associated with increased degradation of RNA was provided by heating bacteria in distilled water under aerobic and anaerobic conditions. Anaerobiosis increased the death-rate but reduced RNA degradation (Fig. 11).

The effects of thermal stress on *Serratia marcescens* were similar to those found

with *Aerobacter aerogenes*. The death-rate of exponential phase *S. marcescens* at 47° was lower in distilled water than in phosphate saline, and Mg^{2+} decreased the death-rate in both diluents, but particularly in the latter. Filtrate from a dense suspension of *S. marcescens* (about 10^{10} bacteria/ml.) protected fresh bacteria (about 10^8/ml.) heated in it. When *A. aerogenes* and *S. marcescens* harvested in the exponential phase from TM broth were washed and heated under similar conditions at 47°, losses of viability and leakage occurred as indicated in Table 3. The loss of viability and the leakage were both greater in the case of *S. marcescens*.

Table 3. *Comparison of the effects of heat stress on exponential phase Aerobacter aerogenes and Serratia marcescens*

Organisms grown in digest broth, washed with phosphate saline by centrifugation and resuspended in phosphate saline (equiv. 0·5 mg. dry wt. bacteria/ml.) were aerated at 47°. Samples were removed at intervals for analyses and viability determinations. Results refer to a suspension equiv. 1 mg. dry wt. bacteria/ml.

	A. aerogenes sampling time (min.)				*S. marcescens* sampling time (min.)			
	0	10	20	30	0	10	20	30
	Analytical results							
Viability (%)	100	—	—	80	100	—	—	40
$E^{1\,cm.}_{255\,m\mu}$ of filtrate	0	0·8	1·3	1·6	0	1·3	1·7	2·0
Ninhydrin-reacting substances as alanine in filtrate (μg./ml.)	4	11	17	20	10	27	30	35

DISCUSSION

Bacterial resistance to heat may alter as a result of environmental changes imposed immediately before thermal stress is applied. One effect of washing *Aerobacter aerogenes* in salt solutions was to desorb magnesium and this may explain the faster death-rate of bacteria at 47° after such treatment compared with bacteria washed in distilled water. Addition of magnesium to the diluent in which bacteria were heated largely eliminated the differences in thermal resistance resulting from the pre-washing treatment. Although stationary phase populations are generally regarded as being more resistant than exponential phase populations to stresses including heat (see review of Winslow & Walker, 1939), this was not true with *A. aerogenes* under all conditions. The influence of the growth phase on thermal resistance depended on several factors but particularly on the pre-washing solution and on bacterial concentration during heating. Thus, when grown in a chemically defined medium, washed in distilled water and heated at a concentration of about 10^8 bacteria/ml. phosphate saline, exponential phase bacteria were much more resistant than stationary phase bacteria; under similar conditions, but after washing in phosphate saline, stationary phase organisms were the more resistant. Assay of bacteria harvested at different times during the growth cycle and washed in distilled water, showed that the magnesium content (on a dry-weight basis) was significantly higher during exponential growth. Thus, the amount of magnesium available after washing with water was probably greater in the case of exponential phase organisms.

The fact that the lethal effect of heating *Aerobacter aerogenes* increased with the exogenous concentration of potassium above 0·1 M may have been due to a progressive

suppression of the diffusion of this ion out of bacteria. In experiments not reported here, leakage of potassium from bacteria at 47° was shown to be progressively decreased with increasing exogenous potassium (but not sodium) concentration up to 0·05M at least; above this concentration, changes caused by leakage of potassium were too small to measure accurately by chemical analysis. Assays of bacterial potassium were not made because of the possibility that some would be removed during washing to remove suspending fluid. At 47°, maintenance of, and possibly addition to, the normal intracellular ionic concentration may lead to ribosomal instability by a competitive displacement of ribosomally bound magnesium. Addition of magnesium to solutions of potassium which at 47° were otherwise highly lethal to bacteria caused a marked decrease in the rates of death and RNA degradation, probably because then the amount of magnesium absorbed by the bacteria was sufficient to counteract the competitive effects. It is of interest that the concentration of exogenous potassium (0·05M) in which *A. aerogenes* was most stable at 47° was also the minimum concentration in which a measurable 'optical effect' (change in turbidity of a suspension as compared with a similar suspension in water; Mager, Kuczynski, Schatzberg & Avi-Dor, 1956) occurred.

The protective effect of high initial bacterial concentration on bacteria subjected to heat stress may be due to many factors among which the heat-stable, dialysable fraction of the leakage products is of major importance. A similar population density phenomenon was observed on chilling exponential phase *A. aerogenes* (Strange & Dark, 1962) and it is of interest that filtrate from a chilled dense suspension (about 10^{10} bacteria/ml.) was found to protect a sparser suspension (about 10^8 bacteria/ml.) not only from the lethal effect of chilling but also from heating at 47°.

In agreement with previous observations by Califano (1952) concerning the effect of heat on various bacteria, the data presented here for suspensions of *Aerobacter aerogenes* heated at 47° under a variety of conditions strongly suggest a relationship between the rates of death and of RNA degradation. Magnesium, which is known to stabilize isolated ribosomes (see review by McQuillen, 1962), decreased both rates, whereas EDTA, which accelerates ribosomal breakdown (Chao, 1957; Wade, 1961), increased both rates. Magnesium, manganese, cobalt and calcium, which afforded good or some protection to *A. aerogenes* during heating, all strongly inhibited the auto-degradation at pH 6·5 of purified ribosomes isolated from *Escherichia coli* by Mr H. E. Wade.

In most cases the shape of the death and leakage curves provided evidence that RNA breakdown preceded bacterial death at 47°, there being an initial period of zero or small death-rate during which RNA degradation occurred at the maximum rate. Other results, not presented here, have shown that at temperatures between 30 and 47° the rate of RNA degradation in *Aerobacter aerogenes* suspensions increased nearly linearly with temperature while, at least up to 44°, the death-rate was negligible for the first hour or more. However, depletion of RNA to the extent that occurs during the initial period of heating at 47° does not account for the lethal effect. Bacteria with their RNA-content substantially diminished by starvation under conditions where there was little loss of viability showed a much greater thermal resistance than did unstarved bacteria. Since the leaked products from heated bacteria are protective, not toxic, one is left with the possibility that the

lethal effect associated with rapid RNA degradation results from an effect on bacterial metabolism of a sudden large increase of RNA fragments within the bacteria. It was shown that the ribose moiety of degraded RNA was metabolized by bacteria at 47° and also that exogenous ribose (or other sugars) accelerated the death-rate during heating. The lethal effect of exogenous sugars was annulled by the presence of magnesium and these findings appear to be related to the phenomenon of 'substrate-accelerated death' which occurs at lower temperatures (Postgate & Hunter, 1963). To what extent the mechanisms are similar remains to be investigated.

We are indebted to Mr F. A. Dark for useful discussion, Mr A. G. Ness for some analyses, Mr B. Phillips for technical assistance and Mr S. Lovett for the use of a scintillation counter.

REFERENCES

Barry, J. M. & Rowland, S. J. (1953). Determination of blood serum potassium by an improved sodium cobalt-nitrite method. *Biochem. J.* **53**, 213.

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.

Califano, L. (1952). Libération d'acide nucléique par les cellules bactériennes sous l'action de la chaleur. *Bull. Wld Hlth Org.* **6**, 19.

Chao, F. C. (1957). Dissociation of macromolecular ribonucleoprotein of yeast. *Arch. Biochem.* **70**, 426.

Chick, H. (1910). The process of disinfection by chemical agencies and hot water. *J. Hyg., Camb.* **10**, 237.

Debney, E. W. (1952). Micro-chemical determination of magnesium, calcium and zinc with disodium ethylene-diamine tetra-acetic acid. *Nature, Lond.* **169**, 1104.

Edwards, O. F. & Rettger, L. F. (1937). The relation of certain respiratory enzymes to the maximum growth temperatures of bacteria. *J. Bact.* **34**, 489.

Elliker, P. R. & Frazier, W. C. (1938). Influence of time and temperature of incubation on heat resistance of *Escherichia coli*. *J. Bact.* **36**, 83.

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.

Gardner, R. J. (1946). Colorimetric determination of magnesium in plasma or serum by means of Titan Yellow. *Biochem. J.* **40**, 828.

Hershey, A. D. (1939). Factors limiting bacterial growth. VII. Respiration and growth properties of *Escherichia coli* surviving sublethal temperatures. *J. Bact.* **38**, 563.

Jordan, R. C. & Jacobs, S. E. (1948). Studies in the dynamics of disinfection. XII. The effect of variation in pH on the rate of disinfection at 51° C. of standard cultures of *Bact. coli*. *J. Hyg., Camb.* **46**, 136.

Lemcke, R. M. & White, H. R. (1959). The heat resistance of *Escherichia coli* cells from cultures of different ages. *J. appl. Bact.* **22**, 193.

Mager, J., Kuczynski, M., Schatzberg, G. & Avi-Dor, Y. (1956). Turbidity changes in bacterial suspensions in relation to osmotic pressure. *J. gen. Microbiol.* **14**, 69.

McQuillen, K. (1962). Ribosomes and the synthesis of proteins. *Progress in Biophys. and Biophysical Chem.* **12**, 69.

Medical Research Council (1931). *System of Bacteriology*. London: H.M.S.O.

Morse, M. L. & Carter, C. E. (1949). The synthesis of nucleic acids in cultures of *Escherichia coli*, strains B and B/R. *J. Bact.* **58**, 317.

Nelson, F. E. (1943). Factors which influence the growth of heat-treated bacteria. A comparison of four agar media. *J. Bact.* **45**, 395.

Postgate, J. R., Crumpton, J. E. & Hunter, J. R. (1961). The determination of bacterial viabilities by slide culture. *J. gen. Microbiol.* **24**, 15.

POSTGATE, J. R. & HUNTER, J. R. (1962). The survival of starved bacteria. *J. gen. Microbiol.* **29**, 233.

POSTGATE, J. R. & HUNTER, J. R. (1963). Acceleration of bacterial death by growth substrates. *Nature, Lond.* **198**, 273.

RAHN, O. (1945). Physical methods of sterilization of micro-organisms. *Bact. Rev.* **9**, 1.

RAHN, O. & SCHROEDER, W. R. (1941). Inactivation of enzymes as the cause of death in bacteria. *Biodynamica*, **3**, 199.

ROTHSTEIN, A., HAYES, A., JENNINGS, D. & HOOPER, D. (1958). The active transport of Mg^{2+} and Mn^{2+} into the yeast cell. *J. gen. Physiol.* **41**, 585.

SHERMAN, J. M. & ALBUS, W. R. (1923). Physiological youth in bacteria. *J. Bact.* **8**, 127.

SHERMAN, J. M. & ALBUS, W. R. (1924). The function of lag in bacterial cultures. *J. Bact.* **9**, 303.

STRANGE, R. E. & DARK, F. A. (1962). Effect of chilling on *Aerobacter aerogenes* in aqueous suspension. *J. gen. Microbiol.* **29**, 719.

STRANGE, R. E., DARK, F. A. & NESS, A. G. (1961). The survival of stationary phase *Aerobacter aerogenes* stored in aqueous suspension. *J. gen. Microbiol.* **25**, 61.

STRANGE, R. E., WADE, H. E. & NESS, A. G. (1963). The catabolism of proteins and nucleic acids in starved *Aerobacter aerogenes*. *Biochem. J.* **86**, 197.

STREHLER, B. L. & TOTTER, J. R. (1952). Firefly luminescence in the study of energy transfer mechanisms. I. Substrate and enzyme determination. *Archs. Biochem. Biophys.* **40**, 28.

WADE, H. E. (1961). The autodegradation of ribonucleoprotein in *Escherichia coli*. *Biochem. J.* **78**, 457.

WATKINS, J. H. & WINSLOW, C.-E. A. (1932). Factors determining the rate of mortality of bacteria exposed to alkalinity and heat. *J. Bact.* **24**, 243.

WHITE, H. R. (1951). Variation with age in the resistance of bacterial cells. *Nature, Lond.* **168**, 828.

WHITE, H. R. (1953). The heat resistance of *Streptococcus faecalis*. *J. gen. Microbiol.* **8** 27.

WHITE, H. R. (1963). The effect of variation in pH on the heat resistance of cultures of *Streptococcus faecalis*. *J. appl. Bact.* **26**, 91.

WINSLOW, C.-E. A. & WALKER, H. H. (1939). The earlier phases of the bacterial culture cycle. *Bact. Rev.* **3**, 147.

WOOD, T. H. (1956). Lethal effects of high and low temperatures on unicellular organisms. *Adv. biol. med. Phys.* IV, 119.

YEMM, E. W. & COCKING, E. C. (1955). The determination of amino-acids with ninhydrin. *Analyst*, **80**, 209.

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The Chemical Composition of Vaccinia Virus

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SUMMARY

Samples of purified vaccinia virus chemically analysed had the following composition (%): N, 14·7; P, 0·49; S, 0·76; DNA, 3·2; total lipid, 5·0 (including cholesterol, 1·2; phospholipid, 2·1); trace constituents detected were: RNA, 0·1; carbohydrate, 0·2; Cu, 0·02; flavin, 5×10^{-4}; biotin, $1{\cdot}3 \times 10^{-5}$. These results are compared with other published figures and the differences discussed. The trace constituents are not considered to be true components of the virus. On the other hand, there is no evidence for regarding cholesterol and the other lipid components as non-essential constituents of the virus.

INTRODUCTION

Twenty years ago Smadel & Hoagland (1942) reviewed their extensive work on vaccinia virus, including a report on its chemical composition. This was the first detailed and comprehensive chemical analysis of an animal virus and the results are still generally quoted. Vaccinia virus had a relatively complex composition in contrast to the plant viruses, which contained only nucleic acid and protein. Thus, in addition to DNA and protein, vaccinia virus was shown to contain cholesterol, phospholipid, neutral fat, carbohydrate, copper, flavin and biotin. All these constituents were considered to be integral components of the virus, except the cholesterol which could be removed from the virus without loss of infectivity. Since this investigation, the only published quantitative results on the composition of vaccinia virus have been a report that the copper content was lower (Joklik, 1962*a*) and several reports on the nucleic acid content. Figures published for DNA have varied over a fourfold range (2·1–7·8 %) but these have not been correlated with phosphorus content. However, there has been general agreement that the RNA content is very small. The present paper reports a chemical analysis of several samples of purified vaccinia virus. Some important differences have been found between these results and those of the earlier workers.

METHODS

Virus samples. Vaccinia virus was obtained from the skin of infected rabbits and purified by centrifugation in sucrose density gradients as described by Zwartouw, Westwood & Appleyard (1962).

Virus infectivity. Virus samples were titrated on chicken embryo chorioallantoic membranes by the method of Westwood, Phipps & Boulter (1957) and infectivity expressed as pock-forming units (p.f.u.)/ml.

Dry weight. Samples of virus were dried to constant weight at 60° and 0·01 mm. Hg over P_2O_5. All analytical results are expressed in terms of dry weight.

Nitrogen. After Kjeldahl digestion, nitrogen was estimated with Nessler's reagent.

Phosphorus. Phosphorus was assayed as described by King (1946).

Sulphur. Samples were digested with $HClO_4$ and assayed by the method of Spencer (1960).

Lipid. Dried virus (15–20 mg.) was extracted for 1 hr. with a boiling mixture of ethanol + ether (3 + 1, by vol.). Further extraction with the same solvent, or with a mixture of chloroform and methanol, did not remove any more lipid. The solvent was evaporated from the lipid extract and the residue of lipid extracted with light petroleum (boiling range 40–60°); this left a trace of insoluble material. The lipid obtained after evaporation of the light petroleum was dried at 20 mm. Hg and 20° and weighed as total lipid. This was dissolved in chloroform and samples were used for estimation of cholesterol by the method of Green, Lowe & Morton (1955) and for phosphorus. Phospholipid was calculated from the lipid-P results by assuming that the phospholipid contained 4·0 % P. Neutral fat was calculated by subtracting the cholesterol + phospholipid figures from the total lipid.

DNA. Virus samples were extracted with 0·5 N-$HClO_4$ at 90° for 20 min. These conditions produced the maximum DNA in solution obtainable by a single acid extraction. A second similar extract contained about 5 % of the amount of DNA in the first and further extracts did not contain detectable amounts of DNA. Routinely, DNA was measured in two successive hot acid extracts by the diphenylamine reaction as modified by Burton (1956). A standard solution of calf thymus DNA was adjusted by P content to correspond to the theoretical value (10·04 % P) for a DNA tetranucleotide and heated in 0·5 N-$HClO_4$ in the same manner as the virus samples.

RNA. To facilitate the measurement of small amounts of RNA in virus material which contained much larger amounts of DNA, the nucleic acids were separated by a modification of the method of Schmidt & Thannhauser (1945). Virus was suspended in 0·3 N-KOH and maintained at 37° for 16 hr. After acidification with $HClO_4$, the hydrolysed RNA in solution was assayed by the ribose method of Bolognani, Coppi & Zambotti (1961). A standard solution of yeast RNA was adjusted by P content to correspond to the theoretical value (9·5 % P) for an RNA tetranucleotide.

Copper. Virus samples were digested with a mixture of sulphuric and nitric acids, diluted with water, boiled to hydrolyse any pyrophosphate, neutralized and the copper present determined by the method of Kolthoff & Sandell (1952).

Flavin. 'Free' and 'combined' flavin was assayed by the method of Bessey, Lowry & Love (1949) and expressed as riboflavin by using a riboflavin standard.

Biotin. Virus samples were refluxed with 4 N-H_2SO_4 for 30 min. and biotin assayed by the procedure of Wright & Skeggs (1944).

Carbohydrate. The quantitative Molisch reaction as described by Kabat & Mayer (1948) was used.

RESULTS

Principal components

The analytical values for the principal components of six samples of purified vaccinia virus are shown in Table 1. They agree well with the results of Smadel & Hoagland (1942) except for the DNA content.

DNA. Since our value for the DNA content (3·2 %) was considerably lower than the original figure (5·6 %) of Hoagland, Lavin, Smadel & Rivers (1940), we tested three other methods of extracting the DNA for assay. (1) Virus was treated successively with cold $HClO_4$, solvent (ethanol + ether), 0·3 N-KOH at 37° and finally 0·5 N-$HClO_4$ at 90° as in the modified Schmidt–Thannhauser method. (2) Virus was partially digested with pepsin, dissolved in hot alkali and the precipitate obtained with acetic acid and methanol assayed for DNA. This was the original procedure of Hoagland, Lavin *et al.* (1940). (3) Virus was dissolved in hot alkali and assayed directly as described by Joklik (1962*a*). None of these procedures gave higher values for the DNA content.

Table 1. *Chemical analysis of vaccinia virus samples*

Virus sample	% of dry weight						
	N	P	S	DNA	Total lipid	Cholesterol	Phospholipid
1	14·8	0·51	0·73	3·3	4·5	0·7	1·9
2	14·2	0·55	0·93	3·0	4·1	1·5	1·6
3	15·0	0·50	0·71	3·0	7·2	1·6	3·4
4	14·3	0·50	0·72	3·1	4·5	1·1	1·7
5	14·9	0·51	0·77	3·3	4·8	1·2	1·9
6	14·9	0·56	0·70	3·3	5·1	1·2	2·1
Mean value	14·7	0·52	0·76	3·2	5·0	1·2	2·1

Table 2. *Infectivity of dried and solvent-extracted vaccinia virus*

Virus sample	Infectivity*	
	p.f.u.†/ml.	% of untreated
Untreated	$6·9 \times 10^9$	100
Freeze dried	$5·6 \times 10^8$	8
Freeze dried and extracted with ether 4 hr. at −10°	$3·4 \times 10^7$	0·5
Freeze dried and extracted with ethanol + ether (3 + 1 by vol.) 4 hr. at −10°	$2·7 \times 10^6$	0·04

* Each treated virus sample (1 ml.) was finally resuspended in 1 ml. dilute buffer by ultrasonic vibration.

† p.f.u. = pock forming unit.

Lipid. Experiments were done to determine whether the removal of lipid from the virus was accompanied by loss of infectivity. The lipid content of the virus was not decreased after attempting further purification by centrifugation in sucrose density gradients. Virus suspended in 5 % Brij 35 (polyoxyethylene lauryl ether) and agitated by ultrasonic vibration for 30 min. lost 50 % of its original lipid (cholesterol, phospholipid and neutral fat were all lost in about the same proportion) but suffered a greater loss (84 %) of its infectivity.

A concentrated aqueous suspension of virus was added to 100 volumes of acetone at −60°. The virus was recovered and washed with acetone by centrifugation at −20° before resuspension in dilute buffer. This cold acetone-extracted virus had only 0·003 % of the original infectivity. Evaporation of the acetone and extraction of the residue with light petroleum showed that, within the limits of measurement,

all the lipid had been removed from the virus. A similar result was obtained by using a mixture of ethanol + ether in place of acetone.

In an experiment similar to that of Hoagland, Smadel & Rivers (1940), freeze-dried virus was extracted with ether or ethanol + ether mixture. Ethanol + ether caused a considerable loss of infectivity and although ether alone had less effect, it nevertheless caused a 16-fold decrease in infectivity (Table 2). This is contrary to the results the earlier workers found after ether extraction.

Vaccinia virus has been classified as 'ether-stable' by Andrewes & Horstmann (1949). Using their conditions, i.e. adding ether and 10 % serum to an aqueous virus suspension and leaving the mixture at 4°, we confirmed that no infectivity was lost in 24 hr. However, when virus suspended in dilute buffer was incubated with ether at 37°, infectivity rapidly decreased (1000-fold in 4 hr) while the infectivity of incubated controls without ether did not. In general, all our experiments showed that vaccinia virus lost infectivity when in contact with fat solvents.

Trace substances

RNA. Samples of purified virus consistently contained 0·3–0·5 % RNA. This was most easily measured after separation from DNA by alkaline hydrolysis (see Methods). Attempts to eliminate the RNA by repeating the purification procedure (centrifugation in sucrose density gradients) resulted in a decrease of the RNA content to 0·2–0·3 %. To facilitate this re-purification, the virus was first incubated with 0·0001 % trypsin in 0·01 M-phosphate (at pH 8) at 37°; this decreased aggregation of the particles without diminishing infectivity.

However, the RNA content was decreased more effectively by treatment with ribonuclease. Samples of virus (1 mg./ml.) were incubated at 37° for 1 hr. in 0·01 M-phosphate and 0·005 M-ethylenediaminetetraacetic acid (EDTA) at pH 7·6 with 0·1 mg. ribonuclease/ml. After washing in the centrifuge, the recovered virus had suffered no loss of infectivity and contained only 0·1 % RNA. Further treatment with ribonuclease did not diminish the RNA content below 0·1 %.

Copper. The copper content of different virus samples varied over a fourfold range (0·009–0·035 %), with a mean content of 0·021 %. Crude virus obtained as skin scrapings from infected rabbits contained only 0·0003 % copper. The copper content of purified virus was not decreased by suspending the virus in 0·005 M-EDTA at pH 7·5 for 1 hr at 37°.

When virus was suspended (1 mg./ml.) in a solution of copper sulphate (0·1 mg. copper/ml.) at 37° for 1 hr and subsequently recovered and washed three times by centrifugation, the copper content of the virus had increased 20-fold. Although this increased copper content was not diminished by washing the virus with water, two-thirds of it was removed by treatment with 0·05 M-EDTA at pH 7·5. The remaining one-third (equal to 8 times the original copper content of the virus) was not removed by repeated treatment with EDTA (Table 3). These experiments showed that copper was readily adsorbed by vaccinia virus and was not easily removed.

Spectroscopic examination of purified virus detected only one other metal, namely calcium; its amount varied widely, from less than 0·02 % to about 0·1 %.

Flavin. Small quantities of flavin were present (3–7 μg./g., expressed as riboflavin); 90 % of this was in a conjugated form (i.e. liberated after acid hydrolysis).

Crude virus before purification contained 8 μg. flavin/g., 25 % of which was in a conjugated form.

Biotin. Different samples of purified virus contained between 0·06 and 0·21 μg. biotin/g., with a mean content of 0·13 μg./g. Crude virus contained 0·25 μg./g.

Carbohydrate. The Molisch reaction, after allowing for the measured RNA content of the samples, indicated that purified virus samples contained 0·1–0·3 % carbohydrate expressed as glucose.

Table 3. *Adsorption and attempted removal of copper from vaccinia virus*

Virus sample	Treatment of sample	Copper content (%)
1	Untreated	0·022
2	Cu^{2+} solution then washed three times with water	0·47
3	Sample 2 after one EDTA treatment	0·17
4	Sample 2 after two EDTA treatments	0·15
5	Sample 2 after three EDTA treatments	0·18

Table 4. *Phosphorus distribution in vaccinia virus*

	% of total phosphorus	
Phosphorus fraction of virus	Uncorrected	Corrected for RNA removable by ribonuclease*
Calculated from DNA content	61	65
Lipid phosphorus	16	17
Cold acid soluble	3	3
Liberated by warm alkali and not present as nucleic acid	8	9
Not extracted by hot acid or warm alkali	5	5
Calculated from RNA content	8	2

* 75 % of the RNA was removed by ribonuclease treatment.

Phosphorus distribution in vaccinia virus

The mean analytical figures for DNA, phospholipid and RNA (without ribonuclease treatment) accounted for 61, 16 and 8 %, respectively, of the total phosphorus found in the virus. Experiments were done to determine the nature of the remaining 15 %. Cold acid (0·5 N-$HClO_4$ at 0°) extracted 3 % of the total P from the virus. In the Schmidt–Thannhauser extraction, the RNA fraction (i.e. material which was acid-soluble after alkaline hydrolysis) always contained more phosphorus than was accounted for by its nucleic acid content. This extra phosphorus, which was not present as hydrolysed RNA or DNA, amounted to 8 % of the total P. The same figure for alkali-labile P was obtained in another way. Virus was serially extracted with cold acid, ethanol + ether and finally hot 0·5 N-$HClO_4$ thus removing successively acid-soluble P, lipid P and nucleic acid P (RNA and DNA). The residue, which contained 13 % of the total P, was then hydrolysed in 0·3 N-KOH for 16 hr at 37° and separated again into acid-soluble and insoluble fractions. These contained 8 and 5 % of the total P, respectively. Table 4 shows that all of the phosphorus was accounted for in the different components. After correction for the RNA removable

by ribonuclease treatment, the results suggest that 14 % of the total P was present as phosphoprotein, of which 9 % was liberated by warm alkali and 5 % was resistant to such hydrolysis.

DISCUSSION

Table 5 compares our summary of vaccinia virus composition with the results of Smadel & Hoagland (1942). Agreement between the two sets of figures for nitrogen content and for the lipid components is very close. The earlier workers considered that the cholesterol found was not an integral part of the virus since they claimed that it could be extracted by ether with almost no loss of infectivity. We were not able to confirm this and we therefore consider that cholesterol must be regarded as a component of the virus in the same way as phospholipid and neutral fat. Although vaccinia virus has been referred to as 'ether-stable', it should be noted that the term is only relative and that the virus is sensitive to ether when the test conditions are suitably adjusted.

Table 5. *Comparison of analytical values for vaccinia virus*

	% of dry weight	
	Present work	Smadel & Hoagland (1942)
Principal components		
Nitrogen	14·7	15·3
Phosphorus	0·49	0·57
Sulphur	0·76	—
DNA	3·2	5·6
Cholesterol	1·2	1·4
Phospholipid	2·1	2·2
Neutral fat	1·7	2·2
Trace substances		
Carbohydrate	0·2	2·8*
Copper	0·02	0·05
Riboflavin	$0{\cdot}5 \times 10^{-3}$	$1{\cdot}3 \times 10^{-3}$
Biotin	$1{\cdot}3 \times 10^{-5}$	Present
RNA	0·1	—

* Reducing sugar after hydrolysis.

The constituents carbohydrate, copper, flavin and biotin, which were found in the earlier work, have also been detected in our virus preparations. However, the amounts of these constituents were considerably less than those claimed earlier (Table 5). The small positive carbohydrate reaction might have been due to the large amount of non-carbohydrate material necessarily present in the carbohydrate assay.

The mean result for the copper content (0·02 %) agrees closely with the figure of 0·015 % reported by Joklik (1962*a*). We have confirmed the claim that copper is concentrated in the virus fraction during purification (Hoagland, Ward, Smadel & Rivers, 1941) and have found additional evidence (treatment with EDTA) that the copper could not be removed from the virus. However the copper content we found was lower than that originally claimed and our figures show variation over a fourfold range. It seemed most likely that copper was strongly adsorbed by some component of the virus and that the amount found in purified virus samples varied because

different amounts or concentrations of copper were available for adsorption in the crude virus preparations. In support of this argument we found that copper was adsorbed from solution by vaccinia virus, and adsorbed copper was not removed by washing with water, although part of it was removed by EDTA. Even after repeated treatment with EDTA, the treated virus contained a relatively large amount of adsorbed copper.

Flavin did not appear to concentrate in the virus during purification and the small amount detected is almost certainly of doubtful significance. Biotin was detected in vaccinia virus by Hoagland, Ward, Smadel & Rivers (1940) and although they reported no quantitative results they claimed the amount present was comparable to other biotin-rich materials, such as egg yolk and liver. The amount of biotin present in our purified virus samples, which was smaller than the amount detected in crude virus, was less than one-twentieth the biotin content of liver. Thus there is no reason to regard the trace of biotin found in the virus as anything but impurity.

Table 6. *Analytical values for nucleic acid in vaccinia virus*

Reference	DNA (%)	RNA (%)
Hoagland *et al.* (1940)	5·6	—
Wyatt & Cohen (1953)	2·1	—
Planterose *et al.* (1962)	—	0·1 to 0·2
Joklik (1962*a*, *b*)	5·25*	(0·1)†
Allison & Burke (1962)	7·8	< 0·26‡
Present work	3·2	0·1

* Calculated as % from reported 5·8 parts DNA/100 parts protein and allowing for 5 % lipid content.

† Quoted for rabbit-pox virus as a maximum of 2·5 % of DNA content.

‡ Quoted as < 1/30 of DNA content.

The only vaccinia virus component which has received much attention is the nucleic acid. Several values have been published for the DNA content together with attempts to demonstrate the absence of RNA. These results are compared in Table 6. In general the values for DNA are higher than our results, but there are possible explanations for this since some results are only indirectly related to the virus dry weight. The highest reported value (7·8 %) was calculated by Allison & Burke (1962) from the measured DNA content per virus particle and the particle weight was considered to be $3{\cdot}6 \times 10^{-15}$ g. Our preliminary measurements indicate that this value is too low for the particle weight. Joklik (1962*a*) reported 5·8 parts of DNA in relation to 100 parts of vaccinia virus protein, the latter being measured by the colour reaction of Lowry, Rosebrough, Farr & Randall (1951). It is arbitrary to assume that equal weights of the relatively insoluble vaccinia virus protein and an unrelated protein standard produce equal colours in the protein assay. From measurements of the distribution of ^{32}P in fractions from labelled rabbit-pox virus, after removing various impurities containing ^{32}P by cold acid and enzyme treatments, Joklik (1962*b*) concluded that 70 % of the label represented DNA. This figure together with his result for DNA content of rabbit-pox virus (5·4 parts of DNA/100 parts protein; Joklik, 1962*a*) requires the virus to contain at least 0·7 % total-P. This is significantly higher than the total-P found in the closely related vaccinia virus. In the earliest chemical investigations, Hoagland, Lavin, Smadel &

Rivers (1940) reported 5·6 % DNA and Hoagland, Smadel & Rivers (1940) 0·57 % total-P in vaccinia virus. Smadel, Rivers & Hoagland (1942) extracted a 'nucleoprotein antigen' from vaccinia virus with sodium hydroxide solution. This material was equal to about half the weight of the original virus and was reported to contain 6 % DNA and 1·8 % P. If sodium hydroxide solution extracted all the nucleic acid from the virus, the original virus should have contained about 3 % DNA and 0·9 % P.

The colorimetric estimation of DNA, which has been used by all the investigators, depends on comparison with a DNA standard. Previous authors have not indicated whether they had made allowance for the water and metal cations in their standard DNA. Phosphorus content is the most reliable basis for preparing the standard solution and usually indicates a 25–30 % correction by weight. In view of the possible errors just discussed, we consider that our result of 3·2 % DNA in vaccinia virus is the most accurate value so far reported.

The important question whether DNA is the only nucleic acid present has only been partly answered. The lowest figures for RNA quoted by various authors show that the amount is not more than 0·1 %. This low figure has usually been obtained only after ribonuclease treatment of the virus. Direct measurement of this small quantity of RNA is difficult and uncertain because of interference by the 99·9 % of other material. Application of the Schmidt–Thannhauser method to vaccinia virus produced a hydrolysed RNA fraction in which ribose could be assayed, but it contained phosphorus in excess of one equivalent for the ribose and had a large non-specific ultraviolet absorption. Thus the ribose assay for RNA could not be confirmed by phosphorus analysis or by absorption at 260 mμ. Joklik (1962*b*) and Planterose, Nishimura & Salzman (1962) have devised ingenious experiments with radioactive tracers but do not appear to have obtained any greater accuracy, and their results depend on the assumption that different viral components are uniformly labelled. Although the remote possibility must be recognized that vaccinia virus contains a small RNA component, it seems reasonable to conclude that RNA is not an essential part of the virus.

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REFERENCES

ALLISON, A. C. & BURKE, D. C. (1962). The nucleic acid contents of viruses. *J. gen. Microbiol.* **27**, 181.

ANDREWES, C. H. & HORSTMANN, D. M. (1949). The susceptibility of viruses to ethyl ether. *J. gen. Microbiol.* **3**, 290.

BESSEY, O. A., LOWRY, O. H. & LOVE, R. H. (1949). The fluorimetric measurement of the nucleotides of riboflavin and their concentration in tissues. *J. biol. Chem.* **180**, 755.

BOLOGNANI, L., COPPI, G. & ZAMBOTTI, V. (1961). Determination of pentoses. *Experientia*, **17**, 67.

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.

GREEN, B., LOWE, J. S. & MORTON, R. A. (1955). The effect of vitamin A deficiency on the cholesterol levels of the plasma and liver of the rat. *Biochem. J.* **61**, 447.

HOAGLAND, C. L., LAVIN, G. I., SMADEL, J. E. & RIVERS, T. M. (1940). Constituents of elementary bodies of vaccinia. II. Properties of nucleic acid obtained from vaccine virus. *J. exp. Med.* **72**, 139.

HOAGLAND, C. L., SMADEL, J. E. & RIVERS, T. M. (1940). Constituents of elementary bodies of vaccinia. I. Certain basic analyses and observations on lipid components of the virus. *J. exp. Med.* **71**, 737.

HOAGLAND, C. L., WARD, S. M., SMADEL, J. E. & RIVERS, T. M. (1940). Biotin in elementary bodies of vaccinia. *Proc. Soc. exp. Biol., N.Y.* **45**, 669.

HOAGLAND, C. L., WARD, S. M., SMADEL, J. E. & RIVERS, T. M. (1941). Constituents of elementary bodies of vaccinia. IV. Demonstration of copper in the purified virus. *J. exp. Med.* **74**, 69.

JOKLIK, W. K. (1962*a*). The purification of four strains of poxvirus. *Virology*, **18**, 9.

JOKLIK, W. K. (1962*b*). The preparation and characteristics of highly purified radioactively labelled poxvirus. *Biochim. biophys. Acta*. **61**, 290.

KABAT, E. A. & MAYER, M. M. (1948). *Experimental Immunochemistry*, 1st ed., p. 315. Springfield. Illinois: Charles C. Thomas.

KING, E. J. (1946). *Microanalysis in Medical Biochemistry*, 1st ed.. p. 51. London: Churchill.

KOLTHOFF, I. M. & SANDELL, E. B. (1952). *Textbook of Quantitative Inorganic Analysis*, 3rd ed., p. 637. New York: Macmillan.

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

PLANTEROSE, D. N., NISHIMURA, C. & SALZMAN, N. P. (1962). The purification of vaccinia virus from cell cultures. *Virology*, **18**, 294.

SCHMIDT, G. & THANNHAUSER, S. J. (1945). A method for the determination of desoxyribonucleic acid, ribonucleic acid and phosphoproteins in animal tissues. *J. biol. Chem.* **161**, 83.

SMADEL, J. E. & HOAGLAND, C. L. (1942). Elementary bodies of vaccinia. *Bact. Rev.* **6**, 79.

SMADEL, J. E., RIVERS, T. M. & HOAGLAND, C. L. (1942). Nucleoprotein antigen of vaccine virus. I. A new antigen obtained from elementary bodies of vaccinia. *Arch. Path.* **34**, 275.

SPENCER, B. (1960). The ultramicro determination of inorganic sulphur. *Biochem. J.* **75**, 435.

WESTWOOD, J. C. N., PHIPPS, P. H. & BOULTER, E. A. (1957). The titration of vaccinia virus on the chorioallantoic membrane of the developing chick embryo. *J. Hyg., Camb.* **55**, 123.

WRIGHT, L. D. & SKEGGS, H. R. (1944). Determination of biotin with *Lactobacillus arabinosus*. *Proc. Soc. exp. Biol., N.Y.* **56**, 95.

WYATT, G. R. & COHEN, S. S. (1953). The bases of the nucleic acids of some bacterial and animal viruses: The occurrence of 5-hydroxymethyl-cytosine. *Biochem. J.* **55**, 774.

ZWARTOUW, H. T., WESTWOOD, J. C. N. & APPLEYARD, G. (1962). Purification of poxviruses by density gradient centrifugation. *J. gen. Microbiol.* **29**. 523.

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Time–Temperature Interaction in the Induction of Pleomorphism in *Trichophyton mentagrophytes*

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SUMMARY

The frequency of appearance of mutant pleomorphic patches on the surface growth of granular strains of *Trichophyton mentagrophytes* was markedly increased by incubation at 36° as compared to the number seen at 26°. A minimum of 8–9 days of incubation at the higher temperature was required to induce the higher frequency of pleomorphic mutants and, as the number of days at 36° increased, the number and relative size of the pleomorphic patches also increased. Typical mutants could be isolated whether the granular culture was incubated for 8–9 or 14 days at 36°. Visual scoring was relatively accurate since most of those patches visually scored as pleomorphic were found on isolation to be typical or atypical mutants. Three phenotypic characters were used here to define pleomorphism: type of surface mycelium, pigmentation on reverse side of colony, and effect of methionine. These characters could change independently of one another. Thus, pleomorphism may be the result of alterations in more than one closely linked chromosome-borne locus.

INTRODUCTION

Sabouraud (1910) described an unusual mutation, pleomorphism, manifested as tufts or patches of fluffy white aerial mycelium on the appressed powdery surface growth of dermatophytic fungi. He found that a medium with a high concentration of carbohydrate (5 % glucose, maltose or mannitol) and incubation at 30–37° favoured the development of the pleomorphic patch in all species of Trichophyton studied. Sabouraud emphasized the critical nature of these two parameters of the phenomenon, since no patches developed when he used a medium lacking sugar or an incubation temperature below 30°. Robbins (1950) and Bistis (1959, 1960) also reported the occurrence of pleomorphic patches when cultures of *T. mentagrophytes* were grown at 35° on a medium containing 5 % glucose. Bistis (1959) noted the absence of pleomorphic mutants in cultures of *T. mentagrophytes* at 25°. To define the phenomenon of pleomorphism and to classify presumptive pleomorphic isolates as many stable morphological and biochemical criteria of pleomorphism as possible should be used. Thus, the original definition of pleomorphism in Trichophyton advanced by Sabouraud (1910) and extended by Klein (1963) will be used here: a typical granular culture has a granular (grainy) surface growth, is pigmented on the reverse side of the colony, and is methionine sensitive. A typical pleomorphic mutant exhibits a fluffy white surface growth, is unpigmented on the reverse, and is methionine insensitive. *T. mentagrophytes* forms few macroconidia and many

microconidia while the pleomorphic mutant loses the ability to form macroconidia but continues to produce microconidia, sometimes in large numbers (Sabouraud, 1910; Bistis, 1959; Klein, unpublished). Since cultural conditions also influence the number of both macro- and micro-conidia produced (Bistis, 1959; Chin & Knight, 1957), sporulation is not included in this working definition of pleomorphism in *T. mentagrophytes*.

In delimiting the phenomenon of pleomorphism it is important to determine the environmental parameters under which pleomorphic mutants are formed and to determine whether the induction and growth of these mutants can be placed under precise environmental control. The work reported describes the relation of time and temperature of incubation to appearance of the pleomorphic mutation and advances the conclusion that frequency of mutation is modified by temperature.

METHODS

Organisms. Granular strains of *Trichophyton mentagrophytes* tested were M12-4, 4a5, T 689 N, T 16-4, T 16-141, T-9, T 17-3 (from the New York Botanical Garden, NYBG); X-1, X-7, X-26, X-28, X-31, X-44, X-205, X-404 (from Communicable Disease Center, CDC, Atlanta, Georgia, U.S.A.); 9129, 9633 (from American Type Culture Collection, ATCC, Washington, D.C., U.S.A.). The typical pleomorphic strain of *T. mentagrophytes* used was T16-12 (New York Botanical Garden).

Culture media. The basal medium contained (g. or ml./l.) $MgSO_4.7H_2O$, 0·5; KH_2PO_4, 1·5; K_2HPO_4, 0·15; trace element solution, 0·5; thiamine, 0·01; agar, 15. The nitrogen source was usually 0·2 % (w/v) Difco Neopeptone. In certain experiments vitamin-free acid-hydrolysed casein (Nutritional Biochemicals Corp., Cleveland, Ohio), 20 ml./l., or a mixture of 19 L-amino acids (California Corporation for Biochemical Research, Los Angeles, California, U.S.A.), each at the same concentration as found in hydrolysed casein, and including tryptophan, was substituted for the Neopeptone (Klein, 1963). The carbon source was usually 2 % (w/v) glucose; it was replaced in certain experiments by 4 % (w/v) glucose or mannitol or 2 % (w/v) soluble starch. All media were adjusted to pH 5·7.

Conditions of growth. Stock cultures were grown in test tubes on slopes of Neopeptone 2 % glucose agar medium and stored at 5°. Cultures used as a source of inoculum were grown in Petri dishes on Neopeptone 2 % glucose agar medium at 26°. Inocula for all experiments were 4 mm. diam. discs of agar cut from the advancing edge of mycelial growth with a sterile cork borer and placed, one disc per plate, in the centre of the agar medium. Cultures were incubated at 26° or 36° or in a sequence of these two temperatures. A humid atmosphere was maintained and the agar medium did not dry out during the extended incubation periods. Two or three plates were used per variable and each experiment was repeated at least once.

Isolation from pleomorphic patches. At the end of most experiments isolations were made from patches visually scored as pleomorphs. As small an amount of mycelium as possible was taken from each patch with a glass needle since isolation of too much mycelium, particularly too deeply in the patch, usually resulted in a mixed growth of pleomorph and granular. The isolates were grown on Neopeptone 2 % glucose agar medium and incubated at 26° for 10–14 days when each colony was scored for type of surface growth and presence of pigmentation on the reverse

side. Subcultures were then made from each colony to a 19 L-amino acid 2 % glucose agar medium and incubated at 26° for 7–10 days at which time each was scored for methionine effect (Klein, 1963). The few isolates which proved to be mixtures of granular and pleomorphic growth were eliminated from further evaluation because their occurrence was random.

RESULTS

Time–temperature interaction

When granular strains of *Trichophyton mentagrophytes* were grown on Neopeptone 2 % glucose (or 4 % glucose or 2 % soluble starch) agar medium at 26° for up to 35 days pleomorphic patches were rarely seen. With strain M 12-4 an average of one patch was seen per 4–5 Petri dishes; this was considered to be the spontaneous frequency. When granular strains were incubated at 36° for 21 days the following results were observed. All NYBG strains showed pleomorphic patches; certain strains (M12-4, T689N, T16-141, T17-3) exhibited an average of 6–9 patches per plate. The carbon source did not affect the number of patches formed. The other NYBG strains showed fewer patches. The CDC strains were more variable in response; strains X-28, X-44 and X-205 showed pleomorphs (2–4 per plate), the latter two only when the carbon source was soluble starch. ATCC strain 9129 showed patches comparable in number to NYBG strain M12-4; strain 9533 showed no patches.

Granular strain M12-4 was chosen for use in the following experiments because the number of pleomorphs which occurred on the surface growth was consistently high. The length of time of incubation at 36° required to induce the development of pleomorphic patches was determined. Neopeptone 4 % glucose agar plates were inoculated with the granular culture and kept at 36°. Control plates were incubated at 26°. Daily, up to 14 days, two or three plates were transferred from 36° to 26°. Thus, samples of the fungus were grown from 1 to 14 days at 36°, with subsequent incubation at 26° (Table 1; Pl. 1, figs. 1–4). No pleomorphs were seen on plates incubated at 26° (Pl. 1, fig. 2); about 8–9 days of continuous incubation at 36° were required to induce development of pleomorphs. As the time at 36° increased, the size and number of individual patches increased (Pl. 1, figs. 1, 3, 4). The order of presentation of the higher temperature was not a factor in the final response, since the typical number and average size of pleomorphs was seen at 21 days even when the 8–9 days at 36° came at the end rather than at the beginning (Table 1) of the 14-day incubation period.

A number of modifications of the medium, including addition of 0·5 % (w/v) Difco yeast extract, 2 % glucose or 4 % mannitol instead of 4 % glucose, hydrolysed casein or the mixture of 19 L-amino acids in place of Neopeptone, did not change the pattern of response in that no patches were observed on plates at 26° while 8–9 days at 36° induced the development of typical pleomorphic patches. No pleomorphs developed, however, when the carbohydrate was omitted from the medium although there was still an appreciable amount of growth; this observation confirms that of Sabouraud (1910). Thus, temperature seems to be the key inducing-factor of pleomorphism in many strains of *Trichophyton mentagrophytes* as long as the medium is not a limiting factor.

Table 1. *Relation between number of days of incubation at 36° and frequency of appearance of pleomorphic patches on the surface of granular cultures of Trichophyton mentagrophytes strain M12-4; subsequent incubation was at 26°*

Observations were made at 14 and 21 days after inoculation. Results summarize the average of 4 experiments.

Time of incubation at 36° (days)	Pleomorphic patches observed					
	14 days			21 days		
	Present	Average no./plate	Size* range	Present	Average no./plate	Size* range
0	0	—	—	0	—	—
1–7	0	—	—	0	—	—
8	±	0·1	S	±	0·3	S
9	±	0·1	S	+	0·6	S
10	±	0·4	S	+	2·4	S–M
11–13	+	1·2	S–M	+	3·2	S–L
14	+	1·6	S–L	+	5·0	S–L

* S are small, M are medium, and L are large sized patches.

Table 2. *Classification of cultures isolated from pleomorphic patches which formed on the surface of granular cultures of Trichophyton mentagrophytes strain M12-4 following increasing number of days of incubation at 36°*

Data presented summarize four experiments. The + indicates presence of pigmentation and methionine sensitivity; the 0 indicates absence of pigmentation and methionine insensitivity.

Time of incubation at 36° (days)	No. pleomorphs isolated	Classification		
		Type of surface growth*	Pigmentation	Methionine effect
1–7	0			
8	1	Fuzzy	0	0
9	3	Fuzzy	0	+
		Fluffy (2)	0	0
10	6	Granular (3)	+	+
		Fuzzy (2)	0	0
		Fuzzy	0	+
11	10	Granular (3)	+	+
		Fuzzy (4)	0	0
		Fuzzy	+	0
		Fluffy (2)	0	0
12	6	Granular	+	+
		Fuzzy (2)	0	0
		Fuzzy (3)	+	0
13	15	Granular (2)	+	+
		Fuzzy (4)	0	0
		Fuzzy (3)	+	0
		Fuzzy (2)	+	+
		Fluffy (4)	0	0
14	15	Granular (3)	+	+
		Fuzzy (2)	0	0
		Fuzzy (4)	+	0
		Fluffy (6)	0	0

* Those described as granular are identical with parental granular appearance.

Classification of isolated pleomorphic patches

Longer incubation at 36° did not result in the induction of a higher frequency of typical pleomorphs (Table 2); furthermore, typical and atypical pleomorphs were isolated from patches which formed after 9 or 14 days exposure of strain M12-4 to 36°. With few exceptions, pleomorphs with atypical (fuzzy) surface growth were methionine insensitive; isolates showing typical pleomorphic surface growth (fluffy) were invariably insensitive to methionine. These observations confirm the previous report on methionine effect (Klein, 1963).

There was no correlation between type of isolated colony and appearance of the patch; most cultures scored as granular, for example, were isolated from small, flat, or fuzzy beige patches, but typical and atypical pleomorphs also came from similar small patches. There was a greater variation in appearance of the patches than in appearance of the resulting cultures.

DISCUSSION

Elevated temperatures have been shown to increase the frequencies of gene mutation (Plough, 1941), chromosomal aberration (Sax, 1937) and cytoplasmic mutation (Ogur, Ogur & St John, 1960; Papazian, 1955). It would appear that the pleomorphic alteration in many strains of *Trichophyton mentagrophytes* is yet another genetic system in which temperature plays a vital role. Whether elevated temperatures increase the frequency of the pleomorphic alteration or, on the other hand, serve only to activate the growth of a pre-existing mutant cell is not known. Bistis (1959) concluded, without experimental evidence, that the mutation may occur at 25° but that the mutant cell does not grow at this temperature. The data presented in this report do not shed any light on this important question.

The site of the mutational event is also an important unsolved problem since it is not known whether the mutation occurs in the nucleus of the conidium, the nucleus of a hyphal cell, or whether it is non-nuclear. The simplest explanation, at the moment, is that the pleomorphic mutation involves the alteration of more than one chromosome-borne locus. This is supported by the data which show that alterations of the surface character of the mycelium, capacity to form pigment, and methionine sensitivity, may or may not occur simultaneously. This fact has been commented on previously (Klein, 1962). Mutants have been isolated in this study and previously (Klein, 1961) which are non-pigmented, methionine insensitive, but not truly fluffy; or which are fuzzy and non-pigmented, but are methionine sensitive; or which are fuzzy and methionine sensitive, but are pigmented. These observations argue against pleomorphism's being the result of the mutation of a pleiotropic gene (Sinnott, Dunn & Dobzhansky, 1958), but do not preclude the possibility that typical pleomorphism in *Trichophyton mentagrophytes* might be the result of a chromosomal aberration.

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REFERENCES

BISTIS, G. N. (1959). Pleomorphism in the dermatophytes. *Mycologia*, **51**, 440.

BISTIS, G. N. (1960). Pleomorphism and growth cycles in *Trichophyton mentagrophytes*. *Mycologia*, **52**, 394.

CHIN, B. & KNIGHT, S. G. (1957). Growth of *Trichophyton mentagrophytes* and *Trichophyton rubrum* in increased carbon dioxide tensions. *J. gen. Microbiol.* **16**, 642.

KLEIN, D. T. (1961). Effect of ionizing radiation on Trichophyton. *Amer. J. Bot.* **48**, 539.

KLEIN, D. T. (1962). Kinetics of pleomorph induction in Trichophyton. *Amer. J. Bot.* **49**, 655.

KLEIN, D. T. (1963). Morphological response of *Trichophyton mentagrophytes* to methionine. *J. gen. Microbiol.* **31**, 91.

OGUR, M., OGUR, S. & ST JOHN, R. (1960). Temperature dependence of the spontaneous mutation rate to respiratory deficiency in Saccharomyces. *Genetics*, **45**, 189.

PAPAZIAN, H. P. (1955). Sectoring variants in Schizophyllum. *Amer. J. Bot.* **42**, 394.

PLOUGH, H. H. (1941). Spontaneous mutability in Drosophila. *Cold Spr. Harb. Symp. quant. Biol.* **9**, 127.

ROBBINS, W. J. (1950). Growth requirements of dermatophytes. *Ann. N.Y. Acad. Sci.* **50**, 1357.

SABOURAUD, R. (1910). *Les Teignes*. Paris: Masson et Cie.

SAX, K. (1937). Chromosome behaviour and nuclear development in Tradescantia. *Genetics*, **22**, 523.

SINNOTT, E. W., DUNN, L. C. & DOBZHANSKY, T. (1958). *Principles of Genetics*, 5th ed. New York: McGraw-Hill Book Co., Inc.

EXPLANATION OF PLATE

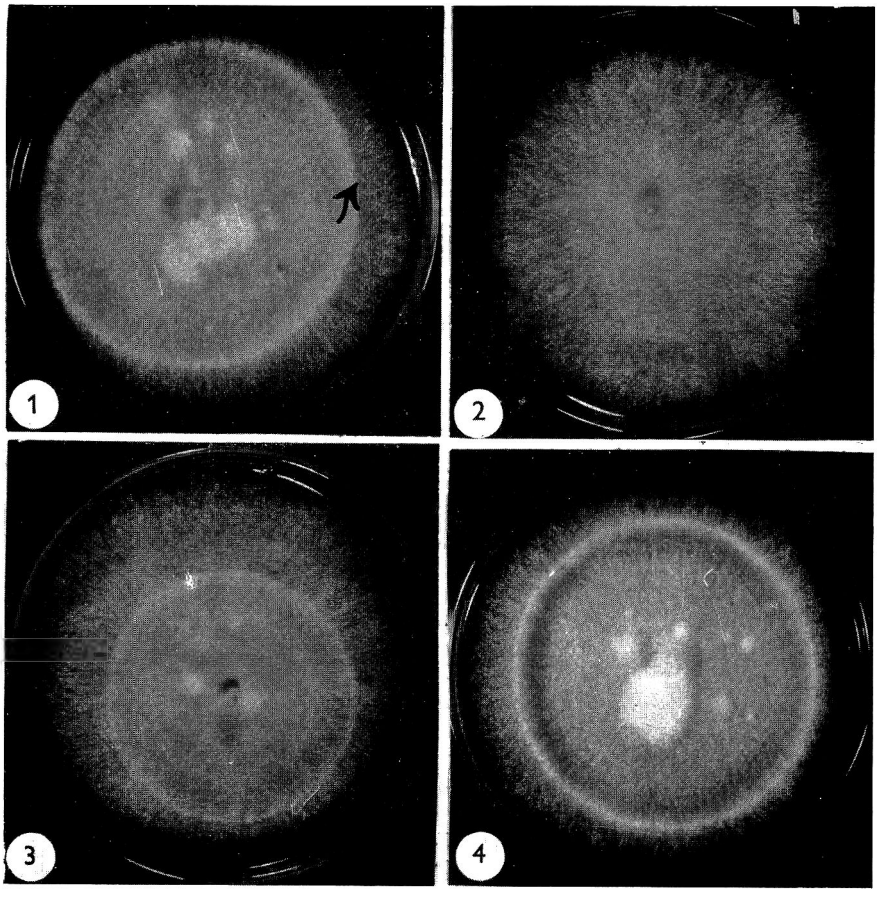
Figs. 1–4. Appearance of pleomorphic patches on surface growth of granular cultures of *Trichophyton mentagrophytes* strain M12-4 after various times at 36°. Medium was Neopeptone 4 % glucose agar. Photographs were taken 21 days after inoculation.

Fig. 1. Fourteen days at 36° followed by 7 days at 26°; arrow indicates the change in growth habit between 36° and 26°.

Fig. 2. Twenty-one days at 26°.

Fig. 3. Ten days at 36° followed by 11 days at 26°.

Fig. 4. Twelve days at 36° followed by 9 days at 26°.



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Relationships between Certain Pyrimidine and Arginine Mutants of Neurospora, as Revealed by their Response to Carbon Dioxide

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SUMMARY

A pyrimidine-requiring mutant of Neurospora was found to grow on minimal medium when the proportion of CO_2 in the gas-phase (air) was increased to 30 % (v/v); arginine prevented growth in 30 % CO_2, but did not prevent growth when pyrimidines were present. An arginine-requiring mutant also grew on minimal medium in the presence of 30 % CO_2. The response of this mutant to CO_2 was annulled by pyrimidines. It is proposed that these mutants have defects in carbamoyl phosphate synthesis. Wild-type Neurospora may have two pathways for making carbamoyl phosphate from CO_2, one pathway subject to feed-back inhibition or repression by arginine, the other pathway subject to feed-back inhibition or repression by pyrimidines. The pyrimidine mutant may lack an enzyme which produces carbamoyl phosphate for pyrimidine synthesis, and the arginine mutant may lack an enzyme which produces carbamoyl phosphate for arginine synthesis. Thus each mutant may have only one effective mechanism for carbamoyl phosphate synthesis. It is suggested that 30 % CO_2 causes increased synthesis of carbamoyl phosphate by the remaining pathway in each mutant, thereby providing sufficient carbamoyl phosphate to overcome the nutritional deficiency. The inhibition of the pyrimidine mutant by arginine, and the inhibition of the arginine mutant by pyrimidines, are explained as feed-back inhibition or repression of the remaining pathway for carbamoyl phosphate synthesis.

INTRODUCTION

This paper describes interrelations between certain uridine-requiring and arginine-requiring mutants of *Neurospora crassa*. The interrelations are expressed in two ways. First, both mutants grow vigorously on minimal medium when the proportion of carbon dioxide (CO_2) in the surrounding air is increased to 30 % (v/v). Secondly, growth of the uridine mutant in 30 % CO_2 in air is prevented by arginine; and the growth of the arginine mutant in 30 % CO_2 in air is prevented by uridine. Some of these results have been presented elsewhere (Charles, 1962) but will be re-considered here in so far as they are relevant to a discussion of the relationship between uridine and arginine metabolism.

METHODS

Carbon dioxide and air mixtures. Carbon dioxide was generated by dilute hydrochloric acid and marble chips in a Kipps apparatus, any HCl being removed by bubbling the gas through sodium bicarbonate solution. Petri dish cultures were

supplied with CO_2 by placing them in 5 l. Pyrex vacuum desiccators, reducing the air pressure in the desiccator appropriately with an air pump, and then admitting CO_2 until atmospheric pressure was restored. Restoration of atmospheric pressure was indicated by the swelling of a thin inflatable polythene bag interposed in the delivery tube from the Kipps apparatus.

Desiccators were charged at room temperature. When incubation was above room temperatures excess gas was released by quickly opening and closing the tap after the desiccators had stood in the incubator for a few minutes. When necessary, CO_2-free air was obtained by placing about 100 ml. 50 % (w/v) potassium hydroxide solution in the appropriate desiccator. Tufts of glass wool were partially immersed in the potassium hydroxide solution to increase the area of CO_2-absorbing surface. All desiccators were opened and refilled with fresh gas mixture every 24 hr. It is not claimed that the methods used for regulating CO_2 pressure were exact; they were satisfactory in that they gave reproducible results.

Media. Solid media were used in all experiments. Liquid cultures were not used because special apparatus would have been required for mixing and bubbling the gas mixtures. Solid media also had advantages in that they enabled use of the auxanographic method for observing nutritional requirements, and permitted the observation of the behaviour of individual conidia.

The minimal culture medium was Fries 3 as modified by Ryan, Beadle & Tatum (1943) solidified with 1 % (w/v) Difco Bacto agar. The sucrose concentration was 0·5 %, and 1 % sorbose was added to induce colonial growth. The sorbose solution was autoclaved (120°, 20 min.) separately in concentrated solution and added to the rest of the medium immediately before it was poured into 9 cm. Petri dishes.

Growth factor requirements, inhibitions and antagonisms were investigated by the auxanographic method (Pontecorvo, 1949). Unsterilized crystals of compounds were used in preliminary tests, but all important observations were confirmed with sterile solutions of known concentration. All solutions were adjusted to pH 5·4.

Solid media in Petri dishes were inoculated by spreading a suspension of conidia over the dried surface of the medium or by suspending conidia in the molten medium just before it was distributed into Petri dishes. The second method was preferred because it gave a more uniform distribution of conidia, but it was usually unsuitable for experiments in which the effect of the gas-phase on growth was being examined.

In experiments with yeasts certain Petri-dish cultures were found to be more easily deprived of CO_2 than others when potassium hydroxide was present in the desiccator. This was because the halves of some Petri dishes fitted together tightly enough to impede diffusion of gases. An attempt was made to overcome this difficulty by using glass rods 10 cm. long or S-shaped wire hooks to hold the halves of the dishes slightly apart at one edge. This method was unsatisfactory because the piles of Petri dishes were unstable and occupied more desiccator space. A better method found was to remove the outer halves (i.e. the lids) from the dishes and to incubate only the inner halves containing medium in the desiccators, with the exposed agar surfaces downwards. The first layer of three dishes rested on a wire shelf ($\frac{3}{4}$ in. mesh) which covered the well of the desiccator. Successive layers of three dishes were stacked so that each dish rested equally on two of the dishes in the layer below. This arrangement permitted adequate diffusion of gases, and more dishes could be placed in each desiccator than by other stackings. Contamination

of cultures did not increase when this method was used, and it was used whenever the effect of gas-phase composition on growth was being investigated. It was important to exclude air bubbles from the medium, particularly when attempting to free it from CO_2. Some CO_2-requiring mutants grew vigorously around air bubbles, even when incubated in a desiccator which contained potassium hydroxide. Presumably sufficient CO_2 was then trapped in the air bubbles to enable growth to begin.

RESULTS

Fairley (1954) reported that a pyrimidine-requiring mutant of Neurospora (mutant 1298) grew when supplied with α-aminobutyrate or threonine instead of the usual pyrimidines; Fairley's experiments were done in liquid media. Attempts by the present author to repeat Fairley's experiments on solid media were at first unsuccessful. When conidia of Neurospora mutant 1298 were plated on minimal medium + α-aminobutyrate or threonine about one colony appeared for every 10^4 viable conidia plated. Eventually it was found that when the conidia were incubated at 20° they all gave colonies, whereas at 30° no conidia grew. Thus the mutant was temperature sensitive when growing with α-aminobutyrate or threonine. When the temperature of incubation was increased from 20° to 30°, fewer and fewer conidia gave vigorous colonies. In contrast, the mutant was not temperature sensitive when supplied with pyrimidines, and then grew vigorously even at 30°. The most satisfactory explanation of this behaviour of mutant 1298 seemed to be that some kind of adaptive process was necessary to enable utilization of α-aminobutyrate or threonine, and that the chances of the adaptation occurring decreased with increasing temperature. Mutation was excluded as an explanation because the rare colonies which appeared at high temperature gave cultures which were identical in all respects with mutant 1298.

Experiments with mutant 1298 were next done to determine whether any other environmental factors, apart from temperature, influenced the temperature-sensitive process. These experiments were unsuccessful until discovery of a reference by Haldane (1954) to the work of Borek & Waelsch (1951) led to an examination of the effect of CO_2 pressure on the mutant. Borek & Waelsch showed that an instance of temperature sensitivity in certain lactobacilli was attributable to a requirement for CO_2; as the incubation temperature was increased the CO_2 concentration in the medium gradually decreased until at 30° insufficient CO_2 was dissolved for growth to occur. Borek & Waelsch suggested that some instances of temperature sensitivity in mutants might be due 'to a need for CO_2 in the medium'.

When incubated in a vacuum desiccator containing air + 15 % (v/v) CO_2 conidia of Neurospora mutant 1298 grew vigorously on α-aminobutyrate or threonine medium; germination of conidia and growth at 30° were the same as at 20°. It thus appeared that Borek & Waelsch's suggestion was applicable to mutant 1298, and that CO_2 was required for growth on α-aminobutyrate medium; with increasing temperature, less and less CO_2 would be dissolved in the medium, and growth would become increasingly difficult. Possibly the rare conidia which grew at the higher temperatures did so because they were near air bubbles trapped in the agar. Once conidia have started to grow it is possible that they generate sufficient CO_2 to enable growth to continue. The ability of rare conidia to grow in air at higher temperatures

was not investigated experimentally: attention was directed towards other aspects of the CO_2 effect.

It is suggested above that a certain concentration of CO_2 is necessary to enable the mutant to respond to α-aminobutyrate or threonine. If this be true, the mutant should not respond to α-aminobutyrate when CO_2 is completely removed from the air, even at 20°. Experiment confirmed this suggestion. Mutant 1298 did not respond to α-aminobutyrate when incubated at 20° in a 5 l. desiccator containing 100 ml. 50 % (w/v) potassium hydroxide. In contrast, the response of mutant 1298 to added uridine or other pyrimidines was not prevented by removing CO_2 in this way. Wild-type conidia grew vigorously on minimal medium or on α-aminobutyrate medium in the absence of CO_2. The requirement for CO_2 thus appeared to be characteristic of mutant 1298 when growing on α-aminobutyrate or threonine medium. Further experiments revealed that mutant 1298 grew vigorously on minimal medium without supplement when the proportion of CO_2 in the gas-phase was increased to 30 % (v/v). This mutant was not temperature-sensitive when incubated in an atmosphere containing 30 % (v/v) CO_2. It was possible that the observed effects of CO_2 resulted from an alteration of the pH value of the medium. However, growth of the mutant did not occur in air when the pH value of the minimal medium was adjusted in other ways. It therefore seems that CO_2 did not act simply by disturbing the pH value of the medium.

To establish whether 30 % (v/v) CO_2 in air completely overcomes the block in mutant 1298 it would be necessary to compare the growth rates of mutant and wild-type on minimal medium in air + 30 % CO_2. This has not been done because of the technical difficulty of controlling the gas-phase in tubes of liquid media. However, it was clear from experiments with Petri-dish cultures that 30 % CO_2 overcame most or all of the metabolic block in mutant 1298.

The above experiments show that Neurospora mutant 1298 may justifiably be referred to as a carbon dioxide-requiring mutant. Before the discovery of these CO_2 effects it had been observed that yeast extract contained something which prevented growth of mutant 1298 on α-aminobutyrate or threonine medium. These experiments were made at 20° to obtain a vigorous growth response to α-aminobutyrate in the control cultures. Arginine was found to reproduce the inhibitory effects of yeast extract. A low concentration of arginine (less than 1 mg./l.) caused complete inhibition of growth of mutant 1298 on α-aminobutyrate or threonine media. The arginine inhibition was not competitive, in the sense that there was no concentration of arginine whose inhibitory effect could be overcome by increasing the concentration of α-aminobutyrate or threonine. This conclusion was based on auxanographic experiments; when a spot of arginine solution was placed very near a spot of α-aminobutyrate solution a zone of inhibition was obtained the radius of which was independent of α-aminobutyrate concentration and dependent only on the arginine concentration.

Arginine did not prevent growth in the presence of uridine or other appropriate pyrimidines. Exposure of mutant 1298 to arginine therefore had the same result as depriving it of CO_2, in that both treatments prevented growth on α-aminobutyrate but did not prevent growth on pyrimidines. This suggested that arginine acted by preventing the mutant from responding to CO_2. It was possible to test this hypothesis because the mutant responds to 30 % CO_2 in the absence of α-aminobutyrate

Experiment confirmed that mutant 1298 was unable to grow in 30 % CO_2 when arginine was present. The growth-promoting effects of α-aminobutyrate and threonine were not investigated further.

The next question was: do any other Neurospora mutants respond to CO_2? In principle CO_2 might exert its effect on mutant 1298 in one of two ways. First, it might act as an essential precursor of some metabolite, such as uridine. Secondly, it might act in a physico-chemical way, perhaps by altering intracellular pH values or by combining with a protein and so altering its conformation. On either explanation it seemed that, if other CO_2-requiring mutants occurred, some of them might be temperature sensitive when growing in air, in the same way that mutant 1298 growing on α-aminobutyrate medium in air seemed to be temperature sensitive because of a relative deficiency of dissolved CO_2 at the higher temperatures. Several temperature sensitive Neurospora mutants described by other workers were therefore tested, but none responded to CO_2. A list of all mutants which have been tested and which did not respond to 30 % CO_2 is shown in Table 1. For reasons to be discussed later arginine mutants were also tested for response to 30 % CO_2. Of three mutants available, one (mutant 30300) grew vigorously on minimal medium in air + 30 % (v/v) CO_2. This arginine mutant is 'leaky' in the sense that it grows slowly on minimal medium in ordinary air in the absence of arginine. When growing slowly on minimal medium in air, the hyphae are characteristically more submerged in the agar than would be the hyphae of wild-type Neurospora. The submerged growth of the hyphae of mutant 30300, and the knowledge that this mutant responded to CO_2, suggested that the 'leaky' growth on minimal medium might be a response to the CO_2 present (about 0·03 %, v/v) in ordinary air and medium. This suggestion was confirmed by finding that mutant 30300 did not grow on minimal medium when the Petri dishes of medium were incubated in a desiccator containing KOH. Mutant 30300 did not respond to α-aminobutyrate or to threonine, under any conditions.

Since the response of the uridine mutant 1298 to CO_2 was specifically prevented by arginine, a search was made to see whether the arginine mutant 30300 was similarly inhibited by any common substance. Of many substances tested, uridine, cytidine, uracil, lysine and canavanine prevented growth of mutant 30300 on minimal medium in air + 30 % CO_2. The three pyrimidines were not inhibitory when arginine was present; the inhibitions were annulled by arginine in a non-competitive manner. In contrast, the inhibitions by canavanine and lysine were competitively annulled by arginine. The pyrimidines therefore appear to inhibit specifically the response to CO_2, whereas lysine and canavanine may be regarded as inhibiting a different stage, or more than one stage, of arginine metabolism. All these inhibitors prevented the 'leaky' growth of arginine mutant 30300 on minimal medium in air.

Uridine and cytidine were completely inhibitory at 1 mg./l.; a higher concentration of uracil was required for complete inhibition. Other pyrimidines were ineffective or gave weak inhibitions at high concentrations. Those pyrimidines, such as cytosine and thymine, which did not support growth of mutant 1298 also did not inhibit the response of mutant 30300 to CO_2.

Uridine, cytidine and uracil prevented all response to CO_2 by mutant 30300 but they did not prevent growth stimulation by citrulline or arginine. Auxanographic experiments showed that there was no concentration of uridine which prevented

Table 1. *Mutants of Neurospora crassa which did not respond to carbon dioxide (30 %, v/v, in the gas phase). Semi-solid media were used in all experiments. This table includes results obtained by Mr J. A. Broadbent*

Locus	Mutant	Locus	Mutant	Locus	Mutant
thr-1	46003	*ad*-3B	Y112M2A	*pyr*-3a	37301a
thr-2	35423A	*ad*-4	44206a	*al*-2	15300a
thr-3	44104	*ad*-5	71104a	*asp*	C123a
ser-1	H605A	*ad*-6	28610A	*for*	C24A
ser-2	65004A	*ad*-7	44411a	*pdx*	44602a
thi-1	56501a	*ad*-8	Y193M22A	*pdx*-1	37803A
thi-3	18558A	*prol*-1	21863a	*cot*	C102A
ol	R23660	*arg*-5	27947	*sfo*	E15172a
ad-1	3254a	*arg*-10	B317A		

Table 2. *Growth requirements of Neurospora crassa mutants* 1298 *and* 30300. *Data from many experiments; semi-solid media and the auxonographic method were used in all experiments. Vigorous growth is indicated by the sign + ; failure to grow is indicated by the sign −. Where growth was very slow it is described as 'leaky'*

Temperature (°)	Additions to growth medium	Mutant 1298 Gas-phase			Mutant 30300 Gas-phase		
		CO_2-free air	Air	Air + 30 % CO_2	CO_2-free air	Air	Air + 30 % CO_2
20–35	Nil	−	−	+	−	Leaky	+
20	α-Aminobutyrate (250 mg./l.)	−	+	+	−	Leaky	+
35	α-Aminobutyrate (250 mg./l.)	−	−	+	−	Leaky	+
20–35	Arginine (5 mg./l.)	−	−	−	+	+	+
20–35	Uridine (5 mg./l.)	+	+	+	−	−	−
20–35	Arginine + uridine (5 + 5 mg./l.)	+	+	+	+	+	+

the growth response of mutant 30300 to citrulline or arginine. These experiments were made in a CO_2-free atmosphere; in ordinary air growth in presence of arginine was slightly inhibited by the pyrimidines. This slight inhibition is probably an inhibition of that part of the growth which is attributable to the CO_2 of ordinary air.

The effects of CO_2, arginine and uridine on mutants 1298 and 30300 are summarized in Table 2.

DISCUSSION

The experiments described above show that a Neurospora pyrimidine mutant (1298) and an arginine mutant (30300), unable to grow on a minimal medium alone, grew vigorously when the cultures were incubated in air + 30 % (v/v) CO_2. The pyrimidine mutant 1298 did not grow on minimal medium in 30 % CO_2 when arginine (1 mg./l.) was added; but when uridine was added mutant 1298 grew vigorously, whether or not CO_2 was present in the air, and arginine was not inhibitory at any concentration. Conversely, arginine mutant 30300 did not grow on minimal medium + uridine in 30 % CO_2. With minimal medium + arginine, mutant 30300 grew vigorously, whether or not CO_2 was present, and uridine was not inhibitory. The two mutants therefore exhibit an interesting symmetrical relationship. A similar relationship between certain pyrimidine and arginine mutants of Neurospora has also been shown through the study of suppressors of these mutations (Reissig, 1960; Davis, 1962).

The nature of the metabolic lesions in the mutants is not known. Davis (1960) studied Neurospora mutants allelic with mutant 1298 and found that the enzymes of the pyrimidine-synthesis pathway were unimpaired; Davis did not examine the enzymes which produce carbamoyl phosphate (see below). The first question is: how can an increase in the partial pressure of CO_2 enable mutants 1298 and 30300 to grow? CO_2 might act in two ways; it might act as a precursor of uridine or arginine, or it might act, for example, by altering intracellular pH value or by combining with a protein and altering its conformation. Of these two alternatives, only the 'precursor' hypothesis led to profitable experiments.

In developing the 'precursor' hypothesis, the obvious biochemical process through which CO_2 might act is in the synthesis of carbamoyl phosphate, for which CO_2 is known to be required; and carbamoyl phosphate in turn is a precursor of uridine and arginine (Jones, Spector & Lipmann, 1955). Thus mutants 1298 and 30300 may have defects in the synthetic pathway to carbamoyl phosphate. The nutritional behaviour of arginine mutant 30300 is consistent with the suggestion that it is blocked in the synthesis of carbamoyl phosphate, in that it grows when provided with citrulline but not when provided with ornithine. With regard to the pyrimidine mutant, as already pointed out, it is known that in Neurospora mutants similar to mutant 1298 the enzymes of the main pyrimidine pathway are all present (Davis, 1960) so that it is quite probable that one of the subsidiary processes which provide substances such as carbamoyl phosphate for the main pathway may be damaged.

The hypothesis of a defect in carbamoyl phosphate synthesis was originally developed to explain the behaviour of the pyrimidine mutant 1298, which was the first mutant found to respond to CO_2 in place of pyrimidines. Since carbamoyl phosphate is involved in arginine metabolism as well as in pyrimidine metabolism, there seemed no reason why an arginine CO_2-mutant should not also be found, if the

explanation of the behaviour of the pyrimidine mutant were correct. This hypothesis therefore led to the testing of arginine mutants and the discovery that mutant 30300 grew on minimal medium in air + 30 % CO_2.

If these mutants are defective in carbamoyl phosphate synthesis, there still remains the problem of how an increase in CO_2 concentration enables these mutants to grow. The simplest explanation is that an enzyme which 'fixes' CO_2 for carbamoyl phosphate synthesis has a decreased affinity for CO_2, such that sufficient fixation of CO_2 only occurs at a high CO_2 concentration. This hypothesis is inadequate in two ways. First, the hypothesis leads one to expect that a single mutation which affects carbamoyl phosphate synthesis would lead to a mutant strain requiring arginine + a pyrimidine. It does not explain the occurrence of two different CO_2-mutants, one of which responds alternatively to pyrimidine, the other alternatively to arginine. Secondly, the hypothesis does not explain why the growth of the pyrimidine mutant in air + 30 % CO_2 is inhibited by arginine, nor why the growth of the arginine mutant in air + 30 % CO_2 is inhibited by pyrimidines.

These considerations apparently require rejection of the hypothesis that both mutants are defective in carbamoyl phosphate synthesis because of an inadequate mechanism for fixing CO_2. However, the fact that substances which the hypothesis predicts to be alternative growth factors turn out to be specific inhibitors seems unlikely to be mere coincidence. The hypothesis probably requires modification rather than rejection. Work by Stadtman, Cohen, Lebras & de Robichon-Szulmajster (1961) on the biosynthesis of lysine, methionine and threonine by *Escherichia coli* provides a basis on which may be constructed a more satisfactory hypothesis. In *E. coli*, lysine, methionine and threonine all have the same early precursors. Stadtman *et al.* (1961) obtained evidence that one of the common precursors, aspartyl phosphate, can be formed by three different enzymes (aspartokinases). One of these aspartokinases was specifically repressed and inhibited by lysine; the second aspartokinase was specifically inhibited by threonine; the third aspartokinase, for which the evidence was less complete, appeared to be inhibited by homoserine, a precursor of methionine Different enzymes which catalyse the same chemical reaction are referred to as isozymes or isoenzymes (Markert & Møller, 1959). By analogy, it is suggested that there may be two enzyme systems which synthesize carbamoyl phosphate in Neurospora, one system providing carbamoyl phosphate for uridine synthesis, and the other system providing carbamoyl phosphate for arginine synthesis. Such distinct pathways might be expected to be subject to regulation only by their own specific end-products. With certain further assumptions these Neurospora mutants can be understood if one assumes that the uridine mutant lacks an enzyme which produces carbamoyl phosphate for uridine synthesis, and that the arginine mutant lacks an enzyme which produces carbamoyl phosphate for arginine synthesis. Thus each of these mutants is considered to retain intact one mechanism for synthesizing carbamoyl phosphate, whereas the wild-type organism is considered to have both mechanisms (see Fig. 1). Spatial separation of the two enzyme systems appears to be necessary to explain why carbamoyl phosphate from one pathway is not freely available to the other defective pathway in these mutants.

Growth in air + 30 % CO_2 would then be explained if the increased concentration of CO_2 caused increased carbamoyl phosphate synthesis by the intact pathway, so

that excess carbamoyl phosphate 'overflowed' and became available to the defective pathway. Thus the uridine mutant 1298 may be considered to respond to CO_2 by utilizing excess carbamoyl phosphate synthesized by the arginine pathway. In the presence of exogenous arginine the latter pathway might be shut again by repression of enzyme formation or by feed-back inhibition, so that carbamoyl phosphate would no longer be synthesized from CO_2. Thus the uridine mutant 1298 should be unable to respond to CO_2 in the presence of arginine, although its ability to respond to pyrimidines should remain unchanged. Conversely, the arginine mutant 30300, when utilizing CO_2, would be dependent upon the uridine pathway for formation

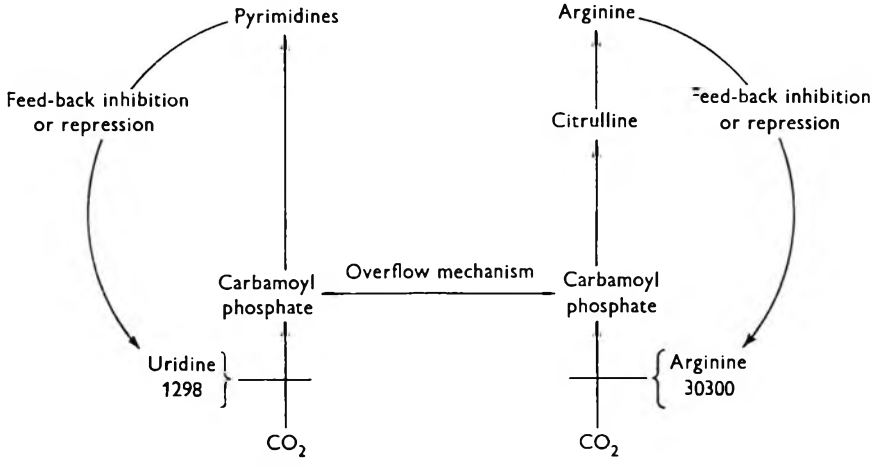

Fig. 1. To show the suggested mechanism for synthesizing carbamoyl phosphate by two separate pathways, one pathway subject to feed-back control by arginine, the other pathway subject to feed-back control by pyrimidines. The figure also shows the 'overflow' mechanism which may operate when the CO_2 concentration is high.

of excess carbamoyl phosphate, and should therefore be susceptible to inhibition by uridine; response of the arginine mutant 30300 to arginine should not be prevented by uridine. Thus, the apparent cross-inhibitions of growth between uridine and arginine can be explained in terms of isozymes and orthodox feed-back processes.

The arginine mutant 30300 grows slowly in air (0·03 % CO_2) but not in CO_2-free air; presumably the CO_2 concentration in ordinary air leads to some overflow of carbamoyl phosphate from the uridine-specific pathway. This suggestion is supported by the fact that this 'leaky' growth does not occur when uridine is present. The uridine mutant 1298 does not show 'leaky' growth, suggesting that there is no overflow of carbamoyl phosphate from the arginine pathway when this mutant is incubated in air.

Enzymological and genetical studies by Davis (1960, 1961) and Davis & Woodward (1962) on 'suppressed' pyrimidine mutants support the hypothesis presented here. These workers studied a suppressor mutation which enables mutants similar to pyrimidine mutant 1298 to grow on minimal medium (Houlahan & Mitchell, 1947, 1948; Mitchell & Mitchell, 1952). Davis & Woodward did not study the enzymes responsible for carbamoyl phosphate synthesis, but they found that the enzymes catalysing the subsequent steps of the pyrimidine pathway were not

changed by the original mutations, regardless of whether the suppressor mutation was present or not. However, when the suppressor mutation was present the mutants showed a considerable decrease in ornithine transcarbamylase activity, having less than 2 % of the enzymatic activity of the unsuppressed mutants and of wild-type cultures. One of the effects of the suppressor was thus to limit the amount of free arginine in the mycelium, without actually leading to an arginine deficiency. As Davis (1961) pointed out, growth of the pyrimidine-less mutants when arginine synthesis is diminished suggests that the unsuppressed mutants do not grow because of an internal inhibition by endogenously produced arginine. In terms of the hypothesis developed here, synthesis of carbamoyl phosphate for the arginine pathway would normally be regulated by an arginine feed-back mechanism such that sufficient carbamoyl phosphate is produced to meet only the needs of the arginine pathway. Diminution of the free arginine pool by the suppressor mutation might well permit synthesis of excess carbamoyl phosphate such that some becomes available to the uridine pathway, thereby enabling the pyrimidine mutant to grow. Significantly, the suppressed mutants are very susceptible to inhibition by exogenous arginine (Houlahan & Mitchell, 1947). Davis (1962) suggested that there might be two sources of carbamoyl phosphate in Neurospora, one specific for uridine synthesis, the other specific for arginine synthesis.

Other observations which may be explicable in terms of mutations in isozymic systems are worth consideration. Miller & Harrison (1950) described a yeast which was unusual in being strongly inhibited by uracil; this uracil inhibition was annulled non-competitively by arginine. This yeast thus behaved rather like the Neurospora arginine mutant 30300. It is possible that the yeast of Miller & Harrison was deficient in a carbamoyl phosphate-synthesizing system which was specific for arginine synthesis and had therefore become completely dependent on a carbamoyl phosphate-synthesizing system normally specific for pyrimidine metabolism. That uracil was the most efficient inhibitor suggests that uracil might be a feed-back regulator of pyrimidine synthesis in this yeast; it would be necessary to assume that the regulatory system in this yeast is such that adequate carbamoyl phosphate normally overflows to supply the arginine pathway.

Doudney & Wagner (1952) described a Neurospora mutant which grew well on minimal media but differed from the wild-type in being strongly inhibited by threonine, and the threonine inhibition was annulled non-competitively by methionine. Reference has already been made to the work of Stadtman *et al.* (1961) on the synthesis of threonine and methionine by *Escherichia coli*. By adapting the hypothesis developed above for the arginine and uridine CO_2-mutants of Neurospora, the mutant of Doudney & Wagner may be readily understood as having lost an isozyme from the early part of the methionine pathway. Since vigorous growth occurred on minimal medium, it would seem that adequate precursors overflowed from the intact threonine pathway, where the early steps are the same as in methionine synthesis. An external supply of threonine would be expected to prevent the production of these precursors, thereby explaining the inhibitory effect of threonine and its non-competitive annulment by methionine.

The situations discussed above suggest certain general principles applicable to the study of the genetics of mutations in isozymic systems. The simplest case may be considered, namely, the occurrence of two isozymic pathways within a cell, but

the argument is applicable to multiple pathways such as those studied by Stadtman *et al.* First, if 'overflow' of identical precursors from one pathway to another does not occur, then mutation resulting in loss of an enzyme from one pathway should give a typical biochemical mutant which cannot grow on minimal medium. Since one isozyme would still be present, however, crude enzymological studies would not detect an enzyme deficiency in the mutant. Secondly, if restricted overflow takes place between the pathways, partially by-passing the block, then a slow-growing or 'leaky' mutant should result. Thirdly, if considerable overflow should occur, loss of one isozyme should give a mutant which grows vigorously on minimal medium. In both cases where overflow occurs the growth on minimal medium should be very sensitive to inhibition by the end-product of the intact pathway; this inhibition, in turn, should be overcome by the end-product of the damaged pathway, usually without competition.

At first sight it would seem that one cannot study the genetics of those isozymic systems where considerable overflow occurs, because the mutants should grow vigorously on minimal medium, and therefore would not be isolated by the usual screening techniques for obtaining mutants. However, sensitivity to inhibition by the appropriate end-product should offer a valuable method for increasing the efficiency of selection of 'leaky' mutants, and for detecting them once obtained. Stadtman *et al.* (1961) have already pointed out that the occurrence of 'leaky' mutants at a locus may offer useful evidence that an isozymic system is involved. Susceptibility of such 'leaky' mutants to inhibition by an appropriate end-product should be valuable supporting evidence for the presence of an isozymic system, especially when the inhibition is non-competitively annulled by a possible end-product. Not many enzyme systems have been studied genetically. In some cases this is probably because the appropriate mutants are not isolated by the usual isolation procedures. The presence of isozymic systems, and consequent 'leaky' or 'very leaky' mutants, may account for the failure to isolate some of these mutants. This is probably true, for instance, of the early stages of lysine, threonine and methionine synthesis in micro-organisms.

Another situation which may be explicable in terms of isozymes is the occurrence of more gene loci than would be expected from the number of enzymes which control a given metabolic pathway. For example, there seem to be more genetic loci than known enzymes concerned with arginine synthesis in Neurospora. Since the arginine pathway may have other functions apart from the synthesis of arginine for proteins, it is possible that two or more isozymic pathways for synthesizing arginine occur in Neurospora. If this be the case, there must be little overflow between the pathways, otherwise the mutants would be too 'leaky' to be detected. It is interesting in this respect that arginine mutants seem to be uncommon in Salmonella. Perhaps in Salmonella two or more pathways for arginine synthesis occur, with considerable overflow of intermediate products between the two pathways, so that mutants deficient in an enzyme from one pathway are usually very leaky, and therefore not distinguishable from the wild-type organism.

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REFERENCES

BOREK, E. & WAELSCH, H. (1951). The effect of temperature on the nutritional requirement of micro-organisms. *J. biol. Chem.* **190**, 191.

CHARLES, H. P. (1962). Response of Neurospora mutants to carbon dioxide. *Nature, Lond.* **195**, 359.

DAVIS, R. H. (1960). An enzymatic difference among *pyr*-3 mutants of *Neurospora crassa*. *Proc. nat. Acad. Sci., Wash.* **46**, 677.

DAVIS, R. H. (1961). Suppressor of pyrimidine-3 mutants of Neurospora and its relation to arginine synthesis. *Science*, **134**, 470.

DAVIS, R. H. (1962). Consequences of a suppressor gene effective with pyrimidine and proline mutants of Neurospora. *Genetics*, **47**, 351.

DAVIS, R. H. & WOODWARD, V. W. (1962). The relationship between gene suppression and aspartate transcarbamylase activity in *pyr*-3 mutants of Neurospora. *Genetics*, **47**, 1075.

DOUDNEY, C. O. & WAGNER, R. P. (1952). Threonine inhibition in a strain of Neurospora. *Proc. nat. Acad. Sci., Wash* **38**, 196.

FAIRLEY, J. L. (1954). The growth-promoting effect of certain amino acids for pyrimidine-less Neurospora mutants. *J. biol. Chem.* **210**, 347.

HALDANE, J. B. S. (1954). *The Biochemistry of Genetics*. London: George Allen and Unwin.

HOULAHAN, M. B. & MITCHELL, H. K. (1947). A suppressor in Neurospora and its use as evidence for allelism. *Proc. nat. Acad. Sci., Wash.* **33**, 223.

HOULAHAN, M. B. & MITCHELL, H. K. (1948). Evidence for an interrelation in the metabolism of lysine, arginine and pyrimidines in Neurospora. *Proc. nat. Acad. Sci., Wash.* **34**, 465.

JONES, M. E., SPECTOR, L. & LIPMANN, F. (1955). Carbamyl phosphate, the carbamyl donor in enzymatic citrulline synthesis. *J. Amer. Chem. Soc.* **77**, 819.

MARKERT, C. L. & MØLLER, F. (1959). Multiple forms of enzymes: tissue, ontogenetic and species specific patterns. *Proc. nat. Acad. Sci., Wash.* **45**, 753.

MILLER, E. J. & HARRISON, J. S. (1950). Growth inhibition of a yeast by uracil, and its reversal by arginine. *Nature, Lond.* **166**, 1035.

MITCHELL, M. B. & MITCHELL, H. K. (1952). Observations on the behaviour of suppressors in Neurospora. *Proc. nat. Acad. Sci., Wash.* **38**, 205.

PONTECORVO, G. (1949). Auxanographic techniques in biochemical genetics. *J. gen. Microbiol.* **3**, 122.

REISSIG, J. L. (1960). Forward and back mutation in the *pyr*-3 region of Neurospora. I. Mutations from arginine dependence to prototrophy. *Genet. Res.* **1**, 356.

RYAN, F. J., BEADLE, G. W. & TATUM, E. L. (1943). The tube method of measuring the growth rate of Neurospora. *Amer. J. Bot.* **30**, 784.

STADTMAN, E. R., COHEN, G. N., LEBRAS, G. & DE ROBICHON-SZULMAJSTER, H. (1961). Feed-back inhibition and repression of aspartokinase activity in *Escherichia coli* and *Saccharomyces cerevisiae*. *J. biol. Chem.* **236**, 2033.

J. gen. Microbiol. (1964), **34**, 143–151
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Physiological Characteristics and Vitamin Requirements of Lactobacilli Isolated from Milk and Cheese

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SUMMARY

The vitamin requirements and physiological characteristics of 179 strains of lactobacilli isolated from milk and cheese have been studied and their relationship to classification discussed. The vitamin requirements showed good correlation with the results of the physiological and biochemical tests with strains of the subgenus *Streptobacterium* but the correlation was poor among the beta bacteria. It was not possible to differentiate the physiologically similar *Lactobacillus brevis* and *L. buchneri* species by their vitamin requirements. The nutritional data indicated that the species *L. plantarum* probably consists of more than one variety, and confirmed the reliability of a thiamine requirement as a means of differentiating heterofermentative and homofermentative lactobacilli. The nutritional data also proved useful with some strains by verifying their unclassifiable status or by making possible their classification when they could not be identified with certainty on the basis of biochemical tests alone. The nitratase activities of strains of *L. casei*, *L. plantarum*, *L. brevis*, *L. buchneri* and pediococci were also studied. Four of the *L. plantarum* and 2 of the *L. casei* strains were able to reduce nitrate but none of the other species tested showed this characteristic.

INTRODUCTION

In a recent study of the bacterial flora of cheese milk and Cheddar cheese (Franklin & Sharpe, 1963), 1755 strains of lactobacilli were isolated and classified by using a variety of biochemical and physiological tests. Whilst most of these strains were satisfactorily assigned to appropriate species, a few strains could not be identified because their biochemical characteristics were intermediate between species. Rogosa and co-workers (1947, 1953, 1961) found that the biochemical and cultural characteristics of members of the genus *Lactobacillus* correlated well with their vitamin requirements. Accordingly, the vitamin requirements of representative strains of these milk and cheese isolates were investigated to determine whether a similar correlation occurred with these organisms and also to see whether knowledge of their vitamin requirements assisted in the identification of the unclassified strains.

Costilow & Humphreys (1955) observed that some strains of *Lactobacillus plantarum* reduced nitrates under certain conditions and this was later confirmed by Rogosa (1961). Because of the ready availability, in this investigation, of a large number of strains of lactobacilli and pediococci from different sources, the oppor-

tunity was taken of examining these organisms for nitratase activity to determine the extent to which these observations were confirmed. The strains tested, however, were not always the same as those used in the nutritional study.

METHODS

Lactobacilli isolated from milk and cheese as described by Franklin & Sharpe (1963) were classified according to the schemes of Rogosa & Sharpe (1959) and Perry & Sharpe (1960), by using the following tests: microscopic appearance; catalase production; growth at 15° and 45°; production of gas from glucose; production of ammonia from arginine; splitting of aesculin; growth in 0·1 % Teepol (Shell Chemical Co. Ltd., Marlborough House, 15–17 Great Marlborough St, London W. 1.) and fermentation of sugars. Cultures were grown in MRS broth (de Man, Rogosa & Sharpe, 1960) and washed suspensions were used for inoculation of the test media.

In addition to the above tests, some strains were also tested for the reduction of nitrate using a medium of low carbohydrate content maintained at a relatively high pH within the nitratase activity range (Rogosa, 1961). The composition of this medium was as follows (%, w/v): Oxoid peptone, 1·0; Oxoid Lab-Lemco, 1·0; Oxoid yeast extract, 0·5; glucose, 0·2; Tween 80 (Polyoxyethylene sorbitan oleate, Honeywill and Stein Ltd., Devonshire House, Mayfair Place, Piccadilly, London W. 1) 0·1; K_2HPO_4, 0·2; $CH_3COONa.3H_2O$, 1·0; tri-ammonium citrate, 0·2; $MgSO_4.7H_2O$, 0·02; $MnSO_4.4H_2O$, 0·005; KNO_3, 0·1. The pH value was adjusted to 7·5–7·6 before sterilization. The method of testing for nitrate reduction was that used by Rogosa (1961). The vitamin requirements of the lactobacilli were determined by using the medium and methods described by Rogosa, Franklin & Perry (1961).

The taxonomy of the lactobacilli and many of the tests used were discussed recently by Rogosa & Sharpe (1959) and by Sharpe (1962).

RESULTS AND DISCUSSION

The milk and cheese lactobacilli isolates which could be satisfactorily classified on the basis of physiological tests belonged to the four species *L. casei*, *L. plantarum*, *L. brevis* and *L. buchneri*. As it was not practicable to examine the vitamin requirements of all the 1755 strains, representative typical strains were studied from each of these species, together with those strains which could not be classified biochemically into named species or which, although resembling a particular species sufficiently to warrant inclusion in that group, nevertheless differed from the typical reactions of that species in one or more important characteristics. For reasons of simplicity in this publication, the description 'typical' is applied to named strains showing the differentiating physiological characteristics or nutritional requirements of species of lactobacilli as described by Rogosa & Sharpe (1959) and Rogosa *et al.* (1961).

Lactobacillus casei. The vitamin requirements were determined for 74 strains which were classified physiologically as *L. casei* or which resembled this species more than any other. Twenty-two per cent of these, giving the typical reactions of this species listed in Table 1, were able to grow at 45°, whilst 14 % of those giving the typical reactions did not grow at this temperature. Thirty-four per cent differed

Table 1. *The differentiating biochemical and physiological characteristics of species used to classify lactobacilli isolated from milk and Cheddar cheese*

Species of *Lactobacillus*	Growth at 45°	Gas from glucose	NH_3 from arginine	Growth in 0·1 % Teepol	Fermentation of: Arabinose	Cellobiose	Lactose	Mannitol	Melibiose	Raffinose	Salicin	Sucrose	Trehalose	Xylose	Rhamnose	Melezitose	Aesculin cleavage
L. casei	maj +	−	−	−	−	+	+	+	−	−	+	+	+	−	maj −	+	+
L. plantarum	−	−	−	+	maj +	+	+	+	+	+	+	+	+	maj −	maj −	+	+
L. brevis	−	+	+	+	maj +	−	maj −	weak or −	+	maj +	− or weak	maj +	−	maj +	−	−	maj +
L. buchneri	−	+	+	+	+	−	maj +	weak or −	+	+	−	+	−	maj −	−	+	maj +

All species are catalase negative and grow at 15°. maj + = majority of strains positive; maj − = majority of strains negative.

from the typical in one character, 26 % in two characters, 3 % in three characters and 1 % in four characters. The extent to which the different tests gave atypical results with the 74 strains was as follows: sucrose negative, 33 %; trehalose negative, 23 %; cellobiose negative, 10 %; salicin negative 8 %; mannitol negative, 7 %; Teepol positive, 5 %; aesculin negative, 3 %; lactose negative, 3 %; melezitose negative, 3 %; rhamnose positive, 1 %; arabinose positive, 1 %.

The vitamin requirements for the 74 strains of *Lactobacillus casei* tested are given in Table 2. Most of the strains (91 %) had the typical vitamin requirements for the species, and no strain required thiamine. Only one strain had no requirement for riboflavin and this strain differed from the typical physiological and biochemical characteristics of the species only in its failure to ferment sucrose. The remaining strains having atypical vitamin requirements had no other atypical characteristics in common.

Table 2. *The vitamin requirements of* 121 *strains of the sub-genus Streptobacterium Orla-Jensen*

Nicotinic acid and pantothenic acid were required by all strains.

Species	No. of strains	Thiamine	Riboflavin	Pyridoxal	Folic acid
L. casei	62	−	+	+	+*
(74 strains)	5	−	+	S	+*
	3	−	+	S	−
	1	−	+	+	−
	1	−	+	−	+
	1	−	−	+	S
	1	−	+	−	−
L. plantarum	30	−	+	−	−†
(47 strains)	14	−	−	−	−†
	2	−	−	S	−
	1	−	+	S	−

* Typical vitamin requirement pattern for *L. casei* strains (Rogosa *et al.* 1961).
† Typical vitamin requirement pattern for *L. plantarum* strains (Rogosa *et al.* 1961).
+ = requirement; − = no requirement; S = stimulatory.

Lactobacillus plantarum. The vitamin requirements were determined for 47 strains classified physiologically as, or closely related to, *L. plantarum*. Twenty-four per cent of these gave the typical physiological reactions listed in Table 1, 24 % differed from the typical physiological pattern by one character, 9 % by two characters, 4 % by three characters and 39 % by four characters. The proportion of the 47 strains differing in particular characters from the typical were as follows: Teepol negative, 63 %; trehalose negative, 54 %; sucrose negative, 52 %; raffinose negative, 41 %; melibiose negative, 2 % (1 strain). Unlike the *L. casei* group, no strains of *L. plantarum* were able to grow at 45°.

The vitamin requirements of the 47 strains of *Lactobacillus plantarum* tested are given in Table 2. Nearly all the strains (94 %) had the typical vitamin requirements for the species although it is evident that there are two varieties of *L. plantarum*, one requiring riboflavin and one having no requirement for this vitamin. This confirms the findings of Rogosa *et al.* (1953). Three strains only differed from the typical patterns in showing a stimulatory action of pyridoxal.

The strains which had no riboflavin requirement had the typical physiological characteristics of this species or differed only in their failure to grow in 0·1 % Teepol, whereas with the exception of two typical strains and one strain which did not grow in 0·1 % Teepol, the remaining 27 strains which exhibited a requirement for riboflavin also differed from the typical physiological pattern in their failure to ferment one or more of sucrose, trehalose, raffinose and melibiose.

Table 3. *The vitamin requirements of 46 strains of the subgenus Betabacterium Orla–Jensen*

Nicotinic acid and pantothenic acid were required by all strains.

Species	No. of strains	Thiamine	Riboflavin	Pyridoxal	Folic acid
L. brevis	9	+	+	–	+
(27 strains)	6	+	S	+	+
	4	+	+	+	+
	3	+	–	–	+*
	3	+	S	+	+
	2	+	+	–	+
L. buchneri	7	+	–	–	–†
(19 strains)	6	+	–	S	+
	6	+	+	S	+

* Typical vitamin requirement pattern for *L. brevis* strains (Rogosa *et al.* 1961).
† Typical vitamin requirement pattern for *L. buchneri* strains (Rogosa *et al.* 1961).
+ = requirement; – = no requirement; S = stimulatory.

Lactobacillus brevis. Vitamin patterns were determined for 27 strains classified as *L. brevis.* Eight strains gave the typical physiological reactions for this species as listed in Table 1 and 8 strains differed by only one character (Teepol negative, 5 strains; melibiose negative, 6 strains: NH_3 from arginine negative, 1 strain).

The vitamin requirements for the 27 strains of *Lactobacillus brevis* are given in Table 3 which shows that only three strains gave the typical vitamin requirement pattern for this species. All the strains possessed the typical thiamine requirement of the heterofermentative lactobacilli and all required folic acid, but this latter property was also found in other betabacteria. Contrary to the previous findings, riboflavin was essential for growth of, or was stimulatory to, 24 strains, and pyridoxal was essential for the growth of 13 strains. The atypical vitamin-requirement patterns obtained in this group were different combinations of the various vitamin requirements exhibited by so-called typical strains of *L. brevis*, *L. buchneri* and *L. viridescens.* All these strains had the physiological characteristics of *L. brevis*, however. None fermented melezitose and all differed in some respect from typical *L. viridescens* strains.

Lactobacillus buchneri. Vitamin requirements were determined for 19 strains classified physiologically as *L. buchneri.* Four strains gave the typical physiological characteristics for this species as shown in Table 1, 13 strains differed in one character from the typical and 2 strains in two characters. The proportions of the 19 strains differing in particular characters from the typical were as follows: Teepol negative, 13 strains; raffinose negative, 1 strain; sucrose negative, 1 strain; melezitose negative, 1 strain; salicin positive, 1 strain.

Table 4. *The physiological and nutritional characteristics of twelve unclassified lactobacilli isolated from milk and Cheddar cheese*

Nicotinic acid and pantothenic acid were required by all strains.

Strain no.	Catalase	Growth at 15°	Growth at 45°	Gas from glucose	NH_3 from arginine	Growth in 0·1% Teepol	Fermentation of: Arabinose	Cellobiose	Lactose	Mannitol	Melibiose	Raffinose	Salicin	Sucrose	Trehalose	Xylose	Rhamnose	Melezitose	Aesculin cleavage	Requirement for: Thiamine	Riboflavin	Pyridoxal	Folic acid
ML 68	−	+	−	±	−	+	−	−	−	−	−	−	−	−	+	−	−	−	−	+	+	S	−
ML 78	−	+	−	±	−	−	−	−	±	−	−	±	−	+	+	−	−	−	−	+	+	−	−
ML 118	−	+	+	±	+	−	+	−	+	−	+	+	−	+	−	−	−	−	−	+	−	S	−
ML 161	−	−	+	+	+	−	+	−	+	−	+	+	−	+	−	−	−	−	−	+	−	S	−
ML 568	−	+	−	−	−	−	−	+	+	+	−	−	+	±	−	−	−	−	+	−	+	−	−
CL 833	−	+	±	−	−	−	−	+	+	+	−	−	+	−	+	−	−	+	−	−	+	−	−
CL 1025	−	+	−	±	−	−	−	+	+	+	−	−	+	−	−	−	−	+	+	−	+	−	−
CL 1191	−	+	−	−	−	+	+	+	+	+	−	−	+	−	−	−	−	+	+	−	+	−	−
CL 1225	−	+	−	+	±	−	+	−	±	−	−	−	−	+	+	+	−	−	−	+	−	−	−
CL 1226	−	+	±	−	−	−	−	+	+	+	−	−	−	+	±	−	−	+	−	−	+	−	−
CL 1261	−	+	−	±	−	±	−	+	+	+	−	−	+	−	−	−	−	+	+	−	+	−	−
CL 1313	−	+	−	−	−	−	−	+	+	+	−	−	±	−	−	−	−	−	±	−	+	S	−

S = stimulatory, ± = sometimes positive.

The vitamin requirements for the 19 strains of *Lactobacillus buchneri* are given in Table 3. All the strains required thiamine but only 7 strains exhibited the typical vitamin requirement pattern for this species. Pyridoxal was stimulatory and folic acid essential for the growth of the remaining 12 strains which could be equally divided into those which required riboflavin and those which did not. Some strains of *L. buchneri* have been observed to show a requirement for riboflavin and this is confirmed here, although in the present work this character was always accompanied by a requirement for folic acid. The atypical vitamin requirement patterns obtained in the group again appear to be different combinations of the so-called typical patterns for *L. brevis*, *L. buchneri* and *L. viridescens*. However, all the atypical strains produced ammonia from arginine and fermented melezitose, arabinose and melibiose, and could not, therefore, be classified either as *L. brevis* or *L. viridescens* species.

Unclassified Lactobacillus strains

Twelve strains were unclassified physiologically and their physiological and nutritional characteristics are given in Table 4. Some of the implications of these results in relation to their classification are discussed below.

Strain ML *68.* Production of gas from glucose places this strain in the subgenus *Betabacterium*. Failure to produce ammonia from arginine, its inability to grow at 45° and its relative non-fermentative nature suggest that it might be classified as *L. viridescens* and, except that folic acid was not stimulatory, this is supported by the nutritional data.

Strain ML *78.* This strain is also a member of the betabacteria but differs from ML 68 in its failure to grow in 0·1 % Teepol, its fermentation of lactose and raffinose and the lack of growth stimulation by pyridoxal. This strain does not, therefore, fit into any of the recognized species in this subgenus.

Strain ML *118.* The physiological characters of this strain suggest that though capable of growth at 15° it belongs to the species *L. fermenti*. The vitamin requirements supported this classification although pyridoxal was stimulatory contrary to the usual pattern for this group.

Strain ML *161* gave identical results to ML 118 except for its failure to grow at 15°, and would, therefore, also appear to belong to the species *L. fermenti*.

Strain ML *568.* The physiological results, with the exception of the failure to ferment trehalose, indicate that this strain is clearly a member of the species *L. casei*. This is not supported by the nutritional results, however, which were typical of *L. plantarum*.

Strain CL *833.* This fermented few carbohydrates but the typical vitamin requirements of *L. casei* exhibited by this organism suggest that CL 833 should be assigned to this species.

Strain CL *1025.* With the exception of the production of a small amount of gas from glucose and the failure to ferment sucrose, this strain had the typical physiological reactions of *L. casei*; its vitamin requirements, however, were those of *L. plantarum*. The absence of a thiamine requirement excluded it from the heterofermentative lactobacilli and the physiological characteristics, with the exception of gas production, were unlike those of any named *Betabacterium* species.

Strain CL 1261. This was very similar to CL 1025, having physiological characters closely related to *L. casei*, the vitamin requirements of *L. plantarum*, yet producing small quantities of gas from glucose.

Strain CL 1191. This seems to be intermediate between *L. casei* and *L. plantarum*, being unable to ferment melibiose and raffinose but able to grow in 0·1 % Teepol and fermenting arabinose. The vitamin requirements, however, were typical of *L. plantarum*.

Strain CL 1225. Although clearly a member of the subgenus *Betabacterium*, this organism cannot be placed into any of the named species in this group; this was confirmed by the nutritional data.

Strain CL 1226. This physiologically inactive strain showed the typical vitamin requirements of *L. casei* suggesting that it is most closely related to this species.

Strain CL 131. This strain resembles *L. casei* physiologically, although producing small volumes of gas from glucose, but this classification is not entirely supported by the nutritional pattern which was intermediate between those of *L. casei* and *L. plantarum*.

Table 5. *Nitrate reduction by lactobacilli and pediococci isolated from milk and cheese*

Species	No. strains tested	No. strains positive
L. casei	102	2
L. plantarum	12	4
L. brevis	18	0
L. buchneri	8	0
Pediococci	9	0

The results of this investigation confirm the consistency that exists between the physiological characters of strains of *Lactobacillus casei* and *L. plantarum* and their vitamin requirements. The nutritional patterns obtained with *L. plantarum* were mainly of two kinds and this supports the hypothesis (Rogosa *et al.* 1953) that *L. plantarum*, as at present defined, might be a heterogeneous group consisting of more than one variety.

In contrast to the results obtained with the streptobacteria, the correlation between the vitamin requirements and the physiological characteristics of *Lactobacillus brevis* and *L. buchneri* strains was poor. A wide variety of vitamin-requirement patterns were obtained for these species, although no pattern was found to be common to both species. The suggestion of Rogosa *et al.* (1961) that the separation of *L. brevis* and *L. buchneri*, dependent physiologically on the fermentation of melezitose only, could be achieved by use of nutritional data was not supported because, contrary to their observations, many strains of *L. brevis* were found to have a requirement for riboflavin, and folic acid was necessary for the growth of most of the *L. buchneri* strains examined.

A requirement for thiamine by heterofermentative species of lactobacilli and the lack of this requirement by homofermentative species was again confirmed as a very reliable differentiating character, and the usefulness of nutritional studies as an adjunct to the more traditional methods of classifying the lactobacilli was again demonstrated.

Nitrate reduction

Table 5 confirms the ability of certain strains of *Lactobacillus plantarum* to reduce nitrates. Two strains of *L. casei* also reduced nitrate but the proportion of positive strains was very much less than with *L. plantarum* species. None of the strains of the other species tested exhibited nitratase activity. It is evident, therefore, that under conditions of low carbohydrate content and a relatively high pH value, some strains of lactobacilli are able to reduce nitrate; failure to do so cannot be regarded, therefore, as a typical property of the genus *Lactobacillus*.

REFERENCES

Costilow, R. N. & Humphreys, T. W. (1955). Nitrate reduction by certain strains of *Lactobacillus plantarum*. *Science*, **121**, 168.

Franklin, J. G. & Sharpe, M. E. (1963). The incidence of bacteria in cheese milk and Cheddar cheese and their association with flavour. *J. Dairy Res.* **30**, 87.

Man, J. C. de, Rogosa, M. & Sharpe, M. E. (1960). A medium for the cultivation of lactobacilli. *J. appl. Bact.* **23**, 130.

Perry, K. D. & Sharpe, M. E. (1960). Lactobacilli in raw milk and in Cheddar cheese. *J. Dairy Res.* **27**, 267.

Rogosa, M. (1961). Experimental conditions for nitrate reduction by certain strains of the genus *Lactobacillus*. *J. gen. Microbiol.* **24**, 401.

Rogosa, M., Franklin, J. G. & Perry, K. D. (1961). Correlation of the vitamin requirements with cultural and biochemical characters of *Lactobacillus* spp. *J. gen. Microbiol.* **25**, 473.

Rogosa, M. & Sharpe, M. E. (1959). An approach to the classification of the *Lactobacilli*. *J. appl. Bact.* **22**, 329.

Rogosa, M., Tittsler, R. P. & Geib, D. S. (1947). Correlation of vitamin requirements and cultural and biochemical characteristics of the genus *Lactobacillus*. *J. Bact.* **54**. 13.

Rogosa, M., Wiseman, R. F., Mitchell, J. A., Disraely, M. N. & Beaman, A. J. (1953). Species differentiation of oral lactobacilli from man including descriptions of *Lactobacillus salivarius* nov. spec. and *Lactobacillus cellobiosus* nov.spec. *J. Bact.* **65**, 681.

Sharpe, M. E. (1962). Taxonomy of the lactobacilli. *Dairy Sci. Abstr.* **24**, 109.

J. gen. Microbiol. (1964), **34**, 153–164
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The Roles of Serum and Carbon Dioxide in Capsule Formation by *Bacillus anthracis*

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SUMMARY

Capsule formation by virulent strains of *Bacillus anthracis* on nutrient agar is known to depend on incubation in air with added CO_2 as well as the addition of serum or bicarbonate to the medium. The minimum effective concentration of CO_2 varies with the pH of the medium in a way which shows that capsulation depends on a threshold concentration of bicarbonate in the medium. Serum is more effective than bicarbonate and appears to act by binding an agent which inhibits capsule formation since it is replaceable by activated charcoal. The inhibitor might be a fatty acid since certain acids prevented capsule formation. Capsules are formed on nutrient agar containing added bicarbonate only after the culture has become very dense which suggests that the organisms either inactivate the inhibitor or become resistant to its action as their growth rate falls on approaching the stationary phase.

INTRODUCTION

Virulent strains of *Bacillus anthracis* are invariably capsulated *in vivo* but form capsules *in vitro* only under special conditions. These include incubation in air with added CO_2 (Ivánovics, 1937) and the use of nutrient agar containing either serum (Sterne, 1937) or bicarbonate (see Thorne, 1956). Bicarbonate has displaced serum in recent investigations (see Housewright, 1962) but the naked eye appearance of colonies on serum agar and on bicarbonate agar incubated in the same atmosphere immediately suggests that serum agar gives far better capsulation, and that serum and bicarbonate are therefore not equivalent in capsule formation. This observation, which was repeatedly confirmed in studying the genetic control of capsulation (Meynell, 1963), and also the unusually high concentrations of serum and CO_2 said to be required, led to the present work. The results show that serum is replaceable by 0·2 % (w/v) activated charcoal. Both are thought to act by binding an inhibitor which is present in the medium and in their presence 'physiological' concentrations of CO_2 suffice for capsule formation. Capsulation on bicarbonate agar is believed to occur only after the inhibitor has become ineffective. Microscopy confirmed this hypothesis for organisms growing on bicarbonate agar became capsulated many hours after those in comparable cultures on serum or charcoal agar.

METHODS

Organisms. The principal strain, 2160s, was a variant of *Bacillus anthracis* strain 2160, isolated after prolonged incubation of strain 2160 in broth containing 0·025% (w/v) $CaCl_2$ (Renaux, 1952). It formed shorter chains, and therefore smoother colonies, than typical strains of *B. anthracis* (Nungester, 1929), did not form heat-resistant spores (McCloy, 1958), and was virtually avirulent for mice. However, it behaved like typical strains of this species in forming a capsule only when grown on serum or bicarbonate agar in CO_2.

Five typical virulent strains were tested in a few experiments: two capsulated (C+) strains (1444, Hillsborough) from the authors' stocks, and C+ revertants from three strains (Vollum, A 69, A 77) received as C− mutants from Dr G. Ivánovics (Meynell, 1963).

Media. Only solid media were used. The usual formula was (%, w/v): 'Lab-Lemeo', 0·1; Tryptone (Oxoid), 0·2; Peptone (Oxoid), 1; NaCl, 0·7; Davis N.Z. agar, 1·5; pH 7·4. In some experiments, Oxoid Nutrient Broth No. 2, solidified with Oxoid Agar No. 3, 1·5% (w/v) was used, either at pH 7·4 or 8·0, when it also contained 0·1 M 2-amino-2-hydroxymethylpropane-1,3-diol (tris), or at pH 6·2 or 6·8, when inorganic phosphate buffer was added to 0·1 M just before pouring plates. Other supplements were also added to molten agar at this time. Calculated amounts of $NaHCO_3$ were added from a M-solution sterilized by Seitz filtration; the calculations are explained in the legend to Fig. 1. Serum was added to 20% (v/v). Bovine Serum Albumin Fraction V (batches AN 2070 and GC 1170; Armour Laboratories, Hampden Park, Eastbourne, Sussex) was added to 0·7% (w/v) from a 7% (w/v) solution in distilled water or in 0·1 M Sørenson buffer (pH 7·6) sterilized by Seitz filtration. Activated charcoal (Norit A or Hopkin and Williams decolorizing charcoal, code 2992) was added to 0·2% (w/v) from a 10% (w/v) suspension in distilled water previously sterilized by autoclaving at 121° for 15 min.

Incubation of media. Plates were inoculated with a loop to give an area of confluent growth and streaks bearing isolated colonies. Cultures incubated in 5–40% (v/v) CO_2 were held in anaerobic jars from which the necessary volume of air was evacuated and replaced by pure CO_2: thus, 20% CO_2 implies 20% (v/v) CO_2 + 80% (v/v) air. In one of the experiments shown in Fig. 1 and Table 1, each jar contained 60% air, the balance after adding the required amount of CO_2 being made up with N_2. As this did not affect the results, jars simply contained different proportions of air in the later experiments. Cultures in 2·5% CO_2 were set up in candle jars (Nye & Lamb, 1936).

Assessing the degree of capsulation. Plates were examined after incubation for 21–24 hr. at 37°. Capsulation is reflected in a mucoid appearance of the growth, and its degree was estimated from the naked-eye appearance of confluent growth and of colonies which were recorded separately as R, RS, SR, SR—more glistening, M+, M++, M+++ or M++++. SR broadly resembles a smooth salmonella colony, and M+++ resembles *Klebsiella pneumoniae*. The proportion of capsulated organisms was judged from films stained by M'Fadyean's method (1903) with polychrome methylene blue. For degrees of capsulation of M+ or greater, all the organisms appeared capsulated; between R and SR, the proportion of capsulated organisms increased from 10^{-7} to about 0·5.

RESULTS

The role of carbon dioxide

The concentrations of CO_2 customarily used to induce capsulation are exceedingly high (e.g. 20–30%). This suggested that capsule formation depended not on the atmospheric concentration of CO_2 but on the concentration of bicarbonate ion (HCO_3^-) in the medium. This hypothesis was tested by growing cultures on agar of various pH in various concentrations of CO_2, since the Henderson–Hasselbalch equation predicts that a given $[HCO_3^-]$ is produced by less and less CO_2, the higher the pH. The Henderson–Hasselbalch equation can be written

$$\log [HCO_3^-] = pH - pK + \log [CO_2],$$

where the pH is that of the medium; pK is a constant related to the pK_1 of carbonic acid; and $[CO_2]$ is the molarity of dissolved CO_2 and equals $P\alpha(CO_2)\ 5{\cdot}87 \times 10^{-7}$. P is the atmospheric pressure, α is the solubility of CO_2, and (CO_2) is the % (v/v)

Table 1. *Degree of capsulation of Bacillus anthracis strain 2160s on various media of different pH incubated in various concentrations of carbon dioxide*

		% CO_2 in atmosphere									
		2·5		5		10		20		40	
Medium	pH	conf.	col.	conf.	col.	conf.	col.	conf	col.	conf.	col.
Nutrient agar											
+ $NaHCO_3$	6·2	RS	R	RS	R	RS	R	RS	R	RS	R
	6·8	RS	R	RS	R	SR	RS	SR	SR	M+	M+
	7·4	RS	R	SR	RS	M+	M+	M++	M++	M++	M++
	8·0	RS	SR	M+	SR	M++	M++	M++	M++	M++	NG
+ $NaHCO_3$ + albumin or charcoal	6·2	RS	R	RS	R	SR	SR	M+	M+	M++	M++
	6·8	SR	M++	M++	M+++	M+++	M+++	M+++	M+++	M+++	M+++
	7·4	SR	M++	M++	M++	M+++	M+++	M+++	M+++	M+++	M+++
	8·0	SR	M++	M++	M+++	M+++	M+++	M+++	M+++	M+++	M+++
No supplement	6·2	RS	R	RS	R	RS	R	RS	R	SR	R
	6·8	RS	R	RS	R	SR	SR	SR	SR	SR	SR
	7·4	SR	R	SR	RS	SR	SR	SR	SR	SR	SR
	8·0	RS	RS	RS	RS	M+	M+	M+	M+	M+	M−
+ Charcoal	6·2	RS	R	RS	R	RS	RS	M+	M+	M++	M++
	6·8	RS	RS	M+	M++	M++	M++	M+++	M+++	M+++	M+++
	7·4	RS	M+	M+	M++	M+	M++	M++	M++	M++	M+++
	8·0	SR	M++	M++	M++	M++	M++	M+++	M++−	M+++	NG

conf. = confluent growth; col. = isolated colonies; NG = no growth.

of atmospheric CO_2 (see Umbreit, Burris & Stauffer, 1957, chapter 2). It follows that log $[HCO_3^-]$ plotted against log $[CO_2]$ for a given pH gives a straight line and that the plots for different pH form a series of parallel straight lines (Fig. 1). Hence, as stated above, a given $[HCO_3^-]$ is produced by a variety of combinations of $[CO_2]$ and pH.

Cultures on nutrient agar plates, buffered at pH 6·2, 6·8, 7·4, or 8·0, and supplemented with appropriate concentrations of $NaHCO_3$, were incubated overnight in different CO_2 concentrations and examined for capsulation. Figure 1*a* and Table 1 show that capsule production depended entirely on the concentration of HCO_3^- in the medium since it occurred with any combination of pH and CO_2, provided that at least 0·056 M-HCO_3^- was present in the medium.

The role of serum

The early experiments on capsulation used nutrient agar containing a high concentration of horse serum (Sterne, 1937) and concentrations of 20% (v/v) or more have been used ever since. Our initial supposition was that serum acted as a buffer, increasing the concentration of HCO_3^- formed in the medium at a given CO_2 concentration in the same way as an increase in pH or addition of $NaHCO_3$. Two observations, however, were against this explanation. First, the buffering power of the broth used for these experiments was hardly changed by adding 20% (v/v) horse serum and remained less than that of tryptic digest broth which gave less capsulation (Fig. 2). Secondly, overnight cultures on serum agar were far more mucoid than those on bicarbonate agar, the difference being most striking

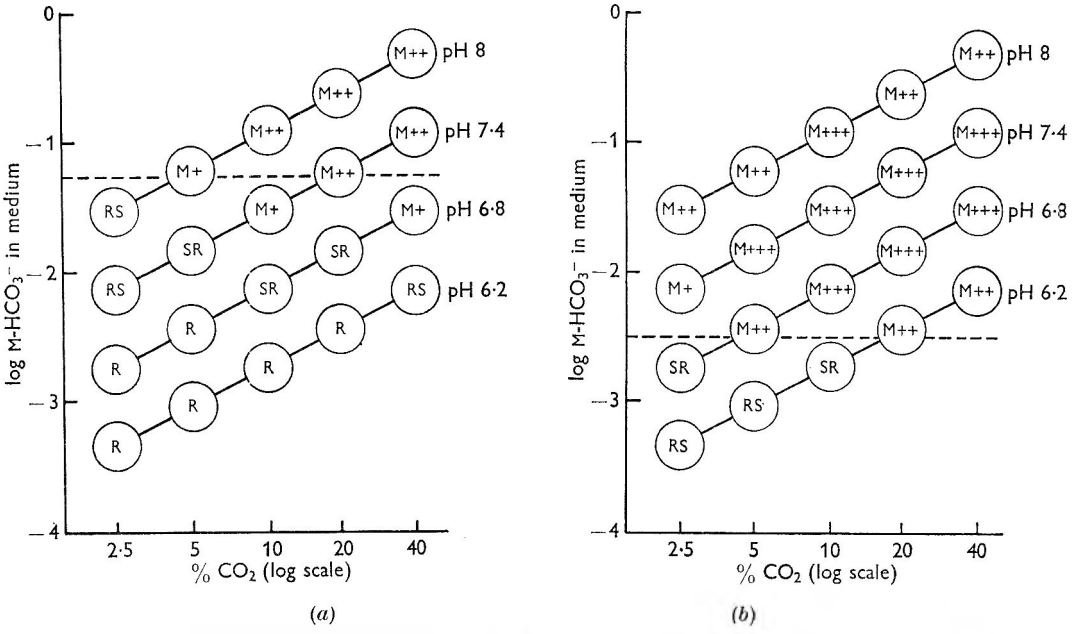

Fig. 1. Relation of bicarbonate ion (HCO_3^-) concentration to atmospheric content of CO_2 at various values of pH. The lines show the relations predicted for pH 6·2, 6·8, 7·4 and 8·0 by the Henderson–Hasselbalch equation, with pK = 6·32; P = 760 mm. Hg; and α = 0·56 ml./ml. at 37°. The amounts of $NaHCO_3$ added to plates were also calculated from these values. The letters enclosed by circles indicate the degree of capsulation produced by strain 2160s after overnight incubation at 37°. Nutrient agar at pH 6·2 and 6·8 was buffered with 0·1 M-inorganic phosphate and at pH 7·4 and 8·0 with 0·1 M-tris. In addition, the calculated amount of $NaHCO_3$ was added to buffer H_2CO_3 formed in the medium by solution of CO_2, leaving the phosphate or tris to maintain the initial pH during growth of the culture. All the media had certain points in common. Capsulation never occurred at any pH after incubation in air (0·03% CO_2). Colonial diameters were respectively half and a quarter those of the controls when the HCO_3^- concentration was in the ranges 0·01–0·07M and 0·07–0·1M. Only pin-point colonies were seen with HCO_3^- concentrations exceeding 0·1M, and thickly inoculated areas of the plates showed only a watery smear of growth. (*a*) Plates with added $NaHCO_3$ only. (*b*) Plates with $NaHCO_3$ and either 0·7% (w/v) albumin or 0·2% (w/v) charcoal. The dashed lines show the threshold HCO_3^- concentration above which mucoid growth occurred. The threshold for bicarbonate agar is 0·056M, about 18 times greater than for albumin or charcoal agar, where it is 0·0032M.

with isolated colonies. In addition, there were quite marked differences in the capsule-promoting activity of different batches of serum; some were almost inactive. The sera of different animal species are known to differ markedly in this respect (Dr H. Smith, personal communication) and, in limited tests with single batches of human, sheep, and calf sera, we found that all were much more active than horse serum. Human serum showed the most activity, followed by calf and sheep serum.

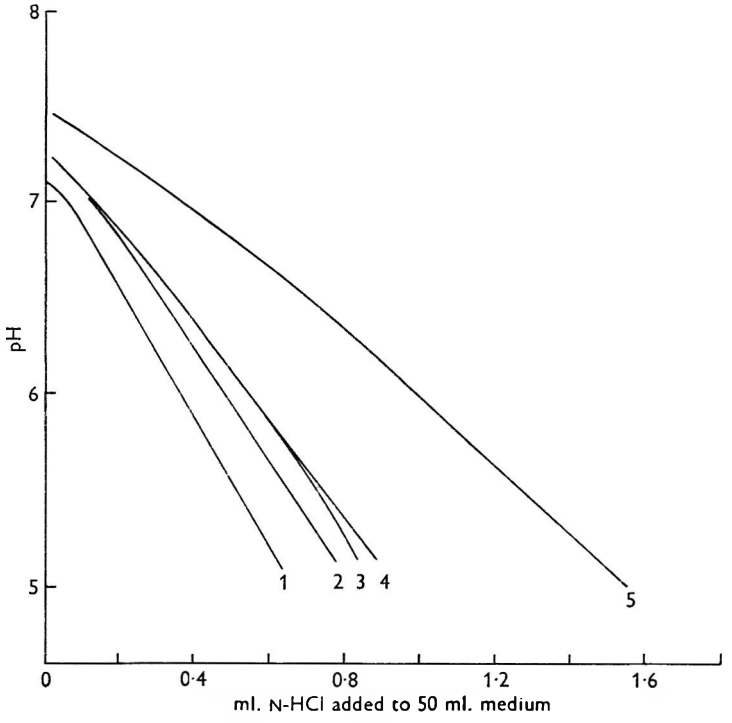

Fig. 2. Titration curves of different liquid media. The buffering capacity is inversely proportional to the slope of the titration curve. Key to curves: 1, Lemco broth + 0·7 % (w/v) albumin dissolved in water; 2, Lemco broth + 20 % (v/v) horse serum; 3, Lemco broth + 0·7 % (w/v) albumin dissolved in 0·1 M-phosphate buffer; 4, Lemco broth alone; 5, Tryptic digest broth.

Massive capsulation resulted when horse serum was replaced by Bovine Serum Albumin Fraction V added to the medium in a concentration equal to that of the albumin provided by whole serum. The same order of capsulation was obtained whether the stock albumin solution was made in distilled water or in 0·1 M-phosphate buffer (pH 7·6). Capsulation occurred on albumin agar when the bacteria were separated from the medium by cellophan (a.p.d., 3 mμ) or by gradocol membranes (a.p.d., 5 mμ), regardless of whether they were inoculated on a membrane placed on the surface of the agar or across unsupplemented agar separated from albumin agar by a strip of cellophan. In the second case, the growth on nutrient agar next to the cellophan was more capsulated than that farther away.

These results suggested that albumin (and presumably serum) acted either by absorbing a low molecular weight inhibitor of capsulation from the medium or by

providing a dialysable factor which promoted capsule formation. Binding of an inhibitor was strongly supported by finding that activated charcoal (0·2%, w/v) was just as efficient as albumin in promoting capsulation. On either medium incubated in 10–20% CO_2, isolated colonies were so mucoid that they coalesced and the confluent growth sometimes poured into the lid of the plate. It seemed very improbable that charcoal contributed a stimulating factor, since either of two brands were active (Norit A or Hopkin and Williams), whether used as supplied or after successively refluxing with equal parts of chloroform and methanol for 6 hr., heating in 6 N-HCl for 30 min. at 121° followed by repeated washing with boiling distilled water, and heating to redness in nitrogen for 1 hr.

The effects of albumin and charcoal were examined in more detail by retesting the influence of CO_2 concentration and pH. Fig. 1*b* shows that, while the general relation found for bicarbonate agar also held for these media, the threshold HCO_3^- concentration required for capsulation was lowered about 18-fold. No capsulation occurred on either medium when incubated in air. The lowering of the threshold explains why capsulation was obtained on unbuffered medium containing albumin or charcoal despite some fall in pH on incubation in CO_2 (Table 1). It appears that capsulation can occur either with a low HCO_3^- threshold even when the pH falls (albumin or charcoal agar) or with a high threshold when the fall in pH is prevented (bicarbonate agar). As expected, the maximum amount of capsulation was produced when the pH was maintained and the HCO_3^- threshold was lowered (albumin or charcoal agar + bicarbonate buffer).

These observations also explain why capsulation does not occur on conventional nutrient agar at pH 7·4. On exposure to CO_2, sufficient carbonic acid forms in the medium to lower its pH and, consequently, to raise still further the concentration of CO_2 needed in the atmosphere to produce the threshold concentration of HCO_3^-. However, capsulation occurs, as expected, when the agar is made exceptionally alkaline before incubation in CO_2, e.g. by raising the pH to 8·5 with an alkali like NaOH (Thorne, Gomez & Housewright, 1952) or by adding bicarbonate (Thorne, 1956).

Other absorbents were also tested (Table 2). Coarse granular charcoal (British Drug Houses Ltd., for gas absorption; Holt, 1962) definitely promoted capsulation, although less efficiently than the finely particulate Norit and Hopkin and Williams charcoals, probably because the coarse granules sank to the bottom of the plate. Florisil was weakly active its particles also tended to sink and its relative inefficiency might also have been due to poor dispersion. Alumina, silicic acid and cholesterol (Lwoff, 1947) produced a slight increase in the proportion of capsulated bacteria in the confluent growth as compared with the controls, but too inconstantly to prove their capsule-promoting activity. The anion-exchangers, DEAE and Amberlite CG-400, had definite activity, while the cation-exchangers, CEC and Amberlite CG-120, appeared to have none. Starch promoted capsulation, though not to the same extent as albumin or charcoal, and isolated colonies were usually more mucoid than the confluent growth and contained a higher proportion of capsulated organisms. The effect of starch might have been lessened owing to its hydrolysis by the amylase produced by the bacteria (Smith, Gordon & Clark, 1952; Knight & Proom, 1950). Alternatively, glucose formed by hydrolysis may have depressed capsule formation (see Discussion).

Table 2. *Action of absorbents in promoting capsulation of Bacillus anthracis strain* 2160S

Each absorbent was incorporated in nutrient agar pH 7·4 which was incubated in 20 % CO_2.

Absorbent and concentration (w/v)	Proportion of capsulated organisms in isolated colonies	Capsulogenic effect
Granular charcoal 2 %	0·05	Slight
Florisil* 1 %	0·01–0·05	Slight
Alumina† 1 %	10^{-8}	None
Silicic acid ‡ 1 %	10^{-8}	None
Lintner's soluble starch 0·6 %	0·1–M++	Inconstant
Cholesterol§ 0·03 %	10^{-6}	None
0·001 %	10^{-6}	None
DEAE 2 %‖	0·8	Probable
CEC¶	10^{-5}	None
Amberlite CG–120** 0·5 %	10^{-8}	None
Amberlite CG–400†† 0·5 %	M++	Probable

* 60–100 mesh magnesium silicate (L. Light and Co.).
† Grade 1 (M. Woelm).
‡ 100 mesh (Mallinckdrodt).
§ Added from a 0·1 %, w/v, emulsion in water.
‖ *N.N*-Diethylaminoethyl cellulose (Eastman Organic Chemicals).
¶ Cellex–CM. Carboxyl methyl cellulose (Bio-Rad laboratories).
** Cation-exchange resin (British Drug Houses Ltd).
†† Anion-exchange resin (British Drug Houses Ltd).

Relation of capsule formation on bicarbonate agar to bacterial concentration

If, in fact, albumin and charcoal removed an inhibitor of capsule formation from the medium, it remained to account for capsule formation on bicarbonate agar. Two possibilities were that the effect of the inhibitor was overcome by HCO_3^- concentrations permitting capsulation so that bicarbonate agar effectively resembled albumin or serum agar; or that the inhibitor ceased to affect the bacteria after their concentration had become fairly high. These alternatives could therefore be distinguished by seeing whether organisms growing on bicarbonate agar in appropriate concentrations of CO_2 were capsulated throughout growth like other species (Meynell, 1961), which is consistent with the first explanation; or whether capsules only appeared late in the growth of the culture after the inhibitor had become inactive. A corollary of the second explanation is that capsulation should appear far earlier in cultures on albumin or charcoal agar in which the inhibitor is assumed to be inactive.

Plates of bicarbonate, albumin, or charcoal agar at pH 7·4 were flooded with non-capsulated bacteria from an overnight culture grown in air, allowed to dry, and then incubated in CO_2 at 37°. Impressions were made every hour from blocks cut from each of three plates of each medium, stained with M'Fadyean's polychrome methylene blue, and examined. Initially, isolated organisms and short chains were seen. In later specimens, when the growth was too dense for impressions, either smears were made or a small amount of the growth was rubbed up in water.

Initially, on all three media, no sign of capsule was present and the bacteria were short, broad and vacuolated, the appearance associated with stationary phase cultures of this strain. After 2–3 hr. fairly long chains of thinner organisms formed, often lying side by side; and after 5–7 hr. a progressively more dense mass of tangled organisms was seen. On bicarbonate agar, about $1/10^6$ fully capsulated organisms or chains were seen from the 4th to the 9th hour but uniform capsulation never appeared before 11 hr., that is, several hours after dense confluent growth had formed. On albumin agar or charcoal agar, most of the bacteria showed capsules after 3 hr. incubation, first seen as a red flush around most of the organisms with sometimes a red granularity. All were definitely capsulated by 4–5 hr. The degree of capsulation after 3 hr. was equivalent to that seen on bicarbonate agar after about 11 hr. Moreover, after 10 hr., the growth on albumin or charcoal agar was glistening and easily suspended in saline, whereas the growth on bicarbonate agar had a dull rough surface and formed a more granular suspension.

The results were the same whether plates with 0·025 M-$NaHCO_3$ were compared with charcoal and albumin plates without $NaHCO_3$ incubated in 30 % CO_2 or whether plates containing 0·03 M added $NaHCO_3$ incubated in 10 % CO_2 were compared with charcoal or albumin plates containing 0·015 M-$NaHCO_3$ incubated in 5 % CO_2, conditions which gave approximately equal capsulation after overnight incubation.

These results therefore support the view that the inhibitor removed from nutrient agar by charcoal or albumin becomes ineffective and ceases to prevent capsulation on bicarbonate agar once the bacteria have become sufficiently dense. Before that, the bacteria grew without capsules on bicarbonate agar. This conclusion was strengthened by the observation that on bicarbonate agar isolated colonies were always less mucoid than confluent growth, and that colonies which happened to be very near confluent growth were often markedly more mucoid than those on other parts of the plate. This was not generally seen on albumin or charcoal agar. Although these were the usual findings, the colonial and confluent growth could differ in three other ways.

(*a*) Where isolated colonies were more mucoid and contained a higher proportion of capsulated bacteria than did confluent growth. This was sometimes observed in the presence of albumin or charcoal with borderline concentrations of HCO_3^- (*c.* 0·004 M), and also on certain other media, such as acid-hydrolysed casein + yeast extract, which gave poor growth. It seems likely that here it was not the inhibitor that limited capsule formation, but either HCO_3^- or a nutrient supplied by the medium.

(*b*) Where a mucoid rim formed round less mucoid, or non-mucoid, confluent growth. This was always seen when albumin or charcoal agar plates were inoculated by flooding and the growth formed after incubation in CO_2 was interrupted by bare areas which either had been left uninoculated or were produced by antibiotics (Table 3). Here again, some factor in the medium other than the inhibitor was probably limiting capsulation. A mucoid rim was also seen with some plates inoculated by loop: when they contained charcoal or albumin, where the isolated colonies were generally mucoid; and when they contained only bicarbonate, where the isolated colonies were rough and capsulation was evidently limited not only by a nutrient in the medium but also depended on there being a sufficient concentration of bacteria.

(*c*) Where mucoid growth only occurred in a few colonies situated very close to confluent growth. This was seen very occasionally on plates without absorbent with calculated concentrations of HCO_3^- of $1{\cdot}8–3{\cdot}2 \times 10^{-3}$ M. It probably indicated a more extreme shortage of the necessary nutrient in the medium and removal of the inhibitor by confluent growth.

Table 3. *Effect of various agents on capsule formation by Bacillus anthracis strain 2160 s*

Nutrient agar plates at pH 7·4 containing Norit A charcoal were inoculated by flooding, and the test agents were placed in wells cut with a cork borer. The plates were incubated in 20 % CO_2.

Agent and concentration (w/v) in wells*	% charcoal in agar (w/v)	Width of growth inhibition zone (mm.)	Rough zone		
			Width (mm.)	Appearance	% capsulated organisms†
Agar extract‡	0·2	0	10	RS	—
	0·04	0	7	RS	*b*
Oleic acid 5 %	0·2	3	5	RS	*a*
	0·08	3	8	R	*a*
Palmitic acid 10 %	0·2	0	5	SR	*b*
	0·08	0	5	RS	*a*
Linolenic acid 12 %	0·2	10	5	R	*a*
	0·08	10	5	R	*a*
Linoleic acid 12 %	0·2	6	0	Rim§	—
	0·08	6	0	Rim	—
Stearic acid 10 %	0·2	0	10	R	*a*
	0·08	0	10	R	*a*
Sodium deoxycholate 10 %	0·2	7	3	R	*a*
	0·04	7	5	R	*a*
Proflavine 1 %	0·2	7	0	—	—
	0·04	7	0	—	—
Streptomycin 0·1 %	0·2	15	0	—	—
	0·04	15	0	—	—
Chloramphenicol 0·1 % Vancomycin 0·1 % Erythromycin 0·1 % Kanamycin 0·1 % Furadantin 0·1 %	0·2–0·04	5–20	0	Rim	—

* Each well contained 0·06–0·08 ml. The pH of the solutions was *c.* 7·2.

† Percentage of capsulated organisms in the rough zone. *a*, < 5 %; *b*, 10–50 %. Controls invariably had 100 %. — = not tested.

‡ Agar extract: 15 g. Davis agar was refluxed with equal parts of chloroform and methanol for 6 hr. The extract was evaporated to dryness and the lipoidal residue warmed in dilute NaOH. Part of the residue did not dissolve. The turbid suspension was neutralized and made up to 10 ml.

§ Rim: raised mucoid rim formed round the inhibition zone.

The nature of the inhibitor

The need for albumin or charcoal immediately suggested that the inhibitor might be a fatty acid present in the medium (see Pollock, 1949; Nieman, 1954). Against this is the fact that although these acids frequently inhibit bacterial growth, they have never been reported to inhibit specifically a function, like

capsulation, unconnected with growth. Various fatty acids and other agents were tested by placing them in wells cut in a plate of inoculated charcoal agar that was then incubated in CO_2 (Table 3). Lipoidal material isolated from agar by refluxing with chloroform + methanol inhibited capsule formation without detectably inhibiting growth, as did the fatty acids, palmitic and stearic. It does not follow, of course, that either acid is the postulated inhibitor, a conclusion which would require analysis of the medium before and during growth of the culture. Moreover, the same effect was produced by sodium deoxycholate which was not likely to be present in the media. Other fatty acids, e.g. linolenic acid, inhibited growth as well as capsule formation; this appears to be a specific effect, since all the chemotherapeutic agents tested inhibited growth alone. A mucoid rim sometimes surrounded the inhibition zone, as already mentioned in the preceding section.

Tests with typical virulent strains

Five typical strains of *Bacillus anthracis* (1444, Hillsborough, Vollum, A69, A77) were grown on the different media to show that strain 2160S was not exceptional. The results were much the same with all six strains, though there may have been some quantitative differences in their requirements. All five typical strains were heavily capsulated (M++++) on media containing 0·03 M-$NaHCO_3$ and either charcoal (0·2%, w/v) or albumin (0·7%, w/v) incubated in 10% CO_2. They also produced capsules on bicarbonate agar and on albumin or charcoal agar, but their growth, particularly that of strain A77, was not nearly so mucoid as when absorbent and bicarbonate were both present.

DISCUSSION

The results shown in Fig. 1 largely account for the high concentrations of CO_2 and of serum customarily used to induce capsule formation *in vitro*. The CO_2 concentration depends on the pH of the medium and on the amount of HCO_3^- required. Serum probably inactivates an inhibitor of capsular synthesis, and on serum or albumin agar at pH 6·8–7·4 excellent capsulation is produced by about 5% CO_2, a concentration of the order found in the mammalian body. The action of purified charcoal makes it extremely unlikely that the function of serum or albumin is to contribute an essential nutrient to the medium. The differing characteristics of growth on bicarbonate agar appear only on examining either young cultures by microscopy or fully grown overnight cultures on solid medium where the confluent growth can be compared with isolated colonies. Many investigations seem to depend solely on chemical estimations of the capsular material, poly-D-glutamic acid, made on confluent growth formed after many hours or days incubation. On bicarbonate agar, capsules only appear after the culture has become very dense, which suggests that the organisms either inactivate the inhibitor (as, for example, other species bind fatty acid or Cu^{2+} (Davis, 1948; von Hofsten, 1962)) or that they become resistant to its action as their growth rate falls. It is not surprising therefore that the bacteria on bicarbonate agar are grossly heterogeneous in respect of capsulation in the early phases of growth, a point clearly brought out by stained films where the majority of organisms are non-capsulated when about $1/10^6$ are fully capsulated, presumably because they come from areas of denser growth. On albumin or charcoal

agar, the organisms are far more uniform, since capsulation begins throughout the population after 3–4 hr. incubation (see Mikhailov, Rozhkov & Tamarin, 1960).

It is evident from Fig. 1 that a higher HCO_3^- concentration is needed on bicarbonate agar, where the inhibitor is assumed to be present initially, than on albumin or charcoal agar. It may be that the inhibitor is competitively antagonized by HCO_3^-. The initial concentration of inhibitor in bicarbonate agar may, however, be so great that its effects are not overcome by any feasible HCO_3^- concentration, but, if it is progressively inactivated by the organisms, the higher concentrations of HCO_3^- used in these experiments succeed in inducing capsulation. Another explanation might be that by the time the inhibitor has become inactive, the culture has become dense and the physiology of the organisms has altered in such a way that capsular synthesis requires more HCO_3^- than with a rapidly growing culture. These alternatives could be distinguished by using steady-state cultures.

The postulated inhibitor has not been identified, though a fatty acid is clearly a possibility (Table 3); nor is it known whether it occurs naturally in the culture media or is produced by the organisms themselves (see Pollock, 1949; Nieman, 1954). The latter seems less likely, for isolated organisms growing on bicarbonate agar would then be producing sufficient inhibitor to prevent their own capsulation.

The action of the inhibitor is evidently linked to the part played by HCO_3^- in capsule formation. In general, CO_2 assimilated by heterotrophs appears to enter the tricarboxylic acid cycle (see Wood & Stjernholm, 1962) and, indeed, *Bacillus anthracis* growing on bicarbonate agar in the presence of $^{14}CO_2$ has been shown to form ^{14}C-labelled aspartate, succinate, etc. (Eastin & Thorne, 1963). Assimilation of CO_2 is known to depend on the action of biotin, which is replaceable by oleic acid. If the inhibitor is indeed a fatty acid, it may therefore act by interfering specifically with this assimilatory pathway; and, judging from studies of the effects of these acids on bacterial growth (e.g. Davis & Dubos, 1947), it would not be surprising if a given acid was stimulatory or inhibitory according to its concentration. The CO_2 requirement of some species is largely removed by adding tricarboxylic acid cycle intermediates to the medium (Lwoff & Monod, 1947; Ajl & Werkman, 1949) but, nevertheless, capsulation did not occur in cultures of strain 2160s incubated in air on either nutrient or charcoal agar containing 0·1 M-aspartate, succinate, glutamate or oxaloacetate at pH 7·4. A related observation was that glucose completely suppressed capsulation. At first, this was presumed to be due to the medium becoming acid and so lowering its content of HCO_3^- (Fig. 1). However, neutral red (pK = 6·85) did not indicate acidity, and, in any case, excellent capsules were formed even at pH 6·2 on charcoal or albumin agar incubated in 20–40% CO_2 (Fig. 1*b*). Glucose may therefore specifically repress capsular synthesis in strain 2160s; this point is still under examination.

It is a pleasure to acknowledge the help of Dr G. M. A. Gray.

REFERENCES

AJL, S. J. & WERKMAN, C. H. (1949). Anaerobic replacement of carbon dioxide. *Proc. Soc. exp. Biol. Med., N.Y.* **70**, 522.

DAVIS, B. D. (1948). Absorption of bacteriostatic quantities of fatty acid from media by large inocula of tubercle bacilli. *Pub. Hlth Rep., Wash.* **63**, 455.

DAVIS, B. D. & DUBOS, R. J. (1947), The binding of fatty acids by serum albumen, a protective growth factor in bacteriological media. *J. exp. Med.* **86**, 215.

EASTIN, J. D. & THORNE, C. B. (1963). Carbon dioxide fixation in *Bacillus anthracis*. *J. Bact.* **85**, 410.

HOFSTEN, B. VON (1962). The effect of copper on the growth of *Escherichia coli*. *Exp. Cell Res.* **26**, 606.

HOLT, L. B. (1962). The culture of *Bordetella pertussis*. *J. gen. Microbiol.* **27**, 323.

HOUSEWRIGHT, R. D. (1962). The biosynthesis of homopolymeric peptides. In *The Bacteria*. Ed. by I. C. Gunsalus and R. Y. Stanier, vol. 3, p. 389. New York and London: Academic Press.

IVÁNOVICS, G. (1937). Unter welchen Bedingungen werden bei der Nährboden-Züchtung der Milzbrandbazillen Kapseln gebildet? *Zbl. Bakt.* (1. *Abt. Orig.*), **138**, 449.

KNIGHT, B. C. J. G. & PROOM, H. (1950). A comparative survey of the nutrition and physiology of mesophilic species in the genus *Bacillus*. *J. gen. Microbiol.* **4**, 508.

LWOFF, A. (1947). Sur le rôle du serum dans le développement de *Moraxella lacunata* et de *Neisseria gonnorrhae*. *Ann. Inst. Pasteur*, **73**, 735.

LWOFF, A. & MONOD, J. (1947). Essai d'analyse du rôle de l'anhydride carbonique dans la croissance microbienne. *Ann. Inst. Pasteur*, **73**, 323.

MCCLOY, E. W. (1958). Lysogenicity and immunity to Bacillus phage W. *J. gen. Microbiol.* **18**, 198.

M'FADYEAN, J. (1903). A peculiar staining reaction of the blood of animals dead of anthrax. *J. comp. Path.* **16**, 35.

MEYNELL, E. W. (1963). Reverting and non-reverting rough variants of *Bacillus anthracis*. *J. gen. Microbiol.* **32**, 55.

MEYNELL, G. G. (1961). Phenotypic variation and bacterial infection. *Symp. Soc. gen. Microbiol.* **11**, 174.

MIKHAILOV, B. Y., ROZHKOV, G. I. & TAMARIN, A. L. (1960). A rapid method for the diagnosis and detection of *Bacillus anthracis*. *J. Microbiol., Moscow*, **31**, 1997.

NIEMAN, C. (1954). Influence of trace amounts of fatty acids on the growth of micro-organisms. *Bact. Rev.* **18**, 147.

NUNGESTER, W. J. (1929). Dissociation of *B. anthracis*. *J. infect. Dis.* **44**, 73.

NYE, R. N. & LAMB, M. E. (1936). Increased carbon dioxide tension as an aid in the primary isolation of certain (mephitibic) pathogenic bacteria. *J. Amer. med. Ass.* **106**, 107.

POLLOCK, M. R. (1949). The effects of long-chain fatty acids on the growth of *Haemophilus pertussis* and other organisms. *Symp. Soc. exp. Biol.* **3**, 193.

RENAUX, E. (1952). Culture de *Bacillus anthracis* en milieu calcique et en milieu oxalaté. *Ann. Inst. Pasteur*, **83**, 38.

SMITH, N. R., GORDON, R. E. & CLARK, E. E. (1952). Aerobic sporeforming bacteria. *U.S. Dep. Agriculture Monograph*, No. 16.

STERNE, M. (1937). Variation in *Bacillus anthracis*. *Onderstepoort J. vet. Sci.* **8**, 271.

THORNE, C. B. (1956). Capsule formation and glutamyl polypeptide synthesis by *Bacillus anthracis* and *Bacillus subtilis*. *Symp. Soc. gen. Microbiol.* **6**, 68.

THORNE, C. B., GOMEZ, C. G. & HOUSEWRIGHT, R. D. (1952). Synthesis of glutamic acid and glutamyl polypeptide by *Bacillus anthracis*. II. The effect of carbon dioxide on peptide production on solid medium. *J. Bact.* **63**, 363.

UMBREIT, W. W., BURRIS, R. H. & STAUFFER, J. F. (1957). *Manometric Techniques*. 3rd ed. Minneapolis: Burgess Publishing Co.

WOOD, H. G. & STJERNHOLM, R. L. (1962). Assimilation of carbon dioxide by heterotrophic organisms. In *The Bacteria*. Ed. by I. C. Gunsalus and R. Y. Stanier, vol. 3, p. 41. New York and London: Academic Press.

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The Cellular Location of Antigens in Streptococci of Groups D, N and Q

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SUMMARY

The absence of Group D antigen from the cell walls and protoplasts of Group D streptococci leads to the conclusion that this antigen is located between the protoplast membrane and the cell wall. The Group N antigen was likewise not present in cell walls of Group N streptococci and probably has a similar location to the Group D antigen. The Group Q strains examined possessed the Group D antigen in the cell contents although these strains did not physiologically correspond to any established Group D species. The antigen upon which Group Q is based is a cell-wall antigen.

INTRODUCTION

Following the work of Elliott (1959) and Jones & Shattock (1960), who reported the unexpected absence of Group D antigen from the cell walls of Group D streptococci, the location of this antigen has been further investigated by examining bacterial bodies lacking cell walls. The presence of the antigen in these bodies would indicate a cytoplasmic location. The Group D antigen is now known to be a glycerol teichoic acid (Wicken, Elliott & Baddiley, 1963) and the cellular location is thus of special interest in relation to the function of these compounds.

Since bacterial protoplasts and L-forms lack cell walls they are suitable material for examining this problem. Hijmans (1962) examined L-forms of Group D streptococci and found no group or type (cell-wall) antigens or precursors in the L-forms or in the culture media. The L-forms used by Dr W. Hijmans (Leiden) were kindly supplied by him and used for some of the work reported here.

Lysozyme is commonly used for the production of bacterial protoplasts. However, *Streptococcus faecalis* (Group D) was originally reported to be resistant to the action of this cell wall-degrading enzyme (Salton, 1953). In our experience many Group D streptococci are sensitive to lysozyme; this has also been reported by Abrams (1959), Salton & Pavlik (1960), Bleiweis & Zimmerman (1961), Chesbro (1961), and Bibb & Straughn (1962). Because of some difficulties in preparing true lysozyme a phage-associated cell-protoplasts by using wall lysin (Bleiweis & Zimmerman, 1961) was also used in the present work.

Because of the physiological similarity of Group N and Group D streptococci it was decided to investigate the location of the Group N antigen by the technique used with the Group D antigen. Slade (1961) reported that with one Group N strain the group antigen was detected in the cell wall and in the residue after removal of

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cell walls from suspensions of disintegrated cocci. In the present work 17 strains of Group N streptococci, including *Streptococcus lactis* and *S. cremoris*, were examined. Group Q (established by Guthof, 1955) constitutes a group of streptococci which have many physiological resemblances to some species of Group D streptococci, and it was therefore of interest to investigate the cellular location of antigens in this group. Slade (1961) reported findings with two Group Q strains similar to his findings with the Group N strain.

METHODS

Organisms. Streptococci of Groups D, N and Q were obtained from the collection maintained in this laboratory. *Streptococcus lactis* strains ML 3 and C 10 and their phages were obtained from Mr B. Reiter (National Institute for Research in Dairying). The Group D phage was isolated from Reading sewage by the method of Adams (1950).

Physiological tests

The nutrient medium for growing organisms for physiological and serological tests was glucose Lemco broth (%, w/v: Evans peptone, 1; Lab. Lemco, 1; NaCl, 0·5; glucose, 0·5). The temperature of incubation was 37° (30° for Group N streptococci). For physiological tests the inoculum was one loopful (4·0 mm. diam.) of 18-hr broth culture. L-forms of Group D streptococci were grown in the liquid medium described by Hijmans (1962).

Sugar reactions. Tubes of peptone water (1 %; 5 ml.) containing 0·5–1·0 % (w/v) test sugar, with bromocresol purple as indicator, were inoculated and examined daily for acid production.

Tolerance of potassium tellurite. Streak plates of glucose nutrient agar containing potassium tellurite (0·04 %, w/v; Skadhauge, 1950) were examined after incubation for 24 hr. Tolerant streptococci (*Streptococcus faecalis*) grew profusely as black colonies; no growth or a dusty grey growth of small colonies was recorded as negative.

Reduction of tetrazolium. The liquid medium of Barnes (1956) was used and examined for a red precipitate after incubation for 18 hr.

Methylene blue. Milk containing methylene blue (0·1 %, w/v) was inoculated with 0·2 ml. of 18-hr glucose Lemco broth culture and examined during 10 days for growth and dye reduction.

Growth at 50°. One loopful of an 18-hr broth culture was inoculated into 5 ml. glucose Lemco broth which was incubated in a water bath at 50°. The culture was examined for growth daily for 3 days.

Serological methods

Preparation of group-specific antisera in rabbits. The technique of Jones & Shattock (1960) was used for preparing Groups D and Q antisera. Group N antisera were prepared by using acetone-extracted ball-milled cocci as antigens (Shattock & Mattick, 1943).

Antigen extraction. The HCl-extraction procedure of Lancefield (1933) was most frequently used.

Precipitin-ring and absorption tests were done as described by Shattock (1949).

Antigen assay. Serial dilutions of antigen extracts in physiological saline were placed in the peripheral wells of slide gel patterns (Mansi, 1958), with antiserum in the central well. The greatest dilution of antigen which showed reaction with antiserum after diffusion for 3 hr was taken as endpoint; the antigen titre was the reciprocal of the endpoint dilution. In some tests the visibility of the precipitation bands was enhanced by immersing the slides in 0·0125 % (w/v) cadmium chloride solution (Crowle, 1958).

Analytical methods

Cell-wall isolation. Cell walls and 'cell contents' were isolated by the method of Cummins & Harris (1956), except that pepsin digestion was omitted.

Rhamnose determination. Rhamnose was assayed by the sulphuric acid + cysteine colour reaction of Dische & Shettles (1948).

Preparation of osmotically fragile bodies

Preparation of phage-associated enzymes. The method of Bleiweis & Zimmerman (1961) was followed for the isolation of phage-associated enzymes from *Streptococcus faecalis* var. *zymogenes* strain H 69 D 5 and *S. lactis* strains ML 3 and C 10.

Production of osmotic fragility. The organisms for spheroplast or protoplast production were grown in 5 ml. glucose Lemco broth for 18 hr; 1 ml. of this culture was then inoculated into 10 ml. glucose Lemco broth, this incubated for 5 hr and the whole added to 100 ml. warm glucose Lemco broth and incubated for a further 2–4 hr. The harvested organisms were washed twice in 0·85 % NaCl solution and suspended in the appropriate buffered solutions. The organisms from a 110 ml. glucose Lemco broth culture in the logarithmic growth phase were suspended in 80 ml. of 1·1 or 2·0 M-sucrose buffered at pH 6·2 with 0·15 M-K_2HPO_4. Lysozyme (Armour Laboratories) was added to a concentration of 720 μg./ml. In each experiment there were control suspensions without sucrose. Turbidity changes were followed by nephelometer readings. For experiments with phage-associated enzyme the same procedure was followed except that enzyme solution (4 %, v/v) was used in place of lysozyme.

RESULTS

Spheroplasts of Group D streptococci

Abrams (1959) reported the preparation of protoplasts from *Streptococcus faecalis* ATCC 9790 by using egg-white lysozyme to remove cell walls and 0·4 M-sucrose to stabilize the osmotically fragile bodies. Two cultures of this organism were obtained (one from Dr A. Abrams, University of Colorado; one from the National Collection of Industrial Bacteria, Aberdeen) and examined physiologically and serologically. The two cultures received, which were both labelled ATCC 9790, were not *S. faecalis* as defined, for example, by Shattock (1962); they resembled *S. faecium* or *S. durans* except that they did not grow in milk + 0·1 % methylene blue. HCl-extracts of these organisms reacted very weakly with Group D antiserum produced with *S. faecalis* strain S 161. This finding correlates with the different structure of the glycerol teichoic acid in this strain as compared to *S. faecalis* strain 39 (Wicken & Baddiley, 1963). It was concluded that the organism received as ATCC 9790 was an unsuitable strain for investigating the location of the Group D antigen.

When other Group D streptococci (in the logarithmic growth phase) were treated with lysozyme, osmotically fragile bodies were produced, but these did not have the appearance of true protoplasts when examined by phase-contrast microscopy. The cocci remained associated in pairs and short chains, indicating the presence of residual cell-wall material. These bodies were concluded to be spheroplasts, that is, osmotically fragile cocci with partially degraded cell walls. Rhamnose assays were impracticable because of the high sucrose concentration.

Table 1. *Group antigen loss from Group D streptococci suspended in saline and in concentrated sucrose, and during formation of spheroplasts by lysozyme*

	Whole organisms in 0·15 M-NaCl	Whole organisms in 1·1 M-sucrose, pH 6·2	Whole organisms producing spheroplasts in 1·1 M-sucrose pH 6·2 + lysozyme
	% diminution of antigen titre* in 2 hr at 37°		
S. faecalis var. *liquefaciens* Elv. 2025 Type 1	nt	20	55
S. faecalis var. *zymogenes* N 37 Type 1	0	15	50
S. faecalis var. *zymogenes* D 76 Type 1	15	35	60
S. faecalis 775 Type 6	25	65	75

nt, not tested; *estimated by gel-diffusion assay against a Group D antiserum prepared from *S. faecalis* S 161.

The Group D antigen in spheroplasts and in supernatant fluids was assayed during lysozyme digestion of *Streptococcus faecalis* var. *liquefaciens* strain Elv. 2025 suspended in 1·1 M-sucrose. Samples were removed from the digestion mixture at 30-min. intervals, centrifuged and the particulate fraction and the supernatant fluid fraction extracted in 0·05 M-HCl at 100° for 10 min. The two extracts were used for antigen assay. The results (Fig. 1) showed that about half the total extractable Group D antigen was released into solution when spheroplasts were produced from whole cocci.

Table 1 shows the results of similar experiments with three other Group D strains. *Streptococcus faecalis* strain 775 lost antigen into solution more readily than did the other strains even in the absence of lysozyme; this was characteristic only of saline-washed cocci.

Streptococcus faecium was more sensitive than *S. faecalis* to lysozyme but was not so suitable for our experiments because of its small content of Group D antigen. Antigen was released when spheroplasts were prepared, but quantitative experiments were not done. A cytoplasmic membrane fraction of *S. faecium* strain HGH 511 prepared by osmotic lysis of spheroplasts did not contain Group D antigen.

Protoplasts of Group D streptococci

A phage-associated enzyme was isolated by the technique of Bleiweis & Zimmerman (1961) from a *Streptococcus faecalis* var. *zymogenes* strain H 69 D 5 phage + host system. *S. faecalis* and varieties were very sensitive to this phage-associated enzyme, lysis occurring within a few minutes; but *S. faecium*, *S. durans*, *S. bovis* and *S. equinus* were not affected. The addition of cysteine hydrochloride was not

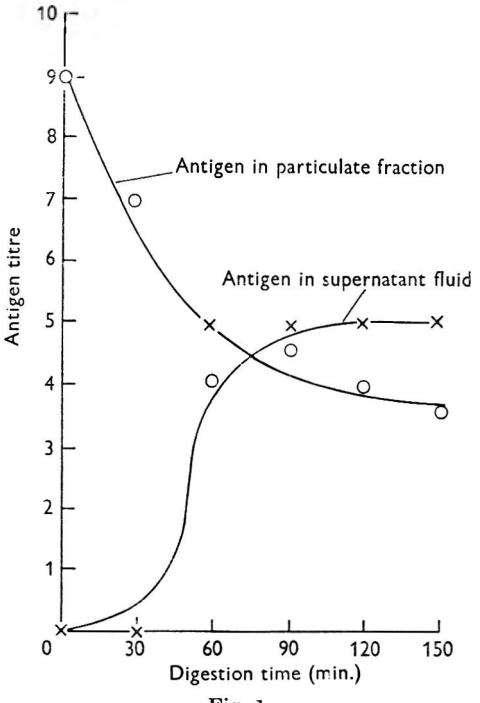

Fig. 1

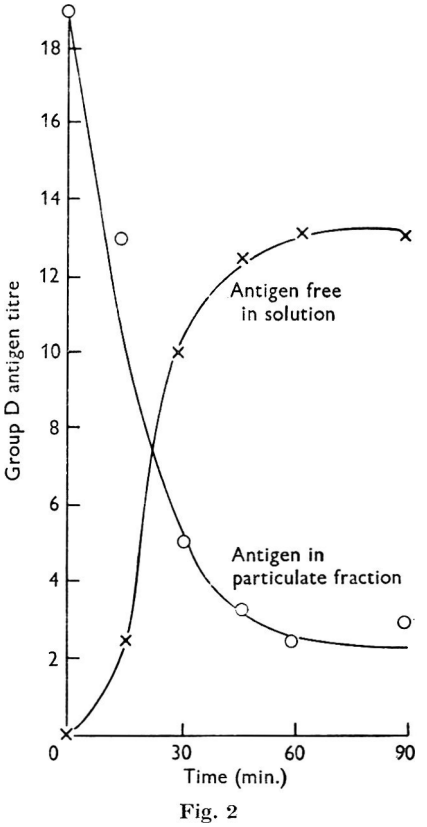

Fig. 2

Fig. 1. Effect of lysozyme (720 μg./ml.) on the distribution of Group D antigen in *Streptococcus faecalis* var. *liquefaciens* Elv. 2025 suspended in 1·1 M-sucrose.

Fig. 2. Effect of phage-associated lysin (4 %, v/v) on the distribution of Group D antigen in *Streptococcus faecalis* var. *liquefaciens* Elv. 2025 suspended in 1·1 M-sucrose.

necessary for the activity of the enzyme preparation. The lysates of bacteria produced by the phage enzyme contained no rhamnose (a cell-wall carbohydrate) sedimentable at 38,000 *g*; it was therefore possible that true protoplasts might be prepared by this method.

When suspended in 1·1 M-sucrose (pH 6·2) *Streptococcus faecalis* and varieties were not completely lysed by the phage enzyme. Phase-contrast microscopy revealed isolated spherical bodies which were concluded to be protoplasts; resuspension of these bodies in distilled water resulted in immediate lysis.

The distribution of Group D antigen between the particulate fraction (protoplasts)

and the supernatant fluid was followed during the treatment of *Streptococcus faecalis* var. *liquefaciens* strain Elv. 2025 with phage enzyme. Samples (5 ml.) were removed at intervals from a reaction mixture of substrate organisms suspended in 1·1 M-sucrose with 4 % (v/v) phage-enzyme solution. The samples were centrifuged at 38,000 *g* for 20 min. and the deposit extracted in 1 ml. 0·05 M-HCl at 100° for 10 min. The supernatant fluids were acidified with M-HCl to a final concentration of 0·05 M-HCl and heated at 100° for 10 min. The neutralized extracts were assayed by gel diffusion against a Group D antiserum. The results (Fig. 2) showed that, when cell wall was removed, about 85 % of the group antigen went into solution where it could be detected. Similar experiments with two other Group D organisms of the same serological type (type 1) gave comparable results; during protoplast formation from *S. faecalis* var. *zymogenes* strains N 37 and D 76, 90 and 80 %, respectively, of the Group D antigen went into solution. With *S. faecalis* 775 (type 6) 90 % of the group antigen similarly went into solution when protoplasts were prepared by using phage enzyme.

L-forms of Group D streptococci

Hijmans (1962) found that L-forms of Group D streptococci produced no Group D antigen when grown in liquid medium containing penicillin. This observation was confirmed in the present work. It was also found that two penicillin-resistant strains of normal streptococci (*Streptococcus faecium*) did not produce the Group D antigen when grown in the L-form medium, but removal of the penicillin stimulated production of the antigen. It was therefore possible that it was the penicillin in the medium which was responsible for the absence of Group D antigen from the L-forms. The L-forms (nos. 27, 30) were stable in the absence of penicillin; no Gram-positive material was detected in the L-forms and there was no synthesis of rhamnose, a carbohydrate characteristic of streptococcal cell walls. When grown in a medium without penicillin and with 0·5 % glucose (a concentration favourable to Group D antigen production; Medrek & Barnes, 1962) instead of the usual 0·1 % glucose, one of the L-forms (no. 27) produced Group D antigen. After about six subcultures in penicillin-free medium L-form no. 27 lost ability to synthesize Group D antigen although it still produced an antigen which reacted with Group D antiserum but did not give a pattern of identity with Group D antigen in gel-diffusion tests. When the Group D antigen was produced quantitative tests showed that 96 to 98 % of the D antigen was free in the culture medium.

Cell-wall isolation from Group N streptococci

Cell walls were isolated from 17 strains of *Streptococcus lactis* and *S. cremoris*. HCl-extracts of the cell-wall fractions in no case reacted with Group N antiserum in gel-diffusion tests. The clear supernatant fluids obtained on centrifuging disintegrated coccal suspensions were rich in Group N antigen (Table 2).

Spheroplasts of Group N streptococci

All 20 strains of *Streptococcus lactis* and *S. cremoris* tested were lysed by egg-white lysozyme and by phage-associated enzyme isolated from *S. lactis* strains ML 3 and C 10 host+phage systems; complete release of rhamnose into solution was not achieved with either enzyme preparation. The stabilized osmotically fragile bodies

were therefore considered to be spheroplasts. Phase-contrast microscopy showed that the osmotically fragile bodies were usually still in pairs or short chains. Spheroplasts of *S. lactis* strain Rice prepared with lysozyme contained the same amount of Group N antigen as did corresponding whole cocci. Table 3 shows the

Table 2. *Serological reactions of HCl extracts of whole cells, cell walls and 'cell contents' of Group N streptococci*

Group N antiserum: prepared by using *S. lactis* OJ as immunizing strain.

	Whole organisms		Cell walls		Cell contents	
	Ring-test	Gel-diffusion test	Ring-test	Gel-diffusion test	Ring-test	Gel-diffusion test
S. lactis strains						
OJ	+	+	−	−	+	+
M 1	+	+	−	−	+	+
H 201	+	+	±	−	+	+
478 dem	+	+	nt	−	+	+
Rice	+	+	∓	−	+	+
H 240	+	+	−	−	+	+
H 211	+	+	−	−	+	+
M 8	+	+	−	−	+	+
85 Edin	+	+	−	−	+	+
92/4	+	+	−	−	+	+
UD Edin	+	+	−	−	+	+
S. cremoris strains						
198/1	+	+	−	−	+	±
95/2	+	+	−	−	+	+
98/54	+	+	−	−	+	+
236/M 1	+	+	∓	−	+	+
133/5	+	+	−	−	+	±
RW	+	+	−	−	+	+

nt, not tested; + (ring test), heavy ring within 5 min.; ± (ring test), slow but definite ring; ∓ (ring test), trace of reaction in 20 min.; + (gel diffusion), strong reaction, pattern of identity with other Group N HCl-extracts; ± (gel diffusion), weaker but definite reaction.

Table 3. *Group N antigen content of normal organisms and spheroplasts of Streptococcus lactis and S. cremoris*

	Whole organisms in sucrose*	Spheroplasts (c10 enzyme)	Spheroplasts (ML3 enzyme)
	Antigen titres		
S. lactis Rice	12	12	8
S. lactis c10	10	8	4
S. cremoris KH	3	1	3
S. cremoris E8	2	1	1

* 1·1 M-sucrose for *S. lactis*; 2 M for *S. cremoris*.

antigen titres of four Group N strains before and after spheroplast production; between 0 and 60 % of the group N antigen went into solution when the spheroplasts were formed.

Physiology of Group Q strains

The five Group Q strains examined did not correspond physiologically with any established Group D species although they resembled them in several ways (growth at 10°, 45°; in 6·5% NaCl; at pH 9·6; fermentation of several carbohydrates). As is seen in Table 4 the Group Q strains had characteristics intermediate between those of *Streptococcus faecalis* and *S. faecium*. Group Q streptococci typically ferment sorbose, but this ability is shared by some Group D and Group N strains: *S. faecium* strain OJ, *S. bovis* strains Lottie 15 and H 6, *S. lactis* strain 84 Edin.

Table 4. *Comparison of physiological characters of Streptococcus faecalis 775, S. faecium HGH 511 and Group Q strains*

	S. faecalis	Group Q	*S. faecium*
Reduction of tetrazolium	+	−	−
Tellurite (0·04%) tolerant	+	−	−
Hydrolysis of Na hippurate	+	−	−
Acid from melezitose	+	+	−
sorbitol	+	+	−
rhamnose	+	+	−
glycerol (anaerobic)	+	+	−
sorbose	−	+	−
arabinose	−	+	+
Methylene blue (0·1%) tolerant	+	−	+
Growth at 50°	−	−	+

Reaction of Group Q strains with Group D antisera

HCl-extracts of the five Group Q strains examined all gave positive precipitin-ring reactions with Group D antisera. The extracts gave patterns of identity with HCl-extracts of Group D strains in gel-diffusion tests against Group D antisera. Strain E 6556 gave an exceptionally strong reaction with Group D antisera; the speed and intensity of the reaction were comparable to the results with *Streptococcus faecalis*. The other strains reacted less strongly and it was usually necessary to incubate the cultures for 48 hr before extraction. The reaction was enhanced by concentrating the extracts by the method of Shattock (1949). The Group Q strains were able to absorb Group D antibodies although the minimal absorbing doses were one to six times greater than with the homologous organism.

Antisera prepared by the inoculation of whole-cell vaccines of Group Q streptococci gave precipitin reactions only with Group Q strains. However, when disintegrated-cell vaccines were used (as for Group D) the resulting antisera reacted with extracts of Group Q and Group D strains. Gel-diffusion analysis showed that the reaction was due to two independent antibodies. Both antibodies were completely absorbed by Group Q organisms, while only the antibody which reacted with Group D extracts was absorbed by Group D organisms. The Group Q antigen was apparently equivalent to the type antigens of Group D. Extracts of the Group Q strains did not react with any of the Group D serological type antisera of Sharpe & Shattock (1952). Strain E 6556, identical in physiology with the other Group Q strains, did not react with Group Q antisera. Two cultures of this strain from different sources gave similar results.

Location of antigens in Group Q streptococci

Suspensions of Group Q organisms were disintegrated and cell walls and cell contents separated by centrifugation at 800 *g* for 60 min. HCl-extracts of cell contents and of cell walls were examined for the presence of Group D and Q antigens; the results are given in Table 5. The Group Q antiserum used in this experiment was prepared by using as vaccine formolized whole organisms of strain E 6749; this antiserum gave no reaction with HCl-extracts of Group D organisms.

Table 5. *Precipitin-ring tests of HCl-extracts of cell walls and HCl-treated 'cell contents' of Group Q streptococci*

	HCl-treated cell contents 100°/10 min. Antiserum		HCl-extract of cell walls Antiserum	
	Gp D	Gp Q	Gp D	Gp Q
E 6749	+ + +	±	−	+ + +
Q2	+ + +	±	−	+ + +
E 6844	+ + +	+	−	+ + +
E 1551	+ + +	±	−	+ + +
E 6556	+ + +	−	−	−

+ + +, Heavy ring within 5 min.; +, moderate ring within 20 min.; ±, slight ring within 20 min.; −, no reaction.

DISCUSSION

Shattock's (1949) demonstration that Group D streptococcal antiserum production was improved when vaccines of disintegrated organisms instead of whole organisms were used was evidence for the internal location of the Group D antigen. McCarty (1952*a*, *b*) showed that the Group A antigen was an integral part of the cell wall and other group antigens were expected to be analogous. However, Elliott (1959) and Jones & Shattock (1960) reported that Group D antigen was not present in the cell walls. The latter authors examined 21 strains of streptococci representing all the species of Group D then recognized (*Streptococcus faecalis* and varieties, *S. faecium*, *S. durans* and *S. bovis*): all these strains lacked Group D antigen in their cell walls.

Elliott (1959) and Jones & Shattock (1960) were of the opinion that the Group D antigen had a cytoplasmic location. Elliott (1960) considered the antigen to be superficially located because of its easy extraction with cold alkali. The present observation that most of the Group D antigen went into solution when protoplasts were prepared, or when L-forms synthesized the antigen, appears to exclude the possibility of a cytoplasmic location. Slade & Shockman (1963) found that isolated protoplast membranes of '*Streptococcus faecalis* ATCC 9790' contained Group D antigen but they did not state whether the antigen associated with the protoplast membrane constituted all the antigen in the cocci.

The observation that Group D antigen was released as the cell wall was removed is considered to be evidence that the D antigen is situated at the surface of the protoplast, beneath the cell wall; this view was expressed by Shattock & Smith (1963). Similar conclusions were drawn by Wicken *et al.* (1963), again with '*Streptococcus faecalis* ATCC 9790'.

From the present results with *Streptococcus lactis* and *S. cremoris* it seems that the Group N antigen has a similar location to the Group D antigen; but the evidence is not conclusive since true protoplasts were not prepared nor were L-forms available. It remains to be seen whether the Group N antigen, like the Group D antigen, is a glycerol teichoic acid.

The five Group Q strains examined formed an homogeneous physiological group which did not correspond to any known species. However the group resembled the 'enterococcus group' of Sherman (1937), with characteristics intermediate between *Streptococcus faecalis* and *S. faecium*. Group Q strains showed serological cross-reactions with Group D strains. The D antigen present in the Group Q strains was absent from the cell walls and possibly is located, as in Group D streptococci, between the cell membrane and the cell wall. The Group Q antigen was detected principally in the cell-wall fraction and therefore corresponds in location with the type antigens of Group D The serological cross-reaction of Groups D and Q has been observed also by Dr R. Wahl (personal communication).

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REFERENCES

ABRAMS, A. (1959). Reversible metabolic swelling of bacterial protoplasts. *J. biol. Chem.* **234**, 383.

ADAMS, M. H. (1950). Methods of study of bacterial viruses. *Meth. med. Res.* **2**, 1.

BARNES, E. M. (1956). Tetrazolium reduction as a means of differentiating *Streptococcus faecalis* from *Streptococcus faecium*. *J. gen. Microbiol.* **14**, 57.

BIBB, W. R. & STRAUGHN, W. R. (1962). Formation of protoplasts from *Streptococcus faecalis* by lysozyme. *J. Bact.* **84**, 1094.

BLEIWEIS, A. S. & ZIMMERMAN, L. N. (1961). Formation of 2 types of osmotically fragile bodies from *Streptococcus faecalis* var. *liquefaciens*. *Canad. J. Microbiol.* **7**, 363.

CHESBRO, W. R. (1961). Lysozyme and the production of osmotic fragility in enterococci. *Canad. J. Microbiol.* **7**, 952.

CROWLE, A. J. (1958). Enhancement by cadmium of double-diffusion precipitin reactions. *J. Immunol.* **81**, 194.

CUMMINS, C. S. & HARRIS, H. (1956). The chemical composition of the cell wall in some Gram-positive bacteria and its possible value as a taxonomic character. *J. gen. Microbiol.* **14**, 583.

DISCHE, Z. & SHETTLES, L. B. (1948). A specific colour reaction of methylpentoses and a spectrophotometric micromethod for their determination. *J. biol. Chem.* **175**, 595.

ELLIOTT, S. D. (1959). Group and type-specific polysaccharides of Group D streptococci. *Nature, Lond.* **184**, 1342.

ELLIOTT, S. D. (1960). Type and group polysaccharides of Group D streptococci. *J. exp. Med.* **111**, 621.

GUTHOF, O. (1955). Über eine neue serologische Gruppe alphahämolytischer Streptokokken (serologische Gruppe Q). *Zbl. Bakt.* (1. *Abt. Orig.*), **164**, 60.

HIJMANS, W. (1962). Absence of the group-specific and the cell wall polysaccharide antigen in L-phase variants of Group D streptococci. *J. gen. Microbiol.* **28**, 177.

JONES, D. & SHATTOCK, P. M. F. (1960). The location of the group antigen of Group D streptococcus. *J. gen. Microbiol.* **23**, 335.

LANCEFIELD, R. C. (1933). A serological differentiation of human and other groups of hemolytic streptococci. *J. exp. Med.* **57**, 571.

MANSI, W. (1958). Slide gel diffusion precipitin test. *Nature, Lond.* **181**, 1289.

McCarty, M. (1952*a*). The lysis of Group A hemolytic streptococci by extracellular enzymes of *Streptomyces albus*. I. Production and fractionation of the lytic enzymes. *J. exp. Med.* **96**, 555.

McCarty, M. (1952*b*). The lysis of Group A hemolytic streptococci by extracellular enzymes of *Streptomyces albus*. II. Nature of the cellular substrate attacked by lytic enzymes. *J. exp. Med.* **96**, 569.

Medrek, T. F. & Barnes, E. M. (1962). The influence of the growth medium on the demonstration of a Group D antigen in faecal streptococci. *J. gen. Microbiol.* **28**, 701.

Salton, M. R. J. (1953). Cell structure and the enzymic lysis of bacteria. *J. gen. Microbiol.* **9**, 512.

Salton, M. R. J. & Pavlik, J. G. (1960). Studies of the bacterial cell wall. VI. Wall composition and sensitivity to lysozyme. *Biochim. biophys. Acta*, **39**, 398.

Sharpe, M. E. & Shattock, P. M. F. (1952). The serological typing of Group D streptococci associated with outbreaks of neonatal diarrhoea. *J. gen. Microbiol.* **6**, 150.

Shattock, P. M. F. (1949). The streptococci of Group D; the serological grouping of *Streptococcus bovis* and observations on serologically refractory Group D strains. *J. gen. Microbiol.* **3**, 80.

Shattock, P. M. F. (1962). *Enterococci* in *Chemical and Biological Hazards in Foods*, p. 303. Ames, Iowa, U.S.A.: Iowa State University Press.

Shattock, P. M. F. & Mattick, A. T. R. (1943). The serological grouping of *Streptococcus lactis* (Group N) and its relationship to *Streptococcus faecalis*. *J. Hyg., Camb.* **43**, 173.

Shattock, P. M. F. & Smith, D. G. (1963). The location of the Group D antigen in a strain of *Streptococcus faecalis* var. *liquefaciens*. *J. gen. Microbiol.* **31**, iv. P.

Sherman, J. M. (1937). The streptococci. *Bact. Rev.* **1**, 1.

Skadhauge, K. (1950). *Studies on Enterococci with Special Reference to the Serological properties*. Copenhagen: Einer Munksgaards.

Slade, H. D. (1961). Structure and biological activity of the streptococci. *Lectures on theoretical and applied aspects of modern microbiology*. University of Maryland.

Slade, H. D. & Shockman, G. D. (1963). The protoplast membrane and the D antigen of *Streptococcus faecalis*. *Bact. Proc.* p. 46.

Wicken, A. J. & Baddiley, J. (1963). Structure of intracellular teichoic acids from Group D streptococci. *Biochem. J.* **87**, 54.

Wicken, A. J., Elliott, S. D. & Baddiley, J. (1963). The identity of streptococcal Group D antigen with teichoic acid. *J. gen. Microbiol.* **31**, 231.