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# The Carotenoid Pigments of Mycoplasma

By P. F. SMITH

*Department of Microbiology, School of Medicine, University of South Dakota, Vermillion, U.S.A.*

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SUMMARY

All the Mycoplasma organisms examined, which did not require a sterol as a nutrient, contained a carotenoid pigment identifiable as the hydrocarbon, neurosporene, and a carotenol. The carotenol occurred as the free alcohol, esterified with fatty acids, and in glycosidic linkage with glucose. The carotenyl glucoside in two organisms was hydrolysable with $\beta$-glucosidase, indicating the glucosidic linkage to be in the $\beta$ configuration. The carotenoid compounds found in these organisms are analogous to the sterol compounds found in sterol-requiring organisms. Cholesterol supplied in the culture medium spared the synthesis of the carotenoids. Carotenols are postulated to serve in reactions which involve substrate transport and end-product transport across the cell membrane.

## INTRODUCTION

Organisms of the Mycoplasma group which require sterol as nutrient are incapable of synthesis of their non-saponifiable lipids (Smith & Rothblat, 1962) and incorporate sterols of appropriate molecular configuration without structural alteration (Rothblat & Smith, 1961; Smith, 1962). Organisms of this group which do not require sterol as nutrient synthesize their non-saponifiable lipids from acetate or mevalonate (Smith & Rothblat, 1962). However, when supplied with cholesterol, these latter organisms incorporate it in the same fashion as do the sterol-requiring organisms. The non-saponifiable lipids of both types of organisms are found primarily in the insoluble fraction (cell membrane) following sonic treatment. The sterol of those organisms incapable of carbohydrate utilization occurs as free alcohol and as esters of fatty acids, and of those organisms which are capable of carbohydrate utilization also in $\beta$-glycosidic linkage with glucose (Rothblat & Smith, 1961). Since the requirement for sterol as nutrient has been advanced as a criterion for classification of the pleuropneumonia group of organisms (Edward & Freundt, 1956), although some organisms encompassed in this schema have no sterol requirement as nutrient, some analogy with the non-saponifiable lipid fraction of these two types of Mycoplasma was sought. Identification of all the components of the non-saponifiable lipid fraction of several non-sterol requiring strains was considered a necessary step in proving or disproving the theory that non-saponifiable lipids are concerned with maintenance of the structural integrity of the cell and are involved in substrate and end product transfer across the cell membrane (Smith, 1959; Smith, 1960; Rothblat & Smith, 1961).

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## METHODS

*Organisms.* *Mycoplasma laidlawii* strain B was originally obtained from Dr D. G. ff. Edward (Wellcome Research Laboratories, Beckenham, Kent, England) and has been maintained in these laboratories. *M. laidlawii* strain A, *M. inocuum* sp.nov. (Adler, Shifrine & Ortmayer, 1961) and an unclassified strain KHS of caprine origin were kindly supplied by Dr M. Shifrine (University of California, Davis, California).

*Media.* A basal medium composed of 2 % Bacto tryptose and 0·5 % NaCl was used. This basal medium was supplemented with 0·5 % Bacto PPLO serum fraction (medium A), 0·5 % glucose (medium B), 0·5 % sodium acetate (medium C), 0·5 % glucose + 0·5 % sodium acetate (medium D) or 0·5 % glucose + 0·5 % sodium acetate + 0·5 % Bacto PPLO serum fraction (medium E) for the determination of the culture medium which gave optimal yields of highly pigmented organisms. Initiation of growth in the basal liquid medium with an inoculum from solid medium (0·3 % beef extract + 0·5 % tryptose + 0·5 % sodium chloride + 1·5 % agar, pH 8·0) required addition of 0·5 % Bacto PPLO serum fraction + 0·5 % glucose. Once growth was initiated in liquid medium, Bacto PPLO serum fraction was no longer required. Experiments designed to demonstrate the sparing effect of sterol on the synthesis of non-saponifiable lipids used culture medium D supplemented with different concentrations of Bacto PPLO serum fraction and 10 $\mu$c. sodium acetate-1-$^{14}$C.

*Cultivation.* Experiments designed to determine the culture medium which gave optimal growth used cultures of 2 l. volume. Growth of organisms in quantity sufficient for isolation and analysis of non-saponifiable lipids required the use of 10–60 l. medium inoculated in batches of 2 or 5 l. Cultures were incubated statically at 37°. The organisms were harvested and washed as previously described (Smith, 1955). Incubated uninoculated culture medium contained no sedimentable material.

*Extraction procedures.* Methods used for extraction of total lipids, saponification and isolation of the non-saponifiable lipid fraction were given in a previous report (Rothblat & Smith, 1961). Fatty acid fractions derived from the carotenyl ester fractions were extracted from the aqueous layer at pH 4 with 3 vol. diethyl ether. Following removal of the ether *in vacuo* and neutralization with NaOH, the extracted acids were distilled at pH 4, volatile acids being collected in standard alkali. Residual non-volatile acids were re-extracted with diethyl ether and titrated with standard alkali. Volatile fatty acid fractions were re-extracted, neutralized with ammonia and used for paper chromatographic analysis.

*Chromatographic procedures.* Chromatography of the non-saponifiable lipid fractions was conducted on 3 g. activated silicic acid (Unisil, 100–200 mesh, Clarkson Chemical Co., Inc., Williamsport, Pa., U.S.A.) packed in a column 10 cm. in diameter. Non-saponifiable lipid or total lipid fractions in amounts of 10–100 mg. were added to the column with two or three 5 ml. lots of light petroleum (b.p. 30°–60°). The series of eluting solvents were modifications of those described by Hirsch & Ahrens (1958). For separation of the components of the non-saponifiable lipid fractions this series consisted of (1) 50 ml. 1 % (v/v) diethyl ether in light petroleum (b.p. 30°–60°); (2) 100 ml. 4 % (v/v) diethyl ether in light petroleum (b.p. 30°–60°); (3) 100 ml. 8 % (v/v) diethyl ether in light petroleum (b.p. 30°–60°); (4) 100 ml.

25 % (v/v) diethyl ether in light petroleum (b.p. 30°–60°); (5) 100 ml. diethyl ether; (6) 50 ml. anhydrous methanol. Separation of the carotenoid pigments in the total lipid fraction was accomplished with the following series of solvent mixtures: (1) 100 ml. 1 % (v/v) benzene in light petroleum (b.p. 30°–60°); (2) 300 ml. 2 % (v/v) benzene in light petroleum (b.p. 30°–60°); (3) 50 ml. 8 % (v/v) diethyl ether in light petroleum (b.p. 30°–60°); (4) 50 ml. 25 % (v/v) diethyl ether in light petroleum (b.p. 30°–60°); (5) 50 ml. diethyl ether. Five ml. samples of effluent were collected by gravity flow, in a fraction collector. Solvents were removed *in vacuo* and tubes containing visible residues were immediately placed under nitrogen or analysed spectrophotometrically. Chromatography of the volatile acids was performed by the method of Reid & Lederer (1951).

*Radioisotope counting.* $^{14}$Carbon counting was performed as previously described (Smith & Rothblat, 1960) except for the use of a Tracerlab Model SC-70 scaler coupled with a Model SC-50B automatic flow counter and Model SC-88 computer with print-out.

*Analytical procedures.* Spectrophotometric measurements were carried out in a Beckman Model DU spectrophotometer, Beckman Model DB recording spectrophotometer, or in a Bausch and Lomb Spectronic 505 recording spectrophotometer. Carotenoids were detected by the Carr–Price reaction (Hawk, Oser & Summerson, 1947) and reducing sugar by the method of Park & Johnson (1949). Acid hydrolysis of non-saponifiable lipids was carried out according to the method of Swift (1952). A determination of pigment synthesized was made by measurement of absorption of the total lipid extract in a volume of 5 ml. of dimethoxymethane (methylal)+ methanol (4+1, by vol.) in the Klett–Summerson photometer with a 420 m$\mu$ filter. Iodine-catalysed photoisomerizations of the carotenoid pigments were carried out by the method described by Magoon & Zechmeister (1957). All other chemical and physical procedures have been described previously (Rothblat & Smith, 1961).

*Enzymic analyses.* Pigments in amounts of about 1 mg. containing chemically detectable reducing sugar were taken up in 0·1 ml. warm ethanol. To this solution was added 2 mg. $\beta$-glucosidase (almond emulsin, Nutritional Biochemicals Corp. Cleveland, Ohio, U.S.A.) and 0·067 M-phosphate buffer (pH 7·0) to a total volume of 1·5 ml. A control without substrate but containing 0·1 ml. ethanol was run. After 3 hr. at 37°, 1·5 mg. adenosine triphosphate (sodium salt), 1·5 mg. hexokinase and 0·067 M-phosphate buffer (pH 7·5) to a final volume of 3·0 ml. were added and the mixture re-incubated for 1 hr. at 37°. To 0·5 ml. portions of the reaction mixtures were added 1·5 mg. glucose-6-phosphate dehydrogenase, 1·5 mg. nicotinamide-adenine dinucleotidephosphate (NADP), and 0·067 M-phosphate buffer (pH 7·5) to a total volume of 3·0 ml. Reduction of NADP was followed spectrophotometrically at 340 m$\mu$. In instances where no hydrolysis occurred in the presence of $\beta$-glucosidase, the aqueous layer, following acid hydrolysis of the pigment and freed from $SO_4^{2-}$ by precipitation with $Ba(OH)_2$, was used as the substrate, omitting the step involving $\beta$-glucosidase.

*Chemicals.* All reagents and enzymes were of the highest purity commercially available. Sodium acetate-1-$^{14}$C with a specific activity of 4 mc./m-mole and acetic-1-$^{14}$C anhydride with a specific activity of 2·73 mc./m-mole were obtained from Nuclear Chicago Corporation (Chicago, Illinois, U.S.A.).

## RESULTS

The necessity for obtaining optimal yields of highly pigmented organisms to permit characterization of the carotenoid pigments required the preliminary determination of the best conditions for growth. Aeration had been shown previously to decrease the pigmentation of the organisms (Rothblat & Smith, 1961). All four organisms were grown in five different culture media (media A to E as described under Methods). Table 1 presents the data on total lipids and the pigment produced/mg. dry wt. organism. The presence of cholesterol (media A and E) resulted in a decrease in amount of total lipid and pigments. Glucose or sodium acetate as the sole supplement permitted only suboptimal pigment production. Best results were achieved with medium D which was therefore selected for the growth of large batches of organisms.

Table 1. *Effect of supplements to culture medium on total lipids and carotenoid pigment production in four strains of Mycoplasma*

| Mycoplasma species | Cell fraction | Culture medium* A | B | C | D | E |
|---|---|---|---|---|---|---|
| *M. laidlawii*, strain A | Cell yield† | 59·6 | 25·5 | 34·1 | 38·1 | 57·5 |
| | Total lipid‡ | 0·07 | 0·24 | 0·13 | 0·12 | 0·06 |
| | Pigment§ | 0·0036 | 0·0094 | 0·0175 | 0·0122 | 0·0076 |
| *M. laidlawii*, strain B | Cell yield† | 15·7 | 14·3 | 12·0 | 18·2 | 37·8 |
| | Total lipid | 0·14 | 0·22 | 0·28 | 0·27 | 0·13 |
| | Pigment | 0·0049 | 0·0237 | 0·0871 | 0·0817 | 0·0172 |
| *M. inocuum* | Cell yield | 39·8 | 26·0 | 21·8 | 17·5 | 67·6 |
| | Total lipid | 0·066 | 0·084 | 0·068 | 0·138 | 0·064 |
| | Pigment | 0·0074 | 0·0138 | 0·0286 | 0·0474 | 0·0065 |
| Caprine strain CHS | Cell yield | 63·5 | 25·5 | 40·9 | 41·7 | 53·8 |
| | Total lipid | 0·074 | 0·136 | 0·083 | 0·147 | 0·082 |
| | Pigment | 0·0033 | 0·0071 | 0·0092 | 0·0163 | 0·0041 |

* Culture media described in Methods.
† Mg. dry wt./l.
‡ Mg. total lipid/mg. dry wt. cells.
§ Optical density at 420 mμ/mg. dry wt. cells.

A further attempt to demonstrate the sparing effect of cholesterol on the synthesis of carotenoid pigments was made by measuring the incorporation of acetate-1-$^{14}$C into the non-saponifiable lipid fraction. Bacto PPLO serum fraction containing 80–100 μg. cholesterol/ml. was the source of sterol supplied to the culture medium. Only one organism, *Mycoplasma laidlawii*, strain B, was used. Table 2 presents the data of this experiment. Increasing amounts of available sterol brought about decreasing synthesis of carotenoid pigments and decreasing incorporation of acetate-1-$^{14}$C into the non-saponifiable lipid fraction.

Column chromatography of the non-saponifiable lipid fraction of each of the four organisms resulted in the separation of three pigmented bands. The first band, pigment A, was eluted with 1 % (v/v) diethyl ether in light petroleum (b.p. 30°–60°); the second band, pigment B, with 25 % (v/v) diethyl ether in light petroleum b.p. 30°–60°); the third band, pigment C, with diethyl ether. Stripping of the

column with methanol resulted in the elution of additional material, especially when the total lipid fractions were chromatographed. No analyses were performed on the methanol fractions except examination of the absorption spectra which revealed nothing significant as regards carotenoids. It was not possible to crystallize any of the pigments, primarily because of the small amounts available. Further chromatography on silicic acid columns with gradient elution technique did not separate any of the three pigmented fractions into additional components. The ratios of weight of the different pigments varied from experiment to experiment but invariably the relative proportions were C > B > A. Table 3 presents results of representative experiments.

Table 2. *Sparing action of cholesterol on synthesis of non-saponifiable lipids by* Mycoplasma laidlawii, *strain* B

| Difco PPLO serum fraction (cholesterol source) (%) | Pigments* | Radioactivity† |
|---|---|---|
| None | 0·014 | 169 |
| 0·05 | 0·012 | 184 |
| 0·10 | 0·011 | 166 |
| 0·50 | 0·010 | 119 |
| 1·00 | 0·009 | 63 |

* Optical density/mg. dry wt. cells; † counts/min./mg. non-saponifiable lipid.

Table 3. *Ratios of weights of carotenoid pigments from various Mycoplasma species*

| | Pigment | | |
|---|---|---|---|
| Mycoplasma species | A (hydrocarbon) | B (hydroxylated) | C (glucoside) |
| *M. laidlawii*, strain B | 1·0 | 2·5 | 12·4 |
| *M. inocuum* | 1·0 | 1·8 | 2·2 |
| Caprine strain KIS | 1·0 | 2·1 | 5·9 |

Pigment A was a brilliant yellow and imparted most of the colour to the sedimented organisms. Exposure of this pigment to air even for a short time resulted in a decrease of the colour intensity. Such changes are common to carotenoid pigments in solution and occur as a result of isomerization from the all *trans* isomer to a mixture of the *trans* and *cis* isomers followed by oxidative destruction (Deuel, 1951). Thus chemical and physical analyses on pigment A were made immediately upon recovery from the column. When stored it was held dry in an atmosphere of nitrogen. Table 4 lists the data describing the properties of pigment A from each of the four organisms. Figure 1 shows the absorption spectra of these pigments. The non-polarity of these pigments, their absorption spectra and coefficients support the supposition that they are hydrocarbons of carotenoid nature. The absorption maxima of this pigment from all four organisms and the absorption coefficients of pigment from two organisms, *Mycoplasma laidlawii*, strains A and B, are almost identical to the values for neurosporene (Haxo, 1949).

Identification of pigment A from *Mycoplasma laidlawii*, strain B, as neurosporene, was made by comparing it with authentic neurosporene isolated from commercial tomato paste and kindly supplied by Dr J. W. Porter (Veterans Administration Hospital, Madison, Wisconsin, U.S.A.). The spectral characteristics of the authentic neurosporene, pigment A, and a mixture of the two, are shown in Table 5. The mixture was subjected to chromatography on a silicic acid (Unisil) column with

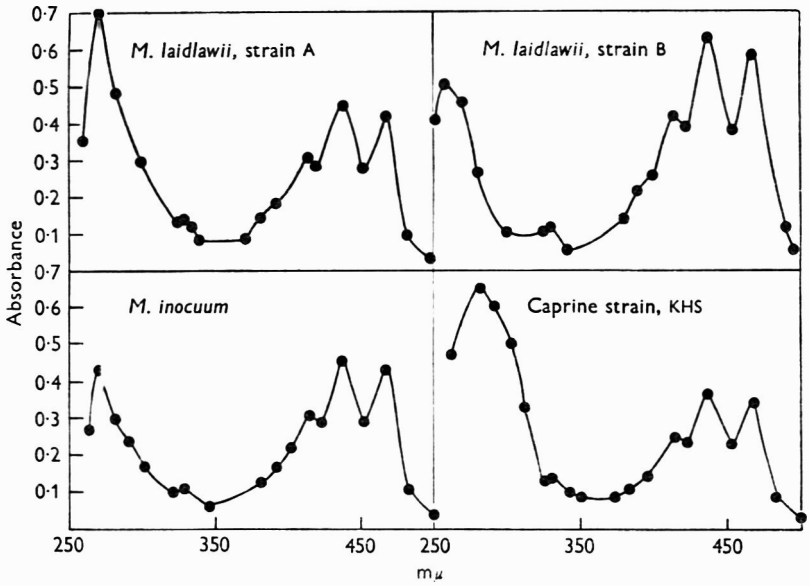


Fig. 1. Absorption spectra of the hydrocarbon carotenoid of Mycoplasma which do not require sterol as nutrient.

Table 4. *Chemical and physical properties of hydrocarbon pigment (A)*

| Property | Mycoplasma species | | | |
|---|---|---|---|---|
| | *M. laidlawii*, A | *M. laidlawii*, B | *M. inocuum* | Caprine strain KHS |
| Absorption maxima (mμ) (hexane) | 275, 414, 437, 467 | 258, 414, 438, 468 | 272, 414, 438, 467 | 273, 414, 438, 467 |
| $E^{1\%}_{1cm.}$ at 438 mμ | 2394 | 2968 | 1164 | 388 |
| $SbCl_3$ (Carr-Price) | + | + | + | + |
| Ethereal $H_2SO_4$ | + | + | + | + |
| Partition: light petroleum/methanol | Epiphasic | Epiphasic | Epiphasic | Epiphasic |

light petroleum (b.p. 30°–60°) as developing solvent. Only one distinct band appeared which was eluted by 170 ml. of the solvent. It was concluded that pigment A was identical to neurosporene and consisted of the all *trans* isomer as indicated by the spectral shift upon iodine catalysed photoisomerization.

Pigment B from each of the organisms was also yellow in colour but distinctly less brilliant than pigment A. It appeared as a more diffuse band with the eluting solvents used. Table 6 gives chemical and physical data for this pigment. In each case the pigment exhibited absorption maxima slightly lower than pigment A,

a shift common to carotenoids upon being oxygenated (Deuel, 1951). Pigment B was more polar than pigment A as indicated by its elution behaviour on silicic acid and its diffusiveness in both layers of the solvent partition mixture. Reaction with acetic-1-$^{14}$C anhydride in pyridine resulted in ready incorporation of radioactivity into the non-polar fraction, and saponification removed the radioactivity from the non-polar fraction. Acetylation resulted in the production of a salmon pink pigment

Table 5. *Comparison of pigment A from* Mycoplasma laidlawii, *strain* B *with authentic neurosporene*

| Property | Hydrocarbon pigment (A) | Neurosporene | Pigment A + neurosporene |
|---|---|---|---|
| Absorption maxima (mμ) (hexane) | 417, 441, 472 | 416, 440, 469 | 417, 440, 470 |
| Absorption minima (mμ) (hexane) | 425, 457 | 422, 456 | 424, 457 |
| Absorption maxima after $I_2$ catalysis (mμ) (hexane) | 330, 414, 437, 466 | 333, 414, 438, 465 | n.d.* |
| Absorption minima after $I_2$ catalysis (mμ) (hexane) | 422, 453 | 422, 455 | n.d. |

* n.d. = Not done.

Table 6. *Chemical and physical properties of hydroxylated pigment (B)*

| Property | Mycoplasma species | | | |
|---|---|---|---|---|
| | *M. laidlawii*, A | *M. laidlawii*, B | *M. inocuum* | Caprine strain KHS |
| Absorption maxima (mμ) (hexane) | 272, 404, 425, 441 | —, 402, 422, 446 | 272, 402, 424, 440 | 272, 405, 424, 440 |
| $E_{1cm.}^{1\%}$ at 424 mμ(hexane) | 263 | 348 | 161 | 64 |
| $SbCl_3$ (Carr-Price) | + | + | + | + |
| Partition: light petroleum/methanol | Epi-hypophasic | Epi-hypophasic | Epi-hypophasic | Epi-hypophasic |
| Radioactivity after acetylation with acetic-1-$^{14}$C-anhydride (counts/min./mg. pigment): | 3144 | 1316 | 2344 | 1973 |
| Radioactivity after saponification of acetylated pigment (counts/min./mg. pigment): | 9 | 274 | 37 | 60 |

which was eluted from silicic acid by benzene or 8 % (v/v) diethyl ether in light petroleum (b.p. 30°–60°). Since pigment B was capable of forming an ester with acetate, it was concluded that this pigment was an hydroxylated carotenoid. Iodine catalysis of pigment B from *Mycoplasma laidlawii*, strain B, resulted in the appearance of a *cis* peak and a spectral shift typical of all *trans* carotenoids, i.e. absorption maxima in hexane before catalysis were 402, 425, and 442 mμ; after catalysis, 356, 400, 423 and 440 mμ.

Two experiments were done to determine whether a relationship existed between the amount of pigment and the amount of acetate in ester linkage. The acetylated

pigment of *Mycoplasma laidlawii*, strain B, purified by chromatography, was weighed and saponified. The non-aqueous phase was recovered and again weighed. The aqueous phase was steam distilled at pH 4, and titrated with standard alkali and acid. In one instance 0·86 mg. free pigment and 3·3 $\mu$mole acetate were recovered; in the other, 0·73 mg. free pigment and 2·8 $\mu$mole acetate. If it be assumed that the pigment was a dihydroxylated C-40 carotenoid, its molecular weight would approximate 570 and the molar ratio of acetate to pigment would approximate 2. Such a molar relationship is valid only when pure pigments of assumed molecular weight are used. Nevertheless, these data suggest that the amount of acetate in ester form was not unreasonable as compared with the unacetylated material.

Table 7. *Chemical and physical properties of naturally esterified pigment (B′)*

| | Mycoplasma species | | | |
|---|---|---|---|---|
| Property | *M. laidlawii*, A | *M. laidlawii*, B | *M. inoculum* | Caprine strain KHS |
| Absorption maxima (m$\mu$) (hexane) | n.d | —, 402, 422, 446 | 272, 405, 422, 440 | n.d. |
| $E^{1\%}_{1cm.}$ at 422 m$\mu$ (hexane) | n.d. | 97 | 73 | n.d. |
| Volatile fatty acids (m equiv.) non-volatile fatty acids (m equiv.) | Volatile only | 2·06 | 0·97 | 3·00 |
| Nature of volatile fatty acids | Acetic | Acetic | Acetic | Acetic |

n.d. = Not done.

The detection of carotenols as components of the non-saponifiable lipid fractions of organisms not requiring sterol as nutrient lead to suspicion that carotenyl esters may occur naturally, analogous to the steryl esters of organisms requiring sterol as nutrient. The total lipid fraction of each of the four organisms was chromatographed as described. Pigment A was eluted with 2 % (v/v) benzene in light petroleum (b.p. 30°–60°); pigment B′, the ester fraction, with 8 % (v/v) diethyl ether in light petroleum (b.p. 30°–60°); pigment B and pigment C with the solvents previously mentioned. Pigment B′ appeared at the top of the column as a distinct salmon pink band but became diffuse and yellowish as it proceeded through the column. The analytical data on this pigment are given in Table 7. The absorption spectra were identical with those of the free carotenols, but the absorption coefficients were decreased because of the greater molecular weight of the esters. The naturally occurring esters contained different amounts of volatile and non-volatile fatty acids as determined by titration. The significant finding was the presence of only acetic acid in the volatile fraction. No identification of the acids comprising the non-volatile fraction was attempted. Non-volatile fatty acids could not be detected in the case of *Mycoplasma laidlawii* strain A.

Pigment C was the most polar of all the pigments. It remained adsorbed to the top of the column until diethyl ether was added as the eluting solvent. It appeared as a distinct brownish yellow band. Total recovery of this component from the saponification mixture required re-extraction of the aqueous layer with diethyl

ether following the initial extraction with light petroleum. The absorption spectrum and a positive Carr–Price test indicated it to be a carotenoid pigment. Its behaviour during extraction procedures and on silicic acid columns bore a similarity to the cholesteryl glucoside present in glucose-fermenting Mycoplasma when supplied cholesterol in the growth medium. Hence this pigment was subjected to analytical techniques directed toward its probable identity as a glycoside of a carotenol. Table 8 presents the data.

Table 8. *Chemical and physical properties of pigment glycoside (C)*

| Property | Mycoplasma species | | | |
|---|---|---|---|---|
| | *M. laidlawii* A | *M. laidlawii* B | *M. inocuum* | Caprine strain KHS |
| Absorption maxima (m$\mu$) (chloroform) | 411, 433, 451 | 408, 430, 454 | 406, 430, 452 | 385, 409, 431 |
| $E$ ($^{1\%}_{1cm}$ chloroform) | 93 (433 m$\mu$) | 112 (430 m$\mu$) | 52 (430 m$\mu$) | 22 (409 m$\mu$) |
| $SbCl_3$ (Carr-Price) | + | + | + | + |
| Partition: light petroleum/methanol | Hypophasic | Hypophasic | Hypophasic | Hypophasic |
| Reducing sugar (Park–Johnson) | + | + | + | + |
| Action of $\beta$-glucosidase | + | + | + | + |
| Enzymic test for glucose | + | + | + | + |

+ = Positive reaction; − = negative reaction.

Pigment C from all four organisms exhibited a plateau in the absorption curve between 280 and 290 m$\mu$. The absorption coefficients are lower than those for the other pigments, again due to the greater molecular weight of the glycosides. The absorption coefficients of pigment C, like those of pigment B, are low and do not permit the relating of pigment C to neurosporene. Iodine-catalysed photoisomerization of pigment C from *Mycoplasma laidlawii*, strain B, resulted in the appearance of a *cis* peak and a spectral shift typical of all *trans* carotenoids, i.e. the absorption maxima in chloroform before catalysis were 409, 433 and 454 m$\mu$; after catalysis, 359, 402, 428 and 451 m$\mu$. The pigment was polar, separating in the methanol phase of the solvent partition mixture. The aqueous fraction following acid hydrolysis gave a positive reaction for reducing sugar. No reducing sugar was detected in the unhydrolysed pigment. Alkaline hydrolysis was ineffective in cleaving the reducing sugar from the pigment. Acid hydrolysis destroyed the pigmented moiety thereby not permitting isolation and identification of the presumed carotenol portion. Pigment C of only two organisms, *Mycoplasma laidlawii*, strain B and *M. inocuum*, was attacked by $\beta$-glucosidase to yield glucose detectable by the coupled hexokinase + glucose-6-phosphate dehydrogenase reaction. However, subjection of the neutralized water soluble fraction following acid hydrolysis of pigment C from *M. laidlawii* strain A and the caprine strain KHS to the action of hexokinase and glucose-6-phosphate dehydrogenase did result in reduction of NADP; Table 9 presents the data. The enzymic determination of glucose was complicated by the presence of contaminating glucose in the hexokinase preparation as evidenced by

substantial reduction of NADP in the control containing no substrate. Nevertheless, NADP reduction in test samples was significantly greater than in the reagent control. It was concluded from this enzymic evidence that the reducing sugar was glucose. In the two organisms containing pigment C hydrolysable by $\beta$-glucosidase, this pigment was considered to be a carotenyl-$\beta$-glucoside. Although pigment C of the other two organisms contained glucose in obvious ether linkage, the isomeric structure could not be deduced from the data obtained.

Table 9. *Reduction of NADP by action of glucose-6-phosphate dehydrogenase on reducing sugar released from pigment C*

Each cuvette contained 0·5 ml. sample with different amount of substrate, 2 mg. hexokinase, 2 mg. disodium salt of adenosine triphosphate, 1·5 mg. glucose-6-phosphate dehydrogenase, 1·5 mg. NADP, 0·067 M-phosphate buffer (pH 7·5) to a total volume of 3·0 ml.

| | | Mycoplasma species | | | |
|---|---|---|---|---|---|
| Time (sec.) | Control | *M. laidlawii*, A* | *M. laidlawii*, B† | *M. inocuum*† | Caprine* strain KHS |
| 0‡ | 0·000 | 0·015 | 0·010 | 0·015 | 0·015 |
| 60 | 0·140§ | 0·480 | 0·347 | 0·220 | 0·370 |
| 90 | 0·155 | 0·565 | 0·405 | 0·245 | 0·475 |
| 120 | 0·165 | 0·638 | 0·432 | 0·260 | 0·535 |
| 150 | 0·172 | 0·675 | 0·452 | 0·270 | 0·570 |
| 180 | 0·180 | 0·703 | 0·480 | 0·277 | 0·605 |
| 600 | 0·213 | 0·900 | 0·515 | 0·335 | 0·762 |

* Reducing sugar released by acid hydrolysis.
† Reducing sugar released by $\beta$-glucosidase.
‡ No glucose-6-phosphate dehydrogenase present.
§ Absorbance (optical density) at 340 m$\mu$.

A relationship between the amount of pigment C and the amount of glucose liberated by the action of $\beta$-glucosidase was shown with *Mycoplasma laidlawii*, strain B. Assuming complete utilization of substrate during the reaction, double the amount of lactose should yield the same value for glucose as the glucose control. These conditions were met by the test system, i.e. 4·0 mg. lactose and 2·0 mg. glucose gave final optical density readings of 0·263 and 0·270. Pigment A in the amount of 7·74 mg. yielded 2·34 mg. glucose as calculated from optical density measurements. If it be assumed that the molecular weight of the pigment approximates that of a dihydroxylated C-40 carotenoid attached to glucose in ether linkage, i.e. about 750, and that complete hydrolysis occurred, the molar ratio of glucose found to pigment tested would approximate 1.

Analysis of the chromatographic behaviour of pigment C from *Mycoplasma laidlawii*, strain B, following the action of $\beta$-glueosidase was performed by extraction of the reaction mixture with light petroleum (b.p. 30°–60°) and subjection of the extract to the same chromatographic procedure as used for the initial separation of the pigments. Two bands appeared, one eluting with the same solvent as pigment B, the other with the same solvent as pigment C. Re-chromatography of the less polar pigment mixed with pigment B resulted in the elution of one band. Further exposure of the more polar pigment to $\beta$-glucosidase resulted in additional formation of two pigments eluting as described above. Complete loss of pigment C as a

result of the action of β-glucosidase was not achieved. However, only a small residual amount of this pigment remained even after the first exposure to β-glucosidase. Thus it appeared that β-glucosidase action on pigment C liberated a pigment with the same chromatographic behaviour as pigment B, an hydroxylated carotenoid.

## DISCUSSION

The present work has shown the presence of three carotenoid pigments in the non-saponifiable lipid fractions of four non-sterol requiring Mycoplasma organisms. These pigments were synthesized by organisms which did not require sterol as nutrient and were not found in the sterol-requiring organisms. One pigment has been identified as a hydrocarbon carotenoid, similar to neurosporene. The other two pigments possess properties suggestive of an hydroxylated carotenoid and a carotenyl glucoside. Besides occurring in glycosidic linkage with glucose, the carotenol occurs naturally in ester linkage with fatty acids.

The absorption maxima of the hydrocarbon pigment of all four organisms are essentially identical. The absorption coefficients of pigments from *Mycoplasma inocuum* and the caprine strain KHS are considerably lower than those of *M. laidlawii*, strains A and B. It is not very probable that these lower absorption coefficients are the result of impure compounds since attempts to further fractionate any of the pigments yielded only one component.

The absorption coefficients of pigments B and C are too low to infer that these pigments are oxygenated derivatives of neurosporene. For example, the decrease in absorption coefficient as a result of oxygenation of β-carotene to zeaxanthin is about 15 % (Deuel, 1951) while the difference between pigments A and B represents a 90 % diminution. Such a wide difference would be explained by the presence of an impurity in pigments B and C, by isomerization to a mixture of *cis-trans* isomers, or lack of similarity to neurosporene. Inability to separate pigments B and C into more than one component by gradient elution chromatography would suggest the absence of a major impurity or of contamination with other isomeric forms. The absence of a *cis* peak in these pigments prior to and the spectral shift following iodine-catalysed photoisomerization ruled out the possibility of their being a mixture of *cis-trans* isomers. Furthermore, isomerization does not result in reduction of absorption to the extent that it would explain the differences in absorption coefficients.

The major difficulty in characterizing all of the pigments detected was to obtain sufficient quantities for analysis. The small amounts obtained significantly affected the accuracy of the gravimetric determinations. Such errors would be magnified in the values calculated for absorption coefficients, thereby making these values less reliable than the other properties examined.

The carotenol as the free alcohol, esterified with fatty acids, and in glycosidic linkage with glucose is analogous to the free cholesterol, esterified cholesterol and the cholesteryl glucoside, found in organisms requiring sterol as nutrient and capable of fermenting glucose and in organisms not requiring sterol as nutrient supplied cholesterol in the growth medium (Rothblat & Smith, 1961). The hydrocarbon carotenoid might be considered a precursor of the carotenol. Most naturally occurring carotenols possess hydroxyl groups on the 3 and 3′ carbon atoms (Deuel, 1951). This position of the hydroxyl groups in carotenols is analogous to the position of the

hydroxyl group in sterols, i.e. on carbon atom 3, capable of supporting growth of sterol-requiring organisms. Any enzymic activity vital to the organism and utilizing this 3-hydroxyl group presumably could function with a 3-hydroxy sterol or a 3-hydroxy carotenol because of the similarities of their molecular structures. Since some organisms are endowed with the enzymic pathway to synthesize a non-saponifiable lipid of appropriate structure, they possess no requirement for 3-hydroxy sterol. On the other hand, those organisms incapable of synthesis of such compounds must be supplied with the preformed lipid.

A vital function for sterols in the sterol requiring organisms has been postulated to be substrate permeability (Smith, 1959; Smith, 1960; Rothblat & Smith, 1961). The carotenols can be postulated to serve this same function in non-sterol-requiring strains, all of which contain the pigment. Thus a glucosidase could serve to transport glucose into the cell via the carotenyl glucoside. An esterase, demonstrated to be active against cholesteryl esters (Smith, 1959) could serve to transport the end product of glucose metabolism, acetate, via a carotenyl acetate, out of the cell. Additional support for this hypothesis is the location of the carotenoid pigments in the insoluble residue (cell membrane) following sonic lysis, and the finding that acetate is the only volatile fatty acid in ester linkage with the carotenol which occurs naturally in the organism. The presence of lipids with a molecular structure analogous to cholesterol in Mycoplasma which do not require sterol as nutrient substantiates a relationship between these organisms and the sterol-requiring Mycoplasma. In addition, the difference in the isomeric structure of the carotenyl glucosides of strains A and B of *Mycoplasma laidlawii* can lead to the presumption that the glucosidases of the two strains are different. This evidence adds credence to the distinction between the two strains.

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# Quantitative Aspects of the Protection of Freeze-Dried *Escherichia coli* Against the Toxic Effect of Oxygen

By M. B. LION
*The Israel Institute for Biological Research, Ness-Ziona, Israel*

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SUMMARY

Various substances protect dry *Escherichia coli* against oxygen. The concentration of these substances in the bacterial suspension, necessary to achieve a given degree of protection, is a function of the concentration of the bacteria in the suspension. The protector seems to act in the dry state. The viability of freeze-dried bacteria, unexposed to oxygen, may also depend on the concentration of the bacterial suspension. A common mechanism is suggested to explain the dependence of killing on population density, both during freeze-drying and during exposure of dried organisms to oxygen. The viability of bacteria during freeze-drying and of dried bacteria exposed to oxygen are both markedly affected by the presence of certain substances such as serum albumin or Bacto-protone.

## INTRODUCTION

*Escherichia coli* organisms, freeze-dried from distilled water, are extremely sensitive to oxygen and are very rapidly killed in its presence (Lion & Bergmann, 1961*a*). Certain inorganic salts, some sugars, and thiourea and some of its derivatives can protect the organisms against the toxic effect of oxygen (Lion & Bergmann, 1961*b*). This effect is further quantitatively explored here. It will be shown that these protective compounds act in the dry state. Some general aspects of the lyophilization of bacteria will also be discussed.

## METHODS

The general methods for the preparation of bacterial suspensions, their lyophilization and the determination of their viability have been described (Lion & Bergmann, 1961*a*, *b*). The strain of *Escherichia coli* used was the same as in the previous studies. The protective substances studied were: thiourea, sodium iodide, glucose.

## RESULTS

*Escherichia coli* suspended in thiourea solution and freeze-dried is protected against oxygen (Lion & Bergmann, 1961*b*). When the bacteria, dried from distilled water, were exposed to oxygen and only then reconstituted in fluid containing thiourea, no protection occurred (Fig. 1). The necessity to preincubate the bacteria in the protective solution before freezing and drying was tested as follows: (1) The bacterial suspension was incubated in either glucose or thiourea solution for 30 min. at room temperature (25°) before freezing. (2) A pre-cooled concentrated solution of either compound was added to a pre-cooled suspension of bacteria in water so

that the final desired concentration of the compound and concentration of bacteria were obtained. The mixture was immediately frozen at −80°; this process took less than 15 sec. There was no significant difference in the protective capacity of glucose or thiourea after either of these treatments (Fig. 2).

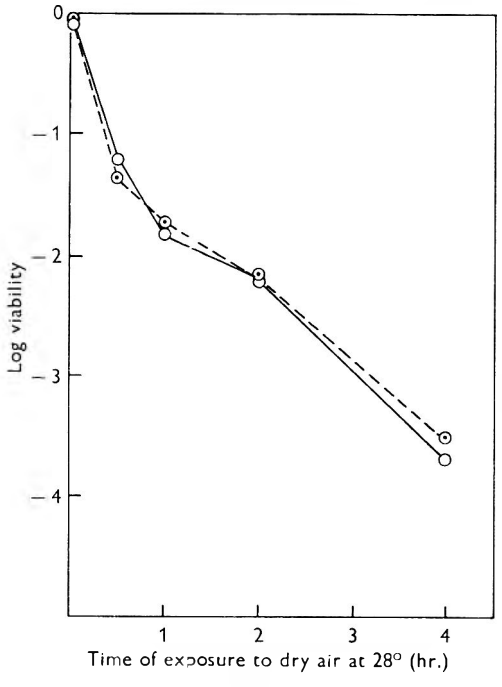


Fig. 1

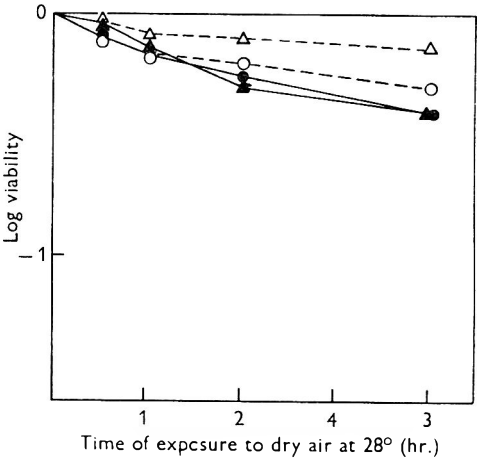


Fig. 2

Fig. 1. Effect of the resuspending medium on the viability of *Escherichia coli* cells, after freeze-drying from distilled water and exposure to air at 28°. ○, Gelatin buffer; ⊙, gelatin buffer + 2% (w/v) thiourea.

Fig. 2. Comparison between the effects of immediate freezing of the bacterial suspension in ○, 1% (w/v) thiourea; △, 2% (w/v) glucose solutions and of preincubation in ●, 1% (w/v) thiourea; ▲, 2% (w/v) glucose before freezing on the viability of *Escherichia coli* cells freeze-dried in the presence of these substances and exposed to air at 28°.

A third experiment was made to see whether the protector against oxygen had to be present before freeze-drying, or whether it could be added in vacuum as a dry powder to the already dried bacteria and still show protective action. A suspension of bacteria in water was frozen on one side of an ampoule and a solution of thiourea frozen on the opposite side, so that there was no contact between the two patches. Some glass beads were added to the ampoules and the ampoules were freeze-dried and sealed in vacuum. The resulting powder was mixed in vacuum and only then exposed to dry air. No protection was detected. It is possible, however, that this finding is not conclusive, since such a mechanical mixing may have been insufficient to bring the bacteria and the protector into intimate contact.

Although direct evidence is lacking, indirect evidence that the protective substance can act in the dry state may be provided by testing bacterial suspensions of various concentrations in different concentrations of protective substance. In a liquid suspension each organism is independently exposed to the same concentration of

protector, provided there is no change in the concentration of the protector by its diffusion into the organisms. If the protector therefore acted by preconditioning bacteria in a liquid suspension to a later resistance to oxygen when in the dry state, then the protective capacity should depend only on the concentration of the protector, but be independent of the concentration of organisms in the suspension. Three suspensions of different bacterial concentrations were tested in each of eight concentrations of three representative protective substances (sodium iodide, thiourea, glucose). Figures 3–5 are plots of the logarithm of the viability of dried bacteria exposed to air under standard conditions in the presence of the protective substances against the concentration of these compounds before freeze-drying. Each point is based on at least two samples. For each of the three protective compounds, the optimum concentration (i.e. that which assured highest viability) is seen to depend on the concentration of the bacterial suspension. In the range of suspension concentrations tested, and for each of the three compounds, the concentration of the protector needed for a given degree of protection seems to be roughly linearly related to the bacterial concentration in the protected suspension.

The concentration of the protective agent in the suspension did not change appreciably by diffusion into the suspended bacteria, even at the high bacterial concentration used. This was shown in the following way. In an earlier small-scale experiment not described here, we have found that a suspension of $1{\cdot}6 \times 10^{10}$ bacteria/ml. is best protected in a 0·175 % (w/v) solution of NaI. The same concentration barely protected a suspension at $1{\cdot}6 \times 10^{11}$ bacteria/ml. The following bacterial suspensions were prepared: (A) $1{\cdot}8 \times 10^{11}$ bacteria/ml. in 0·1 % (w/v). This concentration was used instead of 0·18 % in order to work in the vicinity of that part of the curve where protection decreases sharply with decrease in concentration (cf. Fig. 3). Any depletion of solute from the solution by the high concentration of bacterial suspension would thus be more easily detected. This suspension was incubated for 1 hr. at room temperature and a sample then dried and exposed to air under standard conditions. The rest of the suspension was centrifuged, the sedimented organisms discarded and the supernatant fluid was used to prepare suspension B. (B) $1{\cdot}7 \times 10^{10}$ bacteria/ml. were suspended in the supernatant fluid of A. The suspension was dried and exposed to air as before in A. (C) $1{\cdot}6 \times 10^{10}$ bacteria/ml. were suspended in a fresh solution of 0·1 (w/v) NaI, the suspension dried and then exposed to air. The results presented in Table 1 show that even at high bacterial concentrations in a comparatively low concentration of iodide (suspension A), the concentration of the protective solute was not affected by the presence of the bacteria in the solution. These results provide some evidence that the protector can act in the dry state.

The decrease in protection at higher concentrations of protector above an optimum (indicated by broken lines in Figs. 3 and 4) is not due to loss of protective capacity of the compounds at these concentrations, as the killing observed under these conditions occurred already during freeze-drying and before any exposure to oxygen; this is shown in Figs. 6 and 7. Here the viability of the bacteria after freeze-drying in vacuum is plotted against the concentration of protective solute at different bacterial concentrations. The high mortality of bacteria freeze-dried at an iodide concentration exceeding the optimum for the given bacterial concentration occurred in the absence of oxygen and was therefore due to events during freeze-

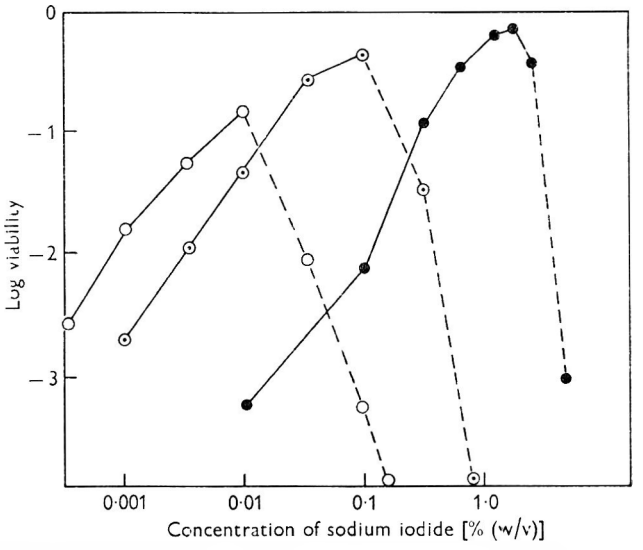


Fig. 3. Viability of *Escherichia coli* cells in suspensions of various densities freeze-dried in sodium iodide solution and exposed to air for 4 hr. at 28°, versus the concentration of sodium iodide in the solution. ●, $1{\cdot}6 \times 10^{11}$ cells/ml.; ⊙, $8{\cdot}0 \times 10^{9}$ cells/ml.; ○, $8{\cdot}2 \times 10^{8}$ cells/ml.

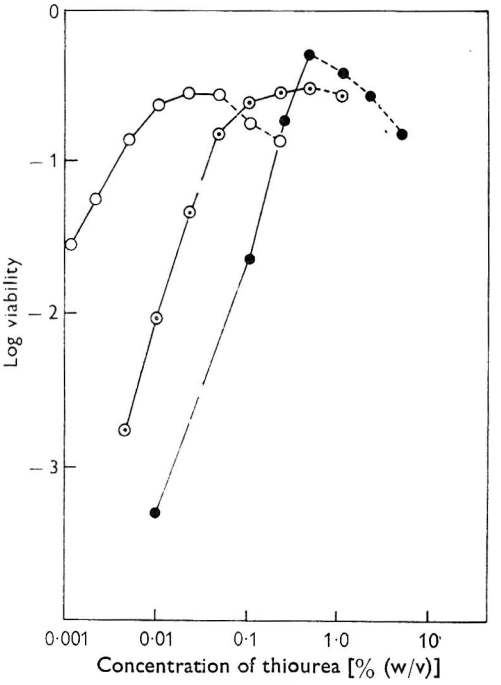


Fig. 4

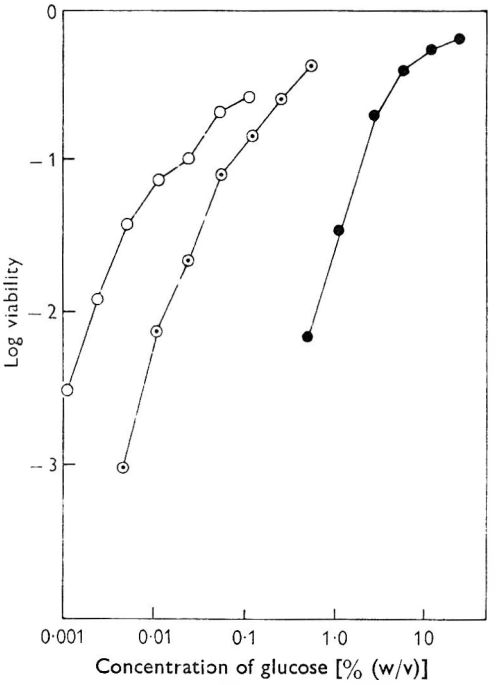


Fig. 5

Fig. 4. Viability of *Escherichia coli* cells in suspensions of various densities freeze-dried in thiourea solution and exposed to air for 4 hr. at 28°, versus the concentration of thiourea in the solution. ●, $1{\cdot}7 \times 10^{11}$ cells/ml.; ⊙, $2{\cdot}8 \times 10^{10}$ cells/ml.; ○, $2{\cdot}4 \times 10^{9}$ cells/ml.

Fig. 5. Viability of *Escherichia coli* cells in suspensions of various densities freeze-dried in glucose solution and exposed to air for 4 hr. at 28°, versus the concentration of glucose, in the solution. ●, $2{\cdot}3 \times 10^{11}$ cells/ml.; ⊙, $1{\cdot}5 \times 10^{10}$ cells/ml.; ○, $1{\cdot}4 \times 10^{9}$ cells/ml.

drying proper (Fig. 6). Drying in excess of thiourea caused a much smaller degree of killing, while glucose even at the highest concentration tested was completely innocuous (Fig. 7).

Table 1. *Viability of suspensions of* Escherichia coli *dried in* 0·1 % *(w/v) NaI and exposed to air (atmospheric pressure) for* 4 *hr. at* 28°

| Suspensions | Absolute concentration before drying | Percentage viability after exposure to air for 4 hr. |
|---|---|---|
| A | $1{\cdot}8 \times 10^{11}$ bacteria/ml. | 1·0 |
| B | $1{\cdot}7 \times 10^{10}$ bacteria/ml. | 35 |
| C | $1{\cdot}6 \times 10^{10}$ bacteria/ml. | 44 |

For make-up of the different suspensions see text.

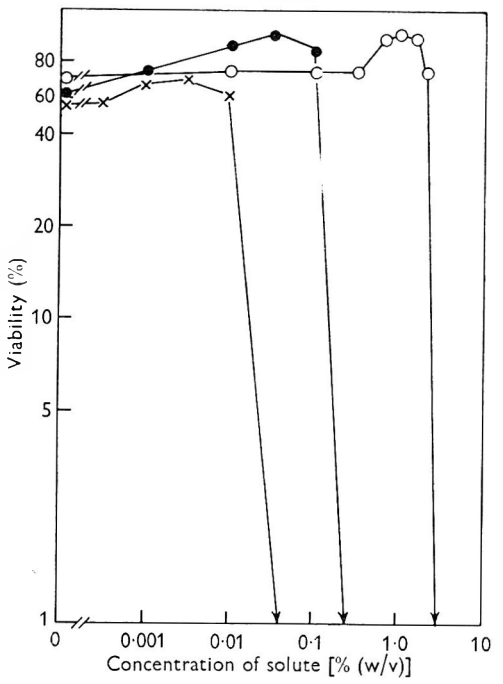


Fig. 6

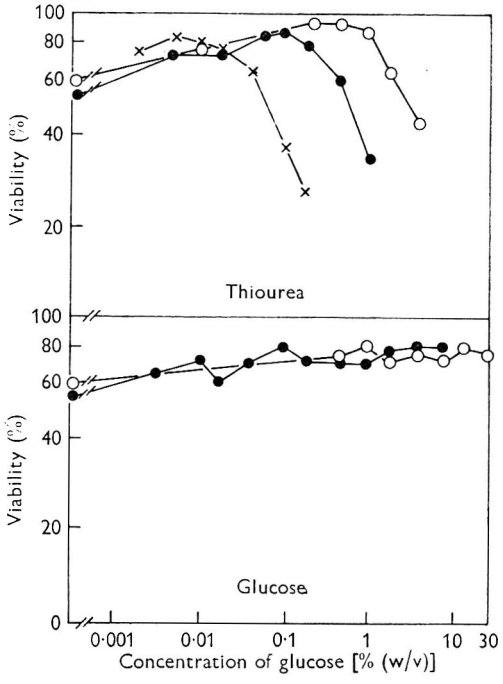


Fig. 7

Fig. 6. Viability of *Escherichia coli* cells in suspensions of various densities freeze-dried in sodium iodide solution and unexposed to air, versus the concentration of sodium iodide in the solution. ○, $1{\cdot}6 \times 10^{11}$ cells/ml.; ●, $8{\cdot}0 \times 10^{9}$ cells/ml.; ×, $8{\cdot}2 \times 10^{8}$ cells/ml.

Fig. 7. Viability of *Escherichia coli* cells in suspensions of various densities freeze-dried in solutions of thiourea or glucose and unexposed to air, versus the concentration of these substances in the solution. Thiourea: ○, $1{\cdot}7 \times 10^{11}$ cells/ml.; ●, $2{\cdot}8 \times 10^{10}$ cells/ml.; ×, $2{\cdot}4 \times 10^{9}$ cells/ml. Glucose: ●, $2{\cdot}3 \times 10^{11}$ cells/ml.; ○, $1{\cdot}5 \times 10^{10}$ cells/ml.

For optimum protection against oxygen, it would appear that each bacterium in the dry state has to be surrounded by a certain amount of protector. If this be so, it should be immaterial how this state is achieved. The same relative survival would be expected, whether some of the bacteria added to the protective solution before the experiment were dead, or whether all the bacteria were alive, as long as the

correct ratio between the amount of protector and the total number of bacteria were maintained. In presence of an excess of protector, addition of an inert 'diluting agent' such as dead bacteria, cell extracts, etc., in the proper amount, should be able to restore the optimum protective ratio. On the other hand, when such an optimum ratio already exists, the addition of any diluting agent should decrease the degree of protection below the optimum. These considerations are borne out by the experiments described in Table 2. A suspension of *c.* $10^{10}$ bacteria/ml. was dried in the presence of NaI or thiourea at two concentrations, one being optimum for $10^{10}$

Table 2. *Viability of suspensions of* Escherichia coli *after exposure to air in the dry state for 4 hr. at 28°*

| Protective agent (%) | Bacterial suspension: $1{\cdot}8 \times 10^{10}$ bacteria/ml. | $1{\cdot}8 \times 10^{11}$ bacteria/ml. | $1{\cdot}8 \times 10^{10}$ living bacteria $+ 2 \times 10^{11}$ dead bacteria/ml. | $1{\cdot}8 \times 10^{10}$ bacteria/ml. $+ 4\%$ bovine serum albumin |
|---|---|---|---|---|
| | Viability (%) | | | |
| Thiourea, 1·0 | (16)* | 32 | 41 | 59 |
| 0·1 | 43 | 3·5 | 2·6 | 2·1 |

| Protective agent (%) | Bacterial suspension: $1{\cdot}5 \times 10^{10}$ bacteria/ml. | $1{\cdot}6 \times 10^{11}$ bacteria/ml. | $1{\cdot}5 \times 10^{10}$ living bacteria $+ 2 \times 10^{11}$ dead bacteria/ml. | $1{\cdot}5 \times 10^{10}$ bacteria $+ 4\%$ bovine serum albumin | $1{\cdot}5 \times 10^{10}$ bacteria/ml. $+ 4\%$ Bacto protone |
|---|---|---|---|---|---|
| | Viability (%) | | | | |
| Sodium iodide, 1·75 | $(10^{-5})$* | 82 | 72 | 40 | 47 |
| 0·175 | 81 | 0·8 | 1·4 | 0·9 | 0·5 |
| None (control) | 0·05 | 0·02 | 0·01 | 0·008 | 0·06 |

* Mortality occurred mainly during freeze-drying; see text.

bacteria/ml., the other being optimum for *c.* $10^{11}$ bacteria/ml., but constituting an excess for the test suspension. The dried preparations were next exposed to air. Three diluting agents were tested: (i) dead bacteria at a concentration of *c.* $10^{11}$ bacteria/ml. (prepared by exposing dried *Escherichia coli* to air for 24 hr.); (ii) 4% (w/v) bovine serum albumin; (iii) 4% (w/v) Bactoprotone, which is produced by Difco Laboratories Inc.; it is rich in higher proteoses but very low in free amino acids, some of which show protection against oxygen. The concentration of 4% for the last two materials was chosen because when dry it gives the same mass/ml. as $1{\cdot}6 \times 10^{11}$ bacteria.

The first two columns in Table 2 are the controls in absence of diluting agent. Brackets around values indicate that the mortality was incurred mainly during freeze-drying in excess of solute (see Figs. 6 and 7); in all other cases mortality during freeze-drying was very small and the values represent the oxygen effect proper. The last row gives % viability in the absence of protectors and proves that the diluting agents were really inert.

Addition of the diluting agents to the suspensions of about $10^{10}$ bacteria/ml. caused this suspension to behave like one of about $10^{11}$ bacteria/ml. In the tests where excess of protector in the controls caused high mortality, the addition of dead bacteria, albumin or protone led to a marked improvement in viability. Under conditions in which originally optimum protection had been obtained in the controls, the viability decreased in the presence of the diluting agents, in agreement with the results obtained in the more concentrated (tenfold) suspension. Noteworthy are the results with iodide; the addition of dead bacteria, albumin or protone increased the viability of the organisms in 1·75% NaI from $10^{-7}$ to nearly 50%, while at the same time the viability in the 0·175% solution was decreased from 80% to about 1%.

## DISCUSSION

The substances which protect freeze-dried bacteria against oxygen may act in one of the following ways: (i) They counteract the oxygen effect during the resuspension of the bacteria after the termination of the oxygen exposure. (ii) They directly interfere with the oxygen reaction in the dry state. (iii) They produce before freeze-drying metabolic changes in bacteria such that bacteria become resistant to oxygen after drying. The most acceptable alternative would be the second. The addition of the protective agent only to the resuspending medium does not protect bacteria against the oxygen death. Bacteria are equally well protected when they are incubated in the protective solution for a long time, or when the solute is added only about 15 sec. before freezing. The diffusion of the protective solute into the bacterial cell required by the third alternative should therefore be completed in 15 sec. This in itself is possible (Cowie & Roberts, 1955), but then the amount of the solute diffusing into the bacterial cell should be dependent only on the concentration of the solute and not on the concentration of the bacteria. Yet the bacterial concentration strongly affects the results. In the case of NaI this cannot be explained by the depletion of the protective solute from the suspension even at the highest bacterial concentrations. Moreover, dead bacteria, as well as inert compounds, can replace live bacteria without affecting the results appreciably. The protective action occurs therefore probably in the dry state and a certain amount of protective material is required for each organism to prevent the reaction between oxygen and some bacterial receptor. Nothing can as yet be said about the nature of this reaction, but free radicals are probably involved (Lion, Kirby-Smith & Randolph, 1961).

When bacterial suspensions are freeze-dried in NaI solutions exceeding a critical concentration, considerable killing occurs even without oxygen exposure. The critical concentration of NaI depends on the bacterial concentration. Freeze-drying in thiourea affects the viability only to a small extent and in glucose not at all. NaI differs from the last two compounds in that solutions exceeding *c.* 1 M concentration are very toxic to bacterial suspension in liquid at 0° (unpublished results). Freeze-drying of $8 \times 10^9$ bacteria/ml. in 0·5% NaI solution causes a high mortality. If the same toxic effect in liquid suspensions is also responsible for the killing during freezing and drying, we have to suppose that the actual concentration of NaI during freezing and drying of $8 \times 10^9$ bacteria/ml. is much higher than the starting concentration. Physically this is actually to be expected. During freezing of the solution pure ice separates leaving behind a concentrated solution which solidifies

only at the eutectic temperature ($-32°$ for pure NaI) (Luyet, 1960). Such concentrated solution will also form when a rapidly frozen solution is dried above the eutectic temperature. In both cases the bacteria probably remain in the unfrozen solution and are exposed to a high concentration of NaI. When now more bacteria are added to the same solution, a point is reached when the volume of the solution at the eutectic concentration is too small to submerge all the bacterial cells and the toxic effect will disappear. Thus the critical concentration of NaI is that starting concentration, at which the volume of the unfrozen part of the solution near the eutectic temperature is just large enough to submerge all or most of the organism in the suspension. This critical concentration depends on the bacterial concentration in the suspension. This point is illustrated by the following rough calculation.

The eutectic concentration of NaI is 390 g./1000 g. solution (Intern. Crit. Tabl.). Its specific gravity is *c.* 1·5 g./ml. at $-32°$. In a suspension of $8 \times 10^9$ bacteria/ml. in 0·5% NaI there are $6 \times 10^{-13}$ g. NaI/organism. At eutectic concentration the weight of the pool surrounding the organism is $1{\cdot}5 \times 10^{-12}$ g. and its volume is $1{\cdot}0 \times 10^{-12}$ ml. Even for a spherical pool its diameter would be at least 1·2 $\mu$ and in case of partial overlap between pools even larger than that. This would be enough to submerge all the bacteria in the suspension. When the starting concentration of NaI is now decreased to 0·1 %, or when the bacterial concentration is increased to $1{\cdot}5 \times 10^{11}$ bacteria/ml., the average diameter of the pool surrounding each organism will be decreased by a factor of 1·7 and 2·7 respectively. The pool will then be too small on the average to submerge any single bacterium. There will be no difference if the increase in bacterial concentration is caused by live or dead organisms, or if an equivalent volume of the submerging pool is occupied by inert compounds such as serum albumin.

The accumulation of the solute round the bacteria assumed to be caused by freeze-drying might be a prerequisite for effective protection against oxygen in the dry state. This could explain the failure to achieve protection when dry bacteria were mixed with the dry protective compound before exposure to oxygen. Analogously, Gordy & Myagawa (1960) obtained radioprotection of dry proteins by cysteine only when the two compounds were freeze-dried together, but not when they were mixed in the dry state before radiation.

Our results may also explain the so-called 'protective colloid' effect in freeze-drying. Usually low concentrations of bacteria have been freeze-dried in isotonic solutions (Heckley, 1961). The resulting low viabilities were considerably improved by increasing the bacterial concentration, as in the case of *Serratia marcescens* and NaCl (Benedict *et al.* 1958). Similar results may be obtained by addition of high molecular substances, as shown in our experiments. This effect has been ascribed to some protective property of the colloid. Our studies suggest that there is nothing protective *per se* in colloids. On the contrary, when these substances are added to suspensions of bacteria freeze-dried in solutions containing the protector at optimum concentration, the viability of the freeze-dried bacteria exposed to oxygen will be decreased as compared to results without the colloid (cf. Table 2). If the role ascribed here to the protective colloid is correct, then no effect of the colloid would be expected when the bacteria are dried from distilled water. This is also demonstrated in Table 2.

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# The pH Value at the Surface of *Bacillus subtilis*

By L. WEISS
*Strangeways Research Laboratory, Cambridge*

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SUMMARY

The reaction between penicillin and the penicillinase of *Bacillus subtilis* strain 749C, in the cell-bound and the free states, was studied over the range pH 3·9–9·0. The results suggested that the bound penicillinase behaved as though it were reacting in an environment of significantly lower pH value than that of the bulk phase. This effect was not as great as that calculated from electrokinetic data for *B. subtilis* surfaces. Various ways of interpreting the data are discussed.

## INTRODUCTION

Following the work of Peters (1931), Danielli (1937) suggested that owing to charge effects, the pH value at a cell surface might be significantly lower than the pH value of the surrounding environment. McLaren & Babcock (1959) pointed out that enzyme reactions at surfaces might yield information about the surface pH value. In the present communication experiments are described in which the cell bound penicillinase of *Bacillus subtilis* strain 749C was used as such a 'molecular pH-meter' for the surface of this organism, by studying its reaction with penicillin. This particular organism was chosen because previous work had been done on the location of its bound penicillinase (Kushner & Pollock, 1961), and a simple iodometric method was available for penicillinase assay (Perret, 1954).

## METHODS

*Organism.* This was *Bacillus subtilis* strain 749C, a penicillinase-constitutive mutant isolated from *B. subtilis* 749 wild strain (Division of Bacterial Physiology, National Institute for Medical Research, London). Although the penicillinase is largely bound to the bacteria during the log phase of growth, it may be obtained free in the supernatant fluid of older cultures; the release of penicillinase was described by Pollock (1961). The organisms were used in suspensions containing the equivalent of 0·45–0·55 mg. dry weight organism/ml., as determined by measurements of opacity in a Hilger and Watts Spectrophotometer and the use of a concentration curve.

*Reagents.* Buffer solutions were 0·2M-citric acid + sodium hydroxide (pH 3·9–6·5) and 0·2M-potassium dihydrogen phosphate + sodium hydroxide (pH 6·0–8·5).

Benzylpenicillin (crystalline sodium salt, Glaxo, containing 1670 units/mg.) was dissolved (0·25 g.) in 100 ml. 0·2M-phosphate buffer (pH 6·5) and kept in ice. The reagents and method used for the iodometric assay of penicillin were as described by Perret (1954).

The assay of penicillin was carried out in 60 ml. stoppered reagent bottles which were shaken gently in a water bath maintained at 30°. At zero time 1 ml. penicillin solution was added to 4 ml. prewarmed buffer; 5 min. after this either 1 ml. of enzyme solution or 1 ml. of a suspension of washed *Bacillus subtilis* was added. After incubation for 30 min. the residual penicillin was assayed. Twenty-one determinations were made with cell-bound enzyme, and 40 with cell-free enzyme. The penicillinase activity is expressed as percentage maximal activity in each run of experiments.

*Measurements of electrophoretic mobility of* Bacillus subtilis *strain* 749 C. Organisms in the log phase were washed by centrifugation in various buffers and resuspended in similar solutions. The measurements were carried out in the microelectrophoresis apparatus described by Bangham, Flemans, Heard & Seaman (1958). The electrophoretic mobilities of ten intact bacteria which were not actively motile were measured in each buffer.

## RESULTS

*The electrophoretic mobilities of* Bacillus subtilis *strain* 749 C *organisms*

The organisms were suspended in 0·2 M-citrate buffers at 30° and the results are shown in Fig. 1. From these mobilities it is possible to calculate (see later) the

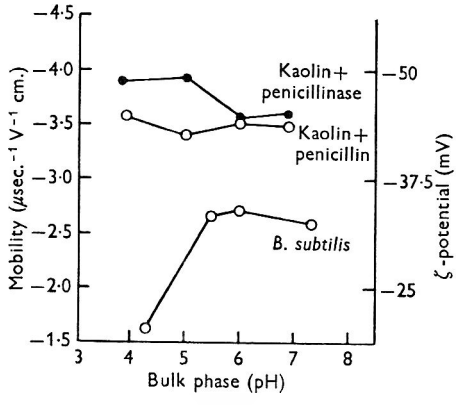


Fig. 1

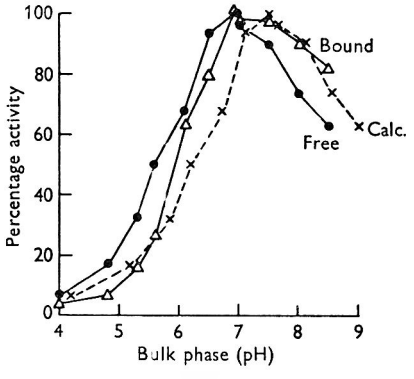


Fig. 2

Fig. 1. Electrophoretic mobilities and calculated ζ-potentials of kaolin particles with adsorbed penicillin (○—○), kaolin particles with adsorbed penicillinase (●—●) and *B. subtilis* (○—○) in citrate buffers of pH values indicated.

Fig. 2. Percentage penicillinase activity at different bulk-phase pH, as observed in the 'free' state (●—●) when bound to the surface of *B. subtilis* (△—△) and as calculated at the surface of *B. subtilis* (× --- ×).

position of the curve for enzyme activity at the bacterial surface from the curve for enzyme activity in the bulk phase. Surface is here used to mean the zone or region in which the pH value is calculated from the ζ-potential (see later). Three curves are therefore plotted in Fig. 2: measured activity in the bulk-phase, measured activity at the bacterial surface, activity at the bacterial surface as calculated from the bulk-phase electrokinetic data.

*Discussion of enzyme activity results*

The relationships were studied in two parts, first, where activity increased with increasing pH value, and secondly where activity decreased with further increase in pH value. There was a significant departure from linearity when points for which enzyme activity was less than 15% were included. Omission of these points produced relations in which there was no significant departure from linearity and regression coefficients adequately summarized the rate of change of activity with pH value; these regression coefficients are given in Table 1.

For the part of the curve where enzyme activity and pH value increased together, the difference between the regression coefficients was not significant at the 5% level ($10\% > P > 5\%$). However, the curves only coincided at the 100% activity level, which is interpreted as meaning either that their slopes were different, or that the lines were parallel until a high degree of activity was reached, at which point they curved over to coincide. If parallelism be assumed, an analysis of covariance shows the lines to be separated by a highly significant amount ($P < 0{\cdot}001$). In either case, therefore, it is concluded that the lines are different. As the pH value increased further, activity decreased from 100% more rapidly for free enzyme than for cell-bound enzyme, the difference between regression coefficients being significant at the 5% level.

Table 1. *Regression coefficients of percentage activity on pH*

| | Cell-bound enzyme | Free enzyme | Calculated reaction at bacterial surface |
|---|---|---|---|
| Activity increasing with increasing pH | | | |
| pH range | 5·3–6·5 | 4·8–6·5 | 5·2–7·1 |
| Activity range | 17·0–79·5 | 16·4–93·7 | 16·4–93·7 |
| Regression coefficient ± S.E. | 55·0 + 4·1 | 45·6 ± 2·4 | 42·6 ± 2·2 |
| Comparison with cell-bound* | — | † | ‡ |
| Activity decreasing with increasing pH | | | |
| pH range | 7·0–8·5 | 7·0–8·5 | 7·5–9·0 |
| Activity range | 98·0–82·0 | 96·6–63·0 | 96·6–63·0 |
| Regression coefficient ± S.E. | −10·7 ± 4·9 | −23·4 ± 3·0 | × 23·5 ± 3·0 |
| Comparison with cell-bound* | — | ‡ | ‡ |

* Significance of difference between regression coefficients.

Significance levels: † not significant at conventional 5% level but $0{\cdot}05 < P < 0{\cdot}10$; ‡ $0{\cdot}01 < P < 0{\cdot}05$.

For small pH values, the enzyme activities were similar but the cell-bound enzyme increased to 100% activity faster, the difference between the regression coefficients being significant at the 5% level. As the pH value increased further, the activity of cell-bound enzyme decreased from 100% more slowly than did the calculated (surface) pH value; this difference between the regression coefficients was again significant at the 5% level. The differences in enzymic activity at different pH values between free and cell-bound enzymes are therefore not explicable only in terms of calculated surface pH value.

Measurements of bulk-phase pH values were made at the beginning and end of the

electrophoretic mobility experiments to determine whether changes in bulk pH values could have contributed to the differences observed in the previous mobility experiments. It was found that, with free and cell-bound enzymes, over the range pH 3·9 to 8·0, the maximal difference between initial and final pH value was 0·1 unit. The differences in the curves shown in Fig. 2, therefore, cannot be attributed to differences in bulk-phase pH values.

To determine whether during the penicillinase assay all of the bound penicillinase was released from the *Bacillus subtilis* organisms into the suspending fluid, assays were done in buffers at pH 3·9, 6·75 and 8·0. Two lots of bacteria were used. (i) Bacteria were kept without penicillin in buffer at 30° for 30 min., then penicillin was added, and their penicillinase activity was compared after 30 min. with that of group (ii). (ii) Bacteria were incubated at 30° for 30 min. with penicillin, then centrifuged, resuspended, more penicillin added and the suspension incubated for a further 30 min. At the end of the respective incubation times the suspensions were assayed for residual penicillin. The enzyme activity remaining on the batch (ii) organisms expressed as percentage of the activity of the batch (i) organisms was 100% at pH 3·9, 65·5% at pH 6·75 and 86% at pH 8·0. The losses due to handling being taken into consideration, it would appear that nearly all of the bound penicillinase remained bound during its interaction with penicillin in the experiments so far described.

Experiments were done, in another laboratory with a fresh sample of enzyme, and with a 0·13M-buffer instead of 0·2M as described earlier. Some experiments were done in the presence of kaolin. The pH optima for free enzymic activity was about 1·0 pH unit lower than in the original experiments. A point to be examined was whether a difference could be observed as between free enzyme and that acting at a kaolin surface, and whether this difference corresponded with the difference calculated from measurements of the electrophoretic mobility of the kaolin particles. These experiments were made as a check on the calculations of surface pH value from measurements of electrophoretic mobility, and also to determine whether the penicillinase + penicillin system could be used to measure surface pH.

### *Experiments with kaolin*

The activity of penicillinase was determined in the presence and absence of kaolin particles. It was hoped that the enzyme adsorbed to kaolin particles would simulate 'cell-bound' enzyme. The assay of free penicillinase was carried out as before except that 2 ml. distilled water were added to each 4 ml. of buffer before warming. The assay of kaolin-bound penicillinase was carried out by adding 2 ml. of a 50% (v/v) suspension of kaolin in distilled water to each 4 ml. of buffer. (The volume of kaolin powder was measured in a measuring cylinder.) After warming, penicillin and later penicillinase were added as previously described. In all, 20 assays were made, each with its own control. Citric acid + sodium hydroxide buffers were used throughout the range pH 3·9–7·0. It was previously found that the presence of kaolin had no detectable effect on the titration of iodine solutions by sodium thiosulphate as in the Perret method.

In another series of experiments penicillinase was added to citrate buffer and suspended kaolin and incubated at 30° for 5 min. The mixtures were then centrifuged at about 3000*g* for 5 min. after which each deposit was resuspended in 4 ml.

of 0·2 M-citrate buffer + 2 ml. distilled water, and about 1 ml. kaolin powder was added to each supernatant fluid sample. Penicillin solution was added after 5 min. incubation, and an assay of residual penicillin made after a further 30 min. The enzymic activity was associated with the centrifuged deposit of kaolin and no enzymic activity was observed in the supernatant fluid, indicating that the enzyme was bound to the kaolin particles.

Two ml. of the 50 % (v/v) suspension of kaolin was incubated with 4 ml. citrate buffer (pH 3·9–6·95) and 1 ml. of penicillin or penicillinase solution for 5 min. at 30°. Samples of these suspensions were then introduced into the electrophoresis apparatus, and the electrophoretic mobilities of the kaolin particles measured; the results are shown in Fig. 1.

### *The results of experiments with kaolin*

The measured activities of the penicillinase in the presence ('bound') and absence ('free') of kaolin are shown in Fig. 3 together with the 'expected' kaolin curve calculated from the 'free' curve and the electrokinetic data. Without statistical analysis it can be seen that the activities of penicillinase are different over the pH range studied in the presence and absence of kaolin, and that the calculated curve for activity in the presence of kaolin fits the observed curve.

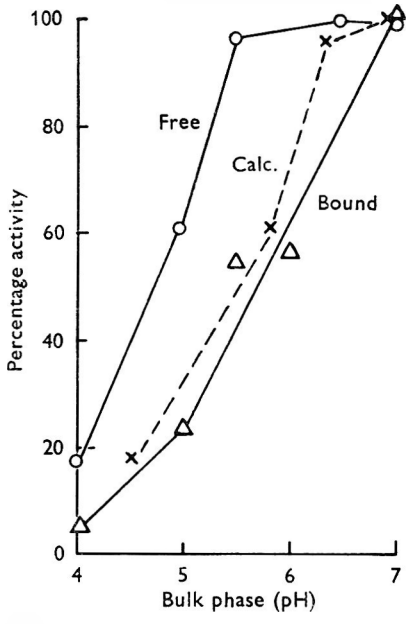


Fig. 3. Percentage penicillinase activity at different bulk-phase pH values, as observed in the 'free' state (○—○) when taking place in the presence of kaolin (△—△) and as calculated in the region of kaolin surfaces (× --- ×).

## DISCUSSION

A discussion of the underlying theory relevant to the concept of 'surface pH value' was given in a review by McLaren & Babcock (1959). Hartley & Roe (1940) proposed that as the local concentration of ions in the 'surface region' of a particle is

determined by its electrokinetic potential ($\zeta$), the 'surface' hydrogen-ion concentration will be

$$[H^+_{surface}] = [H^+_{Bulk}]\, e^{-\epsilon\zeta/kT}, \tag{1}$$

where $\epsilon$ is the electronic charge, $k$ is the Boltzmann constant and $T$ is the absolute temperature. The dissociation constant at the surface ($K_S$) is given by (Hartley & Roe, 1940).

$$K_S = K e^{-\epsilon\zeta/kT} = K e^{-F\zeta/RT}, \tag{2}$$

where $K$ is bulk thermodynamic dissociation constant, $F$ is the faraday and $R$ the gas constant.

At 30° equation (2) may be rewritten as

$$pH_S = pH_B + \zeta/57. \tag{3}$$

(Note that $\zeta$ has a negative value.)

Equation (3 may be transformed into the useful, experimentally determinable form from the Helmholtz–Smoluchowski equation which relates $\zeta$-potential to electrophoretic mobility ($V$).

$$V = \zeta D/4\pi\eta \tag{4}$$

(see Abramson, Moyer & Gorin, 1942), i.e.

$$\zeta = \frac{4\pi\eta}{D} V, \tag{5}$$

where $V$ is mobility towards the cathode in microns/sec/V./cm. of potential gradient, $D$ is the dielectric constant and $\eta$ the viscosity of the medium at the plane of electrophoretic shear.

Under the conditions of electrophoresis in the experiments described, and substituting for $\zeta$ in equation (3) as indicated in equation (5)

$$pH_S = pH_B + 0{\cdot}219\,V. \tag{6}$$

The calculated activity curves of penicillinase at surfaces in Figs. 2 and 3 were obtained by extrapolating the percentage activities of the enzyme in the free state to the pH calculated from equation (6).

The points to be discussed are concerned with the results of the experiments in which the enzyme activity curve obtained experimentally for enzyme bound to the surface of *Bacillus subtilis* differed in position from the curve obtained experimentally for free enzyme and from the calculated activity curve for enzyme located at the bacterial surface. The results with kaolin show that given a pH/activity curve for free penicillinase and the electrophoretic mobility of kaolin particles, it is possible to calculate the pH/activity relationship for penicillinase acting at a kaolin surface. These results also imply that from the aspect of enzyme activity, penicillinase acts in the same way in the bulk-phase as at the kaolin surface, since no allowance is made in the various equations for changes in the enzyme. The results of the experiments with kaolin lend additional support to the idea of using enzymes as molecular pH meters to determine the pH value at surfaces (McLaren & Babcock, 1959), although it must be emphasized that the values obtained are those for the sites at which enzyme activity occurs, and not for the 'surface' as a whole. From the data given, under the described experimental conditions, the pH value in the region of the relevant parts of the kaolin surfaces was about 0·85 pH unit less than in the bulk phase.

There is of course the possibility that kaolin-bound penicillinase is not a good model for penicillinase naturally bound at the surface of *Bacillus subtilis*; in the two situations the type of binding may be different, and this might modify the penicillinase and produce a change in the pH/activity relationships. Indeed in *B. cereus* there are two distinct moieties of penicillinase, that liberated and that bound to the cytoplasmic membrane, which apparently differ only in their tertiary configuration yet have strikingly different immunological and enzymic properties and different

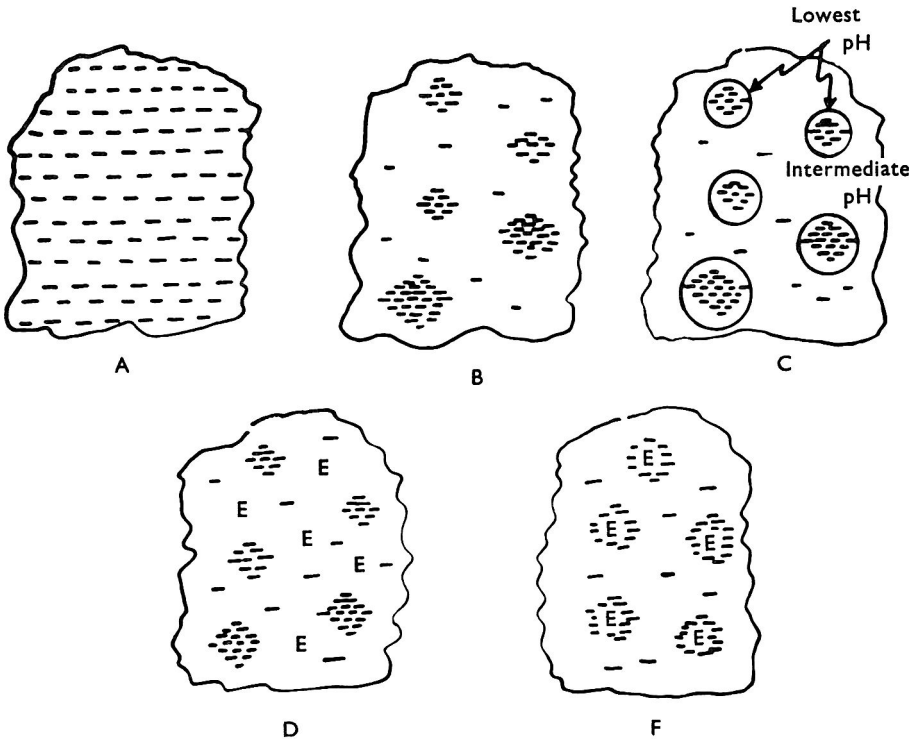


Fig. 4. Schematic representations of the hypothetical distribution of charged groups and bound enzymes (E) at parts of the cell surface. A. Uniform distribution of charged groups, electrophoretically indistinguishable from B, where there is a predominantly zonal distribution of groups resulting in C, zones of lowest pH value and separated by regions of pH values intermediate between 'lowest' pH value and bulk-phase pH value. D, distribution of enzymes relative to zones of lowest pH value and regions of intermediate pH values fitting the data from the experiments shown in Fig. 2. F, possible intrazonal distribution of enzymes to account for activity of surface enzymes in an environment of higher than optimal pH value.

pH/activity relationships. According to Garber & Citri (1962) the distinct properties of the cell-bound ($\gamma$) penicillinase are closely related to its binding to solid surfaces, including the cell envelope. Whatever the effect of binding, the pH/activity curve for penicillinase in the presence of kaolin is 'calculable', whereas the curve for cell-bound enzyme is not.

McLaren (1960) pointed out that Hartley & Roe's equation for surface pH value gives values at the electrophoretic plane of shear. This plane may be spatially different from the plane at which enzyme/substrate interactions occur.

Apart from these considerations the cause of the discrepancy between the observed and calculated pH/activity relationships of cell-bound penicillinase may be that the bound penicillinase is not superficially located at the surface of *Bacillus subtilis*, in the sense that it is deep to the plane of electrophoretic shear, the plane to which the various calculations apply. 'Deep' in the present discussion means inaccessible to the electrical double-layer of ions which follows the surface contours. If an enzyme be imagined as having its active centre in a 'pit' having a diameter less than that of a hydrated counter-ion, then this centre would not directly respond to surface pH value. If bound penicillinase were thus located, it is difficult to understand how it could react with penicillin at all while in the bound state. However, Kushner & Pollock (1961) showed that the cell-bound penicillinase is not free within the cytoplasm, since rupture of the cell envelope does not release it in a soluble state; up to 98% of the bound enzyme can be released from the apparently intact bacillus by trypsin, and up to 40% may be neutralized by specific antiserum.

Another possibility is that under the experimental conditions some of the bound penicillinase had been released and was acting in the free state at a greater pH value than the bound enzyme. Although the possibility that small quantities of enzyme act in this way cannot be ruled out, it seems unlikely that this was occurring to a significant degree, partly because the bacilli were used after washing, when only bound enzyme was present initially, and Kushner & Pollock (1961) have shown this to be firmly bound for short periods of time. Other experiments showed that in spite of handling losses an average of about 84% of the original penicillinase could be demonstrated as bound to bacilli during 30 min. following a preliminary 30 min. reaction with penicillin. During one 30 min. reaction with penicillin and with no intermediate washing it seems likely that much more than 84% of the bound enzyme would remain bound.

The last possibility to account for the differences between the observed and calculated pH/activity relationships of the bound-penicillinase is extremely speculative, but may be considered partly because it lends support to similar speculation about the mammalian cell surface (Weiss, 1962). It is well known that the electrophoretic mobility of particles $V$, is related to their charge density $\sigma$ by

$$V = \frac{\sigma}{\eta\kappa},$$

where $1/\kappa$ is the thickness of the Debye-Hückel electrical double-layer, which is very much less than the radius of curvature of the particle. Measurements of electrophoretic mobility only give an index of charge 'density' and not of charge 'distribution'. If the charged groups of *Bacillus subtilis* were all located at one point on its surface, then the organism's motion through fluid in the electrophoresis chamber would be complex. If, however, the ionogenic groups on the bacterial surface were arranged in zones or clusters having higher charge density than the spaces between them (Fig. 4B) this would be indistinguishable electrophoretically from a surface having uniformly distributed groups (Fig. 4A). It would be expected that the pH values in the region of the high charge-density zones would be lower than that in the intermediate spaces (Fig. 4C). The pH value in the region of the intermediate spaces would be lower than that in the bulk phase, owing to ion-induced dipole formation in the intermediate space leading in turn to increased local cation (including $H^+$

ion) concentration. If the bound penicillinase were located in the intermediate spaces as shown in Fig. 4D, then it would be expected that activity/pH curves would show it to be reacting in a region of smaller pH value than the bulk-phase, but of greater pH value than that calculated on a basis of equal surface-charge distribution. In fact, these expectations are realized, as shown in Fig. 2. If there is any truth in this speculation it may be that other enzymes exist at the cell surface located in the postulated zones of high charge density (Fig. 4F). When examined by the type of experiments described here it might be expected that bound-enzymes, in such a location, would behave as though they were at a smaller pH value than that calculated from measurements of electrophoretic mobility. With mammalian cells such an 'intrazonal' localization might perhaps explain how enzymes with very small pH optima such as the lysosomal acid hydrolases (de Duve, 1959) can function at cell surfaces at 'physiological' bulk-phase pH values.

The techniques described offer the possibility not only of the measurement of pH in regions of the cell surface, but also for the localization of enzymes in relation to other structures.

My sincere thanks are due to Dr M. R. Pollock for his many helpful suggestions and discussions and for providing me with laboratory space at the National Institute for Medical Research during the experiments with bacteria. The measurements of electrophoretic mobilities were carried out by Mr N. G. A. Miller (in Dr A. D. Bangham's laboratory, A. R. C. Laboratories, Babraham), and Dr G. V. F. Seaman (Department of Medicine, Cambridge). I received valuable technical assistance and advice from Miss Joan Fleming. I am grateful to all of these people. I am especially indebted to Dr J. G. Rowell of the A.R.C. Statistics Group for his analysis of the data.

The author is a member of the external scientific staff of the Medical Research Council.

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# The Genetic Basis of Actidione Resistance in Neurospora

By K. S. HSU

*Department of Biological Sciences, Stanford University, Stanford, California, U.S.A.*

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SUMMARY

Seven actidione-resistant mutants recovered from separate strains of *Neurospora crassa* are due to mutations of single genes at either of two loci: *act-1* (located between *nit-1* and *aur* in linkage group I) and *act-2* (located close to *sp* in linkage group V). Mutant alleles at both loci are dominant over their wild-type alleles in heterokaryons. Both genes are located in well marked chromosome arms, and scoring is distinct.

## INTRODUCTION

Antibiotic-resistant mutants have been used routinely in bacterial genetics since Demerec (1945) demonstrated the mutational origin of penicillin resistance in *Staphylococcus aureus*. However, no such mutants have been reported in *Neurospora crassa*. The usefulness of drug-resistant mutants for genetic studies in such fungi as *Aspergillus nidulans* (Roper & Käfer, 1957), and the recent finding that Neurospora mutants resistant to the acridine dye acriflavine are controlled by chromosomal genes which can be used as markers, initiated the attempt to test several categories of drugs, including a number of antifungal antibiotics, with the aim of recovering useful markers. Mutants resistant to actidione have been found. The genetic basis of resistance and sensitivity to this antibiotic is presented in this paper. (A preliminary account of some of these results has been given [Hsu, 1962, and *Neurospora Newsletter*, **1**, 5].) Actidione resistance due to genic difference has previously been reported in *Saccharomyces cerevisiae* (Middlekauff *et al.* 1957) and it was on certain yeasts that actidione was first shown to have high toxicity (Whiffen, 1948).

## METHODS

Throughout the experiments, media containing actidione (Upjohn) were sterilized by autoclaving for 10 min. at 121°. Conidiating colonial strains were selected for use as the starting strains. They were either double mutants or triple mutants, all in the background of St Lawrence stocks 74A and 73a. The genotypes of these strains and the isolation numbers of the alleles involved are listed in Table 1. The wild-type strains used in the present study were STA 4, a vegetative reisolate of 74A, and Pa, derived from St Lawrence stocks by Perkins (1959).

For isolation of mutants, conidia from the sensitive strains were spread on the surface of medium N (Vogel, H. J., *Microb. Genet. Bull.* **13**, 42) containing 10 $\mu$g. actidione/ml., which was supplemented with 1% sucrose and solidified by 1·5% Difco agar. The actidione concentration was the lowest at which no growth was observable when $10^6$ to $10^7$ conidia from the sensitive strains were spread on plates

of graded concentrations of the antibiotic. The plated conidia were exposed to ultraviolet irradiation giving 50–75% killing. Resistant colonies were selected after 5 or 6 days at 25°. Whenever more than one colony was found on the plates receiving conidia from the same strain, only one colony was selected to ensure that the mutants obtained had arisen from separate mutational events.

Each putative mutant was progeny tested by crossing with a standard wild-type strain. Ascospores were collected as unordered tetrads (Strickland, 1960) in order to detect whether the mutant strain was homokaryotic for the mutant allele.

The techniques used for crossing, for isolating random spores, and for linkage detection were similar to those used by Perkins (1959). Dry-weight assays (Ryan, 1950) were employed with modifications to measure quantitatively the sensitivity of different homokaryotic and heterokaryotic strains to this antibiotic. Conidial suspensions were inoculated into 125 ml. Erlenmeyer flasks containing 20 ml. of medium N with or without actidione. Dry weights were measured after four days at 25°.

## RESULTS

*Position of loci.* Seven actidione-resistant strains of independent origin were isolated and subjected to transmission tests. In each case, a 2:2 segregation for resistance and sensitivity was demonstrated in individual unordered tetrads obtained from a mutant × wild-type cross, where two or three known, visible markers were also segregating. It appeared in each case that resistance had arisen through the mutation of a single Mendelian factor. Subsequent tests assigned the seven mutants to two independent loci: *act-1* and *act-2*. The assignment was based on the localization of the allele KH 52 in linkage group I and KH 53 in group V, and by subsequent tests of the remaining mutants with these two for allelism. Two mutants were tentatively considered to be allelic, if no wild type was observed within a small population taken from the progeny of a mutant × mutant cross. (The population sizes were 37, 62, 40, 46 and 29 of the crosses KH 51 × KH 52, KH 54 × KH 52, KH 56 × KH 53, KH 57 × KH 52, and KH 58 × KH 52, respectively.) Table 1 gives the locus to which each mutant was assigned, and the original strain from which each mutant was isolated.

Table 1. *Loci and strains of origin of actidione-resistant mutants*

| Isolation no. | Locus | Strain of origin |
|---|---|---|
| KH 51 | *act-1* | *cr(B123): bal(B56)A* |
| KH 52 | *act-1* | *cr(B123): cot(C102)A* |
| KH 53 | *act-2* | *cr$^{L}$: cot(C102): ylo(Y30539y)-1* |
| KH 54 | *act-1* | *cr(B123): bal(B56)a* |
| KH 56 | *act-2* | *cr(B123): ylo(Y30539y)a* |
| KH 57 | *act-1* | *cr$^{L}$, nit-1(34547), aur(34508)a* |
| KH 58 | *act-1* | *cr(B123), rg(B53)a* |

*Linkage data* Linkage data of *act-1* and *act-2* based on three-point crosses are given in Table 2. The data indicated that the gene *act-1* is located between *nit-1* (nitrate-1) and *aur* (aurescent) in the right arm of linkage group I, and that *act-2* is close to *sp* (spray) in the right arm of linkage group V.

*The double mutant.* The double mutant was synthesized by crossing *act-1* (KH 52) × *act-2* (KH 53). A 3:1 ratio for the resistant and sensitive phenotypes was

observed in the progeny of such a cross, as expected on the basis of independent assortment of genes at these two loci. Among the resistant segregants, one-third were apparently slower in growth in the minimal slants as compared with the

Table 2. *Three-point data on random segregants from crosses involving* act-1 *and* act-2

| Zygote genotype and recombination percentage | Parental combinations | Recombinations: Singles, region 1 | Recombinations: Singles, region 2 | Recombinations: Doubles, regions 1 and 2 | Total and percentage germination | Marker isolation number |
|---|---|---|---|---|---|---|
| + *act-1* + / *cr* + *aur*<br>17·5 17·5 | 24<br>21 | 3<br>4 | 5<br>2 | 3<br>1 | 63<br>63 % | B 123<br>KH 52<br>34508 |
| + *act-1* − / *nit-1* + *aur*<br>6·3 4·7 | 33<br>24 | 2<br>2 | 1<br>2 | 0<br>0 | 64<br>64 % | 34547<br>KH 52<br>34508 |
| + *act-2* + / *lys-1* + *inos*<br>25·7 14·3 | 39<br>47 | 15<br>19 | 9<br>9 | 1<br>1 | 140<br>72 % | 33933<br>KH 53<br>37401 |
| + *sp* *inos* / *act-2* + +<br>3·1 6·2 | 42<br>47 | 1<br>1 | 3<br>2 | 1<br>0 | 97<br>32 % | KH 53<br>B 132<br>37401 |

Numbers of progeny are given in the body of the table. The numbers in the top row of each cross represent the genotypes which contain the plus allele of the left-most marker. Regions are numbered from left to right, and isolation numbers in the last column are listed in the same order. In the last cross the order of *act-2* and *sp* is arbitrary. For descriptions of markers, see Perkins (1959) and Barratt, Newmeyer, Perkins & Garnjobst (1954).

Table 3. *Test crosses identifying double mutants from the cross* act-1 (KH 52) × act-2 (KH 53)

| Cross | Type of cross | Progeny class*: A | B | C |
|---|---|---|---|---|
| 1 | Normal-growing, resistant × sensitive | 28 | 0 | 22 |
| 2 | | 27 | 0 | 28 |
| 3 | | 25 | 0 | 37 |
| 4 | | 34 | 0 | 30 |
| 5 | | 26 | 0 | 32 |
| 6 | | 23 | 0 | 25 |
| 7 | | 38 | 0 | 32 |
| 8 | Slow-growing, resistant × sensitive | 35 | 17 | 14 |
| 9 | | 33 | 24 | 11 |
| 10 | | 26 | 16 | 10 |

* A = Resistant to actidione (10 μg./ml.), normal growth in the minimal slants.
B = Resistant to actidione (10 μg./ml.), slow growth in the minimal slants.
C = Sensitive to actidione (10 μg./ml.), normal growth in the minimal slants.

remaining two-thirds, and with the sensitive segregants. In order to separate the double mutant from the single mutants, seven normal-growing, resistant types and three slow-growing, resistant types were backcrossed to the wild types (Table 3). While the former type of crosses gave 1:1 segregation for resistance and sensitivity and no slow-growing segregants, the latter type gave 3:1 segregation for resistance

and sensitivity, with a recurrence of the slow-growers constituting one-third of the resistant types, indicating that the slow-growing, resistant types are double mutants. The slow-growing segregants were also observed among the progeny of crosses involving other combinations of alleles at *act-1* and *act-2* (KH 52 × KH 56, KH 54 × KH 53, KH 57 × KH 53, KH 58 × KH 53). All the slow growers were resistant, and, in all the cases, they represented about one-fourth of the progeny, as expected if slow growth is characteristic of the double mutant.

Table 4. *Mycelium dry weights (mg) of four actidione genotypes on medium N with different concentrations of actidione**

| Genotype | Actidione concentration (μg./ml.) | | | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | 0 | 0·1 | 0·2 | 0·3 | 0·4 | 0·5 | 1 | 2 | 4 | 10 | 25 | 50 | 100 | 200 | 300 | 400 | 500 |
| Wild-type | 90 | 64 | 43 | 28 | 22 | 18 | 9 | 3 | 0 | 0 | — | — | — | — | — | — | — |
| *act-1* | 93 | — | — | — | — | — | — | — | — | 56 | 34 | 22 | 12 | 10 | 3 | 2 | 1 |
| *act-2* | 88 | — | — | — | — | — | — | — | — | 71 | 38 | 24 | 14 | 10 | 3 | 2 | 2 |
| *act-1; act-2* | 16 | — | — | — | — | — | — | — | — | 20 | 17 | 18 | 21 | 16 | 15 | 14 | 13 |

* Values are averages for three replicate flasks.

*Sensitivity of homokaryons.* The scoring for resistance and sensitivity was carried out on minimal slants into which actidione had been added at the concentration of 10 μg./ml. A clear-cut qualitative difference could be detected this way without paying special attention to the size of inoculum or time of scoring, since the sensitive strains did not adapt appreciably. This was true when either *act-1* or *act-2* strains were compared with the wild-type strains. Between *act-1* and *act-2*, however, there appeared to be little difference in sensitivity to the antibiotic. Quantitative measurement was accomplished by dry-weight assays in a series of actidione concentrations. In addition to wild type (Pa) and the single mutants (KH 52 and KH 53), the double mutant (KH 52; KH 53) was also tested (Table 4). The results showed that while the concentrations the single mutants can withstand are about the same, they are roughly a hundred times higher than those allowing detectable growth of wild type. The double mutant produces approximately as much mycelium in the presence of actidione as in the absence within the concentration ranges tested. The low mycelium production in liquid minimal medium by the double mutant corresponds to its slow growth on minimal agar.

*Sensitivity of heterokaryons.* Three types of heterokaryons were made: (1) *act-1* with +, (2) *act-2* with +, and (3) *act-1* with *act-2*. The alleles KH 52 and KH 53 were used. The auxotrophic alleles used in establishing all three types of heterokaryons were *pan-1* (pantothenic-1, 5531), *inos* (inositol, 37401), and *nt* (nicotinic-tryptophan, C 86). The component nuclei of some heterokaryons were also marked with either *al-2* (albino-2, 15300) or *ylo* (yellow, Y 30539y).

The sensitivity of any particular heterokaryon was tested on minimal slants containing actidione. It was found that both *act-1* with + and *act-2* with + heterokaryons consistently expressed the resistant phenotype when they were tested on actidione. This suggested that both *act-1* and *act-2* are dominant over their corresponding wild-type alleles. The dominance was further supported by the quantitative data obtained from dry-weight assays. As shown in Table 5, the heterokaryons

of *act-1* with + and of *act-2* with + produced almost as much mycelium in actidione as did the *act-1* and *act-2* homokaryons.

The *act-1* with *act-2* heterokaryons were resistant in phenotype, as would be expected if the mutant genes constituting these heterokaryons are dominant. This demonstrates that at least one of the two mutant genes shows a high degree of dominance, i.e. that it is not just partially dominant over its + allele.

Table 5. *Production of mycelium by various actidione genotypes in medium N with and without actidione*

| Genotype | Phenotype† | Dry wt. of mycelium (mg.) in minimal medium* Without actidione | With 10 μg./ml. actidione |
|---|---|---|---|
| Homokaryons | | | |
| Wild-type | S | 113 | 0 |
| *act-1* | R | 108 | 85 |
| *act-2* | R | 110 | 86 |
| *act-1; act-2* | R | 18 | 18 |
| Heterokaryons | | | |
| *act-1*; *pan-1(a)* + *al-2*; *inos(a)* | R | 103 | 73 |
| *act-1*; *nt(A)* + *al-2*; *pan-1(A)* | R | 106 | 80 |
| *act-2*; *pan-1(a)* + *ylo*; *inos(a)* | R | 101 | 75 |
| *act-2*; *pan-1(a)* + *al-2*; *inos(a)* | R | 107 | 71 |
| *act-1*; *nt(A)* + *act-2*; *ylo*; *inos(A)* | R | 95 | 72 |
| *act-1*; *nt(a)* + *act-2*; *pan-1(a)* | R | 95 | 73 |

* Values are averages for two replicate flasks.

† As scored in 3 inch minimal slants containing 10 μg./ml. actidione. S = Sensitive, R = resistant.

## DISCUSSION

The occurrence of a dominant mutation within a single nucleus in a multinucleate conidium produces a heterokaryotic conidium capable of giving rise to a culture with the dominant phenotype. In the present study, heterokaryotic conidia containing both resistant and sensitive nuclei can be picked up from the actidione plates because the alleles for resistance are dominant. On the other hand, if the mutant allele for resistance were recessive, the presence of such an allele in a multinucleate conidium would be phenotypically sensitive, and would, therefore, not be able to grow on the selective medium containing the antibiotic. The method used thus appears to favour the recovery of dominant, resistant mutations. The recovery of recessive mutant alleles for resistance on the selective medium would be possible under the condition that in a single conidium only the nucleus carrying the mutant allele survived u.v.-irradiation. The present result that resistant mutants are dominant does not, therefore, justify the conclusion that recessive mutants conferring resistance do not occur. In fact, recessive resistant mutants are not uncommon. For example, one of the three mutant alleles for acriflavine resistance reported in *Aspergillus nidulans* is a recessive allele (Roper & Käfer, 1957), and, by use of heterogenotes, phage resistance (Lederberg, 1949) and streptomycin resistance (Lederberg, 1951) in *Escherichia coli* have been shown to be recessives.

The mutant genes at both the *act-1* and *act-2* loci are phenotypically indistinguishable. The dominant nature of these mutants prevents testing for allelism in heterokaryons. The applicability of such dominant alleles for resistance to the automatic selection of mitotic segregants arising from diploid nuclei heterozygous for them, if a parasexual cycle does exist in *Neurospora crassa*, would depend upon whether the vegetative segregants can be distinguished from the diploid heterozygotes with respect to level of resistance. Such dominant mutant alleles would not give rise to the pseudo-wild types described by Mitchell, Pittenger & Mitchell (1952) and by Pittenger (1954), since, if the presumed heterozygous disomic strains do arise from a cross, they would be mutant in phenotype.

The presence of at least one wild-type allele at either one of the two actidione loci in a single nucleus appears to be essential for normal growth in the absence of actidione, although when both wild-type alleles are substituted by the mutant alleles, a higher degree of resistance results. This is inferred from the observation that the double mutant is slow-growing while the heterokaryon *act-1*+*act-2* is not.

The actidione-resistant mutants should prove to be useful markers in recombination studies, for they are located in well-marked chromosome arms (Perkins, 1959; Strickland, Perkins & Veatch, 1959), and the scoring for the difference in resistance and sensitivity is clear-cut. Their biochemical and physiological actions, and usefulness in other respects, largely remain to be explored.

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# An Analysis of the Vi-Phage Typing Scheme for *Salmonella typhi*

By A. BERNSTEIN* and E. MAUREEN J. WILSON
*Enteric Reference Laboratory, Public Health Laboratory Service, Colindale Avenue, London N.W. 9*

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SUMMARY

The seventy-two currently recognized Vi-phage types of *Salmonella typhi* have been examined in order to determine, where possible, their structural formulae. The genetic nature of the changes in host range of the corresponding typing adaptations of the Vi-phage II has also been investigated.

Thirty-seven temperate phages have been isolated from the type strains and type-determining ability has been demonstrated in all but five of these phages. Several of the determining phages were found in more than one type strain and it is probable that no more than 16 different determining phages are represented in our collection. Because there are fewer phage reactions to be eliminated in cultures already possessing a restricted sensitivity spectrum to the typing adaptations of Vi-phage II, some of the determining phages ('semi-determining phages') display their full effect only in such cultures.

One determining phage, d1-C, can co-operate with others, especially phages d8 and d9, in multiply lysogenized cultures to produce an effect which is more than a summation of the known type-determining powers of the individual phages concerned. Moreover, the type-determining effects of phages d1-C, d8, and d9 in singly or doubly lysogenized cultures are influenced by the temperature of incubation.

As has been shown by earlier workers, the changes in host-range of Vi-phage II that produce the Vi-typing phages may be phenotypic (host-induced) or genotypic (mutational) in nature, or a combination of these two types of variation. Amongst the current typing phages are represented eighteen different phenotypic modifications and forty-three different host-range mutants. Many of these mutations seem to be related to each other but their chromosomal distribution has not been mapped.

## INTRODUCTION

Vi-phage typing of *Salmonella typhi* has been in routine use for more than 20 years and has continued to justify the initial hopes that were placed in it as an epidemiological tool in the study of typhoid fever (Craigie & Felix, 1947). The typing scheme in current international use contains 72 types of *S. typhi* and their diagnostic reactions with the various typing adaptations of Vi-phage II are shown in Table 1. In this paper, the expressions type, phage-type and Vi-type refer to the bacterial strains recognized by the Vi-typing phages. Similarly, Vi-phage, Vi-

* Present address: Department of Microbiology and Immunology, Marquette University School of Medicine, Milwaukee, Wisconsin, U.S.A.

typing phage and typing phage refer to the typing preparations adapted from Vi-phage II.

In the Vi-phage-typing scheme for *Salmonella typhi*, the phage types of the organism and the corresponding adaptations of Vi-phage II carry the same symbol. Thus, Vi-type A is lysed by phage A, type B1 by phage B1 and so on. In order to avoid confusion, Anderson (1962*a*) recently adopted the practice of italicizing the symbol of the typing phage, and we shall follow this convention.

All the Vi-typing phages are descended from the original Vi-phage II of Craigie & Yen (1938). Anderson (1962*a*) has analysed the genetic mechanisms which underlie the processes of Vi-phage adaptation to, and Vi-type determination in, the various phage-types of *Salmonella typhi*. This analysis is based on several past investigations and in particular those of Anderson & Fraser (1955, 1956). The original paper of Anderson (1962*a*) should be consulted for a fuller account and for a complete list of references, but for our present purposes we summarize it here.

It has been shown (Anderson & Fraser, 1955, 1956) that the Vi-typing preparations fall into four genetic categories: the wild-type of Vi-phage II which is phage *A*; preparations that have undergone only host-induced or phenotypic modification (Luria & Human, 1952; Anderson & Felix, 1952, 1953*a*; Luria, 1953; Bertani & Weigle, 1953; Weigle & Bertani, 1953); those that are pure host-range mutants; and those that represent a combination of phenotypic and mutational changes.

The wild-type of Vi-phage II lyses only type A. This type is also sensitive to all the typing adaptations of Vi-phage II and is regarded as the most primitive Vi-type of *Salmonella typhi*.

Vi-type specificity in *Salmonella typhi*, that is the pattern of reactions with the typing adaptations of Vi-phage II, is in many instances partly dependent on lysogenicity in the types concerned (Craigie, 1946; Felix & Anderson, 1951; Anderson, 1951; Anderson & Felix, 1953*b*), and the phages having this function are called type-determining phages. The remainder of the phage sensitivity in these lysogenic types is controlled by their non-lysogenic ancestors. As the types can thus be regarded as consisting of two components, the non-lysogenic precursor and the determining phage, Anderson & Fraser (1955) suggested that they could be allotted structural formulae consisting of the symbol of the non-lysogenic precursor type followed by that of the determining phage in parenthesis; thus type 29, which is type A carrying phage f2, is structurally A(f2); type F2 is type F1 carrying the same phage, and is structurally F1(f2); type D6 is type A carrying phage d6 and is therefore A(d6); and so on. The structural formulae are useful in the study of this typing system, because they show relationships between types that may not be indicated by the symbols in routine use, and also, as will be seen, give information on the probable genetic nature of the corresponding typing adaptations of Vi-phage II.

Lysogenization of precursor types with determining phages has yielded types identical with those found in nature; and spontaneous loss of determining phages by naturally occurring lysogenic types has revealed the presence of the precursor types they had been postulated to possess (Anderson & Felix, 1953*b*; Anderson & Fraser, 1955). These findings indicate the validity of the structural formulae and suggest the mechanism of formation of the lysogenically determined types in nature.

The commonest Vi-types of *Salmonella typhi* are A, C1 and E1. These are non-lysogenic, and it has been found that they are also the commonest precursors of lysogenically determined types. Anderson & Fraser (1955) showed that typing preparations of Vi-phage II that had undergone a purely phenotypic change were those adapted to non-lysogenic types such as A, C1 and E1. The typing phages consisting of pure host-range mutants of Vi-phage II were those adapted to lysogenically determined types whose bacterial precursors were type A. The phages combining phenotypic with mutational change were those adapted to lysogenically determined types whose precursors were types other than A, such as C1 or E1. Certain types in which lysogenicity could not be demonstrated were lysed only by host-range mutants of Vi-phage II; others were lysed by phages in which phenotypic change was combined with mutation.

The examination of the host-range of a typing phage may suggest to which category the phage belongs. A pure host-range mutant will in general lyse type A, the homologous type strain and possibly a few other types sensitive to the mutation. Its host-range will be unaltered after growth in type A. The pure host-induced modification will almost always attack only type A and the type strain to which it has been adapted. After one cycle of growth in type A it will retain only the ability to lyse type A, that is, it will have reverted to phage *A*. A phage which is a phenotypic modification of a host-range mutant will lyse type A, the homologous type strain, and any type sensitive to the basic mutation. It will also lyse any precursor types such as C1 or E1, which happen to be represented in its homologous type. Growth of such a phage in type A will lead to loss of the ability to attack both the homologous type strain and the precursor type, but not of that to lyse type A and the cultures sensitive to the basic mutation. If no cultures exist which are sensitive to the basic mutation it may be difficult to distinguish this type of phage from a pure phenotypically modified one. However, when the precursor is known this distinction may be made by regrowing the phage which has been propagated on type A in the precursor type. Under these circumstances a phage which was originally a pure host-induced modification will lyse only type A and the precursor type, whereas a phage which was a phenotypic modification of a host-range mutant will have regained all its original potentialities. It may also be possible in some cases to lysogenize type A with a determining phage isolated from the strain to which the Vi-phage had been adapted and so to produce a culture which is resistant to phage *A* but sensitive to both the original phage and that resulting from its growth in type A. This shows that the phage grown on type A has not reverted to phage *A* but must be a host-range mutant of Vi-phage II.

Types E1, E2, E5, and E8 and their corresponding typing phages provide a convenient illustration of the way in which some of the combinations of phenotypic and mutational changes in host-range of Vi-phage II may be characterized. These four types are all members of the E group of the Vi-typing scheme (Table 1) but only E8 is known to be lysogenic. Type E1 is one of the common precursor types and typing phage *E1* has been shown to be a pure phenotypic modification of Vi-phage II (Anderson & Felix, 1952, 1953*a*; Anderson & Fraser, 1955, 1956). Growth of phage *E1* on type A produces a phage indistinguishable from phage *A*.

Type E8 carries a phage identical with phage 26′, which is the determining phage of types 26 and C8. The typing phage *E8* lyses types A, E1, and 26 in addition to

E8. After growth in type A(→*E8*/A) this phage resembles phage *26* and lyses only types A and 26. Fractional symbols such as *X*/Y are used to designate the phage produced by the growth of phage *X* in type Y. The phage resulting from the further propagation of *X*/Y in type Z would be designated *X*/Y/Z, and so on. Growth of phage *E8*/A on type E1, or of phage *26* on type E1, produces a phage indistinguishable from phage *E8*. The host-range of *E8* therefore appears to be due to two changes. One is mutational and is identical with the mutation represented in phage *26* whereas the other is phenotypic and is the same as that in phage *E1*.

Typing phages *E2* and *E5* both lyse types A and E1 in addition to their respective type strains. Phage *E5* also lyses type 32 for reasons at present unknown. After growth on type A (→*E2*/A and *E5*/A respectively) both phages lyse only type A and thus appear to have reverted to phage *A*. However, growth of phage *E5*/A in type E1 gives phage *E1*, whereas growth of *E2*/A in type E1 produces phage *E2*. From this it follows that *E2*/A is not identical with phage *A*, but must represent a host-range mutant of Vi-phage II for which there is at present no indicator strain available other than type A. Phage *E2* is, therefore, a phenotypically modified host-range mutant, the phenotypic change being identical with that in phage *E1*. Phage *E5*, on the other hand, represents a pure phenotypic modification of host-range of Vi-phage II, although the lytic spectrum of phage *E5* is rather wider than is usual with this class of adaptation.

Anderson & Fraser (1955) investigated the various Vi-types of the typing scheme as it then existed and it is from their observations that the principles given above have been developed. At the time of their survey the number of phage types was thirty-three. The number of types has now increased to seventy-two and it was considered worth while, therefore, to record the results of the investigation of the many new types which have been discovered since 1955. In the course of the survey some new determining phages have been isolated and new facts discovered about some of those previously known.

Our techniques, which have developed from those of Anderson & Fraser (1955), are based on the principles presented above and are designed to elicit as much information as possible about the type strains and their typing phages.

## METHODS

*Media.* The liquid medium was Bacto dehydrated nutrient broth (Difco Laboratories) used at a strength of 2% (w/v) in distilled water with 0·85% (w/v) added NaCl.

For solid medium New Zealand powdered agar was added to a final concentration of 1·3% (w/v). The surface layer of agar layer plates contained only 0·4% (w/v) agar.

*Phage-typing.* The methods given by Anderson & Williams (1956) were used for the phage-typing of all cultures. Unless otherwise stated, the phages were used at their Routine Test Dilution (RTD), that is, the highest dilution producing confluent lysis of the homologous type strain. The temperature of incubation was 38·5° unless specifically mentioned.

*Phage titrations.* All phage preparations were titrated by the spot technique. For more accurate assays agar layer titrations were performed (Adams, 1959).

'*Phage sterilization*'. Phage preparations were rendered bacteriologically sterile by one of three techniques All Vi-phage II 'adaptations' were heated in a water

bath at 57° for 40 min. Some temperate phages were also treated in the same way. In many cases, however, the temperate phages were found to be labile at this temperature; they were then sterilized either by treatment with toluene (Anderson & Felix, 1953*b*) or by filtration through sintered glass (a.p.d. 1·2–1·4 $\mu$).

*Determination of lysogeny.* Our usual method for the detection of lysogeny was to inoculate *c.* $10^8$ organisms each of type A and of the strain under examination jointly into 20 ml. of pre-warmed broth. This mixed culture was incubated at 37° with agitation. The supernatant of the culture was examined after 5 and 17 hr. incubation for evidence of phage activity. The indicator strains used for this purpose were type A, the strain under survey, and one or two other selected types. The examination was made by the usual spotting technique and plates were incubated at 36·5° and 38·5° overnight.

If the preparation showed evidence of phage activity, a small portion (*c.* 2 ml.) of the lytic supernatant was heat-sterilized and the original and heated preparations were titrated in parallel on type A and any other sensitive indicators. A phage which was found to be heat-labile was sterilized by toluene treatment or filtration.

Heat-stable phages were purified by cutting several single plaques from the titration on type A. Care was taken to remove as little of the surrounding culture as possible and the plaque was emulsified in 3 ml. of broth which was then heat-sterilized. This preparation was again titrated on type A and similar single-plaque selection was made from at least one more titration. In cutting plaques from the final titration, some of the surrounding culture was included in the pick and the whole was emulsified in 20 ml. pre-warmed broth. The inoculated broths were incubated at 37° for 5 hr. and the supernatants of these final preparations were sterilized by heat. Purification and propagation of heat-labile phages were essentially the same except that sterilization was by either filtration or the use of toluene. Some cultures proved to be doubly lysogenic, and in such cases it was usually found that one of the carried phages was stable and the other labile at 57°. The phages isolated from such cultures appeared to be partially heat-sensitive, because of the elimination of the heat-labile component at 57°. This provided an easy method of obtaining pure lines of the heat-stable phage. Titrations of filtered preparations of such mixed phages were carefully explored for diversity of plaque types, in order to identify those of the heat-labile phage.

During the isolation of temperate phages, the various preparations were carefully examined at each stage of plaque selection for evidence of heterogeneity with respect to both plaque morphology and host-range. If such heterogeneity was found, single-plaque selection was made of representatives of each type of variation.

With some temperate phages 'induction' of the lysogenic culture by ultraviolet light was found to improve the phage yield.

*Purification and propagation of typing phages.* The phage concerned was titrated on a suitable host strain and single plaques were emulsified in 3 ml. broth. If the titration showed heterogeneity of plaque morphology, representatives of each plaque type were chosen. The broth preparations were sterilized by heat and titrated on the same host strain and on any other strains which appeared to be suitable indicators of host-range. Further single plaque selections were made and titrated as before. Finally, single plaques representing all of the different plaque and host-range variants were picked and emulsified in 20 ml. pre-warmed broth which was in-

cubated at 37° with agitation until lysis occurred. If lysis was not apparent the incubation was interrupted at 5 hr. The suspensions were centrifuged at 1500*g* and sterilized by heating to 57° for 40 min.

In order to obtain higher titre lysates propagation was also carried out by the agar-layer technique (Swanstrom & Adams, 1951) after initial single-plaque purification.

*Adaptation of Vi-phage II.* The phage to be adapted was titrated on the selected strain and single plaques, if any, were transferred to 3 ml. broth, sterilized by heat, and examined for change in host-range. Further single-plaque selection and final propagation were performed as in the preceding section. In this paper the term 'de-adapted' will be used to refer to any phage, other than *A*, which has been grown on type A. Propagations of a phage on types other than its homologous type will be referred to as 'adaptations'.

*Characterization of host-range of typing phages.* The host-range of the Vi-phages was usually ascertained at the RTD. This dilution is chosen so as to minimize the number of cross-reactions on strains other than the homologous type. The statement that phage *X* does not lyse type Y may only be true, therefore, at the RTD or higher dilutions.

The RTD of a given phage is influenced by the absolute content of plaque-forming particles in the preparation, by the size of the plaques formed by the phage on its type strain, and by the efficiency of plating (EOP) of the phage. The interaction of these factors is discussed later.

The EOP of a phage is a measure of its plaque-forming ability on a given strain relative to some other, usually higher, estimate of phage content (Adams, 1959). For the purposes of this paper, the EOP of a phage on type X is the ratio of plaque count on type X to that on type A. The maximum possible value of the EOP is 1.

*Lysogenization.* Sterilized, undiluted preparations of the various single-plaque lines of the phage concerned were spotted on a lawn of the strain to be lysogenized. After overnight incubation the secondary growth from the central lysed area was inoculated into 20 ml. pre-warmed broth, which was incubated overnight. Serial passages were then made by inoculating 1 ml. of culture into 20 ml. of fresh broth. This was continued for several passages. Subcultures of each passage were made on Dorset's egg medium and retained for reference purposes. Each stage was examined to detect any loss of sensitivity to the lysogenizing phage. Tests were also made of the ability of the cultures to release the temperate phage used for lysogenization and for any change in reaction of the host strain to the typing adaptations of Vi-phage II. For the latter test a restricted range of typing phages was usually sufficient. When these tests suggested that lysogenization had occurred, the culture was plated and several single colonies picked and tested. Serial single-colony selection was carried out until the lines chosen appeared to be stable and homogeneous. The final single-colony lines were tested with all the typing phages in RTD.

In some cases, especially where plaques were minute, secondary growth could not be harvested from surface phage titration. In such cases agar-layer titrations were performed with phage concentrations sufficiently high to yield confluent lysis. Lysogenized secondary growth was usually easy to obtain from these titrations.

## RESULTS

The investigation of various types, including new types, was conducted according to a uniform plan. This derives from the principles outlined earlier and may conveniently be divided into three main stages.

*Stage I.* The type strain was phage typed in the routine way using all the existing typing phages, and, in addition, for the new types, the homologous adapted preparations. The host-range of the typing phage was determined, using all the accepted phage types and the new type.

*Stage II.* Phage *A* was titrated on the type strain and attempts were made to propagate any plaques detected. If no reaction was detected with phage *A*, similar attempts were made with other known typing phages, especially any which had shown evidence of an ability to lyse the new type.

When it was possible to isolate a temperate phage from the new type, this phage was propagated and several clonal lines were used to lysogenize a variety of cultures. These always included type A, one or more of the common precursor types (such as C1, E1, F1), and also any possible precursor types which showed evidence of sensitivity to the new typing phage.

The new typing phage was titrated on the newly lysogenized strains and attempts were made to adapt it to them. It was also de-adapted by growth on type A and then re-adapted to several of the precursor strains.

*Stage III.* A full cross-test was performed using, in addition to all the phages mentioned in stage I, all the adapted preparations used in routine typing, together with other typing phages obtained in stage II. The de-adapted new phage and its various re-adaptations were also included, as were the preparations of any temperate phages isolated from the new type. These phages were tested against all the type strains, including the new type, plus the lysogenized cultures prepared in stage II.

It is usually possible from the results of such a test to deduce the genetic nature of the typing phage, and often also the structural formula of the type strain and the type-determining ability of any temperate phage isolated from it. Possible cross-relationships between the temperate phage and previously known determining phages may also be detected. On the basis of these tests a decision can be made as to which of several single-plaque lines of the typing phage is most suitable for use in routine typing.

Table 2 summarizes all the pertinent information on the structure of the Vi-types and on the genetic nature of the typing phages. We have included for completeness types previously examined by other workers, whether or not we have re-investigated them.

Our system of nomenclature for temperate phages, for lysogenized strains, and for phage adaptations follows that of Felix & Anderson (1951), Anderson & Felix (1953*a*, *b*) and Anderson & Fraser (1955). We have been conservative in the naming of new determining phages; for example the phages isolated from types 26, C8 and E8 are indistinguishable from each other and we prefer to call them all phage 26′ (the phage first isolated from type 26). We believe this to be of use in indicating the relationship between various types, even when full identity of the determining phages has not been established.

Table 2. *Summarized information on the Vi-types of* Salmonella typhi *and their homologous typing phages*

| Vi-type | Type strain: Phages carried | Type strain: Structural§ formula | Nature of change in typing phage: Phenotypic | Nature of change in typing phage: Genotypic | References* |
|---|---|---|---|---|---|
| A | | A | | | |
| B1 | | | | B1 | 8 |
| B2 | b2† | | | B2 | 10 |
| B3 | b3‡ | A(b3) | | B3 | 7, 9, 10 |
| C1 | | C1 | C1 | | 6, 8 |
| C2 | d6 | C1(d6) | C1 | D6 | 2, 4, 8, 10 |
| C3 | f2 | C1(f2) | C1 | 29 | 2, 4, 7, 10 |
| C4 | | | C1 | C4 | 5 |
| C5 | c5† | | C1 | C5 | 5 |
| C6 | c6† | | C1 | C6 | 5 |
| C7 | | | C1 | C7 | 5 |
| C8 | 26′ | C1(26′) | C1 | 26 | 4, 5 |
| C9 | c9‡ | C1(c9) | C1 | B3 | 4, 5 |
| D1 | d1-C or d1-E | A(d1-C) or A(d1-E) | | D1 | 2, 4, 7, 8, 9, 10, 11, 12 |
| D2 | | | D2 | D1 | 8 |
| D4 | d1-C | ?(d1-C) | D4 | D1 | 2, 7, 8, 9, 11, 12 |
| D5 | | | | D5 | 6, 8, 12 |
| D6 | d6 | A(d6) | | D6 | 2, 4, 6, 7, 8, 11, 12 |
| D7 | d1-C | ?(d1-C) | | D7 | 5, 9, 14 |
| D8 | d1-C + d8‡ | A(d1-C) (d8) | | D8 | 4, 5, 9, 14 |
| D9 | d1-C + d9‡ | A(d1-C) (d9) | | D9 | 4, 9, 14 |
| D10 | d1-C + d8‡ | ?(d1-C) (d8) | | D10 | 4, 9, 14 |
| D11 | d6 | ?(d6) | | D11 | |
| E1 | | E1 | E1 | | 6, 8 |
| E2 | | | E1 | C7 | 8 |
| E3 | | | E1 | E3 | 5, 16 |
| E4 | | | E1 | E4 | 5, 16 |
| E5 | | E5 | E5 | | 5 |
| E6 | | | E1 | E6 | 5 |
| E7 | f2 | E1(f2) | E1 | 29 | 4, 5, 7, 11, 15 |
| E8 | 26′ | E1(26′) | E1 | 26 | 2, 4, 5, 8, 12 |
| E9 | d6 | E1(d6) | E1 | D6 | 3, 5 |
| E10 | t-def? | E1(t-def) | E1 | 28 | |
| F1 | | F1 | F1 | | 1, 6 |
| F2 | f2 | F1(f2) | F1 | 29 | 1, 2. 4, 7, 8, 12 |
| F3 | f3‡ | | F1 | F3 | |
| F4 | | | F1 | 43 | |
| F5 | t | F1(t) | F1 | T | |
| G | | G | G | | 8 |
| H | | H | H | | 8 |
| J1 | | J1 | J1 | | 8 |
| J2 | j2 | J1(j2) | J1 | J2 | 4 |
| J3 | | | J1 | J3 | |
| K1 | k† | K1 | | K1 | 7, 8, 11, 12 |
| K2 | d6 | K1(d6) | | K2 | |
| L1 | | L1 | L1 | | 6, 8 |
| L2 | | | L1 | L2 | 8 |
| M1 | | M1 | M1 | | 8, 13 |
| M2 | | | M1 | M2 | 13 |
| M3 | d6 | M1(d6) | M1 | D6 | 13 |
| N | | | | N | 6, 8 |
| O | | | | O | 6, 8 |
| T | t | A(t) | | T | 2, 4, 6, 7, 8, 11. 12 |
| 25 | 25′ | A(25′) | | 25 | 2, 4, 7, 8, 11, 12 |

Table 2 (*cont.*)

| Vi-type | Type strain: Phages carried | Type strain: Structural§ formula | Nature of change in typing phage: Phenotypic | Nature of change in typing phage: Genotypic | References* |
|---|---|---|---|---|---|
| 26 | 26′ | A(26′) | | 26 | 2, 4, 7, 8, 11, 12 |
| 27 | | | 27 | | 8 |
| 28 | t-def+28′‡ | A(t-def) (28′) | | 28 | 7, 8, 11, 12 |
| 29 | f2 | A(f2) | | 29 | 7, 8, 11, 12, 16 |
| 32 | | | 32 | | 8 |
| 34 | | | | 34 | 5 |
| 35 | | | | 35 | 5 |
| 36 | 36′† | | | 36 | 5 |
| 37 | | | 37 | | 5 |
| 38 | | | | 38 | 5 |
| 39 | | | 39 | T | 5 |
| 40 | | | 40 | 36 | 5 |
| 41 | | | 41 | | 5 |
| 42 | | | | 42 | 5 |
| 43 | 43′‡ | ?(43′) | | 43 | |
| 44 | | | | 44 | |
| 45 | | | | 45 | |
| 46 | | | | 46 | |

* References: 1, Anderson (1951); 2, Anderson (1955); 3, Anderson (1956); 4, Anderson (1959); 5, Anderson (1962*b*); 6, Anderson & Felix (1952); 7, Anderson & Felix (1953*b*); 8, Anderson & Fraser (1955); 9, Bernstein (1963); 10, Craigie (1946); 11, Felix & Anderson (1951); 12, Ferguson, Juenker & Ferguson (1955); 13, Nicolle, Hamon & Diverneau (1962); 14, Read & Ferguson (1961); 15, Scholtens (1955); 16, Scholtens (1956).

† Phages which apparently possess no type-determining power.

‡ Semi-determining phages (see text).

§ The structural formulae have been confirmed in most cases by the synthesis of the type concerned. Where the precursor of a phage-determined type is not known, it is indicated by the symbol '?'.

For many of the types nothing can be usefully added to the data recorded in Table 2. There are, however, some types of which a more extensive account of results is necessary and these are considered in detail below. They have been collected into groups, usually on the basis of the determining phages they carry. Some have been grouped because of cross-relationships of the typing phages. Neither grouping necessarily bears any relationship to the practical groupings of the typing scheme.

*D1 group.* We include in this group those cultures lysogenic for phage d1, that is, types D1, D4, D7, D8, D9 and D10. Other members of typing group 'D' are discussed elsewhere. All the typing phages of the group are pure host-range mutants, except phage *D4*, which is a phenotypically modified mutant.

The precursors of types D1, D8, and D9 are all type A and it has been shown by Read & Ferguson (1961) that the differences in the phage sensitivities of these strains are due to the fact that two of them are doubly lysogenic. Read & Ferguson gave the structural formulae of types D8, D9, and D10 as A(d8$\alpha$) (d8$\beta$), A(d9$\alpha$) (d9$\beta$), and ?(d10$\alpha$) (d10$\beta$) respectively. The various '$\alpha$' phages appeared to have identical properties and resembled phage d1. Read & Ferguson also claimed the '$\beta$' phages to be different from any others previously described. Phage d8$\beta$ appeared to be identical with phage d10$\beta$ but d9$\beta$ was completely distinct.

Our investigations of these types have produced results similar to those of Read & Ferguson (1961). Since the three '$\alpha$' phages are indistinguishable from each other and from d1 (but see later) we prefer to give them all the same designation of d1. By doing this the necessity for the '$\alpha$' and '$\beta$' suffixes disappears. We have been unable to detect any difference between phages d8$\beta$ and d10$\beta$ and will call them both d8. Our version of the structural formulae of types D8, D9, and D10 then becomes A(d1)(d8), A(d1)(d9), and ?(d1)(d8). The non-lysogenic precursor of type D10, which is indicated by the '?', in the formula, is not known but is probably the same as that of type D7, which appears to be singly lysogenic for phage d1. Type D4 is also singly lysogenic for phage d1 and we have been unable to identify its non-lysogenic precursor.

The current type strain of D1 is not that originally described by Craigie & Yen (1938). It is a culture substituted at a later date and originally obtained from Dr P. R. Edwards. This strain will be referred to as D1-E to distinguish it from the original strain which we shall call D1-C. The two strains are not identical in their typing reactions, and those given in Table 1 are those of D1-E. Type D1-C differs from D1-E in being less sensitive to most of the phages and in being completely resistant to phages *D6* and *D11* at the standard typing temperature. As the temperature is progressively decreased the sensitivity of D1-C to all the *D* phages is gradually increased, so that at 36·5° all the reactions except those to phages *D6* and *D11* are full. These two phages form minute plaques on D1-C at this low temperature but do not, in RTD, cause confluent lysis.

We have found (Bernstein, 1963) that the first 4 hr. of incubation of typing plates after the application of phage are the most important in determining the final degree of lysis of D1-C by the typing phages. The reaction on a plate incubated at 38·5° for at least 4 hr. will not be affected if it is thereafter transferred to 36·5°. Similarly, a reaction established by primary incubation at 36·5° for the same period will not be reduced by later transfer to 38·5°. The range of temperature fluctuation during this 4 hr. period is critical and should not be more than $\pm$0·50°. It has been found that the temperature affects both plaque size and number. Further investigations of this phenomenon are still in progress and a fuller report will be published later.

Strains D1-C and D1-E are both lysogenic and their determining phages are serologically identical. A culture lysogenized with either phage becomes resistant to the other; we shall call this phenomenon reciprocal interference. In spite of the similarities between the two determining phages, it has been found that the differences between the reactions of strains D1-C and D1-E with the Vi-typing phages of the D group are due to the carriage of phage d1-C by one and d1-E by the other. Lysogenization of type A with phage d1-C (→A(d1-C)) produces a culture apparently identical with D1-C; A(d1-E) prepared artificially in the same way is apparently identical with D1-E (Bernstein, 1963). It is not possible to obtain confluent lysis of A(d1-C) at 38·5° with phages *D1* and *D2* even by using the two phages in concentrations 10 times higher than their respective RTD.

The other type strains of this group also carry phage d1-C. Most of these strains also display a degree of temperature-dependent variability in phage-typing reactions (Table 3). The carriage of phage d1-C and not d1-E by the doubly lysogenic strains plays a significant role in their type determination. We have found that type

A made doubly lysogenic with phage d1-E on the one hand and d8 or d9 on the other is indistinguishable from type D1-E, whereas the association in type A of d1-C with phages d8 and d9 produces cultures identical with D8 and D9 respectively. It thus appears that phage d1-C, but not d1-E, can co-operate with phages d8 and d9 in type determination.

Lysogenization of type D7 with phage d8 produces a culture indistinguishable from type D10 and we presume, therefore, that types D7 and D10 have a common non-lysogenic precursor. Lysogenization of type D7 with phage d9 has no detectable effect on the typing reactions at any temperature.

Table 3. *The effect of temperature on the typing reactions of members of the* D1 *group*

| Vi-type | Structural formula | Incubation temperature (°) | Typing phages in RTD | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | *D1* | *D2* | *D4* | *D5* | *D6* | *D7* | *D8* | *D9* | *D10* | *D11* |
| D1-C | A(d1-C) | 36·5 | CL | CL | CL | CL | +++ | CL | CL | CL | CL | +++ |
| | | 38·5 | +++ | +++ | SCL | +++ | — | CL | CL | CL | CL | + |
| D1-E | A(d1-E) | 36·5 | CL | CL | CL | CL | CL | CL | CL | CL | CL | CL |
| | | 38·5 | CL | CL | CL | CL | CL | CL | CL | CL | CL | CL |
| D4 | ?(dl-C) | 36·5 | ++ | — | CL | — | — | — | — | — | — | — |
| | | 38·5 | — | — | CL | — | — | — | — | — | — | — |
| D7 | ?(d1-C) | 36·5 | — | + | SCL | — | — | CL | ++ | — | CL | — |
| | | 38·5 | — | — | — | — | — | CL | — | — | CL | — |
| D8 | A(d1-C) (d8) | 36·5 | CL | CL | CL | CL | — | CL | CL | ++ | CL | — |
| | | 38·5 | — | — | — | — | — | — | CL | — | SCL | — |
| D9 | A(d1-C) (d9) | 36·5 | ++ | ++ | SCL | ++ | + | CL | CL | CL | CL | ++ |
| | | 38·5 | — | — | +++ | — | — | CL | CL | CL | CL | — |
| D10 | ?(d1-C) (d8) | 36·5 | — | — | +++ | — | — | CL | — | ++ | CL | — |
| | | 38·5 | — | — | — | — | — | — | — | — | CL | — |

For definition of reactions see Table 1.

Phages d8 and d9 appear to be unrelated to each other and to phages d1-C and d1-E, and they do not show reciprocal interference. They are both heat labile whereas both varieties of d1 are heat stable. We have not been able to obtain high titre preparations with either phage d8 or d9 and plaque counts by either spot or agar-layer titrations are usually less than $10^5$/ml. We have, however, had no difficulty in lysogenizing with these preparations. It has been found (Wilson, unpublished) that there is a disparity between the number of plaque-forming units (PFU) detected by titration and the number of particles, apparently of phage, seen in preparations stained with acridine-orange and viewed by fluorescence microscopy (Anderson, Armstrong & Niven, 1959). The particle count in terms of PFU is usually one or more log units less than the count under the microscope. This deficiency in plaque-forming ability may be due to a high efficiency of lysogenization with a concomitantly low production of infective phage particles. It has been found that phages d8 and d9 do not form detectable plaques at 38°, although lysogenization can be performed at this temperature by blind serial passage. All assays of these phages should be conducted at 36·5°–37·0°.

Read & Ferguson (1961) state that neither phage d8 nor d9 has any determining ability in type A. This has not been our experience. In fact A(d8) differs from type

A in that it is completely resistant to typing phage *45* at both 36·5 and 38·5°. At the higher (standard) temperature it also has a much reduced sensitivity to several other typing phages, especially *C2*, *C7*, *E7*, *E9* and *40*, but these reductions are not evident at 36·5°. The distinction between type A and A(d9) is not so obvious and can only be detected at 38·5°. At this temperature A(d9) is relatively insensitive to typing phage *M2*. It is interesting to note that we have examined freshly isolated typhoid cultures showing a typing pattern identical with that of A(d8). These cultures have been found to be lysogenic for a phage indistinguishable from d8.

Type A has been doubly lysogenized with phages d8 and d9 (→A(d8) (d9)); this culture reacts as if it carried only phage d8. Trebly lysogenized type A (A(d1-C) (d8) (d9)), however, resembles type D8 at 38·5° and type D9 at 36·5°. It is evident from this that phages d8 and d9 can co-operate independently with d1-C to produce changes in phage typing pattern; they do not, however, appear to co-operate with each other.

Table 4. *Differentiation of the type-determining effects of phages d6–1691, d6-4274, and f2 by the use of three typing phages in RTD*

| | Typing phages | | |
|---|---|---|---|
| Culture | *D6* | *29* | *46* |
| A(d6-1691) | CL | SCL | CL |
| A(d6-4274) | CL | + + | ± |
| A(f2) | CL | CL | ± |

Thus, phages d8 and d9 have type-determining properties, although d9 is very weak in this respect. Cultures lysogenized with them display a degree of temperature-controlled variability of reaction to the Vi-typing phages.

*The D6 group.* We have included in this group a large number of types: C2, C3, D6, E7, E9, F2, K2, M3 and 29. The criterion for inclusion has been that the strains should be lysogenic for either phage d6 or phage f2. These two type-determining phages were first described by Felix & Anderson (1951) and Anderson & Felix (1953*b*) who found that they were serologically identical and that they resembled each other in host-range, plaque morphology and heat sensitivity; they also displayed reciprocal interference. Differentiation between the two phages could be made only on the basis of their type-determining activity. It was found that A(d6) was sensitive to phage *D6*, whereas A(f2) was fully sensitive to phage *29* in addition to phage *D6* (see Table 4).

In the survey of various types made by Anderson & Fraser (1955) it was shown that the structural formulae of types C2 and C3 were C1(d6) and C1(f2) respectively. The difference in phage sensitivity between these two types is analogous to that between types D6 and 29, i.e. C2 is sensitive only to phage *C2*, whereas C3 is sensitive to phages *C2* and *C3*. Anderson & Fraser also found that the structures of types E7 and E9 were E1(f2) and E1(d6) respectively.

There are, therefore, three pairs of types which have resulted from the lysogenization of precursor types A, C1, and E1 with either phage d6 or f2. However, type F2, which has the structural formulae F1(f2), has not so far been paralleled by a naturally occurring type with the formula F1(d6). Such a culture can nevertheless

be produced in the laboratory. Similarly, types D11, K2 and M3, all lysogenic for a phage resembling d6, have so far no counterparts lysogenic for f2.

It was reported by Anderson & Felix (1953*b*) that the phages, called by them 30′ f2, and 29′ isolated from types C3 (at that time designated type 30), F2, and 29 were identical in type-determining efficiency but differed in the resistance which they produced to phages other than the adapted typing preparations. For the purposes of this paper we will refer only to phage f2. It has been found (E. S. Anderson, unpublished) that there are also differences between some of the phages d6 isolated from different lysogenic types. There are, for example, two members of this phage 'family' which have been isolated from the different type strains of D6. One which we will call d6–1691 is found in culture T1691, which was the original type strain of type D6. The other phage d6-4274 was isolated from a culture, T4274, which was substituted for the type strain at a later date. These two cultures differ in that D6-1691 is sensitive in moderate degree to a wide range of phages which do not, at RTD, lyse D6-4274. These differences between the two strains have been found to be due to the different d6 determining phages which they carry. In many respects phage d6-1691 appears to possess a determining ability about half-way between those of d6-4274 and f2. Thus A(d6-1691) and D6-1691 are partially sensitive to phage 29. A full differentiation between the determining powers of these phages can be made by lysogenizing type A with each and testing the resulting cultures with typing phages *D6*, *29*, and *46* (Table 4). It is probable that there are more forms of d6 than the two we have examined. Indeed, it is probable that there is a group of related phages possessing a spectrum of similar determining abilities ranging from that of d6-4274 to that of f2.

We have found that all the type strains so far examined of the d6-determined types, other than D6-4274, carry the 1691 variant, or ones closely resembling it. Representatives of these types carrying d6-4274 may occur in nature but none has been encountered by us hitherto. In the cases of types C2 and E9, such alternative types have been made by lysogenizing types C1 and E1 respectively with phage d6-4274. These 'artificial' types differ from the natural type strains in their higher degree of specificity.

The non-lysogenic precursors of types D11 and K2 have not yet been isolated. It is possible, however, to produce a culture resembling type K2 by lysogenization of type K1 with phage d6. Culture K1(d6) differs from K2 in that it is lysogenic for phage k as well as d6. Since phage k is not known to possess any determining power it is possible that a non-lysogenic type K1 could exist and that this is the precursor of type K2. It has not yet been possible to synthesize a culture resembling type D11.

All the typing phages of this group are either pure host-range mutants (phages *D6*, *D11*, *K2*, and *29*) or phenotypically modified mutants (phages *C2*, *C3*, *E7*, *E9*, *F2* and *M3*). The phenotypic modifications represented are identical with those produced by types C1 (in phages *C2* and *C3*), E1 (in phages *E7* and *E9*), F1 (in phage *F2*) and M1 (in phage *M3*). The mutations occurring in phages *C2*, *D6*, *E9* and *M3* appear to be identical and we shall call them the *D6* mutation. Similarly, those found in phages *C3*, *E7*, *F2* and *29* may be designated the *29* mutation. The mutations represented by phages *D11* and *K2* are distinct from each other and from the *D6* and *29* mutations. It is possible to distinguish between the basic mutations by an examination of their host ranges on a small number of types (Table 5).

*T Group*: types E10, F5, T, 28 and 39. Felix & Anderson (1951) showed that the specificity of type T was due to a determining phage, t, and its structural formula was represented by Anderson & Fraser (1955) as A(t). Phage t was found to be very unstable even at 4°, and we do not now have an authentic strain of the original phage t in our possession.

Phage *T* was found by Anderson & Felix (1953*b*) to represent a phenotypically modified adaptation of Vi-phage II. Work by Anderson & Fraser (1955) suggested that the Vi-typing phages specific for lysogenically determined cultures were host range mutants of Vi-phage II. There was thus a discrepancy in the case of phage *T* which seemed from earlier experiments to be modified in phenotype only.

Table 5. *Differentiation of the* D6, D11, K2 *and* 29 *mutations of Vi-phage II by an examination of their lytic activity, in RTD, on a restricted number of indicator strains*

| | Basic mutants of Vi-phage II* | | | |
|---|---|---|---|---|
| Vi-types | *D6* | *D11* | *K2* | *29* |
| D6-4274 | CL | CL | CL | + + |
| D11 | — | CL | — | — |
| 29 | CL | CL | CL | CL |
| 42 | — | CL | CL | + + + |

* Basic mutants are the phages obtained after growth in type A. For definition of reactions see Table 1.

It was later shown by E. S. Anderson & A. Fraser (unpublished) that the situation is much more complex. They found that single plaque selection of titration of phage *T* on type A gave lines of phage with a wide range of EOP on type T, that is, the ratios of plaque titres on type T to those on type A were highly variable. The EOP of some lines were close to that of phage *A*, that is, *c.* $10^{-5}$; others resembled phage *T*, and had an EOP of 0·5; and yet others occupied intermediate positions, the range from one extreme to the other being apparently continuous. It seemed as if phage *T* represented a change in host-range of Vi-phage II intermediate in position between host-induced modification and mutation.

We have re-investigated type T and an unexpected finding has been that we have been unable to isolate any temperate phage from the type strain or from several other cultures belonging to the type. We have reason to believe that the strain is still lysogenic, but that the prophage has become defective, that is, it no longer produces mature plaque-forming phage particles. An indication that this is so is provided by the analysis of types E10 and F5.

Type F5 is lysogenic and the temperate phage, f5, appears to possess all the properties originally ascribed to phage t. It displays the same degree of instability, has the same plaque morphology, and is fully neutralized by an antiserum prepared by Anderson & Felix (1953*b*) against phage t. Type A lysogenized with f5 is indistinguishable from type T and type T is insensitive to phage f5. On these grounds we suggest that phages f5 and t are the same; we shall designate both phage t.

Type F1 lysogenized with t is identical with F5. The structural formula of F5 can be represented, therefore, as F1(t). Type E10 has not been found to be lysogenic, but it is resistant to phage t. Lysogenization of type E1 with phage t produces a

culture which reacts as type E10. This suggests that the structural formula of E10 is probably E1(t) but that, as in type T, the determining phage is defective.

Typing phage *F5* appears to be a pure host-induced modification of Vi-phage II and attempts to adapt phage *T* to F1 have hitherto yielded only phage *F1*. Phage *E10*, however, has been found to display a similar diversity of adaptation to that described earlier in phage *T* (B. Parkhouse, unpublished). Any population of phage *E10* yielded some lines behaving as phenotypically modified mutants; such lines produce a phage similar to *T* after de-adaptation, and revert to phage *E10* when re-adapted on type E1. Another portion of the same population was a pure host-induced modification, so that phage *E10*/A was phage *A* and phage *E10*/A/E1 was phage *E1*. Attempts to adapt phage *T* to type E1 have so far yielded only phage *E1*.

The mutation represented in phage *E10* is, however, not the same as that in phage *T* in that phage *E10* can lyse type 28, which phage *T* cannot; moreover, the host-range of the mutant form of phage *E10*/A is indistinguishable from that of phage *28* (see below). It is apparent that there is a diversity of changes possible in the adaptation of Vi-phage II to complexes carrying prophage t.

There are two other types that must be considered as part of this group, in that, in both cases, the typing phage was derived from phage *T*. The first of these types is 28, which was shown to be lysogenic by Anderson & Felix (1953*b*). These authors were not able to show that temperate phage 28′ possessed determining powers. We shall consider this aspect of the type in the next section (see types B3, C9, and 28). Typing phage *28* has an equal EOP on types T and 28, that is, about $10^{-1}$ of that on type A. This EOP is unchanged by growth in type A. The phage is, therefore, a mutant arising in a stock of phage *T*.

The remaining type, 39, has not been shown to be lysogenic. The typing phage has an EOP on type 39 equal to that on type T and about 0·5 of that on type A. On type 28 the EOP is $10^{-2}$ of that on T and 39. After de-adaptation the resulting phage has an EOP on type 39 only $10^{-3}$ of that on A or T. It thus appears that the phage is the result of a host-induced modification of phage *T*. The type strain is resistant to phage t and it is possible that it is lysogenic for defective phage t. No precursor type is known which produces type 39 after lysogenization with phage t.

*Types B3, C9 and 28.* These three types are lysogenic; their temperate phages display reciprocal interference and are serologically identical; the carried phages are designated b3, c9, and 28′ respectively. It was not possible in earlier work to show that phages b3 and 28′ are type-determining (Anderson & Felix, 1953*b*; Anderson & Fraser, 1955; Ferguson, Juenker & Ferguson, 1955) but we now have evidence that they possess this property. All three phages of this group are similar in their determining powers. They will convert type A into type B3 and type C1 into C9. In addition, they will convert type T, or A(t), into type 28. We use separate symbols for them, however, because there are small differences in their type-determining properties. It is possible that they represent only different varieties of the same determining phage, which could then be called b3. The structures of types B3, C9 and 28 are at present represented as A(b3), C1(c9) and A(t)(28) respectively.

Typing phage *B3* is a pure host-range mutant whereas phage *C9* is a phenotypic modification of a mutant, de-adaptation of which yields a phage identical with phage *B3*. Adaptation of phage *C9*/A, or of phage *B3*, to type C1 yields phage *C9*. Phage *C9* may therefore be assumed to be a *C1* modification of phage *B3*.

Phage *28* has been considered earlier and no relationship can be found between it and phages *B3* and *C9*.

*Type J2.* This type is lysogenic and the temperate phage which it carries, j2, is serologically distinct from all others in our possession. Phage j2 resembles d1 in plaque morphology and heat stability, but does not show reciprocal interference with it or with any of the other temperate phages we have examined.

Lysogenization of type J1 with phage j2 produces a culture indistinguishable from type J2 and, although we have not been able to isolate a non-lysogenic precursor from the type strain, we consider its structural formula to be J1(j2). When type A is lysogenized with phage j2 the resultant culture resembles type D5, but differs from it in that A(j2) is sensitive to typing phage *J2* whereas type D5 is not, and that D5 is sensitive to j2 whereas A(j2) is obviously not.

Table 6. *Efficiencies of plating (EOP*) of three different mutant phages produced by the adaptation of phage* A *to Vi-type J2*

| | Phage A/J2 | | |
|---|---|---|---|
| Vi-type | Mutant 1 | Mutant 2 | Mutant 3 |
| D1-E | 1·0 | 0·1 | 0·3 |
| D5 | 1·0 | $< 10^{-6}$ | 0·2 |
| J2 | 1·0 | 1·0 | 1·0 |
| 42 | $< 10^{-6}$ | 0·3 | 0·3 |

* EOP = the ratio of the plaque titre obtained on a given type to that on type A.

We have not been able to demonstrate lysogenicity in the D5 type strain. Ferguson *et al.* (1955) reported the isolation of a phage, which they designated d5, from a culture which appeared to belong to type D5. Phage d5 was serologically identical with phage d1 but did not reciprocally interfere with it. They also found that the D5 type strain was sensitive to d5. When they lysogenized type A with d5 the resulting culture behaved as type D1. It is possible that their identification of A(d5) as type D1 was an error. Anderson & Fraser (1956) showed that the selection of plaques from titration of phage *A* on type D1 yielded approximately equal proportions of mutant lines corresponding to phages *D1* and *D5*. It was found that the 'phage *D1*' in current use at that time was in fact a *D5* mutant which obviously could not distinguish between types *D1* and *D5*.

We do not know the true relationship between type D5 and cultures A(d5) and A(j2). It is interesting, however, that both D5 and A(j2) become resistant to typing phage *D9* if examined at 36·5°. We do not have a culture of A(d5) and so do not know whether it displays a similar variability.

Typing phage *J2* is a phenotypic modification of a hitherto undescribed mutant of Vi-phage II. After de-adaptation the phage will lyse type A and also A(j2), and when re-adapted to type J1 (= *J2*/A/J1) it regains the full lytic activity of phage *J2*. It has been found that adaptation of Vi-phage II to type J2 yields, in equal proportions, three different phages, all of which are phenotypically modified mutations. The host-induced modifications in each seem to be the same as that produced by type J1, but the mutational components differ. Table 6 shows the EOP of the three mutants on types A, D1, D5, J2 and 42. All the phages lyse types A, J1, and J2. In addition, one phage lyses types D1 and D5 fully whereas a second lyses types

D1 and 42 but with a reduced degree of reaction. The third mutant lyses types D1, D5 and 42 with equal but reduced efficiency. The second mutation is that selected for in the preparation of the current typing phage.

Although the three mutants that lyse J2 also lyse D1, none is identical with the *D1* mutation. Adaptation of phage *D1* to type J1 does not yield a phage with the ability to lyse J2. However, phage *D5* adapted to J1 produces a phage closely resembling mutant 1 of Table 6.

*Types E2 and L2.* Anderson & Fraser (1955) stated that these phages were pure phenotypic modifications of Vi-phage II reverting to phage *A* on de-adaptation. Further work has shown, however, that the phages concerned are phenotypically modified host-range mutants. If phage *E2*/A is propagated in type E1 (→*E2*/A/E1) the resultant phage is *E2* and not *E1*, as would be the case if *E2*/A was phage *A*. Similarly, phage *L2*/A grown on type L1 becomes phage *L2*.

We have found that the mutation represented in phage *E2* is the same as that in phage *C7*. There is no phage in the Vi-typing scheme which represents the pure mutant form of phages *E2* and *L2*, nor are there accepted types other than A, which are sensitive to phage *E2*/A or *L2*/A.

As was reported by Anderson & Fraser (1955) these types appear to be non-lysogenic.

*Types E3 and E4.* The typing phages for both these types are host-modified mutants. Neither culture appears to be lysogenic. In each case the host-induced modification is the same as that represented in phage *E1*.

The mutational component of these phages has been previously investigated by Scholtens (1956) who suggested that they might both be phenotypically modified *D1* mutations. We cannot fully characterize the mutations represented in these typing phages but we do not believe them to be *D1*, or at least not the same mutation as that found in the *D1* typing phage. They are, however, clearly related to this mutant.

Both types are sensitive to phages d1-C and d1-E, which indicates that they are not lysogenic for either phage even in defective form. Lysogenization of type E4 with either form of d1 produces no demonstrable effect on typing pattern. When type E3 is lysogenized with phage d1-C, however, it loses its sensitivity to typing phage *E3* (E. S. Anderson, unpublished); phage d1-E however, has no obvious effect in type E3. The change following lysogenization with d1-C is consistent with a prophage co-operation effect such as had been found in several members of the D1 group. No other evidence has been found for a temperate phage in type E3.

Phage *E3* lyses type D1 in addition to its type strain and it might appear from this, and from the fact that the de-adapted phage still lyses D1, that the basic mutation in this phage is that represented in phage *D1*. However, phage *D1* adapted to type E1 produces a phage different from phages *E3* or *E3*/A/E1. Phage *D1*/E1 lyses E3(d1-C) in addition to A, D1 and E3, whereas, as stated above, phage *E3* will not attack strain E3(d1-C). Phages *E3* and *D1*/E1 also lyse E1(d1-C) (E. S. Anderson, unpublished) and E1(d1-E). These two lysogenized cultures resemble type E3 in their sensitivity patterns.

Phage *E4* lyses types D1 and E3 in addition to types A and E4. It has not been possible to obtain from phage *D1* a host-induced modification which has the ability to lyse type E4. Nor has it been possible by lysogenization to produce from type E1 a culture resembling E4.

It is evident that the phenotypic change in phages *E3* and *E4* is the same as that in phage *E1*. The mutational change is different in each phage and neither is the same as that of phage *D1*, although de-adapted phages *E3* and *E4* will lyse type D1. There are, however, several known mutants other than *D1* which have the latter property (e.g. *D5*, *D6*). The specificity of the type strains E3 and E4 is certainly not due to the carriage of either of the forms of phage d1 described earlier.

## DISCUSSION

Our investigation of the various Vi-types and typing phages, both those previously examined and those more recently described, has produced results essentially in agreement with earlier findings and with the hypotheses of type determination and Vi-phage II adaptation as summarized by Anderson (1962*a*). Any additional information has added to, rather than contradicted, the findings of the earlier investigations of Anderson and his co-workers.

Several of the newer types are lysogenic for phages previously described. The results obtained with these types have been fully in accordance with predictions that could have been made from the arguments of Anderson & Fraser (1955) which were based on fewer types.

It may be that several types which we have stated to be non-lysogenic do carry phages, but that our methods are not adequate for their detection. We have suggested that some types, for example E10, are lysogenic for a defective prophage since we have been able to synthesize such types by the lysogenization of suitable precursors with active phage preparations. Defectivity may be the cause of apparent non-lysogenicity in other types, but we have no means of verifying this.

Anderson (1959) has shown that when type A is lysogenized with phage 25′c, a clear-plaque variant of 25′, clones may be isolated which do not yield any temperate phage although they react as type 25 with the Vi-phages and are resistant to phage 25′. It thus seems that the mutant of 25′ they carry has become defective. It is also known from the earlier published work of Anderson & Felix (1953*b*) that type T is lysogenic. However, we have recently been unable to isolate phage t from the type strain of T or from several other representatives of it, although we have isolated a phage which appears to be identical with t from type F5. These results suggest that phage t has become defective in the type T strains examined.

Even when a phage is not defective it may be difficult to detect. Several of the temperate phages form extremely minute, shallow, opaque plaques. Their plaque-forming ability may also be very sensitive to environmental conditions. The plaque-forming efficiency of a temperate phage must be inversely related to its efficiency of lysogenization. A phage with an efficiency of lysogenization approaching 100% would obviously form plaques very rarely. We have already mentioned the evidence that the plaque counts of phage d8 on type A at 36·5° may be only one-tenth, or less, of the true phage particle content of d8 lysates. Several of the known determining phages give rise to mutants with a variety of lysogenic abilities (Anderson, 1959). Some of these, such as 25′c, form clear plaques but are not truly virulent because they lysogenize with low efficiency. Other mutants have a high rate of lysogenization and form very turbid plaques.

It is known from studies of other phages (such as λ, P1) that cultural modifications will alter the efficiency of lysogenization (Lieb, 1953; Bertani & Nice, 1954; Fry,

1959). Similar studies have not been made with the determining phages carried by *Salmonella typhi* but they may exhibit the same phenomenon. We have routinely used two different incubation temperatures and a variety of indicator strains, but only one medium, in our endeavours to isolate temperate phages. However, these conditions may not be adequate for the detection of all temperate phages.

The phages isolated may be broadly classified into those with type-determining ability and those without it, phage k being an example of the latter category. The statement that a given phage has no type-determining power is only true within the framework of reference provided by the Vi-phage typing scheme in its present state. It is possible that the discovery of new typing phages may detect a type-determining property in phages now thought to lack this.

Those phages possessing a determining power may be further subclassified by a consideration of their type-converting efficiency. Many of the phages, such as 25′, may be called fully determining, since lysogenization of type A with one of them produces a highly specific type. Others, which we shall call semi-determining (Anderson & Williams, 1956), do not produce clear-cut types when lysogenizing type A. The alteration in the phage sensitivity of type A may consist solely in the reduction, or loss, of one or more reactions. If a semi-determining phage is used to lysogenize a culture already possessing a restricted phage sensitivity spectrum, it may have a more specific effect.

Some of these semi-determining phages produce an effect only in cultures already lysogenic for another, unrelated phage. Phage d8 produces a minimal, although characteristic change in the typing pattern of type A. If, however, d8 is used to lysogenize type D1-C, or A(d1-C), the resultant culture resembles type D8. The result of this double lysogenization is apparently not a simple summation of the Vi-phage blocking power of the two determining phages. It must be the result of some form of co-operation or synergism between them. This co-operation appears to be highly specific, since phage d8 will interact with phage d1-C but not with the closely related d1-E in the lysogenic cells.

One must distinguish between those synergistic effects shown by phages d8 and d1-C and the additive effects which may be obtained with other double lysogenizations. If type D1-E or A(d1-E), is lysogenized with phage d6, the resultant culture resembles type D6 (Jude, Nicolle & Ducrest, 1951). It is not, however, identical with D6 in that it is resistant to typing phages *36* and *40*. The Vi-phage resistances of A(d1-E)(d6) may be predicted by a superimposition of the typing patterns of types D1 and D6, remembering that resistance is dominant. In some cases, double lysogenization will yield a culture completely insensitive to all the available typing phages. A summation effect is detectable only when the determining abilities of the two phages are suitably complementary.

Some phages with full determining powers may lack a detectable determining effect in certain cultures. For example, types 36 and 40 may be lysogenized with phage d6 without any apparent effect on their typing reactions. The reasons for this are obvious from a study of the phage sensitivities of types 36, 40 and D6 in Table 1. It can be seen that types 36 and 40 are already resistant to typing phage *D6* and its modifications, although phages *36* and *40* will both lyse type D6, the reaction pattern of which is determined by d6. Some cultures of type 40 are lysogenic for phage d6.

The type-determining phages appear to have no effect on their hosts other than to modify their sensitivity to the typing adaptations of Vi-phage II. Any other characteristic of a given precursor, such as its biotype, is found in all its lysogenic descendants. For instance, all the members of types M2 and M3 which have been examined are non-fermenters of xylose, as is the precursor type M1 (Nicolle, Hamon & Diverneau, 1962). Characters unchanged by lysogenization, such as drug resistance, may be used to obtain type conversion with determining-phages which are difficult to isolate. Mixed cultures, in broth, are made of a streptomycin-resistant strain of type A and of a streptomycin-sensitive culture of the type thought to carry the determining phage. After a period of growth the mixed culture is plated on streptomycin agar and the resistant colonies tested for any change in phage type.

All lines of Vi-phage II, whether resulting from mutation or from host-induced modification, are absorbed by, and will kill, any Vi-positive strain of *Salmonella typhi* (Anderson & Fraser, 1956; Anderson, 1962*a*), although there may be no multiplication of the phage. The fact that Vi-phage II is active in transduction (Baron, Formal & Spilman, 1953) suggests that the phage DNA enters the bacterium even in cells which do not support phage growth.

Since all adaptations of Vi-phage II adsorb to any Vi-positive strains of *Salmonella typhi* to produce a lethal effect, one of the most valuable techniques for the detection of phage recombination, the use of mixed indicators, cannot be used in the genetic analysis of Vi-phage II. This is because all lines of phage, parent or recombinant, mixed or pure, produce the appearance of clear plaques on mixed indicator cultures. For these reasons the loci concerned in the adaptation of Vi-phage II to the many types of *S. typhi* have not yet been mapped.

In several other phage-host cell systems it has been shown that cultures able to induce phenotypic modification in the phage are lysogenic. It has been suggested that the modification is the result of interaction between the infecting phage and the temperate prophage (Lederberg, 1957; Christensen, 1961; Drexler & Christensen, 1961). There is no present evidence that the host-range modifications of the Vi-typing phages, whether of mutational or host-induced origin, are due to genetic interaction between Vi-phage II and type-determining phages.

Vi-types which fall into groups in the typing scheme (see Table 1) are so classified because they react as though they possessed a common precursor. Thus, types C8, E8 and 26, although they each carry phage 26′, fall into three different groups in the typing scheme because they have different precursors (C1, E1 and A, respectively). They could, however, be grouped together because they carry phage 26′.

If we were to group types according to the carriage of the same determining phages, the specific typing phages of each group would usually prove to be different phenotypic modifications of the same host-range mutant, the host-induced modifications being those corresponding to the precursor types concerned. There are, however, some groups which are determined by a single temperate phage, but in which the typing phages for the individual types are mostly distinct host-range mutants. These mutants are not identical but are evidently related to each other. Such an example is found in some members of the D group of Vi-types.

As is evident from an examination of the typing scheme (Table 1), many of the types are sensitive to a number of mutants of Vi-phage II. Such types, therefore,

select these mutants indifferently from wild stocks of Vi-phage II, that is from phage *A*, and 'adaptation' of phage *A* to these types without the subsequent characterization and purification of phage clones may result in the production of a mixture of phages of different specificities. As has been mentioned earlier, typing phage *J2* can be represented by any one of at least three different variants of Vi-phage II and the choice of one of these as the typing phage is relatively arbitrary. From the point of view of practical phage-typing convenience it is usual to choose that phage with the most restricted host-range, that is, lysing as few types as possible other than the homologous one.

This possible heterogeneity in the typing preparations is of practical importance in the development and application of the typing scheme. Care must be taken in the maintenance and propagation of phage stocks to ensure completely homogeneous continuity. In the development of a new typing phage the possibility of heterogeneity in the final preparation must be fully investigated. Such heterogeneity may be minimized by testing single-plaque lines in order to select those with the most restricted host-range.

Adaptation may also be carried out by the prolonged joint growth in broth of phage *A* and the cultures under investigation. This may be continued for long periods. Under these conditions heterogeneity in the resultant phage is very likely to occur, especially if the culture is sensitive only to mutants of the parent phage. The relative proportions of the various mutants in the final lysate will bear a direct relationship to their frequency in the starting phage and to their rate of growth and burst-size in the type concerned. Additional mutations may also occur during successive cycles of growth and may be represented in the final lysate.

The host range of typing phages is usually ascertained at a selected RTD. This dilution is used because it yields a reaction approaching the maximum on homologous types whilst minimizing cross-reactions. For purposes other than those of routine phage typing, however, it may be better to characterize the phages not in terms of their activity at the RTD but by a consideration of their EOP on the various indicator strains.

Several of the typing phages are mutants which produce small or minute plaques on their homologous host strains but not always on others. In order to obtain confluent lysis with such a phage it may be necessary to use a high concentration of phage particles, whereas with a large-plaque-forming phage a lesser number would be required. Thus, the RTD of phage *38* contains *c.* $10^6$ phage particles/ml. whereas that of phage *41*, which has a larger plaque size, contains only $10^3$. The fewer particles a phage contains at RTD the fewer cross-reactions it will give. In addition, a phage which requires $10^6$ particles/ml. to produce confluent lysis of its host may cause difficulty in type diagnosis due to the non-specific lethal effect of such a high phage concentration on the bacteria to which it is applied, even if lysis does not occur. This effect is enhanced when several different phages are pooled in order to reduce the number of reagents in routine use. For this reason, care must be taken in the construction of such pools to ensure that their phage content in terms of total particles/ml. is not excessive. Not only may the RTD of different typing phages contain vastly different amounts of phage, but the RTD of any given phage may also vary according to the circumstances under which it is estimated. There are several factors which may affect the sensitivity of the homologous type strain, as

well as of others, towards a given typing phage. Some of these may be intrinsic to the cultures whereas others may be environmental in nature—type of medium, temperature of incubation and so on.

It is often found that the sensitivity of a given type may fluctuate over a period of time. Many types on storage gradually become more resistant to their specific typing phages. The character of the lysis may also change. For example, it may become more opaque because an increase of resistance of the host strain because of an accumulation of Vi-negative or other variant bacterial forms. In such cases it is often possible to regain a fully sensitive (Vi-positive) culture by single colony selection.

Those types which are lysogenically determined may, on storage, lose their determining phages with a consequent change in typing pattern. This is more common with certain phages such as those of the d6-f2 group (Anderson, 1951). Strains which are losing their determining phage become progressively more sensitive to heterologous typing phages. When this occurs it may be possible to re-isolate the specific type strain by single-colony selection. If this fails it may be possible to regain the type by relysogenizing the culture with the requisite phage. Types which are apparently non-lysogenic may display a loss of specificity for reasons at present unknown. This loss may be stepwise in nature, in which case the change in sensitivity to the typing phages resembles that found when a determining phage is lost in a lysogenically determined type. Or it may be gradual, with the widespread acquisition of sensitivity to heterologous typing phages; this change is known as 'degradation'.

In addition to those fluctuations in sensitivity to the Vi-typing phages which may be directly attributed to the culture of a type strain, there may be others which are due to the environmental conditions of the test. In general, anything which causes a decrease in the growth rate of the culture, such as unsuitability of the nutrient medium, will lead to a reduction in phage sensitivity. Conversely, an improvement in growth conditions may result in better lysis.

It is important, therefore, to standardize fully the cultural conditions for selection of the RTD of a phage and for routine phage-typing. For these purposes the medium chosen should display minimal batch-to-batch variation but need not be optimal for growth. The medium which we have used in this survey, and which is that recommended for routine Vi-phage typing, that is Bacto nutrient broth, is certainly not the most nutritious for *Salmonella typhi*. However, it has been remarkably constant during the course of the 20 years during which batch-to-batch comparisons have been made. If phage-typing has to be conducted on a different medium, it is necessary to determine the phage RTD's on it.

If the medium to be used is one which is liable to variation in composition it is advisable to set aside a large quantity derived from either a single production run or from the pooling of several runs. Individual batches should be 'standardized' by comparison with a reference medium.

The reactions listed in Table 1 represent the 'mean' of a number of typing tests. Because of the virtual impossibility of exactly duplicating environmental conditions, the precise reproduction of the reactions listed is almost impossible, however closely the prescribed technique is adhered to. Some of the discrepancies may be due to variation in plaque size, so that reactions have become more or less obvious. Many of the cross-reactions listed are at the limit of visibility with a 10 ×

hand lens and it follows that alteration in plaque size will grossly affect these. It is obvious from the results presented on type D8 that phage typing must be conducted at a well stabilized temperature of 38·5°.

During the phage typing of large numbers of cultures, some are found which differ in only a small degree from some of the listed types. Cultures resembling A but resistant to phage *C5* or *45* are known, as are cultures like C1 but resistant to phage *C5*. Some of these cultures are known to be lysogenic for semi-determining phages such as d8. It is difficult to decide on suitable designations for such cultures, whose typing patterns are as distinct and stable as those of the established types. They would formerly have been classified as 'degraded' but we do not think that this is justifiable. It might be preferable to regard them as subtypes, or varieties, of the types which they most closely resemble.

It is common practice in most typing laboratories to pool groups of phages for routine phage-typing in order to limit the number of phage preparations that have to be applied to a culture. The use of such pools economizes in time and media but may unfortunately lead to errors in type diagnosis by concealing the slight differences in reaction to which we have referred. In particular, a culture may be classified as type A when in fact the use of phage pools has concealed the loss of one reaction, say, to phage *C5*. The true reaction of such a culture can only be detected by the use of all the typing phages individually. It is now our routine practice so to examine any culture which appears to be type A.

There are other cultures which are sensitive in varying degree to many of the typing phages but which show no obvious relationship to any of the standard types. These strains may nevertheless possess a typing pattern which is sufficiently distinct and stable to be valuable for epidemiological purposes. Some of these strains may be descended from type A which has been lysogenized with one of the semi-determining phages mentioned earlier. A few of the existing members of the typing scheme, such as B1 and 45, have typing patterns which might justify their being called degraded. The typing phages corresponding to these strains are, however, highly specific, and it may be possible to obtain equally specific phages for other strains diagnosed as degraded.

It is possible to synthesize many new types by the lysogenization of various precursors with any of the determining phages to which they are sensitive. Many such laboratory 'types' have already been made, and some of them have later been isolated in nature.

We have thought it useful to summarize the information at present available concerning type-determination in *Salmonella typhi* and the host-range specificities of the typing adaptations of the Vi-phage II. This information provides a basis for the better understanding of the principles of Vi-phage typing of *S. typhi*. It is unfortunate that there has been no recombinational analysis of the many host-range adaptations of Vi-phage II. Because of the technical difficulties mentioned earlier, we do not know whether the host-range mutations are located at one or many chromosomal sites, and, if the latter, whether more than one cistron is involved. We are hopeful, however, that a suitable technique will be evolved which will make such analysis possible. Vi-phage II might then be a useful organism for the analysis of genetic fine structure.

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# The Use of Soft Agar in the Study of Conditions Affecting the Utilization of Fermentable Substrates by Lactic Acid Bacteria

By R. WHITTENBURY
*Department of Bacteriology, School of Agriculture, Edinburgh*

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## SUMMARY

Lactobacilli, streptococci, leuconostocs, pediococci and aerococci were examined in a soft agar medium which provided aerobic and anaerobic conditions in the one culture. The relation to oxygen varied in some instances with the energy source and the method by which it was sterilized. Other variations were determined by the temperature of incubation, the pH value of the medium and the salt concentration. Only a proportion of the organisms showed preferences or requirements for diminished aeration or anaerobic conditions. In some cases an aerobic environment was optimal or essential for the utilization of a fermentable substrate. The soft agar medium showed that the fermentation of sugars and other substances might occur as a sequel to mutation. Liquid media were compared with soft agar media; they provided little information about oxygen relationships. This comparison suggested several possible reasons for the variability of lactic acid bacteria reported by investigators who used liquid media in tests for fermentation.

## INTRODUCTION

It is frequently stated in the literature (e.g. in *Bergey's Manual*, 1957), that many lactic acid bacteria are either microaerophilic or anaerobic. However, there is little definite information on this question and the organisms are frequently cultivated without regard to the possible effects of oxygen on their growth and chemical activities. A soft agar medium described below has proved to be suitable for these organisms and to give more information about their fermentative properties than do liquid media.

## METHODS

*Organisms.* Lactobacilli, streptococci, leuconostocs, pediococci and aerococci, in all 250 strains, were examined. They were obtained from several individuals and the National Collection of Type Cultures (NCTC), the National Collection of Dairy Organisms (NCDO), the National Collection of Industrial Bacteria (NCIB), and the Institute of Applied Microbiology, Tokyo (IAM) or were isolated from fresh herbage and silage at the beginning of the investigation. An objective was to secure a set of strains in which the widest possible range of character was represented. The organisms were identified as far as possible by their physiological and morphological properties. The pediococci were classified according to Nakagawa & Kitahara (1959).

*Soft agar medium and preparation of soft agar cultures.* The basal medium contained: meat extract (Lab-Lemco), 0·5 g.; peptone (Evans), 0·5 g.; yeast autolysate

(prepared as in Gibson, Stirling, Keddie & Rosenberger, 1958), 5·0 ml. or yeast extract (Difco), 0·5 g.; Tween 80, 0·05 ml.; agar (Davis), 0·15 g.; tap water to 100 ml. Either bromocresol purple (BCP), 1·4 ml. of a 1·6% (w/v) ethanolic solution, or bromocresol green (BCG), 2·8 ml. of a 0·4% (w/v) aqueous solution, was added per l. medium. Fermentable substrates, as distilled water solutions sterilized by autoclaving at 121° for 15 min., or by Seitz-filtration, were added to the autoclaved (121° for 15 min.) basal medium to give a final concentration of 0·5% (w/v) or, in the case of slightly soluble substances, 0·25% (w/v). The medium used in salt tolerance tests was prepared at double strength and an equal volume of NaCl solution was added after sterilization. Soft agar containing BCP was adjusted to pH 6·8–7·0; that containing BCG to pH 5·4. The basal medium, 90 ml. quantities in bottles, was liquefied by momentarily autoclaving at 115°, allowed to cool to 48° in a water bath and completed by adding 10 ml. of a fermentable substrate solution. The completed medium was replaced in the water bath or kept in an incubator at 50° until required, when it was distributed in 6–7 ml. amounts to sterile $6 \times \frac{5}{8}$ in. test tubes, the amount being judged visually as the medium was poured into the tubes. A set of completed media was placed in the one rack in the water bath so that they could be inoculated with one culture. Immediately before inoculation the media were allowed to cool to 37–40°, a temperature which allowed ample time for inoculation before the agar set. The liquefied medium was inoculated with a drop of a turbid culture added by capillary pipette and then tilted two to three times before being allowed to set. The subsequent growth indicated that the inoculum was uniformly distributed through the medium by this procedure. When for comparative purposes substrates were autoclaved in the medium the same method of inoculation was used.

*Liquid medium.* This was similar in composition to the soft agar medium, but without agar. The medium was distributed in 4·5 or 2·25 ml. amounts in $5 \times \frac{1}{2}$ in. tubes. Substrate solutions, 0·5 ml. in the former instance and 0·25 ml. in the latter, were added subsequently when not included in the medium originally. Inoculation was by capillary pipette.

*Inoculum medium.* This was similar to the liquid medium with the exceptions that glucose, 0·5% (w/v), was autoclaved in the medium, which was adjusted to pH 6·5, and the indicators were omitted.

*Glucose agar.* This was similar to the inoculum medium with the exceptions that agar 1·5% (w/v) and $MnSO_4.4H_2O$ 0·01% (w/v) were added.

*Detection of hydrogen peroxide formation.* Manganese dioxide agar, based on the pyrolusite agar described by Kneteman (1947), was used. Glucose agar was poured as a plate to which was added a very thin layer of glucose agar containing pyrolusite or manganese dioxide (black tech.; Harrington Bros. Ltd.), 4·0% (w/v). The plates were dried and inoculated by streaking; $H_2O_2$ formation was indicated by clearing of the manganese dioxide under and around the growth.

*Incubation temperature.* Unless otherwise stated all incubations were at 30°.

## RESULTS

The soft agar medium provided environments ranging from aerobic to anaerobic in the one culture. This was indicated by the site of growth and by the reduction of methylene blue below the top 0·5–1·0 cm. of the uninoculated medium. Reducing

conditions persisted, at least in the lower half of the medium, throughout the 14-day period of incubation.

The pH indicators, bromocresol purple and bromocresol green, proved satisfactory in the great majority of instances. Sometimes both indicators were bleached by reducing organisms such as *Streptococcus faecalis*. A fourfold increase of the indicator content masked this effect and proved satisfactory in these instances. Doubtful colour changes, unaccompanied by an obvious increase in growth over that in the basal medium, were checked electrometrically.

The sterilization of fermentable substrates by autoclaving in distilled water gave the same results as sterilization by Seitz-filtration. Differences were occasionally observed, however, between growth in the presence of separately sterilized substrate and that on the same substrate autoclaved in the medium. Unless specifically mentioned, the descriptions given below apply to growth not affected by treatment of the substrates.

*Requirement for aerobic conditions*

Although lactic acid bacteria have been described as microaerophilic or anaerobic, a number of these organisms were found to require aerobic conditions when utilizing certain substrates. In such instances, acid-forming growth was restricted to the surface layers of the soft agar culture (Pl. 1, fig. 1). Slight, non-acid forming growth, similar to that which appeared in the basal medium, frequently developed throughout the remainder of the culture. On continued incubation, growth increased in density in the surface layers and occasionally developed as secondary disks below the surface as the medium became oxygenated. Incubation of soft agar cultures under an atmosphere of 95% (v/v) $H_2$ + 5% (v/v) $CO_2$ confirmed that growth restricted to the surface reflected a requirement for aerobic conditions.

When substrates were utilized only aerobically in soft agar, liquid cultures occasionally gave variable results. Obvious acidity and growth were sometimes only observed after 6–8 days of incubation, in contrast to the 2–3 days required in soft agar. When acid production was weak and growth slight but none the less evident in soft agar, no acidity or only slight acidity after 10 or more days was observed in liquid cultures. Shallow shaken cultures became more strongly acid than did deep unshaken cultures.

Examples of a requirement for aerobic conditions were provided by strains of *Streptococcus faecium*, *Pediococcus* spp. and *Lactobacillus plantarum* utilizing glycerol; *Aerococcus viridans* NCTC 8251 and *Pediococcus urinae-equi* IAM 1684 utilizing glycerol, mannitol or sorbitol and, within the first 24 hr. of incubation, glucose; *S. faecalis* strains utilizing *m*-inositol in the absence of fumarate as an exogenous hydrogen acceptor; *L. salivarius* utilizing mannitol or sorbitol; some leuconostocs in the initial stages of hexose utilization; and strains of *L. brevis* and *L. buchneri* utilizing hexoses, gluconate or mannitol which had been sterilized apart from the medium.

A proportion of the organisms, therefore, required or benefited from the presence of oxygen in the utilization of certain substrates. In many cases the action on these substrates involved the formation of $H_2O_2$. Instances of lactic acid bacteria requiring aerobic conditions for the utilization of specific substrates have been recorded previously (Gunsalus & Sherman, 1943; Dobrogosz & Stone, 1959).

*Requirement for anaerobic conditions*

When substrates were fermented only under anaerobic conditions, acid-forming growth was restricted to a sharply defined zone in the anaerobic part of the soft agar culture (Pl. 1, fig. 2). The height of the zone, dependent upon substrate and organism, varied from the bottom fifth of the culture to within 1·0–0·5 cm. of the surface. Examples of anaerobic growth are some organisms of the *Lactobacillus casei-plantarum* group utilizing cellobiose, sucrose or glycerol, and some unclassified streptococci utilizing lactose, amygdalin or raffinose. With other substrates these particular organisms grew well throughout the medium.

Sometimes the pH value of the medium appeared to affect the ability of an organism to grow aerobically. Examples were observed with *Lactobacillus cellobiosus* and *Pediococcus* NCDO 1250. In an originally neutral glucose medium their initial growth and acid production occurred only in the anaerobic part (Pl. 1, figs. 3, 5) but as acid diffused upwards aerobic growth appeared, rapidly in the case of *L. cellobiosus* (Pl. 1, fig. 4), slowly in the case of the pediococcus. In soft agar initially at pH 5·4 both organisms grew uniformly throughout. On neutral glucose agar slopes incubated aerobically these organisms either did not grow or produced very slight growth late in the incubation period, but they grew well when the medium was adjusted to pH 5·4.

Where cases of anaerobic requirements were observed in soft agar comparative liquid cultures produced variable results. Shallow liquid cultures frequently showed no acid formation whilst deep cultures frequently became acid. When incubated anaerobically, however, acid was always formed.

*Requirement for a diminished oxygen concentration*

In the unsupplemented basal medium many organisms produced a thin disk of growth without obvious acid formation about 0·25 cm. below the surface. With a fermentable substance added, acid-forming growth was in certain cases confined to the same position, as for example some strains of *Lactobacillus buchneri* with xylose as the substrate (Pl. 1, fig. 6). In liquid cultures with the same substrates these particular organisms produced slight or questionable reactions.

*No strict aerobic, microaerophilic or anaerobic requirement*

In such instances growth and acidity appeared throughout the whole culture and in most cases uniformly and simultaneously (Pl. 1, fig. 7). A number of leuconostocs, however, exhibited a preference for aerobic conditions since growth and acid formation were initially confined to the surface of the medium. The later-developing anaerobic growth either became as dense as the surface growth or remained slight by comparison. In these circumstances liquid cultures gave constantly positive results.

*Mutant behaviour*

The formation of one or more relatively large, acid-forming colonies surrounded by slight non-acid forming growth was interpreted as mutant behaviour. Old or diluted inocula containing few viable organisms also sometimes gave rise to a few acid-forming colonies, but this could be distinguished by the use of fresh inocula and a control medium containing a substrate which was utilized uniformly by the

organisms. The mutant colonies were frequently distributed throughout the medium (Pl. 1, figs. 8–10), but were sometimes confined to the aerobic portions (Pl. 1, fig. 11) sometimes to the anaerobic portion (Pl. 1, fig. 12) and sometimes to the microaerophilic region (Pl. 1, fig. 13). Occasionally only single colonies were observed in the culture (Pl. 1, fig. 14). Some organisms produced dense growth in the aerobic portion of a medium but only a few mutant colonies in the anaerobic portion—e.g. some strains of *Streptococcus faecalis* when utilizing glycerol. An unusual sequence of events was observed with a strain of the *Lactobacillus plantarum-casei* group when utilizing raffinose. The glucose-grown inoculum produced mutant colonies in the aerobic portion of the raffinose medium and no acid-forming growth in the anaerobic portion. The purified mutant produced dense and uniformly distributed growth in the aerobic portion of the raffinose medium and mutant colonies in the anaerobic portion. These mutants when purified gave dense, acid-forming growth distributed uniformly throughout the aerobic and anaerobic portions of the medium. The pH value of the medium sometimes affected growth—e.g. a strain of the *L. plantarum—casei* group grew uniformly throughout a raffinose medium adjusted to pH 5·4 but only 'mutantly' in the same medium adjusted to pH 7·0. The purified mutant grew uniformly throughout the medium adjusted to pH 7·0.

In all the instances of mutation mentioned, the parent cultures were purified two or three times and the tests repeated; the same behaviour was observed again. The purified mutants were subcultured four to five times through a glucose medium and compared with the parent cultures by using appropriate biochemical tests. The behaviour of the mutant and parent cultures was found to differ only on the substrate which supported mutant growth. No attempt was made to determine the nature of the mutations—e.g. whether or not utilization of the substrate was related to permeability or to the ability to metabolize it, or in the case of glycerol utilization by *Streptococcus faecalis*, the ability to use unidentified exogenous hydrogen acceptors of the basal medium. Fumarate was eliminated in this instance, as its addition to the glycerol medium permitted the formation of uniformly distributed, dense, fermentative growth in the anaerobic portion.

An unsuccessful attempt was made to assess mutation rates. The basal medium supported a slight amount of growth which made it impossible to be certain whether acid-forming colonies arose from mutant organisms in the inoculum or from some which developed later. Frequently 1 ml. of undiluted inoculum and 1 ml. of a 1/100 dilution of the inoculum yielded a similar number of acid-forming colonies. It was suggested by Rogosa *et al.* (1953) that concentration of the substrate may affect the ability of an organism to adapt to its utilization. Increasing substrate concentration up to 2·0 % (w/v) in the present instances did not alter or mask the mutant behaviour.

In contrast to soft agar media which gave constant mutations, liquid media yielded positive results in one experiment and negative in another. In a number of trials with liquid media the proportion of positive results increased with increasing size of inoculum, and it was affected by the depth of the medium in cases requiring aerobic or anaerobic conditions and in some instances by the pH of the medium.

A practical aspect of these observations is their relation to the taxonomy of the organisms. If an organism is mutant on a substrate used for differentiating it

from other species, the value of such a 'characteristic' is diminished. Such an instance was observed with leuconostocs. Three strains of *Leuconostoc citrovorum*, a species separated from *L. dextranicum* by its inability to form acid and dextran from sucrose, produced sucrose-fermenting mutants. These mutants could not be induced to form dextran and so remained distinct from *L. dextranicum* in this characteristic alone.

*The distinction between slow and weak substrate utilization*

Utilization of a substrate in soft agar was regarded as slow when the amount of growth increased over a few days and finally equalled that supported by a rapidly utilized substrate. Utilization was judged to be weak when growth remained only slightly more dense than that which developed in the basal medium and when the pH value decreased by only 1·0–1·5 units in a medium originally neutral, or by 0·5–1·0 unit in a medium originally at pH 5·4. When utilization was weak the growth was often restricted to one portion of the medium, e.g. the anaerobic portion in the case of some leuconostocs when utilizing arabinose or xylose. In a test for acid formation in a liquid medium, weak utilization tended to give an indefinite result.

*Effects of incubation temperature*

The soft agar medium in which glucose was autoclaved was used to test the ability of organisms to grow at various temperatures. Some strains of *Lactobacillus brevis* and *L. buchneri* grew uniformly throughout this medium at 30°, but only in the aerobic portion at 37°. Conversely, *L. hilgardii* NCIB 8040 and some unclassified streptococci and heterofermentative lactobacilli grew uniformly throughout the medium at 30°, but at 37° only in the anaerobic portion. In a liquid medium incubated aerobically at 37° *L. brevis* and *L. buchneri* showed acid formation but *L. hilgardii* and the other organisms did not grow. When glucose was replaced by a fermentable pentose, growth of all these organisms was uniform in soft agar at 37°. Another variation was noted with *L. fructovorans* NCIB 8039; at 30° growth appeared uniformly throughout the medium but as the maximum temperature for growth (37°) was approached, growth and acid production became restricted to a disk about 2 mm. in depth, 1·0–1·5 cm. below the surface (Pl. 2, figs. 19, 20) and did not develop outside this zone on further incubation. At 35° and 37°, the liquid medium incubated aerobically frequently failed to support growth of this organism.

*Effect of different NaCl concentrations*

Three variations were observed in glucose soft agar. The first concerned *Aerococcus viridans* NCTC 8251 and *Pediococcus urinae-equi* IAM 1684. As the NaCl concentration was increased to 10 % (w/v) growth and acid production became restricted to the surface layers of the medium. A second effect observed was the great decrease in the number of organisms of the inoculum which were able to initiate growth at high NaCl concentrations. The growth of *P. soyae* IAM 1673 (Pl. 2, figs. 15–17) illustrates this, comparatively few colonies appearing in a medium containing 18 % (w/v) NaCl. A third variation was observed with some pediococci when growing in the presence of 8–10 % (w/v) NaCl; growth was then restricted to a few colonies which developed in a zone in the middle of the medium (Pl. 2, fig. 18). The manner of growth in NaCl media seemed to be unrelated to mutation. Transfer of

the colonies to a medium lacking NaCl resulted in uniformly dense growth but when they were transferred to a medium containing the same concentration of NaCl the previous result was repeated.

### *Effects of the method of sterilizing media*

The fermentable substrates were sterilized in two ways: (1) by autoclaving in the medium; (2) separately, by autoclaving or Seitz-filtration before being added to the autoclaved basal medium. The only organisms which did not behave in the same way in differently prepared media were all the strains of *Lactobacillus brevis*, *L. buchneri*, unidentified organisms closely similar to these two species, and *L. viridescens*. The growth of these organisms is described in Table 1, which shows that with gluconate, hexoses, di- or tri-saccharides as substrates there was a difference in the two types of media. Aerobic growth was similar in both kinds of media. Anaerobically, growth developed rapidly in a medium in which the substrate had been sterilized, but slowly or not at all in a medium containing separately sterilized substrate. No attempt has yet been made to determine the reasons for these differences. It was clear, however, that a slow anaerobic development with separately sterilized substrates, when it occurred, was not a case of adaptation, since anaerobic growth was equally slow upon transfer to a similar medium. In all this work no instance was recorded of an organism being able to utilize a substrate sterilized by one method but not when sterilized by another.

Table 1. *Growth with substrates sterilized by two procedures*

Type of growth indicated by: aer = aerobic only; pa = aerobic growth with delayed (2–10 days) and slight anaerobic growth; a/san = aerobic growth and simultaneous anaerobic growth which was at first slight; mic = microaerophilic growth; fac = growth uniform throughout aerobic and anaerobic regions of the medium; aerobic growth may finally become heavier than anaerobic growth.

| | Substrate* | | | | | | |
|---|---|---|---|---|---|---|---|
| | Gluconate, aldohexoses, di- and tri-saccharides | | Fructose | | Arabinose | Xylose | Mannitol |
| Organism and no. of strains | Sterilized separately | Sterilized in medium | Sterilized separately | Sterilized in medium | Sterlized separately or in medium | | |
| *Lactobacillus brevis* and similar unidentified organisms (31) | aer or pa | fac | a/san | fac | fac | fac | aer |
| *L. brevis* var. *rudensis* NCIB 4617 | aer | fac | a/san | fac | aer | fac | aer |
| *L. buchneri* (25) | aer or pa | fac | a/san | fac | fac | fac or mic | aer |
| *L. viridescens* (2) | aer | fac | a/san | fac | not utilized | | aer |

* Results apply to organisms able to utilize the energy sources specified.

### *Effect of citrate and manganese*

When potassium citrate (0·1 %, w/v) was added to soft agar, the growth with separately sterilized sugars of both the strains of *Lactobacillus viridescens* studied,

and of some unidentified heterofermentative lactobacilli, was greatly improved. The growth of all the other bacteria was not significantly affected. In the case of *L. viridescens* citrate increased the anaerobic but not the aerobic growth; with the unidentified lactobacilli, aerobic and anaerobic growth developed very slowly in absence of citrate but rapidly in its presence. When pentoses were autoclaved in a medium to which citrate had been added, the anaerobic growth of many strains of *L. brevis* and *L. buchneri* was either completely or partially inhibited. Other organisms were not affected in this way.

The addition of $MnSO_4.4H_2O$ (0·01 %, w/v) to sugar-containing soft agar resulted in a marked increase in the amount of aerobic growth of all the strains of *L. brevis*, *L. buchneri*, unidentified organisms closely similar to these two species and *L. viridescens*. The anaerobic growth of these organisms did not appear to benefit from the presence of manganese. The growth of the other bacteria studied was not obviously improved by adding manganese to the media.

### *A hydrogen peroxide effect*

A number of leuconostocs exhibited a preference for aerobic conditions when utilizing glucose and related compounds. In a soft agar medium containing one of these sugars, aerobic growth was frequently separated from the later-developing anaerobic growth by a growth-free zone (Pl. 2, fig. 21) which often diminished on prolonged incubation (Pl. 2, fig. 22). The addition of catalase to the medium prevented the occurrence of the growth-free zone as did manganese dioxide, which was dissolved in that area. This indicated that $H_2O_2$ was being formed at the surface, and, diffusing downwards, it inhibited the initiation of anaerobic growth.

## DISCUSSION

The soft agar medium proved to have several advantages over liquid media in the study of substrate utilization by lactic acid bacteria. Incubated aerobically, soft agar provides a range of environments from aerobic to anaerobic and permits the suspended inoculum to develop in that region of the medium most suitable for it. With this soft agar medium it is possible to determine when the utilization of an energy source is only the property of a mutant population, and whether growth with a particular substrate is aerobic only, facultative, anaerobic only, or microaerophilic. It was also possible to observe the special effects of temperature of incubation, pH value, sodium chloride concentration, the method of sterilizing energy sources, and the addition to the medium of citrate, manganese and other substances. Liquid media incubated aerobically are much less informative; they tended to give variable results when restricted conditions were required for substrate utilization or when growth was dependent upon a mutation. A question which was not investigated is the effect of the $E_h$ value at different depths of the soft agar medium. Some of the observations concerning growth at particular regions in the medium might indicate differences in $E_h$ requirement by the given organisms. It is evident from the observations made with soft agar cultures that it is not possible to generalize about the oxygen requirements of the lactic acid bacteria. The present survey has indicated that differences in the requirements are especially numerous among leuconostocs related to *Leuconostoc mesenteroides* and among heterofermentative lactobacilli classified as *Lactobacillus brevis*, *L. buchneri* and *L. viridescens*.

The results obtained with soft agar could provide starting points for biochemical investigations as indicated by the following two examples. The first concerns the unusual behaviour of a leuconostoc which grows and ferments sugars only when oxygen is available. Growing cultures form $H_2O_2$ and cell-suspensions rapidly take up oxygen in presence of glucose and form $H_2O_2$. This suggests (*a*) that flavoprotein oxidases play a significant role in the successful growth of this organism and (*b*) that one or more of the dehydrogenases which might be expected to function in anaerobic fermentation are failing to do so or are not being formed. The second case concerns the strictly aerobic growth of some strains of *Lactobacillus brevis* and *L. buchneri* with certain separately sterilized substrates (see Table 1). It may be suggested that one or more of the dehydrogenases which operate in an anaerobic fermentation of gluconate, glucose and related compounds are not functioning or are not being formed when the substrate has been sterilized separately. The rapid development of aerobic growth suggests that flavoprotein oxidases and/or peroxidases may function in place of the missing anaerobic dehydrogenase(s), oxygen being used as a hydrogen acceptor.

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## EXPLANATION OF PLATES

The growth of lactic acid bacteria at 30° in soft agar contained in $6 \times \frac{5}{8}$ in. test tubes.

Plate 1

Fig. 1. A strain of *Streptococcus faecium* utilizing glycerol aerobically; 48 hr.

Fig. 2. A strain of the *Lactobacillus plantarum-casei* group utilizing cellobiose anaerobically; 72 hr.

Fig. 3. *L. cellobiosus* utilizing glucose anaerobically; 24 hr.

Fig. 4. *L. cellobiosus* utilizing glucose; growth beginning to develop in upper part of the medium; 36 hr.

Fig. 5. *Pediococcus* NCDO 1250 utilizing glucose anaerobically; 48 hr.

Fig. 6. A strain of *L. buchneri* utilizing xylose microaerophilically; 72 hr.

Fig. 7. A strain of *L. fermenti* utilizing glucose equally well aerobically and anaerobically; 24 hr.

Fig. 8. A strain of *S. faecium* utilizing sorbitol aerobically and anaerobically after mutation. A haze of non-acid-forming growth surrounds mutant colonies; 6 days.

Fig. 9. A strain of pediococcus utilizing lactose mutantly. A haze of non-acid-forming growth surrounds colonies; 5 days.

Fig. 10. A strain of the *L. plantarum-casei* group utilizing cellobiose mutantly. A haze of non-acid-forming growth surrounds colonies; 5 days.

Fig. 11. A strain of the *L. plantarum-casei* group utilizing sucrose aerobically but mutantly; 4 days.

Fig. 12. A strain of the *L. plantarum-casei* group utilizing cellobiose anaerobically but mutantly; 5 days.

Fig. 13. A strain of the *L. plantarum-casei* group utilizing mannitol mutantly. Sub-surface disk of growth not acid-producing, but acid-forming colonies developed from this disk of growth; 7 days.

Fig. 14. A strain of the *L. plantarum-casei* group; a single mutant sucrose-utilizing colony developing; 7 days.

PLATE 2

Fig. 15. *Pediococcus soyae* IAM 1673 utilizing glucose in the presence of 5% (w/v) NaCl; 4 days.

Fig. 16. *P. soyae* IAM 1673 utilizing glucose in the presence of 15% (w/v) NaCl; 4 days.

Fig. 17. *P. soyae* IAM 1673 utilizing glucose in the presence of 18% (w/v) NaCl; 7 days.

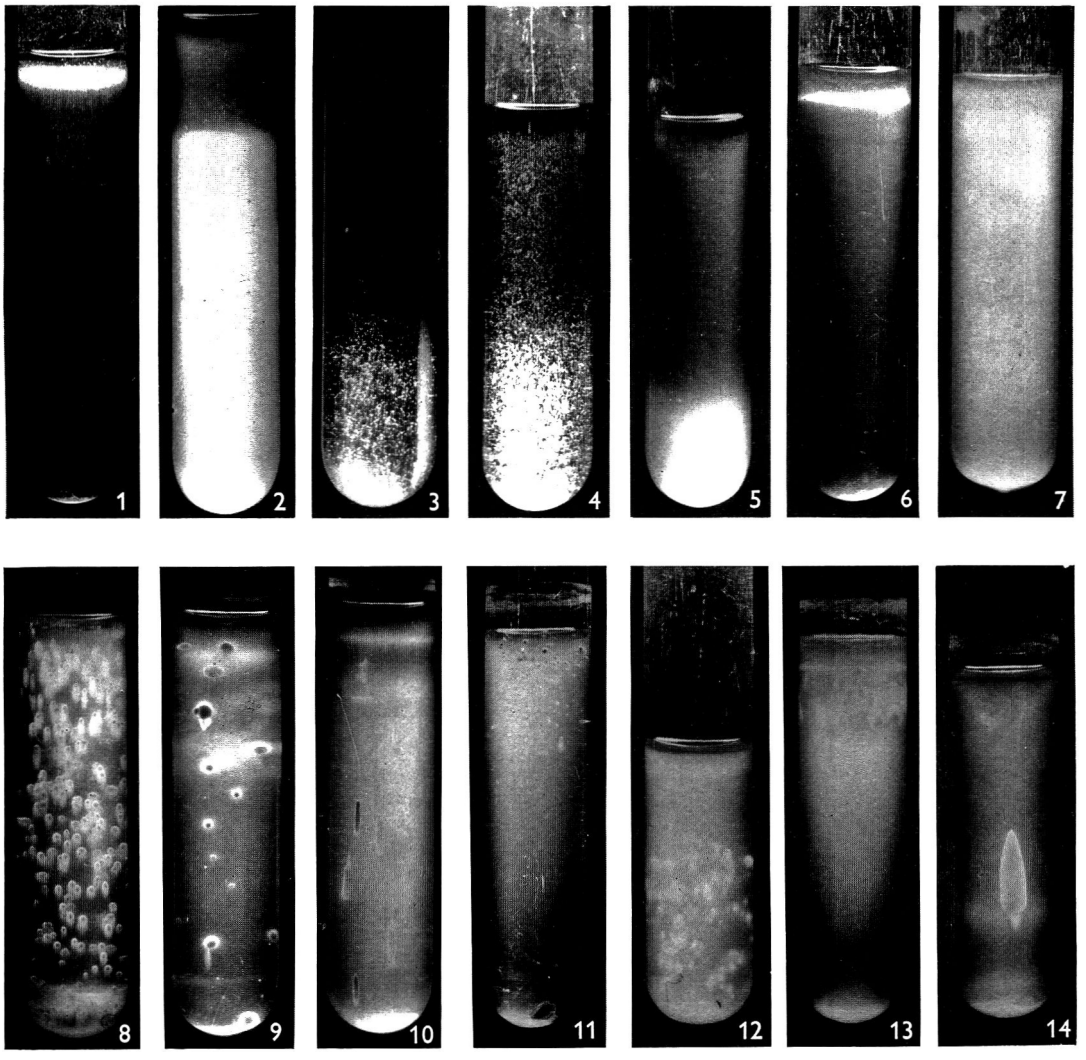
Fig. 18. A strain of pediococcus utilizing glucose in the presence of 10% (w/v) NaCl; 5 days.

Fig. 19. *Lactobacillus fructovorans* NCIB 8039, growth distribution in glucose soft agar; 3 days at 35°.

Fig. 20. *L. fructovorans* NCIB 8039, growth distribution in glucose soft agar; 3 days at 37°.

Fig. 21. A non-dextran-forming leuconostoc utilizing glucose; 3 days. Aerobic (surface) growth is separated from the anaerobic growth by a growth-free zone. Inhibition of growth shown to be caused by $H_2O_2$ diffusing downwards from the aerobic growth.

Fig. 22. As fig. 21, 5 days. The growth-free zone has diminished on continued incubation.

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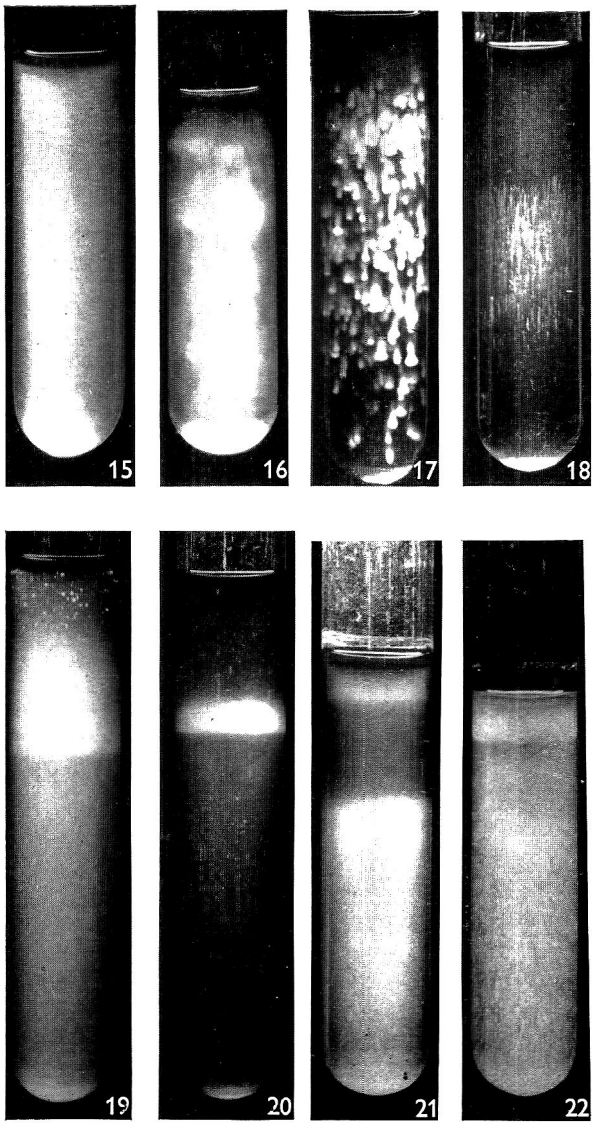
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# The Spore Surface in *Pithomyces chartarum*

By W. S. BERTAUD
*Dominion Physical Laboratory, D.S.I.R., Lower Hutt, New Zealand*

ISOBEL M. MORICE
*Fats Research Laboratory, D.S.I.R., Wellington, New Zealand*

D. W. RUSSELL and A. TAYLOR
*Ruakura Animal Research Station, Hamilton, New Zealand*

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## SUMMARY

Crystalline spicules of sporidesmolides, together with some lipid, form the surface layer of spores of *Pithomyces chartarum*. The formation of the spicules is controlled by the amino acids present in the culture medium.

## INTRODUCTION

Observations by Miss J. M. Dingley indicated that young spores of *Pithomyces chartarum* were echinulate, the echinulation becoming less marked at maturity; electron microscopy showed that a spicular layer covered the surface of the spores. These observations prompted the conjecture of Russell & Brown (1959) that the sporidesmolides might occur as a surface coating. Circumstantial evidence in support of this idea was provided when it was shown that there was a relationship between the number of spores produced in cultures of *P. chartarum* and the weight of sporidesmolides which could be isolated therefrom (Done, Mortimer, Taylor & Russell, 1961; Dingley, Done, Taylor & Russell, 1962). Evidence from electron microscopy, X-ray diffraction, chemical analysis and fermentation experiments which establish the nature of the surface layer are presented here.

## METHODS

*Organisms.* Electron micrographs of spores of the following isolates were kindly provided by Miss J. M. Dingley (Plant Disease Division, D.S.I.R., Auckland, New Zealand) from her collection. Definitions of the terms 'isolate', 'laboratory isolate' and the significance of the numerals are given by Dingley *et al.* (1962). Isolates s68a, s66a and s66 were obtained from pastures at Dargaville, New Zealand, in 1959. Isolate c was described by Dingley *et al.* (1962). Isolate FE4(O) was from pastures at Gippsland, Victoria, Australia obtained in May 1959 and FE4(S) is a laboratory isolate analogous to isolate c derived from FE4(O). Isolate DAOM/57103 was obtained in 1957 during an aerial survey over Manhattan, Riley County, Kansas, U.S.A. and is held in the collection of the Central Experimental Farm, Canada Department of Agriculture, Ottawa. Isolate c was used for all culture work.

*Electron microscopy.* Whole spores were examined by mounting them on carbon-coated collodion films and surface replicas were prepared as follows. Spores were dispersed on microscope slides coated with polyvinyl alcohol. Water vapour was then condensed on the slides to soften the polyvinyl alcohol which was allowed to harden and the spores brushed off, leaving negative impressions on the surface. Collodion (2% w/v, in amyl acetate) was then applied and after drying the composite film was stripped from the slide and floated, collodion uppermost, on hot water to remove polyvinyl alcohol. The collodion film bearing positive impressions of the spores was shadowed with uranium, coated with carbon and mounted on specimen grids. The collodion was removed with amyl acetate and the carbon replica examined in a Philips EM100 electron microscope.

*X-ray diffraction.* The crystalline component was examined by X-ray analysis with Cu$K_\alpha$ radiation collimated to a beam of 0·25 mm. diameter, and a flat film with a specimen to film distance of about 3 mm., accurately calibrated against the X-ray diagram of silver. Powder diagrams were obtained from samples of the total sporidesmolide fraction from whole normal spores, and from pseudo-replicas of spores containing material stripped from the surface. To make the pseudo-replicas a polyvinyl alcohol impression was prepared as for electron microscopy. On brushing off the spores, the spicules were left embedded in the polyvinyl alcohol which was then coated with carbon. When the polyvinyl alcohol was removed with hot water, the spicules were left attached to the carbon film, which was rolled up and inserted in the specimen holder of the diffraction camera.

*Cultivation of organisms.* Spores for benzene extraction were obtained from isolate c, grown on rye (*Secale cereale*) grain media (Lloyd & Clarke, 1959); these were supplied by Dr R. H. Thornton (Soil Bureau, Taita, near Wellington, New Zealand). For the fermentation experiments isolate c was grown in surface culture on potato carrot medium (solids content 20 mg./ml., Done *et al.* 1961) without addition of glucose or ammonium salts. The medium was dispensed into several equal volumes; one volume was used as a control and one of the amino acids (California Corporation for Biochemical Research, Los Angeles, U.S.A.; chromatographically pure) to be investigated was added to each of the other portions. The media thus obtained were dispensed in 100 ml. amounts into pint milk bottles which were then plugged with cottonwool and autoclaved at 120° for 15 min. When cold the bottles containing the various media were randomly distributed, inoculated as described previously (Done *et al.* 1961), laid horizontally in a stack and incubated at 25° for 7 days.

*Benzene extraction of spores.* The fungal tissue (mostly spores, 15·5 g. dry weight) was stirred with benzene (50 ml.) in a sintered glass funnel (no. 4, average pore diameter 5–15 $\mu$) and the extract sucked through; the whole process took about 1 min. A small portion (10–20 mg.) of the residue was removed for electron microscopy. This procedure was carried out 10 times. The residue after the tenth wash was heated under reflux for 15 min. with diethyl ether (25 ml.) + methanol (25 ml.) and filtered hot. This process was performed 5 times and the 5 extracts combined as fraction 11. The residue was heated under reflux with ether (25 ml.) + methanol (25 ml.) + hydrochloric acid (10N; 0·5 ml.) as described for fraction 11 and the 5 extracts were combined as fraction 12. Each of the fractions (1–12) was evaporated to dryness, the residues weighed and extracted with light petroleum (b.p.

40–60°; 6 times with 5 ml.) at 20°. The extracts were evaporated to dryness. The light petroleum-insoluble portions were extracted with chloroform and, in the case of fractions 11 and 12, the chloroform-soluble material recrystallized from acetone. The material soluble in light petroleum from fractions 1–10 was combined, saponified and the fatty acids converted to methyl esters; methyl esters of the fatty acids from fractions 11 and 12 were prepared similarly. The esters were examined by gas-liquid chromatography with a chromatograph constructed in the Fats Research Laboratory as described by Morice (1962).

*Isolation of amino acids from spent medium.* The filtered media from 7-day cultures were lyophilized. The resulting solids (1 g.) in water (20 ml.) was centrifuged (15 min., 1800 rev./min.) and the supernatant fluid run on a column of Amberlite IR 120 ($H^+$ form, 24 g., 22 × 1·9 cm., ≡ 80 ml. N-acid) ion exchange resin. The column was washed with dilute acetic acid (0·5 N; 75 ml.) and then with water (150 ml.). The amino acid was eluted with triethylamine (2 N; in 20%, v/v acetone in water; 280 ml.). The first 40 ml. of eluate were discarded, the remainder collected and evaporated to dryness. The residue was taken up in the minimum volume of warm water, the solution treated with charcoal (0·5 g. Darco G 60, Atlas Powder Co., Wilmington, Delaware, U.S.A.), filtered and the filtrate treated with ethanol until turbid. The colourless crystals which separated after standing for 24 hr. at 2° were collected and identified from their infra-red spectra. All optical rotations quoted were measured on amino acids purified in this way.

Mycelium dry weights, nitrogen determinations, spore counts and sporidesmolide estimations were carried out as described by Done *et al.* (1961). Hydrolysis of depsipeptides and the chromatography of amino acids were carried out as described by Russell, Synge, Taylor & White (1962). The proportion of the various amino acids present in hydrolysates was determined as described by Russell (1960). Infra-red spectra were obtained on specimens dispersed in potassium bromide disks by using a Perkin Elmer 'Infracord' model 137 spectrophotometer.

## RESULTS

When replicas of the spore surface of *Pithomyces chartarum* were examined in the electron microscope, the cuticle was found to be completely covered by a dense coat of fine rod-like structures (Pl. 1, fig. 1). Profile electron micrographs (Pl. 1 fig. 2) showed that these structures were delicate spicules up to 800 mμ long and 30–50 mμ thick, attached by one end to the spore and tending to stand out from the spore surface at right angles. Identical surface structures were observed on all the isolates examined. The appearance of the spore surface in electron micrographs resembled the appearance of the waxy coating seen in electron micrographs of higher plants (Juniper & Bradley, 1958). An attempt was made to remove the spicules by washing the spore with organic solvents; light petroleum had no effect; benzene or diethyl ether removed the spicules completely. The result of such an experiment is shown in Pl. 2, fig. 3; there are no spicules and there is a smooth cuticle, interrupted by the projections seen in the light microscope. It was concluded that the spicules were not an integral part of the spore wall.

To obtain information about the chemical nature of the spicular coating a sample of fungal material consisting mostly of spores was subjected to a series of brief washes with benzene. Electron micrographs of spores removed after each wash made

possible correlation between the disappearance of spicules and the appearance of material in the extract. The weights of the extracts and some of their physical properties are given in Table 1. Fractions 1–10 were completely soluble when treated with light petroleum and then chloroform, but 79 % of fraction 11 and 89 % of fraction 12 were insoluble in these solvents. Only 40 % of that part of fraction 11 soluble in chloroform was sporidesmolides; the nature of the remainder (m.p. 120–135°) was not determined. The infrared spectra of the chloroform-soluble parts

Table 1. *Physical properties of the solutes of benzene washes and of ether-methanol extracts of spores of* Pithomyces chartarum

| Fraction no. | Weight of fraction soluble in light petroleum (mg.) | Sporidesmolide fraction | | |
|---|---|---|---|---|
| | | Weight (mg.) | m.p. (°) | $[\alpha]_D^{19}$ (°) |
| 1 benzene | 50 | 82 | 230–236 | −203 |
| 2 | 15 | 91 | 240–245 | −207 |
| 3 | 5 | 78 | 238–246 | −205 |
| 4 | 2 | 58 | 259–263 | −205 |
| 5 | 2 | 59 | 243–262 | −198 |
| 6 | 1 | 42 | 241–260 | −210 |
| 7 | 1 | 32 | 243–260 | −200 |
| 8 | 1 | 31 | 235–245 | −205 |
| 9 | 1 | 20 | 233–242 | −207 |
| 10 | 1 | 13 | 239–257 | −195 |
| 11 ether+ | 137 | 41 | 245–260 | −185 |
| 12 methanol | 66 | 0 | — | — |

Table 2. *Fatty acid composition (mole %) of lipids found in extracts of spores of* Pithomyces chartarum

$C_{12}$, etc. = straight chain saturated fatty acid having 12 carbon atoms, e.g. stearic acid = $C_{18}$; *$C_{18}^{3=}$ etc. = straight chain unsaturated fatty acid having 18 carbon atoms and 3 double bonds; *(br) = alkyl branched chain; tr. = trace.

| Fatty acid | $C_{12}$ | $C_{44}$ | $C_{15}$ (br)* | $C_{15}$ | $C_{16}^{2=}$* | $C_{16}^{1=}$ | $C_{16}$ | $C_{17}^{1=}$ | $C_{17}$ | $C_{18}^{3=}$ | $C_{18}^{2=}$ | $C_{18}^{1=}$ | $C_{18}$ | $C_{20}^{1=}$ | $C_{20}$ | $C_{22}$ |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | mole % | | | | | | | | | | | | | | | |
| Benzene washes | | | | | | | | | | | | | | | | |
| Fractions 1–10 | 0·3 | 0·8 | 0·4 | 0·6 | 0 | 1·1 | 26·9 | 0·5 | 0·7 | 0·4 | 33·6 | 25·8 | 7·6 | 0 | 0·6 | 0·7 |
| Ether–methanol extracts | | | | | | | | | | | | | | | | |
| Fraction 11 | tr. | 0·5 | 0 | 0·5 | 0·2 | 0·6 | 27·9 | tr. | 0·5 | 0·4 | 51·5 | 12·9 | 4·4 | 0 | 0·6 | tr. |
| Fraction 12 | tr. | 0·6 | 0 | 0·5 | 0 | 0·8 | 23·1 | 0·5 | 0·8 | 0·5 | 51·3 | 15·2 | 4·4 | 0·8 | 1·2 | 0 |

of fractions 1–10 were identical and were indistinguishable from the infrared spectrum of the total sporidesmolide fraction (Russell *et al.* 1962). All of the chloroform-soluble parts of fractions 1–10 provided valine, leucine, *N*-methylleucine and isoleucine after acid hydrolysis and the proportions of these amino acids were the same as those obtained after hydrolysis of the total sporidesmolide fraction. The light petroleum-soluble fraction was principally lipid. The fatty acids present in the lipid from the combined fractions 1–10 and from fractions 11 and 12, and their relative proportions are given in Table 2. The fatty acid composition of fractions 1–10 differed from that of fractions 11 and 12 in that they (fractions 1–10)

contained less linoleic acid and more oleic and stearic acids. The analysis of fractions 11 and 12 was like that reported by Hartman, Morice & Shorland (1962) for the total lipid of spores of *Pithomyces chartarum*. Fractions 1–10 contained 26% of unsaponifiable lipid and fractions 11 and 12, 12%. In Fig. 1 the amount of sporidesmolide remaining on the spore is plotted as a function of the number of washes with benzene.

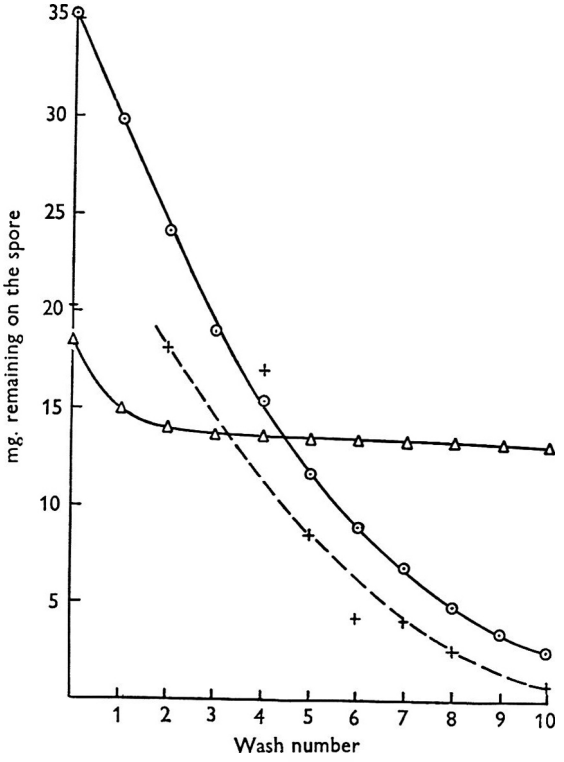


Fig. 1. Extraction curves showing the behaviour of surface constituents during benzene extraction. ⊙——⊙, Sporidesmolides by analysis; △——△, lipids by analysis; +----+, spicule weight by electron microscopy.

Replicas of spores after each benzene wash were examined in the electron microscope; a progressive decrease in the spicular coat was apparent. For comparison with the analytical results estimates were made of the weight of spicular material in each fraction; for this the density of each fraction was assumed to be the same as that of the total sporidesmolide fraction, which was found by centrifugation in a sucrose density gradient to lie between 1·1 and 1·2 g./ml. In making measurements of spicules it was found that length could be ascertained much more readily than thickness. A relationship was found between length and thickness by measuring a large number of clearly defined spicules. Subsequently each spicule was measured for length alone and the relationship used to assign a volume and thus weight. By this means a weight of spicular material per unit area of spore surface was found. The spores were assumed to be prolate spheroids, 15 $\mu \times 10$ $\mu$, of unit density before drying to constant weight; this enabled the amount of spicules to be expressed as a fraction of the spore weight. The results are plotted in Fig. 1. Good agreement

with the weight of sporidesmolides was obtained; most of the points lay on a curve of the same shape as that derived from the chemical analysis. This result is strong evidence that the spicules are composed of sporidesmolide. The displacement of the two curves may indicate the presence of a sporidesmolide component not incorporated in the spicules, but it is doubtful whether the method is sufficiently accurate to make the difference significant.

Further experimental evidence for the nature of the spore coat was sought by making use of the metabolic antagonism of isoleucine and valine (see, for example, Gladstone, 1939). Sporidesmolide I contains two valyl residues but no isoleucine (Russell, 1964). The possibility existed that the organism might be grown in the presence of isoleucine without affecting growth but with inhibition of formation of sporidesmolide I. Electron micrographs of spores of such cultures could then be compared with those of normal spores.

*Effect of* DL-*leucine*, DL-*valine*, *and* DL-*isoleucine on the growth of* Pithomyces chartarum. Adding DL-leucine or DL-isoleucine to cultures increased their growth. The effect was like that reported by Done *et al.* (1961) after addition of ammonium salts and glutamine. The increase in amount of growth was largely independent of the concentration of the starting medium or, in the range examined, of the initial concentration of added amino acid. Only the added amino acid was observed in culture filtrates 7 days after inoculation and quantitative estimation revealed that it accounted for about half the nitrogen present. The isoleucine recovered from culture filtrates was optically active and the direction of the rotation indicated that the L-isomer had been preferentially used by the mould. By contrast, leucine recovered in the same way was optically inactive. Addition of DL-valine also stimulated growth, its effect being greater than that of leucine or isoleucine; it was calculated, on the basis of nitrogen analyses, that 50% of the added valine was incorporated by the mould. The valine recovered from culture filtrates 7 days after inoculation was optically active; in one experiment where the initial DL-valine concentration was 2 mg./ml. the recovered amino acid was almost pure D-valine.

*Effect of* DL-*leucine*, DL-*isoleucine*, *and* DL-*valine on sporidesmolide production*. The yield of sporidesmolides from cultures grown on media supplemented with DL-leucine was the same as that obtained from control cultures grown on unmodified media. The physical properties of the metabolites were identical with those of the total sporidesmolide fraction as described by Russell *et al.* (1962) and the proportion of the amino acid fragments (leucine, valine, *N*-methylleucine, isoleucine) was also the same (1:2:1:0·2). Sporidesmolide production from cultures enriched with DL-valine was greater than that from controls, usually by a factor of 2 or 3, and this ratio was independent of the strength of the starting medium and of the amount of added DL-valine within the range examined. The depsipeptide mixture produced had the same physical properties as the total sporidesmolide fraction from control cultures and gave the same amino acids on hydrolysis.

The addition of isoleucine caused marked changes in the properties and decreased the quantity of depsipeptides produced; in general, weaker starting media produced a greater effect. The isolated depsipeptide fraction from isoleucine cultures had m.p. 221°. It did not sublime and gave an infrared spectrum different from that of the total sporidesmolide fraction obtained from control cultures. On hydrolysis leucine, valine, *N*-methylleucine and isoleucine were obtained in equal proportions.

The quantities of depsipeptides given in Table 5 were calculated from optical rotation data (Done *et al.* 1961) by assuming that the composition of the depsipeptides was the same as that described by Russell *et al.* (1962). As shown above, this assumption is untenable. Therefore the quantity of depsipeptides produced was also determined gravimetrically and was found in all cases to be about 25% higher

Table 3. *Physical properties of amino acids recovered from culture filtrates of* Pithomyces chartarum *after growth for 7 days at 25°*

| | | Recovered amino acid | | | | |
|---|---|---|---|---|---|---|
| Expt. no. | Identity | Weight recovered (mg./ml.) | Weight added (mg./ml.) | $[\alpha]_D^{20}$* (°) | *c* in acetic acid | % D isomer |
| 1 | Isoleucine | 2·3 | 5 | −3·3 | 0·9 | 52·5 |
| 2 | Isoleucine | 1·0 | 2·5 | −15 | 0·5 | 62 |
| 5 | Leucine | 3·0 | 5 | 0·0 | 1·7 | 50 |
| 6 | Valine | 2·0 | 5 | −23 | 0·9 | 66·5 |
| 7 | Valine | 0·15 | 2·5 | −55 | 0·8 | 88 |

* $[\alpha]_D^{20}$ Specific optical rotation using sodium light at 20°.

*c* in acetic acid: optical rotations were determined in acetic acid solution at the concentrations stated in the column.

% D isomer: % enantiomorph having the absolute configuration of the D series.

Table 4. *Sporidesmolide production by* Pithomyces chartarum *in surface culture for 7 days at 25° on media supplemented with 5 mg.* DL-*valine/ml.*

| Expt. no. | Dry weight of fungus at harvest (mg./ml.) | Spores ($\times 10^{-5}$/ml.) | Sporidesmolides (mg./l.) |
|---|---|---|---|
| 7* | 5·9 | 130 | 148 |
| 8 | 10·3 | 117 | 206 |
| 8† | 6·2 | 275 | 279 |
| 6 | 11·4 (7·1) | 380 (125) | 374 (120) |

* Initial concentration of added amino acid 2·5 mg./ml.; initial medium concentration 10 mg./ml.

† 14 days growth.

Values for controls given in parentheses or in Table 5.

Table 5. *Sporidesmolide production by* Pithomyces chartarum *in surface culture for 7 days at 25° on media supplemented with 5 mg.* DL-*isoleucine/ml.*

| Expt. no. | Dry weight of fungus at harvest (mg./ml.) | Spores ($\times 10^{-5}$/ml.) | Sporidesmolides (mg./l.) |
|---|---|---|---|
| 7* | 4·8 (4·1) | 92 (71) | 20 (76) |
| 4 | 5·4 (4·5) | 116 (89) | 28 (45) |
| 1 | 6·6 (7·7) | 145 (134) | 31 (163) |
| 8† | 7·2 (7·6) | 228 (195) | 39 (179) |
| 8 | 9·7 (8·8) | 137 (175) | 35 (147) |
| 3 | 7·9 (7·4) | 230 (228) | 41 (125) |
| 3‡ | 7·9 | 240 | 46 |

* Initial medium concentration 10 mg./ml.

† 14 days' growth.

‡ Initial amino acid concentration 2·5 mg./ml.

Figures in parentheses are results obtained on control cultures, i.e. no supplementary amino acid. Expts. 7 and 4 represent the limits of the observed effect.

than the values given in Table 5. The significance of this result cannot be assessed until more is known about the complexity of this mixture of depsipeptides.

Spores from the isoleucine, valine and control cultures described above were examined by electron microscopy. Estimations of the weight of spicules from the electron micrographs gave the value 7·9 mg. spicules/g. spores for 14-day control cultures (Expt. 8). The values obtained for comparable cultures (Expt. 8, Tables 4 and 5) enriched with valine and isoleucine were 12·4 and 3·2 mg. spicules/g. spores, respectively. All these values are lower than those given in Tables 4 and 5 and it is presumed that this is due to abrasive mechanical loss during the drying and grinding procedures used after harvest. It is clear, nevertheless, that adding isoleucine to the culture medium decreased the amount of spicule material on the spore surface.

*X-ray diffraction.* The total sporidesmolide fraction gave a diffraction pattern with a very strong reflexion at 12·2 Å and strong reflexions at 7·0, 6·1, 5·2, 4·9 and 4·6 Å. The powder diagram from a sample of whole spores (Pl. 2, fig. 4) gave a clear sporidesmolide pattern, together with diffuse haloes which are tentatively attributed to a chitin-like substance in the spore wall. A pseudo-replica containing spicules stripped from the spore surface also gave a clear sporidesmolide pattern but contained no chitin reflexions (Pl. 2, fig. 5).

## DISCUSSION

The presence of spiny processes on the spores of certain strains of Streptomyces was reported by Flaig, Beutelspacher, Küster & Segler-Holzweissig (1952) and several isolates from New Zealand soils have been noted to produce spores with spiny outgrowths (Vernon, 1955). Fragile easily detached spines were observed on the surface of spores of *Streptomyces toyocaensis* so 927 by Arai & Kuroda (1962). Although certain strains of Streptomyces are known to produce depsipeptides (see, for example, Vining & Taber, 1957), no information of this nature is available about the organisms mentioned above and our observations (Vernon & Bertaud, unpublished) indicate that such spines are not removed by washing with organic solvents.

Lipid and sporidesmolides were removed from the surface of spores of *Pithomyces chartarum* at different rates (Fig. 1) by successive washes with benzene; most of the lipid washed from the spores appeared in the first two fractions. Since fractions 1–10 comprised only 28 % of the total lipid and differed in composition from the total spore lipid it seems reasonable to conclude that the washing process removed only material which was present on the surface. By contrast, sporidesmolides were eluted more slowly and 8 % remained at the end of the washing procedure. Because of the very large surface presented by the spicules, of the order of 100 $m^2$./g., equilibrium with solvents is likely to be approached rapidly under the conditions of these experiments; but the solubility of sporidesmolides in benzene is probably too low to account for the observed extraction curve. However, unknown factors such as differences in solubility of the various sporidesmolides and abrasion of colloidal particles during the washing procedure may complicate the results. A second possibility is that the spicules are attached to the spore surface by a benzene-soluble cementing material and pass through the filter as a colloidal suspension. This hypothesis is supported by the fact that little variation was observed in the distribution of spicule lengths during the extraction, whereas if they had been going into

solution a progressive decrease in length would be expected to occur. As the spicules were not removed by washing with light petroleum, the cementing material would presumably appear in the chloroform-soluble fraction and would thus account in part for the discrepancy between weight of sporidesmolides isolated and estimated spicule weight, though no evidence has been obtained for the presence of such an entity.

The numerical correlations between the surface spicules and the amount of sporidesmolides were obtained by two independent experimental approaches. Despite the uncertainty as to the mechanism of extraction with benzene and the reason for the low values of spicule weight on the surface of spores from surface cultures, there is little doubt that the spicules are composed of sporidesmolides and that these compounds are present mainly, if not entirely, on the spore surface. An alternative possibility that the spicules are made of a benzene-soluble precursor which is transformed into sporidesmolides during the isolation procedure is excluded by the X-ray results.

It seems worth while to consider whether the presence of these surface compounds, which are of unusual chemical structure and represent a high (3–4%) proportion of the spore weight, can be used to help understand the sporulation process: does the biosynthesis of the sporidesmolides present unusual features which might provide a clue to the physiology of spore formation? Butler, Russell & Clarke (1962) showed that radioactive valine was incorporated in high yield into sporidesmolide I, and they were unable to show the presence of intermediate products. The effects of DL-valine and DL-isoleucine on sporidesmolide formation recall studies (e.g. Gladstone, 1939) on the role of these amino acids in protein synthesis by micro-organisms, and particularly their effect on the production of actinomycin IV by *Streptomyces chrysomallus* (Katz, Waldron & Meloni, 1961); thus the biosynthesis of sporidesmolide I shows some similarity to protein synthesis. Spore formation is attended by heavy utilization of nitrogen of the medium (Dingley *et al.* 1962); presumably some of this nitrogen metabolism is connected with the synthesis of protein ultimately found in the spores. It seems reasonable to suggest that biosynthesis of sporidesmolides proceeds simultaneously and is an unusual feature of the metabolism of *Pithomyces chartarum* which is associated with sporulation. If this analysis be correct, consideration of the structure of sporidesmolide I suggests that a derivative of valine such as $\alpha$-ketoisovaleric acid may be an important precursor, since residues derivable from such a compound can account for more than 60% of the molecular weight of the metabolite. The precursor might arise during catabolism of mycelium protein.

The spores of *Pithomyces chartarum* are remarkably water-repellent, a property which may be important in their dispersal (Crawley, Campbell & Smith, 1962); in this and in the control of water movement in and out of the spore, the surface sporidesmolides and lipids may well play a part. However, since the sporidesmolides are closely related to antibacterial substances, e.g. valinomycin (Brockmann & Geeren, 1957), one function of the spore coat might be to inhibit the growth of predatory bacteria. Lingappa & Lockwood (1961), discussing the well-known fungistatis of soil, suggested that metabolites with fungistatic properties are produced by micro-organisms whose growth is supported by resting fungal spores. Hence the possibility may be envisaged of an analogous stasis of *P. chartarum* in

pasture as being induced by stimulating the formation of sporidesmolide-deficient spores. Such a biological system would provide a means of determining experimentally the role of *P. chartarum* in facial eczema (Done, Mortimer & Taylor, 1960).

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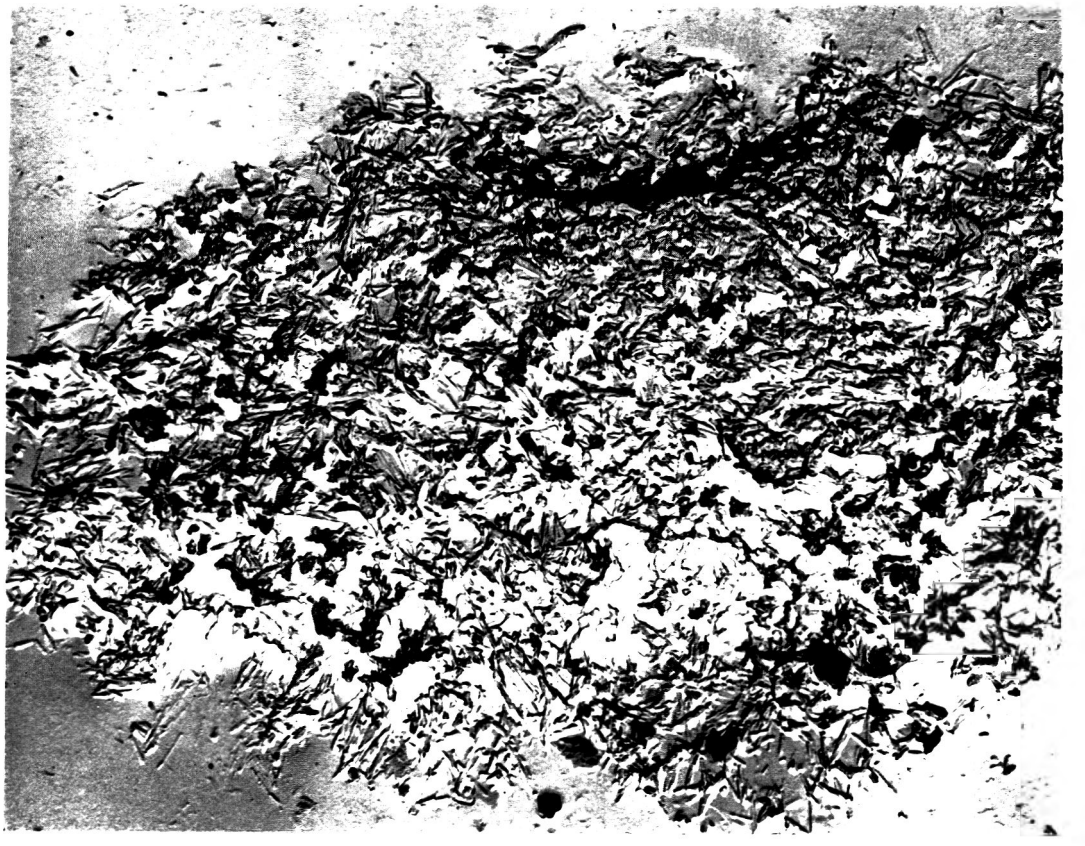

Fig. 1

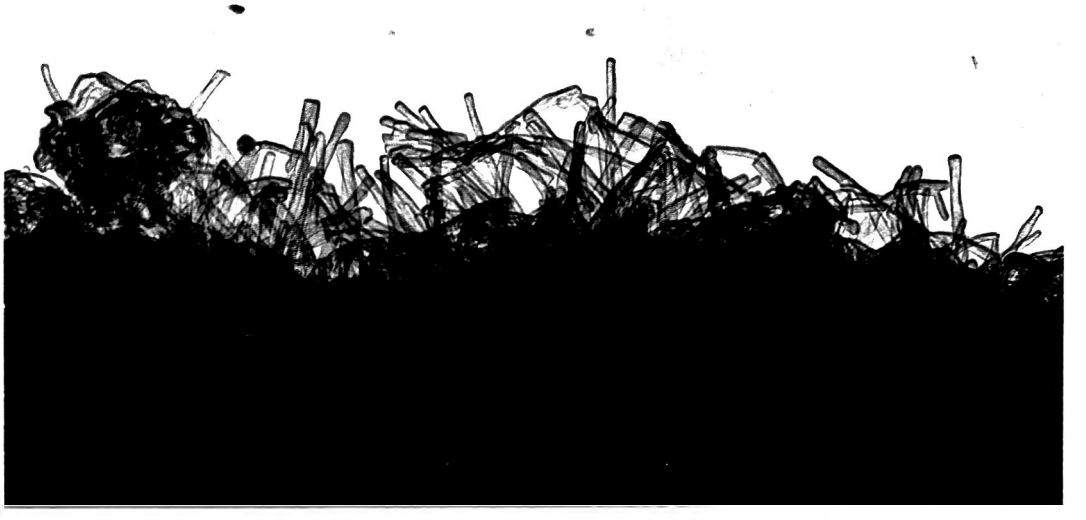

Fig. 2

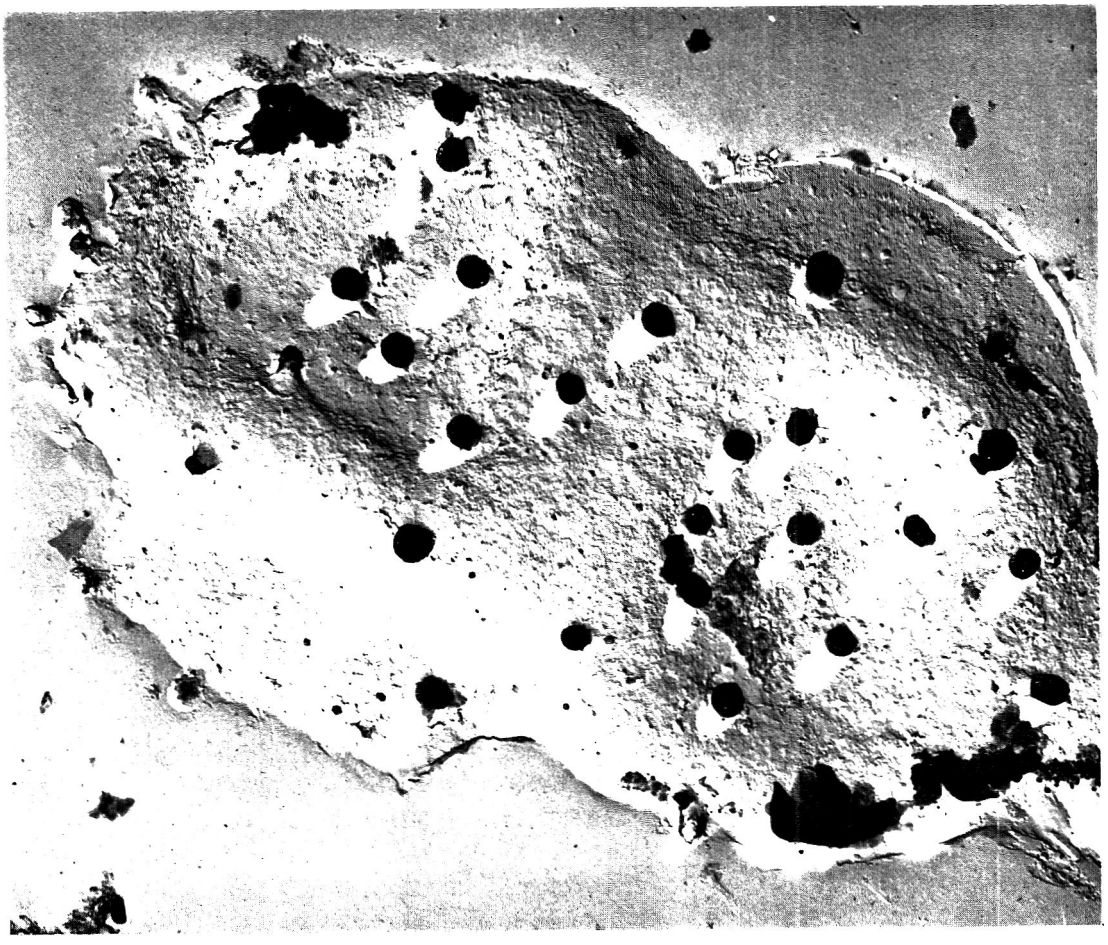

Fig. 3

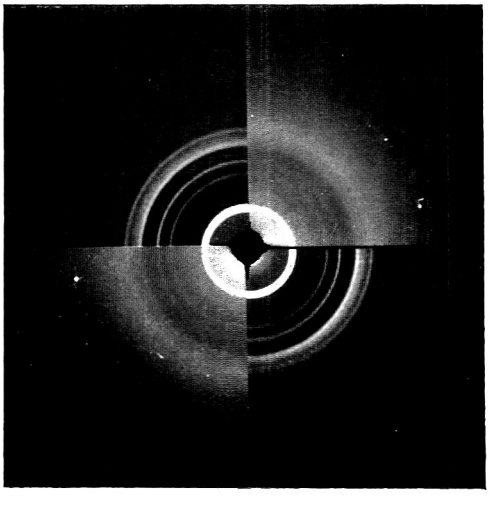

Fig. 4

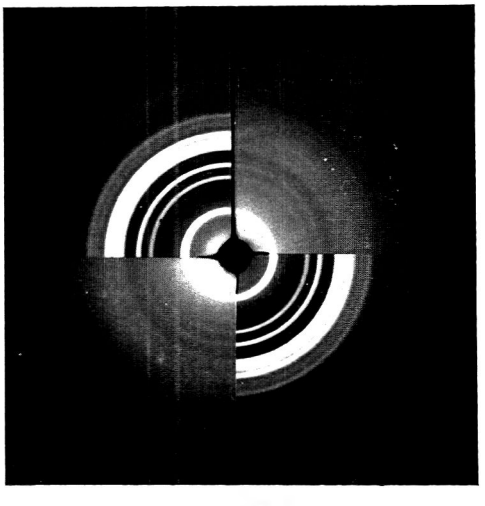

Fig. 5

## EXPLANATION OF PLATES

### Plate 1

Fig. 1. Replica of a natural spore of *Pithomyces chartarum* showing the dense coating of spicules. ( × 13,000.)

Fig. 2. Profile view of a whole natural spore showing how the spicules project from the surface. ( × 30,000.)

### Plate 2

Fig. 3. Replica of a spore washed for 1 hr. in diethyl ether. Many of the cuticular protuberances have been detached and are embedded in the replica. ( × 13,000.)

Fig. 4. X-ray diffraction pattern comparing crystalline total sporidesmolide fraction, top left and bottom right, with whole spores.

Fig. 5. X-ray diffraction pattern comparing crystalline total sporidesmolide fraction, top left and bottom right, with a spore replica containing spicular material as described in the text.

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# Fungi in the Air of Hospital Wards

By W. C. NOBLE*

*Air Hygiene Laboratory,*
*Central Public Health Laboratory, Colindale, London, N.W. 9*

AND YVONNE M. CLAYTON

*Mycology Research Laboratory, Brompton Hospital, London, S.W. 3*

(*Received* 25 *February* 1963)

SUMMARY

The fungal flora of the air of hospital wards was investigated by using slit samplers. Isolations were made on Sabouraud glucose agar and particular attention was paid to the flora which grew at 37°. *Aspergillus fumigatus* was recovered on each of the 78 days of sampling and reached peak of incidence in the autumn and winter months; no other fungal species was recovered at 37° with such regularity. Bed sweeps revealed the presence of these fungi on blankets in the hospital wards. The mean equivalent diameter of the air-borne particles was determined for several fungal species and was found to correspond closely to that of individual spores. No dissemination of fungal particles by four patients with the hypersensitivity type of aspergillosis or by one patient with an aspergillus mycetoma was observed.

## INTRODUCTION

Calendars illustrating the incidence of fungal spores in outdoor air at different times of the year have been prepared by several workers (e.g. Hyde, Richards & Williams, 1956). Examination of the fungi that can be isolated from the air of buildings has, however, been largely confined to those situations where specific fungi might be expected. Thus Ibach, Larsh & Furcolow (1954) reported the isolation of *Histoplasma capsulatum* from the air of a chicken house belonging to a farmer who had recently contracted histoplasmosis, and Stallybrass (1931) reported on the incidence of aspergilli in a grain mill. Clayton & Noble (1963) isolated *Candida albicans*, *Epidermophyton floccosum* and *Trichophyton mentagrophytes* from the air in the vicinity of patients being examined for fungal infections of the skin. Howe, Silva, Marston & Woo (1961) found that of 62 hospital blankets examined, 29 (47 %) yielded unspecified fungi. We know of no other studies carried out on the fungi recoverable from the air of hospital wards. The present report deals with such a study and investigates the size distribution of the fungal particles recovered, particular attention being paid to the flora which grew at 37°. The air of wards at St Bartholomew's Hospital was examined on 78 days between March 1960 and April 1961. Air sampling and blanket sweeps were also carried out at the Brompton Hospital around the beds of four patients with the hypersensitivity type of aspergillosis and one patient with an aspergillus mycetoma.

* Present address: Wright-Fleming Institute of Microbiology, St Mary's Hospital Medical School, London, W. 2.

## METHODS

Slit-samplers were used which sampled at 5 cu.ft./min. on to 6 in. plates and were run for 1 hr. There was no serious overcrowding of the colonies when the plates were incubated at 37° but very much smaller volumes of air had to be sampled when the plates were to be incubated at 20–25°. The slit sampler gives a measure of the mean number of fungal and bacterial particles in the air and the results are conveniently expressed as the number of colonies obtained/cu. ft. of air sampled. The method estimates only the viable and not the total number of spores in the air.

Special samples were also taken on the size-grading slit-sampler (Lidwell, 1959) which divides the particles sampled into four fractions: particles greater than 18 $\mu$ 'equivalent diameter', particles between 10 and 18 $\mu$, particles between 4 and 10 $\mu$ and particles less than 4 $\mu$. From these data the 'mean equivalent diameter' can be calculated. The equivalent diameter of a particle is the diameter of a sphere of unit density having the same settling rate in still air (calculated by using Stokes's Law) as the particle in question (Bourdillon, Lidwell & Lovelock, 1948).

Blankets in use in the wards were examined by the sweep-plate technique of Williams, Blowers, Garrod & Shooter (1960).

The medium used was Sabouraud glucose agar containing streptomycin or neomycin to suppress bacterial growth. Preliminary tests with a slit sampler and with 'settle' plates indicated that more colonies were recovered at 37° on the Sabouraud agar than on malt agar or on Czapek-Dox agar. Since the main interest of the study lay in the Aspergillus species, plates were incubated at 37° but sometimes room temperature (20–25°) was also used.

## RESULTS

### *Isolation of* Aspergillus fumigatus *from the air*

A total of more than 96,000 cu. ft. of air was sampled over 78 days. *Aspergillus fumigatus* was recovered by culture at 37° on each of these days. During the spring and summer months the numbers of *A. fumigatus* particles isolated ranged from 0·01 to 0·2/cu. ft. In the autumn and winter months counts of up to 35 particles/cu.ft. were encountered for periods of up to 2 hr., whilst peak counts of 70 particles/cu.ft. were encountered during 10 min. sampling periods (Fig. 1).

### *Isolation of other fungi*

In the samples recorded above, the only other fungus isolated at 37° with any regularity was *Aspergillus niger*. It was present on 32 of the 78 days, although never in such large numbers as was *A. fumigatus*, the highest count recorded being 0·2 *A. niger* particles/cu.ft. The other fungi recovered from these samples, apart from *Candida albicans*, are given in Table 1. A report upon the prevalence of *C. albicans* in the air is dealt with in a separate paper.

Some sample plates were also incubated at room temperature (20–25°). The various fungi recovered from these 20–25° plates but not from the 37° plates are given in Table 2.

The fungal colonies growing at 37° on Sabouraud agar generally represented about 1 % of the flora which would grow at 20–25°, which averaged about 10 colonies/cu.ft.

This ratio of 37° to room temperature growth fluctuated greatly, depending on the amount of *Aspergillus fumigatus* currently in the air.

*The size-distribution of air-borne fungal particles*

By using the size-grading slit-sampler the mean equivalent diameters of various air-borne fungal particles were calculated and are shown in Table 3. The mean equivalent diameter of these particles approximates to the diameter of spores measured microscopically. This suggests that a number of the air-borne particles exist as single spores and that the spores have a density close to unity.

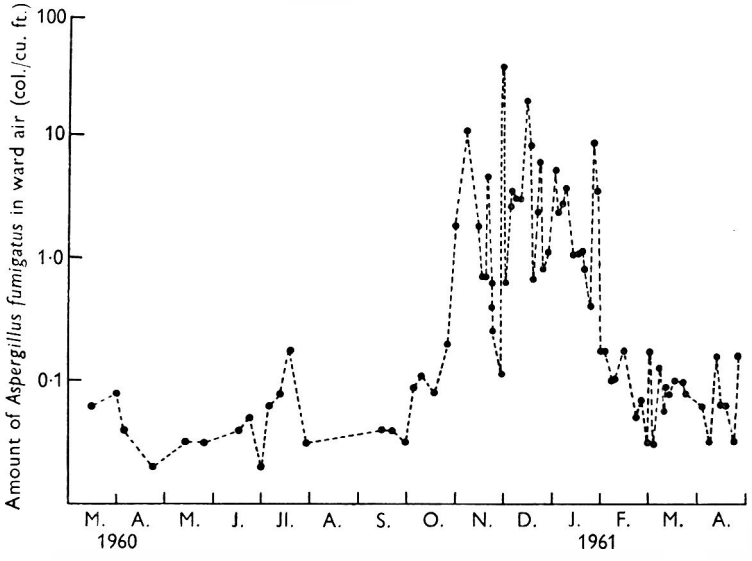


Fig. 1. Number of colonies of *Aspergillus fumigatus* recovered/cu.ft. from ward air over a 2 hr. sampling period

Table 1. *Fungi recovered on a total of* 78 *days' sampling from air samples incubated at* 37°

| Fungus | No. of days fungus isolated | Fungus | No. of days fungus isolated |
|---|---|---|---|
| *Alternaria* | 2 | *Paecilomyces* | 27 |
| *Aspergillus candidus* | 1 | *Penicillium* | 29 |
| *A. flavus* | 21 | *Rhizopus* | 13 |
| *A. fumigatus* | 78 | *Scopulariopsis* | 2 |
| *A. gracilis* | 2 | *Sordaria fumicolor* | 1 |
| *A. nidulans* | 17 | *Syncephalastrum* | 20 |
| *A. niger* | 32 | *Trichoderma koningii* | 5 |
| *A. tamarii* | 3 | *Trichothecium* | 2 |
| *A. terreus* | 13 | *Verticillium* | 2 |
| *A. ustus* | 1 | | |
| *A. versicolor* | 2 | *Chaetomium brasiliensis* | 1 |
| *Botrytis cinerea* | 1 | *Sporobolomyces* | 1 |
| *Cunninghamella* | 1 | *Rhodotorula* | 1 |
| *Epicoccum nigrum* | 1 | *Streptomyces* | 3 |
| *Fusarium* | 1 | *Mycelia sterilia* | — |
| *Gliocladium* | 1 | | |
| *Monilia sitophila* | 17 | | |
| *Mucor racemosus* | 4 | | |

*Examination of blankets*

Over a period of 6 weeks during the autumn and winter, 200 blankets in use in the wards were examined by the sweep-plate method. *Aspergillus fumigatus* was recovered from 79 (39·5 %) of the blankets examined, although the number of beds which yielded the fungus varied from week to week. Other fungi which grew at 37° were recovered from 83 % of the blankets; the majority of fungi recovered from the air were also obtained in small numbers from blankets.

Table 2. *Fungi recovered from air samples incubated at 20–25° but not recovered at 37°*

| | |
|---|---|
| *Aleurisma* | *Phoma* |
| *Aspergillus ochraceus* | *Aureobasidium pullulans* |
| *Cephalosporium* | *Stysanus* |
| *Cladosporium* | *Trichophyton terrestre* |
| *Didymocladium* | *Trichosporon cutaneum* |
| *Isaria* | |
| *Mucor pusillus* | *Candida mycoderma* |
| *M. spinosus* | |

Table 3. *Mean equivalent diameter of air-borne fungal particles*

| Organism | Percentage of particles of given size range ($\mu$) | | | | Mean equivalent diameter of particle ($\mu$) | Size range of single spore ($\mu$) | No. of colonies counted |
|---|---|---|---|---|---|---|---|
| | > 18 | 10–18 | 4–10 | < 4 | | | |
| *Aspergillus fumigatus* | 0·7 | 5·5 | 24·9 | 68·9 | 2·9* | 2·5– 3 | 113,449 |
| *A. niger* (group) | 4·0 | 15·6 | 47·1 | 33·3 | 5·5 | 2·5–10 | 125 |
| *Aspergillus*† | 6·9 | 12·1 | 34·2 | 46·8 | 4·3 | 2·5– 3·5 | 159 |
| *Cladosporium* | 1·4 | 15·5 | 41·1 | 42·0 | 5·2 | (2–6) × (3–20) | 2,651 |
| *Didymocladium* | 14·5 | 49·2 | 31·6 | 4·7 | 10·2 | 5 × (10–15) | 523 |
| *Monilia sitophila* | 8·3 | 39·1 | 49·7 | 2·9 | 8·9 | 3 × (3–10) | 74 |
| *Paecilomyces* | 3·9 | 6·0 | 35·0 | 55·1 | 3·7 | 6 × (2·5–3) | 105 |
| *Penicillium* | 0·7 | 6·3 | 26·8 | 66·2 | 3·0 | 2·5–4·5 | 798 |
| *Rhizopus* | 3·8 | 31·3 | 38·7 | 26·2 | 6·6 | 6–9 on long axis | 45 |
| *Rhodotorula* | 0·3 | 5·3 | 37·9 | 56·5 | 3·8 | (3–5) × (4–7) | 4,039 |
| *Syncephalastrum* | 3·2 | 26·1 | 47·2 | 23·5 | 6·8 | (2·5–5) × (5–20) | 240 |

* This result is based on seven separate evaluations having the following values: 1·8, 2·3, 2·7, 3·2, 3·3, 4·0, 4·1. There is a suggestion that the mean equivalent diameter is greater in winter than in summer.

† These comprise *Aspergillus gracilis*, *A. nidulans*, *A. terreus*, *A. versicolor*.

*Investigations in the environment of patients with aspergillosis*

*Aspergillus fumigatus* was isolated from the blankets of four patients with the hypersensitivity type of aspergillosis and one patient with an aspergillus mycetoma. The number of colonies recovered on the sweep plates was no greater than on those from the surgical wards at St Bartholomew's Hospital. No increase in the mean equivalent diameter of the air-borne spores could be demonstrated when the blankets were shaken, as might have been expected if the spores had become

associated with organic debris or blanket fibre. The incidence of *A. fumigatus* in the air in the environment of these five patients with the fungus in their sputa was no greater than in air samples taken elsewhere in the hospital and outside the hospital at the same time. These particular investigations were carried out in the autumn.

## DISCUSSION

Many workers have used the spore trap to determine the fungus species present in the air (e.g. Hamilton, 1959). Whilst this method is useful for studying allergens, it has the disadvantage that the Aspergilli and Penicillia cannot be differentiated. Another method which has been used was to expose agar plates and to allow organisms to settle on them ('settle' plates). De Vries (1960) and Stallybrass (1961) isolated Aspergilli in this manner, and Friedman, Derbes, Hodges & Sinski (1960) thus recovered dermatophytes from the air of a clinic. However, the number of spores which settle on the surface is dependent not only on their concentration in the

Table 4. *Terminal settling velocity of spherical particles of unit density in still air*

| Mean equivalent particle diameter ($\mu$) | Settling rate (ft./min.) |
|---|---|
| 30 | 5·4 |
| 20 | 2·4 |
| 15 | 1·3 |
| 10 | 0·6 |
| 5 | 0·15 |
| 2 | 0·024 |
| 1 | 0·006 |

The settling rates are calculated from the data given in Bourdillon *et al.* (1948).

air but also on the size of the particles (Table 4). A particle of 20 $\mu$ equivalent diameter settles at a rate of 2·4 ft./min. whilst one of 2 $\mu$ settles at only 0·024 ft./min. in still air, so that even very small air movements will suffice to keep the smaller particles air-borne. Richards (1954) recorded differences in the recovery of moulds indoors and outdoors which he attributed to differences in settling rates of spores of different sizes.

By using the size-grading slit-sampler we found that the mean equivalent fungus particle diameter was close to the diameter of a single spore. It seems reasonable to conclude that some of the air-borne particles are individual spores, though from their size, they might also be fragments of mycelium. Such fragments have been observed in the Hirst spore trap (Hamilton, 1959) but it is not known whether these were viable. Davies (1957) found that more than 80 % of Cladosporium particles recovered indoors were single spores and a further 13 % existed as groups of two spores. Clayton & Noble (report to be published) found that *Candida albicans* in the air of hospital wards had a mean equivalent diameter greater than that of individual organisms. The same is true for dermatophytes (Clayton & Noble, 1963) and for *Staphylococcus aureus* and *Clostridium welchii* in hospital wards (Noble, 1961). These micro-organisms are derived from patients in the wards and may be associated with organic debris.

The number of spores of any given fungus species recovered from the air varied from day to day and from season to season. The peak prevalence for *Aspergillus fumigatus* recorded in these studies was in the autumn, which corresponds with data collected by Hyde *et al.* (1956) by using settle plates. The failure to demonstrate dissemination of *A. fumigatus* by five patients with aspergillosis may have been due to the fact that any increase in fungal spores in their environment was masked by the large numbers of spores already present in the air in the autumn when examinations were made, but equally, such patients may not disperse the fungus.

We are indebted to the Treasurer and Governors of St Bartholomew's Hospital and to the physicians and surgeons of the Brompton Hospital for permission to work in the wards. One of us (Y.M.C.) was in receipt of a grant from the Asthma Council.

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*With* 1 *plate*
*Printed in Great Britain*



# A Systematic Study of the Genus *Spirillum* which Occurs in Oxidation Ponds, with a Description of a New Species

By W. A. PRETORIUS

*National Institute for Water Research, Council for Scientific and Industrial Research, P.O. Box* 395, *Pretoria, Republic of South Africa*

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SUMMARY

A systematic study of the spiral form bacterial flora of oxidation ponds has been made. Three known species, namely *Spirillum itersonii*, *S. serpens* and *S. volutans*, were constantly found. A primary enrichment medium for *S. volutans* from natural sources is given. A new species has been isolated; its main characters are: strictly aerobic, catalase-positive, does not grow under anaerobic conditions in the presence of nitrate, forms microcysts, differs in carbon-source utilization from previous described species. The species has been named *Spirillum peregrinum*.

## INTRODUCTION

With the exception of a few members of the genus *Spirillum* Ehrenberg (Carter, 1888; Cayton & Preston, 1955) all the species so far studied have been isolated from various sources of fresh and salt water containing organic matter (Lewis, 1940; Myers, 1940; Williams & Rittenberg, 1957). Freshwater ponds containing large quantities of organic matter were established when domestic sewage was treated by the oxidation pond system of sewage purification, and it was therefore expected that various species of this genus would be present in such ponds. Apparently a systematic investigation of the spiral form bacterial population of oxidation ponds has not before been attempted. Such a study might prove to be an addition to the existing knowledge of the microbial population of oxidation ponds. Spiral bacterial forms from such ponds were enriched and isolated according to the procedures given below. The cultural characteristics of the isolates obtained are reported and have lead to the clear separation of a new species of the genus *Spirillum*, a description of which is given.

## METHODS

### *Method of primary enrichment*

Samples of oxidation pond water were taken near the inlet and outlet of a primary oxidation pond. The samples were first agitated in a macerator (Townson and Mercer, Croydon, England) for 10 sec. and then enrichment cultures were made in 250 ml. cotton-wool stoppered Erlenmeyer flasks by mixing 50 ml. inoculum with equal volumes of the following media:

*Medium* (*a*). An infusion of 1 g. dried grass (small pieces) in 100 ml. distilled water at 100° for 1 hr., keeping the volume constant. This infusion was used as it was, or with the solids filtered off.

*Medium* (*b*). A defined medium as described by Myers (1940) with calcium lactate as carbon source and $NH_4Cl$ as nitrogen source.

*Medium* (*c*). A basal medium of the following composition: $K_2HPO_4$, 1·0 g.; $(NH_4)_2SO_4$, 1·0 g.; NaCl, 1·0 g.; $MgSO_4.7H_2O$, 1·0 g.; $MnSO_4$ and $FeCl_3$, one drop of a 1 % (w/v) solution of each; peptone (Evans), 1·0 g.; distilled water 1000 ml. Glucose, fructose, ethanol, glycerol, calcium lactate, cellulose powder (Whatman standard grade) and cellobiose were used as sources of carbon, at 1·0 % (w/v). With the exception of glucose, fructose and cellobiose of which solutions were Seitz-filtered and added to the heat-sterilized medium, all other carbon compounds were added before sterilization at 121° for 15 min.

After mixing with the inoculum the pH value of the mixtures were checked and when necessary adjusted to pH 7·0 with diluted NaOH or HCl. All flasks were incubated at 30°.

*Isolation of pure cultures*

All enrichment cultures were first examined microscopically for the presence of spiral bacteria. The different morphological types were noted and an attempt then made to isolate a representative of each of the morphological types.

To allow the spirilla to become dominant it was often necessary to do serial enrichment cultures. This was done by the capillary tube method described by Rittenberg & Rittenberg (1962). This method made it possible in a few cases to separate the spiral forms, to a certain extent, not only from non-motile forms, but also from motile forms other than spirilla. The media used here were (Difco) nutrient broth or medium (*c*) with cellulose as carbon source.

After incubation of these subenrichment cultures for 2 days at 30°, no difficulty was encountered in isolating pure cultures by streaking on nutrient agar and/or cellulose-supplemented medium (*c*) + 1·5 % Bacto agar.

In all the primary medium (*c*) + cellulose enrichment cultures, a giant spiral bacterium was noted. All attempts to isolate this bacterium by the above methods were unsuccessful. However, pure cultures were eventually isolated by using the dialysis bag method of Rittenberg & Rittenberg (1962).

*Identification of isolated spirilla*

*Observations and staining technique.* For the estimation of size (by eye-piece micrometer), suspensions of 24 hr. nutrient broth cultures were used, the organisms being fixed by exposure to 2·0 % (w/v) osmic acid for 30 sec. For the demonstration of flagella, Leifson's (1960) method was used. Volutin granules were demonstrated by the method described by Jörgensen (1948). Hucker's modification (*Manual*, 1957) of Gram's method was used to stain heat-fixed films.

*Colony form.* Cultures were grown on (Difco) nutrient agar, and on potato glucose agar (adjusted to pH 7·0 with sodium carbonate). Colony form was recorded after incubation for 2 days.

*Oxygen requirements.* These were tested by the shake tube method with 15 ml. nutrient agar in $6 \times \frac{5}{8}$ in. test tubes, and examined after 7 days. Nitrate broth (Difco) tubes were placed in boiling water for 15 min., immediately cooled down, inoculated, and sealed with wax.

*Growth temperatures.* Nutrient agar (Difco) slopes were incubated at the following temperatures: 10°, 20°, 30°, 40°, 45° and 50°.

*pH value for growth.* The defined medium of Giesberger (1936; $NH_4Cl$, 0·1 %; $K_2HPO_4$, 0·05 %; $MgSO_4$, 0·05 %) with 0·2 % (w/v) calcium lactate was used, and samples were adjusted with diluted HCl or KOH to give a range of pH values between 5·0 and 9·0 at 1 unit intervals.

*Nitrate reduction.* Nitrate broth (Difco) with Durham fermentation tubes, was used. Cultures were tested with Griess–Ilosvay's reagents (with dimethyl-α-naphthylamine) after 5 days. Zinc metal was added to the negative cultures and when found again negative, tested for ammonia with Nessler's reagent.

*Gelatin hydrolysis.* Stab cultures were made in Difco nutrient gelatin and incubated at 20° for 14 days.

*Catalase production.* Growth from a 24 hr. nutrient agar slope was placed into a drop of '10 vol.' $H_2O_2$ solution and examined for production of gas bubbles.

*Utilization of carbon compounds.* The method of Williams & Rittenberg (1957) was used. Carbon sources were added to 0·2 % (w/v) in Giesberger's basal medium. Organic acid solutions were neutralized with KOH and sterilized separately by autoclaving momentarily at 111° before adding to basal medium.

## RESULTS

### *Primary enrichment media*

By microscopical examination of the primary enrichment cultures, it was observed that the different media differed in selectivity for spiral organisms. For example, medium (*c*) + 1 % (w/v) cellulose powder allowed the growth of a large spiral organism and numerous small spiral bacteria. From the various enrichment media, representative spirilla were eventually obtained in pure cultures which could be classified into four different morphological groups. The number of isolates and some measurements of the different groups are given in Table 1. The organisms of all cultures were Gram-negative with bipolar tufts of flagella and, except for group I organisms, all groups showed volutin granules.

Table 1. *Measurements and some characteristics of isolated spirilla*

| Morphological group | No. of isolates | Measurements of organisms (μ) | | | | Volutin granules | Microcysts formed |
|---|---|---|---|---|---|---|---|
| | | Diameter | Spiral pitch | Spiral width | Length of organism | | |
| I | 4 | 0·5–6·5 | 3·25 | 1·3 | 4·6–5·8 | Not seen | Yes |
| II | 5 | 0·7–8·5 | 6·5–9·0 | 1·5–3·7 | 6·0–3·7 | Present | Yes |
| III | 9 | 0·9–1·3 | 6·5–8·0 | 2·5–3·0 | 10–22 | Present | Not seen |
| IV | 6 | 1·5–2·0 | 6–9 | 9–20 | 21–49 | Present | Not seen |

*Colony form.* The colonies of the spirilla isolated had a characteristic granular appearance. Colony formation was good on nutrient agar and on potato glucose agar. The general appearances corresponded to those described by Williams & Rittenberg (1957). On no occasion, however, was it possible to obtain group IV (Table 1) organisms in pure culture other than in the dialysis bags. This organism did not grow in pure culture in the media tested, so that no growth characteristics have been obtained.

*Oxygen requirements.* By the shake tube method all cultures behaved in the same way: growth was profuse only at the top of the tube. With nitrate broth incubated

anaerobically, only cultures of group I grew well; none of the others showed growth.

*Growth temperatures.* All cultures grew between 10° and 30°; the organisms of group I also grew well at 40° but did not grow at 45°.

*pH range.* The pH ranges over which growth occurred in a defined medium with lactate was as follows: group I, below 5·0 to more than 9·0; group II, from 5·0 to 8·0, not at 9·0; group III, from 6·0 to 8·0, not at 9·0. Group IV organisms grew in cellulose enrichment medium (i.e. not in pure culture) between pH 6·0 and 7·5.

Apart from morphological differences between the groups, the biochemical activities also differed markedly.

*Nitrate reduction.* Group I organisms reduced nitrate, the isolates of the other groups did not. Group I isolates reduced nitrate to nitrite after 24 hr. and completely to ammonia in 48 hr.

*Gelatin hydrolysis.* Only organisms of group III showed weak gelatin hydrolysis after 14 days.

*Catalase reaction.* All cultures were catalase-positive. The catalase reaction of group II organisms was strong, while that of groups I and III was so weak that it was at first thought to be negative.

*Utilization of carbon compounds.* The ability to utilize organic compounds as sole carbon source is summarized in Table 2.

Table 2. *Utilization of compounds as nutrients*

(*a*) Utilization of compounds singly as carbon sources

| Morphological group | Malonate | Citrate | Lactate | Malate | Fumarate | Succinate | Pyruvate |
|---|---|---|---|---|---|---|---|
| I | − | − | + | + | + | + | + |
| II | + | + | + | + | + | + | + |
| III | − | − | + | + | + | − | − |

| Morphological group | Butyrate | Propionate | Acetate | Fructose | Glucose | Glycerol | Ethanol |
|---|---|---|---|---|---|---|---|
| I | + | + | + | + | + | + | + |
| II | + | + | + | + | − | − | + |
| III | + | − | + | − | − | − | − |

(*b*) Utilization of nitrogen sources with succinate as sole carbon source

| | $KNO_3$ | Urea | $NH_4Cl$ | Asparagine |
|---|---|---|---|---|
| Group I | + | + | + | + |
| Group II | + | + | + | + |
| Group III | − | + | + | + |

## DISCUSSION

From the data obtained, the morphological groups I, III and IV were found to be made up of the following species: *Spirillum itersonii*, *S. serpens* and *S. volutans*, respectively. The dimensions of the organisms of group II closely resemble those of *S. mancuniense*, but differed in that they were all strongly catalase-positive, grew well in Difco nutrient broth and potato glucose agar and did not liquefy gelatin. The organisms of group II also differed from those species described and classified by Williams & Rittenberg (1957); they are aerobic, do not grow anaerobically in the presence of nitrate, and form microcysts in old cultures. Their size and metabolic reactions differed from those of other freshwater microcyst-forming spiral bacteria

so far described. It is not known whether these organisms are to be found only in oxidation pond water. Because the organisms of group II are the only unknown spirillum species isolated, the name *S. peregrinum* has been given to these organisms.

It is interesting to note that, apart from the two well-known species *Spirillum itersonii* and *S. serpens*, *S. volutans* also occurred very frequently in the oxidation ponds. The cellulose enrichment cultures, made from samples from various sewage works, were all positive for this organism; such sewages therefore seem to be a natural habitat of this organism.

### *Spirillum peregrinum* sp.nov.

*Growth in nutrient broth and Evans peptone* (1 % *w*/*v*) *water.* In 18–24 hr. cultures the organisms are usually 6·0 $\mu$ long, with a spiral width of 2·3 $\mu$ and a diameter of 0·8 $\mu$. The organisms usually have a S-shape and when not it can be seen that the organism is composed of two or more S-shaped units. Tufts of flagella are formed at these joints (Pl. 1, fig. 1). Only a few organisms showed a granular appearance. In 2- to 3-week cultures, a white sediment formed, consisting of non-motile organisms and microcysts. The supernatant fluid, however, contained organisms of various spiral forms: some were short, thick, fast-moving organisms, others formed perfect rings with the ends open and of diameter 7·5 $\mu$; the others were spiral organisms 22·5 $\mu$ long with a spiral pitch of 9·0 $\mu$ and a spiral width of 3·7 $\mu$. All organisms had a granular appearance, probably due to volutin granules (Pl. 1, fig. 2).

*Growth in defined media containing lactate and pyruvate.* Apart from the above-mentioned forms, numerous long tightly coiled organisms appeared in these media; of length 37 $\mu$, diameter 0·7 $\mu$, spiral pitch 1·5 $\mu$ and spiral width 1·5 $\mu$. Typical appearances are shown in Pl. 1, fig. 3.

*Microcysts.* Microcysts are formed in 1-week and older cultures, are oval to ellipsoidal in early stages of formation, becoming spherical on ageing, the average diameter being 3·5 $\mu$. Although non-motile, these microcysts possessed polar tufts of flagella (Pl. 1, fig. 4). All organisms were Gram-negative with bipolar tufts of flagella.

*Colonies on nutrient agar.* Finely granular, round, greyish, 2–3 mm. diameter.

*Potato glucose agar.* Similar to nutrient agar, except that colonies were yellowish-grey.

*Gelatin.* Not liquefied.

*Nitrogen sources.* Ammonia, nitrate, urea and asparagine used with succinate as carbon source.

pH *range.* Growth occurred between pH 5·0 and 8·0.

*Carbon sources.* Grows well with salts of malonic, citric, lactic, malic, fumaric, succinic, pyruvic, propionic, butyric and acetic acids. Good growth with fructose or ethanol, but very poor or no growth with glucose or glycerol.

Aerobic catalase-positive; no growth under anaerobic conditions in the presence of nitrate.

*Source.* Isolated from primary oxidation pond water. A type culture has been deposited at the National Collection of Industrial Bacteria, Torry Research Station, Aberdeen, Scotland; the number assigned to it is NCIB 9435.

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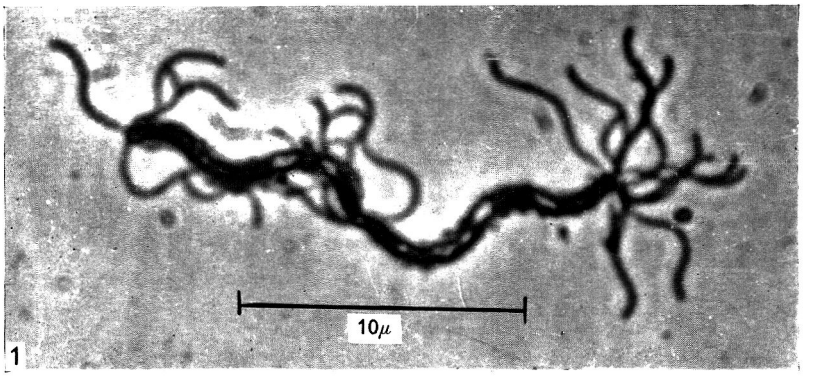
## EXPLANATION OF PLATE

Fig. 1. Organisms from 48 hr. broth culture, showing flagella at poles and intersection. Leifson's flagella stain.

Fig. 2. Typical ring-shaped organism and two longer organisms in 2-week nutrient broth culture. Leifson's flagella stain.

Fig. 3. Long tightly coiled organisms in lactate broth. Methylene blue (1 %) stain.

Fig. 4. Microcyst with bipolar tufts of flagella. Leifson's flagella stain.

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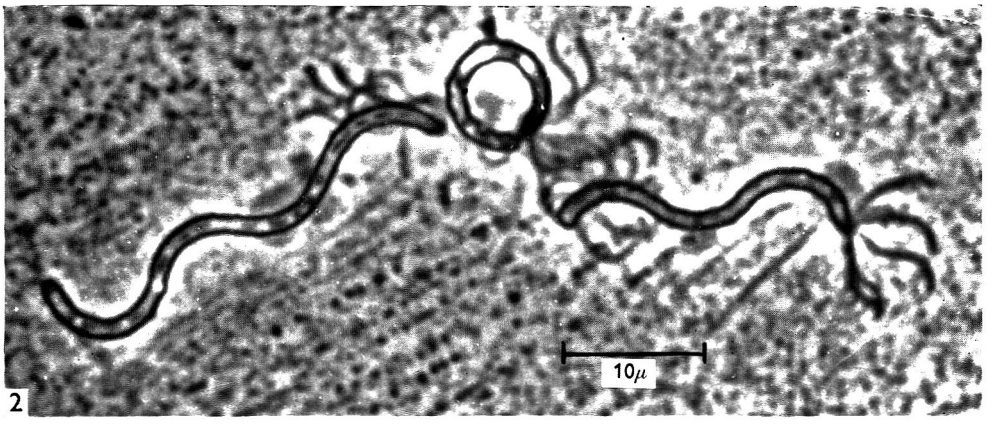


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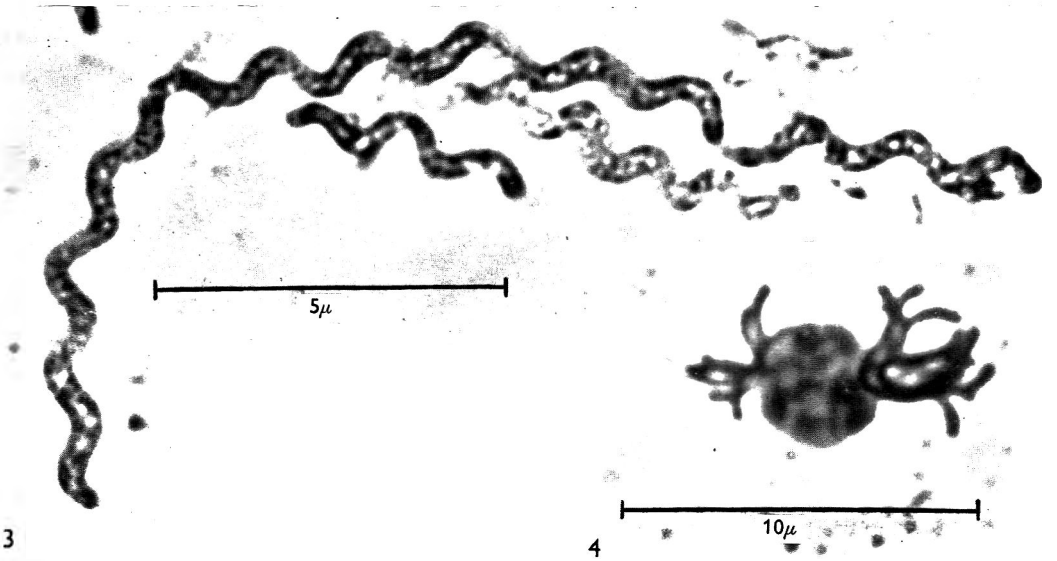


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# The Growth of Myxamoebae of the True Slime Mould, *Didymium nigripes*, in Axenic Culture

By N. S. KERR

*Department of Zoology, University of Minnesota, Minneapolis* 14, *Minnesota, U.S.A.*

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SUMMARY

Myxamoebae of *Didymium nigripes* were grown in a peptone+yeast extract+glucose broth to which formalin-killed *Aerobacter aerogenes* or *Bacillus megaterium* had been added. Either the formalin-killed bacteria or a fraction from broken bacteria was indispensable for growth. The activity of the bacteria was destroyed by heat. The supernatant fluid of sonically disrupted bacteria supported growth; the active fraction of this was non-dialysable and was precipitated by ammonium sulphate. Lysed bacterial protoplasts did not support growth. Numerous compounds were tested unsuccessfully in attempting to eliminate the bacterial factor. When glucose was omitted from the medium the initial growth rate did not change but the population began to decrease rapidly after the fifth day.

## INTRODUCTION

During the life cycle of the true slime moulds several interesting morphogenetic events take place. Spores germinate to yield uninucleate myxamoebae, which are capable of differentiating into flagellated swarm cells. After a period of growth and division the myxamoebae differentiate, probably with sexual fusion, into masses of coenocytic protoplasm called plasmodia; these continue to enlarge for a time before differentiating into fruiting bodies which contain spores. Mycetozoa have usually been cultivated in the laboratory by feeding them with living bacteria or yeast. The presence of living associates makes many studies difficult, and the goal of growing these organisms on a defined medium has long attracted the attention of biologists. One of the first careful nutritional investigations was that of Cohen (1939), who devised techniques for freeing plasmodia of contaminating organisms and growing them on killed yeast. Sobels (1950) and Hok (1954) extended these studies. More recently, Daniel & Rusch (1961) devised a defined medium for plasmodia of *Physarum polycephalum*.

In many ways *Didymium nigripes* is an ideal subject for investigation; it completes its life cycle in less than 7 days (Kerr & Sussman, 1958), making all stages of the life cycle readily accessible. Although *D. nigripes* has been studied extensively, there is no record of previous attempts to grow this organism in axenic culture. The present investigation was designed to overcome this deficiency.

The uninucleate amoeboid stage in the life cycle was chosen for the first attempts at axenic culture. Among the advantages of using this stage is that growth is easily measured as an increase in the number of myxamoebae. Amoebae are more readily subcultured in a reproducible fashion than are plasmodia. Two of the most important

morphogenetic events in the life cycle begin with myxamoebae: flagella formation and plasmodium formation. One reason for working with an axenic medium was to make the isolation of mutant stocks easier. The myxamoebae are presumably haploid, whereas the plasmodia may be diploid.

There are only a few reports of axenic culture of amoebae. Reich (1935) succeeded in growing *Mayorella palistinensis* on a peptone medium. Caillieu (1933) and Neff (1957) did likewise for a species of *Acanthamoeba*. Adam (1959) grew *Acanthamoeba* sp. and Band (1959) grew *Hartmanella rhysoides* on defined media. Sussman (1963) grew *Polysphondylium pallidum* on a medium containing lecithin and skim milk. Other small amoebae have been successfully grown in axenic culture only by including in the medium killed bacteria or a fraction isolated from bacteria. Brent (1954) could obtain axenic growth of *Tetramitus rostratus* only by including heat-killed bacteria in the medium. Sussman & Bradley (1954) isolated a heat-stable protein from *Aerobacter aerogenes* which supported growth of amoebae of *Dictyostelium discoideum*.

## METHODS

For most of the experiments 5 ml. cultures of myxamoebae were grown in 25 ml. Falcon plastic tissue culture flasks. During the latter part of the work 5 ml. cultures in 16 × 150 mm. test tubes, capped with Morton closures, were incubated on a 1 rev./min. roller drum. When 50 ml. cultures were needed spinner flasks or Erlenmeyer flasks on a gyrotatory shaker were used. All cultures were grown at 22°. Growth rates and final yields were comparable in all the containers used.

An increase in number of myxamoebae was used as an index of growth. Counts were made in a Levy counting chamber with Fuchs–Rosenthal rulings, 0·2 mm. deep. Neff, Neff & Taylor (1958) had difficulty in freeing Acanthamoeba from the walls of the culture flask. Myxamoebae of *Didymium nigripes* were easily dislodged from the walls of the culture chamber by swirling or shaking the container.

Heat-stable components of the medium were autoclaved and then dispensed into individual growth containers. Sugars were autoclaved separately. Heat-labile components were sterilized by passing them through an AM-7 Polypore membrane filter.

*Aerobacter aerogenes* and *Bacillus megaterium* were killed with formalin by a modification of the procedure of Weinstein & Jones (1956). Bacteria, grown for 2 days at 22° on glucose + peptone + yeast extract agar, were harvested with water and washed once by differential centrifugation. The washed pellet was resuspended in water to give a suspension with the consistency of thick cream. Sufficient 40 % formaldehyde (USP) was added to make the formaldehyde concentration 7 %. The formaldehyde + bacteria mixture stood at room temperature for a day, and the formaldehyde was then removed by washing the bacteria with water by centrifugation; four washes over a period of 2 days were usually sufficient. The washed formalin-killed bacteria were diluted with sterile water until the final concentration was equivalent to about 25 mg. dry weight/ml. The formalin-killed bacteria could be stored for several months in the refrigerator without loss of activity.

Several procedures were used to fractionate living bacteria in attempting to liberate a growth-promoting fraction. Bacteria were suspended in 0·1 M-phosphate buffer (pH 6·5) and treated for 45 min. in a Raytheon sonicator. The disrupted bacterial suspension was centrifuged to remove the larger particles. The supernatant

fluid was then filter-sterilized. A cooled semi-micro head on a Waring blender was also used to break the bacteria.

Penicillin-induced spheroplasts of *Aerobacter aerogenes* were made by using the procedure of Gebicki & James (1960). Lysozyme-induced protoplasts of *Escherichia coli* were made by using procedures described by Spizizen (1962).

*Purification of myxamoebae. Didymium nigripes* was routinely grown in monoxenic culture on *Aerobacter aerogenes*. The myxomycete was freed from its bacterial associate by one of two procedures. Spores of *D. nigripes* were suspended in glucose + peptone + yeast extract broth to which 1000 units penicillin/ml. and 0·5 mg. streptomycin/ml., filter-sterilized, had been added. The spores were incubated in the antibiotic-containing medium for one day and then removed by centrifugation and used as an inoculum. Because this procedure only occasionally succeeded in freeing the spores of all contaminants, a modification of the migration technique devised by Cohen (1939) was developed. A plasmodium was subcultured to a streak of formalin-killed *A. aerogenes* on plain agar. The plasmodium migrated along the streak of bacteria, consuming them as it moved. When it approached the far end of the streak it was subcultured to plain agar, where it fruited. The resulting spores were almost always free of contaminants. Sterility tests were made frequently to check for contaminants. A drop of the culture was plated on a rich glucose + peptone + yeast extract agar similar in composition to the axenic growth medium. The plate was incubated for at least a week before being discarded. When an axenic culture was first established, it was inoculated on to and stabbed into a variety of other media to test more exhaustively for contamination.

## RESULTS

Spores of *Didymium nigripes*, freed from contaminants as described, were placed in a 1 % peptone + 1 % glucose + 0·1 % yeast extract (w/v) medium buffered with 0·01 M-phosphate (pH 6·5; hereafter called PGY) to which about 500 $\mu$g. dry wt./ml. formalin-killed *Aerobacter aerogenes* had been added. The myxamoebae grew and could be maintained indefinitely with periodical subculture. The one component of the medium essential for growth was the formalin-killed bacteria. When myxamoebae were subcultured to a medium without the bacteria, only a small increase in number resulted (Fig. 1). When these myxamoebae were again subcultured, there was no further growth. Myxamoebae subcultured to phosphate buffer + formalin-killed bacteria grew slowly; the growth rate was markedly improved by adding peptone or yeast extract. Best growth was obtained when all components were present. Aerobic conditions were necessary for growth.

A variety of proteins and protein digests was substituted for the peptone. Concentrations between 0·2 % (w/v) and 2 % of neopeptone, phytone, thiotone, proteose peptone, Casamino acids, gelatin, tryptone, trypticase, and tryptose all resulted in good growth. Since growth was as good on the 1 % Difco Bacto-peptone as on any of the other protein digests this has been routinely included in the medium. Yeast extract, beef extract, and a variety of liver fractions were added to the 1 % peptone singly and in admixtures; growth was improved by adding any one of these. Difco yeast extract (0·1 %, w/v) is now routinely included in the medium. Buffers ranging from pH 4 to 9 were substituted for the pH 6·5 phosphate. Best growth was obtained at pH 6·5.

*Effect of glucose.* Adding 0·05 M-glucose to the medium did not affect the growth rate, but the final yield was greater and large populations of amoebae could be maintained in the stationary phase only when glucose was included. In its absence the population of amoebae began to decrease rapidly on the 5th day of incubation (Fig. 1). When such cultures were left undisturbed for 5–10 days, small plasmodia appeared. No plasmodia were found in cultures containing glucose. Adding filter-sterilized glucose after the other components of the medium had been autoclaved did not affect this result.

Table 1. *The effect of sugars on population size of myxamoebae in the stationary phase*

| Sugar | No. of myxamoebae/ml. expressed as % of number of myxamoebae/ml. at 7 days grown in 0·05 M glucose | | | | Ratio of no. of myxamoebae at 7 days to no. at 10 days | |
|---|---|---|---|---|---|---|
| | 0·01 M | | 0·05 M | | 0·01 M | 0·05 M |
| | 7 days | 10 days | 7 days | 10 days | | |
| None | (31) | (18) | . | . | (1 68) | . |
| D-Glucose | 65 | 60 | 100 | 106 | 1 10 | 0·95 |
| D-Mannose | 31 | 47 | 104 | 136 | 0 81 | 0·76 |
| D-Galactose | 58 | 52 | 90 | 72 | 1 13 | 1·24 |
| D-Fructose | 43 | 21 | 35 | 21 | 2 20 | 1·65 |
| Maltose | 45 | 33 | 55 | 55 | 1 38 | 1·00 |
| Lactose | 49 | 42 | 75 | 51 | 1 17 | 1·48 |
| Sucrose | 31 | 28 | 51 | 32 | 1 11 | 1·56 |
| L-Arabinose | 31 | 10 | 17 | 6 | 3 00 | 2·88 |
| D-Xylose | 44 | 27 | 44 | 22 | 1 62 | 2·00 |
| Raffinose | 31 | 30 | 65 | 49 | 1 14 | 1·31 |
| D-Ribose | 33 | 10 | 17 | 6 | 3 33 | 2·52 |

Each value represents the mean of three experiments.

A number of other sugars were tested to see whether they would mimic the effect of glucose (Table 1). Counts were made of each culture after 3, 7, and 10 days. After 3 days the myxamoebae were nearing the end of logarithmic growth (Fig. 1), but there was no significant difference between the cultures at this time. After 7 days, only galactose and mannose permitted yields comparable to those obtained with glucose. Mannose, galactose, lactose, sucrose, and raffinose were the only sugars able to maintain the population at a given value between 7 and 10 days. Only when mannose or glucose was present did the population continue to increase up to the 10th day. None of the sugars tested supported growth in the absence of formalin-killed bacteria.

*Bacterial preparations tested for growth promotion.* When different amounts of formalin-killed *Aerobacter aerogenes* were added to the PGY medium, the growth rate was independent of bacterial concentration (Fig. 1). At 22° the generation time was about 11 hr., as compared with a generation time of 4·5 hr. when the amoebae were grown on agar with living *A. aerogenes* (Kerr, 1961). The maximum number of amoebae/ml. was proportional to the amount of bacteria added. Similar results were obtained with formalin-killed *Bacillus megaterium* (Fig. 2), with a generation time of about 13 hr. The growth-supporting activity of formalin-killed bacteria was heat-labile. Formalin-killed *A. aerogenes* were added to 5 ml. tubes of PGY and

heated at various temperatures for 15 min. in a water bath. The activity was decreased by about one half by heating to 80° and completely destroyed at 90°.

Penicillin-induced spheroplasts of *Aerobacter aerogenes* were lysed in 0·1 M-phosphate (pH 6·5), filter sterilized and added to PGY, but the myxamoebae did not grow. Nor were washed, lysed, lysozyme protoplasts of *Escherichia coli* able to support growth. The recipe for producing lysozyme protoplasts was modified by omitting sucrose. Under these conditions, the bacteria were lysed instead of being

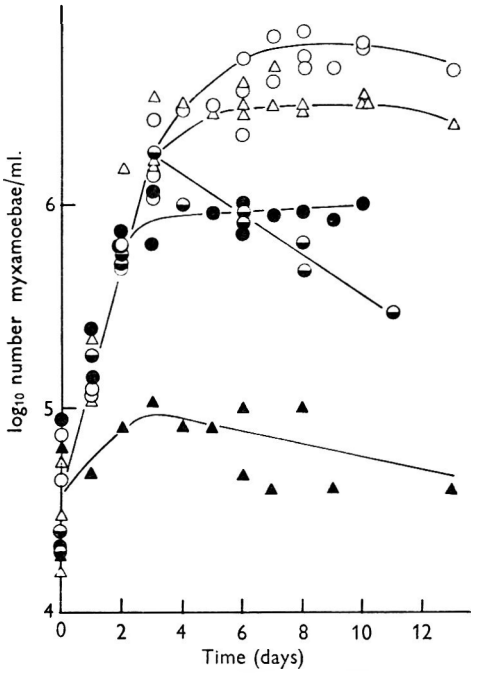


Fig. 1

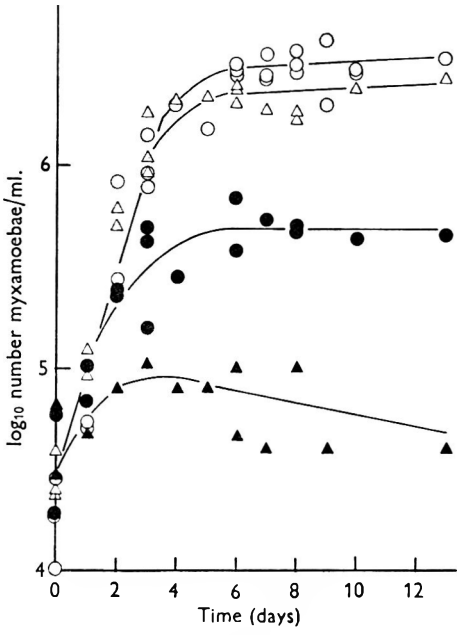


Fig. 2

Fig. 1. The growth of *Didymium nigripes* on formalin-killed *Aerobacter aerogenes*.

Fig. 2. The growth of *Didymium nigripes* on formalin-killed *Bacillus megaterium*. ○ = PGY medium + 1000 μg. dry wt. bacteria/ml.; △ = PGY medium + 500 μg. dry wt. bacteria/ml.; ● = PGY medium + 50 μg. dry wt. bacteria/ml.; ◒ = PY (no glucose) medium + 500 μg. dry wt. bacteria/ml.; ▲ = PGY medium alone.

converted to protoplasts. Sufficient $CaCl_2$ was then added to bind with the ethylene-diamine tetra-acetate necessary for lysis, but this mixture did not support growth of myxamoebae. All of the above preparations permitted growth when formalin-killed bacteria were added to them, showing that they were not toxic.

When *Aerobacter aerogenes* was treated exhaustively in a sonic-oscillator or in a Waring blender, the filter-sterilized supernatant fluid supported growth with a generation time of 24 hr. When dialysed, the activity remained in the dialysis residue. The active fraction was precipitated by ammonium sulphate.

*Aerobacter aerogenes* were dried with solid $CO_2$ + acetone and subsequently sterilized by formalin treatment. The acetone-dried bacteria supported growth of myxamoebae but the dried acetone extract was inactive.

*Attempts to replace the bacterial fraction.* Several media, reported to support axenic growth of other amoeboid organisms, were tested. The medium devised by Daniel & Rusch (1961) for plasmodia of *Physarum polycephalum* and that reported by Sussman (1963) to support growth of *Polysphondylium pallidum* were both inactive. Tissue culture media, such as medium 199 and Scherer's medium (with or without serum) did not support growth. Any of the above supported growth after the addition of formalin-killed bacteria.

Supernatant fluids of axenic cultures of *Acanthamoeba* sp. and *Tetrahymena pyriformis* were tested to see whether these organisms produced a diffusible metabolite that would replace the bacterial factor. No positive results were obtained.

Different compounds were added to PGY medium to see whether they would replace the formalin-killed bacteria. Ribonucleic acid, deoxyribonucleic acid, serum albumin, embryo extracts, liver fractions, haemin, haematin, Eagle vitamin mix + vitamin $B_{12}$, bacitracin, Tween 20, Tween 80, whole egg, lecithin, $\beta$-sitosterol, menadione sulphate and lysine were among the compounds tested. None supported growth.

That lysed protoplasts could not support growth, whereas bacteria broken by sonic treatment did, suggested that the active component might be in the bacterial cell wall or capsule. Several commercially available constituents of bacterial cell walls, including L-rhamnose, D-arabinose, glucuronic acid lactone, D-alanine, D-glutamic acid and polygalacturonic acid were tested singly and in mixtures. No growth resulted in any case

## DISCUSSION

All attempts to eliminate bacteria from the culture medium which supported the growth of *Didymium nigripes* failed. The bacterial extract or formalin-killed bacteria might serve one of three functions. First, the bacteria might inactivate, possibly by absorption, a toxic material in the medium. This seems unlikely, but, if true, one would hardly expect the bacterial activity to be heat-labile. Furthermore, growth would not be proportional to bacterial concentration. Rather, little or no growth would occur until a certain amount of bacteria had been added and growth might then be expected to be maximal. Neither would we expect to observe one or two divisions when amoebae grown on formalin-killed bacteria were subcultured to medium without bacteria. A second possible function of the bacteria might be to stimulate the formation of food vacuoles by amoebae. Although this possibility cannot be ruled out, it seems unlikely that the requirement would be so specific. Pinocytosis, a process similar to food vacuole formation, is induced by a variety of salts and proteins. The third and most likely possibility is that the bacteria supply one or more essential metabolites. Because washed bacterial protoplasts did not support growth, the active material may be located either in the capsule or the cell wall of the bacteria. Active material is found in more than one family of bacteria but not in yeasts. It exhibits characteristics of a protein or mucopolysaccharide, being heat-labile and non-dialysable. Alternatively, it might be tightly bound to such a macromolecule. The polypeptide isolated by Sussman & Bradley (1954), necessary for growth of *Dictyostelium discoideum*, differs from the present factor in that the former was stable to heat at neutral pH values.

The effect of glucose is interesting in that it had no effect on the growth rate of the myxamoebae. However, in its absence the size of the population underwent a

precipitous decline, with plasmodia occasionally forming afterwards. The effect was restricted to optical isomers of glucose and to certain disaccharides containing glucose. Kerr & Sussman (1958) noted that adding high concentrations of glucose to the agar on which myxamoebae were growing in monoxenic culture with *Aerobacter aerogenes* inhibited plasmodium formation. The plasmodium formation spreading technique (Kerr, 1961) was tried with axenic myxamoebae grown in the presence or absence of glucose; in neither case were plasmodial clones formed. The possibility that the axenic culture had selected a plasmodia-less strain (Kerr, 1959) was tested by subculturing axenically-grown myxamoebae to agar with living bacteria. These myxamoebae formed plasmodia normally. Hence, if in the absence of glucose plasmodia are suddenly forming, their viability in liquid culture on killed bacteria must be extremely low.

This study was begun to see whether a defined medium could be devised, making possible the selection of auxotrophic mutants for genetic studies. To date this goal has not been realized. Even if the bacterial fraction cannot be eliminated, it can be purified and characterized, making it possible to select nutritional mutants for other components of the medium. Even in its present crude form with formalin-killed bacteria, the axenic medium has opened the possibility of selecting mutants resistant to a variety of drugs and antibiotics without first selecting resistant strains of bacteria. This is now being attempted. The axenic culture will also make metabolic studies practicable. For example, it will now be possible to compare respiratory rates and changes in amount of a variety of enzymes and substrates in different stages of the life cycle.

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# The Relation of Polyphenoloxidase in Leaf Extracts to the Instability of Cucumber Mosaic and Other Plant Viruses

By B. D. HARRISON and W. S. PIERPOINT
*Rothamsted Experimental Station, Harpenden, Hertfordshire*

(*Received* 15 *March* 1963)

SUMMARY

Extracts made with buffered sodium diethyldithiocarbamate (DIECA) or potassium ethylxanthate from tobacco leaves infected with cucumber mosaic virus (CMV) were 5–500 times more infective than those made in buffer alone, or in buffer containing three other metal chelators which did not prevent the extracts going brown. DIECA preserved infectivity slightly better than did potassium ethylxanthate; both prevented browning equally. With DIECA in the extraction fluid, infectivity was not increased by other substances that enabled mitochondrial enzyme systems to be removed intact. CMV was inactivated by leaf polyphenols only when these were being oxidized. Oxidized polyphenols from virus-free leaves did not inactivate CMV when added together with copper, whereas deproteinized extracts of leaves crushed in an atmosphere of nitrogen did. When copper and chlorogenic acid, the main polyphenol in tobacco leaves, were added to infective extracts that contained polyphenoloxidase, the chlorogenic acid was oxidized and CMV was inactivated. A tobacco necrosis virus was slightly inactivated by incubating leaf extracts with chlorogenic acid and copper, but four other viruses were not. The tobacco necrosis virus was also the only one of these five to be at all stabilized by DIECA in the extraction fluid. The reported instability of some other plant viruses in leaf sap may mean that they also are susceptible to inactivation by polyphenoloxidase systems.

## INTRODUCTION

During their extraction from leaves plant viruses are exposed to enzymes and the products of enzyme reactions. The ease with which viruses can be purified probably depends on their resistance to these systems. Some of the enzymes involved in virus-inactivating systems may be contained in subcellular organelles (Bawden & Pirie, 1957). We therefore examined whether a labile virus, cucumber mosaic, could be stabilized by making extracts from leaves by a method that preserves the integrity of chloroplasts and mitochondria, and permits their removal. When infected tobacco leaves were crushed in a medium containing 2-amino-2-hydroxymethylpropane-1,3-diol (tris) buffer, sucrose, ethylenediaminetetra-acetate (EDTA), citrate and phosphate, moderately to highly infective preparations were obtained, and 80–90 % of their infectivity was retained after the chloroplasts and mitochondria were removed by centrifugation. When sodium diethyldithiocarbamate (DIECA) was added to this extraction medium, to prevent browning of the leaf extracts, the infectivity was still greater. Indeed, when DIECA was used, most of the other components of the extraction medium proved to be unnecessary. In this paper we describe experiments made to investigate the effect of DIECA.

## METHODS

*Viruses and plants.* A derivative of Price's cucumber yellow mosaic virus was used unless otherwise stated; a strain from Britain that produces a green mosaic in systemically infected leaves of tobacco was used in some experiments. Both strains were cultured in tobacco (*Nicotiana tabacum* var. Xanthi-nc), and systemically infected leaves were used as source of virus. Infectivity was assayed by counting the local lesions produced in inoculated half-leaves of French bean (*Phaseolus vulgaris* var. Prince) or whole leaves of *Chenopodium amaranticolor*. *C. amaranticolor* gave less accurate results than French bean and was used only in summer, when local lesions do not develop on French bean (Bhargava, 1951). Tobacco mosaic virus was obtained from systemically infected leaves of Java tobacco and assayed in Xanthi tobacco. Lucerne mosaic and tomato black ring viruses were obtained from systemically infected leaves of Xanthi tobacco, tobacco necrosis 'b' virus from inoculated leaves of Xanthi tobacco, and carnation ringspot virus from inoculated leaves of French bean; tomato black ring virus was assayed in Xanthi tobacco and the other three viruses in French bean. Inocula to be compared were distributed among the leaves or half-leaves of the assay plants so that each inoculum occurred equally often in equivalent positions. Superfloss Celite (Johns-Manville Ltd.) was added to all inocula to increase the number of lesions. The plants were grown in insect-proof glasshouses in which the temperature averaged 18° but in summer sometimes exceeded 25°.

*Preparation of leaf extracts.* After removing the midribs, about 10 g. of leaf-blade tissue from plants infected with any of the viruses was cut into strips and ground in a chilled mortar containing the extraction fluid (usually 5 ml./g. leaf) and about 5 g. acid-washed sand. The suspension was freed from sand and fibre by squeezing through two layers of butter muslin, and the filtrate centrifuged at 10,000 rev./min. (8000 *g*) for 30 min. The almost clear supernatant fluid was dialysed overnight in Visking tubing against two 4 l. lots of about 5 mM-phosphate buffer (pH 7·4). The whole procedure was done in a cold room at 0–5°. Extraction fluids which contained either DIECA (10 mM) or ascorbic acid (10 mM) were freshly made by dissolving these compounds in the appropriate buffer immediately before grinding the leaves.

Deproteinized extracts of tobacco leaves were made in three different ways. In the first, the leaf blades (25 g.) were crushed in an atmosphere of nitrogen by using the press described by Pirie (1961). The juice was collected in 40 ml. of phosphate buffer (10 mM; pH 7·4) and mixed, still under nitrogen, with 5 ml. N-hydrochloric acid, then centrifuged at 8000 *g* for 30 min. to remove the precipitate, the pH value adjusted to pH 7 with N-sodium hydroxide, and centrifuged again when a precipitate formed. In the second method, comparable extracts were made similarly, but in air.

The third method consisted of grinding the leaves in 3–4 times their own weight of 0·1 N-hydrochloric acid through which nitrogen had been bubbled to remove oxygen. The extract was then adjusted to pH 5·5 with N-sodium hydroxide and the precipitate removed by centrifugation (8000 *g* for 20 min.). More sodium hydroxide was then added to the supernatant fluid to bring to pH 7.

*Buffers.* Phosphate buffers were prepared from M-$NaH_2PO_4.2H_2O$ by adding conc. KOH solution, and diluting to the specified molarity of phosphate. Tris

buffers were similarly made from M-2-amino-2-hydroxymethylpropane-1:3 diol and HCl, and were diluted to the specified molarity of the tris buffer.

*Extraction, detection and estimation of polyphenols.* Leaves (25 g.) were cut into small pieces and dropped immediately into 200 ml. boiling methanol. After 10 min. the extract was filtered and the tissue re-extracted with 60 ml. boiling methanol. The two clear extracts were pooled, placed in a water bath at 40°, and evaporated to dryness with a water pump. The dry residue was then extracted overnight with a few ml. of water to which 0·1 N-sodium hydroxide was occasionally added to bring to between pH 6 and 7. Solutions containing polyphenols were spotted on to sheets of Whatman no. 1 filter paper and chromatograms developed for 4 hr. in either butan-1-ol + acetic acid + water (6 + 1 + 1 by vol.; Hillis & Carle, 1962) or ethyl acetate + pyridine + water (2 + 1 + 2 by vol.; Weaving, 1958). After being air dried, the papers were examined in ultraviolet light before and after exposure to ammonia vapour, and were sprayed with either ferric chloride + potassium ferricyanide reagent (Barton, Evans & Gardner, 1952) or a solution of sodium nitrite (1 %, w/v) in acetic acid (10 %, v/v) followed by N-sodium hydroxide (Roberts & Wood, 1951).

Marker spots of chlorogenic acid, caffeic acid and rutin, three of the major polyphenol components of tobacco leaves, were added to most chromatograms. These substances were satisfactorily separated from each other in both solvents, although the spots tended to be rather streaked. They could be distinguished by their relative positions, by their appearance in ultraviolet light and by the colours they developed with the nitrite spray.

Rough estimations of the concentration of chlorogenic acid in leaf extracts were made from the appearance of chromatograms developed in the butanol solvent. For this purpose various volumes of extracts (usually 1, 2, 5, 7 and 10 $\mu$l.) were chromatographed on the same sheet of paper with similar volumes of a stock solution (4 mg./ml.) of authentic chlorogenic acid, and the size and intensity of the chlorogenic acid spots were compared visually in ultraviolet light and again after spraying with ferric chloride + potassium ferricyanide solution. This comparison was expected to give only approximate values; two estimations done on one leaf extract on different days gave results that differed by 50 %.

## RESULTS

### *Effects of the extraction medium*

A medium previously used to extract mitochondria (Pierpoint, 1959) contained sucrose, phosphate, citrate, a chelating agent (EDTA), and was buffered at pH 7·8 with tris. Preparations obtained by crushing leaves in this medium were considerably more infective than water extracts. When the crude extracts were centrifuged at 1000 *g* for 7 min. to remove chloroplasts and cell-wall fragments, centrifuged at 8000 *g* for 30 min. to sediment mitochondria and then dialysed overnight against distilled water, the dialysis residue was only slightly less infective than the unclarified extract which had been stored at 2°. Only 5–10 % of the total infectivity was recovered from the chloroplast and mitochondrial fractions. The clarified extracts were brown, probably as the result of polyphenoloxidase activity (Shiroya, Shiroya & Hattori, 1955). Browning was prevented and infectivity was greater when 10 mM-DIECA, which is a metal-chelating agent and oxidase inhibitor,

or ascorbic acid, was added to the extraction medium. Table 1 shows that when DIECA was used, ascorbic acid made no difference to infectivity and that some of the other components of the medium decreased it. In different experiments, extracts made with DIECA were 5–500 times more infective than comparable extracts made with buffer alone, and the difference was usually 10- to 20-fold.

Table 1. *Effect of the composition of the extraction medium on the infectivity of CMV preparations from tobacco leaves*

Infected leaves were randomly divided into equal lots, and each lot ground in extraction medium (8 ml./g. leaf) of the specified composition. The concentration of each component of the medium, when present, was: sucrose 0·4 M; tris buffer 0·2 M; citrate 20 mM; EDTA, 5 mM; potassium phosphate, ascorbic acid and DIECA, all 10 mM. The pH of the medium was 7·7, and the extracts 7·2. The extracts were passed through muslin and centrifuged at 8000 *g* for 30 min. before being dialysed against 5 mM-phosphate (pH 7·4) and assayed for infectivity. The infectivity figures are total numbers of lesions in 8 half-leaves of French bean. Figures in parentheses are the dilutions of the extracts used as inocula. The green strain of CMV was used in Expt. 2, and the yellow strain in the other experiments.

| Composition of extraction medium | | | | | Infectivity of extract | | | |
|---|---|---|---|---|---|---|---|---|
| Tris | DIECA | Ascorbic acid | Sucrose | EDTA, citrate and phosphate | Expt. 1 (1/5) | Expt. 2 (1/5) | Expt. 3 (1/5) | Expt. 4 (1/10) |
| + | − | − | + | + | 8 | 6 | . | . |
| + | − | + | + | + | 159 | 11 | . | . |
| + | + | − | + | + | 795 | 115 | . | . |
| + | + | + | + | + | 645 | 128 | 605 | 10 |
| + | + | + | − | + | . | . | 895 | 49 |
| + | + | + | + | − | . | . | 1970 | 892 |
| + | + | + | − | − | . | . | 3010 | 1100 |

DIECA apparently prevents virus inactivation in leaf extracts and does not act by augmenting infection through an effect on the plant used for assay. Usually all but traces of DIECA were removed by dialysis before inoculation, and adding DIECA to 1 mM immediately before inoculation to extracts made either with or without DIECA did not affect infectivity. Nor does DIECA act by removing an inhibitor of infection. In general, the infectivity of mixtures of virus and inhibitor increased with dilution, whereas that of extracts made without DIECA did not. When DIECA was used, more infectivity was preserved when the extraction medium was at pH 7·3 to 8·1 than at higher or lower pH values (Table 2).

Table 3 compares the effects of DIECA with those of four other metal-chelating agents; potassium ethylxanthate, salicylaldoxime, 2,9-dimethyl-1,10-phenanthroline (neocuproine) and EDTA. These five agents all chelate copper but none is likely to be specific for it; the first three inhibit copper-containing enzymes such as some polyphenoloxidases, ascorbic oxidases (see Dawson & Tarpley, 1951) and diamine oxidase (Mann, 1955; Hill & Mann, 1962). Neither EDTA nor neocuproine inhibits diamine oxidase. DIECA and potassium ethylxanthate each prevented browning; the other three substances did not. Infectivity was preserved only by the substances which prevented browning; infectivity was preserved slightly better by DIECA than by potassium ethylxanthate. Although the latter two substances probably act

by inhibiting a copper-containing enzyme, the results in Table 3 do not prove this. However, the failure of salicylaldoxime to prevent browning or to preserve infectivity does not necessarily mean that copper-containing enzymes are not involved. Albert, Rubbo, Goldacre & Balfour (1947) found evidence that trace metals can be held at a biological surface in such a way that only chelating agents of appropriate architecture have access to them.

Table 2. *Effect of pH value on the stabilization of infectivity of leaf extracts by DIECA*

Forty g. of CMV-infected leaves were randomly separated into four equal lots and each lot was ground in about 100 ml. of DIECA (10 mM) dissolved in tris buffer (0·2 mM) of various pH values. After squeezing through muslin, a sample of each extract was taken for pH measurement and the rest centrifuged at 8000*g* for 30 min. All extracts were then dialysed against 5 mM-phosphate (pH 7·4) for 6 hr. at 2° before they were diluted and assayed for infectivity. The figures given are total numbers of lesions in 8 half-leaves of French bean.

| | Infectivity | |
|---|---|---|
| pH value | Diluted 1/2 | Diluted 1/20 |
| 6·4 | 580 | 86 |
| 7·3 | 1490 | 490 |
| 8·1 | 1590 | 550 |
| 9·0 | 995 | 225 |

Table 3. *Effect of chelators and other substances on the infectivity of leaf extracts*

CMV-infected leaves were randomly divided into three or four equal lots and each lot ground in 0·2 M-tris + HCl buffer (pH 7·8) together with the substances specified. The extracts were then centrifuged and dialysed as usual before their infectivity was assayed. Figures are the total number of lesions in 8 half-leaves of French bean.

| | Infectivity | | | |
|---|---|---|---|---|
| Substance added | Expt. 1 | Expt. 2 | Expt. 3 | Expt. 4 |
| None | 101 | 20 | 38 | 105 |
| DIECA (10 mM) | 680 | 780 | 462 | 1451 |
| DIECA (10 mM) + polyvinylpyrrolidone (2·5 %, w/v) | 1175 | . | . | . |
| Polyvinylpyrrolidone (2·5 %, w/v) | 65 | . | . | . |
| Salicylaldoxime (10 mM) | . | 15 | . | . |
| 2,9-Dimethyl-1,10-phenanthroline (10 mM) | . | 35 | . | . |
| EDTA (10 mM) | . | . | 80 | . |
| *p*-Nitrophenol (10 mM) | . | . | 42 | . |
| Potassium ethylxanthate (10 mM) | . | . | . | 758 |

Attempts were also made to prevent the oxidation of phenolic substances in leaf extracts by using other types of inhibitors. When the extraction medium contained *p*-nitrophenol (a competitive inhibitor of some polyphenoloxidases; Bonner & Wildman, 1946) or polyvinylpyrrolidone (a macromolecule that binds some phenolic compounds; Jones & Hulme, 1961) instead of DIECA, infectivity was not preserved (Table 3). Browning was not prevented by polyvinylpyrrolidone, but the effect of *p*-nitrophenol was difficult to judge because it is itself yellowish brown.

*Effects of extracts of uninfected leaves on virus inactivation*

The effect on infectivity of DIECA seemed likely to be caused by inhibiting polyphenoloxidase and so preventing virus from being inactivated either directly by the enzyme, or by substances formed from components of sap by enzyme action. Many infective preparations, dialysed to remove DIECA, became slightly brown immediately copper sulphate was added to them, but this had little effect on infectivity. The colour is probably caused by a complex formed between the copper and traces of DIECA left after dialysis. These results suggested either that the polyphenoloxidase was irreversibly inhibited, or that some other component of the

Table 4. *Effects of deproteinized extracts of uninfected leaves on infectivity of CMV*

Extracts made with tris+DIECA from CMV-infected leaves, and the three types of deproteinized extracts of uninfected leaves, were prepared as described under Methods. Samples of infective extract were incubated at room temperature for 2·5 hr. with the additions specified. The amounts of the additions differed in the three experiments, the range per 20 ml. or of final concentration being: infective extract 11–14 ml.; tris buffer (pH 7·4), 8·5–17 mM; copper sulphate 42–245 μM; deproteinized extracts 4·2–5·9 ml. All samples were assayed for infectivity after dialysis overnight at 2°. Infectivity values are the numbers of lesions produced in 8 half-leaves of French bean. Figures in parentheses are the number of lesions produced by the same inocula diluted 1/5 with water.

| Additions | Infectivity | | |
|---|---|---|---|
| | Expt. 1 | Expt. 2 | Expt. 3 |
| (1) None | 400 | 627 (55) | 1000 (83) |
| (2) Copper sulphate | 668 | 205 (39) | 751 |
| (3) Deproteinized extract made in $N_2$ | 708 | 333 (59) | . |
| (4) As (3)+copper sulphate | 84 | 17 (2) | . |
| (5) Deproteinized extract made in air | 604 | 807 (53) | . |
| (6) As (5)+copper sulphate | 452 | 299 (29) | . |
| (7) Deproteinized extract made in acid | . | . | 384 |
| (8) As (7)+copper sulphate | . | . | 133 (16) |
| (9) As (7), boiled+copper sulphate | . | . | 157 |
| (10) As (7), dialysed+copper sulphate | . | . | 567 |

inactivating system was missing. To see whether uninfected tobacco leaves could supply a missing component, deproteinized leaf extracts were added with or without copper to infective preparations of CMV. CMV was inactivated in the presence of copper by deproteinized extracts made in nitrogen, but not by extracts made in air or when copper was omitted (Table 4). Deproteinized extracts made by crushing uninfected tobacco leaves in hydrochloric acid, which was later neutralized, also inactivated CMV only when copper was added. There was evidently too little copper in the deproteinized extracts to reactivate the enzyme. The active substance (or substances) supplied by these deproteinized extracts was lost on dialysis; it is stable to acid and to boiling, rapidly changes to an inactive form in leaf extracts made in air, and plays its part in the inactivating system only when accompanied by active enzyme (Table 4). Substrates of polyphenoloxidase have these properties.

*Polyphenols in extracts of tobacco leaves*

The oxidation of polyphenols by polyphenoloxidase appears to have many properties in common with the system that inactivates CMV in leaf extracts. The

polyphenols in tobacco leaves were therefore examined. Chromatograms of methanolic extracts of healthy leaves and of CMV-infected leaves showed one major migrating component, apparently an isomer of chlorogenic acid. It fluoresced blue in ultraviolet light, and gave a deep blue colour with the ferric chloride + potassium ferricyanide spray. It moved the same distance as the main component of commercial chlorogenic acid (L. Light and Co.) in both the solvents used and was not separated from chlorogenic acid when a mixture was chromatographed. It became pale yellow when chromatograms were exposed to ammonia vapour, and its fluorescence in ultraviolet light changed to the 'duck-egg green' characteristic of chlorogenic acid (Hulme, 1953). Like chlorogenic acid it went yellowish pink when sprayed with sodium nitrite + alkali (Roberts & Wood, 1951).

Extracts of infected and healthy leaves each contained two other unidentified substances in smaller amount than chlorogenic acid. On the chromatograms one, which fluoresced blue in ultraviolet light and was stained blue with the ferric chloride + ferricyanide reagent, remained at the origin. The other was blue-green in ultraviolet light; it moved just behind chlorogenic acid in the butanol solvent, but scarcely moved from the origin in ethyl acetate + pyridine + water. There was a slight trace of a material with a mobility similar to caffeic acid in extracts of infected, but not of healthy, leaves. Rutin was not detected in extracts of either.

The amount of chlorogenic acid in a sample of healthy leaves was of the order of 0·05 % of the fresh weight; in a comparable sample of CMV-infected leaves it was 0·07–0·11 %. A virus preparation made from these diseased leaves would therefore contain 0·2–0·4 mM-chlorogenic acid. These values are less than reported for tobacco var. Xanthi leaves (0·26 %) by Shiroya, Shiroya & Hattori (1955), but their value probably refers to field-grown plants, which contain more chlorogenic acid and rutin than tobacco plants grown under glass (Frey-Wyssling & Babler, 1957).

*Effect of chlorogenic acid on infectivity of leaf extracts*

When infective extracts, which were made with tris + DIECA, then dialysed and the dialysis residues incubated with chlorogenic acid and copper sulphate, the solutions became brown and much infectivity was lost. When copper was not added, chlorogenic acid had little effect on the infectivity of most extracts but the infectivity of a few decreased considerably. Many ways to eliminate this variability were tried using different batches of chlorogenic acid, cleaning all glassware with nitric acid, varying slightly the temperature and pH value of the infective extract during incubation with chlorogenic acid; but the variability remained. However, when copper was not added, chlorogenic acid inactivated CMV in more of the extracts made in the summer than in those made in winter.

After 2 hr. incubation at 20° with chlorogenic acid + copper, the infectivity of extracts of leaves infected with CMV had usually decreased to a few % of that of extracts incubated with copper or chlorogenic acid separately (Table 5). Tobacco mosaic, lucerne mosaic, tomato black ring and carnation ringspot, four viruses with very different properties, were not inactivated by this system. The 'b' culture of tobacco necrosis virus, which is the least stable of the tobacco necrosis viruses examined by Kassanis & Nixon (1961), apparently was slightly inactivated (Table 5). The leaf extracts for these experiments were made in the usual way, with the aid of DIECA, and were dialysed before use.

Further tests showed that adding DIECA to the extraction medium did not increase the infectivity of extracts of leaves containing any of the four viruses that were unaffected by the chlorogenic acid system, but that there was an increase with tobacco necrosis 'b' virus. For example, in one experiment an extract made with the aid of DIECA gave 724, 203 and 45 lesions in eight half-leaves of French bean when inoculated at dilutions of 1/5, 1/50 and 1/500, whereas an extract made without DIECA from the opposite halves of the same leaves gave 458, 117 and 13 lesions at the same three dilutions. Susceptibility of viruses to inactivation by the chlorogenic acid system therefore appears to be correlated with the ability of DIECA in the extraction medium to enhance infectivity.

Table 5. *Inactivation of virus in leaf extracts on incubation with chlorogenic acid and copper*

Extracts of infected leaves were prepared in tris+DIECA solution and dialysed overnight at 2°. Samples (5 ml.) were then added to tris buffer (final concentration about 50 mM; pH 7), together with the substances specified below; the final volume was about 8 ml. The mixtures were kept at 21° for 3 hr. (CMV) or for 2·5 hr. (tobacco necrosis virus) and then dialysed overnight against cold phosphate buffer (5 mM; pH 7·4). Infectivity was measured as the total number of lesions produced in 8 half-leaves of French bean. Figures in parentheses are the numbers produced by the same inocula diluted 1/10 with water.

| Additions (final concentration) | Infectivity: Cucumber mosaic virus | Infectivity: Tobacco necrosis 'b' virus |
|---|---|---|
| None | 1000 (193) | 228 (67) |
| Copper sulphate (0·25 mM) | 955 | 273 |
| Chlorogenic acid (2·6 mM) | 872 | 223 |
| Copper sulphate (0·25 mM)+chlorogenic acid (2·6 mM) | 35 | 152 |

## DISCUSSION

The simplest explanation of our results is that CMV is inactivated by the polyphenoloxidase systems in leaf extracts. Substances which prevented the extracts from becoming brown also protected the virus, whereas metal-chelators which did not prevent browning did not prevent virus inactivation. CMV is stable in extracts made with the aid of DIECA and then dialysed, because polyphenoloxidase is inhibited and because the polyphenol substrates diffused away during dialysis. The enzyme was reactivated by adding copper. Thus, when both copper and chlorogenic acid, the main polyphenol in tobacco sap, were added to virus preparations that contained the enzyme, chlorogenic acid was oxidized and CMV was inactivated. CMV is inactivated only while polyphenols are being oxidized. Thus, adding copper +extracts made in air from virus-free leaves to infective preparations, did not inactivate CMV because the polyphenols from the leaves were already oxidized. It is uncertain whether the polyphenols are oxidized by stages and only the intermediate products inactivate CMV, or whether the oxidized polyphenols react so quickly with proteins in extracts of virus-free leaves that they are no longer active when CMV is added.

Many of the virus-inactivating systems previously found in leaf extracts can be distinguished from the polyphenoloxidase system. In one type of system, the rapid

inactivation of grapevine fanleaf virus in sap from grape leaves is caused by the acidity (pH 3–3·5; Dias, 1962), whereas the polyphenoloxidase system inactivates CMV at pH 7–8. In a second type, infectivity of some isolates of tobacco rattle and tobacco necrosis viruses is preserved only when leaves are crushed in water-saturated phenol or in pH 9 buffer (Sänger & Brandenburg, 1961; Cadman, 1962; Babos & Kassanis, 1962). Water extracts of the leaves are often less than 1 % as infective as the extracts made by shaking with phenol. The phenol and pH 9 buffer seem likely to preserve infectivity by inactivating leaf ribonuclease and the other virus-inactivating enzymic systems described by Bawden & Pirie (1959). Schlegel (1960) found that extracts from leaves infected with CMV were 1·5–7 times more infective when made with the aid of phenol and 1 % tetrasodium pyrophosphate than when made with pyrophosphate alone. Whether the phenol preserved initially ribonuclease-sensitive CMV is not clear. However, we find that DIECA does not preserve the infectivity of the unstable tobacco necrosis virus, indicating that DIECA does not inhibit this inactivating system, which is inhibited by phenol.

A third type of inactivation, which occurs when leaf tannins come into contact with virus particles (Cadman, 1959), differs from the polyphenoloxidase system in that infectivity is preserved at pH 8, a condition in which the tannin has little tendency to combine with virus. Another difference is in the range of viruses affected. Lucerne mosaic virus is permanently inactivated and tomato black ring virus is reversibly precipitated by the tannins, whereas neither was affected by the polyphenoloxidase system. Also, Cadman (1959) detected no tannins in tobacco leaves.

The polyphenoloxidase system has greater similarities to the inactivating system obtained by Bawden & Pirie (1957) from leaves infected with tobacco necrosis virus. Neither affects tobacco mosaic virus; both occur in tobacco, need oxygen and involve a substance that is stable to boiling; and inactivation by both is partly prevented by ascorbate. Bawden & Pirie (1957) inactivated tobacco necrosis virus in 20 hr. at 18° and we found that tobacco necrosis virus was slightly affected in 2 hr. The two systems, however, seem to differ, because Bawden & Pirie found most of their system was sedimented at 8000*g*, a procedure we use routinely to obtain infective supernatant fluids in which inactivation can be studied. With leaves infected with CMV, we find sediments obtained at 8000*g* by the method of Bawden & Pirie contain polyphenoloxidase, but little or no substrate. These sediments inactivated CMV in leaf extracts much more slowly than did chlorogenic acid + copper. However, sediments from leaves infected with tobacco necrosis virus might inactivate CMV more rapidly.

DIECA has previously been used to stabilize other plant viruses. Hampton & Fulton (1961) used it to preserve the infectivity of sour cherry necrotic ringspot virus in extracts of cucumber leaves. The virus was inactivated by fungal polyphenoloxidase when a substrate (catechol or tyrosine) was added. Similarly, Mink & Bancroft (1962) found that stable preparations of Tulare apple mosaic virus could be made with DIECA and cysteine HCl, whereas in leaf sap the virus lost half its infectivity every 5 min. (Yarwood, 1955). These two viruses and CMV seem to be particularly sensitive to inactivation by polyphenoloxidase systems. Tomato spotted wilt virus too is sensitive to oxidase systems. Inactivation in sap is greatly

decreased by excluding oxygen, by keeping at 0°, or by adding reducing substances such as cysteine or sodium sulphite. Catechol inactivates the virus in sap kept in air, but not when sodium sulphite is also added (Best & Samuel, 1936). Tobacco streak virus (Fulton, 1949) and rose mosaic virus (Fulton, 1952) also are stabilized by cysteine and sodium sulphite. These six viruses all soon inactivate in sap at room temperature, suggesting that in many instances the standard 'resistance to ageing test' measures the susceptibility of the virus to inactivation by polyphenoloxidase systems. It is possible that these systems also slowly inactivate moderately stable viruses.

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# Copper-dependent and Iron-dependent Inactivations of Cucumber Mosaic Virus by Polyphenols

By W. S. PIERPOINT and B. D. HARRISON
*Rothamsted Experimental Station, Harpenden, Hertfordshire*

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SUMMARY

Extracts made by crushing infected tobacco leaves in buffer solution containing sodium diethyldithiocarbamate (DIECA), centrifuging at 8000*g*, and dialysing the supernatant fluids against dilute buffer, were used to study the inactivation of cucumber mosaic virus (CMV). Incubating such extracts with chlorogenic acid and copper inactivated them; inactivation was usually much less when they were incubated with chlorogenic acid alone. Inactivation did not occur *in vacuo* or when DIECA was added. DIECA did not reactivate inactivated virus. CMV was inactivated rapidly by incubating with caffeic acid and copper, and slowly with catechol and copper, but not with five other phenols. The end-products of oxidation formed when chlorogenic acid was incubated with extracts of uninfected leaves did not inactivate CMV. Some features of the CMV-inactivating system are explained by the properties of tobacco leaf polyphenoloxidase. The compounds most effective in preserving the infectivity of CMV during extraction from leaves, DIECA and potassium ethylxanthate, are those which most strongly inhibit the enzyme. Conversely, the polyphenols that inactivate CMV *in vitro* are those oxidized most rapidly. Inconsistent effects of copper on the *in vitro* inactivating system can be partly explained by the different copper requirement of the polyphenoloxidase in extracts from plants infected for different times. Iron salts also accelerate the inactivation of CMV by chlorogenic acid. The process requires air and is prevented by DIECA; iron did not reactivate the DIECA-inhibited polyphenoloxidase. This system seems different from the one stimulated by copper. The concentration of iron in leaf extracts is usually less than that needed by the inactivating system.

## INTRODUCTION

Stable extracts of cucumber mosaic virus (CMV) can be prepared by grinding infected tobacco leaves in the presence of sodium diethyldithiocarbamate (DIECA) or potassium ethylxanthate, centrifuging at low speed, and then dialysing overnight against dilute buffer (Harrison & Pierpoint, 1963). DIECA and potassium ethylxanthate also prevent the browning of leaf extracts, probably by inhibiting the oxidation of leaf polyphenols by leaf polyphenoloxidase, an enzyme thought to contain copper. We therefore suggested that CMV was inactivated by some product of polyphenol oxidation, and that the virus was stable in these leaf extracts because the enzyme was inhibited and the polyphenol substrates dialysed away. When infective extracts were incubated with chlorogenic acid or deproteinized extracts made anaerobically from tobacco leaves, and copper was added to reactivate the polyphenoloxidase, CMV was inactivated. The *in vitro* inactivation of CMV by

chlorogenic acid and copper salts is the subject of the present paper. The oxidation of polyphenols by leaf polyphenoloxidase was studied manometrically to see how far the properties of the enzyme might account for those of the virus-inactivating system.

## METHODS

*Leaf extracts.* Cucumber mosaic virus (CMV) preparations were made with the aid of DIECA from infected tobacco leaves as described by Harrison & Pierpoint (1963); they will be referred to as infective extracts. Extracts of uninfected leaves were also made in this manner except that the amount of extraction medium used was usually 2–3 ml./g. leaf material.

Extracts required for metal determinations were made from infected leaves using either water or tris HCl buffer (0·2 M; pH 7·8). Most of the protein was removed by adjusting to pH 5 with HCl, and centrifuging; the supernatant fluid was then evapoted to dryness. Iron and copper in the residue were estimated spectroscopically.

*Virus inactivation.* Eight ml. of reaction mixture contained 5 ml. infective extract, 2-amino-2-(hydroxymethyl)-1,3-propanediol (tris) buffer (50 mM; pH 7·1) and, when they were required, chlorogenic acid (2·6 mM adjusted to pH 7) and copper sulphate (0·25 mM). Incubation was either at room temperature (about 18°) or in a water bath at 22°. DIECA dissolved in tris buffer (0·2 M; pH 7·8) was sometimes added at this stage to stop further inactivation. The incubated solutions were then dialysed for 2–3 hr., or occasionally overnight, against phosphate buffer (about 5 mM; pH 7·4) before being tested for infectivity.

To incubate *in vacuo*, the reactants were put into flasks that had ground-glass stoppers with taps. These flasks were divided internally into two shallow compartments by a central ridge. Virus extracts + copper sulphate were put into one of these compartments, and buffer + chlorogenic acid into the other; the two liquids were mixed when the flasks had been evacuated with an oil pump.

Infectivity was assayed as described by Harrison & Pierpoint (1963). The numbers of lesions given in the Tables are the total that formed in eight or more half-leaves of French bean. When the infectivities of more than six samples were to be compared, the samples were divided into batches, with a standard inoculum included in each batch; in such experiments the numbers of lesions quoted are relative to the number given by the standard inoculum.

*Manometric experiments.* The oxidation of polyphenols by extracts of tobacco leaves was followed at 30° in the conventional Warburg apparatus. The centre wells of the flasks contained folded pieces of filter paper and 0·2 ml. 10 % (w/v) KOH. Shaking was at the rate of 120 strokes/min., and a 10 min. equilibration period was allowed before the taps were closed. The final concentrations of reagents in the manometer flasks were similar to those in the virus inactivation experiments: phenolic substrate, 3·2 mM; copper sulphate, 0·36 mM; tris buffer (pH 7), 70 mM; leaf extract, 1·5 ml.; final volume usually 2·8 ml. The phenols were usually added to the manometer side-arms either as solutions or suspensions at pH 7. They were tipped into the main compartments after equilibration. Compounds were tested as polyphenoloxidase inhibitors by adding them in solution at pH 7 to the main compartments of manometer flasks. They were therefore incubated with enzyme but without substrate during the equilibration period; absorption of oxygen was

followed during the next 30 min. In some experiments the carbon dioxide produced during the oxidation was estimated manometrically by the 'direct' method (Umbreit, Burris & Stauffer, 1957).

## RESULTS

### *Inactivation of CMV by chlorogenic acid and copper*

When extracts containing CMV were incubated with chlorogenic acid + copper salts, infectivity disappeared. Table 1 illustrates this, and also shows that chlorogenic acid was effective at 0·1 mM; extracts of infected leaves contain about 0·4 mM (Harrison & Pierpoint, 1963). The minimum effective concentration of copper differed in different experiments (probably depending on the completeness with which DIECA was dialysed away from the extracts); with one extract the effective copper concentration was 6·3 $\mu$M but with another 250 $\mu$M. In about a third of the extracts examined, CMV was inactivated by chlorogenic acid alone, usually only slightly, but sometimes as much as with added copper.

Table 1. *Effect of concentration of chlorogenic acid on the inactivation of CMV*

Samples of infective leaf extract (5 ml.) were added to a series of tubes containing tris buffer (final concentration 50 mM; pH 7), chlorogenic acid and copper sulphate as indicated, and water to 8 ml. The solutions were incubated at 18° for 2 hr. then dialysed at 2° and assayed for infectivity.

| Additions (final concentrations) | | |
|---|---|---|
| Chlorogenic acid (mM) | Copper sulphate (mM) | No. lesions |
| 0 | 0 | 300 |
| 0 | 0·19 | 374 |
| 2·6 | 0 | 240 |
| 2·6 | 0·19 | 11 |
| 1·1 | 0·19 | 4 |
| 0·26 | 0·19 | 10 |
| 0·11 | 0·19 | 46 |
| 0·011 | 0·19 | 338 |
| 0·0011 | 0·19 | 422 |

DIECA, at five or ten times the molar concentration of copper, almost completely prevented CMV from being inactivated. Adding DIECA after incubating infective extracts with chlorogenic acid + copper did not restore infectivity. It was previously shown that the small amounts of DIECA which might be left in solution after dialysis did not affect infectivity (Harrison & Pierpoint, 1963). By making use of DIECA to stop the reaction, the time course of the inactivation of CMV was followed and the effect of air studied. Table 2 shows a time course in which inactivation was complete in 45 min. when copper was added; but without copper inactivation was slight after this time. However, after 2·5 hr., chlorogenic acid alone produced appreciable inactivation. In other experiments done at the same or similar temperatures, CMV was almost completely inactivated with chlorogenic acid + copper in either shorter (15 min.) or longer (120 min.) periods. Table 3 shows

the dependence of inactivation on air. In this experiment, the incubation period was long and there was appreciable inactivation without added copper, but this, like inactivation with copper, required air.

Table 2. *Time course of inactivation of CMV*

Samples of infective leaf extract (5 ml.) were added to a series of solutions containing tris buffer (pH 7; final concentration 50 mM) and either copper sulphate (0·25 mM), chlorogenic acid (2·6 mM), or both. The final volumes were made up to 8 ml. with water and the solutions incubated at 21°. After various times they were cooled, DIECA added (final concentration 1·25 mM) and then dialysed at 2° for at least 3 hr. before being assayed for infectivity.

| Time of incubation (min.) | No. lesions | | |
|---|---|---|---|
| | Copper sulphate added | Chlorogenic acid added | Copper sulphate + chlorogenic acid added |
| 0 | 200 | 231 | 262 |
| 15 | 133 | 197 | 116 |
| 45 | 120 | 116 | 18 |
| 150 | 136 | 53 | 5 |

Table 3. *The effect of air on the inactivation of CMV*

Each tube had 4·5 ml. solution containing 3 ml. infective extract, tris buffer (pH 7; final concentration 4·5 mM), copper sulphate (0·45 mM) and chlorogenic acid (2·3 mM) as specified below. Some tubes were evacuated immediately and all were kept at room temperature (18°) for 2·5 hr. DIECA was then added to all the solutions (final concentration 2·2 mM) and they were dialysed for 3 hr. before being assayed for infectivity.

| Additions to incubation mixture | No. lesions | |
|---|---|---|
| | Incubated aerobically | Incubated *in vacuo* |
| None | 610 | — |
| Copper sulphate | 450 | 510 |
| Chlorogenic acid | 140 | 1060 |
| Copper sulphate + chlorogenic acid | 50 | 660 |

When leaf extracts are made, CMV seems to be inactivated while polyphenols are being oxidized, and not by brown pigments formed as end-products of the reaction (Harrison & Pierpoint, 1963). Table 4 shows that this is also true when CMV is inactivated by chlorogenic acid + copper. Chlorogenic acid was pre-incubated for different times with polyphenoloxidase-containing extracts of healthy leaves before incubation with infective extracts. The longer the period of pre-incubation, the less virus was inactivated. On incubating mixtures of pre-incubated preparations and virus with DIECA added and air excluded (conditions which prevent further oxidative reactions) CMV was inactivated only very slightly even by the preparation that had been pre-incubated for the shortest period. This slight inactivation may be caused by accumulation of virus-inactivating compounds in the early stages of the oxidation, but little importance can be attached to differences of infectivity of this degree. Neither bovine plasma albumin (0·1–1·0 %, w/v) nor polyvinylpyrrolidone (PVP), two substances likely to absorb oxidized or unoxidized poly-

phenols, protected CMV from inactivation during the oxidation of chlorogenic acid. PVP did not preserve infectivity when added to the extraction medium (Harrison & Pierpoint, 1963).

Table 4. *Effect of oxidized chlorogenic acid on CMV*

Samples (2 ml.) of an extract of uninfetced tobacco leaves were incubated for various times in 4·75 ml. of solution containing tris buffer (85 mM), copper sulphate (0·42 mM) and chlorogenic acid (5·0 mM). After this incubation they were added to 5 ml. samples of an infective extract and kept at room temperature (19°) for 90 min. either without further treatment or after adding DIECA (final concentration 1·03 mM) and removing air. All the samples were dialysed overnight at 2° before assaying their infectivity. Suitable controls showed that infectivity was not affected by adding DIECA and removing air.

| Pre-incubation of extracts of uninfected leaves | Conditions of incubation with infective extracts: In air | *In vacuo* with DIECA present |
|---|---|---|
| | No. lesions | |
| 4 hr. at 37° | 3750 | 4360 |
| 2 hr. at 19° | 3120 | 4500 |
| 1 hr. at 19° | 1170 | 4400 |
| 0·3 hr. at 19° | 537 | 1570 |
| 0 hr. | 291 | 2830 |

Table 5. *Effect of polyphenols on infective extracts*

Each tube had 9 ml. of solution containing 6 ml. of infective extract, tris buffer (pH 7; 62 mM in Expt. 1; 35 mM in Expt. 2), polyphenols (2·4 mM) and copper sulphate (0·29 mM) as indicated. The solutions were kept at 18° for 3 hr., dialysed overnight at 2° and then assayed for infectivity.

| Substrate | No. lesions: Expt. 1 Without copper | Expt. 1 With copper | Expt. 2 Without copper | Expt. 2 With copper |
|---|---|---|---|---|
| None | 543 | 370 | 1605 | 1535 |
| Chlorogenic acid | 358 | 54 | 1400 | 56 |
| Caffeic acid | 550 | 62 | 1594 | 4 |
| Catechol | 693 | 161 | 850 | 1100 |
| Gallic acid | 344 | 440 | — | — |

*Inactivation of CMV by other polyphenols*

Seven phenols, some of which occur in tobacco leaves (Roberts & Wood, 1951) were incubated with CMV + copper; caffeic acid inactivated strongly and catechol slightly (Table 5), whereas quercetin, (+)-catechin, rutin, tyrosine and gallic acid were virtually ineffective. In a later section we show that none of the inactive polyphenols, except catechin, was appreciably oxidized by infective extracts; catechin was only slightly oxidized.

*Iron-dependent inactivation of CMV*

CMV was inactivated when incubated with iron salts and chlorogenic acid. Ferrous sulphate was as effective as ferric chloride, possibly because it oxidizes

rapidly in solution. The minimum effective concentration of iron was about 0·25 mM (Table 6). Zinc salts with chlorogenic acid did not inactivate. The iron-dependent inactivation has some properties in common with copper-dependent inactivation. The amount of inactivation increased with time; in one experiment infectivity was decreased by 40, 70 and 96 % after incubation at 22° for 10, 40 and 80 min. Inactivation was largely prevented by adding DIECA (Table 6), or by removing air

Table 6. *Effect of iron and chlorogenic acid on infective extracts*

Samples of infective extracts (5 ml.) were incubated for 2 hr. at 20° with tris buffer (final concentration 49 mM; pH 7·0) and the additions as indicated; the final volume was 8·2 ml. All solutions were then dialysed for 3 hr. at 2° before being assayed for infectivity.

| Additions (final concentration, mM) | | | |
|---|---|---|---|
| Chlorogenic acid | Ferrous sulphate | DIECA | No. lesions |
| 2·6 | — | — | 1000 |
| — | 0·25 | — | 1000 |
| 2·6 | 0·25 | 6·1 | 703 |
| 2·6 | 0·25 | — | 49 |
| 2·6 | 0·025 | — | 363 |
| 2·6 | 0·0025 | — | 503 |
| 0·29 | 0·25 | — | 59 |
| 0·29 | 0·025 | — | 669 |

Table 7. *Effect of air on the iron-dependent inactivation of CMV*

Samples of infective extracts (4 ml.) were added to solutions containing tris buffer (final concentration 36 mM; pH 7·0), chlorogenic acid (2·7 mM) and ferric chloride (0·27 mM) as indicated below; the final volume was 5·5 ml. After some flasks had been evacuated, they were all incubated at 22° for 85 min. DIECA (final concentration 6·3 mM) was then added to each and they were dialysed for 2·5 hr. at 2° before being assayed for infectivity.

| Additions to incubation mixture | No. lesions | |
|---|---|---|
| | Incubated aerobically | Incubated *in vacuo* |
| None | 1400 | — |
| Ferric chloride | 636 | 1365 |
| Chlorogenic acid | 1515 | 1970 |
| Ferric chloride + chlorogenic acid | 150 | 1302 |

(Table 7). Tobacco mosaic virus was not inactivated by this system or by the copper-stimulated one By contrast, nucleic acid prepared from tobacco mosaic virus is inactivated when incubated with iron alone (Bawden & Pirie, 1959).

*Polyphenoloxidase activity of infective extracts*

The conversion of chlorogenic acid to a brown pigment by infective extracts incubated with copper was accompanied by absorption of oxygen (Table 8). Boiling the extracts for 10 min. prevented browning and $O_2$ uptake. The amount of oxygen absorbed in the reaction was almost (86 %) two atoms per molecule of chlorogenic acid present, a value suggested as a theoretical maximum for this oxidation (Rudkin

& Nelson, 1947). Small amounts of carbon dioxide were evolved during the later stages of the reaction, giving a final R.Q. of about 0·2.

The rate at which chlorogenic acid was oxidized by most of the infective extracts was greatly decreased by omitting copper (Fig. 1). Oxidation was completely

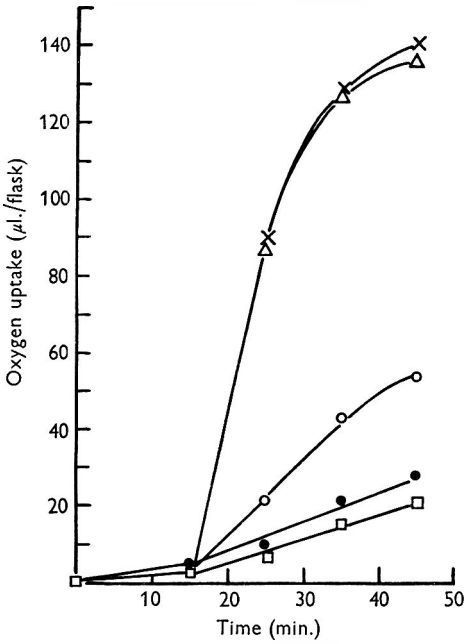


Fig. 1. Effect of copper sulphate on the oxidation of chlorogenic acid by infective extracts. The extract (1·5 ml.) was incubated in manometers with tris buffer (pH 7; final concentration 72 mM) and copper sulphate at the following final concentrations: 360 μM (×); 36 μM (△); 3·6 μM (○); 0·036 μM (●), none (□). After 15 min., chlorogenic acid (final concentration 3·2 mM) was tipped in from the side arms. Oxygen uptake in the absence of leaf extract was negligible.

Table 8. *Oxidation of chlorogenic acid by infective extracts*

The extract was prepared in tris buffer (0·2 M; pH 7·8) containing DIECA (10 mM) and dialysed overnight at 2°. Manometric conditions were as described under Methods, and each flask contained tris buffer (pH 7; 72 mM) and, when specified, fresh or boiled leaf extract (1·5 ml.), chlorogenic acid (3·2 mM) and copper sulphate (0·36 mM).

| Extract | Additions | Oxygen uptake (μl./30 min.) |
|---|---|---|
| Fresh | None | 4·5 |
| Fresh | Copper sulphate | 5·5 |
| Fresh | Chlorogenic acid | 5·0 |
| Fresh | Copper sulphate + chlorogenic acid | 92·0 |
| Boiled | Copper sulphate + chlorogenic acid | 3·3 |
| None | Copper sulphate + chlorogenic acid | 0 |

inhibited when DIECA (10 mM) was added with the copper. However, some preparations oxidized chlorogenic acid without added copper; this occurred with extracts made from the leaves of plants that had been infected with CMV for many weeks. Table 9 contrasts the effect of copper on extracts made from plants infected

for different periods. The enzyme in extracts of leaves infected for 8 weeks recovered spontaneously from inhibition by DIECA; the reason is not clear.

Neither ferrous sulphate nor ferric chloride replaced copper sulphate in the oxidation of chlorogenic acid. In the experiment reported in Fig. 1, 360 $\mu$M ferrous sulphate had as little effect on oxygen uptake of leaf extract + chlorogenic acid as did 0·036 $\mu$M copper sulphate. Iron salts did not interfere with the oxidation that occurred when copper was added, even though highly coloured iron-chlorogenate complexes were formed.

Table 9. *Effect of copper sulphate on the polyphenoloxidase extracted from tobacco leaves at different times after infection with CMV*

Infective extracts were made from plants infected for different times and, after dialysing overnight at 2°, were tested for ability to oxidize chlorogenic acid in the presence and absence of copper sulphate.

| Time after inoculating plants (weeks) | Oxygen uptake ($\mu$l./30 min.) With copper sulphate | Oxygen uptake ($\mu$l./30 min.) Without copper sulphate |
|---|---|---|
| 2·5 | 146 | 24 |
| 5 | 180 | 62 |
| 8 | 186 | 162 |

The rate at which chlorogenic acid was oxidized (Table 10) and the amount of oxygen absorbed depended on pH value and were greatest near pH 7. Above pH 8 chlorogenic acid oxidizes spontaneously without requiring added copper.

The polyphenoloxidase in extracts of infected leaves and in acetone powders of uninfected tobacco leaves (Reid, 1956; Clayton, 1959) have similar specificities. Of the phenols tested only caffeic acid and chlorogenic acid were oxidized rapidly (Table 11). Catechol and catechin were also oxidized, but more slowly. Usually rutin, tyrosine and quercetin were not oxidized, but gave small oxygen uptakes with some infective extracts. Gallic acid oxidized non-enzymically in the presence of copper; leaf extracts slightly diminished this reaction. Phenols extracted from CMV-infected leaves were also oxidized by the polyphenoloxidase. A methanol extract, made as described by Harrison & Pierpoint (1963), absorbed rather more oxygen (77 $\mu$l./0·1 ml.) than was expected (55 $\mu$l./0·1 ml.) from its estimated chlorogenic acid content.

### *Inhibition of tobacco leaf polyphenoloxidase*

Harrison & Pierpoint (1963) judged the effectiveness of polyphenoloxidase inhibitors by their ability to prevent the extracts becoming brown during disruption of the leaves. Substances that prevent browning also affect oxygen uptake when chlorogenic acid is incubated with infective leaf extracts. Extracts of infected leaves, prepared in tris buffer (0·2 M; pH 7·8) without DIECA, oxidized chlorogenic acid at rates similar to that shown in Table 8, and the reaction did not depend on added copper. DIECA (10 mM) inhibited this oxidation by 35–90 %, and prevented the browning. Although potassium ethylxanthate (10 mM) decreased oxygen uptake by only half (37–56 %), it completely prevented the browning. At the same

concentration, 2,9-dimethyl-1,10-phenanthroline decreased oxygen uptake by 15 %, and salicylaldoxime, EDTA, *p*-nitrophenol and sodium azide by less than 10 %. None of these compounds appreciably hindered the browning.

Table 10. *Effect of pH value on the polyphenoloxidase in infective extracts of leaves*

The ability of an infective extract to oxidize chlorogenic acid was tested in the presence and absence of copper sulphate. The experimental conditions were as described under Methods except that the pH of the tris-HCl buffer was varied.

| | Oxygen uptake (μl./30 min.) | |
|---|---|---|
| pH value | Without copper sulphate | With copper sulphate |
| 6·0 | 1·5 | 64 |
| 7·1 | 3·5 | 118 |
| 7·7 | 3 | 95 |
| 8·1 | 6 | 75 |
| 8·9 | 16 | 56 |

Table 11. *Oxidation of polyphenols by infective extracts*

Each substrate, dissolved or suspended at pH 7, was tested manometrically alone, in the presence of copper sulphate, and in the presence of both copper sulphate and the leaf extracts. Conditions were as described under Methods. Oxygen uptake in the absence of substrate was negligible.

| | Oxygen uptake (μl./30 min.) | | |
|---|---|---|---|
| Substrate (3·2 mM) | Alone | With copper sulphate | With copper sulphate and infective extract |
| Chlorogenic acid | — | — | 164 |
| Caffeic acid | 3 | 10 | 161 |
| Catechol | 3 | 11 | 32 |
| (+)-Catechin | 0 | 13 | 30 |
| Gallic acid | 10 | 39 | 30 |
| Quercetin | 3 | 4 | 3 |
| Rutin | — | 0 | 0 |
| L-Tyrosine | 0 | 0 | 1 |

## DISCUSSION

The properties of polyphenoloxidase explain three features of the copper-dependent virus inactivating system. First, the compounds most effective in preserving infectivity when CMV is extracted from leaves, namely DIECA and potassium ethylxanthate, are those which most strongly inhibit the enzyme. Secondly, the polyphenols that inactivate CMV most *in vitro*, namely chlorogenic acid and caffeic acid, are those most rapidly oxidized. Thirdly, the minimum concentration of copper necessary to permit inactivation of CMV *in vitro* is about that required to reactivate the DIECA-treated polyphenoloxidase. The variable copper requirement of the inactivating system is partly explained by the fact that extracts of older leaves contain polyphenoloxidase which recovers spontaneously from inhibition by DIECA (Table 9). However, virus-inactivation experiments with extracts

from leaves of different ages showed that other factors must also be involved. The amount of inactivation without copper probably depends not only on the extent to which the polyphenoloxidase is active without added copper, but also on the amounts of polyphenoloxidase and CMV in the extracts. Whether or not it occurs in a particular experiment also depends on how long the samples are incubated (Table 2).

Some polyphenoloxidases are known to contain copper (Mallette, 1950), and the inhibition of tobacco leaf polyphenoloxidase by DIECA suggests that it does also. This inhibition might be caused by removal of copper from the enzyme protein, or by the formation of an inactive protein–copper–DIECA complex. Unpublished work by Miss P. M. Bell supports the idea that copper is removed, but has not explained the difference (Table 9) between the enzyme in extracts made from leaves infected for different periods. The Michaelis constant of the enzyme in extracts made from leaves which have been infected for 4 weeks is similar to that in extracts from leaves which have been infected for 12 weeks; so is the inhibition constant ($K_i$) with DIECA. Old leaves contain 2–3 times as much copper as young leaves, but so far there is no evidence that these small amounts of copper affect the inhibition of polyphenoloxidase by DIECA.

Tobacco necrosis virus and the nucleic acid prepared from tobacco mosaic virus are inactivated by a range of oxidation systems, some of them enzymic (Bawden & Pirie, 1957, 1959). These oxidations, as well as the oxidation of chlorogenic acid, possibly produce a similar virus inactivating substance, perhaps some form of 'activated' oxygen. However, potassium ethylxanthate preserves the infectivity of CMV when leaves are disrupted, but it only partially inhibits the oxygen absorbing reactions. Thus it seems more probable that CMV is inactivated by a quinone intermediate or an end-product of the oxidation of chlorogenic acid. By analogy with the oxidation of other polyphenols, the initial oxidation product of chlorogenic acid may be a quinone, formation of one molecule of which involves the uptake of one atom of oxygen. A series of oxidation-reduction reactions may then convert half of this quinone to a hydroxyquinone, and regenerate chlorogenic acid from the rest. The hydroxyquinone probably polymerizes, partly by non-enzymic reactions, to produce the dark brown substance and some carbon dioxide. Either the intermediate quinones or the high molecular weight polymers may be responsible for inactivating CMV. Quinones react readily with amino acids and with other nitrogen and sulphur containing compounds; and reactions between polymerized polyphenols and proteins of leaves are suggested by the discovery that the brown compounds of large molecular weight extracted from dried tobacco leaves contain chlorogenic acid, rutin and amino acid residues (Jacobson, 1961).

A possible explanation of the results of the experiment in which chlorogenic acid was incubated with a leaf extract containing polyphenoloxidase and CMV then added (Table 4) is that the virus is inactivated by intermediates of the oxidation. Hampton & Fulton (1961) concluded that *o*-quinone, produced during the oxidation of catechol, was responsible for inactivating prune dwarf and sour cherry necrotic ringspot viruses. However, the idea that the quinone formed from chlorogenic acid inactivates CMV is not easily reconciled with the fact that potassium ethylxanthate preserves the infectivity of CMV during extraction from tobacco leaves; this compound halves the amount of oxygen absorbed during the oxidation of chlorogenic acid, suggesting that it does not inhibit the formation of quinone, but a later stage

in the oxidation. Nor can an effect of the brown end-products of the oxidation on CMV be excluded. These may have been ineffective in the experiment described in Table 4 because they combined with other proteins before CMV was added.

Although the oxidation products of chlorogenic acid probably act on the protein of CMV, it is also possible that they react with its nucleic acid; this reaction might be responsible for loss of infectivity. A few experiments were made to explore this possibility, but the results are difficult to interpret. In one, samples of an infective extract, taken before and after incubation with chlorogenic acid and copper, were extracted with phenol in a manner likely to produce infective nucleic acid (Gierer & Schramm, 1956). Both phenol extracts contained similar proportions (10 %) of the infectivity of the initial samples, suggesting that infective nucleic acid cannot be extracted from inactivated virus. This may indicate that oxidized chlorogenic acid affects virus nucleic acid, or that it strengthens the attachment of virus protein to unaltered nucleic acid. In work with prune dwarf virus, Hampton & Fulton (1961) found that virus inactivated by *o*-quinone still appeared morphologically normal in the electron microscope and retained its ability to react with specific antiserum, suggesting that the surface protein of the inactivated virus had not been grossly altered.

Iron forms at least three complexes with chlorogenic acid (Hughes & Swain, 1962), and between pH 7 and 8 the predominant ones are ferric di- and tri-chlorogenate; whether or how these compounds inactivate CMV is unknown. The inactivation of CMV which occurred with iron, like that involving polyphenoloxidase, requires air and is prevented by DIECA. But iron, unlike copper, does not reactivate the DIECA-inhibited enzyme in leaf extracts. To confirm that there are two separate and unrelated inactivation processes, it will be necessary to show that inactivation by iron + chlorogenic acid occurs in virus preparations free from polyphenoloxidase.

The formation of iron complexes does not prevent the enzymic oxidation of chlorogenic acid. Two inactivating processes may therefore be operating when CMV-infected leaves are disrupted in the absence of DIECA, and it is of interest to estimate the relative effectiveness of the two. The iron contents of extracts made from infected leaves (1 g. in 5 ml. tris buffer) ranged between 2·5 and 6·4 $\mu$M. Inactivation was not detected in the experiment described in Table 6 with 25 $\mu$M iron. Any effect of ferric chlorogenates on CMV in extracts made by grinding 1 g. leaf in 4–5 ml. of liquid will therefore be confined to the period of leaf disintegration, when transitory high concentrations may occur. This inactivation effect will be diminished by iron-sequestering agents from the leaves (e.g. citrate), and will probably be small compared to the inactivation brought about by the enzymic oxidation of polyphenols.

We thank Miss S. Burnett for the metal determinations.

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# The Cellulolytic Activity of Pure Strains of Bacteria from the Rumen of Cattle

By G. HALLIWELL
*Department of Enzymology, Rowett Research Institute, Aberdeen*
AND M. P. BRYANT
*Dairy Cattle Research Branch, Agricultural Research Centre, U.S. Department of Agriculture, Beltsville, Maryland, U.S.A.*

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SUMMARY

The *in vitro* breakdown of degraded and undegraded varieties of cellulose was examined by using pure strains of bacteria isolated from the rumen of cattle. One strain of *Bacteroides succinogenes*, two strains of *Ruminococcus albus* and two strains of *Ruminococcus flavefaciens* were allowed to ferment ground cellulose powder (prepared from filter paper), cellulose powder (Whatman) and undegraded cotton fibres, the extent of breakdown being followed by loss of weight of the insoluble substrate. All five organisms were highly active on degraded ground cellulose powder and dissolved 72–90 %, but only one organism, *B. succinogenes* strain S-85, was equally effective on cellulose powder (Whatman) or on undegraded cotton fibres. *R. flavefaciens* strain FD-1 was somewhat less potent on the latter substrates, achieving 40 and 60 % dissolution, respectively, of cellulose powder (Whatman) and cotton fibres. *R. albus* strain 7 and *R. flavefaciens* strain C-94 had negligible effects on cotton fibres (10 and 0 % solubilization, respectively). *R. albus* strain D-89, producing 40 % solubilization of cotton fibres, was intermediate in activity between *R. albus* strain 7 and *R. flavefaciens* strain FD-1. Cell-free preparations from culture filtrates of *B. succinogenes* strain S-85 gave only 4 % breakdown of ground cellulose powder and up to 9 % breakdown of cellulose powder (Whatman) in 17 days. Cell-free filtrates from the metabolism fluid of *R. flavefaciens* strain FD-1 or from the disintegrated organisms brought about 46 and 36 % solubilization, respectively, of ground cellulose powder, but failed to attack cotton fibres. The results support the view that the capacity of an organism or cell-free enzyme to attack any one particular form of cellulose is no criterion of its ability to attack less degraded or undegraded types of cellulose.

## INTRODUCTION

Several attempts have been made to isolate and describe the activity of rumen cellulolytic micro-organisms by using cellulosic substrates previously degraded by chemical or physical treatments and hence rendered more susceptible to biological attack. Thus, acid-treated and ball-milled cotton has been used by Hungate (1950*a*, *b*), a ball-milled filter paper by Bryant & Burkey (1953*a*) and soluble cellulose derivatives, such as carboxymethylcellulose, by Kitts, Carr & Underkofler (1954) and Underkofler, Kitts & Smith (1953). The micro-organisms in question have not, however, been examined on native undegraded cellulosic substrates such as

cotton fibres, although it is known that this form of cellulose can be readily and completely dissolved *in vitro* by a mixed population from the rumen of sheep (Halliwell, 1957*b*, 1961*a*). The present work compares the *in vitro* bacterial solubilization of different forms of cellulose, extending from degraded ball-milled filter paper through cellulose powder (Whatman) to undegraded, purified cotton fibres. The bacteria used were pure strains, isolated from bovine rumen, of *Bacteroides succinogenes*, *Ruminococcus albus* and *Ruminococcus flavefaciens*.

## METHODS

General procedures for the preparation of media, isolation of bacteria and descriptions of strains (Bryant & Burkey, 1953*a*; Bryant & Doetsch, 1954; Bryant, Small, Bouma & Robinson, 1958*a*), the nature of the three cellulosic substrates, namely, ground cellulose powder prepared by grinding filter paper (Whatman no. 1) in a ball-mill (Bryant & Burkey, 1953*a*), cellulose powder (Whatman) and dewaxed Texas cotton fibres (Halliwell, 1957*a*) and the determination of residual cellulose (Halliwell, 1957*a*), are described in the references cited. Additional details or modifications are given below.

*Bovine bacteria.* *Bacteroides succinogenes* strain S-85, *Ruminococcus albus* strains 7 and D-89 and *Ruminococcus flavefaciens* strains C-94 and FD-1 were isolated from bovine rumen and then maintained as stab cultures in agar at $-50$ to $-70°$. Two or three loopfuls of the stock cultures were transferred to tubes containing 5 ml. cellulose broth and grown at 37° for 24–48 hr. to provide sufficient actively growing inoculum for up to 200 ml. of cellulose broth.

*Culture media.* Cellulose broth was used to grow the organisms and also as a basal medium to examine the cell-free enzymic breakdown of the different forms of cellulose. Broth (100 ml.) contained: 30 ml. whole rumen fluid (prepared by expressing rumen fluid through surgical gauze, allowing to stand overnight at 1° and separating off the middle phase from the lower sediment and upper floating material); 3·75 ml. mineral solution 1 (0·6 %, w/v, $K_2HPO_4$); 3·75 ml. mineral solution 2 (w/v: 0·6 %, $KH_2PO_4$; 1·2, $(NH_4)_2SO_4$; 1·2, NaCl; 0·25, $MgSO_4.7H_2O$; 0·12, $CaCl_2$); 0·1 ml. 0·1 %, w/v, resazurin solution; 5 ml. 8 %, w/v, $Na_2CO_3$; 2 ml. 2·5 %, w/v, cysteine hydrochloride; water to 100 ml. The broth was equilibrated with, and maintained under, $O_2$-free $CO_2$. Ground cellulose powder, cellulose powder (Whatman) or dewaxed Texas cotton fibres were incorporated in the medium at a final concentration of about 0·2 %, w/v.

*Cellulolysis or solubilization of cellulose.* In the standard cellulase assay for whole bacteria, the strains were inoculated under $CO_2$ into test tubes (16 × 150 mm.) of cellulose broth (10 ml.) and incubated anaerobically at 37°. In the standard cellulase assay for cell-free preparations an aqueous suspension of cellulose was added to different amounts of culture filtrate as indicated in the text; the assays were made at 37° under sterile and anaerobic conditions in presence of $CO_2$ and with additional cysteine at the concentration found in the culture medium. Reagents and tubes were sterilized by autoclaving at 120° and enzyme solutions by filtration.

Solubilization of cellulose was followed by gravimetric estimation of residual cellulose by using sintered-glass filter crucibles, porosity M, medium grade (nominal maximum pore size 10–15 $\mu$) for cellulose powders, or porosity C, coarse grade (40–60 $\mu$) for cotton fibres. With the pure cultures used here the washing procedure

with HCl, $NH_4OH$, Teepol and ethanol (Halliwell, 1957*a*), for the removal of contaminating non-cellulosic material from mixed rumen bacterial fermentations, was not required. Residual cellulose was washed copiously with distilled water, dried overnight and weighed: the 'enzyme blank' (bacteria incubated in cellulose-free media) gave negligible values after washing and filtration.

*Cell-free enzyme preparations.* Large-scale cultures with about 400 ml. cellulose broth containing 0·2 % ground cellulose powder, were inoculated from actively growing cultures of *Bacteroides succinogenes* strain S-85 or *Ruminococcus flavefaciens* strain FD-1, grown for the preceding 24–48 hr. in an identical medium. At suitable intervals the large-scale cultures were agitated temporarily with a magnetic stirrer to provide homogeneous suspensions for sampling. The cultures were regassed with $CO_2$ during and after sampling. After the desired stage of fermentation had been reached the cultures were centrifuged at 27000 *g* for 20 min. at 3° (Servall Machine, Model RC2, Rotor SS-34, Ivan Sorvall, Inc., Norwalk, Conn., U.S.A.) and the cell-free supernatant phase removed and maintained under $CO_2$ for assay. In some experiments the precipitated bacteria were also disintegrated in a Mini-Mill (Gifford-Wood Co., Hudson, New York) under $CO_2$ and cooled in ice. Extensive breakage of organisms required 30–50 min. treatment in the Mill, and was judged by safranin stained smears.

*Alkali solubility of cellulosic substrates.* Dewaxed Texas cotton fibres, cellulose powder (Whatman), and a hydrocellulose (prepared by soaking absorbent cotton wool in 11 N-HCl; Halliwell, 1957*a*), were air-dried materials. Ground cellulose powder and ground hydrocellulose, which were prepared in the usual manner in a ball mill (Bryant & Burkey, 1953*a*; Hungate, 1950*a*), were stored and used in aqueous suspension. The hydrocellulose and its ground counterpart had been used as cellulosic substrates in earlier work with rumen micro-organisms, but in the present work they were used only for comparative purposes in the alkaline solubility estimations.

About 50 mg. of each form of cellulose in water was mixed in a test tube with an equal volume (6 ml.) of 2 N-NaOH, the suspension heated in a boiling water bath for 15 min. under a glass pear-bulb, cooled for 5 min. and transferred quantitatively to a sintered glass crucible (porosity M) by washing with 20 ml. N-NaOH. The cellulose was sucked almost dry, washed with 40 ml. N-NaOH, again sucked almost dry, and washed with water, 0·1 N-$H_2SO_4$ and water until neutral. The crucible + cellulose were dried overnight at 105° and weighed.

## RESULTS

### *Bacterial solubilization of ground cellulose powder, cellulose powder and dewaxed cotton fibres*

*Bacteroides succinogenes* strain S-85, *Ruminococcus albus* strains 7 and D-89, *R. flavefaciens* strains C-94 and FD-1 were grown in cellulose broth containing about 0·2 % ground cellulose powder (Whatman) or dewaxed cotton fibres. It is evident from Table 1 that the five organisms attacked ground cellulose powder and, with the exception of *B. succinogenes* strain S-85, attacked this powder, a degraded substrate, more effectively than they did fibres. Only *B. succinogenes* strain S-85, *R. flavefaciens* strain FD-1 and possibly *R. albus* strain D-89 gave significant breakdown of undegraded cellulose as found in cotton fibres.

In an attempt to promote more rapid and extensive attack on cotton fibres we re-examined some of the organisms recorded in Table 1 after incorporating cellobiose at 0·02 % final concentration in the medium. The results (Table 2) confirmed

Table 1. *Solubilization of ground cellulose powder and dewaxed cotton fibres by rumen bacteria*

Conditions: standard cellulase assay (see Methods). Initial weight of ground cellulose powder was 23 mg. (average) and of cotton fibres 20 mg. Period of incubation for ground cellulose powder was 5 days and for cotton fibres 7 days.

| | Substrate | |
|---|---|---|
| | Ground cellulose powder | Cottor. fibres |
| | Cellulose solubilized as % of initial weight | |
| Organisms | | |
| *Bacteroides succinogenes* strain S-85 | 88 | 97 |
| *Ruminococcus albus* strain 7 | 88 | 10 |
| strain D-89 | 88 | 40 |
| *R. flavefaciens* strain C-94 | 72 | 0 |
| strain FD-1 | 90 | 55 |

Table 2. *Solubilization of dewaxed cotton fibres in presence of cellobiose*

Conditions: as in Table 1 but with cellobiose in the medium at 0·02 % final concentration. Initial weight of cotton fibres was 21 mg. (average).

| | Time of action of culture | |
|---|---|---|
| | 4 days | 8 days |
| | Cotton fibres solubilized as % of initial weight | |
| Organisms | | |
| *Bacteroides succinogenes* strain S-85 | 86 | 86 |
| *Ruminococcus albus* strain 7 | 0 | 10 |
| *R. flavefaciens* strain FD-1 | 30 | 60 |

the cellulolytic nature of *B. succinogenes* strain S-85 and of *R. flavefaciens* strain FD-1, whereas *R. albus* strain 7 once again showed little inclination to solubilize undegraded cellulose of cotton fibres. This suggests that the cellulolytic activity of *R. albus* strain 7 is restricted to simpler forms of cellulose (Table 1).

Cellulose powder (Whatman) appears to retain some of the properties of native cellulose more than do certain other powdery forms of cellulose; it is more soluble in N-alkali than cotton fibres (see Discussion) but resists solubilization by cell-free cellulolytic filtrates from fungi although not to the same degree as do cotton fibres (Halliwell, 1961*b*). If particle size were the only obstacle to microbial breakdown of fibrous cellulose we should expect the powdery nature of cellulose powder

(Whatman) to encourage attack by those organisms which find difficulty in metabolizing fibres. Three amounts of cellulose powder (Whatman), about 10, 20 and 50 mg., were supplied to *Bacteroides succinogenes* strain s-85, *Ruminococcus flavefaciens* strain FD-1 and *R. albus* strain 7. At all substrate concentrations the first two strains showed rapid breakdown of cellulose up to the fourth day of fermentation, whilst *R. albus* displayed only feeble activity extending to the second day (Figs. 1, 2).

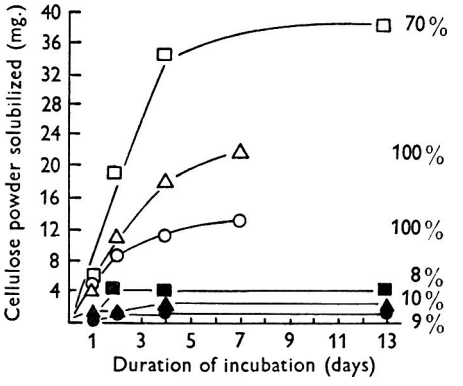


Fig. 1

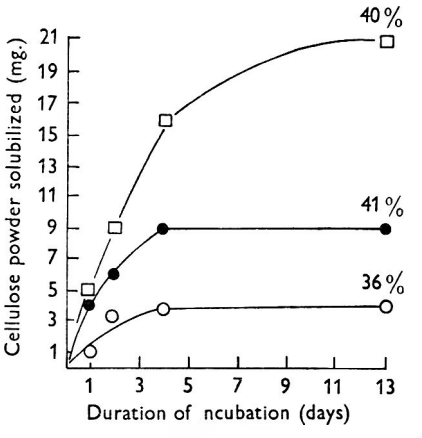


Fig. 2

Fig. 1. The effect of time of incubation on bacterial cellulolysis of cellulose powder (Whatman). Conditions: standard cellulase assay with *Bacteroides succinogenes* strain s-85; initial weight of cellulose 13 mg., ○; 22 mg., △; 54 mg., □. *Ruminococcus albus* strain 7; initial cellulose 11 mg., ●; 20 mg., ▲; 51 mg., ■. Cellulose incubated in absence of bacteria for 13 days lost no weight. Percentages near curves indicate the final extent of solubilization of the initial cellulose.

Fig. 2. The effect of time of incubation on bacterial cellulolysis of different amounts of cellulose powder (Whatman). Conditions: standard cellulase assay with *Ruminococcus flavefaciens* strain FD-1; initial weight of cellulose 11 mg., ○; 22 mg., ●; 53 mg., □. Other details as in Fig. 1.

After the fourth day the rate of solubilization of cellulose decreased with *B. succinogenes* strain s-85 and *R. flavefaciens* strain FD-1 and complete hydrolysis was achieved only by the former organism in 7 days. With *R. flavefaciens* strain FD-1, increasing the fermentation period by 9 days produced little change in the figure of about 40 % breakdown attained after the first 4 days at the lower cellulose concentrations. At all three cellulose concentrations *R. albus* strain 7 dissolved no more than 10 % of the substrate in 2 or 13 days fermentation.

### *Cellulolytic enzymes from* Bacteroides succinogenes *strain s-85 and from* Ruminococcus flavefaciens *strain* FD-1

*The action of cell-free enzyme preparations from* Bacteroides succinogenes *strain s-85 on ground cellulose powder and on cellulose powder (Whatman).* Strain s-85 was grown in 400 ml. broth containing 0·22 % ground cellulose powder, as described in Methods. After 29 hr. the organisms had decreased the amount of cellulose to 37 % of its initial value and to 21 % in a further 12 hr. The culture was allowed a further 23 hr. (total 64 hr.) to attack most of the remaining cellulose and thus to encourage release of any adsorbed enzyme from its cellulosic substrate (see Halliwell, 1961*b*). The

culture was centrifuged to obtain the aqueous phase which was sterilized by filtration; 10 ml. of this cell-free enzyme solution were incubated with 20 mg. ground cellulose powder + cysteine at 37° for 22 days. This produced only a 4 % loss of weight of cellulose. Other cultures of *B. succinogenes* strain s-85, grown under identical conditions to those described, metabolized 86 % of ground cellulose powder in 44 hr. and 88 % in 72 hr. Cell-free culture filtrates were obtained at both stages of attack by the standard procedures and their activities measured by using 20 mg. cellulose powder (Whatman) and 20 ml. of each of the enzymic filtrates. These dissolved only 9 and 3 %, respectively, of the available cellulose powder (Whatman) in 17 days at 37°. The small extent of breakdown of ground cellulose powder and cellulose powder (Whatman) is not considered significant in view of the nature of these substrates (see Discussion).

*The action of cell-free enzyme preparations from* Ruminococcus flavefaciens *strain* FD-1 *on ground cellulose powder and on dewaxed cotton fibres.* Tables 1 and 2 and Figs. 1 and 2 indicate that *R. flavefaciens* strain FD-1 was as active as *Bacteroides succinogenes* strain s-85 on ground cellulose powder, but was not as effective in solubilizing less-degraded substrates such as cellulose powder (Whatman) and cotton fibres. Thus *R. flavefaciens* strain FD-1, like *B. succinogenes* strain s-85, dissolved ground cellulose powder to within 90 % of completion, but gave only 40–60 % loss of weight of cellulose powder (Whatman) and cotton fibres, as compared with the 100 % loss effected by *B. succinogenes* strain s-85. The latter organism did not provide a cell-free cellulase preparation producing marked breakdown of either of the substrates tested and the results indicate that *R. flavefaciens* strain FD-1 was the only remaining bacteria likely to possess this enzyme system.

*Ruminococcus flavefaciens* strain FD-1 was grown by the standard procedure in 400 ml. broth + 0·2 % ground cellulose. Of the cellulose 36 and 80 % was catabolized after 18 and 40 hr., respectively. The older culture supplied two enzyme preparations: the first was the supernatant phase obtained by centrifuging the culture medium; the second was prepared by resuspending the sediment in the minimum necessary volume of some of the above supernatant phase followed by treatment in the Mini-mill disintegrator whereby the enzyme was released into the aqueous phase. This was collected by centrifugation as described earlier. The two enzyme preparations, sterilized by filtration, were incubated in 15 and 5 ml. samples each with 20 mg. ground cellulose powder for 20 days during which period they solubilized 46 and 36 %, respectively, of the substrate. Samples (20 ml.) of the first enzyme preparation were also incubated for 20 days with 10 mg. dewaxed cotton fibres; no loss of weight of cellulose was observed.

## DISCUSSION

In the field of cellulose metabolism by micro-organisms or cell-free enzyme preparations, difficulties of definition are more important than real experimental discrepancies. There is much to be said for adhering only to the terms $\beta$-polyglucosidase or 1,4-$\beta$-glucanase for the enzymes which attack polymers of the cellulose type, and for reserving the term cellulase until such time as the mechanism that accomplishes the initial degradation or sensitization of undegraded cellulose or native cotton fibres has been elucidated. The same difficulties of interpretation arise with the term 'cellulolytic organism' because not all organisms thus styled can

attack cotton fibres. Whilst one is entitled to use the term cellulolytic for an organism that attacks any 1,4-$\beta$-glucan, such organisms are undoubtedly divided into two groups in accordance with their ability or inability to act on cotton fibres.

Soluble cellulose derivatives or similar biologically susceptible forms of insoluble but degraded cellulose are often used as substrates for enzymes in whole organisms or in the cell-free state. The results obtained, however, are difficult to assess in absence of simultaneous studies with an undegraded substrate such as cotton fibre. Whilst all forms of cellulose are believed to be chemically identical in consisting solely of 1,4-$\beta$-linked anhydroglucose units, there are marked differences between them in physical properties such as chain length and the presence of crystalline and amorphous regions. A distinction between various types of cellulose is shown by their relative susceptibilities to alkali. Five cellulosic substrates (dewaxed cotton fibres, cellulose powder (Whatman), a hydrocellulose powder prepared from absorbent cotton wool by soaking in concentrated HCl, the same hydrocellulose after grinding in a ball-mill, ground cellulose powder prepared from filter-paper) were digested with N-NaOH for 15 min. as described in Methods, and lost 0, 17, 16, 17 and 21 % of their weight, respectively (corrected for moisture content). A more comprehensive estimate of the smaller molecular chains of cellulose is given by the $\beta$- and $\gamma$-cellulose contents. These two fractions, arbitrarily defined as the material soluble in 17·5 % (w/w) NaOH at 20°, contain molecules with a degree of polymerization from about 10 to 200. $\alpha$-Cellulose is the fraction insoluble in the same concentration of alkali and has chains with a degree of polymerization greater than 200. The $\beta$- + $\gamma$-cellulose contents of dewaxed Texas cotton fibres and of cellulose powder (Whatman) were 2 and 30 %, respectively, as determined by loss of weight on 50 mg. of each substrate and corrected for moisture contents. This difference in physical properties between celluloses is reflected in the relative activities of the rumen bacteria examined in this report. Five organisms, *Bacteroides succinogenes* strain S-85, both strains of *Ruminococcus albus* and both strains of *R. flavefaciens*, were most effective in solubilizing a large proportion (72–90 %) of a degraded ground cellulose powder (Table 1); but only *B. succinogenes* strain S-85 produced a comparable degree of cellulolysis of undegraded cotton fibres and of cellulose powder (Whatman). In this respect it is similar to mixed rumen micro-organisms (Halliwell, 1957*b*, 1961*a*). *R. flavefaciens* strain FD-1 and *R. albus* strain D-89 were less effective in rate and extent of cellulolysis and were the only other strains to achieve marked breakdown of cotton fibres, 55 and 40 %, respectively (Tables 1, 2). On cellulose powder (Whatman) the action of *R. flavefaciens* strain FD-1, the most effective of the ruminococci in Table 1, was similarly restricted to 40 % solubilization of the substrate, even with prolonged incubation (Fig. 2). *R. albus* strain 7 and *R. flavefaciens* strain C-94 produced extensive solubilization (88 and 72 %) of ground cellulose powder but only 10 and 0 % breakdown of cotton fibres (Table 1). Other strains of *R. albus* which did not attack even the ground cellulose powder have been described (Bryant *et al.* 1958*b*). As only one strain of *B. succinogenes* was available for the present study, it is not known whether this species would, in general, more extensively solubilize fibrous forms of cellulose as compared to ruminococci. It is of interest in this respect that *B. succinogenes* was found in much greater numbers than ruminococci in the rumen of a cow fed wheat-straw, but ruminococci were more numerous when good quality alfalfa hay was fed (Bryant & Burkey, 1953*b*).

It has frequently been suggested that fibrous forms of cellulose are unsuitable substrates for rumen micro-organisms because of the relatively small surface area exposed to attack, and recourse has been made to finely ground varieties of cellulose. An increased susceptibility to biological attack that accompanies the transition from fibrous to powdery cellulose is liable to be associated not only with increased surface area, but also with degradative changes in the cellulose molecules. If the surface area of the cellulose were of paramount importance in favouring bacterial attack then cellulose powder (Whatman) should be broken down to a greater extent than cotton fibres. The results shown in Figs. 1 and 2 do not support this view. *R. albus* strain 7 and *R. flavefaciens* strain FD-1 found cellulose powder (Whatman) just as difficult to solubilize as cotton fibres, suggesting that this powdered cellulose retained some of the characteristics of the fibrous material.

The small degree of cellulolysis of ground cellulose powder (4 %) and cellulose powder (Whatman) (3–9 %) shown by cell-free enzyme preparations from *Bacteroides succinogenes* strain S-85 is probably insignificant as far as undegraded cellulose is concerned, since the effect might be confined to the smaller cellulosic molecules produced in the manufacture. This lack of cellulolytic activity shown by cell-free systems from *B. succinogenes* strain S-85 is in marked contrast to the pronounced activity of the intact organism on all three forms of cellulose (Tables 1, 2; Figs. 1, 2). *R. flavefaciens* strain FD-1, although as active on ground cellulose powder as *B. succinogenes* strain S-85, was only about half as effective as *B. succinogenes* strain S-85 on cellulose powder (Whatman) and on cotton fibres, but yielded cell-free enzyme preparations which solubilized up to 46 % of ground cellulose powder. The same preparations did not attack cotton fibres, suggesting that different mechanisms are responsible for the breakdown of degraded and undegraded cellulose.

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# Thermophilic and Mesophilic Actinomycetes in Mouldy Hay

By R. CORBAZ,* P. H. GREGORY and MAUREEN E. LACEY
*Rothamsted Experimental Station, Harpenden, Hertfordshire*

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SUMMARY

Actinomycetes isolated at 40° and/or 60° from mouldy hay included: *Micromonospora vulgaris* Waksman *et al.*, *Thermopolyspora polyspora* Hens., *T. glauca* sp.nov., *Streptomyces thermoviolaceus* Hens., *S. fradiae* (Waksman et Curtis) Waksman et Henrici, *S. griseoflavus* (Krainsky) Waksman et Henrici, *S. olivaceus* (Waksman) Waksman et Curtis, and *S. griseus* (Krainsky) Waksman et Henrici.

## INTRODUCTION

Mouldy hay associated with farmer's lung disease contained large quantities of actinomycete spores; over $10^9$/g. dry weight hay were reported by Gregory & Lacey (1963*a*). This paper describes the commonest species and gives some information about their occurrence in different grades of hay.

## METHODS

Actinomycetes were isolated with the aid of the Andersen sampler (Andersen, 1958) which was loaded with Petri dishes containing medium and suspended horizontally in a small wind tunnel while hay was shaken up-wind in a perforated drum (Gregory & Lacey, 1962, 1963*a*, *b*).

Three media were used for isolation. For incubation at 40° 0·5 mg. actidione/ml. was added to suppress mould growth.

*Half-strength nutrient agar* contained 14 g. 'Oxoid' nutrient agar granules, 10 g. agar, 1 l. water. *Yeast extract agar* (Pridham *et al.* 1957) contained 4 g. 'Difco' yeast extract, 10 g. malt extract, 4 g. glucose, 20 g. agar, 1 l. water; adjusted to pH 7·3 with KOH. *V8 agar* (Galindo & Gallegly, 1960) contained 200 ml. 'V8' vegetable juice, 4 g. calcium carbonate, 20 g. agar, 800 ml. water; adjusted to pH 7·3 with KOH.

After exposure in the Andersen sampler, Petri dishes were incubated at 40° ($\pm 1°$) and 60° ($\pm 2°$). Colonies were counted after 2, 4 and 8 days. Many isolates were studied on different media and at different temperatures. *Peptone iron agar* (Tresner & Danga, 1958) containing 36 g. peptone iron agar, 1 g. yeast extract, 1 l. water, was used for the melanin test.

## RESULTS

Although very many actinomycete colonies developed on the isolation plates, the number of different species was small: seven grew at 40°, of which three also grew at 60°. At both temperatures a few colonies without aerial mycelium developed, but

* Present address: Stations Fédérales d'Essais Agricoles, Château de Changins, Nyon, Switzerland.

most of these showed good growth with aerial mycelium at lower temperatures. These included *Streptomyces griseus* (Krainsky) Waksman et Henrici; others have not yet been identified.

*Taxonomy*

The taxonomy of thermophilic actinomycetes is now in a state of flux, but like other workers (Tendler, 1959; Erikson, 1952; Henssen, personal communication to P.H.G., 1961) we do not regard thermophily as a good taxonomic character at the generic level. Both thermophilic and mesophilic species are included in the genus *Streptomyces* and we also admit thermophilic species in the genus *Micromonospora*. Tendler (1959) has shown that ability to grow at different temperatures is a function of nutritional requirements rather than a specific character. As morphology seems of primary importance in this group, we follow the classification of Ettlinger *et al.* (1958) for the identification of *Streptomyces*. In the following list those isolates examined in detail are indicated by 'A' and number. Typical cultures have been deposited at the Centraalbureau voor Schimmelcultures (CBS), Baarn, Netherlands, and Eidgenössische Technische Hochschule (ETH), Zürich, Switzerland.

*Micromonospora vulgaris* Waksman *et al.* Aerial mycelium abundant, cottony, white to light lavender; aerial hyphae mostly simple, emerging from the medium and often dipping back in it after a short arch over the agar (Pl. 1, fig. 4); they seem to empty when the spores are formed. Substrate mycelium light brown, sometimes darker. Spores globose, typically sharp-cornered, 0·5–0·8 $\mu$ (Pl. 1, fig. 5*b*), single, sessile (Pl. 1, fig. 5*a*). No reaction on peptone iron agar. Good and rapid growth at 40° and 60°, no growth at 28°. Over 100 isolates were examined which appeared to belong to this species. Their sessile spores disagree with the description of *M. vulgaris*, but with present knowledge of the importance and stability of the sporophore this character seems insufficient for the proposal of a new species. Isolates examined: A 64 (CBS 109.62; ETH 31509), A 65, etc.

*Thermopolyspora polyspora* Hens. Colony yellow. Aerial hyphae whitish, mostly simple, bearing lateral chains of 1 to 10 spores (Pl. 1, fig. 6). Spores globose (Pl. 1, fig. 7) 0·8–1·3 $\mu$; also formed in medium. No reaction on peptone iron agar. No growth at 28°, slow vegetative growth at 40°, and slow vegetative and aerial growth and sporulation at 60°. Isolates examined: A 94 (CBS 100.63; ETH 31520), A 88, A 89, A 90, A 91, A 92, A 95.

*Thermopolyspora glauca* sp.nov. Aerial mycelium abundant, first white, becoming greenish blue and later blue grey; aerial hyphae straight, mostly simple, bearing short lateral spore chains (Pl. 1, figs. 1–3). Vegetative mycelium dark green. Spores oval, 0·8–1·3 × 0·6–0·8 $\mu$, often in pairs, sometimes single, also in chains of 3 or 4; some spores can be found in the medium. No reaction on peptone iron agar. Most isolates were without diffusible pigment but some produced black and some dark brown pigments. Growth very slow but good at 40°, and colonies were first recognizable after 5 or 6 days incubation on nutrient agar. Growth with aerial mycelium also occurs at 28°, but at 60° there is little or no vegetative growth. Type culture: A 66 (CBS 110.62; ETH 28797). Other isolates examined: A 67, A 68, A 69, A 70.

Henssen (1957) described a bluish thermophilic actinomycete with 4–10 spores in chains under the name of *Thermoactinomyces glaucus* Hens. *T. monosporus* (Schütze) Waksman *et al.* (cf. *Bergey's Manual*, 1948) and *T. viridis* Schuurmans, Olson &

San Clemente (1956) have both single spores and bluish aerial mycelium. Cultivated on various media our isolates never showed more than 4 spores; we therefore consider them as a new species of the genus *Thermopolyspora* Hens. (Micropolyspora Lechevalier, Solotorovsky & McDurmont (1961), described four years later, is considered to be a synonym of Thermopolyspora.)

*Streptomyces thermoviolaceus* Hens. The description given by Henssen is completed as follows. Aerial mycelium first brown, later ash grey, not abundant, with open loops (Pl. 1, fig. 8) (cf. Henssen, 1957, figs. 7, 8). Spores oval to cylindrical, 0·8–1·2 × 0·6–0·8 $\mu$, surface covered with small hemispherical particles, 0·03 $\mu$ (Pl. 1, fig. 9), which are reported for the first time. No reaction on peptone iron agar. Growth rapid and excellent at 40°, good at 60°, and slow and usually without aerial mycelium at 28°. A few colonies (under 1 %) had an intense violet diffusible pigment, but as the aerial mycelium and spore morphology are identical with the other isolates they are classified under the same species. Isolates examined: A71 (CBS 111.62; ETH 28745), A73, A74.

*Streptomyces fradiae* (Waksman et Curtis) Waksman et Henrici. Aerial mycelium abundant, woolly, pink. Vegetative mycelium yellowish. Spore chains in open spirals (Pl. 2, fig. 10), monopodial branching with a long straight axis. Spores smooth (Pl. 2, fig. 11), 0·7–1·0 × 0·4–0·6 $\mu$. No reaction on peptone iron agar. Good growth at 40°, slower at 28°, and no growth at 60°. Isolates examined: A75 (CBS 112.62; ETH 28746), A76.

*Streptomyces griseoflavus* (Krainsky) Waksman et Henrici. Aerial mycelium abundant, ash grey. Spore chains in regular spirals (Pl. 2, fig. 12), monopodial branching. Spores 0·8–1·1 × 0·5–0·7 $\mu$, with short (about 0·2 $\mu$) spines (Pl. 2, fig. 13). No reaction on peptone iron agar. Good growth at 40° and 28°, no growth at 60°. Isolates examined: A77 (CBS 113.62; ETH 31510), A78.

*Streptomyces olivaceus* (Waksman) Waksman et Curtis. Aerial mycelium abundant, ash grey. Spore chains in straight hyphae (Pl. 2, fig. 14), monopodial branching. Spores smooth, 0·4–0·6 × 0·7–1·0 $\mu$ (Pl. 2, fig. 15). No reaction on peptone iron agar. Good growth at 40° and 28°, no growth at 60°. Isolate examined: A79 (CBS 114.62; ETH 28748).

Table 1 gives in diagrammatic and tabular form the diagnostic features as an aid to identification.

*Numbers of colonies on different media*

Tables 2 and 3 show the importance of the medium for quantitative studies of the different species of actinomycetes. Except for sample W at 60°, many more colonies of *Micromonospora vulgaris* occurred on nutrient agar than on yeast agar or 'V8' agar; the opposite is true for *Streptomyces thermoviolaceus* and *S. griseoflavus*. More colonies of *Thermopolyspora glauca* developed on nutrient agar than on either yeast or 'V8' agars. More colonies of *S. fradiae* developed on yeast agar than on nutrient agar, but 'V8' agar gave fewer colonies than nutrient agar. Although the occurrence of *T. polyspora* was not recorded in the tests on which Tables 2 and 3 are based, its abundance in self-heated mouldy hay was similar to *M. vulgaris*. Both have been isolated thousands of times and are particularly characteristic of hay associated with farmer's lung disease.

Good hay contains few actinomycetes. In mouldy hays, which have obviously heated, actinomycetes are very abundant, particularly the thermophilic species

Table 1. *Diagnostic features of seven thermophilic actinomycetes*

| Species | Spores | | Colonies | | Temperature and growth rate |
|---|---|---|---|---|---|
| *Micromonospora vulgaris* A 64 | Single | Globose | Flat, large; aerial myc. light pink, veg. myc. brown | | **40–60°**,* fast |
| *Thermopolyspora polyspora* A 94 | Short chains (3–10 spores) | Globose | Very small; aerial myc. whitish, veg. myc. yellow | | *40*–**60°**, slow |
| *T. glauca* A 66 | Short chains (2–4 spores) | Oval, smooth | Small; aerial myc. blue-green, veg. myc. dark green | | (28)–**40°**, slow |
| *Streptomyces thermoviolaceus* A 71 | In long loops | Oval with hemispherical particles | Large; aerial myc. ash grey, veg. myc. dark | | (28)–**40**–*60°*, fast |
| *S. fradiae* A 75 | Spirals on long straight hyphae | Oval, smooth | Middle never flat; aerial myc. woollish, pink, veg. myc. yellow | | 28–**40°**, medium |
| *S. griseoflavus* A 77 | Spirals | Oval, spiny | Flat; aerial myc. ash grey, veg. myc. ± grey | | 28–**40**–*55°*, medium |
| *S. olivaceus* A 79 | Straight hyphae | Oval, smooth | Flat; aerial myc. ash grey, veg. myc. yellowish | | 28–40°, medium |

* (28°), only slight growth; 28°, poor growth; *55°*, fairly good growth; **40°**, optimum growth.

Table 2. *Number of colonies on six Andersen sampler plates exposed to different hay samples shaken in the wind tunnel*

Incubated at 60°.

| Hay sample reference number | Exposure time (sec.) | Sample weight (g.) | Medium* | *Micromonospora vulgaris* No. colonies | *Streptomyces thermoviolaceus* No. colonies |
|---|---|---|---|---|---|
| H 65. Farmer's lung hay | 5 | 41 | N | 46 | 4 |
| | | | Y | 31 | 47 |
| H 65. Farmer's lung hay | 5 | — | N | 161 | 2 |
| | | | V 8 | 22 | 39 |
| F. Mouldy hay | 5 | 39 | N | 236 | 15 |
| | | | Y | 181 | 545 |
| F. Mouldy hay | 5 | — | N | 192 | 1 |
| | | | V 8 | 22 | 17 |
| W. Mouldy hay | 5 | 52 | N | 101 | 6 |
| | | | Y | 134 | 218 |
| G. Good hay | 15 | 41 | N | 61 | 0 |
| | | | Y | 4 | 6 |
| SB. Good hay | 15 | 38 | N | 22 | 2 |
| | | | Y | 6 | 9 |

* N = Nutrient agar, Y = yeast extract agar, V 8 = V 8 agar.

Table 3. *Number of colonies on six Andersen sampler plates exposed to different hay samples shaken in the wind tunnel*

Incubated at 40°. Pairs of results were from the same experiment.

| Hay sample reference number | Exposure time (sec.) | Sample weight (g.) | Medium† | *Micromonospora vulgaris* | *Thermopolyspora glauca* | *Streptomyces fradiae* | *Streptomyces** with greyish aerial mycelium |
|---|---|---|---|---|---|---|---|
| H 65. Farmer's lung hay | 5 | 41 | N | 27 | 10 | 2 | 13 |
| | | | Y | 3 | 5 | 4 | 61 |
| H 65. Farmer's lung hay | 5 | — | N | 30 | 20 | 21 | 2 |
| | | | V 8 | 1 | 1 | 4 | 94 |
| H 44. Farmer's lung hay | 5 | 30 | N | *c.* 700 | 69 | 4 | 89 |
| | | | Y | 30 | 0 | 22 | *c.* 900 |
| H 44. Farmer's lung hay | 5 | — | N | 431 | 52 | 12 | 27 |
| | | | V 8 | 0 | 1 | 26 | 337 |
| F. Mouldy hay | 5 | 39 | N | *c.* 495 | 41 | 8 | 28 |
| | | | Y | 50 | 2 | 14 | *c.* 430 |
| F. Mouldy hay | 5 | — | N | 18 | 2 | 13 | 5 |
| | | | V 8 | 1 | 0 | 8 | 108 |
| W. Mouldy hay | 5 | 52 | N | 105 | 20 | 5 | 9 |
| | | | Y | 39 | 2 | 21 | *c.* 400 |
| G. Good hay | 15 | 41 | N | 12 | 0 | 1 | 1 |
| | | | Y | 7 | 0 | 3 | 7 |
| SB. Good hay | 15 | 38 | N | 19 | 3 | 1 | 4 |
| | | | Y | 3 | 0 | 2 | 7 |

* Mainly *Streptomyces thermoviolaceus* and *S. griseoflavus*.
† N = Nutrient agar, Y = yeast agar, V 8 = V 8 agar.

(Gregory & Lacey, 1963*a*, Table 3), where grey colonies were not counted at 60° because only nutrient agar was used.

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## EXPLANATION OF PLATES

Electron micrographs of spores of Actinomycetes from mouldy hay; and photomicrographs showing aerial growth at edge of colony (photographed in air).

### PLATE 1

Fig. 1. *Thermopolyspora glauca*, edge of colony. × 375.

Fig. 2. *T. glauca*, spore formation. × 13,500.

Fig. 3. *T. glauca*, spores. × 13,500.

Fig. 4. *Micromonospora vulgaris*, edge of colony. × 375.

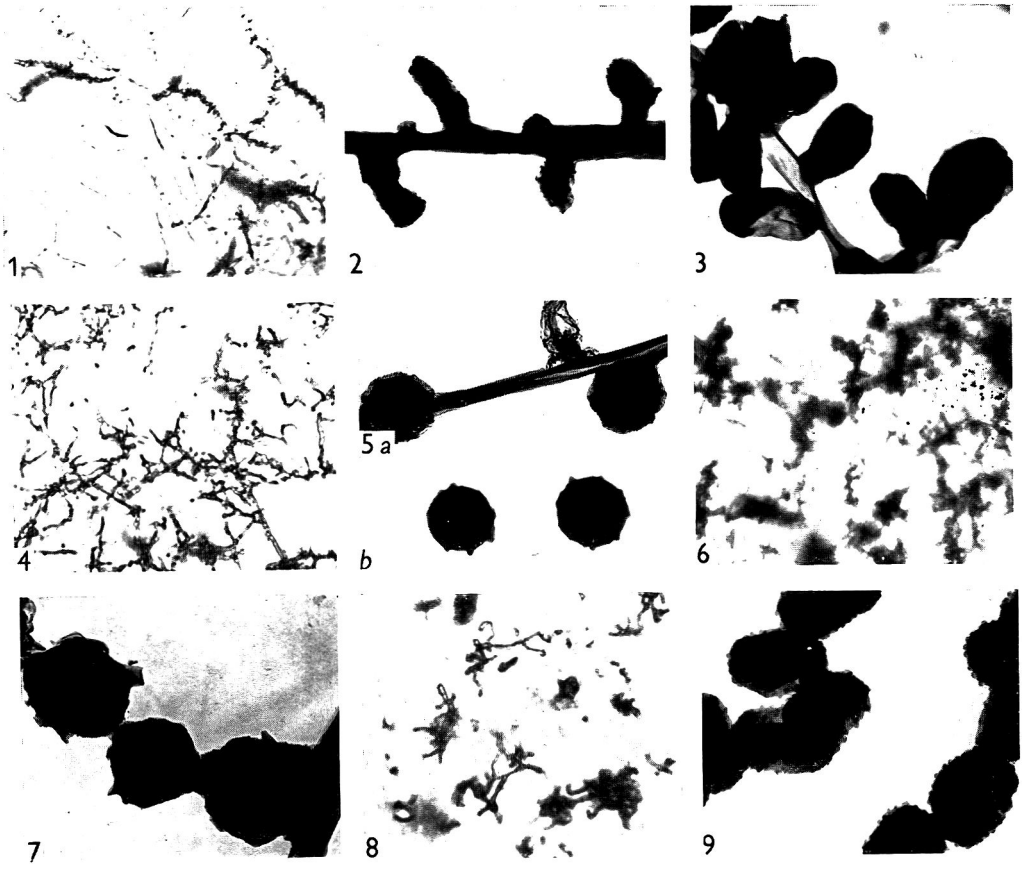
Fig. 5. *M. vulgaris*, spores. × 13,500.

Fig. 6. *Thermopolyspora polyspora*, edge of colony. × 375.

Fig. 7. *T. polyspora*, spores. × 13,500.

Fig. 8. *Streptomyces thermoviolaceus*, edge of colony. × 375.

Fig. 9. *S. thermoviolaceus*, spores. × 13,500.




R. CORBAZ, P. H. GREGORY AND M. E. LACEY

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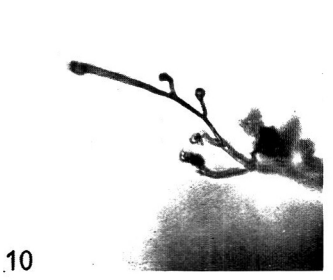
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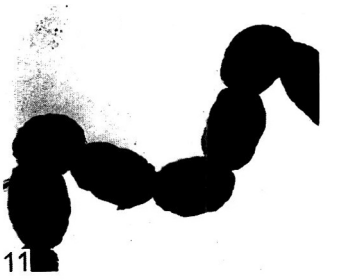
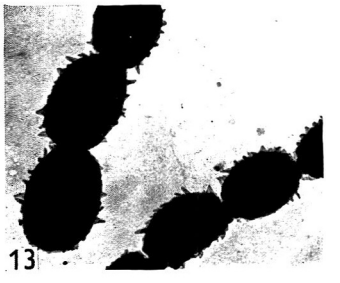

11


12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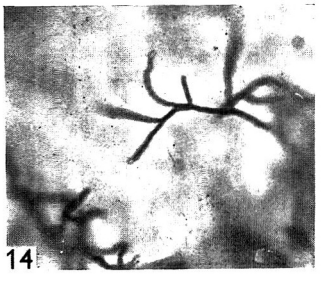
14

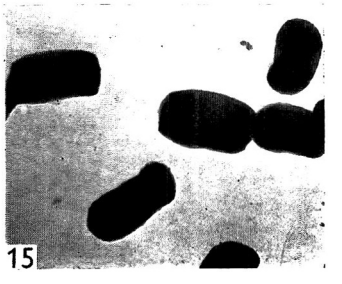
15

PLATE 2

Fig. 10. *S. fradiae*, edge of colony. × 375.

Fig. 11. *S. fradiae*, spores. × 13,500.

Fig. 12. *S. griseoflavus*, edge of colony. × 375.

Fig. 13. *S. griseoflavus*, spores. × 13,500.

Fig. 14. *S. olivaceus*, edge of colony. × 750.

Fig. 15. *S. olivaceus*, spores. × 13,500.

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# Characteristics of Marine Blue-green Algae with Uric Acid as Nitrogen Source

By C. van BAALEN and J. E. MARLER
*Institute of Marine Science, University of Texas, Port Aransas, Texas, U.S.A.*

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SUMMARY

Two types of growth response were found with several species of marine blue-green algae when uric acid was sole nitrogen source for growth. For some species the cell type produced was typical of a blue-green alga and the growth rate was like that with other nitrogen sources. The second cell type was atypical in pigmentation. The organisms had a low nitrogen and ash content and the growth rate was much impaired. The cell type seen with uric acid is considered to be probably a minimum for sustained growth of a blue-green alga. Lipid accumulation was not a characteristic of the nitrogen-deficient condition, rather a carbohydrate reserve of a polyglucose type is indicated. The growth data presented lead to the hypothesis that two different pathways for uric acid utilization may exist in blue-green algae, one probably a normal uricase type and the other a non-specific attack, possibly peroxidative.

## INTRODUCTION

Uric acid has been reported to be utilized as sole nitrogen source for growth for several algae (Droop, 1955; Miller & Fogg, 1958) and might also be an important organic nitrogen source in marine environments, particularly inshore areas. While examining the mechanism of uric acid utilization by several marine species of blue-green algae we found two distinct growth responses. Some species of blue-green algae (e.g. *Plectonema terebrans*) gave a reasonable growth rate with uric acid as compared with nitrate, and had normal pigmentation. With other species (e.g. *Agmenellum quadriplaticum*) the growth rate with uric acid was much lower than with nitrate and the organisms looked as if they were suffering severe nitrogen limitation. We have examined the growth, elementary analysis and pigmentation of the cell type this produced, which appeared to represent an unrecognized, unusual and stable physiological state for a blue-green alga.

## METHODS

The organisms used, except *Phormidium persicinum*, were those previously described by Van Baalen (1962). *P. persicinum* was obtained through the kindness of Dr L. Provasoli; we are indebted to Dr F. Drouet for identification of the cultures.

The growth medium used was ASP-2 (Provasoli, McLaughlin & Droop, 1957) with the following modifications: phosphate increased 10 times and nitrate 20 times; initial pH 8·2, S·3 and vitamin $B_{12}$ added only when needed. For the uric acid medium nitrate was omitted and 0·03% uric acid, sterilized by filtration (Morton

filter apparatus, Corning no. 33990) was added. The uric acid was dissolved by adding tris buffer and gentle heating. Allantoin and urea were also sterilized by filtration.

Growth experiments were done in large test tubes (50 × 400 mm.) incubated in illuminated, thermostatically controlled water baths designed after Myers (1950). Continuous culture of *Agmenellum quadriplaticum*, strain PR-6, was done in apparatus like that described by Myers & Clark (1944). Lighting was by one 13 W. fluorescent lamp inserted in the centre of the chamber. Incubation was at 39°. The chamber was operated manually, one-half the volume of culture was drained and replaced with fresh medium every 2 days. Carbon dioxide was supplied as 1 % (v/v) $CO_2$ in air. Other details of growth conditions are noted in Results.

Uric acid was determined by using a uricase preparation (Worthington Biochemicals, Freehold, New Jersey, U.S.A.). Dry-weight determinations were done by filtering the material on tared Millipore filters, rinsing with distilled water, and drying at 75°.

The chemical analyses of organisms were done by Huffman Microanalytical Lab., Wheatridge, Colorado, U.S.A.

The spectra of whole organisms were measured on a Carey 14 Spectrophotometer (Applied Physics Corp., Monrovia, California, U.S.A.) with Lucite plates ($\frac{1}{8}$ in., no. 7328) inserted in the cuvettes after the method of Shibata (1958). We are indebted to Dr J. Myers for the use of the plates and the Carey Spectrophotometer. Other work on spectra was done with a Beckman DU Spectrophotometer.

Cell extracts were made using a Branson Sonifier, Model LS-75 (Stamford, Connecticut, U.S.A.). Glucose was identified and determined by using 'Glucostat' (Worthington Biochemicals) and by paper chromatography.

## RESULTS

### *Growth responses to uric acid*

Table 1 gives yields of nine marine isolates of blue-green algae grown with uric acid as sole nitrogen source. Two of the organisms, *Plectonema terebrans* strain Jamaica, *Lyngbya Lagerheimii* strain Mont, grew well and gave normal pigmentation. The other organisms which grew were bleached and showed a strong yellow coloration. The growth of strain Jamaica was followed further for comparison with *Agmenellum quadriplaticum* strain PR-6. Strain Jamaica grew with allantoin, urea or nitrate as nitrogen source with yields of organism of: mg. dry weight/20 ml. medium in 72 hr. of 8·3, 9·5 and 6·9, respectively. Strain PR-6 was selected for further study; the data following will deal with it.

### *Growth of* Agmenellum quadriplaticum *strain* PR-6

Figure 1 presents the growth and uric acid utilization of *Agmenellum quadriplaticum* strain PR-6. The specific growth rate constant $k$ in $\log_{10}$ units/day with uric acid as sole nitrogen source was 0·14; with nitrate under the same conditions the value of $k$ was 2·2, and on urea 2·4. Strain PR-6 did not grow with allantoin, adenine, guanine, xanthine, hypoxanthine, uracil, 5-methyl-cytosine, cytosine or thymine as sole nitrogen source; nor when combined nitrogen was omitted from the medium. An uninoculated tube of uric acid medium prepared, incubated, and

treated in the same way as a growth run showed no evidence of uric acid decomposition. Apart from a slight increase in size of organism no microscopic differences between uric acid-grown and nitrate-grown organisms of strain PR-6 were observed.

Table 1. *The growth of certain blue-green algae with uric acid as sole nitrogen source*

| Organism (stock designation in parentheses) | Yield of organism (mg. dry weight/20 ml. medium in 11 days) | Uric acid used (mg.) |
|---|---|---|
| Incubation at 39°, 600 ft.-c., fluorescent | | |
| *Coccochloris elabens* (A-1) | 5·9 | 1·5 |
| *Microcoleus chthnoplastes* (Ba-1)* | 14·4 | 3·3 |
| *Anacystis marina* (6) | 4·6 | 2·2 |
| *Oscillatoria Williansii* (Mev)* | 20·2 | 1·8 |
| *Plectonema terebrans* (Jamaica) | 41·2 | 6·0 |
| *Agmenellum quadriplaticum* (PR-6) | 24·0 | 6·0 |
| Incubation at 30°, 400 ft.-c., fluorescent | | |
| *Lyngbya lagerheimii* (Mont) | 22·6 | 3·8 |
| *Phormidium persicinum* | 0·0 | 0·0 |
| *Plectonema terebrans* (Cal Sp 31) | 0·0 | 0·0 |

* Growth very gelatinous.

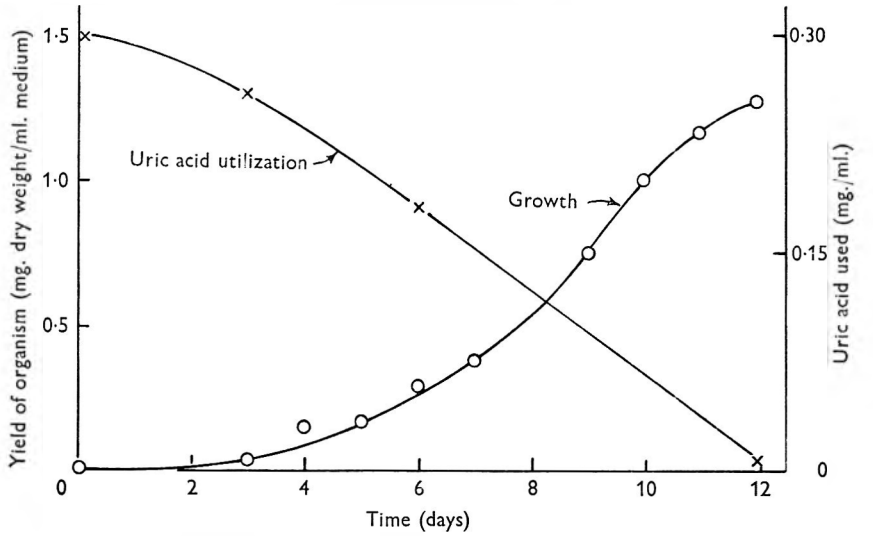


Fig. 1. Growth (○) and uric acid utilization (×) by *Agmenellum quadriplaticum* strain PR-6. Temperature 39°; 600 ft.-c., fluorescent light.

*Elementary analysis of* Agmenellum quadriplaticum *strain* PR-6 *grown on uric acid and nitrate*

Table 2 presents the results of chemical analyses on samples of washed and dried uric acid-grown and nitrate-grown organisms of *Agmenellum quadriplaticum* strain PR-6. The R-value and percentage composition of protein, carbohydrate and lipid were calculated after the method of Spoehr & Milner (1949). The heat of combustion and R-value for uric acid-grown organisms were, as expected, low as compared to nitrate-grown PR-6 organisms and as compared to Spoehr & Milner's values for

Chlorella. A striking feature of the protein, carbohydrate, lipid calculation, even though admittedly an approximation, was the high carbohydrate value of the uric acid-grown organisms. The opposite effect was seen by Spoehr & Milner, namely, with *Chlorella pyrenoidosa* (Emerson) low nitrogen produced a high lipid content.

Table 2. *Cell analyses of* Agmenellum quadriplaticum *strain* PR-6

| | Uric acid-grown organisms | | Nitrate-grown organisms | |
|---|---|---|---|---|
| Carbon (%) | 43·58 | 43·20 | 46·64 | 46·25 |
| Hydrogen (%) | 6·40 | 6·54 | 6·58 | 6·49 |
| Residue (C–H) (%) | 3·6 | 4·4 | 7·6 | 6·4 |
| Nitrogen (%) | 2·69 | 2·90 | 9·70 | 9·61 |
| R-Value | 32·8 | — | 37·3 | — |
| Protein (%) | 18 | — | 64 | — |
| Carbohydrate (%) | 74 | — | 27 | — |
| Lipid (%) | 7 | — | 9 | — |
| $\Delta H_C$ | 4·38 kcal./g | — | 5·03 kcal./g. | — |

A hot water extract of uric acid-grown PR-6 organisms gave no indication of a large soluble carbohydrate concentration. Upon hydrolysis of the residue from the hot water extract with 2N-$H_2SO_4$ for 6 hr. a strong positive test for glucose was found. Quantitative estimation showed that about 40 % of the cell dry weight could be accounted for as glucose; no other sugars were detected. Evidently a poly-glucose material is a main reserve product of uric acid-grown organisms. We have not further examined the nature of the polymer. It is clear, at least with strain PR-6 and perhaps it is a valid generalization for blue-green algae, that nitrogen deficiency does not predispose towards lipid accumulation. The same conclusion was tentatively reached by Fogg (1956) and Collyer & Fogg (1955). Calculation from the cell yield of organism in mg. dry weight shown in Fig. 1 and from the nitrogen content by analysis in Table 2 indicates that uric acid-grown strain PR-6 organisms consume on the average only one-third of the nitrogen of uric acid.

### *Pigment characteristics of* Agmenellum quadriplaticum *strain* PR-6 *on uric acid*

Figure 2 presents three types of measurements of the pigment characteristics of *Agmenellum quadriplaticum* strain PR-6 grown with uric acid. There was slight variation in the curves depending on whether the organisms were batch grown or harvested from the continuous culture chamber, due we believe to slight impurities in the uric acid which can be used as nitrogen sources. By using the calculations of Myers & Kratz (1955) we find 0·29 % chlorophyll, 0·48 % phycocyanin, and 0·5 % carotenoid, for the curves shown. There is some uncertainty about the reliability of the values for phycocyanin. We are not presently prepared to make the statement that uric acid-grown cells of PR-6 contain no phycocyanin. Other blue-green algae we have examined had still lower % chlorophyll/mg. dry-weight values and even slighter indication of any phycocyanin. The data do reflect the fact that chlorophyll is more important for survival and sustained growth under suboptimal conditions. The carotenoid value is one-half the lowest value obtained by Myers & Kratz (1955) with *Anacystis nidulans*.

*Pigment recovery*

Figure 3 shows the results of a typical pigment recovery experiment, when washed uric acid-grown *Agmenellum quadriplaticum* strain PR-6 was supplied with ammonia, the required ions, and vitamin $B_{12}$. When the organisms were suspended in 1·8 % NaCl + 0·16 M-tris buffer (pH 8·1) + 1 mg. $NH_4Cl$/ml. and incubated in the light, only a small increase of pigment was seen. By visual observation of pigment

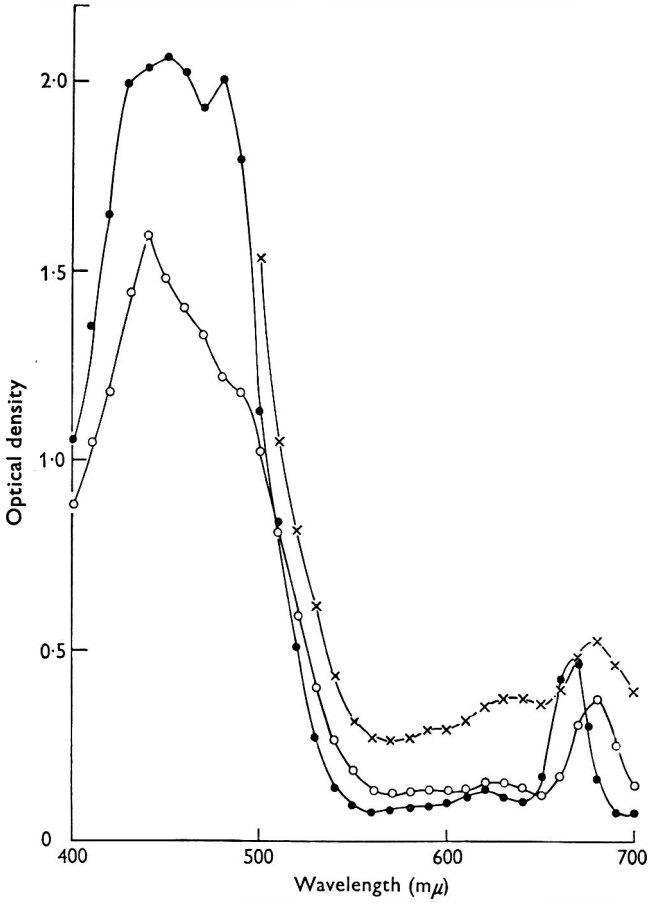


Fig. 2. Pigment curves for uric acid-grown *Agmenellum quadriplaticum* strain PR-6. Curves represent the extract of 2 mg. dry wt. organism/ml.; 1·0 cm. light path. ×, opal glass: ○, organism broken by Branson sonifier: ●, 80 % acetone. Cell source continuous culture chamber, population density at harvest, equivalent to 0·9 mg. dry wt. organism/ml.

recovery we found that, in addition to the above materials, the following ions were also essential, all added at the concentration as in medium ASP-2: $Mg^{2+}$, $SO_4^{2-}$, FeEDTA, $K^+$, $PO_4^{3-}$.

Pigment recovery did not take place when helium or air scrubbed with KOH was passed through the tubes. Recovery was normal with 1 % (v/v) $CO_2$ in nitrogen.

In the first few hours a rapid uptake of ammonia occurred and very little pigment synthesis was evident until after about 7 hr. The 2–3 hr. pigment curve characteristically showed a slight decrease in the 600–700 mμ region.

The greatest degree of recovery can be seen in the phycocyanin area of the curve. The time course for pigment recovery could be varied $\pm 30\%$ depending on the concentration of organisms and the light intensity used. It is of interest that, as shown in these recovery experiments and also in the cell analyses, uric acid-grown organisms in addition to nitrogen deficiency also had a mineral deficiency.

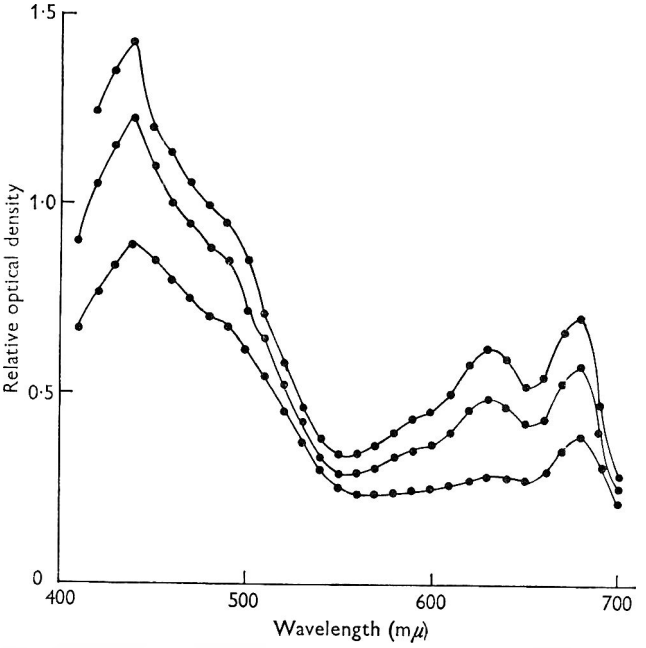


Fig. 3. Recovery curves, conditions: 33°, 400 ft.-c. tungsten. Cells from continuous culture chamber, washed and suspended in recovery medium. Curves are for whole organisms taken with opal glass, Carey 14 Spectrophotometer, *Agmenellum quadriplaticum* strain PR-6. Lower curve 0 time, middle curve after 10 hr. incubation, upper curve after 13 hr. incubation.

## DISCUSSION

The following points about the blue-green algal type seen here when uric acid is sole nitrogen source may be made. As quoted by Fritsch (1952) nitrogen chlorosis of blue-green algae in natural conditions is commonly seen, the implication being that growth is not occurring. The data presented here show that nitrogen deficiency results in a stable physiological state and that continued and reproducible growth can be maintained under these conditions experimentally and probably under natural conditions. It seems probable that uric acid-grown *Agmenellum quadriplaticum* strain PR-6 represents a minimum condition for growth in a blue-green alga. We do not consider that the low growth rate and extreme nitrogen deficiency manifested by strain PR-6 while growing on uric acid is directly related to uric acid but rather to the very limited ability of the organisms to degrade it to a useful nitrogen source. We have searched for other nitrogen sources that would produce a similar effect but have found none. The possibility that uric acid or a product derived from its breakdown is acting as an inhibitor of some stage in nitrogen assimilation seems remote. We have found no evidence that uric acid, allantoin,

alloxanic acid, parabanic acid or oxonic acid (Brandenberger, 1954) affect strain PR-6 when nitrate is the nitrogen source.

A comparison of the growth data for *Agmenellum quadriplaticum* strain PR-6 and the *Plectonema terebrans* strain Jamaica growing on uric acid leads to the hypothesis that there are two different pathways for uric acid breakdown. We are tempted to ascribe to *P. terebrans* strain Jamaica and to *Lyngbya Lagerheimii* strain Mont a normal uricase, uric acid → allantoin → urea system. For *A. quadriplaticum* strain PR-6 and organisms which behave similarly, a non-specific peroxidase activity is a possible explanation (Canellakis, Tuttle & Cohen, 1954).

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# Index of Authors

*Entries marked with an asterisk (*) are those for which only titles are given and of which there are no summaries*
*Small roman numerals refer to pages in the* Proceedings of the Society for General Microbiology


ABBOTT, P. McV., *see* HAWKER, L. E. 295
ADA, G. L., LIND, P. E. & LAVER, W. G. An immunological study of avian, viral and bacterial neuraminidase based on specific inhibition of enzyme by antibody 225
ALEXANDER, M., *see* MACRAE, I. C. 69
*ARBER, W. Bacteriophage lysogeny. *Symp. Soc. gen. Microbiol.* 13, 12

BAALEN, C. VAN & MARLER, J. E. Characteristics of marine blue-green algae with uric acid as nitrogen source 457
BABOS, P. & KASSANIS, B. Serological relationships and some properties of tobacco necrosis virus strains 135
BACQ, C.-M. & HORNE, R. W. Morphology of actinophage $\phi$17 131
BADDILEY, J., *see* DAVISON, A. L. 271
*BAKER, J. M. Ambrosia beetles and their fungi, with particular reference to *Platypus cylindrus* Fab. *Symp. Soc. gen. Microbiol.* 13, 232
BARROW, G. I. The nature of inhibitory activity by *Staphylococcus aureus* type 71 255
BELYAVIN, G. The mechanism of influenza virus haemagglutination as a model of specific receptor adsorption 15
BERNSTEIN, A. & WILSON, E. M. J. An analysis of the vi-phage typing scheme for *Salmonella typhi* 349
BERTAUD, W. S., MORICE, I. M., RUSSELL, D. W. & TAYLOR, A. The spore surface in *Pithomyces chartarum* 385
*BOND, G. The root nodules of non-leguminous angiosperms. *Symp. Soc. gen. Microbiol.* 13, 72
BRAMMAR, W. J. & CLARKE, P. H. Induction and repression of an enzyme in *Pseudomonas aeruginosa* iii
*BROOKS, M. A. Symbiosis and aposymbiosis in arthropods. *Symp. Soc. gen. Microbiol.* 13, 200
BRUMFITT, W., *see* PERCIVAL, A. 77
BRYANT, M. P., *see* HALLIWELL, G. 441
BUCKLAND, F. E. & TYRRELL, D. A. J. A comparative study of virus haemagglutinins. The stability of haemagglutinins and red cell receptors to certain physical and chemical treatments 241
BURNHAM, B. F. Investigations on the action of the iron-containing growth factors, siderамines; and iron-containing antibiotics, sideromycins 117

CENTIFANTO, Y. M. & SILVER, W. S. Some aspects of the leaf-nodule symbiosis of *Psychotria* v
CLARKE, P. H., *see* BRAMMAR, W. J. iii
CLARKE, P. H., *see* LILLY, M. D. 103
CLAYTON, Y. M., *see* NOBLE, W. C. 397
COCITO, C., LADURON, P. & DE SOMER, P. Nucleotide composition of RNA-viruses iii
COETZEE, J. N., *see* DE KLERK, H. C. 61
*COLEMAN, G. S. The growth and metabolism of rumen ciliate protozoa. *Symp. Soc. gen. Microbiol.* 13, 298
CORBAZ, R., GREGORY, P. H. & LACEY, M. E. Thermophilic and mesophilic actinomycetes in mouldy hay 449
CURBY, W. A., SWANTON, E. M. & LIND, H. E. Electrical counting characteristics of several equivolume micro-organisms 33

DAVISON, A. L. & BADDILEY, J. The distribution of teichoic acids in staphylococci 271
DAWES, E. A. Comparative aspects of alcohol formation. 151
DENYS, P. (JUN), LEYTEN. R., VANDEPUTTE, M. & DE SOMER, P. Viral infections in thymectomized animals ii
DINTER, Z., *see* PHILIPSON, L. 277
*DROOP, M. R. Algae and invertebrates in symbiosis. *Symp. Soc. gen. Microbiol.* 13, 171
*DUBOS, R. & KESSLER, A. Integrative and disintegrative factors in symbiotic associations. *Symp. Soc. gen. Microbiol.* 13, 1.

ECHLIN, P. The fine structure of the cell boundary and photosynthetic lamellae of *Anacystis montana* (Lightfoot) f. minor (Wille). Drouet and Daily iv
EVANS, W. C. The microbiological degradation of aromatic compounds 177

FLOOD, A. E., *see* KIRKHAM, D. S. 123
FOLLETT, E. A. C. & GORDON, J. An electron microscope study of vibrio flagella 235

GEZON, H. M., *see* YEE, R. B. 299
GORDON, J., *see* FOLLETT, E. A. C. 235
GREGORY, P. H., *see* CORBAZ, R. 449

HALLIWELL, G. & BRYANT, M. P. The cellulolytic activity of pure strains of bacteria from the rumen of cattle 441
HARRISON, B. D. & PIERPOINT, W. S. The relation of polyphenoloxidase in leaf extract to the instability of cucumber mosaic and other plant viruses 417
HARRISON, B. D., *see* PIERPOINT, W. S. 429
HART, P. D. & VALENTINE, R. C. Growth (without multiplication) of *Mycobacterium lepraemurium* in cell-free medium 43
HAWKER, L. E. & ABBOTT, P. McV. An electron microscope study of maturation and germination of sporangiospores of two species of Rhizopus 295
HOARE, D. S. The breakdown and biosynthesis of glutamic acid. 157

HOŘAK, K., *see* ROSYPAL, S. 189
HORNE, R. W., *see* BACQ, C.-M. 131
TEN HOUTEN, J. G. Obituary Notice Johanna Westerdijk, 1883–1961 1
HSU, K. S. The genetic basis of actidione resistance in Neurospora 341
HUMPHREY, J. H. Some biologically important aspects of adsorption of antibody on to tissue cells 25
*HUNGATE, R. E. Symbiotic associations: The rumen bacteria. *Symp. Soc. gen. Microbiol.* **13**, 266

KASSANIS, B., *see* BABOS, P. 135
KERR, N. S. The growth of myxamoebae of the true slime mould, *Didymium nigripes*, in axenic culture 409
*KESSLER, A., *see* DUBOS, R. *Symp. Soc. gen. Microbiol.* **13**, 1.
KIRKHAM, D. S. & FLOOD, A. E. Some effects of respiration inhibitors and *o*-coumaric acid on the inhibition of sporulation in *Venturia inaequalis* 123
DE KLERK, H. C., COETZEE, J. N. & THERON, J. J. The characterization of a series of Lactobacillus bacteriophages 61
KOCUR, M. & MARTINEC, T. The classification of some violet-prigmented micrococci 185
KOCUR, M., *see* ROSYPAL, S. 189
KRICHEVSKY, M. I. & WRIGHT, B. E. Environmental control of the course of development in *Dictyostelium discoideum* 195

LACEY, M. E., *see* CORBAZ, R. 449
LADURON, P., *see* COCITO, C. iii
LAVER, W. G., *see* ADA, G. L. 225
LENHOFF, H. M., *see* MUSCATINE, L. vi
*LEV, M. Studies on bacterial associations in germ-free animals and animals with defined floras. *Symp. Soc. gen. Microbiol.* **13**, 325
LEYTEN, R., *see* DENYS, P. (JUN) ii
LIE, T. A. Influence of root secretions on the symbiotic association of rhizobium and leguminous plants v
LILLY, M. D., CLARKE, P. H. & MEADOW, P. M. The accumulation of Nucleotides by *Escherichia coli* strain 26–26 103
LIND, H. E., *see* CURBY, W. A. 33
LIND, P. E., *see* ADA, G. L. 225
LION, M. B. Quantitative aspects of the protection of freeze-dried *Escherichia coli* against the toxic effect of oxygen 321
DE LOUVOIS, J., *see* PERCIVAL, A. 77

MACRAE, I. C., ALEXANDER, M. & ROVIRA, A. D. The decomposition of 4-(2,4-Dichlorophenoxy)butyric acid by *Flavobacterium* sp. 69
MAHY, B. W. J., PARR, C. W. & ROWSON, K. E. K. Plasma enzyme levels in mice infected with various viruses i
MARLER, J. E., *see* BAALEN, C. VAN 457
MARR, A. G., *see* SHIFRINE, M. 263
MARTINEC, T., *see* KOCUR, M. 185
MEADOW, P. M., *see* LILLY, M. D. 103
*MELIN, J. B. E. Some effects of forest tree roots on mycorrhizal basidiomycetes. *Symp. Soc. gen. Microbiol.* **13**, 125
MENDOZA, C. G. & VILLANUEVA, J. R. Electron microscopy of membrane prepared from *Candida utilis* i
MEYNELL, E. W. Reverting and non-reverting rough variants of *Bacillus anthracis* 55
MORICE, I. M., *see* BERTAUD, W. S. 385
MORRIS, J. G. The assimilation of 2-C compounds other than acetate 167
*MOSSE, B. Vesicular-arbuscular mycorrhiza: an extreme form of fungal adaptation. *Symp. Soc. gen. Microbiol.* **13**, 146
MUSCATINE, L. & LENHOFF, H. M. Symbiosis of hydra with algae vi

NEWTON, B. A. A bacterial endosymbiote in an osmotrophic flagellate? vi
*NICHOLAS, D. J. D. The biochemistry of nitrogen fixation. *Symp. Soc. gen. Microbiol.* **13**, 92
NOBLE, W. C. & CLAYTON, Y. M. Fungi in the air of hospital wards 397
*NÜESCH, J. Defence reactions in orchid bulbs. *Symp. Soc. gen. Microbiol.* **13**, 335
*NUTMAN, P. S. Factors influencing the balance of mutual advantage in legume symbiosis. *Symp. Soc. gen. Microbiol.* **13**, 51

OVERMAN, J. R., *see* PINE, L. 209

PARR, C. W., *see* MAHY, B. W. J. i
PERCIVAL, A., BRUMFITT, W. & DE LOUVOIS, J. The role of penicillinase in determining natural and acquired resistance of gram-negative bacteria to penicillins 77
PHILIPSON, L. & DINTER, Z. The role of interferon in persistent infection with foot-and-mouth disease virus 277
PIERPOINT, W. S. & HARRISON, B. D. Copper-dependent and iron-dependent inactivations of cucumber mosaic virus by polyphenols 429
PIERPOINT, W. S., *see* HARRISON, B. D. 417
PINE, L. & OVERMAN, J. R. Determination of the structure and composition of the 'sulphur granules' of *Actinomyces bovis* 209
PRETORIUS, W. A. A systematic study of the genus Spirillum which occurs in oxidation ponds, with a description of a new species 403

QUAYLE, J. R. The assimilation of 1-C compounds 163

REITER, B. & ORAM, J. D. Group N streptococcal phage lysin 29
RODWELL, A. W. The steroid growth-requirement of *Mycoplasma mycoides* 91
ROGERS, H. J. The bacterial cell wall. The result of adsorption, structure or selective permeability? 19
ROSYPAL, S., KOCUR, M. & HOŘAK, K. A study of violet-pigmented micrococci. Yellow-pigmented mutants of *Staphylococcus flavocyaneus* 189
ROVIRA, A. D., *see* MACRAE, I. C. 69
ROWSON, K. E. K., *see* MAHY, B. W. J. i
RUINEN, J. Cuticle decomposition by microorganisms in the phyllosphere iv
RUSSELL, D. W., *see* BERTAUD, W. S. 385

SHIFRINE, M. & MARR, A. G. The requirement of fatty acids by *Pityrosporum ovale* 263
SILVER, W. S., *see* CENTIFANTO, Y. M. v

*Smith, D. C. Experimental studies of lichen physiology. *Symp. Soc. gen. Microbiol.* **13**, 31
Smith, P. F. The carotenoid pigments of mycoplasma 307
de Somer, P., *see* Cocito, C. iii
de Somer, P., *see* Denys, P. (Jun). ii
Swanton, E. M., *see* Curby, W. A. 33

Taylor, A., *see* Bertaud, W. S. 385
Theron, J. J., *see* de Klerk, H. C. 61
Tucker, R. G. Effect of metal ions and polyamines on the development of bacteriophage ØR 287
Tyrrell, D. A. J., *see* Buckland, F. E. 241

Valentine, R. C., *see* Hart, P. D. 43
Vandeputte, M., *see* Denys, P. (Jun). ii
Villanueva, J. R., *see* Mendoza, C. G. i

Weiss, L. Some sorption phenomena at mammalian cell surfaces 11
Weiss, L. The pH value at the surface of *Bacillus subtilis* 331
Weitz, B. The specificity of trypanosomal antigens by immunofluorescence 145
Whittenbury, R. The use of soft agar in the study of conditions affecting the utilization of fermentable substrates by lactic acid bacteria 375
Wilkinson, J. F. Carbon and energy storage in bacteria 171
Wilson, E. M. J., *see* Bernstein, A. 349
Wright, B. E., *see* Krichevsky, M. I. 195

Yee, R. B. & Gezon, H. M. Ribonucleic acid of chloramphenicol-treated *Shigella flexneri* 299

# Index of Subjects

*Small roman numerals refer to pages in the* Proceedings of the Society for General Microbiology


Actidione resistance in Neurospora (Hsu) 341
*Actinomyces bovis* 'sulphur granules', structure and composition of (Pine & Overman) 209
Actinomycetes in mouldy hay (Corbaz, Gregory & Lacey) 449
Actinophage $\phi$17, morphology of (Bacq & Horne) 131
Adsorption, cause of bacterial cell wall (Rogers) 19
Adsorption, haemagglutination as model of specific receptor (Belyavin) 15
Adsorption of antibody on to tissue cells (Humphrey) 25
Aerospora of hospital wards (Noble & Clayton) 397
Alcohol formation, comparative aspects of (Dawes) 151
Algae and invertebrates in symbiosis (Droop). *Symp. Soc. gen. Microbiol.* **13**, 171
Algae, blue-green, characteristics of, with uric acid as nitrogen source (van Baalen & Marler) 457
Algae, symbiosis of hydra with (Muscatine & Lenhoff) vi
Ambrosia beetles and their fungi (Baker). *Symp. Soc. gen. Microbiol.* **13**, 232
*Anacystis montana*, fine structure of cell boundary and photosynthetic lameliae of (Echlin) iv
Angiosperms, root nodules of non-leguminous, (Bond). *Symp. Soc. gen. Microbiol.* **13**, 72
Antibiotic, produced by *Staphylococcus aureus* type 71 (Barrow) 255
Antibiotics, iron-containing sideromycins (Burnham) 117
Antibody, adsorption on to tissue cells (Humphrey) 25
Antibody, enzyme neuraminidase inhibited by (Ada, Lind & Laver) 225
Antigens, trypanosomal, by immunofluorescence (Weitz) 145
Aposymbiosis and symbiosis in arthropods (Brooks). *Symp. Soc. gen. Microbiol.* **13**, 200
Apparatus, Coulter counter for micro-organisms (Curby, Swanton & Lind) 33
Aromatic compounds, microbiological degradation of (Evans) 177

*Bacillus anthracis*, reverting and non-reverting rough variants of (Meynell) 55
*Bacillus subtilis*, pH value at the surface of (Weiss) 331
Bacteriophage lysogeny (Arber). *Symp. Soc. gen. Microbiol.* **13**, 12
Bacteriophage ØR, effect of metal ions and polyamines on development of (Tucker) 287
Bacteriophage, Vi typing scheme for *Salmonella typhi* (Bernstein & Wilson) 349
Bacteriophages, lactobacillus, characterization of (de Klerk, Coetzee & Theron) 61
Biochemistry of nitrogen fixation (Nicholas). *Symp. Soc. gen. Microbiol.* **13**, 92

*Candida utilis*, electron microscopy of membrane from (Mendoza & Villanueva) i
Carbon and energy storage in bacteria (Wilkinson) 171
Carotenoid pigments of mycoplasma (Smith) 307
Cations, effect on development of Bacteriophage ØR (Tucker) 287
Cell boundary, fine structure of, of *Anacystis montana* (Echlin) iv
Cell receptors, stability of virus haemagglutinins and (Buckland & Tyrrell) 241
Cell surfaces, some sorption phenomena at mammalian (Weiss) 11
Cell wall, bacterial. Result of adsorption, structure or selective permeability? (Rogers) 19
Cells, tissue, adsorption of antibody on to (Humphrey) 25
Cellulose breakdown by bacteria in rumen of cattle (Halliwell & Bryant) 441
Chloramphenicol-treated *Shigella flexneri*, ribonucleic acid of (Yee & Gezon) 299
Classification of violet-pigmented micrococci (Kocur & Martinec) 185
Composition of 'sulphur granules' of *Actinomyces bovis* (Pine & Overman) 209
1-C Compounds, the assimilation of (Quayle) 163
2-C compounds other than acetate, the assimilation of (Morris) 167
Copper-dependent inactivations of cucumber mosaic virus by polyphenols (Pierpoint & Harrison 429
*o*-Coumaric acid, effects on inhibition of sporulation in *Venturia inaequalis* (Kirkham & Flood) 123
Counts, electrical, characteristics of equivolume micro-organisms (Curby, Swanton & Lind) 33
Cucumber mosaic virus, relation of polyphenoloxidase to instability (Harrison & Pierpoint) 417
Culture, axenic, of *Didymium nigripes* (Kerr) 409

Decomposition of dichlorophenoxybutyrate (McRae, Alexander & Rovira) 69
Dichlorophenoxybutyrate, decomposition by Flavobacterium (MacRae, Alexander & Rovira) 69
*Dictyostelium discoideum*, environmental control of development in (Krichevsky & Wright) 195
*Didymium nigripes*, growth of myxamoebae in axenic culture (Kerr) 409
Distribution of teichoic acid in staphylococci (Davison & Baddiley) 271

Endosymbiote, bacterial, in osmotrophic flagellate (Newton) vi

Environment, control of development in *Dictyostelium discoideum* (Krichevsky & Wright) 195
Enzyme in *Pseudomonas aeruginosa*, induction and repression of (Brammar & Clarke) iii
Enzyme levels in mice infected with viruses (Mahy, Parr & Rowson) i
Enzyme, neuraminidase, inhibition by antibody (Ada, Lind & Laver) 225
*Escherichia coli*, freeze-dried, protection against effect of oxygen (Lion) 321
*Escherichia coli* strain 26-26, accumulation of nucleotides (Lilly, Clarke & Meadow) 103

Fatty acid requirement by *Pityrosporum ovale* (Shifrine & Marr) 263
Flagella, electron microscope study of vibrio (Follett & Gordon) 235
Flagellate, osmotrophic, a bacterial endosymbiote in an (Newton) vi
Flavobacterium sp., decomposition of dichlorophenoxybutyrate by (MacRae, Alexander & Rovira) 69
Floras, defined, studies on bacterial associations in animals with (Lev). *Symp. Soc. gen. Microbiol.* **13**, 325
Fungi in the air of hospital wards (Noble & Clayton) 397

Genetics, basis of actidione resistance in Neurospora (Hsu) 341
Genus Spirillum, description of a new species (Pretorius) 403
Germ-free animals and animals with defined flora (Lev). *Symp. Soc. gen. Microbiol.* **13**, 325
Glutamic acid, breakdown and biosynthesis (Hoare) 157
Growth and metabolism of rumen ciliate protozoa (Coleman) *Symp. Soc. gen. Microbiol.* **13**, 298
Growth factors, iron-containing, and antibiotics (Burnham) 117
Growth of lactic acid bacteria in soft agar (Whittenbury) 375
Growth of *Mycobacterium lepraemurium* in cell-free medium (Hart & Valentine) 43
Growth of myxamoebae of *Didymium nigripes* in axenic culture (Kerr) 409
Growth, steroid, requirement of *Mycoplasma mycoides* (Rodwell) 91

Haemagglutination model of specific receptor adsorption (Belyavin) 15
Hay, mouldy, thermophilic and mesophilic actinomycetes in (Corbaz, Gregory & Lacey) 449

Infection with foot-and-mouth disease virus, interferon in (Philipson & Dinter) 277
Inhibition by *Staphylococcus aureus* Type 71 (Barrow) 255
Inhibition of enzyme by antibody (Ada, Lind & Laver) 225
Inhibition of sporulation in *Venturia inaequalis* (Kirkham & Flood) 123
Interferon in infection with foot-and-mouth disease virus (Philipson & Dinter) 277
Iron-containing growth factors and antibiotics (Burnham) 117
Iron-dependent inactivation of cucumber mosaic virus by polyphenols (Pierpoint & Harrison) 429

Lactic acid bacteria in soft agar (Whittenbury) 375
Lactobacillus bacteriophages, characterization of (de Klerk, Coetzee & Theron) 61
Lamellae, photosynthetic, of *Anacystis montana* (Echlin) iv
Lyophilization and $O_2$ effect in *E. coli* (Lion) 321
Lysogeny, bacteriophage (Arber) *Symp. Soc. gen. Microbiol.* **13**, 12

Medium, cell-free for *Mycobacterium lepraemurium* (Hart & Valentine) 43
Methods, electrical counting of micro-organisms (Curby, Swanton & Lind) 33
Micrococci, violet-pigmented, classification of (Kocur & Martinec) 185
Micrococci, violet-pigmented, yellow mutants (Rosypal, Kocur & Hodak) 189
Micro-organisms, cuticle decomposition by, in the phyllosphere (Ruinen) iv
Microscope, electron, study of structural changes in Rhizopus spores (Hawker & Abbott) 295
Microscope, electron, vibrio flagella (Follett & Gordon) 235
Microscopy, electron of membrane prepared from *Candida utilis* (Mendoza & Villanueva) i
Morphology of actinophage Ø17 (Bacq & Horne) 131
Mutants of *Staphylococcus flavocyaneus*, yellow-pigmented (Rosypal, Kocur & Hodak) 189
*Mycobacterium lepraemurium* growth in cell-free medium (Hart & Valentine) 43
Mycoplasma, carotenoid pigments (Smith) 307
*Mycoplasma mycoides*, steroid growth-requirement (Rodwell) 91
Mycorrhizal basidiomycetes, some effects of tree roots (Melin). *Symp. Soc. gen. Microbiol.* **13**, 125

Nitrogen fixation, biochemistry of (Nicholas). *Symp. Soc. gen. Microbiol.* **13**, 92
Nitrogen source, characteristics of blue-green algae with uric acid as (van Baalen & Marler) 457
Nucleotide composition of RNA-viruses (Cocito, Laduron & de Somer) iii
Nucleotides, accumulation by *Escherichia coli* strain 26-26 (Lilly, Clarke & Meadow) 103
Neuraminidase, avian, viral and bacterial, inhibition by antibody (Ada, Lind & Laver) 225
Neurospora, genetic basis of actidione resistance in (Hsu) 341

Obituary notice: Johanna Westerdijk, 1883–1961 (ten Houten) 1
Orchid bulbs, defence reactions in (Nüesch). *Symp. Soc. gen. Microbiol.* **13**, 335
Oxidation ponds, containing the genus Spirillum (Pretorius) 403
Oxygen, protection of freeze-dried *Escherichia coli* against effect of (Lion) 321

Penicillinase in Gram-negative bacteria (Percival, Brumfitt & de Louvois) 77

Permeability, selective, forming bacterial cell wall (Rogers) 19
pH value at surface of *Bacillus subtilis* (Weiss) 331
Phage lysin, group N streptococcal (Reiter & Oram) 29
Phyllosphere, cuticle decomposition by micro-organisms in (Ruinen) iv
Physiology of lichen (Smith). *Symp. Soc. gen. Microbiol.* **13**, 31
Pigments, carotenoid, of mycoplasma (Smith) 307
*Pithomyces chartarum*, spore surface in (Bertaud Morice, Russell & Taylor) 385
*Pityrosporum ovale*, fatty acid requirement of (Shifrine & Marr) 263
*Platypus cylindrus* Fab.; ambrosia beetles and their fungi (Baker). *Symp. Soc. gen. Microbiol.* **13**, 232
Polyamines, effect on development of Bacteriophage ØR (Tucker) 287
Polyphenol inactivation of cucumber mosaic virus (Pierpoint & Harrison) 429
Polyphenoloxidase, in leaf extracts, relation to the instability of cucumber mosaic (Harrison & Pierpoint) 417
Protozoa, rumen ciliate, growth and metabolism (Coleman). *Symp. Soc. gen. Microbiol.* **13**, 298
*Pseudomonas aeruginosa*, induction and repression of an enzyme in (Brammar & Clarke) iii
*Psychotria*, leaf-nodule symbiosis of (Centifanto & Silver) v

Resistance of Gram-negative bacteria to penicillins (Percival, Brumfitt & de Louvois) 77
Respiration inhibitors of sporulation in *Venturia inaequalis* (Kirkham & Flood) 123
Rhizobium and leguminous plants, effect of root secretions on (Lie) v
Rhizopus spores, structural changes in (Hawker & Abbott) 295
Ribonucleic acid of chloramphenicol-treated *Shigella flexneri* (Yee & Gezon) 299
Root nodules of non-leguminous angiosperms (Bond) *Symp. Soc. gen. Microbiol.* **13**, 72
Root secretions, effect on symbiosis of rhizobium and leguminous plants (Lie) v
Roots, tree, effects on mycorrhizal basidiomycetes (Melin) *Symp. Soc. gen. Microbiol.* **13**, 125
Rumen bacteria, symbiotic associations (Hungate). *Symp. Soc. gen. Microbiol.* **13**, 266
Rumen of cattle, cellulolytic activity of bacteria from (Halliwell & Bryant) 441

*Salmonella typhi*, analysis of the Vi-phage typing scheme for (Bernstein & Wilson) 349
Serology of lactobacillus phages (de Klerk, Coetzee & Theron) 61
Serology of tobacco necrosis virus strains (Babos & Kassanis) 135
Serology of trypanosomal antigens by immunofluorescence (Weitz) 145
*Shigella flexneri*, chloramphenicol-treated, ribonucleic acid of, (Yee & Gezon) 299
Sideramines and sideromycins (Burnham) 117
Sorption phenomena at mammalian cell surfaces (Weiss) 11
Spirillum in oxidation ponds, (Pretorius) 403
Spore surface in *Pithomyces chartarum* (Bertaud, Morice, Russell & Taylor) 385
Stability of virus haemagglutinins and cell receptors (Buckland & Tyrrell) 241
Staphylococci, teichoic acid distribution in (Davison & Baddiley) 271
*Staphylococcus aureus* type 71, inhibition by (Barrow) 255
*Staphylococcus flavocyaneus*, yellow-pigmented mutants of (Rosypal, Kocur & Hodak) 189
Steroids, growth requirement of *Mycoplasma mycoides* (Rodwell) 91
Streptococcal, group N, phage lysin (Reiter & Oram) 29
Structural changes in Rhizopus spores (Hawker & Abbott) 295
Structure of the 'sulphur granules' of *Actinomyces bovis* (Pine & Overman) 209
Substrates, fermentable, conditions affecting utilization by lactic acid bacteria (Whittenbury) 375
'Sulphur granules' of *Actinomyces bovis*, structure and composition of (Pine & Overman) 209
Surface pH of *Bacillus subtilis* (Weiss) 331
Symbiosis, algae and invertebrates in (Droop). *Symp. Soc. gen. Microbiol.* **13**, 171
Symbiosis and aposymbiosis in arthropods (Brooks) *Symp. Soc. gen. Microbiol.* **13**, 200.
Symbiosis, leaf-nodule, of *Psychotria* (Centifanto & Silver) v
Symbiosis, legume, factors of mutual advantage (Nutman). *Symp. Soc. gen. Microbiol.* **13**, 51
Symbiosis of hydra with algae (Muscatine & Lenhoff) vi
Symbiotic associations, integrative and disintegrative factors (Dubos & Kessler). *Symp. Soc. gen. Microbiol.* **13**, 1
Symbiotic associations: rumen bacteria (Hungate). *Symp. Soc. gen. Microbiol.* **13**, 266
Symposium on aspects of cell-surface adsorption phenomena: Some sorption phenomena at mammalian cell surfaces (Weiss); The mechanism of influenza virus haemagglutination as a model of specific receptor adsorption (Belyavin); The bacterial cell wall. The result of adsorption, structure or selective permeability? (Rogers); Some biologically important aspects of adsorption of antibody on to tissue cells (Humphrey); Group N streptococcus phage lysin (Reiter & Oram) 11
Symposium on comparative aspects of intermediary metabolism in micro-organisms: Comparative aspects of alcohol formation (Dawes); The breakdown and biosynthesis of glutamic acid (Hoare); The assimilation of 1-C compounds (Qualye); The assimilation of 2-C compounds other than acetate (Morris); Carbon and energy storage in bacteria (Wilkinson); The microbiological degradation of aromatic compounds (Evans) 151

Teichoic acid distribution in staphylococci (Davison & Baddiley) 271
Thymectomy, viral infections in animals after, (Denys, Leyten, Vandeputte & De Somer) ii

Tobacco necrosis virus strains, serology of (Babos & Kassanis) 135
Trypanosomal antigens by immunofluorescence (Weitz) 145

Uric acid and blue-green algae (van Baalen & Marler) 457

Variants, rough, of *Bacillus anthracis* (Meynell) 55
*Venturia inaequalis*, inhibition of sporulation (Kirkham & Flood) 123
Vesicular-arbuscular mycorrhiza: extreme form of fungal adaptation (Mosse) *Symp. Soc. gen. Microbiol.* **13**, 146
Vibrio flagella, electron microscope study (Follett & Gordon) 235
Vi-phage typing scheme for *Salmonella typhi*, (Bernstein & Wilson) 349
Virus, cucumber mosaic, inactivation by polyphenols and metals (Pierpoint & Harrison) 429
Virus, cucumber mosaic, instability and polyphenoloxidase (Harrison & Pierpoint) 417
Virus, enzyme levels in mice infected with (Mahy, Parr & Rowson) i
Virus, foot-and-mouth disease, interferon in infection with (Philipson & Dinter) 277
Virus haemagglutinins and cell receptors, stability of (Buckland & Tyrrell) 241
Virus, infections in thymectomized animals (Denys, Leyten, Vandeputte & De Somer) ii
Virus, influenza, haemagglutination a model of specific receptor adsorption (Belyavin) 15
Virus, tobacco necrosis, serology of (Babos & Kassanis) 135
Viruses, RNA, nucleotide composition of (Cocito, Laduron & De Somer) iii