THE JOURNAL OF
GENERAL MICROBIOLOGY

EDITED FOR

THE SOCIETY FOR GENERAL MICROBIOLOGY

VOLUME 61, 1970

CAMBRIDGE
AT THE UNIVERSITY PRESS
1970

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Part I *issued* April 1970

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Part 2 *issued* May 1970

Part 3 *issued* June 1970

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THE
JOURNAL OF GENERAL MICROBIOLOGY

THE PREPARATION OF PAPERS

'The more words there are, the more words there are about which doubts may be entertained.'

JEREMY BENTHAM (1748–1832)

'What can be said at all can be said clearly; and whereof one cannot speak thereof one must be silent.'

LUDWIG WITTGENSTEIN: *Tractatus Logico-philosophicus* (tr. C. K. Ogden)

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

(1) Papers should be written with the utmost conciseness consistent with clarity. The best English for the purpose of the *Journal* is that which gives the sense in the fewest short words, spelt according to the *Shorter Oxford English Dictionary*.

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

(3) Authors should state the objective when the work was undertaken, how they did it and the conclusions they draw. A section labelled 'Discussion' should be strictly limited to discussing the results if this is necessary, and not to recapitulation.

(4) Figures and tables should be selected to illustrate the points made if they cannot be described in the text, to summarize, or to record important quantitative results.

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the bibliography by the letters *a*, *b*, etc., following the citation of the year (e.g. 1914*a*, 1914*b*, or 1914*a*, *b*).

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

Description of Solutions. The concentrations of solutions are preferably defined in terms of normality (N) or molarity (M). The term '%' must be used in its correct sense, i.e. g./100 g. of solution; otherwise '%(v/v)' and '%(w/v)' must be used when the figure is larger than 1 %.

Proprietary Substances and Materials. At first mention, the correct designation and the manufacturer's address should be given in the text.

Chemical Nomenclature. Follow the 1969 revision of *Policy of the Journal and Instructions to Authors*, The Biochemical Society, 7 Warwick Court, London, W.C. 1.

Nomenclature of Enzymes. The system published in *Report of the Commission of Enzymes of the International Union of Biochemistry*, Oxford: Pergamon Press, 50*s.*, is used.

Nomenclature in Bacterial Genetics. The proposal by M. Demerec, E. A. Adelberg, A. J. Clark and P. E. Hartman published in *Genetics* (1966), 54, 61 has been reprinted in the *Journal* (1968), 50, 1 as a useful guide to authors. The general principles laid down should be followed wherever practicable.

Nomenclature and Description of Micro-organisms. The correct name of the organisms, conforming with international rules of nomenclature, must be used; if desired, synonyms may be added in brackets when the name is first mentioned. Names of bacteria must conform with the Bacteriological Code and the opinions issued by the International Committee on Bacteriological Nomenclature. Names of algae and fungi must conform with the International Rules of Botanical Nomenclature. Names of protozoa must conform with the International Code of Zoological Nomenclature. Bacteriological Code, Iowa State College Press, Ames, Iowa, U.S.A. (1958); Botanical Code, International Bureau of Plant Taxonomy and Nomenclature, 106 Lange Nieuwstraat, Utrecht, Netherlands (1952); Zoological Code, International Trust for Zoological Nomenclature, London (1961). One or two small changes have been made to these rules at later International Congresses.

The following books may be found useful:

Bergey's Manual of Determinative Bacteriology, 7th ed. (1957), edited by R. S. Breed, E. G. D. Murray and A. P. Hitchens. London: Ballière, Tindall and Cox.

V. B. D. Skerman, *A Guide to the Identification of the Genera of Bacteria with Methods and Digest of Genetic Characteristics* (1959). Baltimore, Maryland, U.S.A.: The Williams and Wilkins Company.

S. T. Cowan, *A Dictionary of Microbial Taxonomic Usage* (1968). Edinburgh: Oliver and Boyd.

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

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

I

Mechanism of Propionate Formation by *Selenomonas ruminantium*, a Rumen Micro-organism

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SUMMARY

The mechanism of propionate formation by two strains of *Selenomonas ruminantium* has been investigated using substrates specifically labelled by ^{14}C. Both strains behaved similarly. When [2-^{14}C]lactate was fermented, the label in propionate was completely randomized in carbons 2 and 3. When cells were grown on lactate in the presence of $^{14}CO_2$, label was fixed exclusively into propionate carboxyl. The results are consistent with propionate being formed by the 'succinate' pathway.

INTRODUCTION

Selenomonas ruminantium was first isolated by Bryant (1956) from rumen contents of cattle which had been fed on a ration high in soluble carbohydrates. The bacterium, which was a significant member of the microbial population, fermented glucose, with the formation of lactate, acetate and propionate. Some strains also fermented lactate to yield acetate and propionate. Thus, under certain conditions, *S. ruminantium* probably plays an important role in the production of propionate, the only major rumen fermentation product known to be glycogenic. For these reasons the mechanism of propionate formation in *S. ruminantium* was studied.

At present the 'succinate' and 'direct reduction' pathways are the two known mechanisms for fermentation of lactate to propionate. The 'succinate' pathway involves heterotrophic fixation of CO_2 to form succinate (Wood & Werkman, 1936; Wood, Werkman, Hemingway & Nier, 1940, 1941; Johns, 1951 b) and this is subsequently decarboxylated to propionate (Delwiche, 1948; Johns, 1949, 1951 a, b, c). In the 'direct reduction' pathway the carbon skeleton of lactate remains intact when it is converted to propionate (Johns, 1952; Lewis & Elsden, 1955; Ladd & Walker, 1959). These two pathways can be distinguished by fermenting [2-^{14}C]lactate and determining the labelling pattern of the propionate produced. Propionate formed via the 'direct reduction' pathway will be [2-^{14}C]propionate, whereas propionate formed by the 'succinate' mechanism will be a mixture of [2-^{14}C]propionate and [3-^{14}C]propionate, which will degrade as [2-3-^{14}C]propionate, carbons 2 and 3 having equal activity. The pathways can also be identified by fermenting lactate in the presence of $^{14}CO_2$. The 'succinate' mechanism will produce [1-^{14}C]propionate, whereas no radioactivity will

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appear in propionate if it arises via the 'direct reduction' pathway. Such experiments have been performed with two lactate-fermenting strains of *Selenomonas ruminantium* and the results are presented here.

METHODS

Organisms and cultural procedure. Strains HD4 and PC18 of *Selenomonas ruminantium* were obtained from Dr M. P. Bryant. They were maintained in test-tubes in a sloppy agar medium containing 1 % (w/v) sodium DL-lactate, 1·5 % (w/v) Oxoid tryptone, 0·5 % (w/v) Difco yeast extract, 0·0001 % (w/v) resazurin, 0·02 % (v/v) thioglycollic acid, 0·2 % (w/v) Davies New Zealand agar and 0·1 M-phosphate buffer (KH$_2$PO$_4$-NaOH), pH 6·8, in distilled water, equilibrated with CO$_2$ at approximately 40°. All ingredients were sterilized together by autoclaving (121°, 15 min.). Cultures were kept anaerobic by placing an alkaline pyrogallol plug at the top of the tube, which was then closed with a rubber stopper. Cultures were incubated at 37° for 10 to 16 hr and then stored at 0 to 5°. Stock cultures were transferred weekly.

Analytical methods. DL-lactate was estimated by the ceric sulphate method of Elsden & Gibson (1954). The growth medium contained interfering substances which were not completely removed by copper-lime treatment. To overcome this, lactate was quantitatively extracted from the medium with 150 ml. freshly distilled ether by the procedure described by Knight (1962). To the ethereal extract were added 2 ml. of 10 % (w/v) Na$_2$CO$_3$, the ether was distilled off and the residue evaporated to approximately 0·5 ml.

Total volatile fatty acid content of a fermentation fluid was measured by titrating a steam distillate with CO$_2$-free 0·1 N-NaOH in a stream of CO$_2$-free air; phenol red was the indicator. When samples contained lactate the distillate was acidified with H$_2$SO$_4$ (final concentration 0·1 N) and redistilled over magnesium sulphate as described by Friedemann (1938).

Individual volatile acids in a mixture were identified and estimated by gas liquid chromatography (g.l.c.) using an apparatus with a manually operated recording burette (James & Martin, 1952). The liquid phase was silicone DC 550–stearic acid–orthophosphoric acid mixture supported on acid-washed celite (James & Martin, 1952). The major difficulty in quantitative estimation of volatile fatty acids by g.l.c. has been the addition of the sample to the column. At the beginning of this work we were introduced by Dr A. T. Johns (at that time Director of the D.S.I.R. Plant Chemistry Division, Palmerston North, New Zealand) to a simple and effective technique which had been developed in New Zealand and which, to our knowledge, has not previously been described. The steam distillate, after titration to measure total volatile fatty acid content, was made alkaline by addition of approximately 1·0 ml. 0·01 M-NaOH and evaporated down in a steam bath to a volume such that the volatile fatty acid concentration was no more than 0·8 m-equiv./ml. A volume of this concentrated solution, not exceeding 30 μl., was placed in a porcelain combustion boat (3 mm. × 66 mm.) and spread along the bottom of the boat to form a thin film of liquid. If the volume of liquid exceeded 30 μl. subsequent separation of the fatty acids was impaired due to formation of azeotropic mixtures. One end of the boat was then lodged in the mouth of the column and evenly and swiftly filled with a mixture (2 vol. + 3 vol.) of powdered sodium bisulphate and acid-washed celite 535. The boat, when full, was pushed into the column and the gas supply connected. After standing

for 5 min. the gas supply (O$_2$-free nitrogen) was turned on. During the 5 min. period the boat and its contents reached the temperature (100°) of the column and acids diffused into the gas phase. Sodium bisulphate liberates the acids, but if it is used alone a crystalline mass is formed which occludes a significant amount of the acids, thus lowering the recoveries.

Additional celite results in the formation of a porous mass which does not retain the acids. The sodium bisulphate + celite mixture was dried at 150° for 2 hr and stored in a glass-stoppered bottle.

Because the volume of sample added to the column was not measured accurately, the record of the analysis obtained was used to calculate the percentage composition of the mixture; the amounts of individual acids were then calculated from the value for the total volatile acid content of the sample obtained by titration of the distillate. Recoveries for acetic, propionic and butyric acid were ± 4 % for each acid.

Measurement of radioactivity. Samples to be counted were converted to CO$_2$ which was plated as BaCO$_3$ (Sakami, 1955; Knight, 1962). Barium carbonate plates were counted with a Packard model 200A gas-flow counter (Packard Instruments, La Grange, Ill., U.S.A.) operated under the conditions described by Knight (1962). Activities, corrected for background (20 to 28 c.p.m.), were expressed as counts per minute at infinite thinness. The total radioactivity of a sample was determined after wet combustion (Sakami, 1955; Knight, 1962).

Degradations. Acetate and propionate were degraded by the Schmidt reaction (Sakami, 1955; Ladd, 1959). Sample and sulphuric acid were cooled in ice-water, prior to mixing, and activated sodium azide added to the cold reaction mixture. The reaction vessel was then quickly attached to the degradation train. The CO$_2$ produced was measured manometrically in a Warburg apparatus. The strongly acid reaction mixture, containing the amine produced by the reaction, was transferred to a Markham (1942) still, made alkaline with 10 N-NaOH (5 ml.) and steam-distilled into N-H2SO$_4$ (2 ml.). The amine sulphate was taken to dryness and combusted.

The CO$_2$ produced was measured manometrically and counted as barium carbonate. The yield of amine was calculated from the amount of CO$_2$ formed by combustion. In some experiments with propionate the ethylamine produced by the Schmidt degradation was oxidized to acetate with alkaline potassium permanganate (Sakami, 1955). The acetate thus formed was recovered by steam distillation, and after titration with N-NaOH the degradation by the Schmidt procedure was repeated.

Reagents. Sodium DL-lactate was prepared by neutralizing boiling AnalaR DL-lactic acid. Amberlite CG-50 resin, methyl ethyl ketone, ceric sulphate, thioglycollic acid and sodium azide were all of laboratory reagent grade (British Drug Houses Ltd., Poole, Dorset). Amberlite CG-50 was fractionated by sedimentation; that portion sedimenting from an aqueous suspension through a height of 17 cm. in 5 min. being used. Resazurin, silicone fluid DC 550 and stearic acid were from Hopkin & Williams Ltd. Celite 535 was from Johns-Manville (London). Acetone (May & Baker Ltd., Dagenham, Essex) was redistilled. Sodium DL[2-^{14}C]lactate and Na$_2$^{14}CO$_3$ were from the Radiochemical Centre, Amersham. All other reagents were of AnalaR grade.

Experimental procedures. Procedures for fermenting DL[2-^{14}C]lactate and lactate plus Na$_2$^{14}CO$_3$ were similar in both cases. Approximately 40 ml. cultures were grown under CO$_2$ in the medium described above (minus agar), in micro-kjeldahl flasks. After inoculation, a sterile solution of sodium DL[2-^{14}C]lactate (10 μmole : 2 μc.) or

$Na_2^{14}CO_3$ (100 μmole) was added. When labelled lactate was used a sample of medium was taken for the estimation of lactate. Vessels were sealed and incubated at 37° until heavy growth was observed, after which cells were centrifuged off and their radio-activity measured. A sample of the supernatant was set aside for estimation of acetate and propionate. Other samples were steam-distilled, neutralized with 0·1 N-NaOH, and evaporated to dryness. The residues were dissolved in 0·2 ml. of a mixture of methyl ethyl ketone, acetone and water (1 + 2 + 9 by vol.), and the volatile acids fractionated on a column of Amberlite CG-50 resin (Seki, 1958). Acetate and propionate fractions eluted from the column were titrated with 0·1 N-NaOH and taken to dryness. The residues were dissolved in water. A portion of each was assayed for volatile acid by steam distillation and titration and a second portion, after mixing with sufficient succinic acid to give a total of 600 μ atoms of carbon, was combusted and the radio-activity measured. The appropriate unlabelled acid was added to a third portion to bring the total acid content to approximately 2 m-mole. The new specific activity was measured, and about 1 m-mole of acid was degraded by the Schmidt reaction, the specific activity of each carbon being determined.

Table 1. *Radioactivity fixed into products when strains HD4 and PC18 of Selenomonas*
ruminantium were grown on DL-lactate in the presence of $Na_2^{14}CO_3$

Cultures were grown for 4 days at 37°. At start each culture contained 100 μmole $Na_2^{14}CO_3$; 3·46 × 10^6 c.p.m. Values given are per culture volume.

	Strain	
	HD4	PC18
Volume of culture (ml.)	36·5	41·4
Radioactivity in cells (c.p.m.)	5·00 × 10³	1·53 × 10³
Total volatile acids formed (μmole)	1,050	983
Total activity in volatile fatty acids (c.p.m.)	4·92 × 10⁵	2·06 × 10⁵
Specific activity of volatile fatty acids (c.p.m./μmole)	469	210
Acetate formed (μmole)	387	388
Activity of acetate (c.p.m.)	7·24 × 10³	1·16 × 10³
Specific activity of acetate (c.p.m./μmole)	18·7	3·0
Propionate formed (μmole)	660	595
Activity of propionate (c.p.m.)	4·80 × 10⁵	1·95 × 10⁵
Specific activity of propionate (c.p.m./μmole)	728	328

RESULTS AND DISCUSSION

Cultures of strains HD4 and PC18 were grown on DL-lactate in the presence of $Na_2^{14}CO_3$, and fermentation products were examined for radioactivity (Table 1). Both strains fixed $^{14}CO_2$ into cell material and volatile fatty acid fraction. Of the iso-tope fixed into volatile fatty acid 97·6 % (HD4) and 94·8 % (PC18) was located in propionate. No significant activity was found in acetate. Degradation of the propionate showed, in both cases, that the specific activity of the carboxyl group was similar to that of the undegraded propionate, and no significant activity was present in carbons 2 and 3 (Table 2). Thus the $^{14}CO_2$ fixed into propionate was located exclusively in the carboxyl group.

Cultures were grown on DL-lactate in the presence of DL[2-^{14}C]lactate. The amounts of radioactivity found in fermentation products are shown in Table 3. One point four per cent or less of the added lactate-label was found in cells, whereas 103 % (HD4) and

20 % (PC18) were located in volatile fatty acid. In both experiments approximately 62 % of the volatile fatty acid-label was in propionate and 43 % in acetate. However, the specific activities of acetate and propionate were approximately equal.

Degradation of acetate produced by the two strains showed, in each case, that the specific activities of the carboxyl group and undegraded acetate were similar (Table 4).

Table 2. *Distribution of radioactivity in propionate produced by the fermentation of* DL-lactate *in the presence of* $Na_2^{14}CO_3$ *by* S. ruminantium *strains* HD4 *and* PC18

	Strain HD4		Strain PC18	
	Specific activity (c.p.m./ μmole)	% of label	Specific activity (c.p.m./ μmole)	% of label
Propionate	37·6	(100)	13·7	(100)
Propionate C-1 (as CO_2)	38·4	102	15·6	114
Propionate C-2 + C-3 (as ethylamine)	0·17	0·5	0·29	2
Total recovery	—	103	—	116

Table 3. *Radioactivity fixed into products when strains* HD4 *and* PC18 *of* S. ruminantium *were grown on* DL[2-^{14}C]lactate

Cultures were grown for 2 (HD4) and 6 (PC18) days at 37°. At the start, HD4-culture contained 61·0 μmole DL-lactate/ml., specific activity 370 c.p.m./μmole; PC 18-culture contained 68·5 μmole DL-lactate/ml., specific activity 307 c.p.m. per μmole. Values given are per culture volume.

	Strain	
	HD4	PC18
Volume (ml.)	34·2	34·5
Radioactivity in cells (c.p.m.)	11,200	396
Total volatile acids formed (μmole)	2,410	724
Total activity in volatile fatty acids (c.p.m.)	$7·96 \times 10^5$	$14·3 \times 10^4$
Specific activity of volatile fatty acids (c.p.m./μmole)	330	198
Acetate formed (μmole)	930	296
Activity of acetate (c.p.m.)	$3·27 \times 10^5$	$6·41 \times 10^4$
Specific activity of acetate (c.p.m./μmole)	352	217
Propionate formed (μmole)	1,480	428
Activity of propionate (c.p.m.)	$5·03 \times 10^5$	$8·94 \times 10^4$
Specific activity of propionate (c.p.m./μmole)	340	209

Table 4. *Distribution of radioactivity in acetate produced by the fermentation of* DL[2-^{14}C]lactate *by* S. ruminantium *strains* HD4 *and* PC18

	Strain HD4		Strain PC18	
	Specific activity (c.p.m./ μmole)	% of label	Specific activity (c.p.m./ μmole)	% of label
Acetate	6·30	(100)	2·82	(100)
Acetate C-1 (as CO_2)	6·35	101	2·99	106
Acetate C-2 (as methylamine)	0·06	1	0·045	2
Total recovery	—	102	—	108

No significant activity was present in the methyl group. Thus, [2-^{14}C]lactate gave rise to [1-^{14}C]acetate.

Table 5 shows results obtained when the two samples of propionate were degraded. The same labelling pattern was produced by both organisms. No activity was found in the carboxyl group. Carbons 2 and 3 were approximately equally labelled, and the sum of their specific activities was approximately equal to the specific activity of the undegraded propionate. Thus, [2-^{14}C]lactate gave rise to [2-3-^{14}C]propionate.

Table 5. *Distribution of radioactivity in propionate produced by the fermentation of DL[2-^{14}C]lactate by S. ruminantium strains HD4 and PC18*

	Strain HD4		Strain PC18	
	Specific activity (c.p.m./ μmole)	% of label	Specific activity (c.p.m./ μmole)	% of label
Propionate	13·3	(100)	4·71	(100)
Propionate C-1 (as CO_2)	0	0	0	0
Propionate C-2 as (CO_2)	6·92	52	2·44	52
Propionate C-3 (as methyl-amine)	6·59	50	2·35	50
Total recovery	—	102	—	102

The results obtained are thus consistent with the hypothesis that *Selenomonas ruminantium* forms propionate via the 'succinate' pathway and acetate by the direct oxidation of lactate.

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Pyrrolidonyl Peptidase in Bacteria: A New Colrimetric Test for Differentiation of Enterobacteriaceae

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SUMMARY

A simple and rapid colorimetric test has been developed for the detection of pyrrolidonpl peptidase (PLP) activity in bacteria. Of 2354 strains belonging to various groups of enteric bacteria tested, 451 were PLP-positive. These included Citrobacter, 223 out of 226 strains, Klebsiella (178/188), Enterobacter (49/52), and one strain of Serratia.

Pyrrolidonyl peptidase was not detected in 1146 Shigella strains, 354 Salmonella strains, 333 Escherichia strains, 21 Arizona strains, nor in 33 strains of Proteus, Providencia and Hafnia.

In addition, 56 cultures of various Gram-negative and Gram-positive bacteria were tested for PLP. Enzyme activity was found in strains belonging to the genera *Bacillus, Streptococcus, Staphylococcus, Micrococcus, Sarcina, Neisseria* and *Pseudomonas*. The possibility of employing the PLP test for differentiation within the family Enterobacteriaceae is discussed.

INTRODUCTION

The classification of some bacteria may be based upon their peptidase activity (Muftic, 1965, 1967; Muftic & Schroder, 1966; Westley *et al.* 1967). In a previous paper (Szewczuk & Mulczyk, 1969) we reported new pyrrolidonyl naphthlyamides synthesized in our laboratory which could be used as chromogenic substrates for colorimetric determination of pyrrolidonyl peptidase activity. The enzyme was found in Gram-positive and Gram-negative bacteria. Pyrrolidonyl peptidase from *Bacillus subtilis* was purified and its properties and specificity were described.

This paper describes a simple colorimetric test for detection of pyrrolidonyl peptidase activity (PLP test) in bacteria and its adaptation as a new method for the differentiation of the various groups of Enterobacteriaceae.

METHODS

Reagents. (1) Substrate solution: 0·01 M-L-pyrrolidonyl-β-naphthylamide in methanol (2·5 mg./ml.). This substance was synthesized by the method previously described (Szewczuk & Mulczyk, 1969). (2) 0·5 M-tris/HCl buffer, pH 7·7. (3) 10 % (v/v) acetic acid solution in water. (4) 0·1 % tetra-azotized *o*-dianisidine (Fast Blue B) solution in water.

Strains. A total of 2354 cultures that belonged to the various groups of Enterobacteriaceae were tested for PLP. The strains had been collected for several years
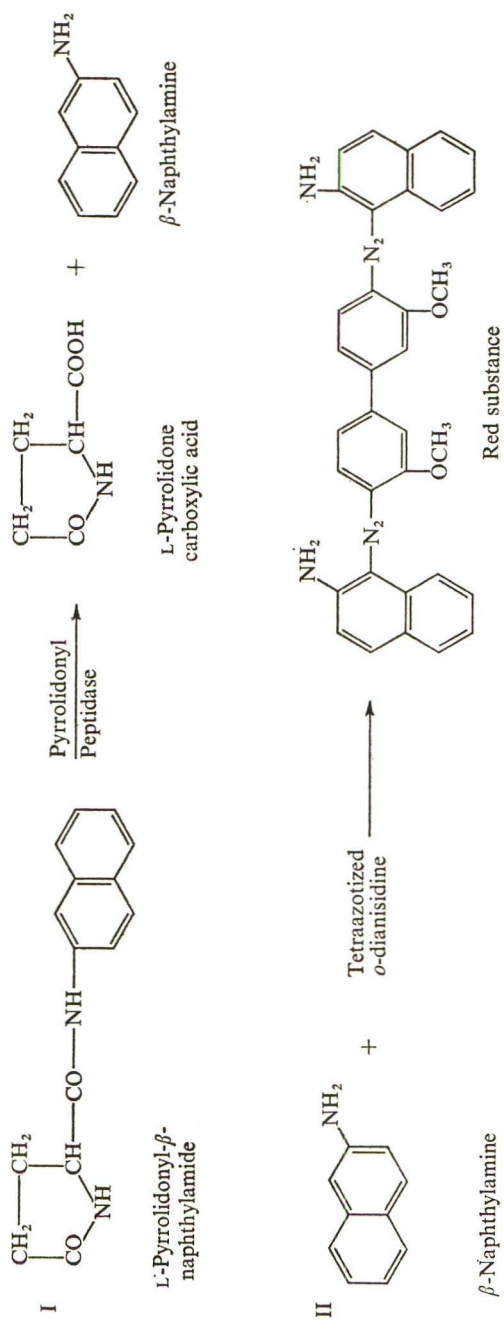
Fig. 1

and had been received from different sources. Recently isolated strains from diagnostic material were also included. In addition, 56 strains of different groups of Gram-positive and Gram-negative bacteria were examined.

Table 1. *The occurrence of PLP in enteric bacteria*

Group	Positive strains/tested strains	
	Group	Genera
Shigella dysenteriae, serotypes 1–8	0/34	
S. flexneri, all serotypes	0/867	
S. boydii, serotypes 1–15	0/45	0/1146[a]
S. sonnei	0/200	
Salmonella typhi	0/76	
S. paratyphi B	0/14	
S. typhimurium	0/67	
S. enteritidis	0/131	
S. anatum	0/19	0/354[b]
S. newington	0/20	
S. heidelberg	0/10	
S. brandenburg	0/2	
S. morbificans	0/15	
Escherichia,		
O antigens 1–149	0/143	
H antigens 1–49	0/49	0/333[c]
K antigens 1–91	0/91	
From diagnostic material	0/50	
Klebsiella aerogenes	60/61	
K. pneumoniae	10/10	
K. edwardsii var. *edwardsii*	19/20	
K. edwardsii var. *atlantae*	0/8	178/188[d]
K. ozaenae	28/28	
K. rhinoscleromatis	13/13	
K. oxytoca	48/48[e]	
Citrobacter,		
O antigens 1–32	87/88[f]	
O antigens 33–42	10/10[g]	223/226[h]
From diagnostic material	126/128	
Arizona, O antigens 1–21	0/21	0/21[h]
Enterobacter aerogenes	34/35	
E. liquefaciens	4/5	49/52[i]
E. cloacae	11/12	
Proteus species	0/20	0/20
Providencia species	0/10	0/10
Hafnia species	0/3	0/3
Serratia species	1/1	1/1
Total		451/2354

NOTES

[a] Including strains from the Dysentery Reference Laboratory, London, and from Dr A. J. Weil, New York.

[b] Received from Professor Z. Buczowski, Gdańsk, and Professor J. Chomiczewski, Łódź.

[c] Including strains from Dr F. Ørskov, Copenhagen.

[d] Including Kauffmann's strains, received from Dr I. Ørskov, Copenhagen, and eight new serotypes (Maresz-Babczyszyn, 1962; Durlakowa *et al.* 1963).

[e] Received from Dr S. Kałuzewski, Warsaw.

[f] Including NCDC strains.

[g] Sedlak & Slajsova (1966).

[h] Received from Professor J. Sedlak, Prague.

[i] Received from Dr L. Le Minor, Paris.

The pyrrolidonyl peptidase activity test (PLP test). This test is based on the following two reactions: pyrrolidonyl peptidase present in bacteria catalyses the hydrolysis of L-pyrrolidonyl-β-naphthylamide liberating free L-pyrrolidone carboxylic acid and β-naphthylamine (reaction I). The latter substance, after coupling with tetra-azotized *o*-dianisidine (Fast Blue B), gives the red azo substance (reaction II) (Fig. 1).

Procedure. The culture to be tested is inoculated on to an agar slant and incubated for 18 to 24 hr at 37°. The bacteria are washed off with 1·5 ml. saline and 0·4 ml. suspension is added to a test-tube. Then 0·05 ml. 0·5-M tris/HCl buffer and 0·05 ml. substrate (L-pyrrolidonyl-β-naphthylamide) are added and mixed immediately. The suspension is incubated in a water bath at 37° for 3 hr and then 0·2 ml. of acetic acid is added. The colour is developed by addition of 0·1 ml. Fast Blue solution followed by incubation at 37° in a water bath for 1 hr. A positive reaction is indicated by a strong red colour of the suspension, stable for at least one day at room temperature.

RESULTS

The PLP activities of 2354 cultures from various groups of Enterobacteriaceae are presented in Table 1. It can be seen that Shigella, Salmonella, Escherichia, Arizona, Proteus, Providence and Hafnia species gave a uniformly negative result in PLP test. In contrast, Klebsiella species gave a positive result except for all strains of *K. edwardsii* var. *atlantae* tested and one strain each of *K. aerogenes* and *K. edwardsii* var. *edwardsii*. With few exceptions (see Table 1) Citrobacter and Enterobacter strains were also positive. The one Serratia strain tested gave a positive result.

In addition to the above-mentioned cultures of Enterobacteriaceae, 56 Gram-positive and Gram-negative strains were examined for PLP. The results are recorded in Table 2: except for the strains belonging to the genera *Corynebacterium* and *Saccharomyces*, strains that gave positive reactions were found in all remaining groups tested.

Table 2. *The occurrence of PLP in some Gram-positive and*
*Gram-negative bacteria**

Genus	No. of cultures tested	PLP-positive
Pseudomonas	8	4
Neisseria	4	4
Micrococcus	1	1
Bacillus	9	8
Corynebacterium	3	0
Streptococcus	7	5
Sarcina	3	3
Staphylococcus	19	16
Saccharomyces	2	0

* Strains from Polish Collection of Micro-organisms, Wrocław, and from diagnostic material.

DISCUSSION

Different biochemical tests have been described that have distinct taxonomic value within the family of Enterobacteriaceae. Some tests may be readily applied in laboratory diagnosis and were found useful in the differentiation of enteric bacteria (Edwards & Ewing, 1962; Cowan & Steel, 1965). Our data indicate that the simple test for detection of pyrrolidonyl peptidase activity in bacteria described in this paper may

be considered as an additional one in the classification and differentiation of bacteria. This is supported by the fact that the cultures of some species had PLP and the others were devoid of the enzyme. Thus the PLP test may be applied in the differentiation of strains which still constitute diagnostic problems. These are strains that are closely related biochemically and serologically and that compose the Salmonella–Arizona–Citrobacter division. Since the great majority of Citrobacter strains are positive in the PLP test and those of Salmonella and Arizona are negative, the introduction of the test may be of great value in the differentiation of these organisms in addition to the tests for lysine decarboxylase (Møller, 1955) and KCN tolerance (Møller, 1954).

The lack of PLP in all eight strains of *Klebsiella edwardsii* var. *atlantae* should also be noted. However, only further studies of a larger number of Klebsiella cultures can show whether negative strains in the PLP test are limited to the subgroup of *K. edwardsii* var. *atlantae*.

Considering that the number of strains of some species that were tested was small, no attempts were made to evaluate the usefulness of the PLP test in routine diagnostic work. To do so, further studies are required on a larger number of strains of different serotypes of Enterobacteriaceae including original stock cultures and those isolated from diagnostic material. These studies are now in progress in our laboratory and the results will be the subject of a separate communication. It must be stressed here, however, that as the distribution of pyrrolidonyl peptidase is characteristic as a rule of the individual bacterial groups, the PLP test may be of great diagnostic value in differentiation within the family of Enterobacteriaceae.

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Chemical Characteristics of Endotoxin from
Bacteroides fragilis NCTC 9343

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SUMMARY

Phenol-water extracted lipopolysaccharides (LPS) from *Bacteroides fragilis* NCTC 9343 did not contain heptose or 2-keto-3-deoxy-octonate. The sugar components identified were glucosamine, galactosamine, glucose, galactose, fucose, rhamnose and traces of mannose. An unidentified amino compound was found. The preparations were free of nucleic acids and protein constituted only a minor fraction of the LPS. The LPS preparations had endotoxic effect when tested in rabbits, but the endotoxic potency was low.

INTRODUCTION

Endotoxic lipopolysaccharide (LPS) from *Bacteroides melaninogenicus* lacks heptose and 2-keto-3-deoxy-octonate (KDO) (Hofstad, 1968). With a few exceptions (Hickman & Ashwell, 1966; Volk, 1966; Kasai & Nowotny, 1967; Adams, Tornabene & Yaguchi, 1969) these sugars are present as essential constituents in LPS from aerobic Gram-negative bacteria (Osborn, 1963; Lüderitz, Staub & Westphal, 1966). The unexpected finding that both sugars were absent in *B. melaninogenicus* LPS has prompted a survey of the chemistry of LPS from related non-sporing anaerobic Gram-negative bacteria. The present report deals with the chemical composition of LPS from *B. fragilis* NCTC 9343.

METHODS

Bacterial strains. The *Bacteroides fragilis* strain NCTC 9343 was furnished by Dr Ella Barnes, Food Research Institute, Norwich, England. Cultivation was performed as described earlier (Hofstad, 1968) in an enriched fluid medium based on beef extract.

Extraction of LPS. Extraction of LPS with phenol-water (Westphal, Lüderitz & Bister, 1952) and further purification by ultracentrifugation were carried out as before (Hofstad, 1968). Extractions were made at room temperature from acetone-dried whole cells. After ultracentrifugation (100,000 g for 1 hr) the pellet was suspended in 0.1 M phosphate buffer pH 7.0 containing ribonuclease (5 × cryst., ex-bovine pancreas, Sigma Chemical Company, St Louis, Missouri, U.S.A.) and deoxyribonuclease (25% activity of crystalline material deoxyribonuclease, ex-bovine pancreas, L. Light & Co. Ltd., Colnbrook, England) to give an enzyme:substrate ratio of about 1:50, and incubated at 37° for 1 hr. The ultracentrifugation was repeated, and the washed LPS finally taken up in distilled water and lyophilized.

Paper chromatography. Acid hydrolysis was performed in sealed tubes at 100° with 4 N-HCl for 4 hr, or with N-H$_2$SO$_4$ for 10 min., 4 or 16 hr, as required. Acid was removed from HCl hydrolysates by evaporation *in vacuo* in the presence of NaOH pellets. The H$_2$SO$_4$ hydrolysates were neutralized by passage through a column of Dowex-1 in the formate form. Circular paper chromatography was carried out with *n*-butanol + pyridine + water (6 + 4 + 3) or *n*-propanol + 1 % aqueous ammonium hydroxide (7 + 3). Sugars were stained with silver nitrate or aniline hydrogen phthalate. Amino sugars were also detected with the Elson–Morgan reagent (Partridge, 1948) or with ninhydrin.

Chemical analyses. Nitrogen was determined by the Micro-Kjeldahl method as described by Kabat & Mayer (1961). Samples were digested for 4 hr. Hexosamines were estimated with the Rondle & Morgan method (1955), with glucosamine.HCl as standard. Samples were hydrolysed at 100° with 3 N-HCl for 4 hr. Amino sugars were also separated on columns of Dowex-50 w × 8 (Gardell, 1953). Protein was estimated by the Folin-Ciocalteau phenol method (Lowry, Rosebrough, Farr & Randall, 1951) with bovine serum albumin as standard. Fatty acid esters were determined as tripalmitin by the method of Snyder & Stephens (1959). Neutral sugars were measured by the Winzler orcinol method (Winzler, 1955) with glucose + galactose (1 + 1) as standard. Methyl pentose was estimated by the sulphuric acid + cysteine method as described by Dische (1955). The sulphuric acid + cysteine reaction was also used as a test for heptoses (Dische, 1953). The presence of KDO was investigated by the malonealdehyde + thiobarbituric acid method as modified by Weissbach & Hurwitz (1959).

Shwartzman reaction. Albino rabbits of either sex, 6 months old, and weighing 2·6–3·7 kg. were used. The animals were fed commercial pellets and water *ad libitum*. Serial dilutions of LPS were made in sterile saline. Doses of 0·2 ml. of each dilution were injected intradermally on alternative sites into the shaved abdomen. Provocative injections of LPS were given 24 hr later, and the animals were examined for haemorrhagic lesions on the following day.

RESULTS

Quantitative chemical analyses were carried out on three different batches obtained after extraction of acetone-dried cells with phenol-water for 15 or 30 min. (Table 1). Extraction for 15 min. gave a product which contained only small amounts of protein. The u.v. absorption curve of this LPS showed no peak or shoulder in the region of 260 nm. indicating that the LPS was free of or virtually free of nucleic acids. The yield was rather low, varying between 1 and 2 %. By extending the extraction to 30 or 45 min. the yield could be more than doubled. However, LPS obtained by such prolonged extraction was contaminated with protein, and possibly also with intracellular glycogen as judged by a relatively strong glucose spot on chromatograms made with acid hydrolysates.

Chromatographic analysis of sulphuric acid hydrolysates (N-H$_2$SO$_4$, 4 hr) of LPS obtained after extraction for 15 or 30 min. revealed bands corresponding to glucose, galactose, fucose, rhamnose and traces of mannose. Glucosamine and galactosamine were identified in hydrochloric acid hydrolysates. In addition, an unknown amino compound with an R$_{glucosamine}$ value of 1·64 in *n*-butanol + pyridine + water was detected on paper chromatograms developed with the Elson–Morgan reagent. The
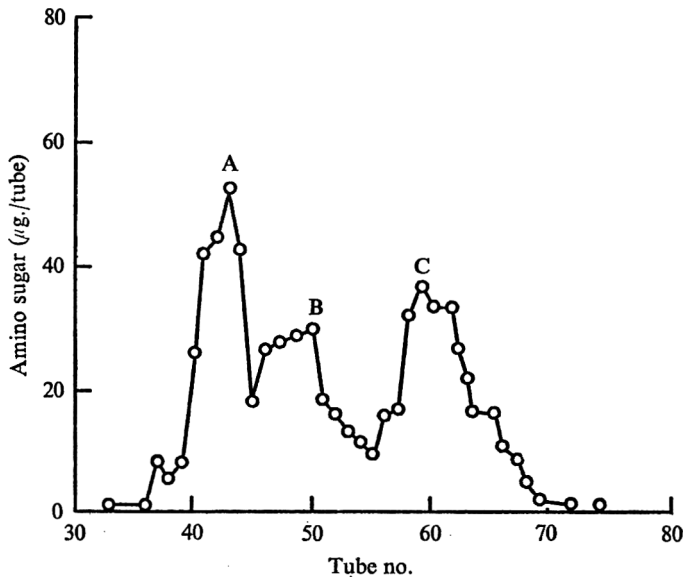
Fig. 1. Elution pattern of amino sugars and the unidentified amino compound from *Bacteroides fragilis* (Batch 3, 40 mg.) on a Dowex-50 column. Each fraction contained 2 ml. Amino sugar concentrations are quoted as though they were glucosamine. A, glucosamine; B, galactosamine; C, unidentified amino sugar.

Table 1. *Chemical composition of lipopolysaccharide isolated from Bacteroides fragilis* NCTC 9343 *by extraction of acetone-dried cells with phenol-water for 15 min. (Batch 1 and 2) or 30 min. (Batch 3)*

	Neutral sugar (%)	Methyl-pentose (%)	Hexosamine (%)	Fatty acid ester (%)	Nitrogen (%)	Protein (%)
Batch 1	27·1	8·9	14·8	24·7	2·2	3·4
Batch 2	29·3	9·3	14·8	21·6	2·3	2·6
Batch 3	32·9	3·7	7·3	14·6	6·0	40·3

compound reduced silver nitrate, but gave no colour reaction with aniline hydrogen phthalate. On Dowex-50 the compound had a chromatographic mobility of 1·37 relative to glucosamine when eluted with 0·3 N-HCl (Fig. 1).

KDO could not be demonstrated when unhydrolysed samples, or samples of LPS hydrolysed for varying periods of time with 0·02 N-sulphuric acid, were examined by the thiobarbituric acid method.

Unhydrolysed samples of LPS were examined in the sulphuric acid–cysteine reaction of Dische (1953). The chromogenes gave an absorption maximum at 400 nm., and smaller peaks at 540 and 600 nm. No peak was observed at 510 nm., thus excluding the presence of heptose, nor was heptose demonstrated in the 16 hr sulphuric acid hydrolysates by paper chromatography. Acid labile deoxysugars were not detected in samples of LPS hydrolysed with N-sulphuric acid for 10 min.

The endotoxic potency of LPS was estimated by the local Shwartzman reaction in 12 rabbits. The animals were each prepared with different doses of LPS obtained by

extraction for 15 min. (6 rabbits) or for 30 min. (6 rabbits). Provocation was performed by intravenous administration of 400 μg. LPS. The results have been listed in Table 2.

Table 2. *Local Shwartzman reaction elicited by lipopolysaccharide isolated from Bacteroides fragilis* NCTC *9343 by extraction of acetone-dried cells with phenol-water for 15 min.* (*Batch 1*) *or 30 min.* (*Batch 3*)

	Preparative injection (μg.)						
	400	200	100	50	25	12·5	Saline
Batch 1	6/6*	6/6	3/6	1/6	0/6	0/6	0/6
Batch 3	4/6	3/6	3/6	3/6	1/6	0/6	0/6

* Positive Shwartzman reaction/total, 18 hr after provocative injection.

DISCUSSION

The outstanding feature of the present study is the lack of heptose and KDO in LPS prepared from *Bacteroides fragilis* NCTC 9343. These constituents are also lacking in LPS isolated from oral strains of *B. melaninogenicus* (Hofstad, 1968). This suggests that endotoxic LPS of Bacteroides species has a core structure differing from that of LPS of Gram-negative aerobic bacilli where heptose is the basal sugar of the core (Lüderitz *et al.* 1966) and KDO provides the bridge between the polysaccharide and lipid moieties of the LPS (Osborn, 1963). Another rather unusual finding is the demonstration of fucose.

Apparently LPS of *Bacteroides fragilis* NCTC 9343 contains a wider range of sugar components than does LPS of *B. melaninogenicus* (Hofstad, 1968). However, further chemical studies have shown that the LPS preparations from *B. melaninogenicus* were heavily contaminated with intracellular glycogen (unpublished data), which may have masked the presence in acid hydrolysates of small amounts of other sugars.

Because of the low recovery and the limitations of the methods used, the quantitative analyses performed cannot give more than a rough estimation of the quantitative composition of the isolated LPS. However, the results are comparable with those obtained by quantitative analyses of LPS from other bacteria.

To our knowledge an amino sugar with properties similar to those of the unidentified amino compound has not previously been found in material isolated from bacteria. The chromatographic data obtained are not consistent with those reported for fucosamine (Crumpton & Davies, 1958; Sharon, Shif & Zehavi, 1964) or for the fast-moving diaminohexose isolated from *Bacillus subtilis* ATCC 9945 by Sharon & Jeanloz (1960). The possibility that the compound is part of a macromolecular structure other than LPS cannot as yet be excluded.

The *Bacteroides fragilis* LPS proved to be toxic for rabbits, but the endotoxic potency was low.

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Stability of DNA in Dark-repair Mutants of *Escherichia coli* B Treated with Nalidixic Acid

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SUMMARY

Breakdown of DNA to acid-soluble fragments induced in strains of *Escherichia coli* B by nalidixic acid (10 μg./ml.) differed in linear rate according to the ability of the strain to repair DNA lesions. Strains which were *exr* (BS-2) and *exr, uvr* (BS-1) exhibited excessive DNA breakdown following nalidixic acid treatment. The excision-defective *uvr* strains, BS-8, BS-12 and WWP-2 *hcr*, also degraded their DNA to acid-soluble fragments at a rate which initially was greater than that of the parental strain (*E. coli* B). This degradation was unaffected by the *rad* mutation. Removal of nalidixic acid from the system considerably lowered the rate of DNA breakdown. The responses to nalidixic acid and other agents potentiating DNA breakdown are compared.

INTRODUCTION

The antibiotic nalidixic acid selectively inhibits DNA synthesis in growing bacteria without impairing the synthesis of other macromolecules (Goss, Deitz & Cook, 1964, 1965). Its deleterious effects on viability and on DNA synthesis require active growth and are absent in stationary phase cells. Nalidixic acid causes bacteria to grow as long snakes and to degrade portions of their DNA to acid-soluble fragments (Cook, Brown, Boyle & Goss, 1966). A number of other treatments which impair DNA synthesis, such as u.v.- and x-irradiation, and the antibiotics Mitomycin *C* and Phleomycin, also cause DNA breakdown. With these, the extent of the breakdown observed and its kinetics are influenced by the genotypes of the bacterial strains exposed to the treatments; mutants blocked in various steps of dark-repair processes varying widely from wild type in sensitivity to DNA breakdown.

In this paper the patterns of DNA breakdown in mutants blocked in either the ability to excise thymine dimers (UVR) or in a step in the recombinational-repair process (EXR, Witkin, 1968; Rupp & Howard-Flanders, 1968), or in both (UVR, EXR), are compared with those of *E. coli* B.

METHODS

The strains of bacteria used, together with their genotypes and source are shown in Table 1.

Thymine-requiring mutants of the strains listed in Table 1 were selected by the procedure of Okada, Yanagisawa & Ryan (1960). The bacteria were radioactively labelled by growing them at 37° with shaking in the glucose salts medium G.T.

(Grigg, 1969), supplemented with [^{14}C]thymidine (20 μg./ml.), overnight. The ^{14}C-labelled organisms were washed and transferred to fresh medium containing non-radioactive thymidine and allowed to divide once before being used as exponentially growing cells in the experiments. The bacterial concentration at this stage was about 2×10^7/ml. In determining the proportion of DNA degraded to acid-soluble fragments the procedure of Aoki, Boyce & Howard-Flanders (1966) was followed. Nalidixic acid was bought from Sterling, Winthrop. Unless otherwise specified it was used at a concentration of 10 μg./ml.

Table 1. *Repair deficient mutants of Escherichia coli*
used in this investigation

Strain	Source	Genotype	Reference
B	R. Hill	*lon*	
BS-1	R. Hill	*lon, uvr, exr*	Mattern, Zwenk & Rörsch (1966)
BS-2	R. Hill	*lon, exr*	
BS-8	R. Hill	*lon, uvrB, rad*	Donch & Greenberg (1968)
BS-12	R. Hill	*lon, uvr-12*	
WWP-2 *hcr*	R. Hill ex E. Witkin	*lon, uvrA, rad, trp*	(Greenberg, private communication)

RESULTS

DNA breakdown in all the bacterial populations studied commenced about 15 min. after addition of nalidixic acid and proceeded at a linear rate for at least 2 hr.

The highest rate of degradation of DNA to acid-soluble fragments was observed in the two *exr* strains BS-2 and BS-1 (Fig. 1). The latter also carries the *uvr* mutation but its response to nalidixic acid-induced breakdown of DNA was identical to that of the *uvr*$^+$ *exr* mutant BS-2. 50 % of the DNA of BS-1 and BS-2 bacteria was degraded in 3 hr. The corresponding figure for strain B bacteria was 17 %.

The pattern of DNA breakdown in the three *uvr exr*$^+$ strains, BS-8, BS-12 and WWP-2 *hcr*, was identical and was not affected by the presence of the *rad* mutation. Initially the rate of breakdown in cultures of *uvr* mutants was appreciably higher than in strain B cultures, but the rate dropped suddenly after 2 hr. After 3 hr the proportion of DNA degraded to small fragments was rather similar to that of *E. coli* B (Fig. 1).

Goss *et al.* (1965) reported that the inhibitory effects of nalidixic acid on DNA synthesis are rapidly reversed by washing cells free of the drug. Both the rate of DNA breakdown and the proportion of DNA degraded decreased immediately upon removal of nalidixic acid. This is illustrated (Fig. 2) by the observations on a culture of BS-1 (*uvr, exr*) exposed for varying periods to nalidixic acid. The rate of DNA breakdown did not fall to zero upon removing the drug from the bacteria by Millipore filtration but continued at a lower rate for the 4 hr period of the experiment.

DISCUSSION

DNA breakdown induced by treatments which themselves do not cause direct strand breakage or do so with low efficiency, e.g. u.v.-irradiation or Phleomycin (2 μg./ml.), is often reduced in *uvr* mutants compared with *uvr*$^+$ strains. This has led

to the suggestion that since the first step in the excision of lesions, such as u.v.-induced pyrimidine dimers (Setlow & Carrier, 1964), from DNA, involves an endonuclease mediated DNA chain break, the *uvr* mutants which display reduced DNA breakdown are defective in this endonuclease. Certainly they exhibit fewer chain breaks following u.v.-irradiation (Setlow, Carrier & Williams, 1967). That the rate of DNA breakdown induced by nalidixic acid in *uvr* mutants exceeded that in *uvr*+ bacteria is therefore unusual.
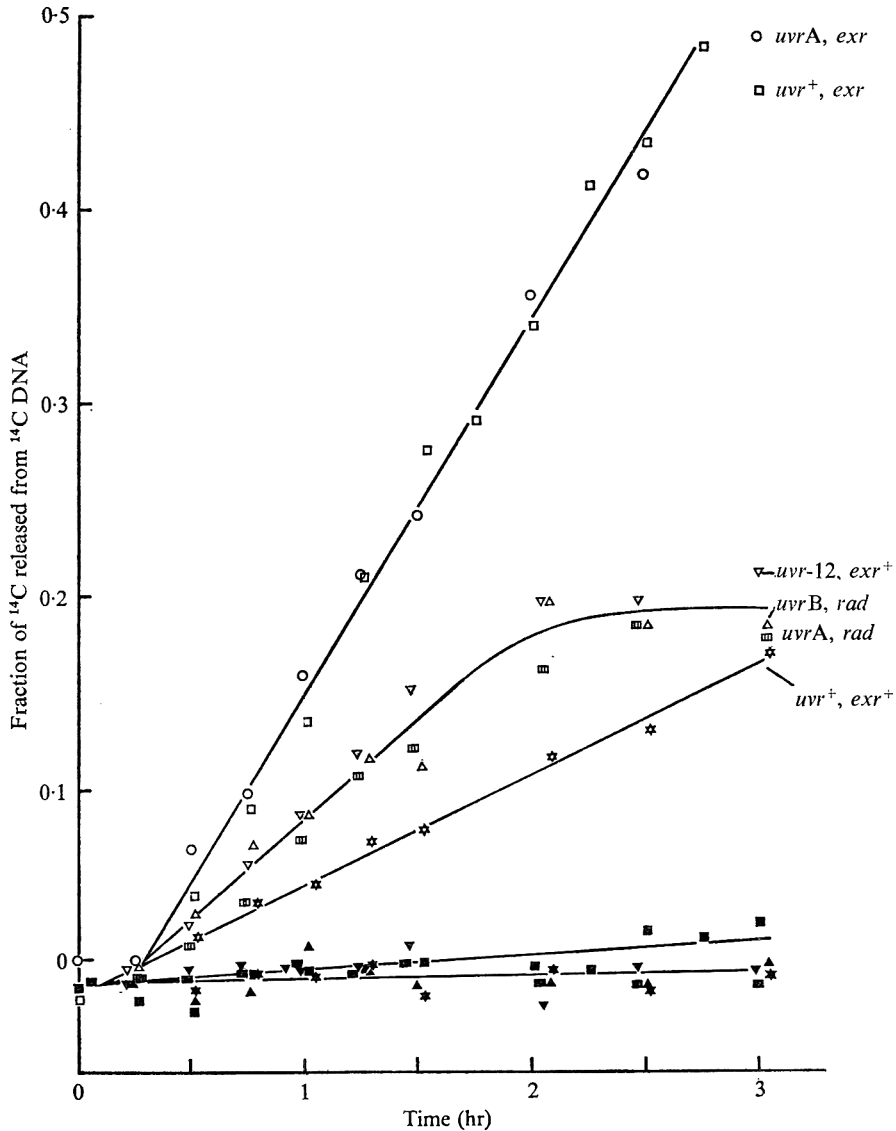
Fig. 1. Effect of genotype on nalidixic acid-induced breakdown of DNA to acid-soluble fragments. Nalidixic acid (10 μg./ml.) was present during the whole of the sampling period (open symbols). The following strains of *E. coli* B were used: *exr*, BS-2; *exr uvr*A, BS-1; *uvr*A, BS-12; *uvr*B *rad*(8), BS-8; *uvr*A *rad*(w), WWP-2 *hcr*; *uvr*+, *exr*+, B. The solid symbols represent untreated controls.

Ionizing radiations differ from the other treatments in that they induce an identical amount of DNA breakdown in *uvr* and *uvr*^{+} cells. The excessive DNA breakdown induced by nalidixic acid in the *exr* mutant BS-2 followed the same pattern as that of other treatments causing breakdown and had the same kinetics in the *uvr exr* mutant BS-1 (cf. Suzuki, Moriguchi & Horii (1966) after u.v. irradiation).
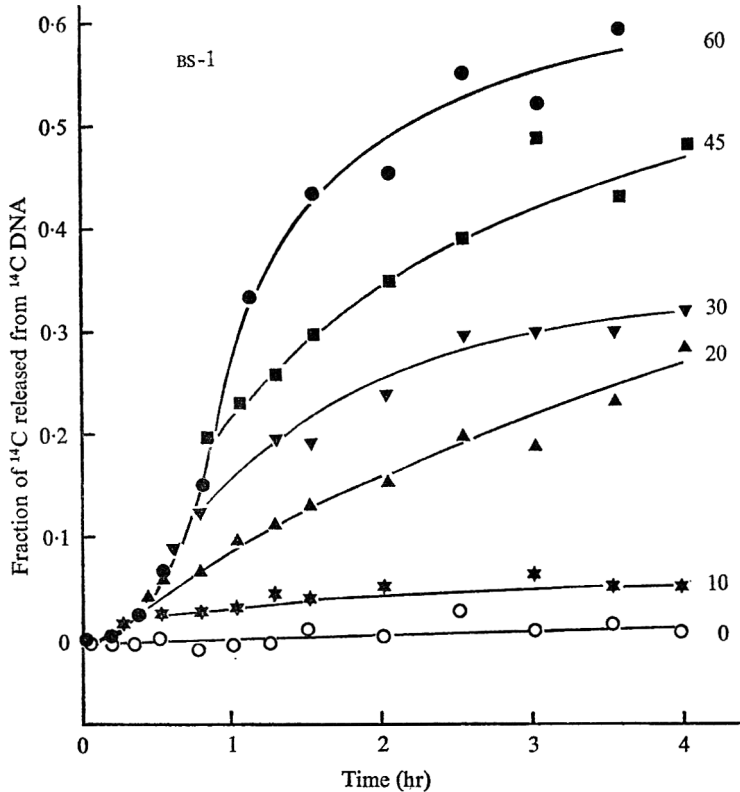
Fig. 2. Relation between period of exposure to nalidixic acid and DNA breakdown in *E. coli* BS-1 *thy* cells. An exponentially growing culture whose DNA was previously labelled with [^{14}C]thymidine was exposed to nalidixic acid (10 μg./ml.) at time 0. After periods of 10, 20, 30, 45 and 60 min. portions of the bacterial population were withdrawn from the nalidixic acid + GT + thymine medium, membrane filtered and resuspended in GT + thymine medium. The DNA breakdown observed in the several portions is expressed as a function of incubation time. The numerals adjacent to the curves indicate period of exposure to the nalidixic acid in min.

The close association of ability of bacteria to repair u.v.-induced lesions in the dark and DNA breakdown, has been commented on before (see e.g. Howard-Flanders & Boyce, 1966; G. W. Grigg, N. Powles & J. Smith, unpublished). It may be significant that of the several methods available of selectively inhibiting DNA synthesis temporarily or permanently, most, including thymine starvation, u.v. and x-irradiation, treatment with Mitomycin, Phleomycin or nalidixic acid, result also in DNA breakdown. Models to account for this correlation (Grigg, 1968; G. W. Grigg, N. Powles & J. Smith, unpublished) predict that any genetic or environmental impairment of repair synthesis or of the reaction rejoining disparate ends at chainbreaks would result

in enhanced attack at unprotected break points by degradative enzymes and the release of acid-soluble fragments from the DNA. As a consequence of this depolymerization single strand nicks would be converted into double strand (chromosome) breaks. DNA breakdown by nalidixic acid may occur by a similar mechanism.

I am greatly indebted to Mrs Jennifer Smith for her very capable assistance with this project.

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Nitrogen Fixation by Sulphate-reducing Bacteria

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SUMMARY

Nitrogen fixation has been obained with strains of *Desulfovibrio vulgaris* and *D. gigas*, organisms hitherto believed to be incapable of using molecular nitrogen. Fixation has been demonstrated by increases in total nitrogen and by uptake of $^{15}N_2$. Fixation of N_2 may be widespread in this genus of the sulphate-reducing bacteria.

INTRODUCTION

The research reported here was begun as a result of Postgate's comment (1965*b*) in a review that 'Most strains of Desulfovibrio do not fix nitrogen'. Enriched cultures from the mud of Lake Mendota, Wisconsin, not completely free of contaminating bacteria, readily fixed N_2; however, the pure cultures intended as negative controls, *Desulfovibrio vulgaris* NCIB 8303 HILDENBOROUGH and *D. gigas* NCIB 9332, also fixed in some experiments. Accordingly, the attempt to purify the new isolates was discontinued in favour of a more thorough examination of this unexpected development. The results, reported briefly at the Colloquium on Nitrogen Fixation held at Sanibel Island, Florida (Silver, 1967), are given in more detail here.

MATERIALS AND METHODS

Cultures. The culture of *Desulfovibrio vulgaris* strain NCIB 8303 (HILDENBOROUGH) originally was obtained from Sister M. Regina Lanigan, Rosary Hill College (Buffalo, N.Y.); later, subcultures of this same strain were obtained from Professors L. L. Campbell (University of Illinois) and J. R. Postgate (University of Sussex). The purity of the cultures was routinely checked by examination in a phase microscope and inoculation of Postgate's medium A (1951). These subcultures are designated by the letters R, C and P after the strain number 8303. Crude cultures of this organism were isolated from Lake Mendota mud using the technique described by Postgate (1965*a*). The sources of the other cultures were: *D. vulgaris* NCIB 8310 (NORWAY 4) from the NCIB; *D. gigas* NCIB 9332 from L. L. Campbell and from H. D. Peck (University of Georgia); *Desulfotomaculum nigrificans* ATCC 8351 from J. M. Akagi (University of Kansas).

Stock cultures were kept in screw cap test-tubes in Starkey liquid medium (1938) with 0·02 % thioglycollate and 0·01 % ascorbic acid added. In the nitrogen fixation

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studies, a modification of this medium was used containing added yeast extract and a trace element mixture prescribed by Dr Y. I. Shethna (personal communication): $ZnSO_4.7H_2O$, 1·1; $MnSO_4.H_2O$, 0·5; $CoSO_4$, 0·005; H_3BO_3, 0·005; Na_2MoO_4, 0·2; $CuSO_4.5H_2O$, 0·0007 mg./100 ml. medium. A 10 % inoculum was routinely used. The Desulfovibrio species were incubated at 30°, the *Desulfotomaculum nigrificans* at 55°.

Analyses. Total nitrogen was estimated with a modified micro-Kjeldahl method based on colorimetric estimation of ammonia. $^{15}N_2$ uptake was determined as follows: 20 ml. of culture in 50 ml. Erlenmeyer flasks was exposed to 0·15 atm. $^{15}N_2$ (33·0 atom % ^{15}N) and 0·75 atm. helium. A partial vacuum was left in the flask to allow for production of gas. The control was an inoculated culture which was boiled or to which 2 ml. of 20 % trichloroacetic acid was added. Burris & Wilson (1957) have described details of the methods of assay.

Table 1. *Nitrogen fixation by Desulfovibrio species*

Experiment	Cultures	Days of incubation	Nitrogen content in μg./ml.		
			Initial	Final	N. fixed
1	*D. vulgaris* 8303 R	3	8·8	16·9	8·1
2		3	13·5	26·2	12·7
3		5	35·6	43·8	8·2
			33·7	39·0	5·3
			35·4	44·0	8·6
4	*D. gigas* 9332 (CAMPBELL)	3	11·6	10·7	—
5		4	8·3	21·0	12·7
			14·0	22·4	8·4
6		5	41·2	51·0	9·8
7	*D. desulphuricans* 8310	4	15·2	31·4	16·2
	(NORWAY 4)		19·6	33·3	13·7
8		6	34·6	42·8	8·2
9	*D. gigas* 9332 (PECK)	5	23·0	26·5	3·5
10		6	29·8	36·9	5·1
			32·2	43·3	13·1

Initial nitrogen in the medium supplied as yeast extract.

RESULTS

Five enrichments from Lake Mendota mud were identified as predominantly strains of *Desulfovibrio vulgaris*. They reduced sulphate, produced desulfoviridin, and were progressively motile vibrios. Although, in nitrogen-fixation experiments, the sulphate-reducing vibrios were the predominant organisms in these isolates, microscopic examination revealed some contaminating bacteria, confirmed by growth on the medium described by Postgate (1951). In older cultures it was sometimes difficult to detect microscopically anything but Desulfovibrio forms; however, transfer of the culture into fresh medium containing combined nitrogen encouraged growth of the contaminants.

Initially, increases in the total nitrogen as measured by the micro-Kjeldahl technique were observed with the pure cultures, but since the medium contained 20 to 40 μg. N/ml. as yeast extract, the gains were often small relative to the initial nitrogen (Table 1). Experiments using $^{15}N_2$ as a tracer indicated that growing cultures of Desulfovibrio

species increased in total nitrogen (Fig. 1) and incorporated $^{15}N_2$ after several days incubation (Fig. 1, Table 2).

Fixation of nitrogen by duplicate cultures varied widely and was not observed in every experiment. For example, in Expt. 12, *Desulfovibrio vulgaris* 8303 P was transferred from a medium containing 0·1 % NH_4Cl to one containing 40 μg. N/ml. as yeast extract; no uptake of $^{15}N_2$ was observed. After two transfers in the yeast extract medium, the experiment was repeated and positive results were obtained. If too
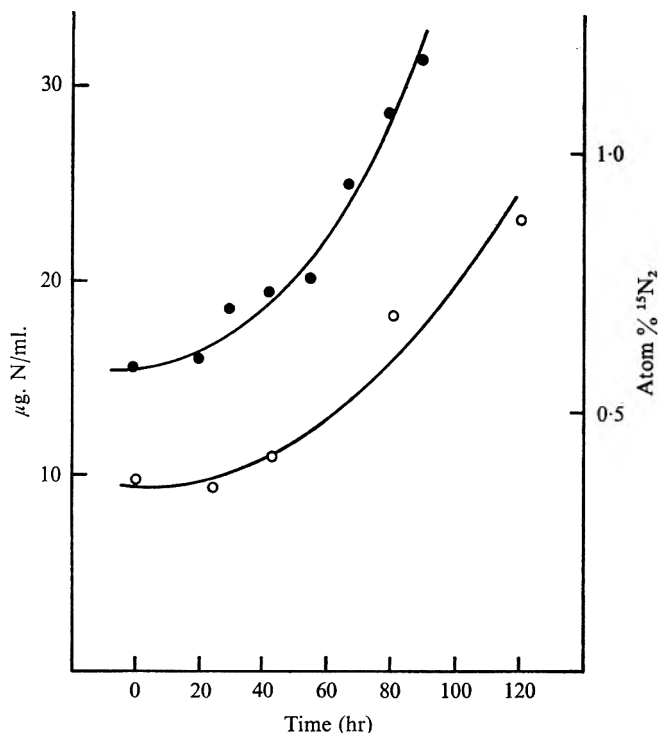
Fig. 1. Time course of nitrogen fixation by *Desulfovibrio vulgaris* 8303 R and *D. gigas* 9332. ●——●, Total nitrogen; ○——○, atom % $^{15}N_2$.

high a concentration of yeast extract was added, however, nitrogen was not always fixed. An initial concentration of 20 to 25 μg. N/ml. appeared to be sufficient to start growth without preventing fixation. We suggest that the function of the added combined nitrogen is to allow the organisms to grow enough to reduce the environment sufficiently for fixation to take place. Fig. 1 illustrates two experiments in which the necessary conditions evidently were achieved for consistent fixation of N_2. In no experiment did we obtain fixation with the thermophilic *Desulfotomaculum nigrificans*.

DISCUSSION

Desulfovibrio vulgaris 8303 had been examined for nitrogen fixation by Bach (1955) and several times by J. R. Postgate (personal communication), always with negative results. At our request, Professor Postgate re-examined this strain and *D. gigas*.

At first the results were negative, but he has now informed us that, using a technique developed by Campbell & Evans (1969), he has observed reproducible acetylene reduction, repressed by ammonium ions, in strains 8303 and 8310 as well as in several other Desulfovibrio strains hitherto believed to be incapable of nitrogen fixation. It thus appears that fixation of N_2 is relatively widespread in this genus.

Table 2. *Incorporation of $^{15}N_2$ by cultures of sulphate-reducing bacteria*

Experiment	Cultures	Atom % ^{15}N	μg. N fixed/ml.
11	Acid control	0·370	—
	D. vulgaris 8303 C	1·476	0·81
		1·675	0·96
		1·892	1·12
	D. gigas 9332	1·855	1·10
12	Boiled control	0·376	—
	Desulfotomaculum nigrificans 8351	0·373	—
		0·377	—
		0·377	—
	D. vulgaris 8303 P	0·374	—
		0·380	—
		0·378	—
13	Acid control	0·470	—
	D. vulgaris 8303 C	1·198	8·80
		0·537	0·81
	D. vulgaris 8303 P	0·748	3·36
		0·835	4·42
		0·839	4·46
	D. nigrificans 8351	0·378	—
		0·369	—
	D. gigas 9332	2·421	23·6
		1·846	16·9
		1·679	14·6
14	Acid control	0·378	—
	D. vulgaris 8303 P	1·369	16·6
		0·395	0·3
	D. vulgaris 8303 R	0·548	2·8
		0·467	1·5
	D. vulgaris 8303 C	1·572	19·9
		1·660	21·6

Expt. 11. Medium contained 20 μg. N/ml. as yeast extract and cultures were incubated 5 days.

Expt. 12. Incubation time was 4 days and the medium contained 40 μg. N/ml. The cultures of *D. vulgaris* 8303 were inoculated with cells grown on N_4^+H–N.

Expt. 13. The incubation time was 5 days and the medium contained 40 μg. N/ml.

Expt. 14. The incubation time was 4 days and the medium contained 20 μg. N/ml.

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The Effect of Chloramphenicol on Growth and Mitochondrial Function of the Flagellate *Polytomella caeca*

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SUMMARY

Chloramphenicol (1 mg./ml.) increased the mean doubling time of *Polytomella caeca* from 3·6 to 5 hr and gave 62 % inhibition of the final yield of organisms on an acetate + mineral salts + thiamine medium. The rates of acetate oxidation and tetrazolium reduction by whole organisms decreased progressively when grown in the presence of increasing chloramphenicol concentrations; no ultrastructural abnormalities of the mitochondria of such organisms were seen in electron micrographs. Succinoxidase and NADH oxidase activities of isolated mitochondria were decreased by 43 % and 72 %, respectively, by growth of organisms with 1 mg. chloramphenicol/ml. The content of cytochromes $(a + a_3)$ was decreased by 40 %, and cytochrome oxidase activity was about 50 % that of normal mitochondria. No alteration in the activities of rotenone-sensitive NADH-cytochrome c oxidoreductase or succinate-cytochrome c oxidoreductase was detected, using mammalian cytochrome c as electron acceptor. However, the rate of reduction of electron-transport components during the aerobic–anaerobic transition (with succinate as substrate) suggested that the supply of electrons to the respiratory chain was rate-limiting and might be more important in leading to respiratory deficiency than the decreased cytochrome oxidase activity. No major cytochrome dislocation was detected, suggesting that the respiratory chain was itself intact.

INTRODUCTION

Chloramphenicol is an inhibitor of growth of several eucaryotic micro-organisms. High concentrations are necessary to inhibit the growth of *Tetrahymena pyriformis* (Mager, 1960; Turner & Lloyd, 1970) and various species of yeast (Clark-Walker & Linnane, 1967), while some strains of *Saccharomyces cerevisiae* are sensitive to lower concentrations of the drug (Wilkie, Saunders & Linnane, 1967). The effect of chloramphenicol in eucaryotes appears to be specific inhibition of mitochondrial protein synthesis: certainly, *in vitro* sensitivity of mitochondrial systems from many sources has been demonstrated (Mager, 1960; Kroon, 1963; Lamb, Clark-Walker & Linnane, 1968).

Mitochondria of yeast cells grown with chloramphenicol have altered morphology, the cristae are less well defined than in normal cells; they also have a lower content of membrane-bound cytochromes (Huang, Biggs, Clark-Walker & Linnane, 1966). Indirect evidence suggests that the primary effect of chloramphenicol as a specific inhibitor of protein synthesis on mitochondrial ribosomes is unlikely to be on cytochrome synthesis itself. The only product of *in vitro* mitochondrial synthesis as yet

identified is a hydrophobic ('structural') protein (Roodyn, 1962; Haldar, Freeman & Work, 1966). Woodward & Munkres (1966) showed that alterations in the primary structure of such a protein resulting from an extrachromosomal mutation in Neurospora can lead to a pleiotropic respiratory deficiency due to altered binding of respiratory chain components. Thus chloramphenicol may act on the synthesis of 'structural' components necessary for the organization of the specific membrane configuration essential for integrated mitochondrial respiratory function.

The present work shows that chloramphenicol at high concentrations also inhibits the growth of the obligately aerobic flagellate *Polytomella caeca*. Although no change in mitochondrial ultrastructure can be seen in the electron microscope, the efficiency of mitochondrial electron transport is markedly decreased and impairment of the mechanism can be located biochemically at the flavoprotein region of the respiratory chain.

METHODS

Maintenance, growth and harvesting of the organism. Polytomella caeca Pringsheim was obtained in pure culture from the Culture Collection of Algae, Department of Botany, Indiana University, Bloomington, Ind., U.S.A. Growth and harvesting procedures have been described previously (Lloyd & Chance, 1968; Lloyd, Evans & Venables, 1968). Chloramphenicol was added as a solid to culture flasks after sterilization of the medium by autoclaving at $121°$ for 20 min.

Preparation of cell-free extracts and isolation of mitochondria. The preparation of mitochondrial suspensions was as described previously (Lloyd & Chance, 1968; Lloyd, Evans & Venables, 1968).

Analytical methods. Measurements of oxygen uptake by whole cell suspensions were made in a conventional Warburg apparatus (Umbreit, Burris & Stauffer, 1957). Polarographic measurements of oxygen uptake by mitochondrial suspensions were made with an oxygen electrode (Lloyd & Brookman, 1967). The reduction of 2,3,5-triphenyl tetrazolium chloride was followed by extraction of the red formazan in acetone and measurement of the extinction of the resulting solutions at 550 nm. ($\epsilon = 4450$). Measurements of the total cytochrome and flavoprotein content of mitochondria were performed by using a Cary model 14 split-beam spectrophotometer fitted with a 0 to $0·1$ extinction slide wire. Values were calculated from difference spectra (dithionite-reduced, oxidized), using the published extinction coefficients for the mammalian electron-transport components. Values for flavoproteins may include a contribution from non-haem iron, which also has an absorption at 465 nm. Steady-state reduction levels and half-times for reduction of components during the aerobic–anaerobic transition (state 4 → state 5, Chance & Williams, 1956) were measured with a dual-wavelength spectrophotometer (Chance, 1951). These measurements and those of oxygen uptake by mitochondrial suspensions were made in a buffer consisting of $0·25$ M-sucrose $+ 2$ mM-EGTA (ethyleneglycol-bis(β-amino-ethyl ether) N,N^1-tetraacetic acid) $+ 15$ mM-KH_2PO_4 + bovine serum albumin $0·15\%$ (w/v) at pH $7·4$. Protein was measured by the method of Lowry, Rosebrough, Farr & Randall (1951).

Enzyme assays. NADH-cytochrome *c* reductase ($NADH_2$: cytochrome *c* oxidoreductase EC $1.6.2.1$) was assayed in the presence of rotenone (20 mμmoles/ mg. protein) in the mitochondrial isolation buffer ($0·32$ M-sucrose $+ 2$ mM-EGTA $+ 20$ mM-tris HCl, pH $7·4$). Succinate-cytochrome *c* oxidoreductase was measured as

previously described. Both these assays were made at various cytochrome *c* concentrations, and a value for rates at infinite concentration of electron acceptor was obtained from a Lineweaver-Burk plot (Lloyd, 1966). Cytochrome *c* oxidase (cytochrome *c* : oxidoreductase EC 1.9.3.1) was measured by the method of Smith (1955), using mammalian cytochrome *c* which had been reduced by exposure to hydrogen and palladized asbestos.

Electron microscopy. Organisms were fixed in phosphate-buffered 1·5 % glutaraldehyde at pH 7·4 for 15 min., rinsed thoroughly in the phosphate buffer before postfixation in 1 % osmium tetroxide in phosphate buffer (pH 7·4) for 1 hr and steeped in 0·5 % (w/v) uranyl acetate for 12 hr. After dehydration in ethanol, embedding was in Araldite. Sections were cut at a nominal 500 Å thickness (grey/silver colour) with an LKB Ultramicrotome, and stained on the grids with lead citrate. Negative-staining was with K phosphotungstate (pH 6·8) (Parsons, 1963). The specimens were examined and photographed in an AEI EM6 at 60 kV.

Materials. Chloramphenicol was obtained both from Boots Pure Drug Co. Ltd., Nottingham, and from Parke-Davis & Co., Hounslow, Middlesex. [14C]chloramphenicol was from the Radiochemical Centre, Amersham, Bucks. NADH and cytochrome *c* were from Sigma Chemical Co., St Louis.
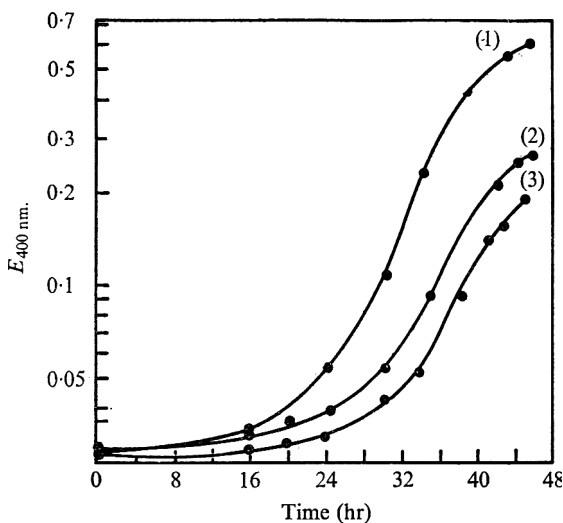
Fig. 1. Growth of *Polytomella caeca* with acetate in the presence of chloramphenicol. Cultures were grown at 25° with gentle forced aeration from standard small inocula (0·05 mg. dry wt) of acetate-grown organisms. (1) No chloramphenicol present; (2) + chloramphenicol 0·5 mg./ml.; (3) + chloramphenicol 1 mg./ml.

RESULTS

Experiments with whole organisms

Growth in the presence of chloramphenicol. In the absence of chloramphenicol the growth rate reached a maximum after about 30 hr; at this stage the organisms were growing exponentially with a mean doubling time of 3·6 hr (Fig. 1). In the presence of chloramphenicol (1 mg./ml.) the mean doubling time was 5·6 hr after a similar lag period. A progressive decrease in yield of organisms (at 42 hr) with increasing

chloramphenicol concentration was also observed; 1 mg. chloramphenicol/ml. led to a 62 % decrease.

Oxidative capacity. Organisms grown with chloramphenicol showed a decreased capacity for the oxidation of acetate (Fig. 2). Organisms taken from a culture containing chloramphenicol 1 mg./ml., when washed and incubated with acetate as a non-proliferating organism suspension, oxidized this substrate at two-thirds of the normal rate. There was a similar decrease in the ability of whole organisms to reduce 2,3,5-triphenyl tetrazolium chloride (Fig. 2).
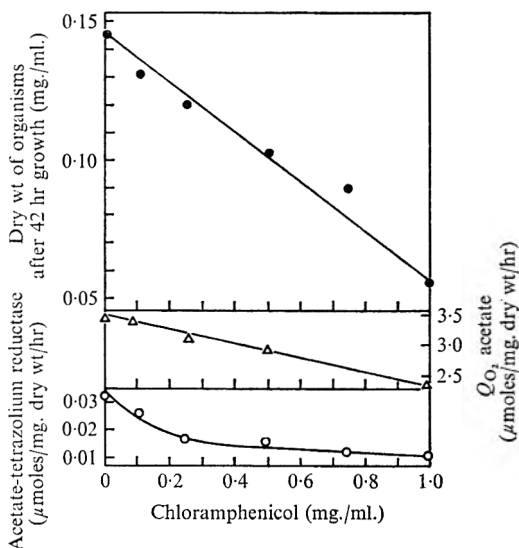
Fig. 2   Fig. 3

Fig. 2. Yields of *Polytomella caeca* after 42 hr growth in acetate medium at various chloramphenicol concentrations, and the rates of acetate oxidation and tetrazolium reduction by such cells. Shake cultures (10 ml.) were grown from small standard (equiv. 0·02 mg. dry wt organism) inocula. Growth was measured by measuring extinctions of suspensions at 400 nm. and converting to dry wt by means of a calibration curve (●). The Q_{O_2} values for acetate oxidation (△) were measured manometrically. The acetate + 2,3,5-triphenyl tetrazolium reductase activities (○) were assayed by following the extinction at 550 nm. of acetone extracts of the red formazan produced as a result of the reduction of the tetrazolium salt.
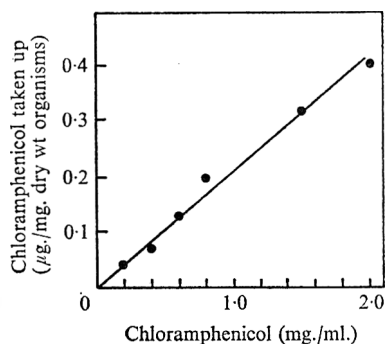
Fig. 3. Uptake of chloramphenicol by acetate-grown *Polytomella caeca*. Flasks containing 5·2 mg. dry wt organisms in acetate medium were incubated for 1 hr in the presence of various concentrations of (methylene-^{14}C)-D-*threo*-chloramphenicol. Samples of whole organisms were washed 4 times with growth medium and then assayed for radioactivity in an I.D.L. 'Tritomat' liquid scintillation counter, using the scintillation mixture described by Bray (1960).

Uptake of chloramphenicol. Organisms incubated in growth medium took up (methylene-^{14}C)-D-threo-chloramphenicol; retention of the label was proportional to the concentration of the antibiotic in the medium (Fig. 3). The packed volume of *Polytomella caeca* has been measured as 13·9 μl./mg. dry wt and of this volume 50 % is extracellular water. The figure of 0·4 μg. chloramphenicol/mg. dry wt of organisms, retained at a chloramphenicol concentration of 0·2 mg./ml. medium,

corresponds to a maximum intracellular concentration of about 70 μg. chloramphenicol/ml. This result suggests that the organisms do not actively accumulate the drug and that there may be a permeability barrier limiting its entry.

Electron microscopy

No marked alteration in mitochondrial morphology was evident in thin sections of organisms grown with chloramphenicol (Pl. 1, fig. 1, 2). As in normal organisms the mitochondria were numerous and showed extremely well developed and extensively invaginated inner (cristae) membranes. Negative staining with phosphotungstate revealed that the inner membranes of mitochondria from organisms grown in the presence of chloramphenicol still possessed their characteristic 9 nm. stalked subunits (Pl. 2, fig. 3).

Table 1. *Cytochrome and flavoprotein content of mitochondria isolated from Polytomella caeca grown with and without chloramphenicol*

Mean values presented together with standard deviations and number of different mitochondrial suspensions assayed.

	Content (mμmoles/mg. protein)	
Electron transport component	Without chloramphenicol	With chloramphenicol (1 mg./ml.)
Cytochrome $(a+a_3)$	0·56±0·03 (4)	0·34±0·04 (5)
Cytochrome b	0·47±0·05 (4)	0·33±0·05 (5)
Cytochrome c	0·23±0·04 (4)	0·23±0·1 (5)
Flavoprotein	0·28±0·04 (3)	0·34±0·05 (3)

Table 2. *Oxidative activities of mitochondria isolated from Polytomella caeca grown with and without chloramphenicol*

Figures presented are the mean values together with standard deviations and number of different mitochondrial suspensions assayed. Figures for succinoxidase and NADH oxidase refer to oxygen uptake rates; other figures are cytochrome redox rates.

	Specific activity (mμmoles/mg. protein/min.)	
Enzyme	Without chloramphenicol	With chloramphenicol (1 mg./ml.)
Succinoxidase	44±4 (5)	25±2 (5)
NADH oxidase	61±2 (4)	18±1 (4)
Succinate-cytochrome c oxidoreductase (V_{max})	61±9 (2)	69±6 (2)
NADH-cytochrome c oxidoreductase (rotenone insensitive) (V_{max})	51±11 (3)	51±9 (3)
Cytochrome c oxidase	1266±350 (6)	581±166 (7)

Experiments with isolated mitochondria

Cytochrome and flavoprotein content. The mitochondrial content of cytochromes and flavoproteins is shown in Table 1. There was a 40 % decrease in the content of cytochromes $(a+a_3)$ in mitochondria isolated from organisms grown with chloramphenicol, while the effect on cytochrome b was hardly significant and the cytochrome c content not measurably affected.

Electron-transport activities. Succinoxidase activity, a measure of the electron-transport capacity of the integrated respiratory chain, was decreased by 43 % in mitochondria from organisms grown with chloramphenicol (Table 2). Succinate-linked reduction of added mammalian cytochrome c was not significantly lower in such mitochondria, whereas the cytochrome c oxidase activity was reduced.

The pathway of electrons from externally added NADH to oxygen in intact mitochondria from *Polytomella caeca* is not precisely established, but there is evidence that the initial steps occur outside the NAD-impermeable barrier (Lloyd & Chance, 1968). A 72 % decrease of NADH-oxidase activity was produced by growth in the presence of choramphenicol, although the rotenone-insensitive NADH-cytochrome c reductase activity of intact mitochondria was unaffected.

Table 3. *Steady-state levels of reduction of electron-transport components (substrate 6 mM-succinate), and half-times for their reduction in the aerobic–anaerobic transition (state 4 → state 5) in mitochondria from Polytomella caeca grown in the absence and presence of chloramphenicol*

100 % reduction of a component taken as anaerobic reduction level minus level in absence of exogenous substrate (state 5–state 2) (Chance & Williams, 1956). Absence of a figure indicates that if any reduction occurred it was below the limit of detection. Figures representative of results obtained with eight different mitochondrial suspensions. Temperature of incubation 20°.

Electron-transport component	Cytochrome				Flavo-protein
	$a(+a_3)$	$a_3(+a)$	c	b	
Wavelength pair (nm.)	605–630	445–455	550–540	430–410	465–510
Without chloramphenicol					
$t\frac{1}{2}$ red^n state 4 → state 5 (sec.)	2·0	2·5	1·0	1·3	1·3
% red^n state 4	5·0	—	21·0	48·0	26·0
% not reduced in state 5 but reduced by dithionite	—	—	—	33·0	39·0
With chloramphenicol					
$t\frac{1}{2}$ red^n state 4 → state 5 (sec.)	7·5	7·5	5·0	7·0	7·0
% red^n state 4	—	6·4	14·0	44·3	23·2
% not reduced in state 5 but reduced by dithionite	—	—	—	37·0	49·0

An investigation of the steady-state levels of reduction of the various electron-transport components during succinate respiration indicated that there are no marked differences in the cytochrome or flavoprotein redox levels between mitochondria from organisms grown normally and with chloramphenicol (Table 3). However, all the electron-transport components studied in mitochondria from organisms grown with chloramphenicol showed a three- to five-fold slower response during the aerobic (state 4) to anaerobic (state 5) transition. In the anaerobic state cytochromes $(a+a_3)$ and c were fully reduced, and the addition of excess dithionite produced no further responses. Further reduction of cytochrome b and flavoprotein was, however, produced on dithionite addition to anaerobic mitochondrial suspensions; the dithionite-reducible flavoprotein accounted for a greater proportion of the total flavoprotein in mitochondria which had been isolated from organisms grown with chloramphenicol.

DISCUSSION

Growth of *Polytomella caeca* in the presence of chloramphenicol, as is the case with various species of both facultatively anerobic and obligately aerobic yeasts (Clark-Walker & Linnane, 1967) and also *Pythium ultimatum* (Marchant & Smith, 1968), leads to respiratory deficiency. Whereas in yeast this deficiency is correlated with disorganized mitochondria and a partial loss of all membrane-bound cytochromes (a, a_3, b, c_1), these organelles in chloramphenicol-grown *Polytomella caeca* are not structurally impaired as seen at the resolution possible in the electron microscope, and are deficient only in cytochrome oxidase. However, the turnover number of cytochrome oxidase is considerably greater than the turnover number required to account for the over-all electron-transport rate or oxygen consumption of isolated mitochondria (Chance, 1967; Lloyd & Chance, 1968); the over-all decreased electron-transport capacity of *Polytomella caeca* mitochondrial suspensions appears to result from an impairment at the substrate end of the respiratory chain, rather than at the level of cytochrome oxidase. Thus, when oxygen is exhausted by respiration and the suspensions become anaerobic, the rates of reduction of *all* the electron-transport components studied are decreased. This, and the fact that the steady-state redox levels during succinate respiration are hardly altered, suggests that flavoprotein–cytochrome and cytochrome–cytochrome interactions are not interrupted in mitochondria from cells grown with the drug. Rather, it is a decreased supply of electrons at the dehydrogenase level which accounts for the lowered respiration rate of isolated mitochondria. Further evidence for the dislocation of flavoprotein (but not of cytochromes with the possible exception of cytochrome b) during growth with chloramphenicol is provided by the finding that the proportion of non-enzymically active flavoprotein (reducible only on dithionite addition) is increased. Tetrazolium salts accept electrons at the flavoprotein–cytochrome b region of the respiratory chain (Lester & Smith, 1961); thus the decreased capacity for dye reduction in intact organisms after growth with chloramphenicol reinforces the view that the lesion of electron transport occurs at the dehydrogenase level.

The characteristic 9 nm. inner mitochondrial membrane subunits have been identified as F_1-ATPase, the terminal enzyme of oxidative phosphorylation in mammalian mitochondria; the oligomycin-sensitivity of this enzyme is dependent on the binding of the F_1 particles to the inner membrane (Kagawa & Racker, 1966). Schatz (1968) observed a decreased oligomycin sensitivity of F_1-ATPase in chloramphenicol-grown yeast, and Marchant & Smith (1968) were unable to detect the stalked subunits in the otherwise ultrastructurally normal mitochondria of chloramphenicol-grown *Pythium ultimatum*. Membrane binding of F_1-ATPase in *Polytomella caeca* mitochondria cannot be assessed by its oligomycin-sensitivity (no inhibition up to 200 μg./mg. protein, D. Lloyd, unpublished) but negative staining clearly revealed the presence of stalked 9 nm. inner membrane subunits. It is of interest that these particles are not detached after growth with chloramphenicol in *Polytomella caeca*; changes in cytochrome redox states on ADP addition (D. Lloyd, unpublished results) confirm that these mitochondria are still capable of carrying out oxidative phosphorylation.

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Plate 1

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0·1 μm.

3
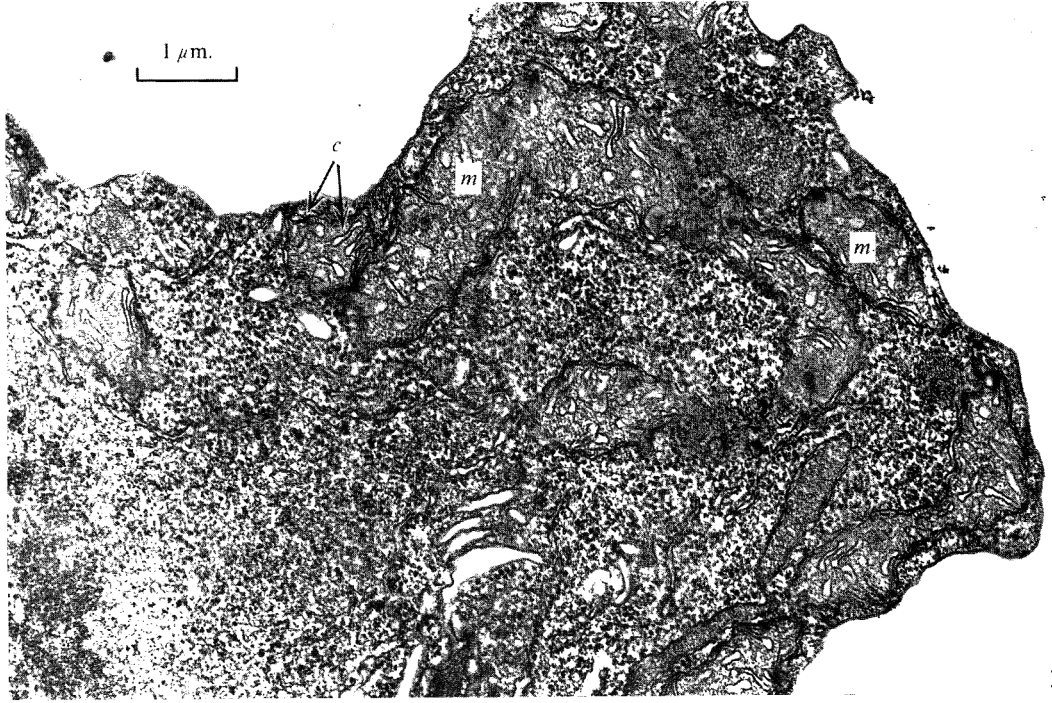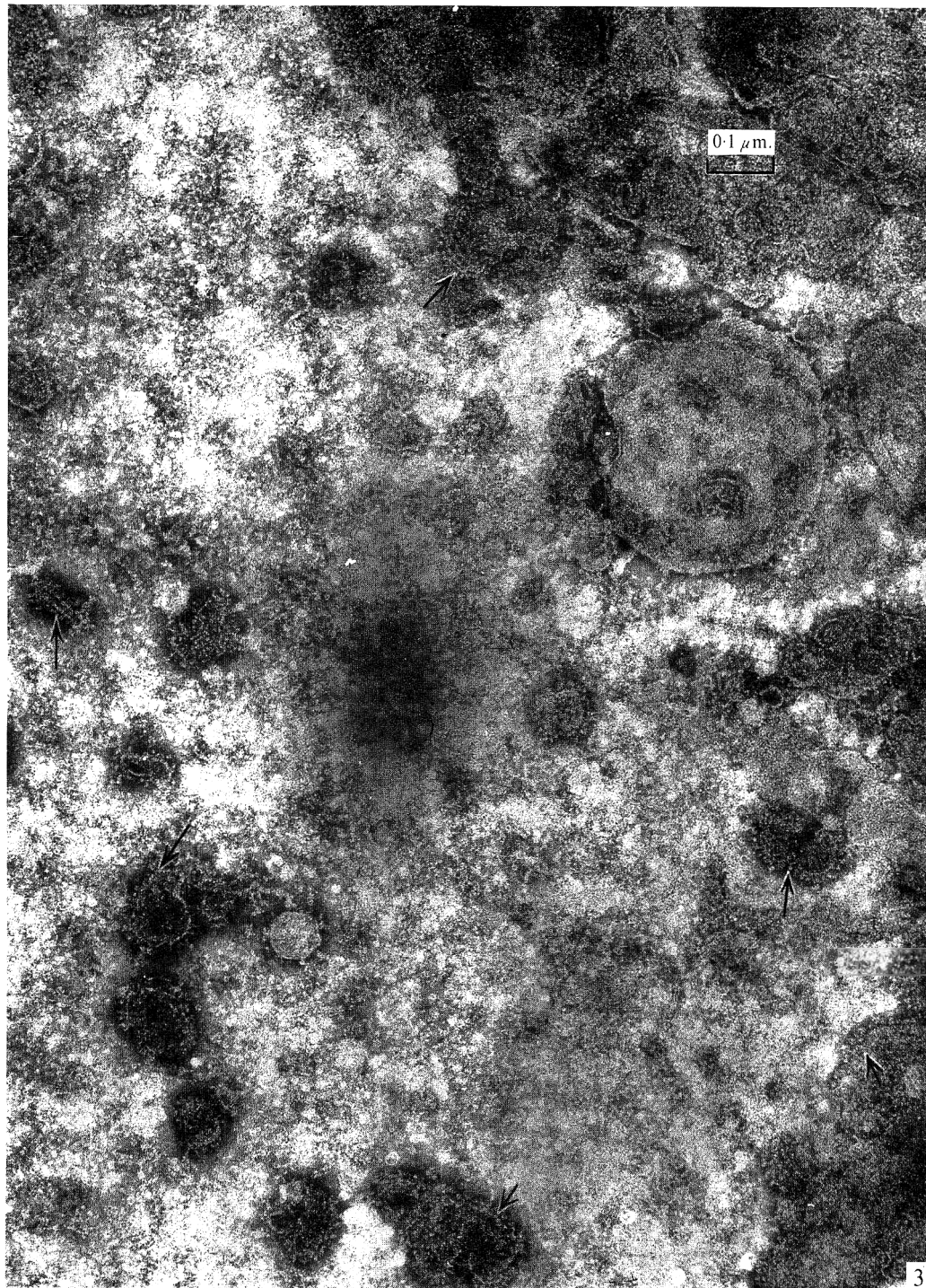
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EXPLANATION OF PLATES

PLATE 1

Fig. 1. *Polytomella caeca:* section of organism grown without chloramphenicol showing contractile vacuole (*cv*), two flagella (*f*), Golgi region (*g*) and mitochondria (*m*). × 18,000.

Fig. 2. Section of organism grown in the presence of chloramphenicol 1 mg./ml., showing mitochondria (*m*) with numerous cristae (*c*). × 18,000.

PLATE 2

Fig. 3. Mitochondria isolated from *Polytomella caeca* grown with chloramphenicol 1 mg./ml. and negatively stained with K phosphotungstate. Arrows indicate rows of stalked particles with 9 nm. heads which cover the inner surface of the inner mitochondrial membrane. × 120,000.

A Comparative Study of Eight Distinct β-Lactamases Synthesized by Gram-negative Bacteria

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SUMMARY

The enzymic properties of 46 strains of β-lactamase-producing enteric bacteria were examined. Eight distinct types of β-lactamase were detected among these strains when substrate profile, sensitivity to *p*-chloromercuribenzoate (*p*CMB) and to cloxacillin inhibition, reaction with antiserum and charge properties are used as parameters of enzyme type. The types of enzyme detected ranged from molecules with a predominantly cephalosporinase profile to those where penicillins were hydrolysed much more rapidly than cephalosporins. The majority of isolates synthesized an enzyme that was almost equally active against penicillins and cephalosporins. To date only three of the eight types of enzyme have been shown to be transferable by conjugation.

INTRODUCTION

So far, three distinct types of β-lactamases (E.C. 3.5.2.6) have been purified from Gram-negative bacterial species. These are the penicillinase synthesized by a strain of *Escherichia coli* carrying the R-factor R_{TEM} (Datta & Kontomichalou, 1965; Datta & Richmond, 1966), the cephalosporinase from *Enterobacter cloacae* strain 214 (Hennessey, 1967; Hennessey & Richmond, 1968) and the enzyme from *E. coli* strain K12 (Lindström, Boman & Steele, 1970). The enzyme from *E. cloacae* 214 is almost certainly identical to the enzyme partially purified from *Aerobacter cloacae*, strain P99, by Goldner, Glass & Fleming (1968). In addition to this, there have been many reports over the last few years that describe the properties of β-lactamases synthesized by a wide range of Gram-negative organisms (e.g. Smith, 1963; Ayliffe, 1965; Hamilton-Miller, 1963, 1967; see also reviews by Citri & Pollock, 1966; Evans, Galindo, Olarte & Falkow, 1968; Sawai, Mitsuhashi & Yamagishi, 1968; Hamilton-Miller, Smith & Knox, 1969). In most cases these studies were made with crude enzyme preparations, or sometimes even on broken cell preparations, and classification was often based on only a narrow range of enzyme characters. Furthermore, where the substrate profile of an enzyme has been investigated, the results are often difficult to interpret since a wide range of different assay techniques has been used by different workers (using the iodometric, manometric and hydroxamate methods, or by bioassay), under various conditions of pH value and temperature. From this information (much of it conflicting) two facts do emerge clearly. First, certain enzymes may be inactivated irreversibly, as far as hydrolysis of ampicillin and benzylpenicillin are concerned at least, by pre-treatment with cloxacillin or methicillin (Hamilton-Miller, Smith & Knox, 1965) in a manner reported earlier for staphylococcal penicillinase (Gourevitch, Pursiano & Lein, 1962). Secondly, some, but not all, β-lactamases from

Gram-negative bacteria may be inhibited by treatment with p-chloromercuribenzoate (pCMB) (Smith, 1963). This last observation implies that some β-lactamases from Gram-negative bacteria contain at least one cysteine residue. It is therefore difficult to be clear how many different types of β-lactamase are to be found among Gram-negative bacteria and what is their distribution among the various species.

In an attempt to clarify this, it was decided to examine the properties of the β-lactamases synthesized by a range of ampicillin-resistant Gram-negative strains (excluding pseudomonads) freshly isolated from a wide variety of sources. For this purpose each enzyme was isolated in crude form and characterized in terms of its substrate profile against benzylpenicillin, ampicillin and cephaloridine; and for its sensitivity to pCMB and to cloxacillin inhibition. In addition each enzyme was tested for its reaction with a rabbit antiserum prepared against purified β-lactamase from *Escherichia coli* carrying R_{TEM}; where a positive result was obtained, the detailed kinetics of interaction were investigated further. A final test was to examine the electrophoretic mobility of partially purified preparations after electrophoresis in starch gel at pH 8·5. This last test also provides a check against the possibility that a single bacterial strain might be synthesizing two distinct lactamase proteins.

On the basis of these parameters, it has been possible to distinguish eight distinct types of β-lactamase among the strains examined here. The enzymes range from a molecule that is almost totally a 'cephalosporinase' in its substrate profile, to one that is almost entirely a 'penicillinase'. Other characteristics show that the strains may be grouped in other ways, the closest correlation being that all enzymes that were sensitive to pCMB were resistant to inhibition by cloxacillin. Only three of the eight types of enzyme examined here were mediated by genes carried on transmissible R-factors present in the strains at the time of isolation.

METHODS

Strains and growth conditions. The β-lactamase producing organisms used in this work, and their origins are shown in Table 1. They were grown routinely in 0·1 % CY medium (Novick, 1963) which has the following composition: sodium β-glycerophosphate, 0·12 M; $MgSO_4.5H_2O$, 1·0 mM; trace metal solution, 0·02 ml./l.; yeast extract (Oxoid), 1·0 % (w/v); Casamino acids (Oxoid), 1·0 % (w/v); glucose, 0·4 % (w/v). The trace metal solution contained (%): $CuSO_4.5H_2O$, 0·5; $ZnSO_4.7H_2O$, 0·5; $FeSO_4.7H_2O$, 0·5; $MnCl_2.4H_4O$, 0·2; conc. HCl, 10 (v/v). The glucose and β-glycerophosphate were sterilized separately and added just before use. Agar (1·5 %, w/v) was added to this medium when required. A mutant of *Escherichia coli* K 12 W 3110 that synthesized alkaline phosphatase at a fully derepressed rate in the presence of inorganic phosphate was used to detect R-factor transfer. This strain was obtained as described by Lee & Richmond (1969).

β-Lactamase assay. β-Lactamase activity was normally assayed iodometrically by the method of Perret (1954) as modified by Novick (1962) except that the assays were done at pH 5·9 rather than pH 7·0. The quantity of benzylpenicillin and ampicillin destroyed in this assay was calculated from the fact that each mole of hydrolysed benzylpenicillin reacted with eight equivalents of iodine (Alicino, 1946); the same equivalence was assumed for ampicillin. The hydrolysis of cephaloridine was followed similarly by assuming that 1 mole of cephaloridine reacted with four equivalents of iodine

Table 1. *The sources and some characteristics of the 46 β-lactamase-producing organisms used in these experiments*

Series no.	Source no.	Tentative identification	Source	Reference (if any)
I	TEM	*E. coli* (R^+_{TEM})	Dr N. Datta	Datta & Kontomichalou (1965)
2	53	*Aerobacter cloacae*	Dr J. T. Smith	Smith (1963)
3	481	*Klebsiella* sp.	U.B.H.	—
4	241	*Aeromonas* sp.	U.B.H.	—
5	418	*K. aerogenes*	Dr J. M. T. Hamilton-Miller	Hamilton-Miller (1963)
6	071	*E. coli*	U of B	—
7	1266	*Proteus morganii*	U.B.H.	—
8	D 539	*Klebsiella* sp.	U.B.H.	—
9	2/37	*Aeromonas* sp.	U.B.H.	—
10	2/46	*Aerobacter* sp.	U.B.H.	—
11	8	*E. coli*	U of B	—
12	18	*Aeromonas* sp.	U of B	—
13	2/40/1	*E. coli*	U.B.H.	—
14	2/40/2	*E. coli*	U.B.H.	—
15	NPL 3	*E. coli*	U of B	—
16	D 31	*E. coli*	Dr H. Boman	Lindström *et al.* (1970)
17	D 535	*Klebsiella* sp.	U.B.H.	—
18	CJL	*E. coli*	U of B	—
19	1082E	*Enterobacter cloacae*	Dr C. O'Callaghan	—
20	466	*Klebsiella* sp.	U.B.H.	—
21	214	*Enterobacter cloacae*	Dr T. Hennessey	Hennessey (1967)
22	P 99	*Aerobacter cloacae*	Dr T. Hennessey	Fleming *et al.* (1963)
23	284	*E. coli*	U.B.H.	—
24	1390	*Aeromonas* sp.	U.B.H.	—
25	1169	*Klebsiella* sp.	U.B.H.	—
26	1758	*E. coli*	U.B.H.	—
27	264	*Klebsiella* sp.	U.B.H.	—
28	D 1144	*E. coli*	U.B.H.	—
29	1316	*E. cloacae*	U.B.H.	—
30	153	*E. cloacae*	U.B.H.	—
31	1929	*E. cloacae*	U.B.H.	—
32	612	*Aerobacter* sp.	U.B.H.	—
33	177	*Enterobacter cloacae*	U.B.H.	—
34	719	*E. coli*	U.B.H.	—
35	533	*Enterobacter cloacae*	U.B.H.	—
36	311	*Klebsiella* sp.	U.B.H.	—
37	347	*Klebsiella* sp.	U.B.H.	—
38	9527	*Klebsiella* sp.	N.C.T.C.	—
39	115	*Klebsiella* sp.	N.C.T.C.	—
40	3117	*Proteus vulgaris*	U.B.H.	—
41	385	*Alkaligenes* sp.	U.B.H.	—
42	Denston 5003	*E. coli*	A.H.C.	—
43	McDonald	*Klebsiella* sp.	A.H.C.	—
44	Shine	*Klebsiella* sp.	A.H.C.	—
45	Wickham	*E. coli*	A.H.C.	—
46	Robinson	*Klebsiella* sp.	A.H.C.	—

Abbreviations: U.B.H., United Bristol Hospitals; U of B, University of Bristol, Department of Bacteriology; A.H.C., Addenbrookes Hospital, Cambridge; N.C.T.C., National Collection of Type Cultures.

All strains were identified according to the criteria of Cowan & Steel (1966) except where reference is quoted.

after hydrolysis as was found for cephalosporin (Alicino, 1961). Activities are expressed in units as defined by Pollock & Torriani (1953).

For certain purposes the hydrolysis of cephaloridine was followed spectrophotometrically by measuring the decrease in absorption at 255 nm. that accompanied the breaking of the β-lactam bond in this antibiotic. The advantage of this method is that it allows the hydrolysis of cephaloridine to be followed in mixtures containing benzylpenicillin, ampicillin or cloxacillin, since these compounds have no significant absorption at 255 nm. For most purposes 3 ml. of a 100 μM solution of cephaloridine was used for this assay. This amount of substrate had an initial extinction of about 1·4 at 255 nm.

Substrate profiles. Substrate profiles were determined with benzylpenicillin, ampicillin and cephaloridine as substrates on crude preparations of enzyme. All these compounds were used at 6 mM. The profiles were calculated against an arbitrary value of 100 for the rate of hydrolysis of benzylpencillin and are quoted in the form of three values separated by a colon. Thus a profile 100:150:175 indicates an enzyme that hydrolyses ampicillin at 1·5 times the rate, and cephaloridine at 1·75 times the rate, of benzylpenicillin.

Anti-TEM serum production. Penicillinase was purified from *Escherichia coli* (R_{TEM}) by the method of Datta & Richmond (1966) and antiserum prepared against this enzyme essentially as described by Pollock (1964). The antigen was injected intramuscularly at 4-day intervals into alternate thigh muscles of rabbits as a suspension of 5 mg. enzyme/ml. Freund's adjuvant (Freund & Bonanto, 1941). Initially 0·2 ml. was injected, but subsequently the dose was increased to 0·5 ml.; 8·5 mg. in all was injected. After 2 weeks, injection in the marginal ear vein was recommenced at 2-day intervals with alum-precipitated enzyme preparation containing about 2·5 mg. antigen/ml.; in this case a total of 7·5 mg. of enzyme was injected. After a further 14 days, blood was taken from the rabbits and serum prepared by allowing the blood to clot for 2 hr at room temperature, followed by standing overnight at 2°. The clot was then loosened and the liquid serum removed, the serum centrifuged at 1500 *g* for 30 min. at 2° in a refrigerated centrifuge and stored at −20°.

Enzyme purifications. Enzyme preparations from *Escherichia coli* (R_{TEM}) and *Enterobacter cloacae* strain 214 were purified by the methods described by Datta & Richmond (1966) and Hennessey & Richmond (1968), respectively. The first of these two enzymes is commonly referred to as TEM-enzyme or TEM-lactamase. Crude preparations of all other enzymes examined here were made as follows. An overnight culture of the organism in question, grown in liquid CY medium, was taken and samples (17 ml. at a time) were disrupted for 2·5 min. at room temperature in a Mullard 25 kcyc./sec. ultrasonic disintegrator (Measuring and Scientific Instruments Ltd.). After breaking, the preparations were stored at 2° until required for use. In certain cases where the amount of lactamase synthesized by the organisms was very small, the original culture was centrifuged at 5000 *g* for 15 min. and the pellet suspended at ten times the original suspension density in 0·1 M-phosphate buffer (pH 5·9) before breaking.

Partially purified preparations of the enzymes studied here were prepared by one of two methods, depending on the ionization properties or the enzyme concerned. Both methods of preparation involved an initial disruption of the organisms in the ultrasonic disintegrator. For this an overnight culture grown in CHY medium and con-

taining about 3 mg. dry wt organisms/ml. was centrifuged for 40 min. at 15,000 *g*.
After centrifugation, the organisms were suspended to about 50 mg. dry wt/ml. in
0·1 M-phosphate buffer (pH 5·9). This suspension was then disrupted as described
above and the broken organisms stored at 2° until breaking was complete. The broken
organisms were then centrifuged at 30,000 *g* for 50 min. at 2° and the pellet discarded.
The supernatant fluid obtained from the 30,000 *g* centrifugation was then dialysed
overnight at 2° against 1000 times its volume of distilled water, centrifuged once more
at 30,000 *g* for 50 min. to remove debris that appeared on dialysis, and stored at 2°
until required for loading on to the appropriate substituted cellulose column.

Two types of substituted cellulose were used to obtain partially purified enzyme:
carboxymethyl-cellulose (CM) and diethyl-amino-ethyl-cellulose (DEAE). Both were
prepared for use in a similar manner, the only difference being that with CM the
phosphate buffer used was at pH 5·9, whereas with DEAE it was at pH 7·0. The
substituted celluloses were suspended in 0·1 M-phosphate buffer of appropriate pH
value and the fines removed. The material was then poured as a column in phosphate
buffer and washed with the appropriate buffer until the effluent had reached the pH
value of the washing buffer. The column was prepared for use by washing with dis-
tilled water until no free phosphate was detected in the effluent. The columns used in
these experiments were normally about 1·5 cm. in diameter and 10 to 20 cm. long;
about 500 ml. distilled water was required to free them of unbound phosphate.
Enzyme preparations were loaded on to these columns in a volume of 2 to 5 ml. and
washed in with distilled water. Distilled water was then used to elute the column
until the effluent contained no protein. This step normally required about 150 ml.
water; during this step much protein and pigmented material, but less than 5 %
of the penicillinase, passed through the column. The enzymes were then eluted from
the column with one of two eluting buffers, depending on the nature of the column.
With DEAE-columns the eluting buffer was 0·1 M-phosphate buffer (pH 7·0); with
CM-columns the buffer was 0·2 M-phosphate buffer (pH 5·9). Elution of u.v. absorbing
material was followed at 254 nm. with a Uvicord I column monitor (LKB Instruments);
β-lactamase in the samples was assayed iodometrically. Chromatography of the
enzymes studied here was normally carried out at room temperature. The only ex-
ceptions were enzymes known to be sensitive to *p*CMB since experience showed
that these enzymes were more labile; in these cases purification was done at 2°.

Starch-gel electrophoresis. Starch blocks for electrophoresis were prepared in 0·03 M-
borate buffer (pH 8·5) by the method of Smithies (1955). Semi-purified enzyme pre-
parations to be examined were loaded into slots cut in the gel, about 1000 units enzyme
in 0·075 ml. being loaded into each slot. The gels were then run for 2·5 hr with a poten-
tial difference of 300 V, using two buffer vessels on each side of the gel joined by a
bridge. The vessel carrying the electrode was filled with saturated NaCl solution and
the vessel next to the gel with 0·3 M borate buffer (pH 8·5). After completion of the
run the gel was sliced into two portions: one was stained for the presence of protein
with the amido-black method (Offord, 1969); the other was used to locate *β*-lactamase
activity; this was achieved by spraying the gel with a solution of 30 mg. benzylpenicillin
in a solution containing 0·016 M-I_2 + 0·12 M-KI + 0·1 M-phosphate buffer (pH 5·9).
The iodine in this reagent coloured the gel blue except where the lactamase converted
benzylpenicillin to penicilloic acid, thus producing a colourless zone. In practice all
the enzymes examined here (including those with a predominant activity against

4

cephalosporins) hydrolysed benzylpenicillin sufficiently rapidly to give a positive test under these conditions. After staining, the position of the enzyme was measured on the gel, its mobility calculated and the value expressed as cm./hr.

Antiserum assays. The effect of anti-TEM serum on purified TEM enzyme was studied iodometrically as described for the reaction between anti-penicillinase serum and purified penicillinase from *Staphylococcus aureus* (Richmond, 1963). The standard curve obtained when increasing quantities of anti-TEM serum were added to a fixed quantity of TEM-lactamase (constant antigen titration) is shown in Fig. 1. The neutralization titre of the serum (used at 1/10 dilution in physiological saline) used in these experiments was 17 units/0·01 ml. serum; the maximum degree of neutralization of the TEM-enzyme was approx. 60 % at the equivalence point (*E*, Fig. 1).
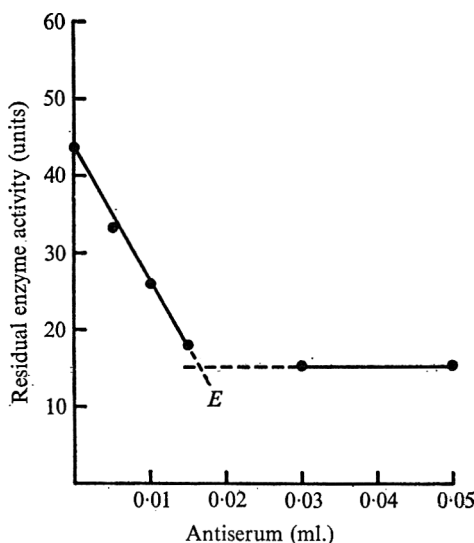
Fig. 1. Neutralization curve obtained when TEM-β-lactamase was titrated with anti-TEM serum (constant antigen titration). *E*: equivalence point.

Preparations of β-lactamase for test were compared with TEM-enzyme by measuring the degree of neutralization obtained with a test quantity of serum. Usually about 50 units enzyme were used in the test and the control assay and the effect of either 0·03 or 0·05 ml. serum determined. These conditions were chosen to give complete neutralization of the 50 units of enzyme used. Only when the effect of the serum on the test enzyme under these conditions was markedly different from that obtained with TEM-enzyme was the reaction between serum and TEM-enzyme examined in greater detail. Although unsatisfactory, this procedure was necessary to conserve serum.

Sensistivity to pCMB. Crude enzyme preparations were mixed with pCMB solutions to give a final concentration of 0·5 mM and the mixture incubated at 30° for 10 min. The residual enzyme activity was then estimated iodometrically. An enzyme sample incubated in absence of inhibitor acted as control. Sensitive enzyme preparations were inhibited by more than 80 % by this treatment.

Sensitivity to cloxacillin. The sensitivity of the enzymes to cloxacillin was measured

by using cephaloridine as substrate since the spectrophotometric method of assaying cephalosporins allowed the hydrolytic activity of the enzyme to be followed against this substrate even when another potentially sensitive β-lactam compound was present in solution. A solution containing 10^{-4} M-cloxacillin and 10^{-4} M-cephaloridine in 0·1 M-phosphate buffer (pH 7·0) was incubated for 5 min. in a cuvette in a continuously recording spectrophotometer and the spontaneous breakdown of the cephaloridine component of the mixture followed. A second cuvette containing cephaloridine alone in 0·1 M buffer was incubated in parallel to give the control rate. At the end of this period about 50 units enzyme were added to each cuvette and the hydrolysis of cephaloridine followed for a further 15 min. The degree of inhibition caused by the presence of the cloxacillin was then calculated by comparing the initial rate of hydrolysis of cephaloridine in the two cuvettes. A 'resistant' enzyme was defined as one that retained more than 65 % of its activity in the presence of cloxacillin. All 'sensitive' enzymes in this series showed less than 25 % of their normal activity in the presence of the inhibitor. It must be stressed both in the case of cloxacillin and pCMB-inhibition that 'sensitivity' of the enzymes was always more significant than 'resistance', since the assays were made with crude enzyme preparations and 'resistance' could be due to the presence of compounds that inactivated the inhibitors.

Transferability of β-lactamase genes. The transferability by conjugation of the genes concerned with β-lactamase synthesis in these strains was determined by mating experiments in which the potential donor culture was mixed with a standard recipient strain (Lee & Richmond, 1969). The recipient used was a mutant of *Escherichia coli* K12 W3110 that was sensitive to ampicillin and that synthesized alkaline phosphatase at a fully de-repressed rate even when grown on media containing high concentrations of inorganic phosphate. The advantage of this recipient is that it is easy to identify by staining the colonies for the presence of alkaline phosphatase. The test was done by mixing exponentially growing cultures (about 10^6 bacteria/ml.) in the ratio of 1 volume donor culture to 10 volumes recipient. The mixture was incubated at 35° in stationary culture, and after overnight growth the organisms were plated on agar containing 25 μg. ampicillin/ml. Any ampicillin-resistant colonies that grew on these plates were derived either from the donors or from recipients that had received the β-lactamase gene from the donor, since transfer of the alkaline phosphatase genes in the opposite direction (i.e. from recipient to donor) did not occur at a detectable rate with the recipient strain used here (Lee & Richmond, 1969). The recipient colonies that had received β-lactamase were distinguished from donor colonies by staining for the presence of alkaline phosphatase with a mixture of α-naphthyl-phosphate and 3,3'-dimethoxybenzidine (Fast Blue B salt; G. T. Gurr Ltd., London; Menten, Junge & Green, 1944) as described by Lee & Richmond (1969). The presence of about 5 colonies/plate in which β-lactamase synthesis was associated with production of alkaline phosphatase was taken to denote the presence of a transferable lactamase gene in the donor.

Buffers. All phosphate buffers were $K_2HPO_4 + KH_2PO_4$ mixtures.

RESULTS

Substrate profile and total amount of protein synthesized

The enzymes from 43 strains were examined for their substrate profiles as described in Methods; the results plotted in Fig. 2. In addition enzymes from *Escherichia coli* (R_{TEM}), *Aerobacter cloacae* strain P99 and *Enterobacter cloacae* strain 214 were examined in the same way and the results also plotted. These preliminary results showed four broad types of substrate profile: group I (including enzyme from *E. coli* (R_{TEM}) and the majority of the other strains tested) had a profile
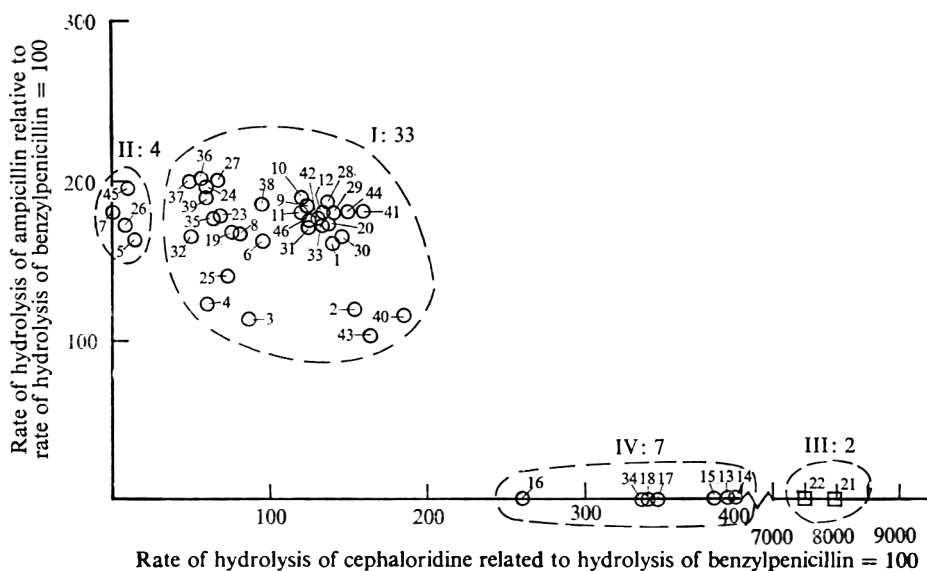
Fig. 2. Substrate profiles of the 46 β-lactamase synthesizing strains examined. The origin of the graph indicates a value for the rate of benzylpenicillin hydrolysis = 100. The numbers appended to each small circle refer to the identification numbers of the strains in the series (see Table 1). The dashed circles enclose strains grouped to show the broad enzyme types (I, II, III, IV) described in the text. The large figures next to the roman numerals indicate the number of strains in the group in question. O, Strains plotted on the basic scale; □, strains plotted on abscissa with scale 12·5 times smaller.

100:120–200:80–150; group II had a profile 100:180:0–15; group III (containing only the enzymes from *A. cloacae*, strain P99 and *E. cloacae* strain 214) had a profile 100: < 10:8000; group IV had a profile 100: < 10:350. Each of these groups is outlined with a dotted line in Fig. 2. The number of strains in each group is also shown in Fig. 2.

Calculation of the specific β-lactamase activity (enzyme units determined with benzylpenicillin as substrate/mg. dry wt organism) of these strains gave the results shown in Table 2. When an arbitrary division is set at a specific activity of 10 enzyme units/mg. dry wt and these results are correlated with substrate profile, an almost complete division of group I into two subgroups can be seen (Fig. 3): almost all strains with a specific activity greater than the limiting value lie on the right-hand side of the group round an average profile of 100:120–200:150, while strains express-
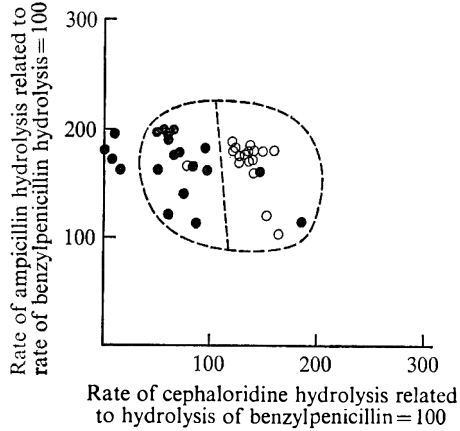
Fig. 3. Fig. 2 replotted to show the strains that synthesize *β*-lactamase at a rate less than 10 units/mg. dry wt organisms with benzylpenicillin (●), and those where enzyme synthesis exceeds this limit (○). The origin of the graph indicates a ratio of benzylpenicillin hydrolysis = 100. This method of plotting is inappropriate for strains where the rate of cephaloridine hydrolysis greatly exceeds that of benzylpenicillin, and these cases are omitted from the figure (cf. Fig. 2).
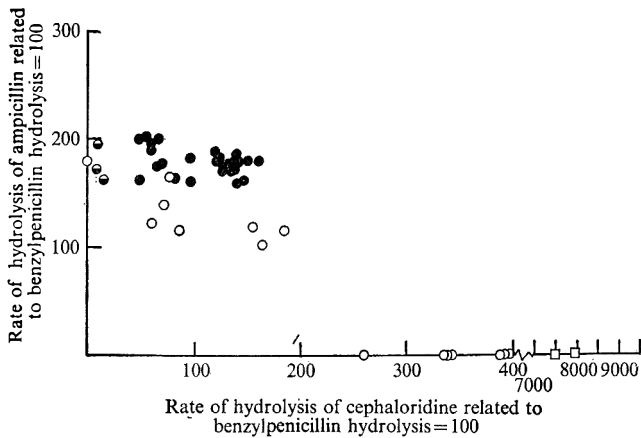
Fig. 4   Fig. 5

Fig. 4. Fig. 2 replotted to show the strains in the series that cross-react with anti-TEM serum. ●, Neutralization identical to TEM-enzyme; ◒, some neutralization but different from the TEM-enzyme/anti-TEM serum reaction; ○, no neutralization.

Fig. 5. The neutralization curves obtained when the reaction between anti-TEM serum and the *β*-lactamases from strain 1758, 418 and Wickham were compared with the neutralization of TEM-enzyme with anti-TEM serum. ○, TEM-enzyme/anti-TEM serum; ●, 1758-enzyme/anti-TEM serum; ▲, 418-enzyme/anti-TEM serum; and △, Wickham/anti-TEM serum.

ing their enzyme below the limit mainly lie to the left-hand side of the group with an average profile of about 100:120–200:90.

This division of group I into two subgroups suggested that there might be two distinct types of enzyme present. However, the difference in average profile between the

Table 2. *Specific enzyme activities (enzyme units/mg. dry wt organisms with benzylpenicillin as substrate) for the 46 β-lactamase producing strains examined here*

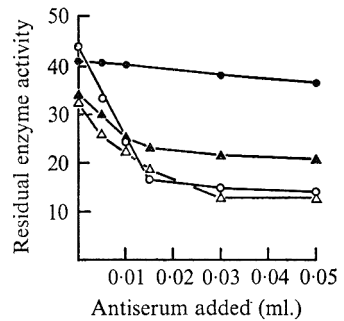| Strain			Strain		
Series no.	Source designation	Specific activity	Series no.	Source designation	Specific activity
1	TEM	55	24	1390	1·0
2	53	160	25	1169	2·1
3	481	1·7	26	1758	6·1
4	241	1·1	27	264	1·0
5	418	1·0	28	1144	110
6	071	5	29	1316	14
7	1266	2·2	30	153	4·3
8	D 539	6·1	31	1929	17
9	2/37	13	32	612	1·5
10	2/46	48	33	177	17·3
11	8	65	34	719	20
12	18	30	35	533	1·0
13	2/40/1	1·2	36	311	1·6
14	2/40/2	2·4	37	347	1·3
15	D 31	3·0	38	9527	1·2
16	D 535	5·2	39	115	1·0
17	NPL 3	16·4	40	3117	0·9
18	CJL	2·0	41	385	50
19	1082 E	76	42	Denston 5003	86
20	466	25	43	McDonald	350
21	214	0·9	44	Shine	127
22	P 99	18	45	Wickham	5
23	284	1·2	46	Robinson	18

Table 3. *Substrate profiles of crude and semi-purified preparations from strain TEM (no. 1, Table 1) and 071 (no. 6, Table 1)*

Strain designation	Strain no. (Table 1)	Approximate specific enzyme activity (enzyme units/μg. protein)	Substrate profile		
			Benzyl-penicillin	Ampicillin	Cephaloridine
TEM	1	0·11	100	160	140
TEM	1	10	100	165	135
071	6	0·015	100	165	95
071	6	2	100	165	135

The specific enzyme activity of pure TEM-β-lactamase is 80 units/μg. protein (Datta & Richmond, 1966).

two subgroups was found to be due to a discrepancy introduced into the iodometric assay of cephalosporinase (but not of penicillinase) by the presence of large amounts of contaminating protein. The effect of this contamination can be seen by comparing the substrate profiles of crude and semi-purified enzyme preparations from strain

TEM (synthesized at 55 units/mg. dry wt organisms, see Table 2) and strain 071 (synthesized at 5 units/mg. dry wt, see Table 2). Partial purification of the TEM-*β*-lactamase had little effect on its substrate profile measured iodometrically (Table 3) but in the case of enzyme from strain 071, partial purification lead to a change in profile from 100:165:95 in the crude material to 100:165:135 after some purification (Table 3).

In summary, therefore, the division of group I into two subgroups probably reflected an imperfection in the assay procedure rather than a fundamental difference in type of enzyme, at least according to the characters determined so far. Subsequent work (described below) showed that there were indeed several different types of enzyme among the strains of group I but they were not distributed within the group as implied by the correlations shown in Fig. 3.

Sensitivity of *β*-lactamases to anti-TEM serum

Samples of crude enzyme from each strain were tested for sensitivity to neutralization by anti-TEM serum; Fig. 4 shows Fig. 2 replotted to denote which of the strains reacted with serum. In all, enzyme from 29 of the strains was neutralized to some extent and two types of response were obtained. In the majority of cases (26/29) the kinetics of neutralization were indistinguishable from those obtained with purified TEM enzyme and anti-TEM serum; but in three cases a shallower and less complete neutralization was obtained (Fig. 5). These three strains (1758, Wickham, 418) all synthesized enzyme with profile 100:150:0–15; their detailed properties will be discussed below. All the strains with neutralization kinetics indistinguishable from the standard TEM/anti-TEM interaction lay in group I, but seven of the strains in this group did not react with anti-TEM serum at all (Fig. 4). Furthermore, these seven strains all lie together towards the bottom of the area occupied by group I in Fig. 2 and 4, suggesting that two fundamentally distinct types of enzyme are found in group I: one with profile of about 100:180:150 that reacts with anti-TEM serum and the other with profile about 100:110:150 distinguished by its insensitivity to antiserum. In general, no strain with an enzyme of predominantly cephalosporinase profile cross-reacted with anti-TEM serum.

Sensitivity to pCMB. Samples of crude enzyme obtained from the test strains were examined for their sensitivity to pCMB as described in Methods; Fig. 6 shows Fig. 2 replotted to show the enzymes susceptible to this inhibitor. Seven strains that synthesized pCMB-sensitive enzyme were found; they were identical with the seven members of group I that did not react with anti-TEM serum (compare Fig. 4 and 6). These results confirmed, therefore, that group I contained at least two distinct types of *β*-lactamase: one reacting with anti-TEM serum, resistant to pCMB and with a profile of 100:180:90 to 180; and the other resistant to anti-TEM serum, sensitive to pCMB and with a profile 100:110:90–180 (compare Fig. 2, 4, 6).

As was the case with the TEM-like enzymes in group I, the seven strains synthesizing pCMB-sensitive enzymes in this group also seemed to be in two subclasses: four forming one subclass with an average profile 100:100:170 and the other three with an average profile 100:100:80. However, unlike the TEM-like enzymes in group I, partial purification of these strains produced no significant change in substrate profile. On the basis of the results obtained so far, therefore, it is only possible to divide the strains in group I into two subgroups: 26 enzymes that react with anti-

TEM serum and are resistant to pCMB, and seven enzymes with the opposite properties. No subdivision of groups II, III and IV is possible on the basis of sensitivities to pCMB.

Sensitivity to cloxacillin. Fig. 7 shows Fig. 2 replotted to denote which of the enzymes were sensitive to cloxacillin by the criteria set out in Methods. No enzymes from group II (Fig. 2) could be tested in this way because of their extremely feeble activity against cephaloridine. The clearest correlation to emerge from these measurements is that
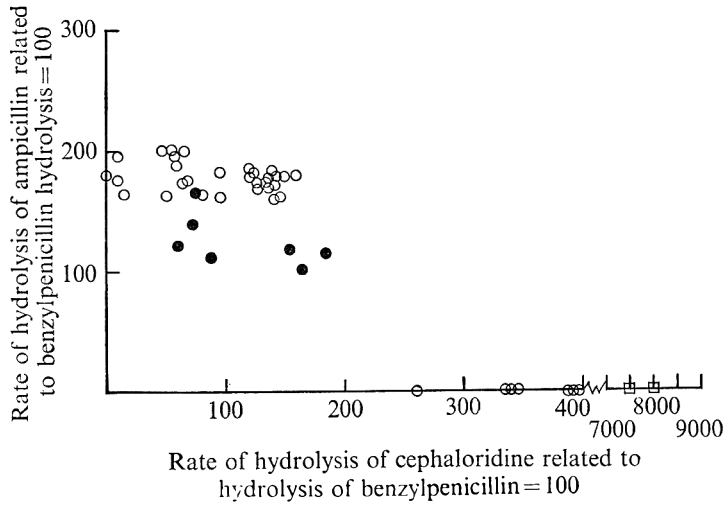

Fig. 6. Fig. 2 replotted to show the strains that synthesize β-lactamases sensitive to inhibition to 5×10^{-3} M pCMB. ●, Strains synthesizing sensitive enzyme; ○, resistant enzyme.
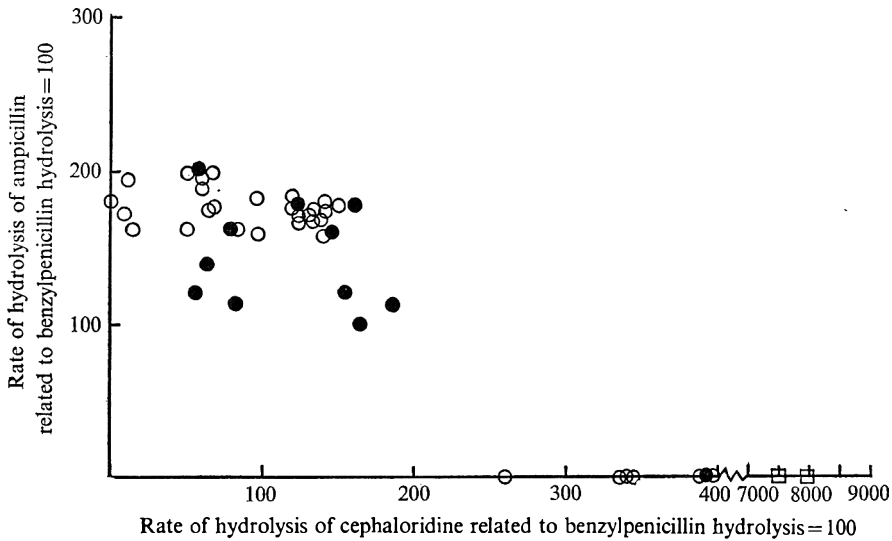

Fig. 7. Fig. 2 replotted to show the strains that synthesize β-lactamase sensitive to cloxacillin inhibition. ○, Sensitive enzymes; ●, resistant enzymes.

all the enzymes sensitive to *p*CMB (see Fig. 5) were resistant to cloxacillin. In addition, two other strains (nos. 30, 41, Table 1) were resistant to this inhibitor; this situation is discussed further below.

Absorption to substituted celluloses and electrophoretic mobility. Two procedures were used to obtain partially purified preparations of the enzymes—one involving the use of CM-cellulose and the other DEAE-cellulose (see Methods). In all cases crude enzyme preparations from the strains tested adsorbed either to one or the other of these celluloses but not to both. Table 4 shows the type of substituted cellulose
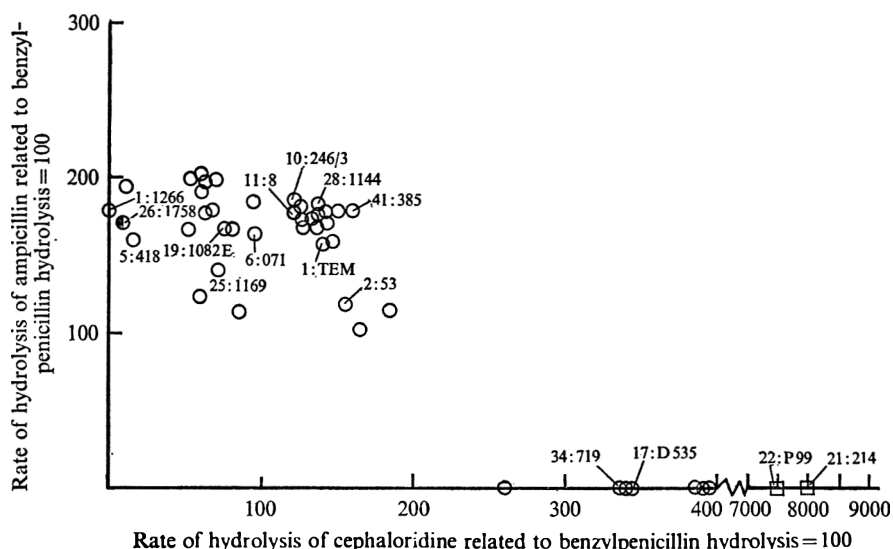

Fig. 8. The location, on a plot of the type used in Fig. 2, of the strains used to purify enzyme for mobility tests in starch gel. The numerals on the graph refer to the series numbers shown in Table 1. ◐, Strain 1758 (which makes two enzymes).

used for purification of the enzymes studied. Although the adsorption characteristics of the enzymes gave some general idea of their charge properties, it was decided to separate the semi-purified enzymes by starch-gel electrophoresis at pH 8·5 to obtain a detailed comparison of their mobility and thus some idea of their degree of ionization at that pH value. Starch-gel electrophoresis was done on partially purified enzymes as described in Methods but, in view of the large number of strains involved in these studies, it was decided to test only a selection from each enzyme group. The relative mobilities of these preparations, together with the strains from which they were derived, are shown in Table 4. In all, 15 strains were chosen; six from group I and the others to include as many representatives of the other groups as possible (see Fig. 8). Six types of mobility were detected. In one case, mobility was towards the cathode (at +0·75 cm./hr) and in three towards the anode (− 1·6, − 1·1, −0·6 cm./hr). With two types of enzyme there was very little mobility at this pH value, but the enzymes could be distinguished because one moved slightly towards the cathode (∼ +0·1 cm./hr) and the other at about the same rate towards the anode. On the basis of mobility alone, therefore, six types of enzyme were distinguished, but when these results were correlated with substrate profile the number of distinct enzyme types rose to eight. Two types of enzyme were present among those migrating with a low positive

mobility at pH 8·5. One of them (no. 2, Table 1) had a profile 100:120:155, while nos. 21 and 22 (Table 1) had profiles 100:0:8000. Similarly, two types of enzyme migrated with a mobility of −0·6 cm./hr; no. 19 (Table 1) had a profile 100:170:75 while no. 7 had a profile 100:180:0.

One of the strains tested by starch-gel electrophoresis (strain 1758, no. 26, Table 1) synthesized two distinct types of β-lactamase. This discovery immediately invalidated the use of substrate profile to characterize this strain since it was not certain how much of the activity against each substrate was contributed by each component enzyme. Fortunately one of the two enzymes in this strain was found to be carried on an R-factor and this provided a means of studying the properties of each component enzyme uncontaminated by the other. The enzyme mediated by the R-factor gene was examined by transferring it to a strain that lacked β-lactamase. The enzyme transferred in this way was found to have a profile 100:180:0, to be insensitive to anti-TEM serum, resistant to pCMB and sensitive to cloxacillin. The other enzyme was examined by obtaining an R⁻ variant of strain 1758. This variant now synthesized only one type of enzyme with profile 100:150:160, sensitive to anti-TEM serum, resistant to pCMB and sensitive to cloxacillin; and in all ways this component was indistinguishable from TEM-β-lactamase.

The over-all substrate profile of strain 1758 (100:170:10) allowed one to infer that the strain as originally isolated synthesized ten times as much enzyme with profile 100:180:0 as it did TEM-lactamase. This mixture is also consistent with the intermediate type of response obtained when the mixed enzymes from this strain were tested for their interaction with anti-TEM serum (see p. 51). The other strains that showed an intermediate response to anti-TEM serum were strains 5 and 45 (Table 1) (approx. profile 100:170:15). Electrophoretic studies gave no indication of inhomogeneity in the enzymes synthesized by these strains and it seems likely that this enzyme is indeed a distinct type of β-lactamase with a heterologous reaction with anti-TEM serum.

Transferability of β-lactamase genes. Twelve of the 46 strains examined here were capable of transferring their β-lactamase genes when grown overnight with the standard recipient strain (see Methods). Ten of the strains shown to have transferable genes by this test synthesized TEM-like enzyme, one synthesized enzyme with profile 100:0:400 and (as described above) one enzyme from strain 1758 (the one with profile 100:180:0) could be transferred by cell-to-cell contact. No transfer of the enzyme from either *Enterobacter cloacae* strain 214 or *Aerobacter cloacae* strain P99 have ever been reported and it seems likely that the gene specifying this type of β-lactamase is chromosomal. Too little is yet known about the transfer properties of the strains that did not transfer the lactamase genes in this test to be dogmatic about their genetic constitution.

DISCUSSION

At least eight different types of β-lactamase can be detected among the enteric bacteria on the basis of the parameters used here; the characteristics of these enzymes are summarized in Table 5. They may be classified in a number of ways depending on the properties chosen for correlation. Enzyme types 2 and 5 (Table 5), for example, are predominantly cephalosporinases while types 6 and 7 are mainly penicillinases. All the remaining enzymes examined here hydrolyse the three substrates at a significant

Table 4. *Electrophoretic mobility and adsorption characteristics of partially purified enzyme preparations from a number of strains*

Strains		Chromoto- graphed on	Mobility (cm./hr)
Series no.	Source no.		
1	TEM	DEAE	−1·6
2	53	CM	+0·1
5	418	DEAE	−0·1
6	071	DEAE	−1·6
7	1266	DEAE	−0·6
10	246/3	DEAE	−1·6
11	8	DEAE	−1·6
17	D 535	CM	+0·7
19	1082E	DEAE	−0·6
21	214	CM	+0·1
22	P 99	CM	+0·1
25	1169	DEAE	−1·1
28	1144	DEAE	−1·6
34	719	CM	+0·7
41	385	DEAE	−1·6

Abbreviations: CM = Carboxymethyl cellulose; DEAE = diethyl-aminoethyl-cellulose; + = mobility towards cathode; − = mobility towards anode.

Table 5. *Properties of the eight types of β-lactamase detected among the enteric bacteria*

Enzyme type	Type species (if any)	Substrate profile (hydrolysis of benzylpenicillin = 100)			Sensitivity to			Chromato- graphed on	Mobility (cm./hr)
		Benzyl- penicillin	Ampi- cillin	Cepha- loridine	Anti- TEM serum	pCMB	Cloxa- cillin		
1	*E. coli* TEM	100	150	180	+	R	S	DEAE	−1·6
2	*Aerobacter cloacae* P99	100	0	8000	−	R	S	CM	+0·1
3	*E. coli* 53	100	120	150	−	S	R	CM	+0·1
4	.	100	125	60	−	S	R	DEAE	−1·0
5	.	100	0	350	−	R	S	CM	+0·7
6	.	100	160	15	±	R	S	DEAE	−0·2
7	.	100	180	0	−	R	S	DEAE	−0·6
8	.	100	170	70	−	S	R	DEAE	−0·6

rate. Enzyme types 2, 3 and 5 are basic proteins since they are still positively charged even at pH 8·5, whereas all the other enzymes are anionic at this pH value. Of the three basic proteins, two are the cephalosporinases mentioned above. Enzyme types 3, 4 and 8 are all pCMB-sensitive and cloxacillin-resistant, whereas all the other types have the opposite properties. Enzyme types 1 and 6 cross-react with anti-TEM serum to some extent, but none of the other enzymes are neutralized to any significant extent by this serum.

The wide range or properties found among the β-lactamases in enteric bacteria is, at first sight, a little surprising, particularly since all the strains examined here were first isolated because of a common property—resistance to ampicillin. However, minor variants of a basic penicillinase structure have been found within a single species in *Bacillus cereus* (Kogut, Pollock & Tridgell, 1956; Pollock, Torriani & Tridgell,

1956), *Bacillus licheniformis* (Pollock, 1964, 1965) and *Staphylococcus aureus*
(Richmond, 1965) and indeed the enzymes from *B. licheniformis* and *S. aureus*
have been shown to have a similar underlying amino acid sequence despite a different
substrate profile, iso-electric point and amino acid composition (Ambler & Meadway,
1969). Similarly, all the β-lactamases in the Gram-negative bacteria may well also
share some basic sequence with minor variations in structure to account for the various
substrate profiles and other properties encountered among the enzymes described here.
The fact that at least six of the eight enzymes have a molecular weight in the range
15,000–20,000 and are sensitive to inhibition by iodine + KI solution (Datta &
Richmond, 1966; Hennessey & Richmond, 1968; Jack & Richmond, unpublished
experiments) is at least consistent with this view. The low value for the molecular weight
of the enzymes from Gram-negative strains suggests, however, that these enzymes
may have relatively little in common with the lactamases from *B. cereus, B. licheniformis*
and *S. aureus*, where the molecular weight of the single polypeptide chain of the
penicillinases is about 30,000 (Citri & Pollock, 1966). If one has to guess any under-
lying molecular classes among the enzymes described in Table 5, the most likely
would seem to be as follows. (1) A basic 'cephalosporinase' (types 2, 5; Table 5).
(2) A general purpose β-lactamase sensitive to *p*CMB but resistant to inhibition by
cloxacillin and anti-TEM serum (Types 3, 4, 8; Table 5). (3) A general-purpose enzyme
resistant to *p*CMB but sensitive to cloxacillin inhibition and to neutralization by anti-
TEM serum (types 1, 6; Table 5).

It is difficult to identify with certainty the enzymes described here among the
earlier reports of other workers, partly because few enzymes have been tested both
against penicillins and cephalosporins and partly because certain tests (e.g. sensitivity
to *p*CMB) have only rarely been done. Apart from the enzymes from *Enterobacter
cloacae* 214 and *Aerobacter cloacae* P99 mentioned earlier, the β-lactamase syn-
thesized by strain 53 (Smith, 1963) is certainly of type 3 since the identical strain
was included in the present survey. Similarly the enzyme synthesized by strain 418
of *Klebsiella aerogenes* (Hamilton-Miller, 1963) was included and is an example of
type 6 enzyme. Sawai, Mitsuhashi & Yamagishi (1969) surveyed a wide range of
β-lactamases from Gram-negative species (excluding pseudomonads) using substrate
profile and immunological cross-reaction to compare the enzymes and concluded
that there were three distinct types of β-lactamase to be found: a 'cephalosporinase'
found in *Enterobacter freundii, Aerobacter aerogenes, Arizona (sic), Proteus morganii,
P. rettgeri, P. inconstans* and one strain of Serratia; a 'cephalosporinase which has a
property of penicillinase' (presumably a mixed penicillinase/cephalosporinase pro-
file) in *P. vulgaris*; and a penicillinase in *K. pneumoniae, P. mirabilis* and *Escherichia
coli*. Of these the 'cephalosporinase' has reasonably uniform properties and may well
be either type 2 or type 5 (or include both) in the classification shown in Table 5.
It seems unlikely, however, that the enzyme classified by the Japanese workers as
'penicillinase' represents a single type of molecule since its substrate profile is re-
ported to range from 100:1:110 in *E. coli* to 100:160:55 in *K. pneumoniae* and to
100:140:5 in *P. mirabilis*. On the basis of these substrate profiles the enzymes found
in *K. pneumoniae* and in *P. mirabilis* would correspond respectively to types 8 and
7 shown in Table 5 above, while the enzyme with profile 100:1:110 has not been
detected by us. The Japanese workers also describe the properties of a 'penicil-
linase' synthesized by *E. coli* W3630 carrying an R-factor (R_{GN14}) (see also Egawa,

Sawai & Mitsuhashi, 1967). This enzyme has a very similar profile to TEM-*β*-lactamase and is therefore probably a representative of enzyme type I (Table 5). The fact that this enzyme is R-factor mediated in the Japanese strains and also commonly found in this state in the strains examined in Britain supports the view that TEM-*β*-lactamase gene is often carried on an R-factor. However, this is not always the case since the TEM-lactamase component in strain 1758 (see p. 56) is not freely transferable whereas the second enzyme component in this strain is.

Although the properties of the eight types of *β*-lactamase described here go some way to account for the wide and variable range of activity of enteric bacteria against the penicillins and cephalosporins, two further factors are also important. The first is the ability of these strains to produce two distinct types of *β*-lactamase at once; and the second is that the level of expression of a given *β*-lactamase gene may vary greatly from strain to strain. Thus type I enzyme is synthesized to a level of only 1 unit/mg. dry wt in strain no. 35 (Table 2) but at more than 100 times the rate in strain no. 44 (Table 2). Furthermore, when the gene in question is carried on an R-factor, its level of expression can vary widely from recipient to recipient (Smith, 1969; G. W. Jack, unpublished observations). This combination of ability to synthesize one or more types of lactamase at a wide variety of rates ensures that any given enteric organism can have a profile ranging from that of an extreme penicillinase to that of an extreme cephalosporinase. Although the resistance of enteric strains to *β*-lactam antibiotics does not depend entirely on the type and quantity of enzyme synthesized, enzymic resistance is undoubtedly an important, perhaps the most important, factor in the resistance of the organisms. The wide range of profile that can be produced by varying the type and quantity of the various lactamases may well be the reason why no one penicillin or cephalosporin is fully effective against all enteric bacteria.

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The Isolation and Properties
of Non-restricting Mutants of Two Different Host Specificities
Associated with Drug Resistance Factors

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Non-restricting (r⁻) mutants of two different host specificities carried by resistance transfer factors have been isolated. As previously found with other host specificities, the non-restricting mutants were of two phenotypes: those that retained the ability to modify DNA, and those which had lost the ability to modify DNA. These mutants were tested for complementation with wild-type host specificities carried either on the *Escherichia coli* chromosome, on resistance transfer factors, or on the phage P1. No complementation was observed and possible explanations for this finding are considered.

INTRODUCTION

The host range of some bacteriophages can be altered as a result of a single cycle of growth in a new host strain, and this alteration is termed 'host controlled modification' (Luria, 1953; Arber & Dussoix, 1962). In *Escherichia coli* strains K12 and B, afterwards called K and B, two distinct host specificity functions, restriction and modification, have been recognized which operate with the same specificity. Restriction involves cleavage of at least one strand of the non-modified phage DNA (Meselson & Yuan, 1968). Modification is specifically associated with the phage DNA also, and protects the phage from this restriction. In the case of the male specific phage fd, modification involves methylation of adenine residues in the DNA (Arber, 1968).

The chromosomal genes determining these two phenotypic characters have been mapped close to *thr* and *serB* in *Escherichia coli*, both by bacterial conjugation and by co-transduction with these markers by phage P1, and the strain specificities K, B and 15 were shown to be allelic (Boyer, 1964; Colson, Glover, Symonds & Stacey, 1965; Hoekstra & De Haan, 1965; Lederberg, 1966; Wood, 1966; Glover & Colson, 1969). However, the elucidation of the genetic fine structure of the host specificity genes has not been feasible, mainly due to the lack of quantitative techniques for selecting clones of different restriction or modification phenotypes. Information concerning the number and function of these genes has come from study of the phenotypes of non-restricting mutants. The non-restricting (r⁻) mutants found have been of two phenotypes, those retaining modification ability (m⁺), and those which were also defective in modification (m⁻) (Colson *et al.* 1965; Wood, 1965, 1966; Lederberg,

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1966). The existence of two phenotypes for non-restricting mutants suggested that the genetic determinants for host specificity involved a minimum of two genes. Conformation of this hypothesis has been obtained as a result of complementation studies in partial diploids constructed with F prime factors. These studies have revealed that at least two genes are involved in restriction; that one of these genes, together with a third, is required for modification; and a fourth gene has been postulated (Boyer & Roulland-Dussoix, 1969; Glover, unpublished results).

Two host specificities, hsI and hsII (Bannister & Glover, 1968), are associated with the drug-resistance factors (R factors) responsible for the transmissible resistance to antibiotics in Enterobacteriaceae (Watanabe, 1963; Meynell, Meynell & Datta, 1968). In this paper we present a genetic analysis of the determinants involved in the control of these host specificities, based upon exploration of the types of restrictionless mutants which can be obtained, and the behaviour of these mutants in complementation tests.

METHODS

Media. Oxoid no. 2 broth: Oxoid no. 2 broth powder 25 g.; distilled water 1·0 l. Oxoid agar: Oxoid no. 2 broth solidified with 12·5 g./l. Davis New Zealand agar. VB agar: VB salts (Vogel & Bonner, 1956) solidified with 15 g./l. Davis New Zealand agar. Water top agar: Difco Bacto agar 6 g.; distilled water 1·0 l. Difco agar: Oxoid tryptone 10 g.; NaCl 8 g.; glucose 1 g.; Difco Bacto agar 10 g.; distilled water 1·0 l. Sugars used at a final concentration of 2 % (w/v). Tetracycline used at a final concentration of 50 μg./ml. Streptomycin used at 20 μg./ml. Amino acids used at 20 μg./ml.

Bacteriophages: phage λ (Lederberg & Lederberg, 1953) and its virulent mutant, λvir (Jacob & Wollman, 1954).

Bacterial strains. The strains of *Escherichia coli* used are listed in Table 1.

Table 1. *Bacterial strains*

Strain	Characteristics	Reference
c600	*thr leu thi lac hsK*	Appleyard, 1954
c600 (p1)	P1 lysogen of c600	Glover, Schell, Symonds & Stacey, 1963
c600 (λ ci857)	λ ci857 lysogen of c600	Sussman & Jacob, 1962
j5–3	*pro met hsK (λ)*	Clowes & Rowley, 1954
4K	*thr leu thi serB lac* r⁻m⁻K	Colson *et al.* 1965
7K	*thr leu thi* r⁻m⁺K	Colson *et al.* 1965
15	*thi thy hsI5*	Stacey, 1965
c	*thr*	Glover & Colson, 1969
B	*trp hsB*	Glover & Colson, 1969
B6	*thr leu ilvA* r⁻m⁺B	Glover & Colson, 1969
B8	*thr leu ilvA* r⁻m⁻B	Glover & Colson, 1969

R factors. R124 *fi⁺* and carrying resistance to tetracycline; R132 *fi⁻* and carrying resistance to streptomycin and sulphonamide; R199 *fi⁻* and carrying resistance to tetracycline; R313-T-1 *fi⁻* and carrying resistance to tetracycline. R124 carries hsI, the other three R factors carry hsII (hsI and hsII define the host specificities determined by these R factors (Bannister, 1969)). The R factors were the gift of Dr Elinor Meynell and Dr Naomi Datta.

Techniques

Overnight cultures of bacteria were obtained by inoculating 5 ml. of Oxoid no. 2 broth from a single colony and incubating at 37°. Efficiency of plating (e.o.p.) is the ratio of the plaque count on the test strain to the plaque count on a permissive strain. A quick test for restriction was employed in the isolation of restrictionless mutants. Colonies were streaked on Difco agar plates and spotted with approximately 0·01 ml. of dilutions of phage λvir.

Transfer of R factors

An overnight culture of the donor strain was diluted 1/10 into fresh broth, and incubated on an inclined rotor at 37° for 1½ hr. This culture was diluted 1/10 into an overnight culture of recipient and incubated at 37° for 30 min., then streaked out on VB agar selective for R^+ clones of the recipient, and incubated at 37° for 24 to 36 hr. Colonies were purified by two serial single-colony isolations, and tested for all the characters of the recipient and the R factor.

RESULTS

Spontaneous restrictionless mutants of hsII

Routinely, R^+ strains were grown overnight from inocula taken from single colonies only. On two separate occasions, the R^+ colony used as an inoculum was found to be a non-restricting mutant, retaining modification ability (i.e. r^-m^+). These mutants were obtained from two different R factors, R199 and R313-T-1, and the two mutant R factors were termed R199-1 and R313-1 respectively.

Restrictionless mutants produced by ethylmethane sulphonate mutagenesis

Although two spontaneous mutants of the hsII host specificity had arisen, screening several hundred single colonies of strain J5-3 carrying R313-T-1 hsII, and of J5-3 R124 hsI, failed to reveal any other restrictionless mutants. To obtain additional mutants of both hsI and hsII, mutagenesis with ethylmethane sulphonate was used, and the mutagenesis was followed by an enrichment procedure developed by Hubachek & Glover (in preparation). This procedure relies on the fact that λ.K can lysogenize restrictionless mutants of hsI or hsII efficiently and cannot readily lysogenize wild-type restricting bacteria. The mutagenesis was performed in R^+ strains lysogenic for the heat-inducible phage λ cI857, and after infection with wild-type λ, the phage was induced by exposure to 42°. Restrictionless mutants which had become lysogenic for wild-type λ were protected against induction, while the restricting bacteria were lysed. The results of five separate experiments, showing the proportion of non-restricting mutants found after enrichment and the phenotypes of the mutants, are presented in Table 2. The e.o.p. of λ.K on hsI$^+$ strains is about 10^{-4}, whereas the e.o.p. of λ.K on hsII$^+$ strains is only 10^{-2}, and this difference in the restriction of λ.K by the two host specificities may account for the greater yield of restrictionless mutants from the strain carrying R124. Two phenotypes, r^-m^- and r^-m^+, were observed, and for hsI, where the total number of mutants scored is higher, these two phenotypes occur in roughly equal proportions.

5-2

The uniqueness of hsI and hsII

It is clear from the results presented in Tables 3 to 6 that the host specificities hsI and hsII are not identical with any of the host specificities previously described. A strain carrying hsI (or hsII) restricts phage λ provided the phage λ was not grown on a strain carrying hsI (or hsII). Likewise, phage λ grown on a strain of *E. coli* C or an r^-m^- mutant of *E. coli* K or B carrying hsI (or hsII) is restricted by *E. coli* K, B, 15 and K (P I).

Table 2. *The production of restrictionless mutants of R factor-carried host specificities*

Overnight cultures of R^+ bacteria were diluted 1/10 into fresh Oxoid no. 2 broth, incubated for $1\frac{1}{2}$ hr, harvested by centrifugation and resuspended in 1/10 the original volume of M9 buffer containing 0·c2 M-ethylmethane sulphonate. After 60 min. the cultures were diluted 1/50 with M9 buffer and centrifuged. The pellet was resuspended in half the centrifugation volume, in Oxoid no. 2 broth, and incubated overnight. The cultures were now diluted 1/10 in fresh broth, and aerated for *c.* 2 hr, followed by a further 1/2 dilution with Oxoid no. 2 broth containing M/100 Mg^{2+}. Wild-type phage λ.K was added, and after 30 min. the culture was transferred to 42° and aerated for 60 min. Appropriate dilutions were made and poured in soft agar overlays on VB supplemented plates, and incubated for 48 hr before picking and testing colonies. Unless otherwise stated, incubation was at 30°.

R factor	No. of colonies tested	No. of r^-m^+	No. of r^-m^-
R 124	40	13	14
R 124	50	7	14
R 132-SS-1	58	3	5
R 313-T-1	40	1	0
R 313-T-1	30	1	0

Table 3. *Efficiency of plating of phage λvir grown on Escherichia coli K, B, C, BRI and CRI*

Host strain*	Phage†					
	λ.K	λ.KRI	λ.B	λ.BRI	λ.C	λ.CRI
K	1·0	1·0	5×10^{-4}	1×10^{-3}	4×10^{-4}	1×10^{-4}
KRI	4×10^{-4}	1·0	7×10^{-5}	5×10^{-3}	4×10^{-4}	6×10^{-4}
KRII	2×10^{-2}	2×10^{-2}	7×10^{-5}	1×10^{-4}	2×10^{-4}	1×10^{-4}
B	2×10^{-4}	5×10^{-5}	1·0	1·0	1×10^{-4}	5×10^{-4}
BRI	2×10^{-4}	1×10^{-3}	7×10^{-5}	1·0	1×10^{-5}	2×10^{-4}
BRII	7×10^{-4}	6×10^{-3}	2×10^{-3}	6×10^{-3}	1×10^{-2}	7×10^{-4}
C	1·0	1·0	1·0	1·0	1·0	1·0
CRI	5×10^{-6}	1·0	7×10^{-6}	1·0	2×10^{-5}	1·0
CRII	4×10^{-3}	1×10^{-2}	1×10^{-4}	1×10^{-4}	1×10^{-2}	1×10^{-2}

* K is C600, B is B*trp*, C is C*thr*, RI is R 124, RII is R 313-T-1.
† λ.K is phage grown on strain K; λ.KRI is phage grown on strain KRI, etc.

Complementation studies

Complementation was tested by transferring the R factor, or an *hs* mutant R factor, to strains carrying the other host specificities, and examining the restriction and modification properties of the resulting strains, with λ*vir*. Controls showing the e.o.p. of variously modified λ*vir* on R^+ and R^- strains are contained in Tables 3 to 6,

the phenotypes of R⁺ strains constructed with hsI and hsII r⁻ mutants are recorded in Tables 7 to 9. Although the wild-type host specificities are always expressed, no complementation of the restrictionless mutants by the wild-type host specificity genes was observed. Restriction was not restored to any hsI or hsII r⁻ mutant in the

Table 4. *Efficiency of plating of phage λvir grown on Escherichia coli*
K, B, C, KRII, BRII and CRII

Host strain	Phage					
	$\lambda.\kappa$	$\lambda.\kappa R II$	$\lambda.\mathrm{B}$	$\lambda.\mathrm{B}R II$	$\lambda.\mathrm{C}$	$\lambda.\mathrm{C}R II$
K	$1\cdot0$	$1\cdot0$	5×10^{-4}	5×10^{-4}	4×10^{-4}	1×10^{-4}
KRI	4×10^{-4}	1×10^{-3}	7×10^{-5}	5×10^{-5}	4×10^{-4}	2×10^{-5}
KRII	2×10^{-2}	$1\cdot0$	7×10^{-5}	7×10^{-3}	2×10^{-4}	1×10^{-3}
B	2×10^{-4}	5×10^{-5}	$1\cdot0$	$1\cdot0$	1×10^{-4}	2×10^{-4}
BRI	2×10^{-1}	7×10^{-5}	7×10^{-5}	7×10^{-4}	1×10^{-5}	1×10^{-4}
BRII	7×10^{-1}	1×10^{-2}	2×10^{-3}	$1\cdot0$	1×10^{-2}	7×10^{-2}
C	$1\cdot0$	$1\cdot0$	$1\cdot0$	$1\cdot0$	$1\cdot0$	$1\cdot0$
CRI	5×10^{-6}	1×10^{-1}	7×10^{-6}	2×10^{-5}	2×10^{-5}	5×10^{-5}
CRII	4×10^{-3}	$1\cdot0$	1×10^{-4}	$1\cdot0$	1×10^{-2}	$1\cdot0$

K is C600, B is B*trp*, C is C*thr*, RI is R 124, RII is R 313-T-1.

Table 5. *Efficiency of plating of phage λvir grown on Escherichia coli*
K, KRI, KRII and K(PI)

Host strain	Phage			
	$\lambda.\kappa$	$\lambda.\kappa R I$	$\lambda.\kappa R II$	$\lambda.\kappa(P I)$
K	$1\cdot0$	$1\cdot0$	$1\cdot0$	$1\cdot0$
KRI	4×10^{-4}	$1\cdot0$	1×10^{-3}	3×10^{-4}
KRII	2×10^{-2}	2×10^{-2}	$1\cdot0$	5×10^{-2}
K(PI)	1×10^{-4}	1×10^{-4}	1×10^{-4}	$1\cdot0$

K is C600, RI is R124, and RII is R313-T-1.

Table 6. *Efficiency of plating of phage λvir grown on Escherichia coli 15,*
15RI, 15RII, C, CRI and CRII

Host strain	Phage					
	$\lambda.15$	$\lambda.15R I$	$\lambda.15R II$	$\lambda.\mathrm{C}$	$\lambda.\mathrm{C}R I$	$\lambda.\mathrm{C}R II$
15	$1\cdot0$	$1\cdot0$	$1\cdot0$	3×10^{-2}	5×10^{-2}	1×10^{-2}
15RI	1×10^{-2}	$1\cdot0$	1×10^{-2}	1×10^{-3}	1×10^{-2}	1×10^{-4}
15RII	1×10^{-2}	1×10^{-2}	$1\cdot0$	1×10^{-2}	1×10^{-2}	1×10^{-2}
C	$1\cdot0$	$1\cdot0$	$1\cdot0$	$1\cdot0$	$1\cdot0$	$1\cdot0$
CRI	1×10^{-3}	$1\cdot0$	1×10^{-5}	5×10^{-4}	$1\cdot0$	1×10^{-4}
CRII	1×10^{-2}	1×10^{-2}	$1\cdot0$	1×10^{-2}	1×10^{-2}	$1\cdot0$

RI is R124, RII is R313-T-1.

presence of intact K, B or PI host specificities (Table 7), or to hsI mutants in the presence of intact hsII (Table 9), or to r⁻ mutants of K and B by intact hsI and hsII (Table 8). Nor is modification restored to any r⁻m⁻ mutants, although the m⁺ phenotype of r⁻m⁺ mutants is expressed (Tables 7 to 9).

Table 7. *Phenotypes displayed by R^+ strains carrying restrictionless mutants of R124, R313-T-1, and R132*

R factor	Host-specificity phenotype of R factor	Host specificity phenotype of the R^+ strain when the host strain is		
		K*	B	K(PI)
R 124	$r_I^+ m_I^+$	$r_I^+ m_I^+$ $r_K^{\pm} m_K^{\pm}$	$r_I^+ m_I^+$ $r_B^+ m_B^+$	$r_I^+ m_I^+$ $r_K^{\pm} m_K^{\pm}$ $r_{P1}^+ m_{P1}^+$
R 124-1	$r_I^- m_I^+$	$r_I^- m_I^+$ $r_K^{\pm} m_K^{\pm}$	$r_I^- m_I^+$ $r_B^+ m_B^+$	$r_I^- m_I^+$ $r_K^{\pm} m_K^{\pm}$ $r_{P1}^+ m_{P1}^+$
R 124-2	$r_I^- m_I^-$	$r_I^- m_I^-$ $r_K^{\pm} m_K^{\pm}$	$r_I^- m_I^-$ $r_B^+ m_B^+$	$r_I^- m_I^-$ $r_K^{\pm} m_K^{\pm}$ $r_{P1}^+ m_{P1}^+$
R 124-3	$r_I^- m_I^+$	$r_I^- m_I^+$ $r_K^+ m_K^+$	$r_I^- m_I^+$ $r_B^+ m_B^+$	$r_I^- m_I^+$ $r_K^+ m_K^+$ $r_{P1}^+ m_{P1}^+$
R 313-T-1	$r_{II}^+ m_{II}^+$	$r_{II}^+ m_{II}^+$ $r_K^{\pm} m_K^{\pm}$	$r_{II}^+ m_{II}^+$ $r_B^+ m_B^+$	$r_{II}^+ m_{II}^+$ $r_K^{\pm} m_K^{\pm}$ $r_{P1}^+ m_{P1}^+$
R 313-1	$r_{II}^- m_{II}^+$	$r_{II}^- m_{II}^+$ $r_K^{\pm} m_K^{\pm}$	$r_{II}^- m_{II}^+$ $r_B^+ m_B^+$	$r_{II}^- m_{II}^+$ $r_K^{\pm} m_K^{\pm}$ $r_{P1}^+ m_{P1}^+$
R 313-2	$r_{II}^- m_{II}^+$	$r_{II}^- m_{II}^+$ $r_K^{\pm} m_K^{\pm}$	$r_{II}^- m_{II}^+$ $r_B^+ m_B^+$	$r_{II}^- m_{II}^+$ $r_K^{\pm} m_K^{\pm}$ $r_{P1}^+ m_{P1}^+$
R 313-3	$r_{II}^- m_{II}^+$	$r_{II}^- m_{II}^+$ $r_K^+ m_K^+$	$r_{II}^- m_{II}^+$ $r_B^+ m_B^+$	$r_{II}^- m_{II}^+$ $r_K^- m_K^-$ $r_{P1}^+ m_{P1}^+$
R 132-1	$r_{II}^- m_{II}^+$	$r_{II}^- m_{II}^+$ $r_K^+ m_K^+$	$r_{II}^- m_{II}^+$ $r_B^+ m_B^+$	$r_{II}^- m_+^{II}$ $r_K^+ m_K^+$ $r_{P1}^+ m_{P1}^+$
R 132-2	$r_{II}^- m_{II}^-$	$r_{II}^- m_{II}^-$ $r_K^+ m_K^+$	$r_{II}^- m_{II}^-$ $r_B^+ m_B^+$	$r_{II}^- m^{II}$ $r_K^+ m_K^+$ $r_{P1}^+ m_{P1}^+$
R 199-1	$r_{II}^- m_{II}^+$	$r_{II}^- m_{II}^+$ $r_K^+ m_K^+$	$r_{II}^- m_{II}^+$ $r_B^- m_B^+$	$r_{II}^- m_{II}^+$ $r_K^+ m_K^+$ $r_{P1}^+ m_{P1}^+$

* K strains used were C600 and J5-3.

The restriction phenotype was determined by measuring the efficiency of plating of variously modified phage λvir on the test strain, and the modification phenotype was determined by measuring the efficiency of plating on standard strains of the phage λvir grown on the test strain.

Table 8. *Phenotypes displayed by restrictionless mutants of Escherichia coli K and B carrying R124 or R313-T-1*

Strain	Phenotype of R^- strain	Phenotype of R^+ carrying	
		R 124 ($r_I^+ m_I^+$)	R 313-T-1 ($r_{II}^+ m_{II}^+$)
C600	$r_K^+ m_K^+$	$r_K^+ m_K^+$ $r_I^+ m_I^+$	$r_K^+ m_K^+$ $r_{II}^+ m_{II}^+$
7K	$r_K^- m_K^+$	$r_K^- m_K^+$ $r_I^+ m_I^+$	$r_K^- m_K^+$ $r_{II}^+ m_{II}^+$
4K	$r_K^- m_{KI}^-$	$r_K^- m_K^-$ $r_I^+ m_I^+$	$r_K^- m_K^-$ $r_{II}^+ m_{II}^+$
B	$r_B^+ m_B^+$	$r_B^+ m_B^+$ $r_I^+ m_I^+$	$r_B^+ m_B^+$ $r_{II}^+ m_{II}^+$
B6	$r_B^- m_B^+$	$r_B^- m_B^+$ $r_I^+ m_I^+$	$r_B^- m_B^+$ $r_{II}^+ m_{II}^+$
B8	$r_B^- m_B^-$	$r_B^- m_B^-$ $r_I^+ m_I^+$	$r_B^- m_B^-$ $r_{II}^+ m_{II}^+$

Table 9. *Phenotypes of C600 R132 strains also carrying R124 or a restrictionless mutant or R124*

Strain	Host-specificity phenotype		
C600 R132 R124	$r_K^+ m_K^+$	$r_{II}^+ m_{II}^+$	$r_I^+ m_I^+$
C600 R132 R124-1	$r_K^+ m_K^{\pm}$	$r_{II}^+ m_{II}^+$	$r_I^- m_I^+$
C600 R132 R124-2	$r_K^+ m_K^{\pm}$	$r_{II}^+ m_{II}^+$	$r_I^- m_I^-$
C600 R132 R124-3	$r_K^+ m_K^+$	$r_{II}^+ m_{II}^+$	$r_I^- m_I^+$

DISCUSSION

The absence of efficient quantitative techniques for selecting different restriction or modification phenotypes seriously limits genetic fine structure analysis of host specificities. Although crude mapping and establishment of the allelic relationships

of the determinants for the host-specificity types κ, b and 15 has been established by bacterial conjugation and cotransduction with phage P1, study of the restriction-less mutants gave the most information concerning the number and function of the genes involved. Complementation studies in partial diploids proved that a minimum of three genes was necessary for the expression of restriction and modification. Provisionally, the following nomenclature for these genes is used: *hsm* is a gene specifying a product essential for modification, *hsr* is a gene specifying a product essential for restriction, and *hss* is a gene specifying a product essential for both restriction and modification (Arber & Linn, 1969).

The host-specificity types hsI and hsII are carried on R factors, whose genomes are relatively small compared with the chromosome of *Escherichia coli*. Mapping of R factors by time of entry studies is not possible since all R factor-carried determinants appear to enter the recipient at the same time (Meynell *et al.* 1968). Mapping by phage P1 transduction is also excluded, since phage P1 normally transduces the entire R factor (Watanabe & Fukasawa, 1961; Kondo, Harada & Mitsuhashi, 1962). Within these limitations, the present analysis of hsI and hsII shows that the phenotypes r⁻m⁻ and r⁻m⁺ were found for restrictionless mutants of hsI and hsII, the r⁻m⁻ class being observed approximately as frequently as the r⁻m⁺ class, and we conclude that both arose as a result of single mutations. By analogy with the κ and b host specificities, we anticipate that the hsI and hsII host specificities are controlled by at least three genes, *hss, hsr* and *hsm* and that the two phenotypes found, r⁻m⁻ and r⁻m⁺ are caused by mutations in *hss* and *hsr* respectively.

Further investigation of these host specificities is dependent on complementation studies. Unfortunately, there is incompatibility between R factors of the same *fi* type, such that two *fi⁻*, or two *fi⁺*, R factors will not co-exist stably in the same bacterium (Harada *et al.* 1961; Mitsuhashi *et al.* 1962; Watanabe *et al.* 1964). Only one R factor (R124) is known to carry the hsI host specificity, and all R factors carrying hsII are *fi⁻* (Bannister, 1969). Thus it is not possible to test complementation between mutants of hsI, or between mutants of hsII. The only complementation tests possible were between two different host-specificity systems. Complementation does occur between mutants of κ and b (Boyer & Roulland-Dussoix, 1969; Arber & Linn 1969; Glover, unpublished results) and it was therefore reasonable to test for complementation between hsI and hsII, and between either hsI or hsII and the host specificities κ, b and that carried by phage P1. However, in no instance was complementation observed.

The assumption was made that the most suitable test for complementation was the restoration of restriction to an r⁻m⁺ (*hsr⁻*) mutant in the presence of the wild-type host specificity of a different type. In addition, r⁻m⁻ (presumed *hss⁻*) mutants were included, and modification was also scored. This assumption requires that the genetic control of host specificities hsI and hsII is analogous to the genetic control of κ and b host specificities. On the basis of the three-gene model, complementation would involve the association of dissimilar gene products from two host-specificity systems to form a functional enzyme. If the analogous gene products from different host-specificity types were slightly different, association of the gene products might not occur, or the product of association might not function correctly, and complementation would not be observed. A second explanation of the absence of complementation is possible. The apparent similarity of the hsI and hsII host specificities to the κ and b host

specificities may be only superficial, and at the molecular level there may be fundamental differences.

It has been argued, in relation to host specificity mutants of phage P1, that the absence of complementation with the intact K and B host specificities is no indication that complementation could not occur, because such restrictionless mutants of phage P1 are normally selected in K strains. Hence, the phage P1 mutants are preselected for the inability to complement with the K host specificity, and, it may be assumed, with the B host specificity also. This criticism is also true of the mutants of hsI and hsII, which have been selected in a K strain. However, mutants of K and B host specificities, known to complement other mutants (7K and B6 in Table 8), failed to complement with the intact hsI and hsII host specificities. Hence it may be assumed that the absence of complementation is indicative of an inherent difference between the host specificity systems, rather than to the nature of the mutants.

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Imperfect Forms and the Taxonomy of Mallomonas

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SUMMARY

This paper deals with taxonomic problems caused by the occurrence in nature of forms of Mallomonas species which are considered to be stages in development. They are called 'imperfect' forms; that is, forms that lack features that have been included in the diagnosis. These individuals might be described as separate species except when intermediate forms are noticed which link them with the perfect form of diagnosed species. The fine details of their scales are the same as in the perfect form.

INTRODUCTION

Mallomonas is a large genus of the Chrysophyceae, a class of algae with golden green chromatophores. Authors differ in the way they subdivide the Chrysophyceae but the genus *Mallomonas* is usually placed near such familiar genera as the colonial *Synura* and less close to *Dinobryon*. Mallomonas is an elegant flagellate possessing a single flagellum and a cell covered with silica scales whose shape and elaborate markings have proved to be specific in their pattern. The scales may have a rigid prolongation, a spine, and may bear a single mobile hinged bristle which is readily detachable. Certain stages of the organism differ considerably from the mature forms. The development of such 'imperfect' forms has been traced as far as it could be in the wild and in samples of natural waters kept under observation for a few weeks. These forms may have caused a good deal of taxonomic confusion. Imperfect forms may be found in all Mallomonas species. They are mainly seen during a period of rapid cell division or 'swarming'. I describe a few of the most commonly found forms having selected them chiefly because they might lead to taxonomic confusion. Undoubtedly these forms have their place in the life-cycle of the species concerned, but this must be studied by other methods than those used here.

Before the advent of the electron microscope the species were based on the shape and size of the cell together with the character and distribution of the bristles and such information about the scales as the microscope of the day could provide. In 1955 Fott published the first electron micrograph of a Mallomonas scale; this was followed by a similar publication by Asmund (1955). Thus a whole new range of taxonomic facts was revealed, the scale and its markings becoming the ultimate criterion of the species. In my view the diagnosis of a Mallomonas species without an electron micrograph of the scale is now out of date.

OBSERVATIONS

One may base the study of a Mallomonas species on a large population of well-developed (perfect) individuals and if a few small or unusual ones are present these will be disregarded. However, imperfect organisms are sometimes abundant and I have noticed that when such a sample is kept in a shady place out of doors the imperfect forms may become few and the perfect ones predominate.

All the torquata group of species have an armour made of three sorts of scale, a few triangular scales with or without bristles, forming a collar, a number of rhomboidal scales forming the body and a few small scales at the rear. Often the rearmost of these has a corner drawn out into a point, ridge or spine. In the commonest imperfect form the collar and rear scales are the same in size and number as in the perfect form but the body scales though of the normal size are few, so that such an organism will be small. The perfect cell also has scales that fit together and form a coherent armour which does not collapse on drying while the scales of the imperfect form overlap and the armour collapses on drying unless the cell has first been fixed. Plate 1, fig. 1–4 illustrate these forms of *Mallomonas pumilio*, Pl. 1, fig. 2, a replica, shows the way the scales fit together, and Pl. 1, fig. 4, a fixed cell, shows the overlap. Plate 1, fig. 5 and 6 show *M. mangofera* in imperfect and perfect forms.

Fig. 1*a*, and *b*, illustrate another type of imperfection which has been seen in *Mallomonas pumilio* and in other species of Mallomonas, though much more rarely. In this the scales appear in the front only, leaving the rear naked. I think it likely that these organisms have emerged recently from the cyst.

Mallomonas heterospina Lund 1942 e.m. Asmund 1956 is a species of another section. It was described as having short bristles at the rear end which bear curiously shaped hooks. Under the electron microscope these hooks can be seen in unmistakable detail. Sometimes, however, imperfect forms occur in which these hooked bristles are absent and the long straight bristles normally occurring at the anterior portion may occur all round the organism. Electron micrographs Pl. 2, fig. 7, 8 illustrate the imperfect and the perfect forms. There are drawings of Mallomonas under various names which might be hookless forms of *M. heterospina*.

Mallomonas akrokomos Pascher of another section was diagnosed by its shape, its 6 to 8 anterior bristles and its size. The scales are so small that they were barely seen clearly until the advent of the electron microscope. Several similar species or varieties have been described since 1913 (see below) which differ in the number and length of the bristles and length of the organism. In my experience the size of *M. akrokomos* from the same pond may vary greatly, the largest form appearing early in the year and the smaller ones during swarming and before encystment. The number of bristles varies from eight down to none, and of such bristles as are present a variable number may be long and others short. This variation is met both in large and in small cells (Harris, 1958, p. 61). An imperfect form not figured before is shown in Fig. 1*c*. It is unusual in lacking the long tail formed by two attenuated scales. I imagine it is an individual recently produced by cell division. When a Mallomonas divides it loses all its old scales and new ones appear on the daughter cells. But this organism has not yet produced its rear scales. Such organisms have been seen occasionally in a few ponds where *M. akrokomus* is swarming.

The following species and varieties may well be imperfect forms of *Mallomonas*

akrokomos: *M. pauciseta* Nauman; *M. quadricornis* Wermel; *M. akrokomos* var. *parvula* Conrad; *M. paucispina* Conrad. See Huber-Pestalozzi (1941) or Matvienko (1965) for figures and further references.

Mallomonas insignis Penard of another section; see Harris (1958) for e.m. illustrations and for imperfect forms. It is very common in some ponds near Reading, Berkshire, England. Its scale proves constant but imperfect forms of cell which look very unusual, are frequent. I suggest that the following species may be imperfect forms of *M. insignis* since the published figures look very much like my own drawings: *M. torulosa* Kisselew; *M. insignis* var. *lacustris* Bourrelly; *M. mesolepis* Skuja; *M. mesolepsis* var. *spinosa* Matvienko (see Huber-Pestalozzi, 1941, or Matvienko, 1965, for figures and further references).
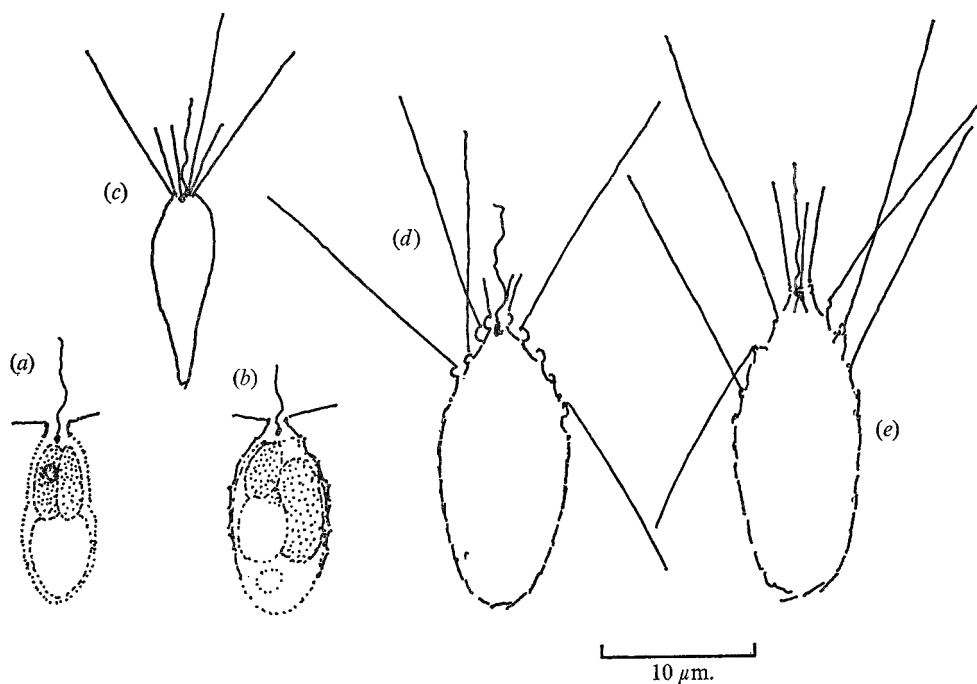
Fig. 1. (*a*) Imperfect form of *Mallomonas pumilio*, possibly occurring soon after germination. (*b*) Imperfect form of *M. pumilio*, at a later stage than (*a*). (*c*) Imperfect form of *M. akrokomos* which lacks two long rear scales. (*d*) Imperfect form of *M. tonsurata* lacking rear bristles. (*e*) Imperfect form of *M. intermedia* lacking rear bristles.

In the section tripartitae, the acaroides group is particularly difficult taxonomically. One of the main characters supposed to distinguish the species is the fraction of the cell covered by bristle-bearing scales but even in a species which normally has bristles all over, imperfect forms occur without bristles at the rear. The bristles themselves have specific characters and in imperfect forms they may be simpler than normal. For example, the perfect cell of *Mallomonas intermedia* of this group has bristles all over but that of *M. tonsurata* var. *alpina* lacks them at the rear. Another difference is that *M. intermedia* has bristles with refractive tips. In both species imperfect forms are met with bristles confined to a varying portion of the front of the organism. In *M. intermedia* these imperfect forms may lack the refractive tip to the bristle and the

size of the imperfect forms may be the same in the two species so that they look almost alike under the light microscope. Compare Fig. 1*d*, *M. tonsurata*, with Fig. 1*e*, *M. intermedia*.

I thank Professor R. M. Ditchburne, F.R.S., and Professor Trevor Evans for allowing me to use the electron microscope in the J. J. Thomson Laboratory, University of Reading.

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EXPLANATION OF PLATE

PLATE 1

Fig. 1. Direct electron micrograph of perfect cell armour of *Mallomonas pumilio*. × 4300. Unfixed material, cytoplasm removed.

Fig. 2. Replica of cell armour of *M. pumilio* var. slightly damaged at rear. One rear scale shows a small point. × 4300. Unfixed material.

Fig. 3. Replica of armour of imperfect form of *M. pumilio* var. × 4300. Unfixed material.

Fig. 4. Replica of imperfect form of *M. pumilio* var. × 4300. Fixed in vapour of osmium tetroxide.

Fig. 5. Direct electron micrograph of imperfect form of *M. mangofera*. × 4300. Unfixed material, cytoplasm removed.

Fig. 6. Replica of perfect form of *M. mangofera*. × 4300. Unfixed material.

PLATE 2

Fig. 7. Direct electron micrograph of imperfect cell of *M. heterospina*. × 5000. Material fixed in osmium tetroxide vapour, cytoplasm removed.

Fig. 8. Direct electron micrograph of perfect cell of *M. heterospina*. × 5000. Fixed osmium tetroxide vapour.
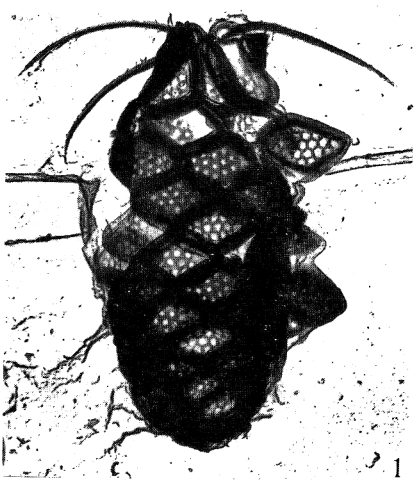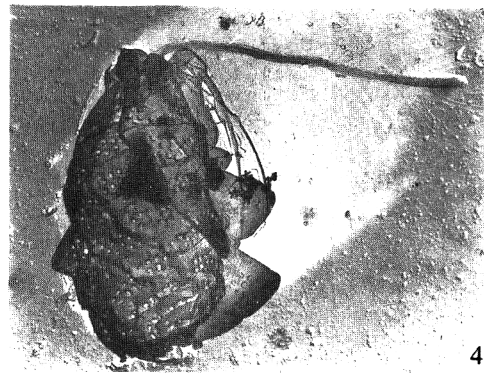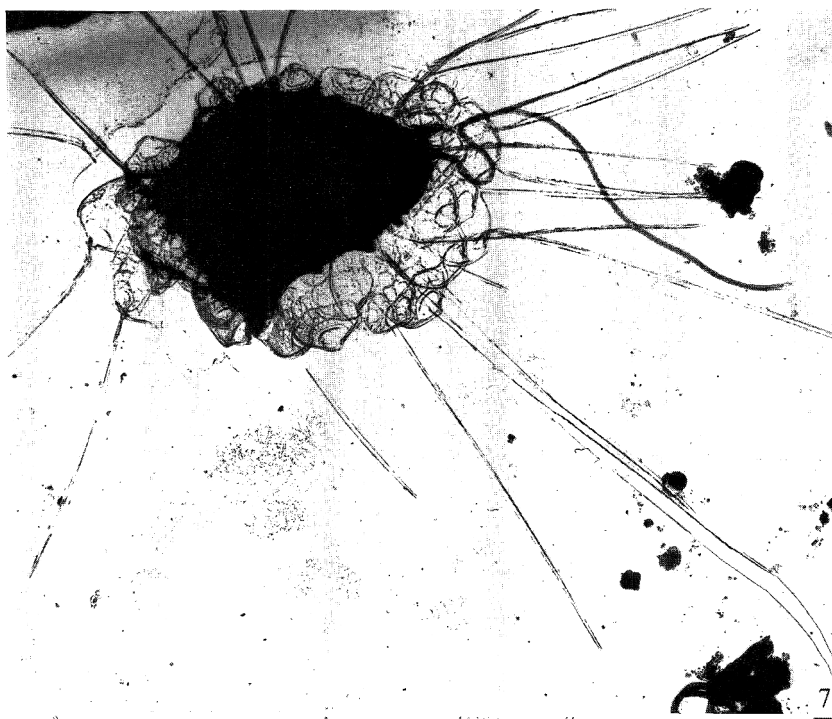
K. HARRIS

Species of the Torquata Group of Mallomonas

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SUMMARY

A new species of the torquata group of the genus *Mallomonas* is described by light and electron microscopes. A list is given of those members of the torquata group which have been published with illustrations obtained with the use of light and electron microscopes. A few of the torquata group which have been published only with light microscope illustrations are listed and discussed. It is considered that no clear decision about their nomenclature can be reached without an electron micrograph of the scales.

OBSERVATIONS

Mallomonas allantoides sp.nov. (Fig. 3 to 6; Pl. 1, fig. 1 to 5)

Holotype (Pl. 1, fig. 1)

Latin diagnosis. Cellula ferme valde elongata, evidente flectens, aliquando formam commutans in momenta temporis aliquantum. Flagellum breve. Squamae in factinones tres divisae: circa quinque squamae collaris, permultae squamae corporis et squamae postremae paucae. Squamae collaris erectes, triquetrae alliquanto procerae, latus sinistrum paulum concavum, latus dextrum convexum, cupola forte constituta, cuspidata, nonnumquam setam brevem ferens. Scutum signatum squamarum corporis simile, margo recurvata, lata in latere convexo et basi scuti, finem faciens prope basim lateris concavi, sed continuata ad cupolam appendice scuti. Squamae corporis rhomboidales, productae transverse, cupola invalida, margo recurvata, praesens in latera due cupolae opposita. Scutum ora crassa et superficie crassitudinibus reticulatis inscriptum, macula una quaeque in tres aut quattuor divise. Squamae postremae minores, squamae ultimae minimae, aliquae squamarum postremarum reticulo inscriptae. Squamae ultimae ferme spinas attenuatas ferentes, non scripta ferme. Cellula 13 ad 47 μm. longa, 5 ad 9 μm. lata, squamae collaris et corporis 2·5 × 4 μm. Cystum 12 ad 15 μm. in arta cellula materna. Setae 6 ad 8 μm. longa, spina 3 ad 9 μm. longa.

English diagnosis. Cell normally much elongated, distinctly flexible, sometimes changing its shape in minutes. Flagellum short. Scales comprising three groups: about five collar scales; numerous body scales; a few rear scales. Collar scales erect, triangular, rather long; left side slightly concave, right side convex, dome strongly developed, pointed, sometimes bearing short bristle. Shield marked as shields of body scales. Flange recurved, broad on convex side and base of shield, ending near base of concave side but joined to dome by appendage. Body scales rhomboidal, transversely elongated, dome feebly developed, flange recurved, developed on two sides opposite dome, shield with thickened edge and surface marked with reticulate thickenings, each mesh divided into three or four. Rear scales smaller, last scales smallest, some rear scales with

reticulate marks. Last scales usually unmarked but bearing tapering spines. Cell 13 to 47 μm. long, 5 to 9 μm. wide, collar and body scales 2·5 × 4 μm. Cyst 12 to 15 μm. in diameter in close fitting mother cell. Bristles 6 to 8 μm. long, spines 3 to 9 μm. long.

Occurrence. The species is rare in the Reading district (Berkshire, England) and I have found it in only two localities. The type locality was a roadside pond now filled-in; the other pond was on gravel and surrounded by trees and rhododendrons. Both ponds are on what appeared to be poor land. The specific epithet *allantoides* means sausage-like, to suggest the organism's shape.
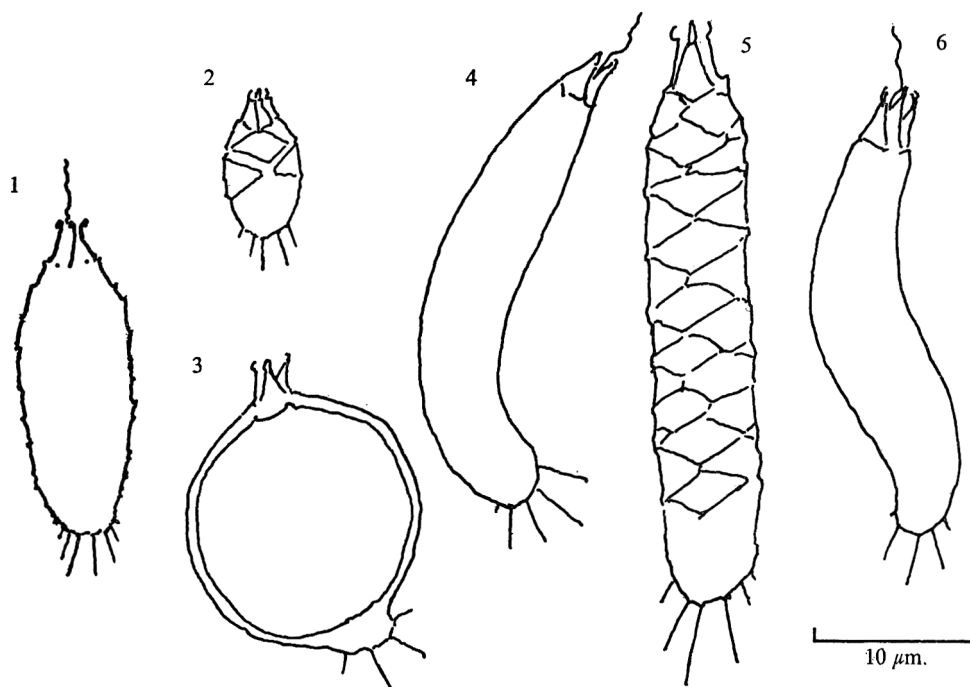
Fig. 1 to 6. 1, *Mallomonas doignonii* perfect form. 2, *M. doignonii*, imperfect form. 3, *M. allantoides*, encysted. Fig. 4 to 6, *M. allantoides*, perfect forms showing changes of shape.

DISCUSSION

Mallomonas allantoides is of interest in being the most flexible Mallomonas species that I have seen. It has a long slender cell which does not change much in width until it approaches encystment. Then the armour stretches until it is nearly spherical, enclosing a spherical cyst.

There are only two other species of the torquata group of the genus *Mallomonas* bearing spines which have been examined with both light and electron microscopes. These are *M. doignonii* Bourrelly, 1951; Harris & Bradley, 1957; and *M. eoa* Takahashi, 1963. Of these species *M. allantoides* has the longest cell and its cell has unusual flexibility; *M. eoa* has the longest rear spines, while *M. doignonii* is much the same length as *M. eoa* but has much shorter spines; *M. doignonii* also has a marked rigidity in its armour which is difficult to break up, at least in its perfect form. The three species may be distinguished at once by the electron micrograph of the scales. The *M.*

doignonii scale has thickened striations, usually vertical; *M. allantoides* scale gives the impression of having one network superimposed upon another which is less well marked; *M. eoa* has a single close network of circular holes. *M. doignonii* Bourrelly 1951 is the same as that figured by Harris & Bradley (1957) as *M. coronata*, Pl. 1, fig. 7, and discussed in Harris & Bradley (1960).

An imperfect form of *M. allantoides* (Pl. 1, fig. 2) has some well-developed rear spines, but as is usual in a dried specimen at this stage, the armour has collapsed and partly disintegrated. Fig. 1 is a perfect form of *M. doignonii* and Fig. 2 an imperfect form of it which although short and with fewer body scales has fully developed rear spines.

Species of the torquata group which have been studied with both light and electron microscope

Species with rear spines over 1 μm. long:

    M. doignonii Bourrelly 1951, Harris & Bradley 1957, 1960;

    M. eoa Takahashi, 1963; this includes *M. clavus* Bradley, 1964;

    M. allantoides, present paper.

Species without rear spines but often showing rear points less than 1 μm. long:

    M. pumilio var. *schwemmlei* Glenk, 1956; electron-microscope illustrations, Fott
        & Ettl, 1959, see below;

    M. pumilio Harris & Bradley, 1957, 1960;

    M. pumilio var. *silvicola* Harris & Bradley, 1960;

    M. pumilio many unnamed varieties, this periodical;

    M. phasma Harris & Bradley, 1960;

    M. mangofera Harris & Bradley, 1960;

    M. grata Takahashi, 1963.

M. pumilio var. *schwemmlei* was first described with the light microscope by Glenk (1956) as *Mallomonas schwemmlei*. Its electron micrograph was then published, together with a good light micrograph showing its general habit, by Fott & Ettl (1959). This was before it was fully realized that the rear point was a rigid part of the scale and not capable of change into a spine. I have therefore renamed it *M. pumilio* var. *schwemmlei*.

There are three species of *Mallomonas* of the torquata group with rear spines that have been described since 1940 but with the light microscope only. They all have a certain similarity, and bearing in mind the variation in shape which may occur in one species and the variation in interpretation of very small objects, I believe that an electron micrograph of the scale is the only way to compare them.

These three species are:

    M. coronifera Matvienko, 1941. Ukrainian material;

    M. lefeuvrier Villeret, 1954. French material;

    M. coronifera Perman & Vinnikova, 1955. Czech material.

There is a species with a small cell and no spines, *M. potsdamiensis* Bethge, 1954. We have information with the light microscope only but it deserves electron microscope study.

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EXPLANATION OF PLATE

Mallomonas allantoides

Fig. 1. Whole perfect cell. × 4000. Direct electron micrograph.

Fig. 2. Whole imperfect cell. × 4000. Replica.

Fig. 3. Collar scale. × 10,000.

Fig. 4. Body scale. × 10,000.

Fig. 5. Rear scales, two with spines. × 10,000.
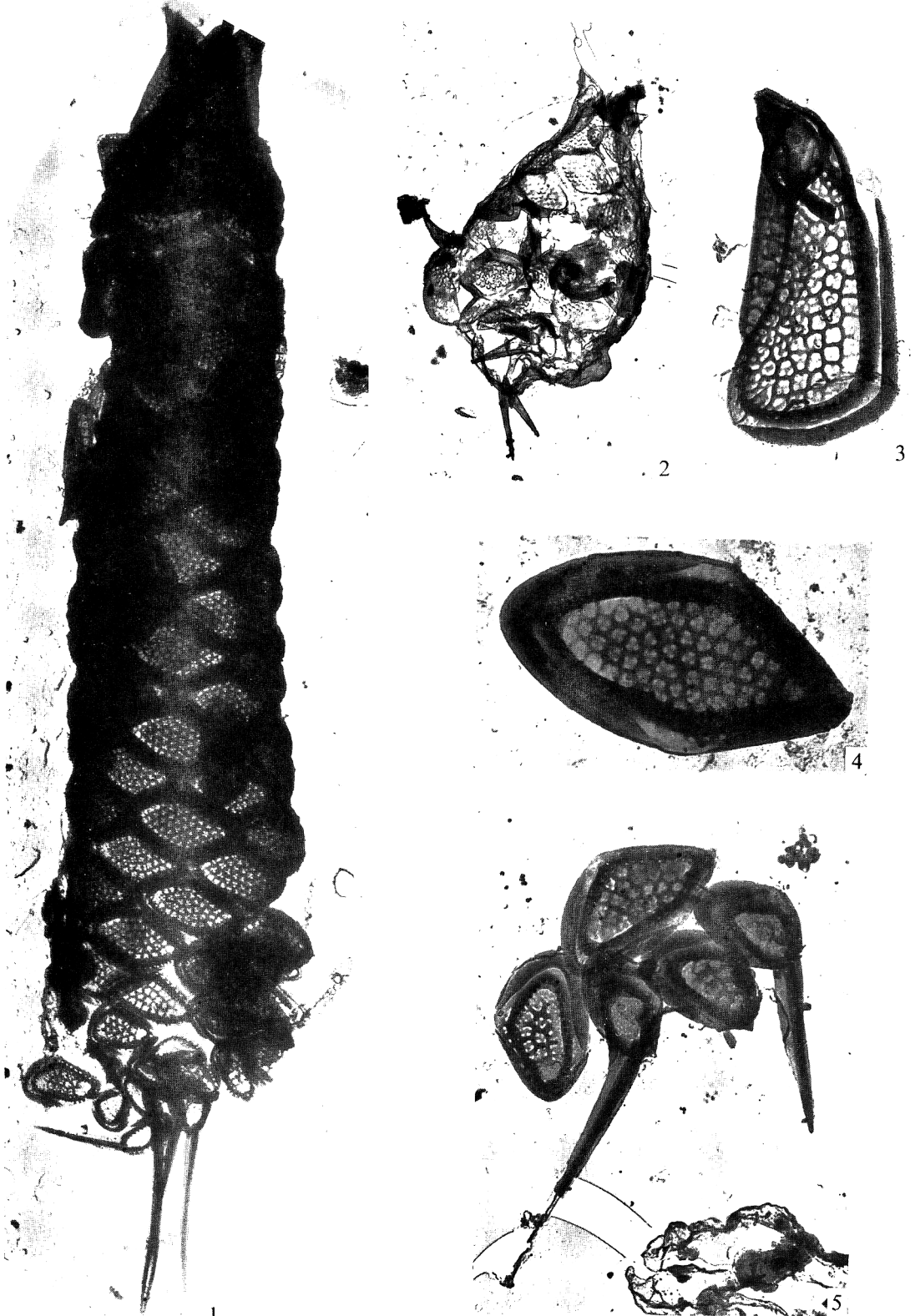
Aflatoxin and Kojic Acid Production by Resting Cells of *Aspergillus flavus* Link

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SUMMARY

Resting *Aspergillus flavus* synthesized more aflatoxin on a medium with glucose than they did with any other substrate tested. D-Xylose and ethanol fostered the formation of kojic acid but not aflatoxin. Yields of kojic acid and aflatoxin responded differently to alteration in temperature, pH, surface–volume ratio of the culture medium, and the presence of various chemicals in the medium. [^{14}C]Acetate as a substrate led to strongly labelled aflatoxin being formed. The simultaneous addition of unlabelled kojic acid did not lower the relative isotope content of the synthesized toxin. On the other hand, addition of unlabelled acetate to medium with [^{14}C]kojic acid did reduce the relative isotope content of the toxin synthesized. It is concluded that the synthesis of kojic acid and aflatoxin follow separate pathways, and that kojic acid is not an intermediate in aflatoxin synthesis by resting cells of *A. flavus*.

INTRODUCTION

Amongst the toxic metabolites of *Aspergillus flavus*, kojic acid has been known for several decades, and aflatoxin since 1961. The observations of Davis (1963), that an aflatoxin-forming strain of *A. flavus* produced kojic acid suggested a biosynthetic relationship between the two compounds. This is further supported by the fact that the bisdihydrofuran moiety is seen in aflatoxin as well as in sterigmatocystin in whose biosynthesis kojic acid has a key position. This lead Holker & Underwood (1964) and Heathcote, Child & Dutton (1965) to postulate that aflatoxin might be derived biogenetically from a compound similar in structure to sterigmatocystin. Parrish Wiley, Simmons & Long (1966) found that all aflatoxin-forming strains of *A. flavus* produced kojic acid but not all kojic acid-forming strains produced aflatoxin. This close association of kojic acid with aflatoxin in toxin-forming strains of *A. flavus* posed the question whether kojic acid formation is an intermediate step in toxin biosynthesis. This point has been examined with the use of resting *A. flavus*.

METHODS

The organism. An isolate obtained from infected groundnuts and identified as *Aspergillus flavus* was used. It was preserved in sterile soil and stored at 5°.

Inoculum. A loopful of the soil was transferred to Czapek–Dox agar slant and incubated at 25° for 5 days. Heavy mycelial growth and sporulation were obtained. The conidia formed were suspended in sterile distillation water and used as the inoculum.

Preparation of resting organisms. Czapek–Dox–Casein–thiamine medium (Basappa,

Jayaraman, Sreenivasamurthy & Parpia, 1967) inoculated with the conidia was incubated without agitation at 25° for 48 hr. The mycelium was washed with cold sterile distilled water and finally with cold, sterile, resting-mycelium medium. The composition of this was as described by Adye & Mateles (1964) but without glucose. The washed mycelium, in four times its weight of resting mycelium medium was blended in a Waring Blender for 30 sec. Twenty ml. of this was placed in each 100 ml. Erlenmeyer conical flask for experimental treatment. In experiments where ^{14}C-compounds were used, 5 ml. was placed in a 50 ml. flask.

Substrates, inhibitors and stimulants. Concentrated solutions of substrates, inhibitors and stimulants were sterilized at 10 lb./in. pressure for 20 min. and dispensed into flasks prior to the addition of resting cells. [^{14}C]Kojic acid was prepared biosynthetically by the resting cells of *Aspergillus flavus* using [1-^{14}C]acetate as carbon source. It was separated and purified on thin layer chromatoplates by the method described below.

Incubation. Preliminary studies showed that toxin began to form after 36 hr incubation and reached its maximum at 72 hr. Resting mycelia were therefore incubated always for a period of 72 hr at 25°. The pH of the medium, unless otherwise stated, was at 6·0 before incubation. All treatments were in triplicate and all results were confirmed by repeating at least once. The means of triplicated data from one experiment are reported.

Estimation of aflatoxin. After 72 hr incubation the medium with the resting cells was repeatedly extracted with chloroform. Pooled extracts were cleaned by a procedure outlined by Mayura & Sreenivasamurthy (1969) and concentrated. Concentrated extract was streaked on a number of thin-layer chromatoplates coated with silica gel G. The plates were developed with chloroform containing 10 % ethanol. The fluorescent band corresponding to aflatoxin B was scraped off and the toxin eluted with methanol. The clear methanol solution was heated in a water bath under reduced pressure to remove the solvent. The residue was dissolved in 5 ml. methanol and its absorption at 363 μm. in a D.U. spectrophotometer determined. Toxin concentration was obtained by watching the optical density against a standard curve.

Estimation of kojic acid. Kojic acid was estimated by the colorimetric method described by Bentley (1957) with ferric chloride as the reagent.

Isolation and purification of [^{14}C]*aflatoxin.* Aflatoxin was extracted, purified and dissolved in methanol as described above. Eluate was transferred to a planchet and heated under infrared light to evaporate the solvent. Radioactivity counts were then determined using a windowless gasflow counter (Tracer Lab. U.S.A.).

Isolation and purification of [^{14}C]*kojic acid.* The aqueous phase remaining after chloroform extraction was streaked on a thin layer chromatoplate. A small quantity of kojic acid was spotted at one end of the streak to serve as the reference spot. The plates were developed first with 10 % ethanol in chloroform to separate any residual toxin, then with 4 + 1 + 1 mixture of butanol + acetate acid + water to separate kojic acid. The kojic-acid band corresponding to the reference spot, which was detected by coloration with ferric chloride, was scraped off, eluted with water, and concentrated in a nitrogen atmosphere. A sample of the concentrate was evaporated on a planchet and its radioactivity count determined with a windowless gasflow counter. [1-^{14}C]Acetate was obtained from Babha Atomic Research Centre, Trombay, Bombay, India.

RESULTS

The yields of aflatoxin and kojic acid by *Aspergillus flavus* grown with different carbon sources are presented in Table 1. On an equimolar basis glucose yielded more toxin than any other substrate. Sodium acetate was next best. Kojic acid yielded little toxin. D-Xylose and ethanol supported the formation of kojic acid, but yielded little toxin. The effects of altered temperature, pH and ratio of surface area/volume on synthesis of aflatoxin and kojic acid by resting mycelium are presented in Tables 2, 3 and 4. Kojic acid synthesis with acetate as the substrate was maximal at pH 6·0 and 7·0 while aflatoxin yields were highest at pH 5·0 and 6·0. Similarly, kojic acid yield was highest at 37° while aflatoxin yield was highest at 25°. Alteration in the ratio of surface area to volume markedly affected synthesis of aflatoxin but not of kojic acid.

Table 1. *Yields of aflatoxin and kojic acid by resting Aspergillus flavus in media with different carbon substrates*

Substrate 100 μmole/ml.	Kojic acid (μg./ml.)	Aflatoxin (μg./ml.)
None	30·0	2·5
Na acetate	80·0	21·0
D-Glucose	300·0	56·0
Kojic acid	550·0	12·0
D-Xylose	200·0	10·0
Ethanol	160·0	2·5

Table 2. *Effect of pH on yields of aflatoxin and kojic acid by resting Aspergillus flavus*

Substrate 100 μmole/ml.	pH	Kojic acid (μg./ml.)	Aflatoxin (μg./ml.)
Control (no substrate)	5·0	5·0	0·1
Control (no substrate)	6·0	5·0	0·2
Acetate	4·0	0·0	0·3
Acetate	5·0	90·0	26·4
Acetate	6·0	100·0	26·6
Acetate	7·0	100·0	0·2
Acetate	8·0	44·0	0·1

Table 3. *Effect of temperature on yields of kojic acid and aflatoxin by resting Aspergillus flavus*

Substrate: 100 μmole/ml. acetate

Temperature	Kojic acid (μg./ml.)	Aflatoxin (μg./ml.)
20	0·0	1·0
25	80·0	29·0
28	100·0	6·5
37	130·0	0·1

The results in Table 5 show that $Na_2S_2O_4$ increased the yield of the toxin, but not of kojic acid. Malonate on the other hand reduced the yield of kojic acid, but not of toxin. Hydroxylamine reduced the yield of both. Under an atmosphere of nitrogen,

however, kojic acid yield was significantly increased while yield of toxin was slightly reduced. When kojic acid was the substrate instead of acetate, none of these treatments influenced yield of toxin.

Table 4. *The effect of changes in the ratio of surface area to volume on yields of kojic acid and aflatoxin by resting Aspergillus flavus*

Substrate: 100 μmole/ml. acetate/ml.

Volume of medium in 100 ml. flask (ml.)	Kojic acid (μg./ml.)	Aflatoxin (μg./ml.)
10	150	39
20	170	38
30	140	12
40	130	6·0

Table 5. *Effect of inhibitors and stimulants on yields of aflatoxin and kojic acid by resting Aspergillus flavus*

Inhibitor/stimulant	Kojic acid (μg./ml.)	Aflatoxin (μg./ml.)
Substrate: 100 μmole acetate/ml.		
Control	85·0	21·0
+ Na$_2$S$_2$O$_4$ 1 mg./ml.	70·0	70·0
+ Malonate 5 mg./ml.	5·0	22·0
+ Sulphanilamide 0·5 mg./ml.	28·0	1·0
+ Hydroxylamine 0·1 mg./ml.	5·0	1·0
+ Under nitrogen	160·0	12·0
Substrate: 50 μmole kojic acid/ml.		
Control	2400·0	13·0
+ Na$_2$S$_2$O$_4$ 1 mg./ml.	2250·0	13·0
+ Sulphanilamide 0·5 mg./ml.	3000·0	12·0
+ Hydroxylamine	3500·0	12·0
+ Malonate 5·0 mg./ml.	3000·0	13·0

Table 6. *Incorporation of [1-^{14}C]acetate into kojic acid by resting Aspergillus flavus*

Substrate*	μmole of kojic acid/5 ml.	Specific activity in kojic acid (mμc/μmole)	r.i.c.† of kojic acid
[1-^{14}C]Acetate 7·5 μc + unlabelled acetate 250 250 μmole	2·1	75·3	2·5
[1-^{14}C]Acetate 7·5 μc + unlabelled acetate 125 μmole + unlabelled kojic acid 125 μmole	2·4	71·7	2·4

* 1 mg./ml. NaHSO$_3$ added to the substrate for stimulation.
† Relative isotope content calculated as described by Adye & Mateles (1964).

Data on formation of aflatoxin and kojic acid by *Aspergillus flavus* using labelled substrates are presented in Tables 6, 7 and 8. The specific activity and relative isotope content of the toxin were very high when labelled acetate was used as the substrate. With labelled kojic acid toxin formation was slight. Addition of unlabelled kojic acid to the medium containing labelled acetate as the substrate did not lower the specific

activity or the relative isotope content of the synthesized toxin. Indeed there was a slight increase. Addition of unlabelled acetate to the medium with labelled kojic acid significantly reduced specific activity as well as the relative isotope content of the toxin synthesized. The results in Table 6 show that incorporation of the labelled carbon from [¹⁴C]acetate into kojic acid is not influenced by adding unlabelled kojic acid to the medium.

Table 7. *Influence of kojic acid on the incorporation of* ¹⁴C *from acetate into aflatoxin by resting Aspergillus flavus*

Substrate*	μmole of aflatoxin/5 ml.	Specific activity in aflatoxin B mμc/μmole	r.i.c.† of aflatoxin
[1-¹⁴C]Acetate 7·5 μc + unlabelled acetate 250 μmole	0·64	157·0	5·2
[1-¹⁴C]Acetate 7·5 μc + unlabelled acetate 125 μmole + unlabelled kojic acid 125 μmole	0·64	204·0	6·8

* 1 mg./ml. NaHSO₃ added to the substrate for stimulation.
† Relative isotope content calculated as described by Adye & Mateles (1964).

Table 8. *Influence of acetate on the incorporation of* ¹⁴C *from kojic acid into aflatoxin by resting Aspergillus flavus*

Substrate*	μmole of aflatoxin/5 ml.	Specific activity in aflatoxin B mμc/μmole	r.i.c.† of aflatoxin
[¹⁴C]Kojic acid 0·3 μc + unlabelled kojic acid 150 μmole	0·3	3·0	1·5
[¹⁴C]Kojic acid 0·3 μc + unlabelled acetate 250 μmole	0·7	0·9	0·75

* 1 mg./ml. NaHSO₃ added to the substrate for stimulation.
† Relative isotope content calculated as described by Adye & Mateles (1964).

DISCUSSION

The data on the influence of substrates, pH, temperature and surface area/volume ratio on toxin and kojic acid synthesis by *Aspergillus flavus* show that the syntheses of the two compounds follow two different pathways controlled by different enzyme systems and differently influenced by various chemical and physical factors. This conclusion is supported by the data on the influence of dithionite, malonate and nitrogen atmosphere on toxin and kojic acid formation. Even in those cases where kojic acid synthesis was high, there was no enhancement of toxin formation. This was true with the substrates alcohol and xylose and also at high incubation temperature. This shows the unsuitability of kojic acid as a substrate for aflatoxin synthesis and hence kojic acid does not seem to be an intermediate in aflatoxin synthesis. This conclusion is also supported by the data with ¹⁴C-compounds. The relative isotope content of the toxin is very high with labelled acetate as the substrate. If kojic acid formation were an intermediate step in toxin synthesis, unlabelled kojic acid added to the medium with labelled acetate should enter into the synthetic pathway and lower the specific activity

or the relative isotope content of the toxin. The results show that unlabelled kojic acid had no such effect, even though as much as 90 % of it was utilized by the organism. The data with [^{14}C]kojic acid further substantiate this view. Inclusion of unlabelled acetate with [^{14}C]kojic acid markedly reduced the specific activity of the toxin but considerably enhanced aflatoxin formation thus showing that acetate, but not kojic acid was the preferred substrate for toxin synthesis. Since ^{14}C incorporation from acetate into kojic acid was quite high, this compound must be one of the normal metabolites of acetate metabolism and have no role in aflatoxin synthesis.

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Sporidesmin Production and Sporulation in
Pithomyces chartarum

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SUMMARY

Sporidesmin production by 37 recently isolated strains and one heavily sporing laboratory isolate of *Pithomyces chartarum* was assayed by toxicity *in vitro* to tissue culture cells and related to sporulation and growth. Under standard cultural conditions strains varied greatly in their ability to produce sporidesmin and spores. Heavily sporing cultures produced most sporidesmin and the level of sporidesmin production by a strain could be changed by manipulation of cultures in ways which also stimulated or depressed sporulation. Ultraviolet radiation increased sporulation and sporidesmin production in 33 strains. Shaken cultures, in which growth was good but sporulation suppressed, produced no sporidesmin. The close association of sporidesmin production with sporulation supports the reliability of spore counts as an index of pasture toxicity. Most and probably all strains of *P. chartarum* are potentially able to produce moderate to high levels of sporidesmin. Ultraviolet radiation may stimulate production of sporidesmin by the growing fungus but destroys it in aqueous solutions leached from the senescent mycelium.

INTRODUCTION

Strain variability in production of sporidesmin by *Pithomyces chartarum* (Berk. & Curt.) M. B. Ellis was observed soon after this fungus, growing on pasture debris, was recognized as the cause of the mycotoxicosis of sheep and cattle called facial eczema (Percival & Thornton, 1958). Ross & Thornton (1962) and Ross (1962), using several strains, found that different isolates grown under similar conditions varied in the amount of sporidesmin they produced and that there was a general relationship between spore numbers and sporidesmin production. Dingley, Done, Taylor & Russell (1962) using six strains, some of which did not spore freely, found more than hundredfold differences in amounts of sporidesmin produced under standard conditions. Davison & Marbrook (1965), using ten strains which spored well in culture, found only sevenfold differences.

The following is an account of assay by toxicity *in vitro* to tissue culture cells of sporidesmin production by 38 strains of *Pithomyces chartarum* grown under various conditions. The relationship between sporidesmin production and sporulation was examined by manipulation of cultures in ways affecting sporulation; ultraviolet radiation was used to stimulate sporulation, shaken cultures to suppress it. A second purpose of the work was to determine the range of toxicities in a group of recently isolated strains in an attempt to find whether high and low toxin-producing strains occurred randomly in *P. chartarum* populations in the field, or whether strains of

similar toxicity occurred together in time or place. A third was to select for routine sporidesmin production those strains which produced most sporidesmin on sterilized ryecorn, a medium more suitable for large scale extraction than broth or agar cultures.

For the reasons given by Mortimer & Collins (1968) we assumed that virtually all the toxin assayed in the *Pithomyces chartarum* cultures was sporidesmin.

METHODS

Strains used

Thirty-seven of the 38 *Pithomyces chartarum* strains used were isolated between September 1966 and June 1968 during a survey of moulds on leaves of pasture plants (di Menna & Parle, 1970). Nineteen were from samples collected in the Hamilton area and five from places on the East Coast of the North Island, *c.* 150 miles from Hamilton. Thirteen were from material collected from one place on one day, from Thames 40 miles from Hamilton, on 1 March 1967. Seven of the Hamilton strains were recovered in 1966 and 1967 during a period when field conditions were unfavourable for the growth of *P. chartarum* and only isolated cases of facial eczema occurred. The remaining 12 Hamilton strains were recovered in the first half of 1968 when there was a widespread facial eczema outbreak. The last strain used, C, a laboratory isolate from a heavily sporing sector of a culture isolated in 1958, has been used for sporidesmin production.

Single spore isolates were used, except for a few very poorly sporing strains from which such preparations were not practicable. Primary cultures, from which subcultures were made for the preparation of inocula, were held in sterilized soil.

Culture techniques

Media. Semi-synthetic broth (SSB): Glucose, 20 g.; Difco yeast extract, 2 g.; Oxoid peptone, 2 g.; maltose, 2 g.; NH_4NO_3, 2 g.; $NaNO_3$, 2 g.; $MgSO_4$, 2 g.; KH_2PO_4, 2 g.; distilled water 1 l.; pH adjusted to 6·5–7·0; sterilized by autoclaving for 15 min. at 120° (Bohner, Fetz, Harri, Sigg, Stoll & Tamm, 1965). Sterilized ryecorn: Ryecorn (*Secale cereale*), 75 g. and distilled water, 45 ml./pint milk bottle; soaked overnight, sterilized by autoclaving for 30 min. at 120° (Done, Mortimer, Taylor & Russell, 1961). Maintenance medium for dilution of culture filtrates: Tissue culture medium 199 (Burroughs Wellcome & Co., London) with 5 % pooled calf serum; 0·16 % $NaHCO_3$, penicillin (100 units/ml.) and streptomycin (100 μg./ml.).

Preparation of cultures. SSB cultures to be irradiated and incubated in parallel in the dark were inoculated in bulk lots with suspensions of spores and hyphae and dispensed in 15 ml. quantities into disposable plastic Petri dishes 8·5 cm. in diameter. Inoculated broth to be incubated on a rotary shaker in the dark and, in parallel, unshaken, was dispensed in 200 ml. quantities into 500 ml. Erlenmeyer flasks. Cultures for each treatment were set up in triplicate; one culture was used for sporidesmin assay, one for a spore count, and one for estimation of the dry weight of mycelium.

Ryecorn in ten bottle batches was inoculated with each strain, incubated in the dark, harvested, and mixed thoroughly before aliquots were taken for analysis.

All strains were cultured in SSB with and without ultraviolet (u.v.) radiation and in ryecorn. Three strains were used for shaken and static cultures.

Incubation temperature. Cultures were incubated at 20°, the optimum temperature

for sporidesmin production on potato–carrot broth (Ross & Thornton, 1962), and on propylene oxide sterilized ryegrass leaves (Davison & Marbrook, 1965).

Incubation times. SSB cultures in Petri dishes were routinely incubated for 2 weeks but, to follow the course of the production of sporidesmin and spores, three strains were grown with and without u.v. radiation and harvested after 3, 6, 9, 12 and 14 days incubation.

SSB shaken and static flask cultures were incubated for 4, 7 and 14 days. A further set was incubated on the shaker for 7 days and then static for 7 days. Collars of growth forming on the sides of the shaken flasks were loosened into the medium every second day.

Ryecorn cultures were incubated for 4 weeks. Preliminary work with strain c showed that there was a tenfold increase in sporidesmin content between the second and third week of incubation and a twofold increase between the third and fourth week, but only a slight increase between the fourth and fifth week.

Ultraviolet radiation. The fixture used was a fluorescent tube giving radiation at 350 nm. mounted between two cool white fluorescent tubes at a distance of 12 in. above bench height. The plastic Petri dishes used were transparent to radiation of 295 nm. or more. Cultures were irradiated for 10 hr/day between the second and eighth incubation days, a total of 70 hr.

In addition, two strains incubated in SSB for 14 days were given u.v. radiation doses of 20–130 hr. The effect of time of dosage was also examined. A total of 60 hr u.v. radiation was given, either on the second to seventh incubation days or on the eighth to thirteenth days.

Assay techniques

Spore counts. SSB cultures from Petri dishes or flasks were first frozen and thawed to improve spore dispersal, then shaken with glass beads for 10 min. on a wrist action shaker. The resultant spore suspension, diluted where necessary, was counted in a haemocytometer. One g. of ryecorn culture suspended in 10 ml. water was treated in the same way. Spores were counted in 2 cu. mm. of each preparation.

Dry weight of mycelium. SSB cultures were centrifuged to remove excess broth and mycelial mats dried at 105° for 24 hr before weighing.

Sporidesmin assay. SSB cultures were homogenized for 30 sec. in a Sorval Omni-mixer and vacuum filtered successively through Whatman no. 41 and no. 1 filter papers. The filtrates were then diluted to 10^{-2} with maintenance medium, sterilized by pressure filtration (Millipore G.S. $0.22\,\mu$), and diluted decimally to 10^{-7}. Non-inoculated SSB was prepared similarly for assay controls.

Fifty g. of each ryecorn culture were weighed into a 500 ml. Erlenmeyer flask and shaken with 125 ml. 80 % (v/v) methanol/water on a rotary shaker at 20° for 20 hr. The extracts were vacuum filtered through paper as above, a 5 ml. portion from each diluted to 10^{-2} with maintenance medium, filter sterilized, and diluted decimally to 10^{-7}.

To assay the relatively small amount of sporidesmin produced by some strains, 10^{-1} dilutions were prepared. For these 1 ml. portions of each extract were evaporated *in vacuo* in the dark. Each residue was dissolved in 0.1 ml. ethanol, the volume made up to 10 ml. with maintenance medium, and sterilized by filtration. Previous work in this laboratory had shown that ethanol at 1 % (v/v) or less in water had no effect

on the HEp2 cells in the culture system used, and in our study none of the test filtrates contained more than this. Non-inoculated ryecorn was extracted similarly and used as an assay control.

Tissue culture. Cells were cultured as described by Mortimer & Collins (1968), who showed that a typical cytotoxic reaction was produced on HEp2 cell mono-layers with a least recognizable toxic dose of 0·4 ng. sporidesmin/ml. In this investigation, solutions of purified crystalline sporidesmin of known concentration were tested at the same time as the *Pithomyces chartarum* culture filtrates and gave a typical reaction at a least toxic dose (l.t.d.) of 0·5 ng./l. All tubes were roller cultured and examined at 48 and 72 hr. Four tubes were used at the 10^{-1} dilution and 5–7 at all other dilutions.

The greatest dilution at which a definite cytotoxic dose was produced was taken to be the l.t.d. and equivalent to 0·5 ng. sporidesmin/ml. The concentrations in the original SSB culture or methanol extract of ryecorn were then calculated and expressed as mg. sporidesmin/l.
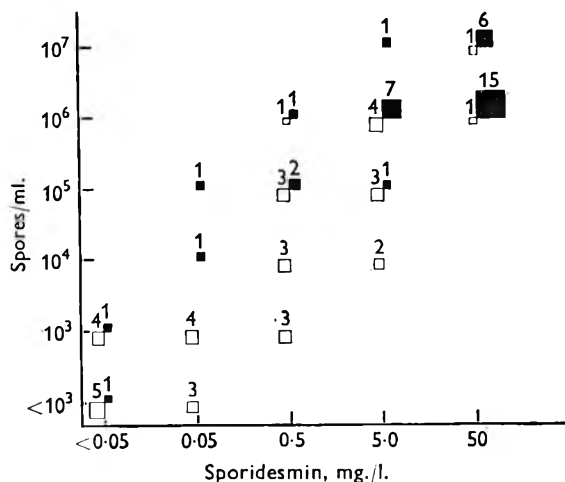
Fig. 1. Relationship between sporidesmin concentration and spore numbers in 37 field strains of *Pithomyces chartarum* grown on SSB for 14 days at 20° in the dark or with 70 hr u.v. radiation. Numerals are number of strains at each level. □, cultures incubated in the dark. ■, cultures given u.v. radiation.

RESULTS

SSB cultures

Effect of u.v. radiation. The effect of 70 hr u.v. radiation on production of spores and sporidesmin in 14-day SSB Petri dish cultures is shown in Fig. 1. Without u.v. radiation only 11 field strains produced 5 or more mg. sporidesmin/l. With u.v. radiation 30 produced sporidesmin at this level, 18 producing 50 mg./l. and three producing an amount in excess of 50 but less than 500 mg./l. Spore numbers were similarly affected; u.v. radiation increasing the number of strains producing 10^6 or more spores/ml. from seven to thirty. There was a general but not exact relationship between sporidesmin production and spore numbers. A few cultures produced low levels of sporidesmin with no spores, or few spores but no detectable sporidesmin.

Strain C and four field strains were not affected by u.v. radiation. Strain C produced

sporidesmin at a level of more than 50 but less than 500 mg./l. and 5×10^6 spores/ml. in both irradiated and non-irradiated cultures. Two field strains also had high sporidesmin levels and spore counts in both sets of cultures, and two produced few or no spores and no detectable sporidesmin.

The dry weight of mycelium produced varied with strain, in non-irradiated SSB cultures from 0·82 to 1·62 (mean value 1·26) g./100 ml. of medium. In irradiated cultures the yield was always less, from 0·62 to 1·19 (mean value 1·02) g./100 ml.
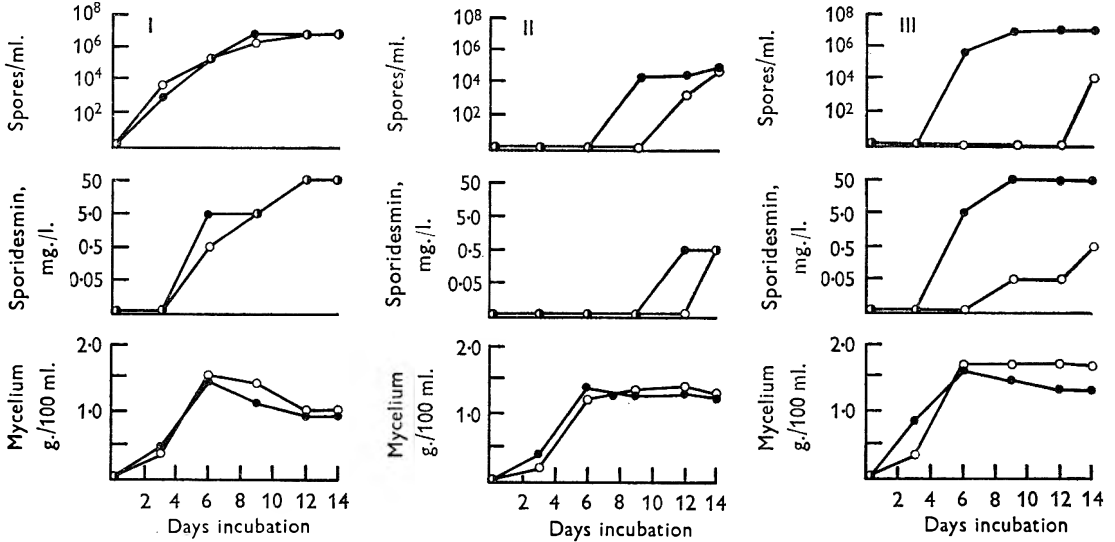
Fig. 2. Rate of production of sporidesmin, spores and dry weight of mycelium by three strains of *Pithomyces chartarum* grown on SSB at 20°. O———O incubated in dark. ●———● given 10 hr daily doses of u.v. radiation from the second to eighth incubation days inclusive. I, strain C; II, strain WS(1)1367; III, strain 2R(15)4468.

Times at which sporidesmin and spores were produced in SSB Petri dish cultures varied with strain and treatment (Fig. 2). The maximum dry weight of mycelium was reached after 6 days incubation; subsequent to this it remained constant or decreased a little. Sporidesmin production was not related to growth for it was first detected after periods of from 6 to 14 days, after, before or at the same time that spores were first seen.

The effect of increasing doses of u.v. radiation upon two strains is shown in Table 1. Twenty hr radiation had little effect on sporidesmin or spore production but reduced the dry weight of mycelium. Forty to 60 hr exposure gave the maximum increase in production of sporidesmin and spores, and after this the response did not change.

Whether u.v. radiation was given in the first week of incubation when the mycelium was growing actively, or in the second week when the dry weight was no longer increasing, did not alter the final quantity of sporidesmin or spores produced by the two strains examined (Table 2).

Shaken and static cultures. Results from these are given in Table 3. Sporidesmin and spore production were both less in the SSB static flask cultures than in the same strains in the shallower Petri dish cultures. Growth also was less and after 14 days

Table 1. *Effect of increasing doses of u.v. radiation on sporidesmin production, spore numbers and dry weight of mycelium by two strains of Pithomyces chartarum grown on SSB for 14 days at 20°*

Radiation was begun on the second incubation day and given in 10 hr doses. There was no change in response to dosage at 70–130 hr so these values are not given.

| U.v. radiation dosage | Strain 6L 17167 | | | Strain R₂(39)22568 | | |
	Spori-desmin (mg./l.)	Spores/ml.	Dry wt mycelium (g./100 ml.)	Spori-desmin (mg./l.)	Spores/ml.	Dry wt mycelium (g./100 ml.)
0 hr	0·5	70,000	1·61	0·5	11,000	1·62
20	0·5	200,000	1·33	0·5	1,000,000	1·09
40	50	2,000,000	1·19	50	7,000,000	1·03
50	50	4,000,000	1·11	50	8,000,000	1·23
60	50	8,000,000	1·06	50	9,000,000	1·23
70	50	10,000,000	1·00	50	8,000,000	1·22

Table 2. *Effect of time of u.v. radiation dose on sporidesmin production, spore numbers and dry weight of mycelium by two strains of Pithomyces chartarum grown on SSB for 14 days at 20°*

U.v. radiation was given in 10 hr doses, on the 2nd to 7th incubation days during the 1st week, on the 8th to 13th during the 2nd week.

| U.v. radiation dosage | Strain 6L 17167 | | | Strain NB(10)1367 | | |
	Spori-desmin (mg./l.)	Spores/ml.	Dry wt mycelium (g./100 ml.)	Spori-desmin (mg./l.)	Spores/ml.	Dry wt mycelium (g./100 ml.)
0 hr	0·5	200,000	1·60	0·5	70,000	1·59
60 hr (1st week)	50	10,000,000	1·09	50	10,000,000	1·08
60 hr (2nd week)	50	10,000,000	1·31	50	9,000,000	1·23

Table 3. *Sporidesmin, spores and dry weight of mycelium produced in shaken and static SSB cultures of three strains of Pithomyces chartarum*

| Days incubated... | 4 | | 7 | | 14 | | |
Treatment...	Shaken	Static	Shaken	Static	Shaken	Static	Shaken 7/ Static 7
Strain C							
Sporidesmin (mg./l.)	0	0	0	5·0	0	5·0	5·0
Spores/ml.	0	5,000	0	400,000	0	400,000	100,000
Dry wt (g./100 ml.)	0·71	0·19	1·36	0·56	0·72	0·74	0·69
Strain WS(1)1367							
Sporidesmin (mg./l.)	0	0	0	0	0	0	0
Spores/ml.	0	0	0	0	0	0	0
Dry wt (g./100 ml.)	0·70	0·07	1·15	0·23	0·60	0·43	0·69
Strain 2R(15)4468							
Sporidesmin (mg./l.)	0	0	0	0	0	0·5	0·05
Spores/ml.	0	0	0	0	0	0	0
Dry wt (g./100 ml.)	0·37	0·14	1·34	0·36	0·98	0·77	0·65

had not reached the maximum yield to be expected. In shaken cultures growth was good and reached a maximum in 7 days; there was considerable decline in dry weight by the 14th incubation day. Spores and sporidesmin were not produced in shaken cultures, but both sporidesmin and spores were present in cultures shaken for seven days and then held static for seven.

Ryecorn cultures

Strain c produced more than 50 but less than 500 mg. sporidesmin/l. of methanol extract of ryecorn culture, more than any of the field strains examined, and remains
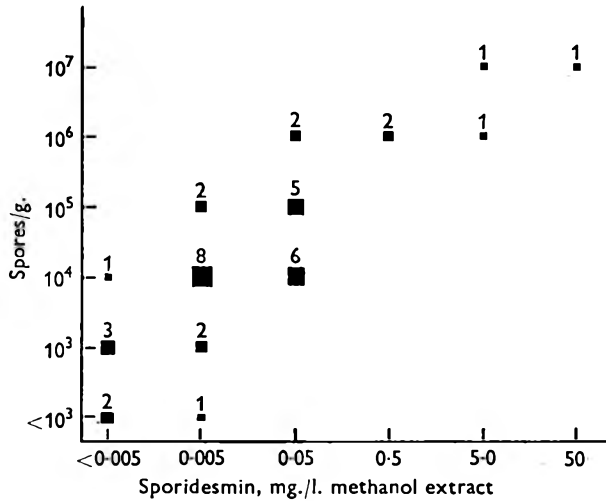
Fig. 3. Relationship between sporidesmin concentration and spore numbers in 37 field strains of *Pithomyces chartarum* grown on ryecorn for 4 weeks at 20°. Numerals are number of strains at each level.

Table 4. *Sporidesmin production and spore numbers on ryecorn and SSB in 13 strains of Pithomyces chartarum isolated on 1 March 1967 from a site at Thames*

Sporidesmin is measured in mg./l. of methanol extract of ryecorn culture, in mg./l. of SSB.

	Sporidesmin (mg./l.)			Spores/g. or ml. of medium		
Strain	Ryecorn	SSB	SSB+u.v.r.	Ryecorn	SSB	SSB+u.v.r.
NB(7)	0·5	0·05	0·5	10^6	9,000	1,000,000
NB(8)	0·005	0·05	0·05	0	0	70,000
NB(9)	0·005	0·05	0·05	10^5	0	300,000
NB(10)	0·005	5	50	10^4	200,000	8,000,000
NB(16)	0·05	0·5	50	10^4	200,000	9,000,000
NB(22)	0·05	0·05	5	10^5	3,000	4,000,000
NB(27)	0·005	0·5	5	10^4	1,000	2,000,000
NL(4)	0·005	0·05	0·5	10^5	0	500,000
NL(14)	0·005	0·5	5	0	200,000	1,000,000
WL(10)	50	50	50	10^7	10,000,000	6,000,000
WB(1)	5	0·5	50	10^6	100,000	8,000,000
WB(27)	0·05	0·5	5	10^5	2,000	7,000,000
WS(1)	0·005	50	50	10^4	1,000,000	1,000,000

the most suitable isolate for routine sporidesmin production. Only one field strain produced 50 mg./l., the majority 0·05 to 0·005 mg./l., and six no detectable sporidesmin.

Spore counts varied from 10^8/g. of culture in strain C to less than 5000/g. in three field strains. Sporidesmin production is plotted against spore numbers in Fig. 3 and, as with the SSB cultures, there is a general relationship between spore numbers and amount of sporidesmin produced. Four strains produced no detectable sporidesmin but 10^{3-4} spores/g. and one strain produced no spores but 0·005 mg. sporidesmin/l.

Strain response to treatment. Sporidesmin and spore production by 13 strains of *Pithomyces chartarum* isolated on 1 March 1967 from a site near Thames is given in Table 4. Each strain responded differently to culture on ryecorn and on SSB with and without u.v. radiation. From the data available the pattern of response of a strain was not predictable. Proportions of high and low sporidesmin and spore producers on the different treatments were the same in this group of isolates as in the whole range examined. There was no evidence that strains of similar toxicity occurred together in one place at one time.

DISCUSSION

Done, Mortimer, Taylor & Russell (1961), Ross & Thornton (1962), Ross (1962), Dingley *et al.* (1962) and Davison & Marbrook (1965) measured sporidesmin production by a number of strains of *Pithomyces chartarum*. Because there were differences in the media, incubation times and temperatures, and in the methods of sporidesmin assay used by each group, including the present authors, the absolute values for sporidesmin production are not comparable and the relative toxities of strains used by different workers cannot be determined. However, there was almost always a general relationship between the number of spores in a culture and the amount of sporidesmin present. There were two exceptions. A freely sporing American strain of *P. chartarum* produced no detectable sporidesmin (Brook & Matthews, 1960; Ross & Thornton, 1962). Two strains, C and S73a, grown in shaken potato–carrot broth for 4 days at 25° produced 0·3 and 1·5 mg. sporidesmin/l. respectively, but also some spores (Dingley *et al.* 1962). The sporidesmin produced under these conditions had disappeared by the seventh incubation day, and four other strains grown under similar conditions produced neither spores nor sporidesmin.

In the present work production of sporidesmin could not be dissociated from sporulation. Although the relationship between sporidesmin levels and spore numbers within a culture was not strictly quantitative, cultures which contained many spores had moderate to high sporidesmin levels and cultures with few spores contained little or undetectable sporidesmin. Within strains, u.v. radiation increased sporidesmin production where it increased sporulation; sporidesmin and sporulation were both suppressed in shaken cultures, and sporidesmin was first detectable in cultures at about the time that spores were produced. The association of sporidesmin with sporulation, although not exact, was sufficiently close to support the view that spore counts in herbage samples are a reliable index of pasture toxicity.

The way in which the level of sporidesmin production could be modified by the limited and somewhat arbitrary treatments used here suggests that strains may not have characteristically high or low sporidesmin producing potentials but rather may vary in the conditions under which they produce most sporidesmin. There was no

evidence in this work that proportions of high to low toxin producing strains in field populations of *Pithomyces chartarum* varied with time or place. At all events it now appears more significant that sporidesmin production is much influenced by the chemical and physical state of the environment. In the field, substrate, temperature, humidity and ultraviolet dosage vary, within limits, with time and place. Conditions would favour sporidesmin production now by one strain and now by another.

Two of the strains examined produced little or no sporidesmin and few spores with any treatment used. It is possible that in any cirumstances they would be weakly toxic but probable that under some cultural conditions they would produce sporidesmin at a moderate level at least. In this work it was not practicable to fit incubation period to strain, although it was known that time taken to produce spores varies greatly with strain, from two days to over a month under standard, apparently most favourable, conditions, and it is reasonable to assume that variation in time of sporidesmin production would be of the same order. Another important factor not examined was the effect on production of sporidesmin and spores of the fluctuations in temperature and humidity normal to pasture.

Turning to the practical importance of u.v. radiation, irradiation increases the rate of destruction of sporidesmin in aqueous solution (Clare & Gumbley, 1962; Clare & Mortimer, 1964). In the field the interplay between the stimulation by u.v. radiation of fungal production of sporidesmin and the destructive effect of u.v. radiation on aqueous sporidesmin solutions must be considered. The stimulatory role would result in rapid build up of spores and sporidesmin in pasture when other conditions were optimal. The destructive role would hasten the loss of pasture toxicity when conditions for growth became less favourable.

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Influence of Mode of Steroid Substrate Addition on Conversion of Steroid and Growth Characteristics in a Mixed Culture Fermentation

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SUMMARY

The mode of addition of the steroid substrate, 16α-hydroxycortexolone 16,17-acetonide, was shown to influence markedly the rate of enzymic conversion of the steroid as well as the form of growth of the fungal organism, in a mixed culture of *Arthrobacter simplex* (1-dehydrogenator) and *Curvularia lunata* (11β-hydroxylator). The effects observed were apparently related to the size and solubility of the steroid particles added in suspension or precipitated in the medium by addition of the steroid in any of several water-miscible non-aqueous solvents. The best rates of steroid conversion were observed when the mould grew in elongated pellets rather than in filamentous form.

INTRODUCTION

Shull (1959) and Kimura (1962) reported, respectively, on the multiple conversion of cortexolone to prednisolone by mixed cultures of *Curvularia lunata* and *Mycobacterium phlei* and of *C. lunata* and *Bacillus sphaericus*. Subsequently, we published a paper dealing with the bioconversion of 9α-fluorohydrocortisone to 1-dehydro-16α-hydroxy-9α-fluorohydrocortisone (triamcinolone) by mixed cultures of *Arthrobacter simplex* and *Streptomyces roseochromogenes* (Lee, Ryu, Thoma & Brown, 1969). In the present paper we report results obtained from the use of a mixed culture of *Arthrobacter simplex* (1-dehydrogenator) and *C. lunata* (11β-hydroxylator) for the transformation of 16α-hydroxycortexolone 16,17-acetonide to its 1-dehydro-11β-hydroxy derivative, an intermediate in the synthesis of triamcinolone acetonide (Fig. 1).

16α-hydroxycortexolone 16,17-acetonide   1-dehydro-11β-hydroxy product
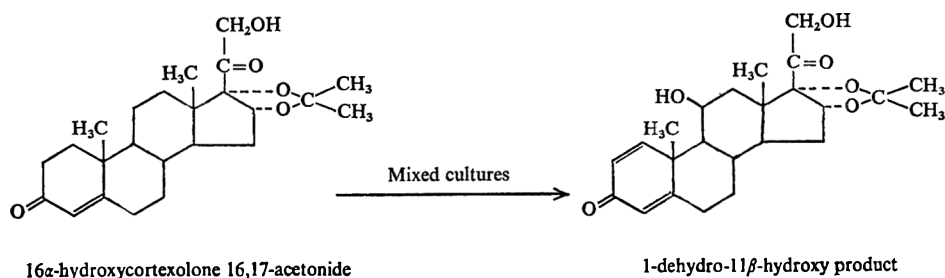
Fig. 1.

In the early stages of our studies we added the steroid substrate as a suspension in aqueous Tween 80 solution (polyoxyethylene sorbitan mono-oleate) and achieved a substrate concentration in the culture of not more than 250 μg./ml. Later, we found that, when we added the steroid in hot ethanol–water containing sodium tetraborate, we were able not only to increase the substrate concentration from 300 to 1000 μg./ml., but also to shorten the time of the mixed-culture fermentation from 120 to 72 hr. These results led us to compare still other methods of steroid addⁱtion. We found that the mode of addition of steroid substrate affects its solubility, and that there is a relationship between mode of addition, rate of bioconversion of steroid, and form of growth of the fungal organism in the pair.

METHODS

Organisms. Arthrobacter simplex (Squibb Culture 6062-v3) and *Curvularia lunata* (Squibb Culture 5471) were used. *Arthrobacter simplex* was maintained on yeast beef-agar slants and *Curvularia lunata* on Gould agar slants. Stock cultures were stored over liquid nitrogen at −150°.

Steroid substrate. 16α-Hydroxycortexolone 16,17-acetonide used throughout the studies was a product of Syntex Laboratories, Mexico. The steroid substrate was suspended in cold (room temperature) 0·1 % (w/v) aqueous Tween 80 or cold solvent, or dissolved in hot solvent; heating was effected by immersing a tube containing steroid and solvent in boiling water. Each suspension or solution of the substrate was made to a concentration of 100 mg./ml. The hot borate+ethanol+water solution was made by combining 1240 mg. of the steroid substrate with 2·29 ml. of aqueous sodium tetraborate solution (62·5 mg./ml.) and ethanol to a final substrate concentration of 100 mg./ml., and by heating the mixture gently until a clear solution was obtained.

Cultures. All cultures were grown in 100 ml. portions of medium in 500 ml. Erlenmeyer flasks and shaken at 25° on a Gyrotory G-52 shaking machine (New Brunswick Scientific Co.) at 280 rev./min. in a 2 in. diameter circle. The inoculum was grown in two 48 hr stages. For the fermentation stages, portions (1 ml. and 5 ml. respectively) of vegetative cultures of *Arthrobacter simplex* and *Curvularia lunata* were used to inoculate 100 ml. of the broth. When the steroid substrate was added at 500 μg./ml. or less, substrate and organisms were added at zero time. When a total substrate concentration of 1000 μg./ml. was used, the first portion of substrate (500 μg./ml.) was added at zero time and the remainder after 24 hr incubation.

Each organism was grown separately in two stages of E 33 and E 2B medium or E 24 medium for both germination stages. For fermentation, the two organisms were mixed and grown in E 24 medium. E 33 medium contained (g./l. of medium) soybean meal (Archer–Daniels–Midland), 15; soybean oil, 2·2; glucose, 11; $CaCO_3$, 2·5. E 2B medium was made of (g./l. of medium) Staley's soybean meal, 20; glucose, 30; soybean oil, 2·2; $CaCO_3$, 2·5. E 24 medium contained (g./l. of medium) cornsteep liquor, 6; glucose, 10; $NH_4H_2PO_4$, 3; Difco yeast extract, 2·5; $CaCO_3$, 2·5. All media were made with distilled water.

Preparation of samples for steroid measurement. Samples (5 ml.) were drawn at intervals from the culture and each sample was extracted with 2 ml. of 4-methyl-2-pentanone (methylisobutylketone, MIBK).

Paper chromatography. Portions (0·1 ml.) of MIBK extracts were spotted on Whatman no. 1 paper impregnated with propylene glycol and developed by descending chromatography with toluene saturated with propylene glycol.

Steroid measurement. Following development of the chromatograms, steroid spots were detected by ultraviolet scanning. The spots were marked with a tin–lead stylus, cut out, eluted with 95 % (v/v) ethanol, and the extinction of the extract measured at 240 μm. in a Beckman DU spectrophotometer. The concentration of steroid in each sample was then estimated using a standard solution equilibrated with MIBK and chromatographed in the same way as the samples.

Qualitative identification. Steroid spots were located by ultraviolet scanning and the papers sprayed with triphenyl-tetrazolium chloride (TTC). Steroids were then identified on the basis of mobility (Table 1) and TTC reduction.

Centrifugation of samples for dissolution studies. Samples (4·0 ml.) were taken at intervals, centrifuged for 10 min. at 25° at 39,900 g with a Sorval RC-2 centrifuge (head type SM-24, 4·34 in.), and the steroid concentration was measured separately in the supernatant and precipitate from each sample.

Table 1. *Relative mobility values of steroids separated by paper chromatography in toluene-propylene glycol*

Steroid	Relative mobility (R_F)
16α-hydroxycortexolone 16,17-acetonide	0·81
1-dehydro-16α-hydroxycortexolone 16,17-acetonide	0·67
11β,16α-dihydroxycortexolone 16,17-acetonide	0·30
1-dehydro-11β,16α-dihydroxycortexolone 16,17-acetonide	0·15

Preparation of slides and photographs of crystals of 16α-hydroxycortexolone 16,17-acetonide. 16α-Hydroxy-cortexolone 16,17-acetonide (300 μg./ml.) was added to the supernatant of E 24 medium in cold Tween 80 suspension, in hot ethanol and in hot borate+ethanol+water, and mixed well by shaking. Slides were prepared (wet with no stain), and photographs were taken at a magnification of ×430, with Polaroid black and white/3000 speed film (type 107).

Particle-size analysis. A Coulter Counter (model B) was used to measure particle-size distributions in artificial systems (Fig. 2), because similar analyses on cultures presented major difficulties. It was believed that information gained from the artificial systems would be indicative of occurrences in the fermentation. Consequently the steroid was suspended in a prefiltered solution containing 0·1 % (w/v) Tween 80 and 1 % (w/v) sodium chloride (the latter being a requirement for operation of the counter). The 400 μm. aperture tube that was used is capable of detecting particles 6 to 200 μm. in diameter. Analyses were also made of suspensions resulting from the addition of solutions of the steroid in hot ethanol and in hot borate+ethanol+water to appropriate amounts of prefiltered 1 % sodium chloride solutions. The concentration of steroid in the resultant suspensions was 500 μg./ml. Samples were withdrawn and analysed using a 30 μm. aperture tube capable of measuring particles 0·6 to 15 μm. in diameter.

RESULTS

Effects of the mode of substrate addition on steroid conversion

The multiple conversion of 16α-hydroxycortexolone 16,17-acetonide to its 1-dehydro-11β-hydroxy derivative was followed under conditions in which the substrate was charged, in two portions of 500 μg./ml. each at zero time and 24 hr, in various forms. The conversion obtained with each form of substrate addition was, in descending order of efficiency, hot borate + ethanol + water solution (90 %), hot ethanol + acetone solution (67 %), hot ethanol solution (65 %), cold dimethylformamide (DMF) solution (60 %), cold ethanol + acetone suspension (30 %), cold ethanol suspension (20 %), and cold aqueous Tween suspension (10 %) (Fig. 3). When the substrate solubilized in hot solvents (borate + ethanol + water, ethanol + acetone, ethanol) or in cold DMF was added to the culture, it precipitated immediately. The resultant precipitates appeared to be different in crystal form and size from the 'raw' or original material added in cold aqueous Tween suspension. When added in solution, in general, the substrate was dispersed in forms much finer than that of the original crystalline material (Pl. 1, fig. 1, 2, 3). Thus, more surface area was provided for contact with cells and for dissolution.
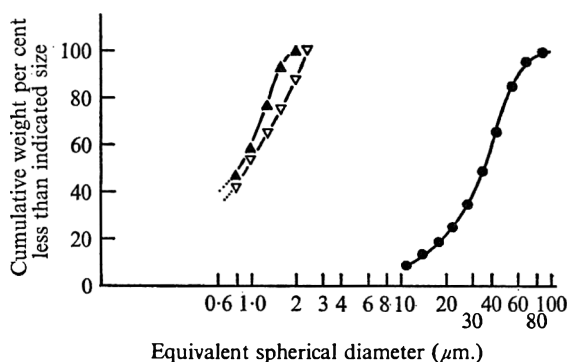
Fig. 2   Fig. 3

Fig. 2. Particle-size distribution as determined by the Coulter counter. ▲ indicates data from particles generated by addition of a hot ethanol solution of steroid to a prefiltered solution containing 0·1 % (w/v) Tween 80 and 1 % (w/v) sodium chloride; ▽, particles generated by addition of a hot ethanol + borate + water solution of steroid to the prefiltered solution; ●, particles obtained by addition of steroid suspension to the prefiltered solution.
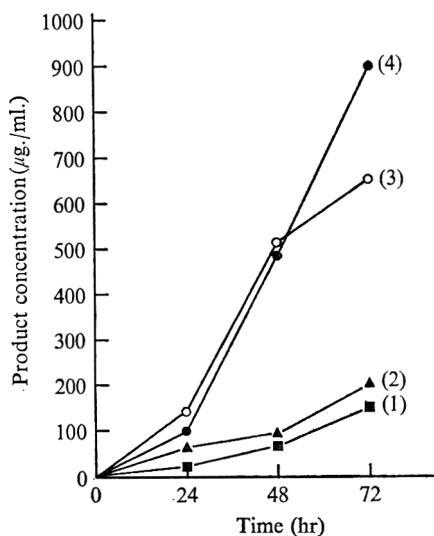
Fig. 3. Effects of the mode of substrate addition on the multiple transformation of 16α-hydroxycortexolone 16,17-acetonide to the 1-dehydro-11β-hydroxy product by a mixed culture of *Arthrobacter simplex* and *Curvularia lunata*. Each of the organisms was separately germinated in E 24 medium and then grown in the same medium with the steroid substrate added in different forms. (1) indicates final product formed when the steroid substrate was added in cold Tween suspension; (2), final product formed when the steroid substrate was added in cold ethanol suspension; (3), final product formed when the steroid substrate was added in hot ethanol solution; (4), final product formed when the steroid substrate was added in hot borate + ethanol + water solution.

Borate was tested in several concentrations in the mixture. Fig. 4 shows three productivity curves obtained with 3, 30, or 90 μmole of borate/100 ml. of culture broth. In each case, 1·0 ml. of stock solution was added to 100 ml. of culture broth. From these curves it is seen that 30 μmole/100 ml. was better than the higher or lower concentrations. However, when 30 μmole of borate/100 ml. was added separately from the appropriate amounts of suspended steroid and ethanol, the productivity was much lower (Fig. 4, curve 4).
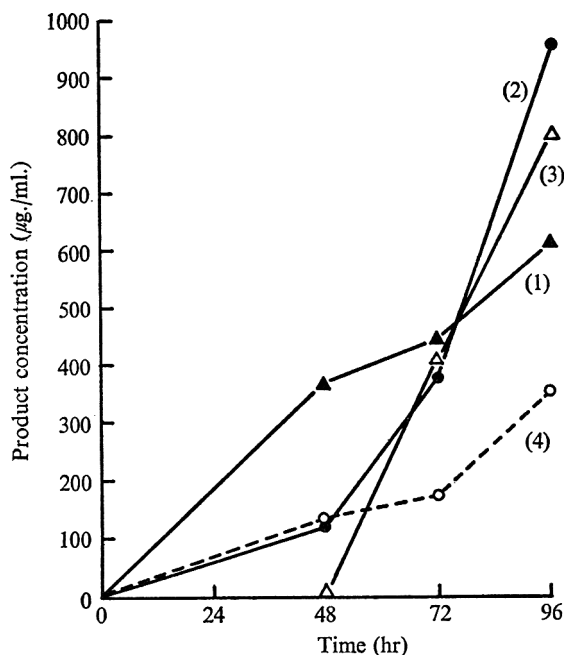
Fig. 4. Productivity, in the multiple conversion of 16α-hydroxycortexolone 16,17-acetonide to 1-dehydro-11β,16α-dihydroxycortexolone 16,17-acetonide, from different concentrations of borate. (1) From 3 μmole of borate incorporated in the solubilized stock solution of steroid substrate + borate + ethanol + water; (2) from 30 μmole of borate, as (1); (3) from 90 μmole of borate, as (1); (4) from 30 μmole of borate added separately following separate addition of steroid substrate and of ethanol into the medium.

Effects of mode of substrate addition on growth characteristics

The differences in the effects of the mode of substrate addition on growth characteristics of the fungal organism mixed in the pair were also striking. Cold Tween suspension resulted in a tan filamentous type of growth, cold ethanol suspension in tan granular-filaments, hot ethanolic solution in brown elongated pellets, cold ethanol + acetone suspension in brown granular filaments, hot ethanol + acetone solution in brown-black elongated pellets, hot borate + ethanol + water solution in black elongated pellets, and cold DMF solution in brown granular filamentous growth mixed with elongated pellets (Pl. 2). As a general rule, the elongated pellet form of growth was more often associated with higher conversion than was the filamentous type of growth.

The appearance of the untreated control culture was tan granular-filaments, indistinguishable from the culture treated with steroid in Tween suspension. Moreover,

the appearance of the culture was not modified when 30 μmole of borate/100 ml. culture broth and the appropriate amounts of ethanol and steroid in suspension were added separately.

No change in the morphology of *Arthrobacter simplex* due to steroid solution or suspension was detected.

Solubility studies

The solubility of 16α-hydroxycortexolone 16,17-acetonide, added at 500 μg./ml. in cold aqueous Tween suspension and in hot borate+ethanol+water solution, were
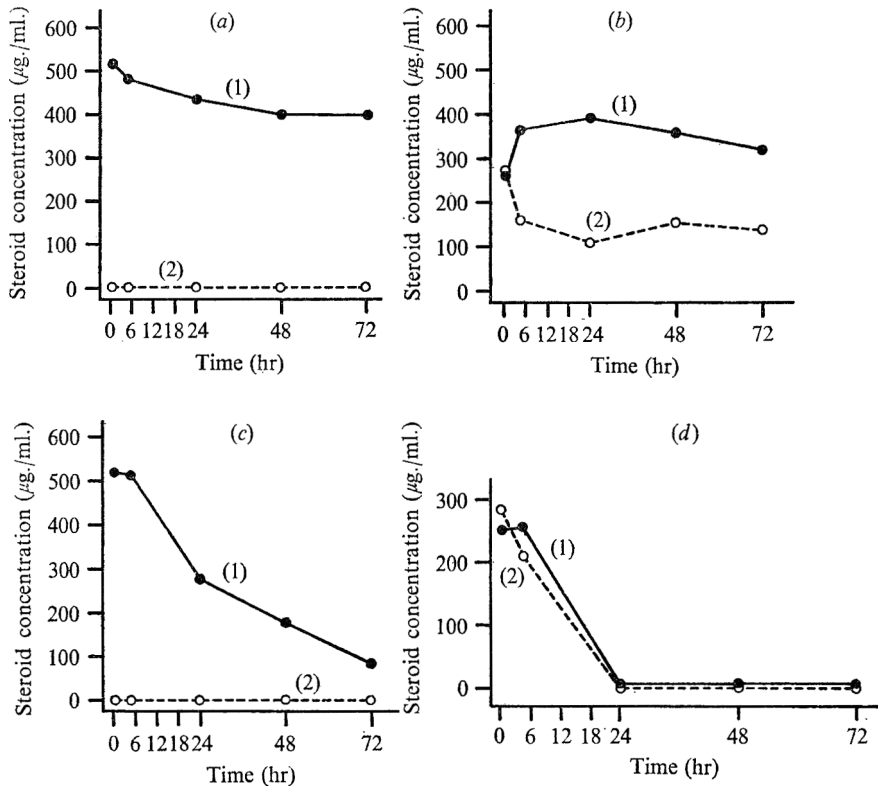
Fig. 5(*a*). Dissolution rate of 16α-hydroxycortexolone 16,17-acetonide added in cold Tween suspension into E 24 medium minus organisms. (1) indicates the steroid concentration in precipitate (undissolved steroid); (2), the steroid concentration in supernatant (dissolved steroid).

Fig. 5(*b*). Dissolution rate of 16α-hydroxycortexolone 16,17-acetonide added in hot borate + ethanol + water solution into E 24 medium minus organisms. (1) indicates the steroid concentration in precipitate (undissolved steroid); (2), the steroid concentration in supernatant (dissolved steroid).

Fig. 5(*c*). Dissolution rate of 16α-hydroxycortexolone 16,17-acetonide added in cold Tween suspension into E 24 medium including the mixed culture of *Arthrobacter simplex* and *Curvularia lunata*. (1) indicates the steroid substrate concentration in precipitate (undissolved steroid substrate); (2), the steroid substrate concentration in supernatant (dissolved steroid substrate).

Fig. 5(*d*). Dissolution rate of 16α-hydroxycortexolone 16,17-acetonide added in hot borate + ethanol + water solution into E 24 medium including the mixed culture of *Arthrobacter simplex* and *Curvularia lunata*. (1) indicates the steroid substrate concentration in precipitate (undissolved steroid substrate): (2), the steroid substrate concentration in supernatant (dissolved steroid substrate).

followed in the cornsteep liquor medium (E 24) with and without the mixed cultures of *Arthrobacter simplex* and *Curvularia lunata.* The solubility of the steroid substrate added in cold Tween suspension was negligible as compared to that added in hot borate + ethanol + water solution (Fig. 5*a, b*). When added in cold Tween suspension, the substrate was slowly transformed by the mixed culture without any substrate in solution being detectable. A substantial amount of the substrate was left untouched up to 72 hr incubation (Fig. 5*c*). On the other hand, the substrate added in hot borate + ethanol + water solution was dissolved, up to 280 μg./ml., in less than 30 min. Faster conversion was observed and no trace of the substrate was seen either in the supernatant or in the precipitate of the culture filtrate at or after 24 hr (Fig. 5*d*). Thus, the results show a direct relationship between solubility of the substrate and the rate of steroid conversion.

Particle-size studies

The particle-size distributions as measured by the Coulter counter are shown in Fig. 2. The lowest dimension that could be measured with the counter was 0·6 μm., and it can be presumed that smaller particles were present. However, the data clearly show the large differences that result when the steroid is added directly as a suspension and when the steroid is first dissolved in a limited volume of solvent and then added to an aqueous system. The range of particle sizes obtained by use of a hot borate + ethanol + water solution is slightly greater than that obtained by use of a hot ethanol solution (Pl. 1, fig. 2, 3). Even though these results were obtained using an artificial system, we have good reason to believe that the results are representative of the situation in the culture. The appearance of crystals in the culture supports the particle-size data as do the more detailed photomicrographs (Pl. 1, fig. 1, 2, 3).

DISCUSSION

Weaver *et al.* 1960 & Weaver (1962), used *Rhizopus nigricans* or *Aspergillus ochraceus* and carried out 11α-hydroxylation of progesterone by adding the steroid substrate in the original 'raw' form and in a ground 'smooth' form, and found, at a concentration of 50 g. progesterone/l., 65 % and 40 % conversion, respectively, from ground and unground substrate. He postulated that, by grinding the substrate, not only was the organism provided with a smooth non-injurious surface, but a higher degree of interfacial contact between the substrate particles and the organisms was obtained.

Our data clearly show that the rate of steroid bioconversion is much influenced by the mode of steroid substrate addition. A relatively rapid rate is obtained when the steroid concentrate is added as a solution, notwithstanding the immediate precipitation that occurs upon its addition to the culture. Clearly, in this instance more of the steroid substrate is available to the organisms than when a relatively coarse suspension is added.

The enhanced availability is directly related to the markedly greater solubility of the resultant fine suspension than that of the coarse material (Fig. 5*a, b*). It is known that fine particles are more soluble than relatively coarse ones. Furthermore, the material that precipitates appears by microscopic observation to be amorphous (Pl. 1, fig. 2, 3), and it is also well known that different polymorphic forms have different solubilites, the amorphous forms often being considerably more soluble than a crystalline form. The existence of the amorphous form is also supported by the

failure of the fine suspension obtained in the artificial system to rotate polarized light, whereas the coarse suspension does.

Both phenomena are metastable. With time the coarse particles will grow at the expense of the fine, and the more stable polymorphic species will form at the expense of the less stable species. However, the greater solubility persists for at least 72 hr in fine suspension (Fig. 5*b*).

In addition to the aforementioned solubility effect, the fine particles also have a more rapid dissolution rate. In the presence of the organisms that are utilizing dissolved steroid, replenishment of dissolved steroid is very rapid from the fine suspension (Fig. 5*d*). With the coarse suspension the amount of steroid in solution is low and practically at a steady state (Fig. 5*c*).

Growth characteristics of the fungal organism in the pair were also influenced by the mode of steroid substrate addition. In general, pellet and filamentous forms of growth were brought about, respectively, by addition of the steroid in solution and in suspension. When the steroid and an appropriate amount of solvent were added separately to a single flask containing inoculated medium, the filamentous form of growth was observed, as in the case of adding the steroid in suspension. Further, different means of steroid addition did not have any significant effect on the pH value of the growth medium. Thus, neither solvent nor pH value was a factor in directly altering the changes in growth of the fungal organism. Apparently the differences in growth characteristics observed are related to the differences in size and shape of the steroid particles that result from the different modes of substrate addition. One would expect the large crystals to have a different abrasive effect on the fungal mycelium as it grows than do the smaller particles. Possibly this then is the reason for the development of the filamentous and pellet types of mycelium in the presence of large crystals and small particles, respectively.

We believe that the greater rate of steroid transformation is related not only to the greater solubility and more rapid dissolution of the substrate but also to the presence of the pellet form of fungal mycelium. The latter being more dispersed than the filamentous form provides a greater surface area and thereby greatly enhances the contact between organism and steroid.

It is to be noted from Fig. 4 that the initial rates of conversion are inversely proportional to the amounts of borate used. This observation implies that borate is toxic to at least one of the organisms. At 30 μmole borate/100 ml. culture broth, delayed transformation is observed during the initial phase of the fermentation. This initial toxic effect is then apparently overcome, since a rapid rate of conversion is eventually achieved.

The observation that use of borate + ethanol + water for solubilization makes possible continuing rapid conversion beyond 48 hr as contrasted to the use of ethanol alone is of interest (Fig. 3, curves 3 and 4). Since essentially only intermediates and final product are present after 48 hr, it is conjectured that the presence of borate maintains the permeability of the organisms to the intermediates and that, as a consequence, a high rate of conversion is maintained. In the absence of borate the organisms may lose permeability with age and the conversion slow down. However, no experimental data have been obtained to support these speculations.
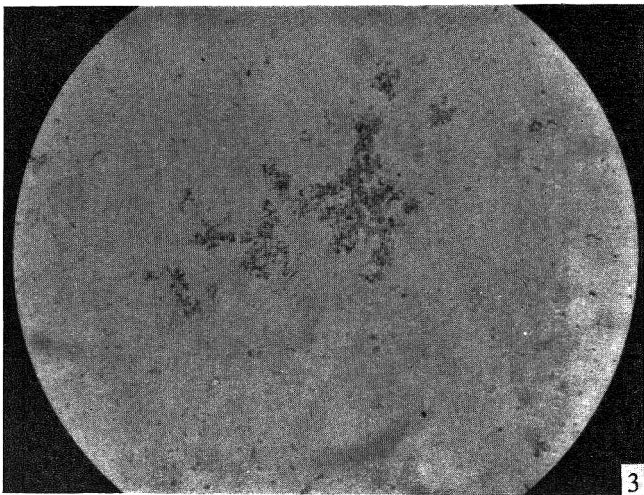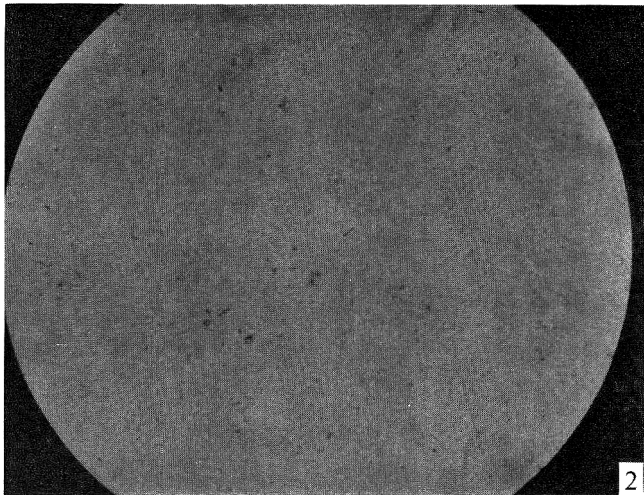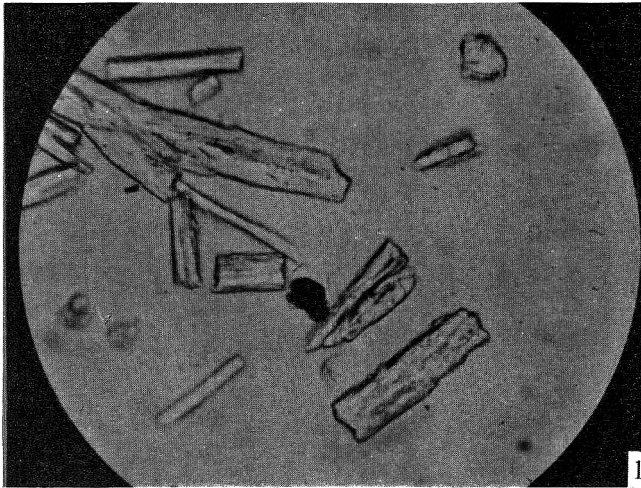
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EXPLANATION OF PLATES

PLATE 1

Fig. 1. Photomicrograph of crystals of 16α-hydroxycortexolone 16,17-acetonide seen immediately after addition of cold Tween-80 suspension to the supernatant of E 24 medium. × 430.

Fig. 2. Photomicrograph of crystals of 16α-hydroxycortexolone 16,17-acetonide seen immediately after addition of solution in hot ethanol to the supernatant of E 24 medium. × 430.

Fig. 3. Photomicrograph of crystals of 16α-hydroxycortexolone 16,17-acetonide seen immediately after addition of solution in hot borate + ethanol + water mixture to the supernatant of E 24 medium. × 430.

PLATE 2

Effects of the mode of the steroid substrate addition on growth characteristics of the fungal organism in the pair (*Arthrobacter simplex* and *Curvularia lunata*). The figure shows photographs looking downward on samples taken from 72 hr shaken flask cultures, placed in test-tubes, and propped at a low angle for better visualization: (*a*) addition in cold dimethylformamide solution resulted in a brown granular filamentous form of growth mixed with elongated pellets; (*b*) addition in hot borate + ethanol + water solution resulted in black elongated pellets; (*c*) addition in hot ethanol + acetone (1 + 1) solution resulted in brownish-black elongated pellets; (*d*) addition in cold ethanol + acetone suspension resulted in a brown granular filamentous form of growth; (*e*) addition in hot ethanol solution resulted in brown elongated pellets; (*f*) addition in cold ethanol suspension resulted in a tan granular filamentous form of growth; (*g*) addition in cold Tween suspension resulted in a tan filamentous form of growth.

Hydrogen Sulphide Production by Yeast under Conditions of Methionine, Pantothenate or Vitamin B₆ Deficiency

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SUMMARY

Methionine-requiring mutants of *Saccharomyces cerevisiae* produce large amounts of hydrogen sulphide from sulphate, sulphite or thiosulphate when grown in the presence of suboptimum concentrations of methionine. *O*-Acetylhomoserine and homocysteine act like methionine with a methionine-requiring mutant which can use them for growth. Wild-type strains of *S. cerevisiae* and *S. carlsbergensis* also form large amounts of hydrogen sulphide from inorganic sulphur sources when the yeast is deficient in either pantothenate or vitamin B₆. This excess sulphide production is inhibited by methionine or its immediate precursors, suggesting that both vitamins are required for methionine biosynthesis. *O*-Acetylhomoserine is a normal precursor of homocysteine and methionine in *S. cerevisiae* and *S. carlsbergensis*. The effect of pantothenate on sulphide production by these yeasts is probably due to its involvement in the formation of *O*-acetylhomoserine.

INTRODUCTION

Pantothenate-deficient yeasts produce large amounts of sulphide from inorganic sulphur compounds (Kodaira, Ito & Uemura, 1958). With all strains tested, and not merely Saccharomyces species, yeasts which require pantothenate for growth produce abnormally large amounts of sulphide when grown with suboptimal concentrations of pantothenate (Wainwright, 1962). This effect of pantothenate deficiency has been reported only with yeasts and not with other micro-organisms.

A number of hypotheses have been suggested to explain the action of pantothenate, but none is entirely satisfactory and all propose a rather indirect role for the vitamin. Wiebers & Garner (1967) showed that homocysteine, which is considered to be a methionine precursor in yeast, is formed by yeast enzyme preparations from *O*-acetyl-homoserine and hydrogen sulphide in the presence of pyridoxal phosphate. One methionine-requiring mutant of *Saccharomyces cerevisiae* has also been described which grows on *O*-acetylhomoserine and apparently lacks homoserine transacetylase (de Robichon-Szulmajster, 1967; de Robichon-Szulmajster & Cherest, 1967). The present work was undertaken to determine if the effect of pantothenate is due to its involvement, presumably as coenzyme A, in the biosynthesis of *O*-acetylhomoserine.

METHODS

Organisms. Saccharomyces cerevisiae Guinness strain 1164 was used in most of the experiments. *Saccharomyces carlsbergensis* NCYC4228 is a yeast employed in the laboratory for vitamin B₆ assays. *Saccharomyces cerevisiae* CC92-17A (lacking

homoserine transacetylase) and *S. cerevisiae* ME-8 (lacking homocysteine synthetase) were kindly supplied by Dr H. de Robichon-Szulmajster, *S. cerevisiae* ME-3 by Dr L. W. Parks and the thiosulphate-utilizing mutants of *S. cerevisiae* 6, 21 and 24 by Dr N. Naiki. The succinate-requiring mutant of *Escherichia coli*, W 1485-SUC-1, was obtained from Dr J. K. Guest and the pantothenate-requiring mutants of *E. coli* from the late Professor D. D. Woods.

Growth measurement. Growth was measured with an EEL colorimeter (Evans Electroselenium Ltd., Harlow, Essex) fitted with a neutral density filter. Calibration curves were made relating dry weight of organism and colorimeter reading.

Media. The basal liquid medium for yeast growth contained glucose, 50 g.; $(NH_4)_2SO_4$, 2·5 g.; KCl, 425 mg.; KH_2PO_4, 275 mg.; $MgSO_4.7H_2O$, 125 mg.; $CaCl_2.2H_2O$, 125 mg.; $MnSO_4$, 2·5 mg.; $FeSO_4$, 2·5 mg.; potassium citrate, 5 g.; citric acid, 1 g.; biotin, 0·1 mg.; inositol, 25 mg.; thiamin, 0·5 mg.; distilled water to 1 l.; pH 5·2. Unless otherwise stated the medium also contained 2 mg. calcium pantothenate and 0·5 mg. pyridoxine hydrochloride/l. In experiments on thiosulphate reduction to sulphide, the inorganic sulphate constituents were replaced by the corresponding chlorides and 4 mM thiosulphate was included in the medium. The basal medium for the two methionine-requiring mutants was supplemented with 20 mg. adenine, 10 mg. uracil and, except when stated, 300 mg. methionine/l.

The basal medium for *Escherichia coli* was that of Davis & Mingioli (1950). Calcium pantothenate (5×10^{-7} M) was used to obtain full growth of the pantothenate-requiring mutants and 4 mM succinate for the succinate-requiring mutant.

Growth conditions. Yeast growth experiments were performed using 20 ml. quantities of medium in 50 ml. conical flasks incubated statically at 24° for about 42 hr. The flasks were inoculated with a suspension of washed organisms equivalent to 0·9 mg. dry wt of yeast. This was derived from a 42 hr subculture on a suitable liquid medium as indicated in the section of Results.

Bacterial growth experiments were performed at 30° using 20 ml. quantities in static 50 ml. flasks. The inoculum, derived from liquid subculture in basal medium containing enough supplement to allow full growth, was equivalent to 0·4 μg. dry wt.

Cell suspensions. Organisms were subcultured in basal medium with 40 μg. calcium pantothenate/l. Washed organisms were suspended to give 9 mg. dry wt/ml. in a medium, pH 5·0, containing 10 g. glucose, 50 mg. $MgCl_2$, 24·3 ml. 0·1 M-citric acid, 25·7 ml. 0·2 M-Na_2HPO_4 and 50 ml. water. Three ml. quantities of this suspension were incubated with 0·4 ml. 2 % (w/v) zinc acetate and substrates in 1·0 ml. water at 30° on an orbital shaker at 115 rev./min.

Hydrogen sulphide determination. Hydrogen sulphide was determined by the formation of methylene blue (Brenner, Owades & Golyzniak, 1954). The results presented here were obtained from experiments in which 0·2 % (w/v) zinc acetate was present in the medium throughout the growth or incubation period. At the end of the incubation, the medium was acidified and, after the addition of a drop of capryl alcohol, nitrogen gas was blown through the culture flask for 2 hr to displace hydrogen sulphide through a capillary tube into a 2 % (w/v) zinc acetate trap. The sulphide content of the trap was then determined.

Since it is known that heavy-metal ions can under certain conditions markedly increase the amounts of hydrogen sulphide formed by yeast (Rankine, 1963; Lawrence & Cole, 1968) many of the results have been checked by omitting zinc acetate from the

culture medium and using either a closed vessel or one with an external zinc acetate trap. The results were never qualitatively different from those obtained with zinc ions in the medium, but the quantities of hydrogen sulphide recovered were much lower and much more variable, probably due to oxidation of the sulphide formed.

Chemicals. *O*-Acetyl-DL-homoserine was prepared by the method of Nagai & Flavin (1967). Other chemicals were the best grade commercially available.
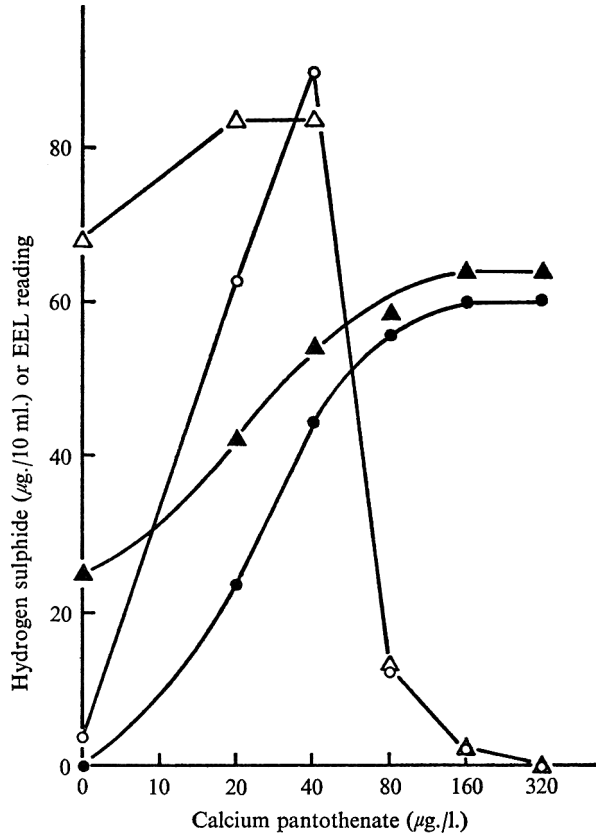
Fig. 1. Growth and H_2S production by pantothenate-deficient *Saccharomyces cerevisiae*. ○ Indicates hydrogen sulphide production with inoculum grown on 10 μg. pantothenate/l., and ● the corresponding EEL reading; △ indicates hydrogen sulphide production with inoculum grown on 320 μg. pantothenate/l., and ▲ the corresponding EEL reading.

RESULTS

Effect of supplements on hydrogen sulphide production by wild-type strains

Pantothenate

Growth of the yeast and the amount of sulphide formed depend on the pantothenate concentration in the medium (Fig. 1). With sufficient pantothenate (> 160 μg./l.) no hydrogen sulphide is formed. With lower concentrations there is a peak production of sulphide usually at about 40 μg. pantothenate/l. The exact shape of the production curve depends on the concentration of pantothenate present during growth of the inoculum A more deficient inoculum gives less growth at low pantothenate concentrations

and consequently less hydrogen sulphide. This dependence of sulphide production on the amount of growth is shown if the growth is limited at any one suboptimal concentration of pantothenate by the amount of glucose, sulphate or nitrogen source available, or if the course of growth and hydrogen sulphide production is plotted against time. It is essential to allow for this dependence on the amount of growth when considering the effect of added compounds such as methionine on the amount of hydrogen sulphide produced.

Methionine

Kodaira & Uemura (1960) using Saké yeast claimed that a variety of amino acids, notably methionine and leucine, decreased the amount of sulphide formed by pantothenate-deficient yeast. Methionine under certain conditions inhibits sulphide formation by brewer's yeast but the effect depends both on the methionine concentration and on the yeast strain. As shown in Table 1, with some yeasts increasing concentrations of methionine progressively suppress the amount of sulphide formed, although they do not inhibit hydrogen sulphide formation completely. With other yeasts, although higher concentrations of methionine inhibit hydrogen sulphide formation, below a certain concentration methionine actually stimulates production (Table 1).

Table 1. *Effect of methionine on hydrogen sulphide production by different strains of pantothenate-deficient Saccharomyces cerevisiae*

Yeast strain	DL-Methionine (M)	Growth (g. dry wt./l.)	Hydrogen sulphide (mg./l.)
1164	0	3·1	4·6
	2×10^{-3}	2·7	3·35
	5×10^{-3}	2·7	1·35
	10^{-2}	2·35	0·6
1408	0	2·7	0·875
	2×10^{-3}	2·6	1·35
	5×10^{-3}	2·35	0·275
	10^{-2}	2·3	0·125

Two Guinness strains of *Saccharomyces cerevisiae* were grown 44 hr in basal medium containing 40 μg. calcium pantothenate/l., using inocula previously grown in the same medium with no methionine.

Both types of effect—the progressive inhibition of hydrogen sulphide formation at higher methionine concentrations and the stimulation at intermediate concentrations—have been obtained with several strains of *Saccharomyces cerevisiae*. With 5 mM DL-methionine, inhibition occurs in all yeasts. The D-isomer by itself is inactive, and L-methionine is twice as active as DL-methionine on a molar basis.

O-Acetylhomoserine, homoserine and homocysteine

Table 2 shows that *O*-acetyl-DL-homoserine suppresses hydrogen sulphide formation by yeast. On a molar basis it is equivalent to DL-methionine. Homoserine, the precursor of *O*-acetylhomoserine, does not inhibit the formation of hydrogen sulphide by pantothenate-deficient yeast. Since both methionine and *O*-acetylhomoserine inhibit hydrogen sulphide formation by pantothenate-deficient yeast, one might expect inhibition by homocysteine, which is thought to be a metabolic intermediate between

these compounds. Some inhibition is observed although the result is not clear-cut. Homocysteine can itself give rise to hydrogen sulphide production and, at high concentrations of the amino acid, this obscures the inhibiting effect on reduction of inorganic sulphur compounds. If homocystine is used in place of homocysteine, the expected inhibition is obtained (Table 2).

Table 2. *Inhibition by O-acetylhomoserine and homocystine of hydrogen sulphide formation by pantothenate-deficient yeast*

DL-O-Acetyl-homoserine (M)	Growth (g. dry wt/l.)	Hydrogen sulphide (mg./l.)
0	2·45	2·3
2×10^{-3}	2·35	2·25
4×10^{-3}	2·3	1·55
10^{-2}	2·3	0·1

DL-Homocystine (M)	Growth (g. dry wt/l.)	Hydrogen sulphide (mg./l.)
0	2·1	2·8
2×10^{-3}	2·0	1·35
5×10^{-3}	2·0	0·62
10^{-2}	2·1	0·25

Strain 1164 was grown 42 hr in basal medium containing 40 μg. calcium pantothenate/l. The inoculum was also grown in this medium.

Table 3. *Effect of methionine on hydrogen sulphide production by Saccharomyces cerevisiae CC 92-17 A*

Pantothenate (μg./l.)	DL-Methionine (M)	Growth (g. dry wt/l.)	Hydrogen sulphide (mg./l.)
500	10^{-3}	1·9	2·45
	3×10^{-3}	2·85	2·65
	5×10^{-3}	2·5	0·4
0	10^{-3}	1·7	2·5
	3×10^{-3}	2·5	2·4
	5×10^{-3}	1·65	0·15

Growth was for 48 hr in appropriate basal medium with the indicated supplements. The inoculum was grown in basal medium containing 40 μg. calcium pantothenate/l. and the normal methionine supplement.

Effect of supplements on hydrogen sulphide production by mutants

When grown with 5 mM-DL-methionine, mutant CC92-17A produces relatively little hydrogen sulphide (Table 3). However, if the methionine concentration is decreased, large amounts are formed, even though growth is hardly affected and the pantothenate concentration is well above that required to suppress hydrogen sulphide formation by pantothenate-deficient brewery yeast. Increased hydrogen sulphide production at lower methionine concentration is also shown in the absence of pantothenate (Table 3). With this mutant, added pantothenate does not decrease the amount of hydrogen sulphide formed; in fact it increases it, but this increase can be related to the increased growth.

O-Acetylhomoserine inhibits hydrogen sulphide formation by mutant CC92-17A. It is equivalent to methionine on a molar basis and the effects are additive. Under

8

certain conditions, homocysteine decreases the amount of hydrogen sulphide formed (Table 4) but again the fact that this compound is itself a source of hydrogen sulphide obscures its inhibitory action. The effects of homocysteine and methionine, although not additive, are synergistic.

Two different methionine-requiring mutants, ME-3 (Pigg, Spence & Parks, 1962) and ME-8 (Cherest, Eichler & de Robichon-Szulmajster, 1969), would not grow on O-acetylhomoserine. As with strain CC92-17A, more hydrogen sulphide was produced during growth on 2 mM-DL-methionine than at higher concentrations. The addition of 5 mM-O-acetylhomoserine did not decrease the amount of hydrogen sulphide formed in the presence of 2 mM-methionine, suggesting that O-acetylhomoserine itself does not inhibit sulphide formation but must first be converted into an active compound, such as methionine. The amount of hydrogen sulphide produced by these mutants growing on 2 mM-DL-methionine was the same whether 40 or 1000 μg. calcium pantothenate/l. was present.

Table 4. *Inhibition by O-acetyl-homoserine and homocysteine of hydrogen sulphide production by Saccharomyces cerevisiae CC92-17A*

DL-Methionine (M)	O-Acetyl-homoserine (M)	Growth (g. dry wt/l.)	Hydrogen sulphide (mg./l.)
2×10^{-3}	0	2·65	1·9
0	2×10^{-3}	3·2	1·55
0	4×10^{-3}	2·9	0·05
2×10^{-3}	2×10^{-3}	2·45	0·05

DL-Methionine (M)	DL-Homocysteine (M)	Growth (g. dry wt/l.)	Hydrogen sulphide (mg./l.)
2×10^{-3}	0	3·75	1·4
0	2×10^{-3}	4·0	0·65
0	4×10^{-3}	3·65	1·25
2×10^{-3}	2×10^{-3}	4·8	0·55

Growth was for 42 hr. in basal medium containing no methionine and 400 μg. calcium pantothenate/l.

Hydrogen sulphide production by suspensions of non-growing organisms

Production of sulphide from sulphite or sulphate by non-growing suspensions of mutant CC92-17A and of pantothenate-deficient brewery yeast was also studied. The buffered suspension contained glucose but no nitrogen source except that introduced by the amino-acid supplement.

With the mutant, methionine, homocysteine, and O-acetylhomoserine all stopped hydrogen sulphide formation both from the added sulphite (or sulphate) and from endogenous sulphur compounds (Table 5).

Suspensions of pantothenate-deficient yeast produced more hydrogen sulphide from sulphite (or sulphate) than the mutant, but there was no endogenous formation. Added methionine or O-acetylhomoserine inhibited hydrogen sulphide formation, and in every experiment O-acetylhomoserine was the more potent inhibitor. Homocysteine itself gave so much hydrogen sulphide that there was no evidence of inhibition of formation by this compound (Table 5).

In experiments in which the suspensions were incubated for 15 min. with actidione before addition of the sulphur source and either methionine or O-acetylhomoserine,

the latter compounds still inhibited sulphide formation. This suggests that they act upon preformed enzymes rather than after incorporation into enzyme formed *de novo*.

Reduction of thiosulphate to sulphide

Pantothenate-deficient brewery yeast also forms hydrogen sulphide when thiosulphate is used as the inorganic sulphur source. Hydrogen sulphide production is inhibited by pantothenate or methionine.

It is well known that inorganic sulphur compounds can be oxidized in air to sulphate, so the results on thiosulphate reduction were checked by using mutants of *Saccharomyces cerevisiae* which are known to be unable to reduce sulphate or sulphite to sulphide (Naiki, 1965). Three such mutants were used with similar results. Pantothenate-deficient mutants produced hydrogen sulphide from thiosulphate and this was inhibited by pantothenate or methionine (Table 6).

Table 5. *Inhibition of hydrogen sulphide formation in cell suspensions of Saccharomyces cerevisiae*

Yeast strain	Na_2SO_3 (M)	DL-Methionine (M)	DL-Homocysteine (M)	DL-O-Acetyl-homoserine (M)	Hydrogen sulphide (μg.)
CC92-17A	0	—	—	—	2·2
	0	10^{-2}	—	—	0·3
	0	—	10^{-3}	—	0·75
	0	—	—	10^{-2}	0
	10^{-3}	—	—	—	5·6
	10^{-3}	10^{-2}	—	—	0·9
	10^{-3}	—	10^{-2}	—	1·0
	10^{-3}	—	—	10^{-2}	0
1164	0	—	—	—	0
	0	10^{-2}	—	—	1·5
	0	—	10^{-3}	—	12·5
	0	—	—	10^{-3}	0·9
	10^{-3}	—	—	—	12·0
	10^{-3}	10^{-2}	—	—	7·0
	10^{-3}	—	10^{-2}	—	16·0
	10^{-3}	—	—	10^{-2}	1·4

Incubation was for 3 hr at 30° using 4·4 ml. liquid in shaken 25 ml. conical flasks. The 1164 yeast was pantothenate-deficient.

Table 6. *Effect of pantothenate and methionine on production of hydrogen sulphide from thiosulphate by Saccharomyces cerevisiae*

Calcium pantothenate (μg./l.)	DL-Methionine (M)	Growth (g. dry wt/l.)	Hydrogen sulphide (μg./l.)
40	0	1·5	295
40	2×10^{-3}	1·85	135
40	4×10^{-3}	1·5	72
40	10^{-2}	1·35	20
400	0	1·5	10

The thiosulphate-requiring mutant 21 was grown for 42 hr in sulphate-free basal medium containing 0·4 mM-sodium thiosulphate. The inoculum was grown with 40 μg. calcium pantothenate/l.

Vitamin B_6-deficient yeast

When *Saccharomyces cerevisiae* Guinness strains are grown on limiting quantities of vitamin B_6, they produce small amounts of hydrogen sulphide. This effect is more pronounced with *S. carlsbergensis* NCYC4228 employed for B_6 assays in beer (Fig. 2). A similar effect has been observed by Kodaira, Ito & Uemura (1958).
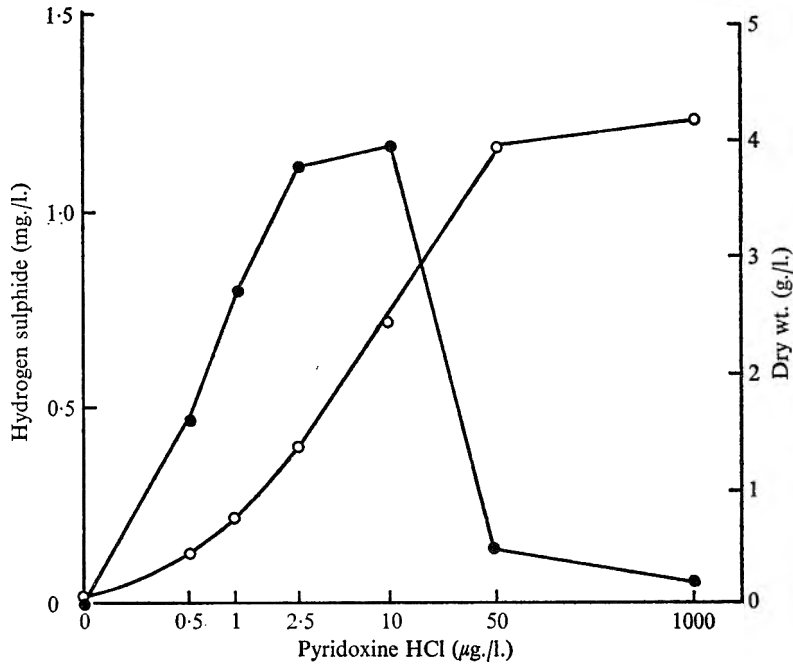
Fig. 2. Effect of pyridoxine on hydrogen sulphide production by *Saccharomyces carlsbergensis*. ● Indicates hydrogen sulphide production, and ○, growth.

Table 7. *Hydrogen sulphide production by vitamin B_6-deficient yeast*

Supplement (5 mM)	Growth (g. dry wt/l.)	Hydrogen sulphide (mg./l.)
None	2	1·18
DL-Methionine	2·5	0·075
DL-Homocysteine	2·3	0·8
DL-O-Acetylhomoserine	2	0·2

Saccharomyces carlsbergensis 4228 was grown for 42 hr with 10 μg. pyridoxine HCl/l. using an inoculum after three subcultures on basal medium with 5 μg. pyridoxine HCl/l.

DL-Methionine at 5 mM, the concentration which inhibits sulphide formation by the mutants or by pantothenate-deficient yeast, inhibits sulphide formation by vitamin B_6-deficient yeast. Acetylhomoserine also inhibits hydrogen sulphide formation markedly, and homocysteine inhibits slightly (Table 7).

Saccharomyces cerevisiae CC92-17A can grow with mM-DL-methionine in the absence of vitamin B_6 although the vitamin stimulates growth slightly (Table 8). In contrast to the results in vitamin B_6 assays with normal yeast (Fig. 2), high concentra-

tions of pyridoxine increase the yield of hydrogen sulphide. Wi:h mutant ᴍᴇ-3, pyridoxine has no effect on growth, and neither its absence nor its presence in abnormally high concentrations evokes hydrogen sulphide formation (Table 8).

Excess pantothenate does not prevent hydrogen sulphide formation by vitamin B_6-deficient yeast nor can excess pyridoxine overcome pantothenate deficiency. With concentrations of pantothenate greater than those giving maximum yields, but insufficient to inhibit hydrogen sulphide production completely (e.g. 80 μg./l.; see Fig. 1), pyridoxine deficiency increases the amount of hydrogen sulphide formed (Table 9). Methionine inhibits production when both vitamins are present in inadequate concentrations as well as when only one is lacking (Table 9).

Table 8. *Hydrogen sulphide production by vitamin B_6-deficient methionine-requiring mutants Saccharomyces cerevisiae CC92-17A and ME-3*

Yeast strain	DL-Methionine (M)	Pyridoxine HCl (μg./l.)	Growth (g. dry wt/l.)	Hydrogen sulphide (mg./l.)
CC92-17A	10^{-3}	0	1·9	2·23
	10^{-3}	10	2·0	2·93
	10^{-3}	1000	2·1	4·25
	5×10^{-3}	10	2·05	0·4
ME-3	10^{-3}	0	1·1	0
	10^{-3}	10	1·1	0
	10^{-3}	1000	1·1	0
	5×10^{-3}	10	1·55	0

The basal medium for the experiment contained 400 μg. calcium pantothenate/l. but no methionine or pyridoxine. The inoculum had been subcultured twice in medium with 5 mᴍ-DL-methionine, 5 μg. pyridoxine HCl/l. and 400 μg. calcium pantothenate/l.

Table 9. *Hydrogen sulphide production by Saccharomyces cerevisiae 1164 deficient in both pantothenate and vitamin B_6*

Pantothenate (μg./l.)	Pyridoxine (μg./l.)	DL-Methionine (M)	Growth (g./l.)	Hydrogen sulphide (mg./l.)
40	10	0	1·67	2·4
40	10	10^{-2}	1·98	1·25
40	1000	0	2·28	2·5
80	10	0	1·85	1·85
80	10	10^{-2}	2	0·33
80	1000	0	2·3	0·7
1000	10	0	1·7	0·8
1000	10	10^{-2}	2	0
1000	1000	0	2·3	0

Growth was for 42 hr using a basal medium containing no pantothenate or pyridoxine. The inoculum was grown with 40 μg. calcium pantothenate/l. in a medium containing no pyridoxine.

Hydrogen sulphide production by Escherichia coli

As mentioned in the Introduction, the pantothenate effect on hydrogen sulphide production has been observed only in yeasts. In experiments with three pantothenate-requiring mutants of *Escherichia coli*, very little hydrogen sulphide was detected, but the cultures always reverted to pantothenate independence during the growth period. In *E. coli*, succinyl-homoserine and not acetylhomoserine is the normal precursor of methionine (Wiebers & Garner, 1967). In preliminary experiments with the succinate-

requiring mutant W 1485-suc-1 of *E. coli* (described by Herbert & Guest, 1968), more hydrogen sulphide is produced at suboptimal succinate concentrations. The inference is that hydrogen sulphide may be produced when succinyl-homoserine is lacking.

DISCUSSION

The fact that pantothenate deficiency results in the production of hydrogen sulphide by yeasts has been known for some time, but hitherto no satisfactory explanation has been apparent. Kodaira & Uemura (1960) found that methionine inhibited sulphide production by pantothenate-deficient yeast and suggested that pantothenate was somehow required for the conversion of cysteine to methionine. They suggested that if this could not occur, excess cysteine was converted to hydrogen sulphide by cysteine desulphydrase. Five years later, Okuda & Uemura (1965) suggested, as a result of testing crude enzyme fractions, that in pantothenate deficiency a sulphite reductase was formed which differed from that in normal cells. Methionine inhibited the formation of this reductase. Our own work on the purification of yeast sulphite reductase from normal and pantothenate-deficient yeast does not support this (Wainwright, 1962, and unpublished observations), and De Vito & Dreyfus (1964) also reported that methionine did not repress the formation of yeast sulphite reductase. More recently, Aida, Tokuyama & Uemura (1969) have suggested that the cysteine desulphydrase of baker's yeast is more active in pantothenate-deficient organisms than in normal yeast. They suggested that this was because pantothenic acid deficiency causes a decrease in the ATP content in the organism and that this results in less allosteric inhibition of cysteine desulphydrase by ATP. They suggest that methionine suppresses hydrogen sulphide formation because *S*-adenosylmethionine inhibits cysteine desulphydrase.

The results in the present paper confirm that methionine (or a derivative) plays a key role in the regulation of sulphide production from inorganic sulphur compounds by yeast under three quite different conditions—deficiency of methionine, vitamin B_6, or pantothenate. These results have been obtained with many strains of yeast and not only those described in the paper. It is also obvious from the results that O-acetyl-homoserine can be an intermediate in methionine biosynthesis by *Saccharomyces cerevisiae* and *S. carlsbergensis*. Again the results have been confirmed on several strains additional to those mentioned in the paper. The evidence for the role of O-acetyl-homoserine in methionine biosynthesis, although strong, has previously been obtained with relatively few strains of Saccharomyces. In the case of the mutant ME-3, which apparently cannot form methionine from acetylhomoserine, it was found that acetyl-homoserine did not inhibit hydrogen sulphide formation. The fact that in all other experiments it was at least as active as methionine on a molar basis suggests that it is probably a normal intermediate in Saccharomyces species.

An obvious explanation for the effect of pantothenate is that it is required in the form of coenzyme A for acetylhomoserine synthesis. Thus the same effect can be obtained with growing organisms or in non-growing suspensions by pantothenate or O-acetylhomoserine. Pantothenate had little effect on growth of the mutant CC92-17A and certainly pantothenate deficiency did not increase sulphide production. Obviously if the role of pantothenate is in the formation of acetylhomoserine, and the yeast cannot for other reasons form acetylhomoserine, then pantothenate should have no

effect either on growth or on hydrogen sulphide formation. Pantothenate likewise had no effect on hydrogen sulphide production by mutants ME-3 or ME-8. *O*-Acetylhomoserine also had no effect so that its precursors would not be expected to show activity.

The part which vitamin B_6 plays in the regulation of sulphide formation is less obvious although most of the results suggest an involvement in methionine biosynthesis. Methionine inhibits hydrogen sulphide production by vitamin B_6-deficient brewery yeast whether or not it is pantothenate-deficient (Table 9). This amino acid also increases the amount of growth of *Saccharomyces cerevisiae* (and of *S. carlsbergensis*) at low vitamin B_6 concentrations (Table 9), as might be expected if it is an anabolic product of vitamin B_6 metabolism (Woods, 1953). This is supported by the fact that both the methionine-requiring mutants grew quite well without vitamin B_6 (Table 8).

When the mutant CC92-17A is growing on mM-DL-methionine, hydrogen sulphide is formed to the largest extent in a pyridoxine-rich medium (Table 8) and growth is augmented. This is consistent with the possibility that some methionine is synthesized by the mutant in the presence of pyridoxine since, as shown in Table 3, this is one of the strains of yeast with which methionine at certain concentrations increases hydrogen sulphide production. Mutant ME-3, which cannot grow on *O*-acetylhomoserine, is blocked at one of the later steps in methionine biosynthesis. If pyridoxine cannot be used for methionine biosynthesis in this mutant, it should have no effect on growth or hydrogen sulphide formation. In fact (Table 8) pyridoxine has no effect.

Whilst these results suggest that vitamin B_6 affects hydrogen sulphide formation because it is involved in methionine biosynthesis, the site of involvement is not obvious. It is known that pyridoxal phosphate is required for the condensation of acetylhomoserine and hydrogen sulphide to form homocysteine (Wiebers & Garner, 1967). Lack of vitamin B_6 could result in excretion of hydrogen sulphide because it cannot be utilized for this reaction. However *O*-acetylhomoserine itself inhibits sulphide formation and, whilst it is possible that large amounts of substrate drive the reaction through a mass action effect, it is more likely that vitamin B_6 does not directly affect homocysteine biosynthesis.

In *Saccharomyces cerevisiae*, pyridoxal phosphate is required for the formation from serine of the methyl group of methionine (Botsford & Parks, 1969). Vitamin B_6 deficiency may cause methionine deficiency through shortage of methyl groups. Again, however, acetylhomoserine should not cure such a defect. It is also possible that the direct effect of vitamin B_6 deficiency is on cysteine biosynthesis. Pyridoxal phosphate is required for the formation of cysteine from serine (Schløssmann & Lynen, 1957) or acetylserine (Wiebers & Garner, 1967). If hydrogen sulphide cannot be utilized for the reaction, it may be excreted. Moreover, since cysteine represses yeast sulphite reductase formation (De Vito & Dreyfus, 1964), a deficiency in cysteine biosynthesis might augment hydrogen sulphide production. The effect of methionine in vitamin B_6 deficiency might be due to the known ability of yeast to form cysteine from methionine. Methionine, however, also has a marked action on hydrogen sulphide formation in conditions other than vitamin B_6 deficiency, and in these circumstances cysteine biosynthesis is probably not limited.

It is difficult to explain the results with methionine as due to an inhibition of sulphite reduction since, with many pantothenate-deficient strains, appropriate methionine

concentrations actually increase the amount of sulphide formed (Table 1). Moreover pantothenate, *O*-acetylhomoserine and methionine all have no effect on purified yeast sulphite reductase (T. Wainwright, unpublished observations) and the extracts from pantothenate-rich or pantothenate-starved organisms are equally active in reducing sulphite to sulphide (Wainwright, 1962). The pathways for reduction of sulphite (or sulphate) and thiosulphate in yeast are quite distinct (Naiki, 1965). Since pantothenate and methionine inhibit hydrogen sulphide accumulation when yeast (normal or mutant) is grown either on sulphate or on thiosulphate, it is probable that methionine regulates the metabolism of hydrogen sulphide which has been formed rather than that it prevents hydrogen sulphide formation by two independent pathways.

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Determination of Multiple Forms of Esterases in Rhizobium by Paper Electrophoresis

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SUMMARY

Fifty-two strains, comprising six Rhizobium species, were examined for their esterase patterns using electrophoresis on cellulose acetate. Esterase activity was detected in five Rhizobium species. The sixth species, *R. japonicum*, was characterized by the absence of esterase activity in all but one of the strains examined. *Rhizobium trifolii* and *R. leguminosarum* strains showed similarities in their esterase profiles. *Rhizobium meliloti* strains formed a group distinct from these on the basis of their esterase patterns. *Rhizobium lotus* sp. and *R. phaseoli* also exhibited esterase activity.

Heat denaturation and metal inhibition studies suggest that the esterase activity is truly enzymic. The inability of the bacterial esterase to react with a synthetic peptide suggests that residual esterase activity associated with certain proteolytic enzymes is not involved. Heat tests revealed differences in the sensitivity of the multiple forms of esterases in rhizobia to inactivation.

INTRODUCTION

Studies on enzyme profiles in bacteria is a recent technique of considerable importance, which has been applied by a number of workers concerned with the classification of bacteria (Norris, 1964; Lund, 1965; Cann & Willox, 1965; Nakayama & Takeya, 1967; Morichi, Sharpe & Reiter, 1968; Hogan & Colwell, 1969). The relatively high concentration of esterase activity in bacteria accompanied by the multiple nature and ease of detection of these enzymes has been responsible for their choice as markers in this process. In the present investigation, the intensity and electrophoretic nature of the esterase patterns of 52 strains from six Rhizobium species were determined to establish the relationship of these patterns to the present classification of these organisms. It was also intended to explore the possibility of using esterase patterns as identification markers for strains with similar nodulation and nitrogen fixation properties. Classically six Rhizobium species are recognized, comprising *R. trifolii, R. leguminosarum, R. meliloti, R. phaseoli, R. japonicum* and *R. lupini* (*Bergey's Manual* 1957). Currently, however, with the advent of improved techniques for studying cell components and the analysis of the resulting information by computers, certain changes in the original classification have been suggested (Lange, 1961; Graham, 1964; De Ley & Rassel, 1965; 't Mannetje, 1967; Moffett & Colwell, 1968).

Although esterase activity in bacteria and fungi has been the subject of investigation by a number of workers (Norris, 1964; Lund, 1965; Cann & Willox, 1965; Robinson, 1966; Moustafa & Greenwood, 1967; Nakayama & Takeya, 1967; Peberdy & Turner,

1968; Hogan & Colwell, 1969) there is little information available on the nature of the activity, particularly with respect to rhizobia. Such information is desirable especially in view of the existence of non-enzymic esterase activity (Downey & Andrews, 1965) and residual esterase activity associated with certain proteolytic enzymes (Kimmel & Smith, 1954). This report contains results of preliminary investigations on the effects of mercurial and thermal treatments on the bacterial esterase with a view to establishing the nature of this activity in rhizobia.

METHODS

Origin and number of strains. Fifty-two Rhizobium strains, comprising several species were used in this investigation. The sources of the test strains and their nitrogen fixation performance are given in Table 1.

Culture of rhizobia. Test cultures were maintained on slopes of yeast-extract-mannitol-agar (YEMA) at 4° after 72 hr growth at 25° (Allen, 1957). A suspension of

Table 1. *Characteristics and sources of Rhizobium strains*

Species	Strain no.	Source*	Test host	N-Fixation†	Zymogram no.‡
R. trifolii	J728	J.C.		+	1
	J752	J.C.		+	2
	CORYN	R.		−	3
	AF 12	R.		−	4
	J755	J.C.		+	5
	J735	J.C.		+	6
	J708	J.C.		+	7
	J731	J.C.		−	8
	J729	J.C.	*Trifolium repens*	−	9
	J733	J.C.		+	10
	J736	J.C.		+	11
	J738	J.C.		−	12
	J732	J.C.		−	13
	J730	J.C.		−	14
	J734	J.C.		+	15
	J737	J.C.		−	16
	J739	J.C.		−	17
	J740	J.C.		−	18
R. leguminosarum	1001	R.		+	19
	1004	R.		−	20
	1013	R.	*Vicia hirsuta*	+	21
	1016	R.		−	22
	1019	R.		−	23
	1010	R.		−	24
R. leguminosarum	1007	R.	*Pisum sativum*	+	25
	307	W.	.	n.d.	26
	337	W.	.	n.d.	27
	341	W.	.	n.d.	28
R. meliloti	2009	R.		−	29
	2010	R.		+	30
	2011	R.		+	31
	2012	R.	*Medicago sativa*	+	32
	2013	R.		+	33
	2006	R.		−	34
	2007	R.		+	35
R. phaseoli	3601	R.	*Phaseolus vulgaris*	+	36
	3603	R.	*Phaseolus vulgaris*	−	37
	3604	R.	*Phaseolus vulgaris*	−	38

Table 1 *cont.*

Species	Strain no.	Source*	Test host	N-Fixation†	Zymogram no.‡
R. japonicum	3401	R.	*Glycine max*	−	39
	3402	R.	*Glycine max*	+	40
	3404	R.	*Desmodium uncinatum*	−	41
	3405	R.	*Desmodium uncinatum*	−	42
	511	W.	.	n.d.	43
	5527	W.	.	n.d.	44
R. lotus sp.	A166	H.	*Lotus corniculatus*	+	45
	A168	H.	*Lotus corniculatus*	+	46
	J202	J.C.	.	n.d.	47
	J204	J.C.	*Lotus corniculatus*	+	48
R. lotus sp.	J201	J.C.	.	n.d.	49
	3001	R.	*Lotus corniculatus*	+	50
	3002	R.	*Lotus corniculatus*	+	51
	3206	R.	*Lotus corniculatus*	−	52

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† +, Effective in nitrogen fixation; −, ineffective in nitrogen fixation; n.d., nitrogen fixation characteristic not determined.

‡ Zymogram no. refers to esterase pattern number in Fig. 1.

freshly grown rhizobia was incubated in 1 l. quantities of yeast-extract-mannitol-broth on a Brunswick gyrotory shaker for 72 hr at 25°, when required for electrophoresis.

Preparation of cell-free extracts. The rhizobia were harvested by centrifugation at 23,000 *g* for 30 min. using an M.S.E. high speed centrifuge. They were washed twice with distilled water and 1 g. rhizobia suspended in 3 ml. 0·05 M-phosphate buffer, pH 7·0. Cell-free extracts were prepared by sonication of the cooled suspension for 2 min. at 4° using an M.S.E. 60 watt sonic disintegrator. Sonicated extracts were centrifuged at 38,000 *g* at 4° for 40 min. to remove cell debris.

Electrophoresis. The cell-free extracts were analysed by paper electrophoresis (Preston, Briere & Batsakis, 1965; Mager, Blatt & Abelman, 1966; Murphy *et al.* 1969). Eight-inch square sheets of cellulose acetate (Gelman Instrument Co., Michigan, U.S.A.), which permitted 8 samples to be processed simultaneously, were used. Ten *μ*l. samples were applied and electrophoresis conducted at 15° using a continuous phosphate buffer system 0·05 M, pH 7·0. The initial current applied was 15 mA and duration of electrophoresis was 90 min. Results presented are the mean of at least two determinations. With all species examined preliminary tests were made to determine the direction of migration of the esterase bands.

Qualitative detection of esterases. On completion of electrophoresis the cellulose acetate sheets were immersed in a solution of α-naphthyl acetate and fast blue salt for 30 min. (Lawrence, Melnick & Weimer, 1960). It was found that subsequent treatment of the stained sheet with 0·1 N-NaOH accentuated the colour of the dye in the areas of enzymic activity, which greatly facilitated the detection of weak esterase bands in the zymogram.

Peptidase activity. The synthetic peptide *N*-α-benzoyl-DL-arginine-*p*-nitroanilide-HCl (BAPNA) was substituted for α-naphthyl acetate and the same procedure followed as described for esterase determination.
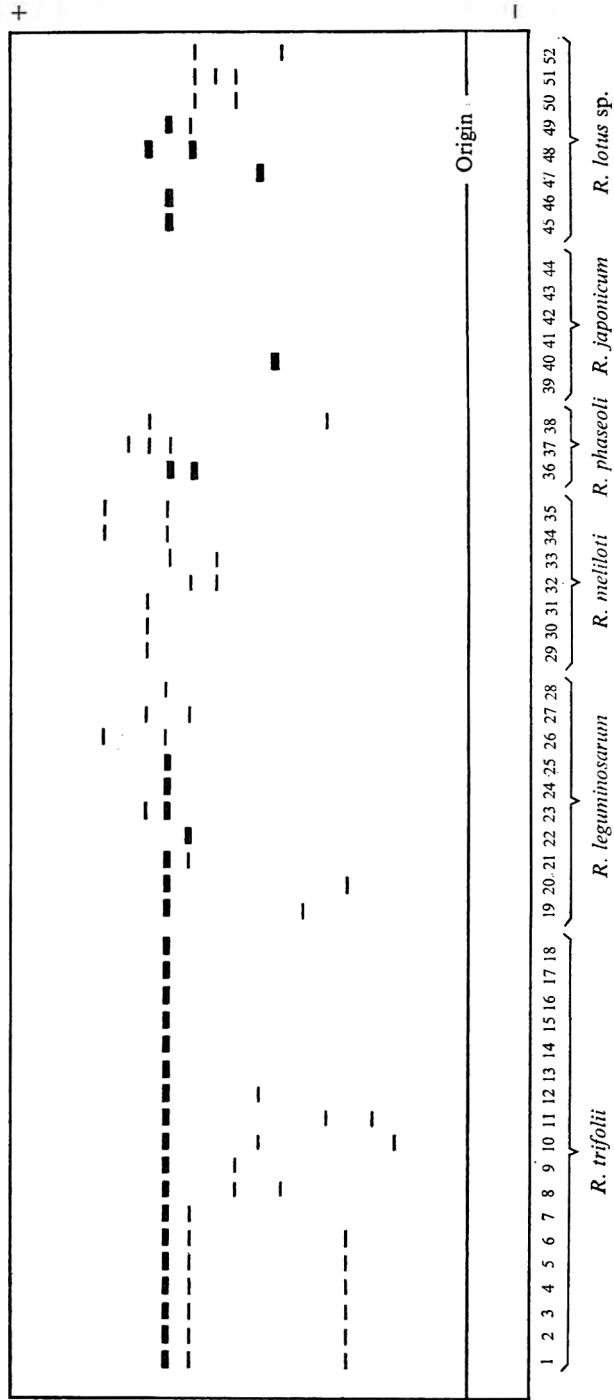
Fig. 1. Esterase patterns of five Rhizobium species.

Quantitative determination of esterase activity. Esterase activity was determined spectrophotometrically at 410 nm. in 0·05 M-phosphate buffer, pH 7·0, at 15° by measuring the formation of *p*-nitrophenol from *p*-nitrophenol acetate (Downey & Andrews, 1965).

Protein determination. Protein concentrations were measured using the biuret reaction (Gornall, Bardawill & David, 1949).

Heat inactivation of esterase activity. This was determined both quantitatively and qualitatively. For the former measurement enzyme solutions (1 mg. protein/ml.) were heated at 45° for various periods of time and esterase activity determined spectrophotometrically. For the latter measurement, enzyme solutions (10 mg. protein/ml.) were heated at various temperatures (45° to 75°) for 10 min. and the effect on the esterase electrophoretic pattern examined.

Effect of p-chloromercuribenzoate (pCMB) on esterase activity. A stock solution of *p*CMB (1×10^{-3} M) was prepared in 0·05 M phosphate buffer, pH 7·0. Enzyme solutions (1 mg. protein/ml.) were incubated with *p*CMB (final molarity 8×10^{-4} M) for various periods of time and esterase activity determined spectrophotometrically.

RESULTS

Esterase patterns. The esterase patterns obtained from the Rhizobium cultures examined in this work are shown in Fig. 1. Considerable esterase activity was found in all species with the exception of *R. japonicum*. In the latter species no activity could be detected in five strains, while the sixth strain tested showed one band of low mobility. Multiple esterase bands were obtained with most strains, the maximum number being three. In all instances, the direction of migration of the bands was from the cathode to the anode. It is evident from Fig. 1 that differences in esterase pattern exist not only between species but also between strains within species. In the case of *R. trifolii* (Fig. 1, 1 to 18) these differences are evident only in the minor esterase bands as the major, fastest moving component, is common to all strains of this species. This component is quite prominent also in *R. leguminosarum*, occurring in six of the ten strains studied (Fig. 1, 19 to 28). It is totally absent from strains of *R. meliloti* (Fig. 1, 29 to 35) and *R. japonicum* (Fig. 1, 39 to 44) and occurs in one of the *R. phaseoli* strains examined (Fig. 1, 36 to 38) and in three of eight strains of *R. lotus* sp. (Fig. 1, 45 to 52).

Peptidase activity. No peptidase activity was detected with any of the species using BAPNA as substrate.

Effect of heat on the esterase electrophoretic pattern. Figure 2 shows the effect of various temperature treatments, over a period of 10 min., on the esterase electrophoretic patterns of four Rhizobium species. The esterases of the strain of *R. trifolii* studied were largely inactivated on heating to 45° for 10 min. The fastest moving component was totally inactivated and only a trace of the second component remained (Fig. 2). The *R. lotus* sp. strain, however, was resistant to inactivation at 45°. At 50°. the slowest moving component was inactivated but the fastest moving fraction was unaffected. This component was not inactivated until a temperature of 75° was reached,

Similarly with the *R. leguminosarum* strain, the fastest moving component was unaffected at 45° though the other two components were largely inactivated. No activity remained at a temperature of 75°. The esterase components of the *R. meliloti* strain were inactivated at 45°, only a trace of the fastest moving component remaining.
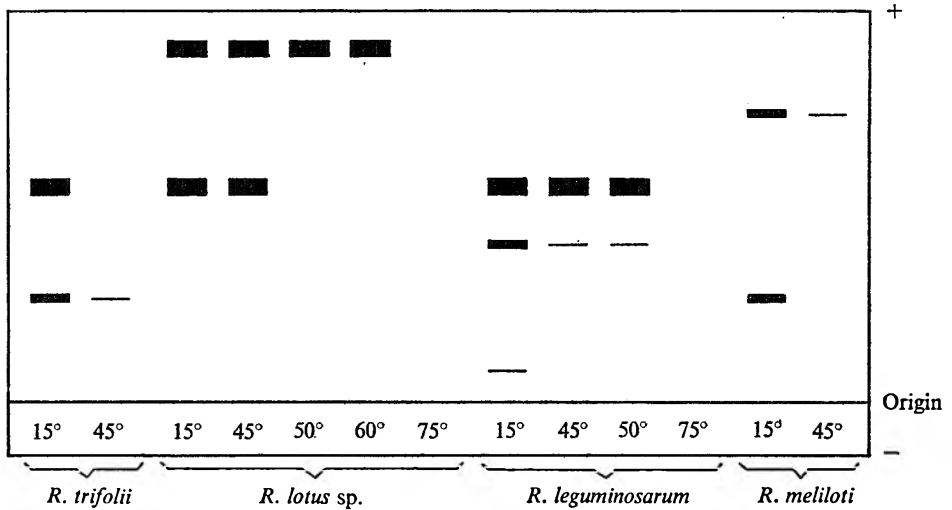
Fig. 2. Effect of heat on the electrophoretic esterase patterns of four Rhizobium species. Enzyme solutions (10 mg. protein/ml.) were heated at temperatures between 45° and 75° for 10 min. and the electrophoretic esterase patterns then determined. The control sample (15°) was unheated.
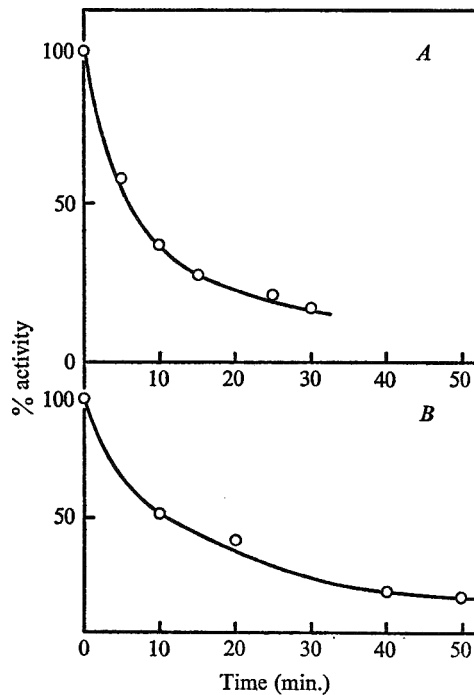
Fig. 3. (A). Heat inactivation of esterase activity. Cell-free extracts (1 mg. protein/ml.) of a *Rhizobium trifolii* strain were heated at 45° and esterase activity with time, determined spectrophotometrically at 410 nm. (B). Inhibition of esterase activity by pCMB. Cell-free extracts (1 mg. protein/ml.) were treated with pCMB (8 × 10⁻⁴ M) and esterase activity determined with time.

Figure 3*A* shows the effect of heat on bacterial esterase activity of *Rhizobium trifolii* determined spectrophotometrically. The activity is very sensitive to heat being reduced by 50 % in 7 min. at 45°, a result which is in agreement with that depicted in the zymogram in Fig. 2. Figure 3*B* shows the effect of *p*CMB on esterase activity with time. Esterase activity is rapidly inactivated by this organomercurial reagent.

DISCUSSION

Results obtained in this work and illustrated in Fig. 1 reveal that in general the leguminous root nodule bacteria have high esterase activity. Fottrell (1968) demonstrated the presence of multiple esterase forms in the root nodule system, where up to ten bands of activity, comprising both anodic and cathodic fractions, were detected. The present report shows that fewer esterase components are present in the free-living bacteria and these are anodic in nature.

The zymogram patterns obtained from the strains tested reveal variation, especially between species, but also within species. *Rhizobium trifolii* and *R. leguminosarum* strains show the greatest similarity in esterase patterns and it is interesting to note that other workers have reported the occurrence of compatible characteristics in these two species (Graham, 1964; Moffett & Colwell, 1968; Fottrell & O'Hora, 1969). *Rhizobium meliloti* strains appear to form a distinct grouping in agreement with the currently accepted classification of this species. The absence of esterase activity in most strains of *R. japonicum* is quite striking and in contrast to the presence of high activity in *R. lotus* sp. Moustafa & Greenwood (1967) studied esterase and phosphatase activity in four strains of *R. lotus* sp. and found that the free-living rhizobia had similar enzyme patterns but differences in esterase profiles were encountered in the bacteroidal form.

The use of esterase patterns as a means for typing strains of rhizobia may have useful application. Patterns obtained are reproducible provided that experimental conditions are strictly adhered to. This is particularly true at the sonication step in the preparation of cell-free extracts, where excessive heat build-up may result in partial or total loss of enzyme activity.

There is no correlation between nitrogen-fixing abilities of the bacteria and their esterase patterns. This is illustrated in the case of *Rhizobium trifolii* (Fig. 1, 1 to 6) where identical esterase patterns were obtained for strains showing different symbiotic responses (Table 1). These strains also represented isolates from mineral and peat soils.

Esterase activity has been associated with non-enzymic proteins (Downey & Andrews, 1965) and some proteolytic enzymes also show esterase activity (Kimmel & Smith, 1954). Conventional heat tests, which have demonstrated the susceptibility of the rhizobial esterase to denaturation coupled with the fact that no proteolytic activity was detected using BAPNA as substrate, suggest that 'true esterase' activity is involved. Esterases of *R. trifolii* and *R. meliloti* strains are very sensitive to heat denaturation being inactivated on heating at 45° for 10 min. Morichi *et al.* (1968) studied esterases of lactic acid bacteria and reported denaturation temperatures of 60° and 65° for 10 min. Cann & Willox (1965) reported on the presence of esterases in mycobacteria capable of withstanding 100° for 10 min. Esterases of *R. lotus* sp. bacteria were resistant to denaturation at 45° and showed selective denaturation above this temperature. *Rhizobium leguminosarum* strains followed a similar pattern, a final temperature of 75°

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being necessary to achieve complete denaturation of the esterase enzyme. Multiple forms of esterases in *R. lotus* sp. and *R. leguminosarum*, therefore, differ not only in electric charge but also in sensitivity to heat denaturation. However, the comparative ease of denaturation of the esterase activity coupled with the absence of proteolytic activity suggests that the multiple forms are different expressions of a single enzyme activity. The sensitivity of the rhizobial esterases to heat denaturation, particularly *R. trifolii* and *R. meliloti* strains, emphasizes the need for carefully controlled temperature conditions when using sonication for cell disintegration. The present report has shown the sensitivity of the bacterial esterase to inhibition by *p*CMB. Morichi *et al.* (1968) reported that the esterases in lactic acid bacteria were resistant to 10^{-4} M-*p*CMB.

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Nutritional Requirements for Growth of *Aerococcus viridans*

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SUMMARY

Vitamin requirements for growth in a casein hydrolysate medium were determined for 27 strains of *Aerococcus viridans* from diverse sources. Pantothenic acid, nicotinic acid and biotin were either absolutely required by, or markedly stimulatory to, all strains; none required thiamin, riboflavin, pyridoxine, folic acid or folinic acid. Tween 80 replaced the biotin requirement of most strains. Amino acid requirements were not sharply defined and varied from strain to strain. As the amino acid composition of the medium was simplified the amount of growth was decreased and most strains would not grow when biotin was replaced by Tween 80. A single purine base was required: either guanine or xanthine alone satisfied this requirement for each of the strains tested; adenine was a suitable alternative source for some strains. Exogenous pyrimidine was not required.

INTRODUCTION

There is increasing evidence that a relatively homogenous group of Gram-positive tetrad-forming cocci is widely distributed in our environment. These organisms may be referred to as *Aerococcus viridans* (Williams, Hirch & Cowan, 1953) or *Pediococcus homari* (Deibel & Niven, 1960), and appear to be indistinguishable from the lobster pathogen *Gaffkya homari* (Aaronson, 1956; Deibel & Niven, 1960). These organisms are found in the air of occupied rooms (Williams *et al.* 1953), in meat curing brines (Deibel & Niven, 1960), in medicine bottles (Clausen, 1964), on raw and processed vegetables (Mundt, Graham & McCarty, 1967), in hospital environments (Kerbaugh & Evans, 1968) and in a variety of human infections (Colman, 1967). There have not been detailed reports of nutritional studies of these organisms, referred to here as *Aerococcus viridans*, although undocumented references to the requirements of one or two strains are included in papers by Aaronson (1956) and Sakaguchi & Mori (1969). The present study was undertaken to provide a survey of the nutritional requirements of a relatively large collection of cultures from diverse sources.

METHODS

Sources of strains. Included in this study were 14 strains isolated in our laboratory (MK-series) from the hospital environment (Kerbaugh & Evans, 1968), three strains

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received from Baird-Parker (strains 301, 302 and 303), four strains from Clausen (c2, c6, c7 and c9), five strains from Mundt (49-3, 49-7, 49-8, 61-8 and 62-4) and two strains from the American Type Culture Collection (10400 and 11563). Included as control cultures in some experiments were *Gaffkya tetragena* (ATCC 10875), *Pediococcus cerevisiae* (ATCC 8081) and enterococci from our departmental collection.

Media. Stock solutions of vitamins, amino acids, purines, pyrimidines and mineral salts were made in distilled water in acid-cleaned bottles. One ml. of a volatile preservative (Hutner, Cury & Baker, 1958) was added to 100 ml. volumes of these solutions, which were stored at 5°. Test media were adjusted to pH 7·0 to 7·2, dispensed in 10 ml. amounts in 18 mm. wide acid-cleaned borosilicate test-tubes, capped with Morton type stainless steel caps and sterilized at 121° for 13 min. The composition of the casein hydrolysate medium that served as the 'complete' medium for most of these studies is given in Table 1. Folinic acid (1 μg./l.) was added in some experiments. Amino acids (100 mg./l.) of the L-isomeric form replaced the hydrolysed casein in some experiments.

Table 1. *Casein hydrolysate medium for Aerococcus viridans*

Component	Per litre	Component	Per litre
Casein (acid-hydrolysed)	50 ml.	Biotin	100 μg.
L-Cystine	100 mg.	Folic acid	10 μg.
L-Tryptophan	100 mg.	Nicotinic acid	2 mg.
D-Glucose	10 g.	Calcium pantothenate	1 mg.
NH_4Cl	1 g.	Pyridoxine HCl	2 mg.
K_2HPO_4	4 g.	Riboflavin	1 mg.
Adenine	5 mg.	Thiamin	1 mg.
Guanine	5 mg.	$MgSO_4.7H_2O$	200 mg.
Uracil	5 mg.	$FeSO_4.7H_2O$	10 mg.
Xanthine	5 mg.	$MnSO_4.4H_2O$	10 mg.

Inoculation and serial transfer. Cultures were transferred three times at 24 hr intervals in BBL Trypticase Soy Broth. Test media were inoculated with one drop/tube and incubated at 30°. Growth was measured turbidimetrically at 24 hr intervals for 3 days, using a B & L Spectronic 20 colorimeter at 520 nm. From each tube of test medium that exhibited growth (optical density of at least 0·10) one drop was serially transferred to another tube of the same test medium. When growth occurred within 24 hr in this second transfer, a drop was transferred from it to a third tube of the medium. This procedure eliminated errors due to carry-over of nutrients in the inoculum. Results are generally presented as the optical density of the third transfer after 72 hr. When growth in the first or second transfer was insufficient to warrant serial transfer, the growth was recorded as 0.

RESULTS

Vitamin requirements. The vitamin requirements were determined for 27 of the 28 strains of *Aerococcus viridans*. One strain (c9) grew too poorly in the casein hydrolysate medium to determine its requirements and addition to the medium of folinic acid and Tween 80 did not elicit improved growth. Data for seven of the cultures are presented in Table 2; similar results were obtained with the other 20 strains. Control cultures that required thiamin, riboflavin, pyridoxine, folic acid and folinic acid were also included in the study to prove that the basal medium was free from contamination with these vitamins.

A three-vitamin casein hydrolysate medium was prepared (with only pantothenic acid, nicotinic acid and biotin) and 24 of the 27 cultures produced essentially the same growth as in the seven-vitamin medium (optical densities within ±0·10). Of the other three cultures, two grew better in the three-vitamin medium and one grew better in the seven-vitamin medium. Addition of folinic acid to the three-vitamin medium did not stimulate growth of any of the cultures. Tween 80 (0·1 % (v/v)) completely eliminated the biotin requirement of most strains.

Table 2. *Vitamin requirements of representative strains of Aerococcus viridans*

All figures are optical density readings on the third transfer after 72 hr at 30°.

			Vitamin omitted						
Strain	TSB*	Complete medium†	Thi-amin	Ribo-flavin	Pyri-doxine	Panto-thenate	Nicotinic acid	Folate	Biotin
MK-32	0·79	0·53	0·45	0·64	0·52	0	0	0·54	0·24
MK-80	0·84	0·58	0·49	0·56	0·54	0	0	0·60	0
MK-95	0·98	0·70	0·56	0·80	0·75	0	0	0·68	0·37
MK-165	0·85	0·62	0·62	0·64	0·57	0	0	0·59	0
49-3	0·80	0·79	0·69	0·75	0·73	0	0	0·75	0·24
61-8	0·82	0·59	0·55	0·57	0·59	0	0	0·54	0
11563	0·62	0·31	0·21	0·29	0·35	0	0	0·34	0

* BBL Trypticase Soy Broth
† Medium described in Table 1.

Table 3. *Purine and pyrimidine requirements of representative strains of Aerococcus viridans*

All figures are optical density readings.

	Strain number					
Medium	MK-3	MK-53	MK-95	MK-132	MK-201	303
Complete*	0·54	0·53	0·63	0·55	0·55	0·69
Basal†	0·18	0	0·07	0	0·09	0·23
+adenine	0·44	0	0·62	0	0	0·42
+guanine	0·45	0·58	0·55	0·56	0·46	0·50
+xanthine	0·46	0·47	0·54	0·47	0·41	0·48
+uracil	0·16	0	0	0	0	0·29

* Medium described in Table 1 with 0·1 % Tween 80 added.
† Medium without purines or pyrimidine.

Amino acid requirements. It was determined that most cultures grew well in a medium with 18 L-amino acids as the nitrogen source, producing 80 to 100 % of the growth in the casein hydrolysate medium. One strain (MK-155) did not grow in the amino acid medium. An attempt was made to determine the specific amino acid requirements of three test strains by omitting individual amino acids from the medium. Cystine, methionine and tryptophan were absolute requirements of these three strains, and valine was required by two strains. Omission of several other amino acids markedly decreased the growth, but the three cultures differed in their response.

A medium containing the 10 amino acids required or apparently stimulatory to one or more of the three test organisms (arginine, glutamic acid, tyrosine, phenylalanine,

leucine, isoleucine, in addition to the four amino acids noted above) supported moderate growth (about 50 % of the growth in the casein hydrolysate medium) of 15 of 20 strains tested with both biotin and Tween 80 in the medium. Omission of biotin from this medium prevented growth of all strains.

Purine and pyrimidine requirements. Omission of the purines and pyrimidine singly from the casein hydrolysate medium did not show any requirement. However, as illustrated in Table 3, simultaneous omission of the purines and pyrimidine allowed little or no growth. Addition of either guanine or xanthine alone produced good growth of all strains tested; adenine alone sufficed for about half of the strains. Uracil was without apparent effect.

DISCUSSION

Despite individual differences among the various strains tested the nutritional requirements of this collection of aerococci seemed to be relatively homogeneous. Their vitamin requirements were particularly uniform, with pantothenic acid, nicotinic acid and biotin required by or markedly stimulatory to all strains and there was no evidence of other vitamin requirements. These vitamin requirements tended to differentiate these organisms from other species of Gram-positive cocci; they lacked the requirement for folinic acid that is characteristic of pediococci (Jensen & Seeley, 1954). Their lack of a requirement for thiamin or riboflavin differed from most streptococci and staphylococci.

Of interest were our results with *Gaffkya tetragena* ATCC 10875, which was found to have a low GC content (33 %) and to differ in other ways from the aerococci (Evans & Schultes, 1969); this culture required only nicotinic acid and thiamin. However, other strains received as *G. tetragena* had quite different vitamin requirements (unpublished observation).

In view of our results it is impossible to give credence to the report of Sakaguchi & Mori (1969) that two strains of aerococci required biotin, pantothenic acid, nicotinic acid, *p*-aminobenzoic acid, pyridoxine, riboflavin, thiamin, folic acid and folinic acid. They did not describe their methods, verify the identity of their cultures or present any data. We also question the report of Aaronson (1956), who implied, also without details of procedure or results, that the two strains he studied required pantothenic acid, nicotinic acid, biotin and thiamin. From our results one might infer a slight stimulatory effect of thiamin on some strains (e.g. MK-95); different basal media and techniques might enhance this effect.

Numerous efforts to select combinations of amino acid supplements that more specifically meet the nitrogen requirement of these organisms have not been successful. Hence, we have not devised what one could call a minimal defined medium for the aerococci. Further studies on the interrelationships between various nutrients such as amino acids and vitamin requirements may be warranted.

Agricultural Experiment Station, Raleigh, North Carolina. This work is taken from a dissertation submitted by T.L.M. in partial fulfilment of the requirements for the M.S. Degree.

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SHORT COMMUNICATIONS

The Transformation of Both Recipient Strands of Pneumococcal DNA

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It has been established that, in pneumococcus, donor sites on transforming deoxyribonucleic acid (DNA) can be divided into at least two types, known as low-efficiency (LE) sites and high-efficiency (HE) sites, by comparing the number of transformants of a particular character with that obtained for a standard reference gene (Ephrussi-Taylor, Sicard & Kamen, 1965; Lacks, 1966). The reference gene used by Ephrussi-Taylor and her colleagues was *str-41*, whilst Lacks used the *sulf-d* gene. Ephrussi-Taylor & Gray (1966) proposed that LE markers are integrated into both chains of the recipient DNA by an excision and repair mechanism, whilst HE markers have to wait for a further division before both the recipient strands become altered. Evidence that the LE markers are transmitted into both strands whilst HE markers are transmitted to only one strand has been put forward by Ephrussi-Taylor (1966), Gray & Ephrussi-Taylor (1967) and Louarn & Sicard (1968). Louarn & Sicard (1969) have taken advantage of this difference between the integration of the markers to show that either of the recipient strands of DNA can be transformed by the HE marker. They calculated the ratio $R = (D.N)/(A.B)$, where D = number of doubles transformed for genes A and B, N = the total colony-forming units (c.f.u.) present, and A and B the number of transformants for genes A and B respectively. By comparing the ratios obtained for pairs of markers having the composition HE–HE, LE–LE, or HE–LE, Louarn and Sicard showed that the results were consistent with either strand being transformed by an HE marker. They also discussed the effect of replication events on the value of R, particularly with respect to the location on the chromosome of such events in relation to the markers. The results reported below support these conclusions.

METHODS

Organisms and markers. *Diplococcus pneumoniae* strain C13 and strain *amiA-r1* were used as recipient organisms. Genetic markers used were *str-41* (HE), resistant to 2 mg./ml. streptomycin; *amiA-r1* (HE), resistant to 10^{-5} M-aminopterin; *ery-r2* (HE), resistant to 1 μg./ml. erythromycin; and *opt-r2* (LE), resistant to 5 μg./ml. optochin. Purified solutions of DNA possessing these markers were prepared from *D. pneumoniae* strain *ery-r2 str-41 opt-r2* and strain *amiA-r1 ery-r2 str-41 opt-r2*. Strain *amiA-r1 ery-r2 str-41 opt-r2* had been derived from strain *amiA-r1* by transformation

with the DNA of strain *ery-r2str-41opt-r2*. Evidence will be presented elsewhere showing that these markers are unlinked, as indicated by Rolfe & Ephrussi-Taylor (1961), Martin & Ephrussi-Taylor (1964) and Louarn & Sicard (1969).

Transformation procedure. The method used was essentially as described previously (Butler, 1965), except that 1·0 ml. of the competent culture was added to 0·1 ml. of suitable dilutions of the DNA solution. These dilutions were at both saturating and non-saturating concentrations. Double transformants were assayed by the addition of of a top layer of nutrient agar containing the two antibiotics after full expression had been allowed for the gene having the longest expression time. The same concentrations of antibiotics were used for double as for single transformants.

Assay of colony-forming units. An aliquot of the competent culture was taken and suitable dilutions were plated in triplicate in deep culture in nutrient agar containing horse blood. After overnight incubation the colonies were counted.

RESULTS

Transformation experiments were carried out using two recipient strains and preparations of DNA made from two different strains of pneumococcus. Assays were made for the number of colony-forming units present in the suspension, and for the single transformants to erythromycin, streptomycin, and optochin resistances, and for the three sets of doubles possible with these markers. DNA concentrations were arranged to be either saturating or just below saturating. The ratio R was calculated for the H–H (i.e. HE–HE) pair of markers and for the two H–L combinations, and the ratio R_{H-H}/R_{H-L} was calculated for each combination. The results are given in Table 1, and it can be seen that this ratio was consistent within an experiment but varied from experiment to experiment between 0·32 and 0·84, with an average figure of 0·54.

DISCUSSION

Louarn and Sicard have shown that values of the ratio R_{H-H}/R_{H-L} of below 1·0 can be explained if the H marker can affect either chain of the recipient DNA and also that there is a likelihood that a replication fork can exist between the markers at the time of integration. A high-efficiency marker is integrated into one chain only of the recipient DNA; if integration can be effected into either chain, then the probability of obtaining a double transformant by the integration of a pair of high-efficiency markers in the absence of a replication fork is 0·5, because only one of the two possible combinations of the two markers will give rise to a double transformant, whereas if only one of the chains can be affected, then both markers must always be integrated into the same chain and both possibilities can give rise to a double transformant with a probability of 1·0. When considering H–L pairs, the probability of obtaining double transformants is always 1·0 since the L marker always involves both DNA strands. Hence, the ratio of H–H/H–L is either 0·5/1·0 if either strand is transformable by an H marker, or 1·0/1·0 if only one strand is transformable. It can be shown that the presence of a replication fork increases the ratio to between 1 and 2 if only one strand is transformable, whereas it would increase the ratio to between 0·5 and 1·0 if either strand is transformable. The results given in Table 1 all lie below 1·0, with only three values below 0·5, giving an average of 0·54. Hence, the results are not in support of

Table 1. *Values of R and of R_{H-H}/R_{H-L}*

Transformations were carried out in the crosses indicated and scored for single (A and B) and double (D) transformants, and for the total colony-forming units (N). The ratio $R = D \cdot N / A \cdot B$ was calculated and compared for different pairs of markers.

Recipient	DNA	Conc. of DNA	Pairs of markers	Efficiency of markers	R	$\dfrac{R_{H-H}}{R_{H-L}}$
amiA-r1	*ery-r2 str-41 opt-r2*	Sat.	*str-41–ery-r2*	H–H	0·39	—
			str-41–opt-r2	H–L	0·68	0·57
			ery-r2–opt-r2	H–L	0·67	0·58
amiA-r1	*ery-r2 str-41 opt-r2*	Sat.	*str-41–ery-r2*	H–H	0·35	—
			str-41–opt-r2	H–L	0·61	0·57
			ery-r2–opt-r2	H–L	0·73	0·48
Cl3	*amiA-r1 ery-r2 str-41 opt-r2**	Sat.	*str-41–ery-r2*	H–H	0·12	—
			str-41–opt-r2	H–L	0·37	0·32
			ery-r2–opt-r2	H–L	0·32	0·37
Cl3	*amiA-r1 ery-r2 str-41 opt-r2†*	Sat.	*str-41–ery-r2*	H–H	0·12	—
			str-41–opt-r2	H–L	0·15	0·84
			ery-r2–opt-r2	H–L	0·17	0·71
		Unsat.	*str-41–ery-r2*	H–H	0·093	—
			str-41–opt-r2	H–L	0·19	0·49
			opt-r2–opt-r2	H–L	0·19	0·49
Cl3	*amiA-r1 ery-r2 str-41 opt-r2†*	Sat.	*str-41–ery-r2*	H–H	0·29	—
			str-41–opt-r2	H–L	0·46	0·63
			ery-r2–opt-r2	H–L	0·73	0·40
		Unsat.	*str-41–ery-r2*	H–H	0·43	—
			str-41–opt-r2	H–L	0·67	0·64
			ery-r2–opt-r2	H–L	0·89	0·48
Cl3	*amiA-r1 ery-r2 str-41 opt-r2‡*	Sat.	*str-41–amiA-r1*	H–H	0·26 ⎫	
			ery-r2–amiA-r1	H–H	0·34 ⎬	—
			str-41–ery-r2	H–H	0·18 ⎭	
				(Av.) 0·26		—
			amiA-r1–opt-r2	H–L	0·81 ⎫	
			str-41–opt-r2	H–L	0·36 ⎬	—
			ery-r2–opt-r2	H–L	0·26 ⎭	
				(Av.) 0·48		0·54

Over-all average 0·54

*, †, ‡ Different preparations of *amiA-r1 ery-r2 str-41 opt-r* DNA.

only one strand of the duplex being transformable, but indeed are consistent with the explanation that the H marker is able to affect either chain of the recipient duplex.

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The Action of Nalidixic Acid on Euglena Plastids

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When *Euglena gracilis* is grown in the presence of some antibiotics (Provasoli, Hutner & Schatz, 1948; Ebringer, 1962, 1966; Celmer & Ebringer, 1967), organisms with no chloroplasts are soon produced. These aplastidic organisms can then be isolated and maintained as permanently colourless races. The mechanism of the bleaching action is still in dispute. We have suggested an interaction with plastid DNA or some hereditary apparatus responsible for chloroplast replication (Ebringer, Mego & Jurášek, 1969). To test this hypothesis, we have examined some antibacterial agents which inhibit DNA synthesis in bacteria or in other organisms. Nalidixic acid inhibits specifically the synthesis of DNA in bacteria (Goss, Deitz & Cook, 1965 *a*, *b*; Cook *et al.* 1966) and exerts a mutagenic effect upon proliferating bacteria (Cook, Goss & Deitz, 1966). Some other agents which affect DNA synthesis in bacteria also exert a bleaching action on *Euglena gracilis* (to be published), so the question arises whether nalidixic acid is able to produce permanent colourless races of this organism.

METHODS

Euglena gracilis, strain z, was used in this study. Cultivation conditions and all methods of evaluation were as previously described (Ebringer *et al.* 1969). Chloroplasts were counted in samples of exactly 200 organisms. Identification of chloroplasts was made on the presence of a discrete structure containing chlorophyll which showed definite absorption of blue light by fluorescence microscopy. The source of u.v. light used was a mercury lamp (Vorschaltgerat HBO 50, Carl Zeiss, Jena); blue filters nos. BG 3/2, BG 3/4 and BG 12/2 were used and for the eyepiece protecting filter no. GG 9 was employed. This combination of filters gave the advantage that leucoplasts, paramylon grains, cell walls and other details could be distinguished in addition to chloroplasts.

RESULTS

Nalidixic acid caused the mean number of plastids per organism to decrease gradually until on the fourth day of cultivation the organisms almost completely lacked chloroplasts (Table 1). In the control culture without nalidixic acid the mean number of plastids per organism decreased slightly on the first day, but the number returned to the original value of about 8.

After treatment with nalidixic acid the chlorophyll content per 10^7 organisms gradually declined and on the fourth day of cultivation only traces remained (Table 2), but in the control culture the chlorophyll content increased continuously so that on the fourth day it was around 20 times higher than in a parallel drug-treated culture.

Table 1. *Chloroplast counts per organism at intervals during growth of Euglena gracilis with 500 µg./ml. of nalidixic acid and in control without the drug. Initial pH 7·8*

| No. of plastids | Frequency (no. of organisms) | | | | | | | |
| | Nalidixic acid | | | | Control | | | |
	Day 1	Day 2	Day 3	Day 4	Day 1	Day 2	Day 3	Day 4
0	—	1	85	199	—	—	—	—
1	—	4	24	—	—	—	—	—
2	—	28	43	1	—	1	—	—
3	1	67	31	—	3	—	—	—
4	36	67	10	—	19	18	14	2
5	71	30	5	—	62	45	22	6
6	38	2	—	—	46	47	29	23
7	42	1	2	—	41	56	42	44
8	11	—	—	—	14	24	41	42
9	—	—	—	—	9	9	20	27
10	1	—	—	—	3	—	12	19
11	—	—	—	—	1	—	8	12
12	—	—	—	—	1	—	7	13
13	—	—	—	—	1	—	4	4
14	—	—	—	—	—	—	—	2
15	—	—	—	—	—	—	1	5
18	—	—	—	—	—	—	—	1
Total organisms counted	200	200	200	200	200	200	200	200
Plastids per organism								
Range	3–10	0–7	0–7	0–2	3–13	2–9	4–15	4–18
Mean	5·5	3·4	1·4	0·01	5·5	6·2	7·5	8·5

Table 2. *Concentration of organisms, chlorophyll content and percentage of permanently bleached colonies after treatment with nalidixic acid at 500 µg./ml. in medium of initial pH 7·8. '% bleached colonies' refers to the % colonies which were bleached after washing the organisms and plating on solid drug-free medium. Colonies were counted on the 9th day after plating*

| Day | Number of cells × 10³ per ml. | | µg. chlorophyll per 10⁷ cells | | % bleached colonies | |
	Test	Control	Test	Control	Test	Control
1	93	86	20	26	17	0
2	194	184	19	28	86	0
3	493	501	13	56	92	0
4	1388	1175	8	158	96	0

Nalidixic acid required more than 4 days to produce 100 % bleached colonies at 500 µg./ml. and at an initial pH of 7·8. In a medium with an initial pH of 6·0, the bleaching action of nalidixic acid was weaker: higher concentrations of drug and longer periods of exposure were required to produce 100 % bleaching. Concentrations of nalidixic acid lower than 500 µg./ml. bleached only a certain number of organisms within 4 days.

To obtain 100 % bleaching with lower doses it was necessary to prolong the cultivation time.

During cultivation in the presence of nalidixic acid, the plastids became increasingly abnormal in appearance, smaller and less dense (Pl. 1, fig. 1 to 4). By the third day the number of plastids was definitely decreased and they were clumped in the centre; on the fourth day all traces of plastids had disappeared. In the control cells (Pl. 1, fig. 5) on the fourth day of cultivation fully developed chloroplasts could be counted.

DISCUSSION

Nalidixic acid, an inhibitor of bacterial DNA synthesis, apparently blocks replication of plastids without concomitant blocking of cell division. After about six divisions in the presence of the drug the organisms become aplastidic. The weaker effect of nalidixic acid at pH 6·0 is similar to what has been found in the antibacterial action of this drug. Fortunately *Euglena gracilis* grows at the same rate in media of widely different pH values. Like other antibacterial agents, nalidixic acid bleaches only dividing organisms; non-growing cultures are not bleached. This finding correlates well with the antibacterial activity of nalidixic acid, which is lethal only for proliferating cultures of bacteria (Goss, Deitz & Cook, 1965). The drug does not inhibit multiplication of *E. gracilis*. It is relatively non-toxic to euglenas and is very well tolerated by laboratory animals and man. These factors favour the use of nalidixic acid as a bleaching agent for, in order to be effective, such a substance must be less toxic for the whole organism than for the plastids.

It has been suggested that plastids originated from exogenous photosynthetic bacteria or blue-green algae which became symbionts in eukaryotic cells (Mereschkowski, 1905). This is why we decided to compare the actions of various antibacterial drugs against plastids of *Euglena gracilis*. All inhibitors of bacterial DNA synthesis (rubiflavin, sarkomycin, edeine, porfiromycin, anthramycin, novobiocin, streptonigrin and nalidixic acid) inhibit replication of plastids so that after several cell divisions bleached organisms are obtained (to be published). We emphasize that this loss of plastids is permanent and hereditary. Mutants obtained by nalidixic acid are white and permanently heterotrophic. Inhibitors of protein and RNA synthesis often produce temporary loss of chlorophyll but, with the exception of streptomycin and erythromycin, this effect is never permanent (to be published). We consider this indirect evidence that streptomycinoid antibiotics and the macrolides produce bleaching by an interaction with plastid DNA synthesis. Stern, Barner & Cohen (1966) showed that streptomycin does in fact inhibit DNA replication in bacteria and consider this process to be the primary site of action of streptomycin. Alteration of DNA appears to be a more reasonable explanation of permanent hereditary changes than interference with RNA or protein synthesis. Studies of the effect of these drugs on isolated Euglena plastids might help to clarify this question.

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EXPLANATION OF PLATE

Fig. 1–4. *Euglena gracilis* on the first, second, third and fourth day after addition of nalidixic acid.

Fig. 5. Control *Euglena gracilis* on the fourth day of cultivation on the drug-free medium.

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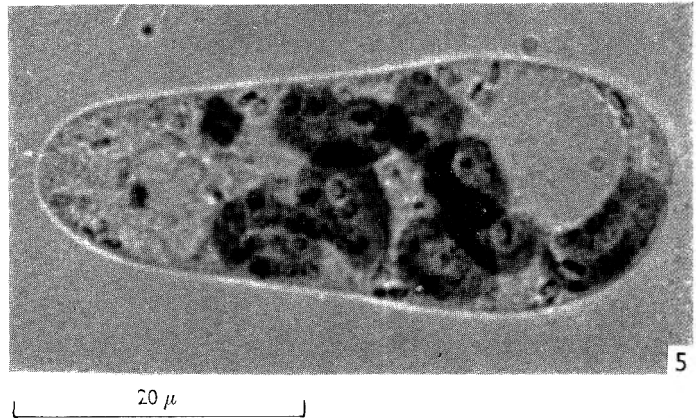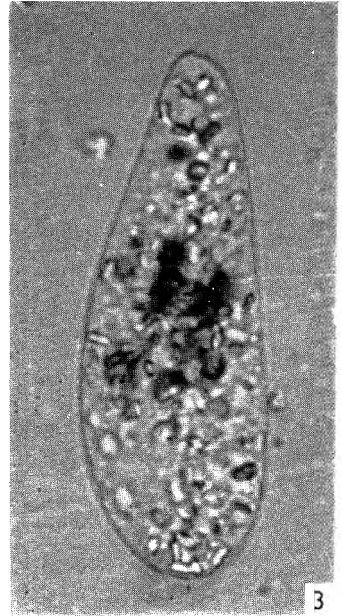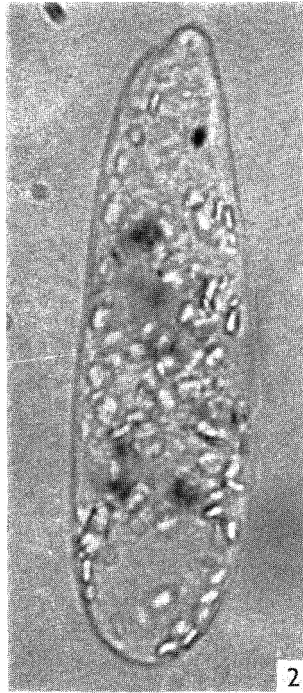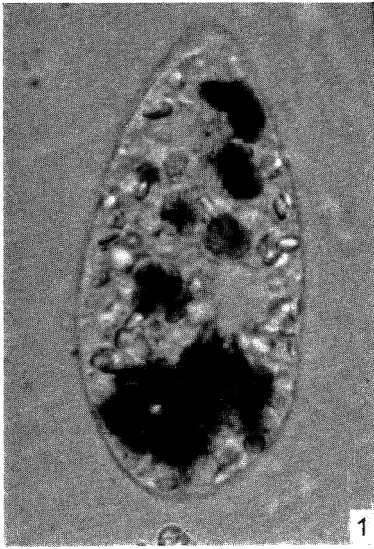
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