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Utilization of L-Glutamic and 2-Oxoglutaric Acid as Sole Sources of Carbon by *Escherichia coli*

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SUMMARY

A wild-type strain of *Escherichia coli* (W) did not utilize glutamate or oxoglutarate as sole source of carbon for growth, but mutants able to grow on each of these compounds were isolated. The abilities to utilize glutamate and oxoglutarate did not necessarily accompany each other. The evidence presented supports the view that both kinds of mutant involve changes in a permeation mechanism. The mutation to growth on glutamate was always accompanied by appearance of sensitivity to inhibition by 2-methyl-DL-glutamic acid and by partial or even complete loss of glutamic acid decarboxylase. It is proposed that the permeation mechanism for glutamate also allows entry of 2-methylglutamate, a compound which prevents glutamine formation. The loss of glutamate decarboxylase remains unexplained.

INTRODUCTION

In the course of studies on the mechanism of utilization of ammonia by cultures of *Escherichia coli* it was noted that various strains differed in their ability to utilize L-glutamate and 2-oxoglutarate. Some of the strains used, including strain W, were unable to grow on minimal media in which either of these compounds served as the carbon source (Halpern & Umbarger, 1960). Later, mutants capable of utilizing glutamate or oxoglutarate were selected. The acquisition of the ability to grow on one of the two compounds appeared to be independent of the ability to grow on the other. It was of interest that all the wild-type *E. coli* strains which were unable to grow on glutamate possessed a highly active L-glutamic acid decarboxylase, while those that could utilize glutamate for growth showed no such activity. Similarly, mutants selected for ability to grow on glutamate lost most or all of their decarboxylase activity. Whereas no direct causal relationship between growth on glutamate and the absence of glutamic acid decarboxylase could be demonstrated, evidence has

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been obtained which implicates specific permeation systems as prerequisites for the utilization of glutamate and oxoglutarate. These experiments are described in this paper. A preliminary report has been given elsewhere (Halpern & Umbarger, 1959).

METHODS

Micro-organisms. The organisms used in this work were: *Escherichia coli* strains W, K-12, B and H; *Aerobacter aerogenes* strain 1033; a strain of *Pseudomonas aeruginosa* from the stock culture collection of the Department of Bacteriology and Immunology, Harvard Medical School.

Media. The minimal medium of Davis & Mingioli (1950), with the omission of citrate, was used throughout. The various compounds used as carbon sources were prepared in separate solutions and added aseptically to the basal medium after autoclaving.

Selection of mutants. E. coli strain W was inoculated (about 5×10^7 organisms/ml.) into 100 ml. amounts of minimal medium with 0·5% (w/v) L-glutamic or 2-oxoglutaric acid as the carbon source. The cultures were incubated at 37° with shaking. Usually there was no detectable growth even after prolonged incubation (72 hr.). However, rapid increase in optical density ensued on the 5th day of incubation, the final concentrations of organisms equalling those obtained with succinate as carbon source. Such cultures were plated on eosin + methylene blue agar and single colonies were picked and tested for their ability to grow on the respective carbon source. Three glutamate-utilizing mutants were obtained on separate occasions by this method, and were designated W/Gl$_1$, W/Gl$_2$ and W/Gl$_3$. One mutant utilizing oxoglutarate was also obtained and was designated W/KG. From strain W/Gl$_1$ a secondary mutant was later selected for ability to utilize oxoglutarate in addition to glutamate; this mutant was designated *E. coli* W/Gl$_1$/KG. Similarly, two glutamate-utilizing mutants of *E. coli* H were obtained using this method, designated H/Gl$_1$ and H/Gl$_2$.

Growth experiments. For the determination of growth rates inocula from log-phase cultures were transferred to 20 ml. of appropriate medium in 250 ml. Erlenmeyer flasks with cuvette side arms. Such cultures were incubated at 37° in a New Brunswick gyratory water-bath shaker. The changes in optical density were followed in a Klett–Summerson photoelectric colorimeter with a No. 42 filter.

Oxygen uptake measurements. Conventional manometric techniques were employed for the determination of oxygen uptake by cell suspensions.

Reduction of triphenyltetrazolium chloride. The capacity of bacterial suspensions, and bacteria disintegrated by sonic oscillation (15 ml. of a cell suspension of OD $\times$ 10 = 60 at 550 mμ in M/15 phosphate buffer, pH 7·5 was treated for 8 min. in a Raytheon 10 KC magnetostrictive sonic oscillator), to reduce triphenyltetrazolium chloride in the presence of glutamate was determined under both aerobic and anaerobic conditions. For the anaerobic experiments Thunberg tubes were used. The reaction was stopped by the addition of 0·1 ml. 4 N-HCl. The formazan formed was extracted with 2 ml. isobutanol and the colour intensity measured at 485 mμ in a Coleman Junior spectrophotometer.

Preparation of cell-free extracts. Cell-free extracts used in the determinations of the enzymic activities studied were prepared by disintegration of bacterial suspen-

sions (OD $\times$ 10 = 60 at 550 mμ) in a Raytheon 10 KC magnetostrictive sonic oscillator in the cold for 8 min. The cell debris was removed by centrifugation in the cold (4–6°) for 15 min. at 28,000 *g*. The protein content of the supernatant liquid was determined by Mehl's biuret method (Mehl, 1945).

Assay of glutamic acid dehydrogenase. Glutamic acid dehydrogenase activity of cell-free extracts was determined by reduction of triphosphopyridine nucleotide (TPN) in the presence of L-glutamate, and its rate was followed at 340 mμ in a Beckman model DU spectrophotometer. The reaction mixture contained: bacterial protein, 0·3 mg., different concentrations of L-glutamate (pH 8·7), TPN, 0·3 μmole, phosphate buffer (pH 8·7) 120 μmole, in a total volume of 3 ml. Increase in absorption at 340 mμ was followed for 5 min. at room temperature. Enzyme activity was calculated from the reaction rate for 1 min. between the initial 30 and 90 sec. after addition of glutamate.

Assay of glutamine synthetase. Glutamine synthetase activity of cell-free extracts was determined in a system similar to that described by Fry (1955). The amounts of glutamine formed and glutamic acid utilized were estimated after paper chromatography, according to Giri, Radhakrishan & Vaidyanathan (1952).

Assay of glutamic acid decarboxylase. Glutamic acid decarboxylase activity of extracts of cells grown in the presence of glutamate was determined manometrically or by paper chromatography as previously described (Halpern & Grossowicz, 1956).

Determination of ^{14}C-L-glutamate uptake by logarithmic phase cultures of Escherichia coli *strains W and W/Gl$_1$.* *E. coli* W and W/Gl$_1$ were grown overnight (inoculum about 10^7 organisms/ml.) in 20 ml. minimal medium containing a limiting amount of glucose (0·05%, w/v). Logarithmic growth was immediately resumed upon addition of 0·3% (w/v) glucose. When the cultures reached a turbidity of 0·36 (at 550 mμ), 1·5 ml. samples were withdrawn and incubated with various concentrations of ^{14}C-uniformly labelled L-glutamate (The Radiochemical Centre, Amersham, Bucks), in the presence and in the absence of 2-methyl-DL-glutamic acid, $2{\cdot}79 \times 10^{-1}$M, for 4 min. at 37° in a total volume of 2 ml. The reaction mixture was filtered in the cold through a bacterial membrane filter, 25 mm. in diameter (Membranfilter, Göttingen, Gruppe:3) and rinsed with about 80 ml. ice-cold water. The filters were air-dried and their radioactivity measured in a Geiger–Muller counter (Tracerlab, Inc.).

RESULTS

Growth studies

Following the observation that growth of *Escherichia coli* strain W on glutamate or on 2-oxoglutarate was possible only following selection of variants from a large population, experiments were performed to compare the growth rates of the variants with that of the wild strain on each of several carbon sources. Table 1 gives the results of such experiments. It was noted that with the acquisition of the ability to utilize glutamate or 2-oxoglutarate, there was little or no change in the growth rate on glucose or on another tricarboxylic acid cycle intermediate, succinate. In addition, it was apparent that the ability to grow on either glutamate or 2-oxoglutarate had no effect on the ability to grow on the other. Furthermore, for those strains which were able to grow on them, succinate, glutamate or 2-oxoglutarate were utilized for growth about equally effectively. Although the wild-type parent utilized glutamate as the source of nitrogen, it did so only poorly (column 6, Table 1).

However, the strain which utilized glutamate as a source of carbon (strain W/Gl$_1$) also used it better as a source of nitrogen, although it was able to utilize ammonia more effectively as a nitrogen source (column 2, Table 1).

Table 1. *Growth of* Escherichia coli *strain W and its mutants on different compounds serving as a sole source of carbon*

The experiment was performed in minimal medium, to which 0·5 % (w/v) of the respective carbon source was added. The inocula were prepared from overnight cultures on succinate. The initial concentrations were about 2×10^8 organisms/ml. For other conditions, see Methods.

	Carbon source				
Strain	Glucose	Succinate	L-glutamate	2-oxo-glutarate	Glucose + glutamate ($(NH_4)_2SO_4$ omitted)
	Generation time (hr.)*				
E. coli W	1·00	1·50	> 6	> 16	3·00
E. coli W/KG	1·00	1·50	> 7	1·30	—
E. coli W/Gl$_1$	1·25	1·65	1·55	> 16	1·70
E. coli W/Gl$_1$/KG	—	—	1·55	1·40	—

* Time required for doubling of the optical density of the culture in the logarithmic phase of growth.

Table 2. *Rates of oxygen uptake by suspensions of* Escherichia coli *W and its mutants on various substrates*

The bacteria were grown overnight on succinate as the carbon source, washed and resuspended in 0·05 M phosphate buffer (pH 8·7). Each Warburg vessel contained about 15 mg. equiv. dry weight organism, $MgCl_2$, 10 μmole, and phosphate buffer (pH 8·7) 50 μmole in the main compartment; 10 μmole of substrate in the side arm, and KOH 40 % (w/v) 0·2 ml., in the centre well; total volume 1·5 ml.; incubation at 30°.

	Substrate		
Organisms	Succinate Q_{O_2}*	L-Glutamate Q_{O_2}*	2-Oxoglutarate Q_{O_2}*
E. coli W	540	49 (93)†	90
E. coli W/KG	540	8	264
E. coli W/Gl$_1$	522	402 (18)†	100
E. coli W/Gl$_1$/KG	468	336	234

* The values obtained for endogenous respiration were subtracted from those obtained in the presence of substrate.

† Numbers in parentheses represent the amount of glutamic acid recovered at the end of the experiment, as % of initial value.

Rates of oxygen uptake

Experiments were performed to determine the oxygen uptake of washed suspensions of the different strains. There was a very good correlation between respiratory activity towards the different carbon sources and the ability of a given strain to utilize these substances for growth (Table 2). When glutamate was the substrate, over 80 % of it disappeared in the presence of strain W/Gl$_1$, whereas in the presence of the wild-type organism practically all of the added glutamate remained at the end of the experiment.

Glutamic acid dehydrogenase activity

Since glutamic acid dehydrogenase might be involved in the early steps of glutamic and 2-oxoglutaric acid metabolism, the activity of this enzyme was investigated in extracts of *Escherichia coli* W and of the glutamate and oxoglutarate utilizing mutants. No significant differences between the activities of the various strains were detected.

Reduction of triphenyltetrazolium chloride

Different results were obtained when suspensions of *Escherichia coli* strains W and W/Gl$_1$ were tested for their ability to catalyse the transfer of electrons from glutamate to triphenyltetrazolium chloride. It can be seen that with intact organisms (Fig. 1) the apparent affinity of the wild strain for glutamate was much

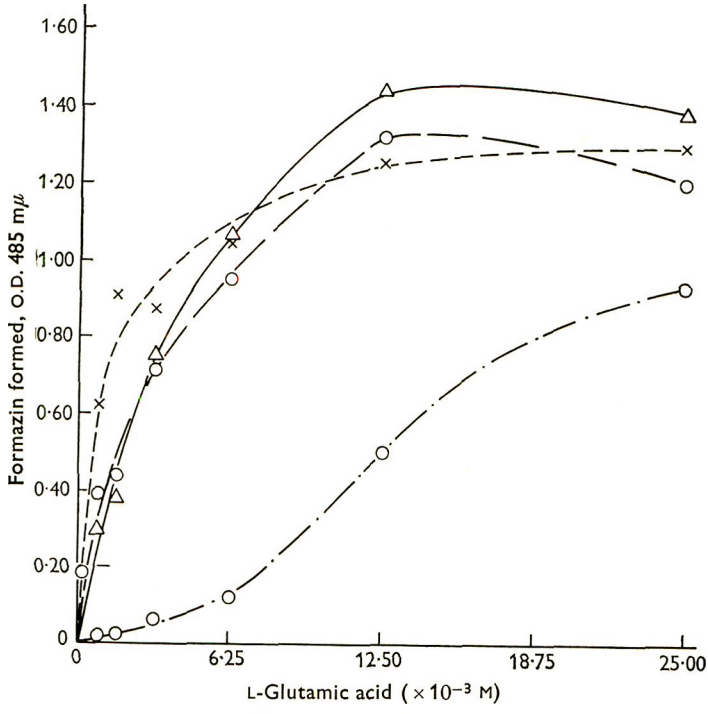

Fig. 1. Reduction of triphenyltetrazolium chloride by whole cell suspensions and by sonically disintegrated cells of *E. coli* W and W/Gl$_1$ with glutamate as the electron donor. Overnight cultures of *E. coli* W and W/Gl$_1$ on minimal medium, containing 1% (w/v) Na-succinate and 0·5% (w/v) L-glutamic acid, were washed and resuspended in M/15 phosphate buffer, pH 7·5. Both suspensions were adjusted to the same optical density (OD × 10 = 60 at 550 mμ). Part of each suspension (15 ml.) was disrupted in a Raytheon 10 KC magnetostrictive sonic oscillator for 8 min., and the remaining part was diluted with an equal volume of M/15 phosphate buffer, pH 7·5. The reaction mixtures contained: whole cell suspension (0·945 mg. dry weight of bacteria) or sonically disintegrated cells (4·413 mg. dry weight of bacteria), L-glutamic acid as indicated, triphenyltetrazolium chloride, 0·06% (w/v), phosphate buffer pH 7·5, 467 μmoles, in a total volume of 1·6 ml.; incubated aerobically at 37° for 10 min. The reaction was stopped by addition of 0·1 ml. 4 N-HCl; the formazan formed was extracted with isobutyl alcohol, 2 ml., and read at 485 mμ. –·– ○ –·–, W whole cells; — ○ —, W broken cells; —×—, Gl$_1$ whole cells; —△—, Gl$_1$ broken cells.

lower than that of the Gl_1 mutant. In contrast, when sonically disrupted suspensions were used, the differences in the reaction rates of the two strains at either low or higher concentrations of glutamate were slight and perhaps not significant.

Effect of 2-methyl-DL-glutamic acid

Further evidence on the nature of the mutation to glutamate utilization was obtained by using a glutamate analogue, 2-methyl-DL-glutamate. This compound has been shown (Ayengar & Roberts, 1952) to inhibit the growth of certain lactic acid bacteria, presumably by inhibiting the conversion of glutamate to glutamine. As shown in Table 3, when 2-methylglutamate was added to a glucose minimal medium, those *Escherichia coli* strains able to use glutamate as sole carbon source (W/Gl_1, W/Gl_2, B, H/Gl_1) were inhibited by the analogue. Strains unable to grow on glutamate (W, K-12, H) were resistant.

The correlation suggested by the results shown in Table 3 was further supported by isolating from the glutamate-utilizing mutant strain W/Gl_1 a variant, strain

Table 3. *Inhibition of growth of different* Escherichia coli *strains by 2-methyl-DL-glutamic acid*

For conditions of growth see Methods.

		In the presence of 2-methyl-DL-glutamic acid			
		0·15 % (w/v)		0·50 % (w/v)	
Strain	No additions (control) Klett reading	Klett reading	Inhibition (%)	Klett reading	Inhibition (%)
E. coli W	375	313	16·5	290	22·7
E. coli W/Gl_1	443	Not tested		26	94·1
E. coli W/Gl_2	242	31	87·2	5	97·9
E. coli K-12	280	345	0	Not tested	
E. coli B	325	23	92·9	10	96·9
E. coli H	415	275	33·7	202	51·3
E. coli H/Gl_1	510	12	97·6	15	97·1

Table 4. *Uptake of* ^{14}C-L-*glutamate by* Escherichia coli *strains W and* W/Gl_1 *in the presence and in the absence of 2-methyl-DL-glutamic acid*

The reaction mixture contained: logarithmic phase organisms equiv. 0·567 mg. dry wt. bacteria, in minimal medium containing 0·3 % (w/v) glucose, ^{14}C-L-glutamate (uniformly labelled, 107 c.p.m./μmole) and 2-methyl-DL-glutamic acid as indicated, in a total volume of 2 ml.; incubation in a water-bath with shaking (about 200 oscillations/min.) at 37° for 4 min. For other conditions see Methods.

	Uptake of ^{14}C-L-glutamate (c.p.m.)					
	E. coli W			*E. coli* W/Gl_1		
	2-Methyl-DL-glutamate added			2-Methyl-DL-glutamate added		
^{14}C-L-glutamate added (M)	None	$2·79 \times 10^{-1}$M	% Inhibition	None	$2·79 \times 10^{-1}$M	% Inhibition
$9·3 \times 10^{-6}$	287	230	19·9	455	225	50·1
$2·8 \times 10^{-5}$	631	523	17·1	1024	491	52·0
$8·4 \times 10^{-5}$	1070	992	7·3	1703	1251	26·5

W/Gl_1/MG, selected for ability to grow in glucose minimal medium + 2-methylglutamate. This variant was found to have lost its ability to grow on glutamate. Similarly, another derivative, strain W/Gl_1-8, was selected by means of the penicillin selection method (Davis, 1948) for restoration of the original inability to grow on glutamate; this strain was found to have lost its sensitivity to 2-methylglutamate. In contrast, when the glutamine-synthesizing systems were tested in extracts it was observed that the activity in extracts of both *Escherichia coli* strains W and W/Gl_1 was inhibited by the analogue (78–88 % inhibition by 0·011 M-2-methylglutamate).

Further evidence which stresses the relationship between the ability of the organism to utilize L-glutamate and the inhibitory effect of 2-methyl-DL-glutamate on its growth was obtained by uptake experiments described in Table 4. As shown in Table 4, uptake of ^{14}C-labelled L-glutamic acid by *Escherichia coli* W/Gl_1 was considerably higher than that by the W strain and was inhibited to the extent of 50 % in the presence of the 2-methyl analogue. On the other hand, addition of 2-methyl-DL-glutamic acid caused an inhibition of L-glutamate uptake by strain W of less than 20 %. The residual (uninhibited) uptake was virtually the same in both strains.

Table 5. *Comparison of* L-*glutamic acid decarboxylase activities of various mutants of* Escherichia coli

The reaction mixtures in Warburg flasks contained: bacterial extract equiv. 0·75 mg. protein, L-glutamic acid, 15 μmole; acetate buffer (pH 4·5) 90 μmole; pyridoxal phosphate, 150 μg; in a total volume of 1·5 ml. Incubation was at 30°. The extract in each case was prepared from organisms harvested in the logarithmic phase from a medium containing initially 1·0 % (w/v) glucose and 0·5 % (w/v) L-glutamate.

Strain	Glutamate as sole carbon source	Q_{O_2}	Strain	Glutamate as sole carbon source	Q_{O_2}
E. coli W	−	592	*E. coli* H/Gl_2	+	0
E. coli W/Gl_1	+	20	*E. coli* K-12	−	200
E. coli W/Gl_2	+	224	*E. coli* B	+	0
E. coli W/Gl_3	+	20	*A. aerogenes* 1033	+	0
E. coli H	−	1467	*Ps. aeruginosa*	+	0
E. coli H/Gl_1	+	452			

Glutamate decarboxylase activity in glutamate-utilizing strains

The only enzymic difference noted between extracts of organisms unable to grow on glutamate and extracts of those able to utilize glutamate was in the decarboxylation of glutamate. Thus *Escherichia coli* strains W, K-12 and H exhibited strong glutamic acid decarboxylase activity, whereas variants of these strains which can grow on glutamate, showed either a partial or a complete loss of the decarboxylase. It was also found that the three wild-type organisms which were observed to utilize glutamate as a carbon source, *E. coli* strain B, *Aerobacter aerogenes* strain 1033 and the strain of *Pseudomonas aeruginosa*, exhibited no glutamic acid decarboxylase activity (Table 5). At present, the mechanism underlying this apparent correlation is not clear. The relationship, however, does not appear to be a direct one. For example, selection for loss of the ability to utilize glutamate or for resistance to 2-methylglutamate (such as strains W/Gl_1-8 and W/Gl_1/MG described above) did not select organisms which had gained the ability to decarboxylate glutamate.

DISCUSSION

The difference between *Escherichia coli* W and its mutants, with respect to their ability to utilize glutamate and oxoglutarate for growth, might have been due either to changes in the respective metabolic pathways or to changes in the ability of the organisms to take up the substrates. Although glutamate oxidation by strains selected for growth on glutamate was much higher than that in strains unable to grow on glutamate, the glutamic dehydrogenase activities of the extracts prepared from the two kinds of strains were not significantly different. This difference must be accounted for, therefore, by a difference in the ease of entry of glutamate into the cells of the two strains. This hypothesis is directly supported by the observations on the difference in tetrazolium reduction as between whole organisms and extracts. Thus, at low concentrations of glutamate there was a distinct difference in the rates of tetrazolium reduction by intact organisms of the two strains. This difference could be essentially abolished by disrupting the organisms or by using higher concentrations of glutamate. The change in the glutamate-utilizing mutant could probably best be explained by an increased capacity to transport glutamate into the cell.

A difference between the penetration of glutamate into organisms of strain W and into organisms of strain W/Gl$_1$ could also explain the difference in the sensitivity of the two kinds of organism to 2-methylglutamate, a compound which inhibits glutamine formation by extracts of both strains. It would appear that the same mechanism which is responsible for the transport of glutamate into the cell also facilitates the penetration of the analogue.

Although it is not clear how the loss of decarboxylase activity is related to the utilization of glutamate, the two phenomena nevertheless seem to be connected. In every mutant examined the appearance of the ability to grow on glutamate was accompanied by a total or at least partial loss of glutamic decarboxylase activity. Even the relatively high decarboxylase activity of strain H/Gl$_1$ (Q_{CO_2} = 452) comprises only 30% of the activity of the parent H strain. The same argument holds also for the decarboxylase of strain W/Gl$_2$, which, although being as active as that of *Escherichia coli* K-12 unable to grow on glutamate, is nevertheless only one-third as active as that of its parent W strain.

Finally, it may be pointed out that the data in Table 1 provide evidence that the mutation to growth on 2-oxoglutarate was also due to a change in a penetration mechanism. The present evidence for a similar system in *Escherichia coli* is less conclusive than in the case of glutamate. However, if it may be assumed that glutamate utilization proceeds via 2-oxoglutarate, it is clear that an organism which metabolizes glutamate at a rate sufficient for growth must also metabolize endogenous oxoglutarate at a rate sufficient for growth. Since such strains do not grow on exogenous oxoglutarate, their failure to do so must be attributed to the inability of this compound to enter the cell. It is of interest that Kogut & Podoski (1953) presented evidence for the existence of an oxoglutarate permeation system in a Pseudomonas strain.

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The Cultural and Physiological Characters of the Pediococci

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SUMMARY

The cultural and physiological characters of 89 strains of pediococci have been studied. Proposals are made for extension of the genus and its subdivision into two and possibly three groups. The reactions of eleven strains of *Aerococcus viridans* were also investigated.

INTRODUCTION

Organisms described as pediococci have been studied for some considerable time, mainly in relation to problems in the brewing industry (Balcke, 1884; Mees, 1934; Shimwell & Kirkpatrick, 1939). More recently these organisms have been found in appreciable numbers in fermenting vegetable material (Pederson, 1929; Pederson & Albury, 1950; Pederson, Albury & Breed, 1954), in 'caecal faeces' of turkeys (Harrison & Hansen, 1950), in the rumen of cows (Bauman & Foster, 1956), in summer sausage (Deibel & Niven, 1957), and in cheese (Naylor & Sharpe, 1958; Dacre, 1958*a*, *b*).

Systematic studies of the physiological characters (Pederson, 1949; Felton & Niven, 1953; Jensen & Seeley, 1954; Pederson *et al.* 1954) and of the nutritional requirements (Jensen & Seeley, 1954) have led to recognition of the pediococci as belonging to a separate genus and to its classification within the family Lactobacillaceae in the tribe Streptococceae. This view has been incorporated in the seventh edition of *Bergey's Manual* (1957), where *Pediococcus* is described as a genus of Gram-positive cocci occurring singly and in tetrads, pairs and short chains, microaerophilic, generally catalase-negative, homofermentative, producing optically inactive lactic acid from carbohydrates, producing acidity and cloudiness in beer, and found as saprophytes in fermenting vegetable juices. Within the genus thus described two species only are recognized, *P. cerevisiae* Balcke, 1884, and *P. acidilactici* Lindner, 1887, distinguished by their optimum growth temperatures and ability to grow in beer.

In the course of a study of bacterial changes occurring during the ensilage process (Hoffman, Wolf & Barker, 1957) a large number of Gram-positive cocci was isolated which resembled the pediococci in certain characters. However, some of the silage isolates differed sufficiently from the descriptions of pediococci to suggest that the genus might be wider than previously thought. We have therefore made a survey of the cultural, physiological and serological characters of strains from a wide range of sources. Such an investigation seems to be particularly appropriate at the present time since the possibility of utilizing pediococci in fermentation processes has recently been proposed (Pederson & Albury, 1950; Dacre, 1958*a*; Dr C. F. Niven

Jun., personal communication). A possible relationship between the pediococci and members of the genus *Aerococcus* (Williams, Hirch & Cowan, 1953) was suggested by Jensen & Seeley (1954) and by Dr Ellen I. Garvie (personal communication). We have therefore included in the survey some *Aerococcus* strains.

METHODS

Sources of cultures. Eighty-nine isolates (including 39 new isolates from silage) were collected; 31 of these were representatives of named species. Most of the experimental work was carried out with all isolates; where this was not practicable isolates considered to be representative were selected for investigation.

In addition to the pediococcus cultures, 11 isolates of *Aerococcus viridans* were studied. A list of the isolates is given in Table 1, together with the references for the named species.

Maintenance of stock cultures and methods of cultivation. For maintenance of stock cultures, preparation of inocula and in all experimental work, 'Oxoid' tomato juice (TJ) broth or tomato juice (TJ) agar, adjusted to pH 6·6, were used unless otherwise stated. The following were exceptions to this rule: for strain Tc. 1 sodium chloride (5 %, w/v) was added to the medium; and for the aerococci glucose Lemco broth (Shattock & Hirsch, 1947) or glucose yeast extract (GY) agar (containing, as %, w/v; peptone, 1·0; Yeastrel, 0·3; glucose, 1·0; NaCl, 0·25; agar, 1·0; at pH 7·4) was used.

Cultures were incubated aerobically except where otherwise stated. The normal incubation temperature was 30° except for isolates M-1, 8519 and 8520, for which it was 22°.

Stock cultures were maintained as stab cultures and stored at 4°. New transfers were made at 3-monthly intervals.

For use as inocula, vigorously growing cultures were obtained by making at least three successive subcultures. The incubation period was 24 hr. except for strains M-1, 8519, 8520 and Tc. 1 which required 72 hr. A 'standard' inoculum consisted of one loopful (about 4 mm. diameter) of such a vigorously growing culture/5 ml. test medium, and was used except where otherwise stated.

Morphology and staining reactions. The shape, arrangement and size of individual organisms were determined in Gram-stained smears prepared from 24 hr. cultures fixed by heat in the usual way. The Gram reaction (Jensen's modification; Mackie & McCartney, 1953) of 24 and 48 hr. liquid cultures was determined. Hanging drop preparations of 24 hr. broth cultures were used to determine motility. Smears from 72 hr. agar cultures were examined for the presence of spores according to Fleming's method (Mackie & McCartney, 1953) and Conklin's modification of Wirtz's method (Conklin, 1934). Muir's technique (Tanner, 1948) with 24 hr. agar cultures was used to search for capsules.

Cultural characters. The kind of surface colony was noted after incubation for 72 hr. and the form of growth in agar stab cultures observed after 48 or 96 hr. where necessary. Growth characters in liquid cultures were observed after incubation for 48 or 72 hr. Ability to form mucoid colonies on media containing sucrose was tested on the appropriate agar medium to which Seitz-filtered sucrose had been added to a final concentration of 5 % (w/v).

Conditions affecting growth

Except where otherwise stated, all results of experiments to determine the conditions which affected growth were recorded after 24, 48 hr. and 7 days of incubation.

Table 1. *Code number, species name, habitat and sources of cultures*

Code numbers	Species name	Habitat	Source
F-166, E-66, N-82, K-64, K-106, B-168	*P. cerevisiae* (Pederson, 1949)	Fermenting vegetable	(1)
A-1, C-1	*P. cerevisiae* (Pederson, 1949)	Spaghetti sauce	(1)
2-170, 3-124, 3-129, 4-60, 4-89, 5-51, 5-61, 6-107, 6-159, 6-163, D-32, D-95, D-118, N-91	*P. cerevisiae* (Pederson, 1949)	Fermenting vegetable	(2)
FP-1, FP-6	*P. cerevisiae* (Pederson, 1949)	Summer sausage	(3)
P-60	*P. cerevisiae* (Garvie, 1959)	Unknown	NCDO
P.c.	*P. pentosaceus* (Mees, 1934)	Unknown	(4)
M-1	*P. damnosus* (Claussen, 1903)	Beer	(4)
8519, 8520	*P. damnosus* var. *salicinaceus* (Mees, 1934)	Beer	NCIB
Tc.1	*P. halophilus* (Mees, 1934)	Unknown	(4)
PUE	*P. urinae equi* (Mees, 1934)	Horse urine	(4)
EJ-1	—	Fermenting cabbage	(2)
M-31	—	Milk	—
A-140, A-181, B-137, B-190	—	Cheese	—
SS-50, SS-61	—	Air of dairy	—
SS-69, SS-101, SS-128	—	Air of cowshed	(5)
HY-22s	—	Hay	—
BP-1, BP-2	—	Saliva	—
559 (strain A2, Dacre, 1958*a*)	—	Cheese	NCDO
C-1, C-2, C-6, C-14	—	Silage	(6)
P-45, P-128, S-18, S-180, S-182, S-188, S-190, S-191, S-290, S-333, S-334, S-336, S-338, S-339, S-340, S-342, S-344, S-447, S-524, S-525, S-526, S-527, S-532, S-533, L-16, L-20, L-22, L-24, L-92, L-95, L-148, L-171, L-223, L-345, L-347, L-351, L-352, L-354	—	Silage	—
7592, 7595, 7597, 7598, 7599, 7601, 7602 7764, 7765, 7766, 7767	*Aerococcus viridans* (Williams, Hirch & Cowan, 1953)	Air of occupied rooms Milking machines	NCTC

(1) Professor C. S. Pederson, N.Y. State Agriculture Experiment Station, Geneva, N.Y., U.S.A.
(2) Professor H. W. Seeley, Cornell University, Ithaca, New York, U.S.A.
(3) Dr C. F. Niven Jun., University of Chicago, Illinois, U.S.A.
(4) Technische Hoogeschool, Delft, Holland.
(5) Dr M. E. Sharpe, National Institute for Research in Dairying, Shinfield, near Reading.
(6) Dr T. Gibson, The Edinburgh and East of Scotland College of Agriculture, Edinburgh.
NCIB = National Collection of Industrial Bacteria, Torry Research Station, Aberdeen.
NCDO = National Collection of Dairy Organisms, Shinfield, near Reading.
NCTC = National Collection of Type Cultures, London.

Oxygen requirement. Duplicate broth cultures were incubated aerobically, and anaerobically in an atmosphere of 95 % (v/v) hydrogen + 5 % (v/v) carbon dioxide. Visual estimation of growth was made after incubation for 24 or 72 hr. for slow growing strains.

Growth temperatures. To find the optimum growth temperature the amount of

growth after incubation for 24 hr. at 22°, 30° and 37° was estimated visually. With slow growing strains the results were read after 72 hr. of incubation. To indicate the range of growth temperatures, cultures were incubated at 10°, 40° and 45° in water baths controlled to within ±1°.

Growth at pH 9·0 *and* pH 4·2. In these experiments the technique was based on that described by Shattock & Hirsch (1947) for testing growth of streptococci at pH 9·6. The following modifications were made: tomato juice (TJ) broth was substituted for glucose Lemco broth; to obtain the medium at pH 9·0 suitable quantities of the 0·1 M-glycine buffer recommended by Shattock & Hirsch (1947) were added; for the medium at pH 4·2, sodium acetate + acetic acid buffer (Clark, 1928) at 0·04 M was selected, since some inhibitory effects were noted at higher concentrations.

Tolerance to sodium chloride and Teepol. Ability to grow in 4 and 6·5 % (w/v) sodium chloride and in 0·01, 0·05 or 0·1 % Teepol was tested in TJ broth cultures.

Growth in wort, hopped wort and beer. The amount of growth was observed visually in wort, hopped wort and beer. The wort and hopped wort (about 6 % hops) were obtained through the courtesy of Mr C. S. Everitt (Watney Mann Breweries, London) and the beer was commercially available bottled Carlsberg Lager. These media were sterilized by Seitz filtration.

Biochemical tests

Media used for biochemical tests were based on those commonly used for testing lactobacilli since optimal media for pediococci have not yet been devised. Where consistent with satisfactory results the lactobacillus media were simplified. Known positive and negative control cultures were included in each test series.

Catalase activity. Felton, Evans & Niven (1953) found that a medium of low carbohydrate content (YTG) gave a greater number of positive reactions than a medium of high carbohydrate content (APT). Gutekunst, Delwiche & Seeley (1957) recommended that cultures to be used for catalase tests should be neutralized after incubation. In the present work, preliminary tests were carried out with 12 isolates of pediococci to compare nutrient broth (containing (%, w/v): Yeastrel, 0·3; peptone, 1·0; NaCl, 0·5; at pH 7·0) with TJ broth and GY broth as media for catalase tests. No qualitative differences were found but the reactions in nutrient broth were sometimes stronger. In view of this and of the recommendations of the above workers, nutrient broth was retained as the experimental medium. Twice the 'standard inoculum' (above) was used for 5 ml. medium and incubation was carried out for 24 hr., or 72 hr. when necessary. Two ml. of freshly prepared 3 % (10 vol.) hydrogen peroxide were added and the cultures examined up to 30 min. for visible gas bubbles.

Haemolysis. Horse blood (5 %, v/v) agar streak plates were prepared and incubated both aerobically and anaerobically. Pour plates were also made and incubated similarly. Results were read after incubation for 48 hr. and again after overnight storage at 4°.

Liquefaction of gelatin. Stab cultures were incubated at optimum temperature and examined for liquefaction after chilling at 7, 14 and 28 days. The nutrient gelatin medium had the same formula as the nutrient broth, with the addition of 14 % (w/v) gelatin.

Reduction of nitrate. Incubation was carried out for 7 days in the medium of Davis (1955) from which salt solutions 'A' and 'B' had been omitted. Cultures were then tested for the presence of nitrite and of nitrogen gas as described in the *Manual for Pure Culture Study* (1954). The medium was tested for the presence of nitrite before incubation and for residual nitrate after incubation.

Production of ammonia from arginine. The method described by Niven, Smiley & Sherman (1942) was used.

Carbohydrate reactions. Yeast-extract peptone broth (containing, % w/v: peptone, 1·0; yeast extract, 0·5; NaCl, 0·5; $MgSO_4$, 0·05; $MnSO_4$, 0·05; at pH 7·0) was used as a basal medium for fermentation tests, and Seitz-filtered carbohydrate added to give 1 % (w/v) final concentration. Acid and gas production were determined after 7 days of incubation (indicator, 0·04 % (w/v) bromcresol purple, added after incubation), since preliminary results had shown that many isolates, especially fresh ones, were slow in producing acid. Acid once produced was not masked by subsequent production of alkaline substances. The carbohydrates tested were: arabinose, xylose, glucose, fructose, maltose, lactose, sucrose, trehalose, raffinose, inulin, dextrin, glycerol, mannitol, sorbitol, salicin.

Hydrolysis of aesculin. The method of Davis (1955) was used, except that Tween 80 (which according to Jensen & Seeley, 1954 is not required by pediococci) and salt solutions 'A' and 'B' were omitted from the medium, and sodium chloride (0·2 %, w/v), manganese sulphate (0·05 %, w/v) and magnesium sulphate (0·05 %, w/v) were added. The cultures were examined daily for 7 days.

Production of acetylmethylcarbinol from glucose and from lactose. Tests were carried out in the medium of Swartling (1951), modified in one series of experiments by the substitution of glucose for lactose. Cultures were incubated for 6 days and tested for acetylmethylcarbinol by Barritt's (1936) modification of the Voges–Proskauer test.

Final hydrogen ion concentration. Glucose (1 %, w/v) yeast-extract liquid cultures were incubated for 18 days and the final pH values measured electrometrically. Some isolates grew poorly in this medium but the use of tomato juice broth was considered inadvisable because of its natural content of reducing sugar which might have resulted in the production of acids from compounds other than glucose.

Production of carbon dioxide from glucose. The method of Gibson & Abd-el-Malek (1945) was used; cultures were examined daily for gas production during a 2-week incubation period.

Reaction in litmus milk. Litmus milk cultures were examined for reduction of indicator, change in pH value or coagulation during 28 days of incubation.

Type of lactic acid produced. The method of Pederson, Peterson & Fred (1926) was followed except that a continuous ether extraction apparatus was used, extracting the sample for 48 hr. The zinc content of the isolated zinc lactate was determined by the titrimetric method of Kolthoff & Sandell (1950) and the optical rotation determined polarimetrically, using the anhydrous salt in 1 % (w/v) aqueous solution. Six pediococcus strains were examined.

Utilization of ammonium salts as sole source of nitrogen. The medium and method described by Hucker (1924) were used. Incubation continued for 14 days.

Folinic acid requirement. The method used was a modification of that outlined in the *Difco Manual* (1953). Tests were carried out in triplicate in 4 ml. amounts in

12 × 80 mm. EEL colorimeter tubes. Growth after 18 hr. of incubation was measured turbidimetrically with an EEL colorimeter. The folinic acid used in these experiments was supplied as 'leucovorin' by Lederle Laboratory Division Ltd. and was used in concentrations of 0, 0·15, 0·3 and 0·6 mμg./ml. Twenty isolates were examined for this requirement.

RESULTS

A comparison of named pediococcus cultures and unnamed strains showed certain features to be shared by all; these are regarded as characteristic of the genus. A number of additional features was possessed by some but not all isolates examined. On the basis of these additional features, two distinct physiological groups could be differentiated and there was some indication of a less well-defined third group. The results are presented in two sections: (i) characters common to all 'pediococci'; (ii) characters used to differentiate the groups; the results are listed in Table 2. The reactions given by the aerococci are included for comparison. The criteria on which the subdivisions have been based are summarized in Table 3.

Characters common to all pediococci

Morphology and staining reaction. The organisms were spherical, occasionally ovoid, strongly Gram-positive, ranging in diameter from 0·36 to 1·43μ. They were arranged in clusters, tetrads, pairs or singly, were non-motile, non-sporeforming and not encapsulated.

Cultural characters. Surface colonies were greyish-white, smooth, circular, low convex with entire margins. Growth in stab culture was beaded throughout the entire length of the stab, with a small amount of surface growth. Mucoid colonies were not formed on agar media containing sucrose.

Growth conditions. In the media described, growth was similar under aerobic and anaerobic conditions. The optimum temperature was 30° for all but three isolates; for these it was 22°. Growth was initiated at 10°. No strains were able to grow at pH 9·0. Wort provided a suitable substrate but the addition of hops exerted some inhibitory effect; no growth took place in the particular beer used.

Biochemical tests. A small zone of β haemolysis was produced on blood agar by a few isolates only. With the majority of isolates a zone of 'bleaching' similar to that described by Davis & Rogers (1939) for lactobacilli was noted. The organisms did not liquefy gelatin, did not reduce nitrate to nitrite or nitrogen gas, nor did they produce detectable amounts of carbon dioxide from glucose. Litmus milk was reduced, acidified and coagulated only rarely. The lactic acid produced by the six isolates tested was optically inactive. Small amounts of steam-volatile acids were produced in addition to lactic acid. No growth was observable in media in which ammonium salts constituted the sole source of nitrogen.

Characters on which was based differentiation into groups (Tables 2 and 3)

Group I. This group includes 38 isolates, of which 27 were cultures received as *Pediococcus cerevisiae*, one was received as *P. pentosaceus*, and the following were unnamed: C-1, C-2, C-6, EJ-1, SS-50, SS-128, BP-1, BP-2, P-45, P-128.

Members of this group were distinguished readily by: size of surface colonies on tomato juice agar, varying between 0·5 and 1·2 mm. in diameter; abundant growth

Table 2. *Physiological characters of pediococci and aerococci*

	Pediococci					Aerococci		
			Group			Not grouped		
	I	*P. urinae equi* PUE	II*a*	II*b*	III	MI, 8519, 8520	*P. halophilus* Tc. 1	
No. of strains	38	1	8	24	14	3	1	11
Growth on TJB (pH 6·6)	+++	+++	++	+	++	+	++	−
Opt. temp. 22°	−	−	−	−	−	+	−	−
Opt. temp. 30°	+	+	+	+	+	−	+	+
Growth at 40°	+++	+++	++	+ (late) 20/22	++	+ (late)	+ (late)	+
Growth at 45°	+ (26)	−	−	−	−	−	−	−
Growth at pH 4·2	+++	−	+ (late)	+ (late) 14/15	−	−	−	−
Growth in NaCl 4 % (w/v)	+++	+++	+ (late)	+ (late)	++	−	++	++
Growth in NaCl 6·5 %	+ (late)	+ (late)	+ (late) 5	− (21)	− (11)	−	++	+
Growth in Teepol 0·01 %	+++	−	++	+	++	+	.	+
Growth in Teepol 0·05 %	+++	−	+	+	−	±	.	+ (6)
Growth in Teepol 0·1 %	+	−	+ (5)	+ (late) 10/14	+ (late) 7/14	±	.	+ (6)
Catalase	+ (26)	−	−	−	−	−	−	+ (9)
NH_3 from arginine	+	−	−	−	−	−	−	−
Acid from								
Arabinose	+ (31)	−	−	−	− (13)	−	−	−
Xylose	− (27)	−	−	−	− (13)	−	−	−
Glucose	+	+	+	+	+	+	+	+
Fructose	+ (16)	+	+	+ (15)	+ (12)	+	+	+
Maltose	+ (31)	+	+	+ (16)	+ (18)	−	+	+ (10)
Lactose	− (32)	+	− (7)	− (19)	− (12)	−	−	+ (10)
Sucrose	− (31)	+	−	−	−	−	−	+
Trehalose	+ (33)	+	+ (6)	− (22)	−	+	+	+ (9)
Raffinose	− (31)	+	−	− (23)	−	±	−	−
Inulin	− (37)	−	− (7)	−	−	−	−	−
Dextrin	− (36)	−	− (7)	−	+	−	−	− (10)
Glycerol	− (35)	−	−	−	−	±	−	+ (10)
Mannitol	−	+	−	−	−	−	−	− (6)
Sorbitol	−	−	−	−	−	−	−	−
Salicin	+ (25)	+	− (7)	+ (12)	− (8)	−	+	+ (6)
Litmus milk								
Acid	+ (10/25)	−	+ (late)	− (20/20)	+ (8/12)	−	−	+ (10/10)
Dye reduction	+ (14/25)	−	− 6/8	− (20/20)	− (12/12)	−	−	− (10/10)
Coagulation	−	−	−	− (20/20)	− (12/12)	−	−	− (10/10)
Hydrolysis of aesculin	+	+	+ (5)	+	+	−	.	−
AMC from glucose	+ (33)	−	−	−	−	±	.	−
AMC from lactose	+	−	−	−	−	−	−	−
Final pH in GYB after 18 days	3·7–3·9	5·0	3·9–5·8	3·9–6·4	4·4–5·0	.	4·9	4·6–5·1
Leucovorin requirement	+ 8/13	.	− 0/4	− 0/2	− 0/1	.	.	− 0/2

+++ profuse growth; ++ good growth; + moderate growth.

Figures in brackets refer to number of strains giving indicated reaction when all strains were tested and not all gave identical reactions. When only a proportion of strains was tested the number positive/the number tested are given as a 'fraction'.

TJB = tomato juice broth; AMC = acetylmethylcarbinol; GYB = glucose yeast-extract broth.

in tomato juice broth; consistently low final pH value (3·7–3·9) in GY broth; vigorous growth at pH 4·2; production of ammonia from arginine. Thus, these isolates form a well-defined and easily recognizable group. In addition, 25 of these isolates were capable of growth at 45°, and 33 produced acetylmethylcarbinol from glucose and lactose. Catalase activity was demonstrated in 26 isolates. All isolates were tolerant of 4 % (w/v) sodium chloride but a concentration of 6·5 % delayed the growth of 27 isolates, to some degree. The addition of Teepol at 0·01 and 0·05 % (v/v) did not affect growth but at 0·1 % initiation of growth was delayed by 24 hr. for 3 isolates. Eight isolates in this group exhibited a specific requirement for leucovorin.

Group II. This group consisted of the cultures received as *Pediococcus halophilus* (Tc. 1) and the following 32 unnamed isolates: M-31, A-140, A-181, B-137, B-190, SS-61, SS-101, HY-22s, S-18, S-180, S-182, S-188, S-190, S-191, S-290, S-333, S-334, S-335, S-338, S-339, S-340, S-342, S-344, S-447, S-524, S-525, S-526, S-527, S-532, S-533, C-14, SS-69. Its members differed from group I in: producing surface colonies on tomato juice agar of only 0·3–0·4 mm. diameter; showing less vigorous growth in tomato juice broth; absence of catalase activity; failure to produce ammonia from arginine; no acetylmethylcarbinol from glucose or lactose. They were unable to grow at 45° or in 4 % (w/v) NaCl within 24 hr. and grew at pH 4·2 only after prolonged incubation (up to 7 days). Two subdivisions were recognized within this group. The 24 isolates listed (S-18, SS-69), unlike the others, grew poorly in tomato juice broth, failed to grow at 40° within 24 hr. and generally did not produce acid from trehalose.

Table 3. *Main characters on which subdivision of pediococci may be based*

Species name	Group	Optimum growth temp.	Diameter of surface colonies on TJA (mm.)	Growth in TJB	Growth pH 4·2	Growth in 4 % (w/v) NaCl 24 hr.	Growth in 4 % (w/v) NaCl 48 hr.	NH_3 from arginine	AMC from glucose	Acid from dextrin
P. cerevisiae	1	30°	0·6–1·2	+ + +	+ + +	+ + +	+ + +	+	+ almost always	–
P. 'parvulus'	11	30°	0·3–0·4	+	Late	–	±	–	–	–
	111	30°	0·6–1·0	+ +	–	+ +	+ +	–	–	+
P. damnosus?		22°	0·3–0·6	Late	–			–	+	–
P. halophilus?		30°	0·6–0·8	Late	–	5 % essential	–	–	–	–

TJA = tomato juice agar; TJB = tomato juice broth; AMC = acetylmethylcarbinol.
+ + + Growth abundant; + + good growth; + moderate growth.

Possible group III. This group was less well defined but is tentatively suggested for the following 14 unnamed isolates: L-16, L-20, L-22, L-24, L-92, L-95, L-148, L-171, L-223, L-345, L-347, L-351, L-352, L-354. These isolates resembled group I in size of surface colonies on tomato juice agar and abundant growth in tomato juice broth but could be differentiated by their inability to grow at pH 4·2 even when incubated for 7 days and by their failure to produce ammonia from arginine. They differed also from the majority of group I isolates in: failure to grow at 45°; absence of catalase activity; failure to produce acetylmethylcarbinol from glucose or lactose. They could be separated from group II isolates by: colony size; absence

of growth at pH 4·2; ability to grow in media containing 4 % (w/v) NaCl within 24 hr.; acid production from dextrin.

Other isolates. Some isolates did not fall readily into any of the above three groups. *Pediococcus urinae equi* was indistinguishable in cultural characteristics from members of group I but differed from the majority of those in failure to grow at pH 4·2, inability to produce ammonia from arginine and acetylmethylcarbinol from glucose or lactose, higher final pH value in glucose broth (pH 5·0) and lower resistance to Teepol. It may be regarded as a member of a possible subgroup of group I.

Pediococcus damnosus strain M-1 and *P. damnosus* var. *salicinaceus* strains 8519 and 8520 were readily distinguishable from the other pediococci by their lower optimum growth temperature of 22°. They did not produce ammonia from arginine and *P. damnosus* strain M-I produced acetylmethylcarbinol from glucose but not from lactose.

Pediococcus halophilus strain Tc. 1 was exceptional in its requirement for 5 % (w/v) sodium chloride; in most of its other reactions it resembled members of group III.

Aerococci. These organisms were fairly uniform in character. They resembled the pediococci, as defined above, in morphology, failure to hydrolyse gelatin, to reduce nitrate to nitrite or to utilize ammonia salts as sole source of nitrogen, and in absence of gas formation from glucose. Unlike the pediococci, however, the aerococci grew well in nutrient broth, grew vigorously at pH 9·0 and were highly sensitive to acidity (even pH 6·8 exerted an inhibitory effect). Nine of the 11 isolates were catalase-positive, one was negative and one gave a variable reaction. However, in the media used all these aerococci were strictly aerobic.

DISCUSSION

Relationship of pediococci to other genera

The results of the present work provide additional evidence in support of the recognition of a separate genus *Pediococcus* as suggested by Balcke (1884), Mees (1934) and more recently by Pederson (1949), Felton & Niven (1953) and Jensen & Seeley (1954). All members of this genus are easily recognizable by their morphology, mode of division and high lactic acid-producing capacity. We think, however, that the genus should include a rather wider range of organisms than suggested by Pederson *et al.* (1954) and by Jensen & Seeley (1954).

The present results show that the leucovorin (folinic acid) requirement, suggested by Felton & Niven (1953) and by Jensen & Seeley (1954) to be typical of all pediococci, is, in fact, restricted only to some isolates within our group I. Another property considered by some investigators to be an outstanding character of pediococci (Balcke, 1884; Lindner, 1887; Claussen, 1903) was the ability to multiply vigorously in beer, although more recently strains have been described which failed to multiply in this medium (Mees, 1934; Shimwell, 1949; Pederson, 1949). Pederson (1957) in *Bergey's Manual* (7th ed.) describes the genus as producing acidification and some degree of clouding in beer, and the two species listed are separated according to their optimum growth temperature and ability to grow in beer and hopped wort. *Pediococcus cerevisiae* Balcke 1884, the type species, is regarded as capable of growth in wort, hopped wort and beer, while *P. acidilactici* Lindner 1887 will grow in unhopped

wort but not in beer. Of the isolates studied in the present work, none showed growth in the particular beer used for the test. The use of the criterion 'growth in beer', without further qualifications as to the kind in which growth is tested, is however of little value, as beers may show wide variation in acidity, in hop content, in ethanol and carbon dioxide concentration, and in the degree of 'attenuation' which influences the quantity of nutrients available in the medium.

The present investigation confirms the separation of the genus *Pediococcus* from the other closely related genera. It resembles the lactic acid streptococci in requiring complex media for growth as shown by Jensen & Seeley (1954), and in being homofermentative as demonstrated by Pederson *et al.* (1954) and confirmed in this study. However, as reported previously (Günther, 1959), the pediococci are also clearly distinguishable from streptococci on the basis of morphology and mode of division. Morphology, homofermentative character and production of optically inactive lactic acid, as found in the present work and previously by Pederson *et al.* (1954) serve to differentiate the pediococci from the genus *Leuconostoc.*

The pediococci closely resemble micrococci in morphology and mode of division but may be separated from them by consideration of their biochemical characters. Although biochemical characters are often found to be variable and therefore unreliable as diagnostic criteria, all of those investigated in the present study have been shown to be stable over a period of at least 18 months. Where isolates obtained by other authors were investigated, the results were in general the same as those previously described. Therefore it seems justifiable to use such characters as differential criteria. Thus pediococci can be differentiated from micrococci on the basis of their failure to: grow on simple media; utilize ammonium salts as sole source of nitrogen; reduce nitrate to nitrite or nitrogen gas; liquefy gelatin.

The differentiation between pediococci and aerococci is less satisfactory on the basis of present results. Their morphology and mode of division is similar and neither group is capable of reducing nitrate, hydrolysing gelatin or utilizing ammonium salts as sources of nitrogen. Jensen & Seeley (1954) and Dr Ellen I. Garvie (personal communication) have suggested that the two groups may be related sufficiently to be included in the same genus. However, in contrast to the pediococci, the aerococci grew well on simpler media, grew only under aerobic conditions, were highly sensitive to acid, but grew profusely in alkaline media (pH 9·0). Such differences provide sufficient evidence for separating the two groups but further experimental data are yet required before establishing their separation at generic or specific level.

Subdivision of the pediococci

A subdivision of the pediococci into three groups, as indicated in the section on results, is suggested. Although there is some variability within each proposed group, experience in the laboratory handling of these organisms has enabled us to recognize easily the three groups by their cultural characters and we feel justified in suggesting the subdivision of the pediococci in this way.

Pederson (1949) and Jensen & Seeley (1954) recognized only one species, *Pediococcus cerevisiae* Balcke, the type species. In a later paper, Pederson *et al.* (1954) suggested two possible additional species of which the first (*a*) produced slime, and the second (*b*) possessed a higher optimum growth temperature. For organism (*a*) the name *P. viscosus* Lindner was suggested, and for organism (*b*) either *P. acidilactici*

Lindner or *P. hennebergi* Sollied. In the seventh edition of *Bergey's Manual*, Pederson (1957) lists two species: the type species *P. cerevisiae* Balcke; *P. acidilactici* Lindner characterized by an optimum growth temperature of 40° and failure to grow in beer. In the present survey no isolates were observed which produced slime (in presence of sucrose) or had a high optimum temperature.

The reactions characteristic of the group I organisms of this study are in general the same as those described for the strains of *Pediococcus cerevisiae* Balcke studied by Pederson (1949), Felton & Niven (1953), Jensen & Seeley (1954) and Dacre (1958*a*). It would appear justifiable, therefore, to apply the species name *P. cerevisiae* Balcke to this group, which includes the isolates received as *P. pentosaceus* (Mees), strain A2 (NCDO 559) of Dacre (1958*a*), and strain P-60 previously known as *Leuconostoc mesenteroides* P-60 but classified recently with the pediococci by Garvie (1959).

Pederson *et al.* (1954) compared strain *Pediococcus urinae equi* with their culture of *P. cerevisiae* and concluded that this organism should not be included in the pediococci because of lower acid-production properties. The present study confirmed the inability of *P. urinae equi* to produce a low final pH value but showed that it was indistinguishable from *P. cerevisiae* in most of its morphological and cultural characters. It can therefore only be regarded as a variant of that species. The features of the group II isolates are sufficiently distinct to warrant the recognition of a separate species. No description could be found in the literature of such a species and the name *Pediococcus parvulus* is suggested.

Group III is much less well defined. The characters of this group resemble those described by Andrews & Gilliland (1952) for a dextrin-fermenting organism which they named *Streptococcus damnosus* var. *diastaticus*. A culture of the latter organism was not available for comparative study. It is felt that insufficient evidence is at present available on which to base the establishment of this group at specific rank.

Three isolates received as *Pediococcus damnosus* strain M.1 and *P. damnosus* var. *salicinaceus* strains 8519 and 8520 failed to fit into any of the three groups described. None of them fermented salicin. Many authors have found the fermentation of pentoses and salicin to be variable and Pederson (1949) classified such strains as *P. cerevisiae*. However, the three isolates we received resembled each other and differed from *P. cerevisiae* (group I) in a number of characters (see Table 2; it may be noted that they all have a low optimum temperature). They might form the nucleus of a fourth group should other isolates with such characters be noted in future and should be regarded as members of a species *P. damnosus*.

The culture received as *Pediococcus halophilus* (Tc.1) was quite distinct in character (especially in its requirement for 5 %, w/v, NaCl) and, should additional strains be isolated, a fifth group might be recognized. Deibel & Niven (1960) described strains of pediococci isolated from meat-curing brines which were salt tolerant and produced dextrorotatory lactic acid from glucose. Deibel & Niven considered that their strains may be closely related to the marine micrococcus *Gaffkya homari* (Sniesko & Taylor, 1947) and also to *Aerococcus viridans*. They suggested that all these organisms should be placed in the genus *Pediococcus* with the species name *Pediococcus homari* nov.comb. Further investigation is needed before it can be decided whether this species and the *P. halophilus* of Mees (1934) are the same. It

would seem that a group of salt-tolerant or even halophilic pediococci might indeed constitute a fifth species.

Serological work to be published in the following paper (Günther & White, 1961) confirms a subdivision into at least three groups as here suggested.

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Serological Characters of the Pediococci

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SUMMARY

Rabbit antisera were prepared against 12 pediococcus isolates and tested by precipitin, precipitin-absorption and agglutination tests. Common precipitins for the three species *Pediococcus cerevisiae*, *P. parvulus* n.sp. and *P. damnosus* were demonstrated. An antiserum prepared against *Aerococcus viridans* (NCTC 7599) reacted with extracts of the homologous strain and 7 out of 10 heterologous *Aerococcus viridans*.

INTRODUCTION

Renewed interest in the genus *Pediococcus* has led in recent years to extensive studies of the cultural and physiological characters of its members (Pederson, 1949; Felton & Niven, 1953; Pederson, Albury & Breed, 1954; Jensen & Seeley, 1954) but, so far, no study has been reported of the serological properties of the organisms. In view of the successful application of precipitin and agglutination tests in the characterization of other lactic acid bacteria it was decided to explore the possibility of using serological methods in the classification of pediococci. Some preliminary results of this investigation are reported in this paper.

METHODS

The cultures and methods of cultivation are described in our previous paper (Günther & White, 1961).

Organisms. Twelve strains representative of the physiological groups described in the same previous publication were selected for antisera production.

Pediococcus cerevisiae (group I) was represented by strains 8081, F-166, 2-170, N-91, A-1, FP-1; *P. parvulus* (group II) by strains M-31, A-181, S-190, SS-69; *P. damnosus* (possible group IV) by strain M-1, and *P. halophilus* (possible group V) by strain Tc.1.

We intended to prepare an antiserum for strain L-148, which belongs to the unnamed group (group III), but this proved unsatisfactory and no further attempts have as yet been made to prepare antisera against strains belonging to this group.

One antiserum against *Aerococcus viridans* H-9 (NCTC 7599) was prepared.

Preparation of antisera. Intravenous injections of (*a*) whole living organisms, or (*b*) disintegrated suspensions, were given to rabbits which had been tested previously for the absence of naturally occurring antibodies. For method (*a*) 40 ml. glucose Lemco (GL) broth (Shattock & Hirsch, 1947) were inoculated with 0·5 ml. of a 24 hr. 'Oxoid' tomato juice (TJ) broth culture and incubated for 24 hr. at 30°. The culture was centrifuged, washed twice in 0·9 % (w/v) sodium chloride solution

resuspended in 0·9 % sodium chloride solution to give an opacity equivalent to Brown's tube No. 7 and then stored at 4° for use in one course of animal injections. In method (*b*) fresh suspensions of organisms were prepared for each injection. Organisms from 20 ml. of a culture in GL broth were washed and adjusted to the same optical density as in method (*a*). They were then disrupted for 30 min. at room temperature in a tissue disintegrator (Mickle, 1948) in a vessel containing 5 g. sterile Ballotini beads grade No. 12. After centrifugation the supernatant fluid was found to be sterile and was used for animal injection.

When the two methods of antibody production were compared it was noted that the disintegration of the organism before inoculation did not result in a speedier production of antibodies, nor was the potency of the resulting antiserum increased. Suspensions of whole living organisms were therefore used for the routine preparation of antisera; the results recorded below refer in all instances to sera prepared by this method.

The experimental animals received 1 ml. volumes of suspensions of organisms prepared as above at 3–4 days intervals. One course of injections consisted of five such inoculations. When a test bleeding at this stage indicated that the antibody produced was of insufficient potency, a second course was given. Occasionally, a third course had to be administered before satisfactory antibody was obtained. The animals were bled 3 or 4 days after the last injection. Sera were stored at 4° without preservative.

The presence of non-specific antibodies was excluded by precipitin tests with each serum against extracts of streptococci (representative of Lancefield's groups A to N) and against extracts of 7 staphylococcus and 3 leuconostoc strains.

Preparation of extracts. (*a*) *Crude extracts*: Lancefield's (1933) HCl extraction method was used as modified by Sharpe (1955). The cultures were incubated for 48 hr. at 30° in tomato juice broth. The reactions of at least two extracts, prepared at different times, were tested to ensure reproducibility of results. (*b*) *Ethanol precipitation.* The difficulty in reading the results with some of the sera and crude extracts prepared as above made it desirable to concentrate the antigenically active material in the extracts by ethanol precipitation as suggested by Shattock (1949) for group D streptococci. A slight increase in activity in the precipitate was noted with most of the extracts, but in a few this treatment resulted in the complete loss of activity in the precipitate as well as in the supernatant fluid. Ethanol precipitation was not used, therefore, for the routine preparation of extracts.

Precipitin tests. Ring tests were carried out as described by Sharpe (1955). Observations were continued for 30 min., or up to 60 min. when a loss of potency in the antiserum was noted after prolonged storage. Results obtained with antisera which had been stored for a long time were always regarded as unreliable but were useful in preliminary work.

Precipitin-absorption tests. The organisms were cultivated in tomato juice broth for 24 hr. at 30°. The technique outlined by Sharpe (1955) was followed.

Agglutination tests. Tube agglutination, employing suspensions of organisms from 16 hr. tomato juice broth cultures in 0·9 % (w/v) sodium chloride solution, was used. The tubes were read after overnight incubation at 50°.

RESULTS

The selected pediococcus isolates differed in their capacity to induce antibody response in the rabbit and the choice of suitable isolates for antiserum production was of great importance. The difficulty experienced in obtaining active extracts with some isolates merits further investigation. In the future, modifications may

Table 1. *Precipitin tests with antisera of pediococci and* Aerococcus viridans

	Antisera												
	P. cerevisiae						*P. parvulus*				*P. damnosus*	*P. halophilus*	*A. viridans*
	F-166	2-170	8081	N-91	A-1	FP-1	M-31	A-181	S-190	SS-69	M-1	Tc.1	H-9
P. cerevisiae (group I)													
F-166	++	+	+	+	+	+	+	+	+	+	±	−	−
2-170	+	++	+	±	±	+	+	+	+	±	−	−	−
8081	+	+	++	+	+	+	+	+	+	+	+	−	−
N-91	+	+	+	++	+	+	+	±	−	+	−	−	−
A-1	+	+	+	−	++	+	−	−	−	−	−	−	−
FP-1	+	+	+	−	−	++	+	−	−	+	−	−	−
Others	+ 30/31	+ 29/31	+ 27/31	+ 24/30	+ 26/30	+ 29/31	+ 24/30	+ 14/16	+ 18/30	+ 28/30	+ 21/28	− 28/28	− 30/30
P. parvulus (group II)													
M-31	+	+	+	+	.	+	++	+	−	+	+	−	−
A-181	+	+	±	−	+	±	+	++	+	+	+	−	−
S-190	+	+	+	+	+	+	+	.	++	−	−	−	−
SS-69	+	+	+	+	±	±	+	.	−	++	+	−	−
Others	+ 20/24	+ 21/24	− 17/24	− 17/24	− 14/18	+ 10/24	+ 22/24	− 9/13	− 18/23	+ 18/24	+ 18/24	− 24/24	− 24/24
P. damnosus (? group IV)													
M-1	+	±	±	−	−	−	+	.	−	+	++	−	−
Others	+ 2/2	+ 2/2	+ 1/2	+ 1/2	.	− 2/2	+ 2/2	.	− 2/2	+ 1/2	.	− 2/2	− 2/2
P. halophilus Tc.1 (? group V)	−	−	−	−	.	−	−	.	−	−	−	++	±
A. viridans													
H-9	−	−	−	−	−	−	−	−	−	−	−	−	++
Others	− 8/10	− 10/10	− 9/10	− 10/10	− 3/3	− 9/9	− 9/9	− 1/1	− 8/10	− 9/10	.	− 10/10	+ 7/10
Leuconostocs	− 3/3	− 2/3	− 3/3	.	− 3/3	− 3/3	.	− 3/3	.	.	.	.	.
Streptococci	− 24/24	− 24/24	− 24/24	− 12/24	− 12/24	− 24/24	− 12/24	− 24/24	− 12/24	− 12/24	− 12/24	− 12/24	− 24/24
Staphylococci													
Staph. aureus 7447	−	−	−	−	.	−	−	.	−	−	−	−	+
Staph. albus 7292	−	−	−	−	.	−	−	.	−	−	−	+	+
Others	− 5/5	− 5/5	− 5/5	− 5/5	.	− 5/5	− 5/5	.	− 5/5	− 5/5	− 4/5	− 3/5	− 4/5

++ = strong precipitate, + = precipitate, ± = weak reaction, − = no reaction, . = not tested.

The figures are $\frac{\text{no. of strains giving reaction}}{\text{no. of strains tested}}$.

have to be made in the method of preparation in order to obtain satisfactory extracts of consistently high activity.

Precipitin tests. These results are summarized in Table 1. Positive reactions were obtained with extracts of the homologous and with the majority of heterologous strains of the same species. Antisera against *Pediococcus cerevisiae* strains F-166, 2-170, and FP-1 also precipitated consistently with extracts of *P. parvulus*. Antisera against *P. cerevisiae* strains 8081, N-91, and A-1 were less consistent in their reactions with extracts of *P. parvulus*, but they too gave positive reactions with a large number of extracts of that species.

Antisera against *Pediococcus parvulus* and *P. damnosus* reacted with the homologous and with the majority of the heterologous extracts of the same species as well as with extracts of *P. cerevisiae*. Cross-reactions occurred also between *P. parvulus* and *P. damnosus*.

Reactions could not be observed between sera against *Pediococcus cerevisiae*, *P. parvulus* or *P. damnosus* on the one hand, and between 'group III' isolates or *P. halophilus* on the other. The serum against *P. halophilus* was not precipitated by extracts of any of the other pediococci.

Table 2. *Absorption of antisera of* Pediococcus cerevisiae *strains F-166 and 2-170 with cells of homologous and heterologous* P. cerevisiae *strains*

	Antiserum									
	F-166				2-170					
		Absorbed with strains				Absorbed with strains				
Antigen (extracts)	Unabsorbed	F-166	D-32	PUE	Unabsorbed	2-170	F-166	8081	FP-1	A-1
P. cerevisiae (group I)										
F-166	++	−	−	+	+	−	−	−	−	−
2-170	+	−	.	−	++	−	−	.	.	.
PUE	+	−	.	−	+	.	.	.	.	.
Others	+	−	−	−	+	−	−	−	−	−
P. parvulus (group II)	+	−	−	−	+	.	.	−	−	−

Symbols as Table 1.

Six of the pediococcus antisera gave positive reactions with one or the other aerococcus extract, but in these instances ring formation was markedly delayed and considerably less intense than that observed with the pediococci.

The *Aerococcus viridans* H-9 serum reacted with extracts of the homologous and with 7 of the 10 other strains of *A. viridans* tested. A weak cross-reaction was obtained also with extracts of *Pediococcus halophilus* and with strain L-171 of the unclassified group III.

Cross-reactions were not found between the pediococcus antisera used and the extracts of streptococci, staphylococci and leuconostoc strains, with the exception of one reaction between a strain of *Staphylococcus albus* and *Pediococcus halophilus* antiserum.

Precipitin-absorption tests. Four antisera were selected for absorption tests. The results are shown in Tables 2, 3, 4. These results indicate that certain antisera contained more than one antibody, one of which was absorbed by heterologous

Table 3. *Absorption of antiserum* Pediococcus cerevisiae *strain F-166 with cells of* P. parvulus *and strain L-148 (group III)*

	Antiserum F-166 absorbed with strains *P. parvulus*					
Antigen	Un-absorbed	A-181	S-190	SS-69	S-182	L-148
P. cerevisiae (group I)						
F-166	++	+	–	+	–	+
2-170	+	–	.	–	.	+
PUE	+	.	–	.	.	.
Others	+	–	–	–	–	.
P. parvulus (group II)	+	–	–	–	–	+

Symbols as Table 1.

Table 4. *Absorption of antisera* Pediococcus parvulus *strains S-190* and *SS-69 with cells of* P. cerevisiae *and* P. parvulus

	Antiserum							
	S-190				SS-69			
		Absorbed with				Absorbed with		
		P. parvulus		*P. cerevisiae*		*P. parvulus*	*P. cerevisiae*	
Antigen	Unabsorbed	S-190	SS-69	F-166	Unabsorbed	SS-69	F-166	PUE
P. parvulus (group II)								
S-190	+	–	+	+	+	–	–	–
SS-69	+	.	.	.	+	–	+	+
Others	+	–	–	–	+	–	–	–
P. cerevisiae (group I)	+	–	–	–	+	–	–	–

Symbols as Table 1.

Table 5. *Agglutination tests with pediococcus and aerococcus antisera*

	Antisera							
	P. cerevisiae					*P. parvulus*		*A. viridans*
Antigen	F-166	2-170	8081	N-91	FP-1	M-31	SS-69	H-9
P. cerevisiae (group I)								
F-166	1280+	–	–	–	–	–	–	–
2-170	80	1280+	–	40	80	–	–	–
8081	20	40	1280+	40	40	40	40	20
N-91	–	–	–	640	–	–	–	–
FP-1	40	80	–	40	1280+	–	160	–
P. parvulus (group II)								
M-31	–	–	–	–	–	1280+	–	–
SS-69	320	40	40	40	160	80	1280	40
A. viridans								
H-9	–	–	–	–	–	–	320	1280+

Figures are the reciprocals of the highest titres read. – = > 20.

strains and the other absorbed only by the homologous strain. Antibodies in *Pediococcus cerevisiae* antiserum were absorbed by *P. parvulus* strains and vice versa, indicating that these two species share at least one common antigen. Absorption with strain L-148 organisms, with strains of *Aerococcus viridans*, streptococci and staphylococci did not result in loss of antibodies from the antisera of *P. cerevisiae* or *P. parvulus*.

Agglutination tests. The results are shown in Table 5. With homologous strains titres of more than 1/1280 were obtained generally but with heterologous strains, when any reaction at all was obtained, the titres seldom exceeded 1/80. A number of cross-reactions occurred between antisera of *Pediococcus cerevisiae* and *P. parvulus*. In one instance cross-reactions occurred between *P. parvulus* strain SS-69 and *Aerococcus viridans* strain H-9.

DISCUSSION

The present investigation indicates that serological techniques may be applied successfully to the separation and classification of the pediococci. Antisera prepared against pediococci did not react with extracts of organisms from the closely related genera *Streptococcus* and *Leuconostoc* and generally not with extracts from aerococci. It becomes evident that pediococci possess precipitins which are common to members of more than one species. Such antigens were demonstrated in *Pediococcus cerevisiae*, *P. parvulus* and *P. damnosus*, but not in *P. halophilus* and in members of the as yet unclassified subgroup (III). As these antigens were shared by more than one species they may be referred to as 'group' antigens by analogy to the group antigens of streprococci.

Some pediococcus strains showed an additional antigen which appeared to be more strain specific and may therefore represent a 'type' antigen. The presence of more than one antigen was also demonstrated in agglutination tests. These tests revealed relationships apparently much narrower than were demonstrated in precipitin tests. It is probable, then, that pediococci possess a 'group' antigen demonstrable by precipitin tests and a 'type' antigen which can be shown by precipitin and agglutination tests, as with streptococci and lactobacilli. No study has as yet been made to determine whether the 'type' antigens demonstrable in the two tests were identical. The possibility that the organisms possess more than one 'type' antigen cannot be excluded.

Some indication that the 'group' antigen may be protein in nature was obtained from ethanol precipitation of some of the extracts since the antigenically active material had been found in the precipitate. However, the antigen might be carbohydrate carried down with the protein.

The serological position of *Pediococcus halophilus* and of the unnamed group III could not be established satisfactorily. Absorption of *P. cerevisiae* antiserum with one strain of group III did not remove the 'group' antigen from the serum. This indicates that members of group III are not related antigenically to *P. cerevisiae* (group I) and *P. parvulus* (group II). They may possibly represent a separate serological group.

The reaction of strain PUE is of interest. Pederson *et al.* (1954) excluded it from the pediococci but the strain was classified by us (Günther & White, 1961) as *Pediococcus cerevisiae*. This organism gave strong precipitin reactions with antisera

of *P. cerevisiae*, *P. parvulus* and *P. damnosus*. When PUE was used for absorption of *P. cerevisiae* antiserum the 'group' antigen was absorbed. This is further evidence that the correct classification of this strain is with *P. cerevisiae*.

The serological work also confirms the classification of strain A-2 isolated by Dacre and classified by him tentatively as *P. cerevisiae* (Dacre, 1958) and of the old '*Leuconostoc mesenteroides* P-60' reclassified by Garvie (1959) as *P. cerevisiae*.

The cross-reactions between the aerococci and pediococci might have been due to non-specific antibodies, but more experimental evidence is required. The work is as yet incomplete but it is being continued and we hope to clarify at least some of the issues raised in this and the preceding paper.

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The Production of Sporidesmin and Sporidesmolides by *Pithomyces chartarum*

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SUMMARY

Methods for the assay of sporidesmin and the sporidesmolides were worked out and used in the study of the production of these metabolites by *Pithomyces chartarum* (Commonwealth Mycological Institute, Kew, England, Herbarium no. 74473) growing on an enriched potato carrot medium. High yields of sporidesmin and the sporidesmolides were associated with high utilization of medium constituents. No increase in the quantity of sporidesmin isolated from unit volume of medium was observed after the third day of incubation, despite further rapid growth of the fungus; by contrast good correlation was observed between the weight of sporidesmolides produced and fungal growth. These results were successfully applied to the production of experimental quantities of these materials.

INTRODUCTION

Recently the suggestion was made (Percival & Thornton, 1958; Thornton & Percival, 1959) that the production of toxic material by a micro-organism growing on pasture flora was an important part of a disease of sheep and cattle which occurs in North Island, New Zealand, and is known as facial eczema. The disease occurs under climatological conditions which are conducive to fungal growth. Sheltered warm low-lying pastures have frequently had a history of facial eczema outbreaks. In a survey Thornton & Ross (1959) showed that certain micro-organisms were predominant on pastures where the disease commonly occurred. One of these micro-organisms *Pithomyces chartarum* (Berk. & Curt.) Ellis (1960) (syn. *Sporidesmium bakeri* Syd.; Commonwealth Mycological Institute Herbarium no. 74473) after surface-culture on potato carrot medium for 7 days was fed to sheep. The livers of these animals showed pathological changes similar to those observed in clinical cases of facial eczema (Percival, 1959). Chemical examination (Russell, 1960) of such cultures showed that depsipeptides (Shemyakin, 1960) probably identical with those isolated from cut grass from pastures where facial eczema had occurred (White, 1958) were present. Synge & White (1959) isolated from ethereal extracts of cultures of this fungus a crystalline material which they named sporidesmin. This material, administered orally to guinea-pigs, produced pathological changes in the livers which resembled those observed when similar experimental animals were fed

samples of dried grass from pastures known to have been associated with the disease. The potential importance of factors of this kind seemed to warrant an attempt to find what conditions of laboratory cultivation were conducive to the growth of the fungus and whether these were related to the formation of sporidesmin. As the sporidesmolides belong to a group of natural products which commonly possess antibiotic activity (Shemyakin, 1960) it was also of interest to find whether their appearance in the cultures was related to the production of sporidesmin and to the growth of the mould. Such experiments might find application in pasture management, in comparisons of the ability of different strains of the fungus to produce toxic metabolites and in the production of adequate quantities of sporidesmin and the sporidesmolides for biochemical and pathological comparisons of the disease as observed in the field with that produced experimentally.

METHODS

Organisms. There is at present some controversy about the nomenclature of the organism used in this work (see Hughes, 1958; Ellis, 1960). With one exception the strain used in the present experiments was disposed by the Commonwealth Mycological Institute (Kew, England) as *Sporidesmium bakeri* Syd. (Hughes, 1953), C.M.I. Herbarium no. 74473. The name *Pithomyces chartarum* is used in this paper on the basis of Ellis's (1960) work. Miss J. M. Dingley provided us with a strain of this organism that she had isolated at Mount Albert, Auckland, New Zealand. This strain (Ellis, 1960) was used in the experiments recorded in Table 3.

Inocula. Sterile 0·05% (w/v) Lissapol N samples (10 ml.) were transferred aseptically to a 10- to 21-day culture of the fungus on potato carrot agar prepared from the medium described below diluted to half strength with tap water with omission of the additional glucose. The liquid was washed over the slope until this was thoroughly wetted. The suspension was decanted into a sterile container attached to an automatic pipette and sterile water added to make the spore concentration 5×10^6 spores/ml. For larger quantities of inocula sterile Lissapol N solution (50 ml., 0·05%, w/v; I.C.I. Ltd.) was added to a 21-day culture of the organism on rye (*Secale cereale*) and the spore suspension prepared as before. The suspension (0·1 ml.) was inoculated to the medium (100 ml.) and to each milk bottle of rye grain or bran (see below).

Surface cultures. Potatoes (30 kg.) and carrots (30 kg.) were scrubbed and cut mechanically into 3 cm. cubes. The mixed vegetables were covered with 60–70 l. tap water in a stainless steel vessel equipped with a stainless steel stirrer (120 rev./min.) and three 2 kW. immersion heaters. The stirred mixture was boiled for 30 min. after which the semi-solid material was allowed to settle and the supernatant liquor decanted. The liquor was made up to the required volume (about 100 l.) and sugar and nitrogen determinations carried out. The sugar concentration was then adjusted to 1% (w/v) by adding glucose and the nitrogen content was made to 0·05% (w/v) by adding ammonium sulphate. The medium was adjusted to pH 5·0 by adding dilute sulphuric acid. After filling the culture vessels the medium was heated at about 120° for 20 min. All surface cultures were incubated at 24° ± 0·5° for 7 days.

Cultures on rye grain and on bran. In each of 200 pint milk bottles were put rye grain (75 g.) and tap water (45 ml.) (or 50 g. bran and 20 ml. water) and the bottles

allowed to stand overnight. The bottles were then plugged with non-absorbent cotton wool, the plugs being protected with aluminium foil. The bottles were autoclaved at about 120° for 2 hr. After standing in the incubation room for 48 hr. three samples selected at random were removed aseptieally, transferred to potato + carrot agar slopes and incubated at 24° for 72 hr. These slopes were examined from time to time. Meanwhile, when no obvious contamination was present, the bottles were inoculated and incubated at 24° ± 0·5° for 28 days.

Extraction. After incubation for 28 days 80 % (v/v) methanol in water (250 ml.) were added to each bottle, the fungus and grain broken up with a stout glass rod and the mixture poured into a stainless steel vessel. The mixture was stirred for 20 hr. at room temperature and then filtered through a large nutsche dressed with Terylene cloth (coarse-grade sail cloth available in New Zealand was used, precise specifications not known). The residue that remained on the filter was replaced in the stainless steel vessel, 80 % (v/v) methanol in water (50 l.) added and the mixture stirred for 3 days at room temperature. The liquid that had passed through the Terylene was filtered through Whatman no. 31 filter paper and the perfectly clear filtrate was evaporated down to 3 l. in a cyclone evaporator. The concentration process was operated at 2–5 mm. Hg pressure, the heating heat-exchanger was maintained at 80° and the condensing heat-exchanger at −40°. The rate of flow into the heating heat-exchanger was such that the emergent fluid from this part of the apparatus did not exceed 35°. The concentrate was then extracted with diethyl ether (peroxide free, 1 l.) continuously in a Hilditch-type fat extractor for 18 hr. The concentrate may also be extracted by hand in a separating funnel in an efficient fume cupboard but even so the process is hazardous; severe dermatitis was experienced by workers using this technique in the early stages of this work. The ethereal extract was evaporated to dryness *in vacuo*. The second methanolic extract was processed similarly. Gas chromatographic analysis of the methanolic extracting solvent indicated this to be 60–70 % (v/v) methanol in water. The average yield at this stage for 20 batches was 1·2 g. sporidesmin as a 10–20 % (w/v) concentrate. Concentrates of about 20 % (w/v) were obtained when *iso*propyl ether was used as the second extractant. The lowest yield was 0·6 g. and the highest 1·9 g. Alumina chromatography of this concentrate, partition of the diethyl ether eluate between 80 % (v/v) methanol in water and light petroleum (b.p. 60–80°) and finally partition chromatography on Hyflo-supercel with the system carbon disulphide + methanol + water (5, 4, 1, by vol.) as described by Synge & White (1959), provided crystalline material in 80–90 % overall recovery. This material was used in animal experiments to be described elsewhere.

The rye grain and fungus from the second methanolic extraction was stirred and boiled with methanol (20 l.) for 3 hr. and the mixture filtered hot. The hot filtrate was concentrated to 4 l. in a cyclone evaporator. The concentration process was operated at 20 mm. Hg, the heating heat-exchanger was maintained at 110° and the condensing heat-exchanger at −40°. The concentrate was kept in the cold room at 2° for 24 hr. and the solid that had separated was collected and dried *in vacuo*. The average yield for ten batches was 27 g. containing 50–70 % sporidesmolides. Further purification was achieved as described below.

Analytical methods. As only about 50 % of the sporidesmin produced by the growth of *Pithomyces chartarum* was found in culture filtrates all analyses were

done on extracts of culture medium and mycelium. Extracts were prepared as follows. The fungus separated from the culture medium was macerated with Whatman no. 31 filter paper (one 12·5 cm. filter paper/100 g. wet wt. fungus) in a top-drive macerator; the resulting mixture was filtered and the residue sucked dry. The filtrate was added to the culture medium and the residue macerated under diethyl ether (400 ml./500 g. wet wt. residue). This process was repeated three times and the combined ethereal solutions, residual fungus and culture medium were continuously extracted in a Hilditch-type fat extractor for 20 hr. At least 1 l. of ethereal extract was kept in the boiling flask which was heated in a water bath at 80°; when quantities of culture medium between 5 and 20 l. were extracted 2 l. of ethereal extract were kept in the boiling flask. Further extraction produced only traces of sporidesmin. The ethereal extract was evaporated almost to dryness at 0° and the residue cooled to −80°. The ice and sporidesmolides (about 50 mg. of the latter/l. were obtained by this procedure) were collected, washed with diethyl ether and the filtrate and washings evaporated to dryness in a tared vessel *in vacuo*. The resulting brown gum (1 g./10 l.) was assayed by at least two of the following procedures.

Tissue culture toxicity tests. Human malignant epithelial cells (strain HeLa, Gey) were obtained from Mr A. M. Murphy (Auckland General Hospital) in May 1959 and transformed pig kidney cells from Glaxo Laboratories Ltd. in October 1959. The medium described by Murphy & Worker (1960) was modified by increasing the calf serum content to 10% (v/v) and by the addition of penicillin (6 μg./ml. = 100 i.u./ml.) and streptomycin (100 μg./ml.). Phosphate buffered saline (PBS) was made up with sodium chloride (A.R., 1·15 g.), potassium chloride (A.R., 0·2 g.), disodium hydrogen phosphate (A.R., 1·15 g.), potassium dihydrogen phosphate (0·2 g.) and water to 1 l. A 0·1% (w/v) 'Bactotrypsin 1:250' solution in PBS was used for trypsination. Water for the medium, PBS and trypsin was twice glass-distilled. Stock cultures were maintained in 150 ml. square Pyrex screw-cap bottles and test cultures were grown in 125 × 16 mm. Pyrex screw-cap test-tubes. After use these vessels were soaked overnight in 5% (w/v) potassium hydroxide solution in ethanol; they were then washed in tap water, with dilute hydrochloric acid (about 2%, w/v, HCl in water) and finally with distilled water.

The medium was decanted from a 7- to 10-day bottle culture and the cells treated with the trypsin solution (8 ml.). After incubation for 10–15 min. the contents of the bottles were gently mixed and the vessel clamped so that the cells settled in one corner of the base. The supernatant fluid was sucked off and fresh medium (5 ml.) added. A uniform suspension was made by making the cells enter and leave a pipette 5–10 times (the efficiency of a pipette in this operation seems to depend on the characteristics of its tip); then a further 10 ml. portion of medium was added. A sample (0·5 ml.) of the suspension was centrifuged at low speed for a few minutes and the cells washed twice with PBS (2 × 1 ml.). Protein was estimated by the Folin colorimetric method (see below). Medium was then added to the suspension to give a final cell-protein content of 50 μg./ml. and 1 ml. amounts were dispensed into the test-tubes, with an automatic pipette, with magnetic stirring of the suspension. In the preparation of small batches the suspension was dispensed by a 5 ml. graduated pipette. The tubes were placed in trays and the inclination adjusted until the medium reached 5–6 cm. from the bottom of the tube. The trays were rocked

gently to obtain a uniform suspension and were incubated at 36° for 24 hr. at which time extracts were added in amounts up to 0·1 ml.

Extracts were dissolved in ethanol at 20 mg./ml., and these solutions were serially diluted with PBS. Doses of 4, 2, 1, 0·5, 0·25, 0·12 and 0·06 μg. were used for extracts from surface cultures and a tenth of these amounts for extracts from rye grain and bran cultures. Sporidesmin was assayed over the range 24, 12, 6, 3, 1·5 and 0·75 mμg./ml. from dilutions of a 50 μg./ml. ethanolic solution.

The effects of the test solutions were usually determined by comparing the morphology of the cells in the test cultures with those in the controls after 1, 2, 3 and 4 days. The sporidesmin present was assessed on the basis of the least dose that gave a marked effect (least toxic dose). There was usually a clear difference between the effect of this dose and that of half the dose; on the other hand, the effect of twice and four times the dose appeared to be similar.

The estimation of protein in the cultures was based on the method of Oyama & Eagle (1956) and took account of the modified procedure of Miller (1959). It was found that the more concentrated sodium hydroxide solution of the modified method facilitated dissolution of the cells.

Corneal opacity test. About 10 mg. of an extract containing about 1% (w/v) sporidesmin (e.g. from a surface culture) was dispersed in 0·5 ml. of 1% (v/v) 'Tween 80' solution (L. Light and Co., Colnbrook, England) in a Griffith's tube; two four-fold serial dilutions were made and 0·05 ml. volumes of these solutions applied to the eyeballs of small New Zealand White rabbits (weighing 800–1600 g.) whilst the eyelids were held open and elevated from the eyeball. The instilled material was made to flow over the eyeball and the conjunctival membranes by gentle massage, using the elevated eyelids to disperse the material. On the following day similar amounts of the same dilutions were instilled in like manner. The eye lesions at these concentrations were compared with the lesions observed after instillation of 20, 5 and 1·25 μg. crystalline sporidesmin applied in 1% (v/v) 'Tween 80' solution in the same way. No reaction was noticed at the greatest dilution; at the smallest dilution congestion of the scleral vessels, with oedema and inflammation of the conjunctival membranes, was observed after 2–3 days. Four to five days after instillation this concentration produced marked corneal opacity which lasted for several weeks. A similar corneal opacity was observed at the intermediate dilution 5–7 days after instillation. Controls were not normally used because no lesions were observed after instillation of 0·1 ml. 5% (w/v) 'Tween 80' solution.

Iodometric estimations. The method was essentially the same as that used for penicillin (Clarke, Johnson & Robinson, 1949). Fifty mg. extract were accurately weighed, dissolved in ethanol and the solution made up to 20 ml. with ethanol. A 5 ml. volume was treated with 5 ml. 20% (w/v) sodium hydroxide solution and the mixture allowed to stand for 15 min. at room temperature. The resulting mixture was cooled in ice-water, acidified with 30% (v/v) acetic acid in water, 10 ml. 0·01 N-iodine added and the excess iodine titrated with standard sodium thiosulphate. Starch must be used for this end-point; otherwise it is obscure because of a yellow pigment which is present in the extract. A further 5 ml. volume of the extract solution was acidified with 30% (v/v) acetic acid in water, treated with 10 ml. 0·01 N-iodine and the iodine titrated with standard sodium thiosulphate immediately. Under these conditions 1 ml. 0·01 N-iodine was equivalent to 0·7 mg.

sporidesmin and the percentage sporidesmin in the sample was calculated from the expression $(x-y)280/z\,\%$, where x is the titre of the blank, y the titre of the alkali-treated sample and z the weight of the original sample. Sporidesmin frequently crystallizes with one molecule of solvent (Synge & White, 1959). The conversion factor given here for the iodine titration is based on the non-solvated molecule of molecular weight 475·5.

Sporidesmolides. The solvents used were of reagent grade. Activated charcoal (Darco, Grade G. 60) was obtained from the Darco Corporation, New York, U.S.A. The total sporidesmolide fraction, prepared as described by Russell & Brown (1960), was recrystallized from 70 % (v/v) acetic acid in water (m.p. 256–259°; corr.; $[\alpha]_D^{20} = -205°$, $c = 1\,\%$, chloroform). Diethyl ether for this determination was shaken with excess of the total sporidesmolide fraction for 24 hr. and filtered before use. When sufficient mycelium was available 10 g. were used; when less was available, the volumes of the reagents, etc., were all decreased in proportion. The final volume of chloroform filtrate and washings was also adjusted as follows: for 8–10 g. mycelium, 25 ml.; 6–8 g., 20 ml.; 4–6 g., 15 ml.; less than 4 g., 10 ml.

The dried mycelial felt was weighed and extracted with methanol in a Soxhlet apparatus for 16 hr. The methanol was removed on a rotatory evaporator at 50° and the residue transferred to a separating funnel in solution in a mixture of methanol (70 ml.), chloroform (100 ml.) and water (30 ml.). The mixture was well shaken, the phases allowed to separate, the lower layer collected and the solvents removed on a rotatory evaporator as before. The residue was dissolved in a mixture of chloroform (50 ml.) and ethanol (50 ml.); then benzene (50 ml.) was added and the solvents once more evaporated. The cooled residue was treated with sporidesmolide-saturated ether (80 ml.), mixed by gentle swirling and allowed to stand at room temperature overnight. The precipitate was collected quantitatively, washed with ether saturated with sporidesmolides and treated with chloroform (about 5 ml.) at 50°. Solution of the sporidesmolides was completed by gentle agitation for 1 hr. at room temperature. Activated carbon (0·1 g.) was formed into a pad on the surface of a sintered glass funnel and the chloroform solution (containing suspended particles) of the sporidesmolides was passed through under positive pressure. The vessel that contained the unfiltered chloroform solution and the filter were washed successively with further small quantities of chloroform until the volume of the combined filtrates was 25 ml. The solution was mixed and its optical rotation determined. The weight (mg.) of total sporidesmolides (W) was calculated from the expression $W = 4{\cdot}9\,\alpha\,V/l$, where α is the observed rotation, V the volume of the solution (ml.) and l the length (dm.) of the solution.

Spore counts were made as follows. The fungus filtered off was washed on the filter with distilled water and dried to constant weight in a vacuum oven at room temperature and 0·1 mm. Hg pressure over solid sodium hydroxide. This material was also used for the determination of the amount of sporidesmolides. A sample (about 50 mg.) was weighed and ground with 0·05 % (v/v) Lissapol N solution (5 ml.) until thoroughly dispersed; a duplicate sample was similarly treated. Several spore counts were done on each suspension by using a haemocytometer. The results given are the average of these counts.

Ash and dry weights. Media and filtered culture fluids were lyophilized in tared flasks to determine dry weights. The resulting solid was dissolved in the minimum

quantity of distilled water and transferred to tared silica crucibles. Solutions were evaporated to dryness in a desiccator and ashed to constant weight in a muffle furnace at 1000°.

Nitrogen was determined by the Kjeldahl method. The results (Table 4) refer to the total-N in the sample since the digests were not de-proteinized.

Sugars were determined by the method of Hanes (1929).

RESULTS

Estimations of sporidesmin and sporidesmolides were based on extracted material; thus some information was required about losses during this procedure. The sporidesmolides, because of their insolubility in water, were almost wholly present in fungal tissue. They were high melting point, cyclic depsipeptides, stable under the extraction conditions used and thus unlikely to undergo serious losses during the extraction process. Sporidesmin is unstable in alkaline solution, and parallel experiments showed decreased yields when solutions were held above 40°. Sporidesmin (5 %, w/v, extract from a previous batch added in ethanolic solution to an equivalent volume of a bulked surface culture) added to a culture before the extraction process was completely recovered when submitted to the isolation procedure; further extraction of the raffinate gave only traces of sporidesmin. When a rye grain culture had been extracted five times with 80 % (v/v) methanol in water some sporidesmin remained in the residue as it could be isolated by a sixth extraction. A more rapid extraction was achieved by using 80 % (v/v) ethanol in water but the extract was considerably cruder. Extraction was similarly facilitated in the case of bran cultures but it was not complete and the crude isolate was only 7 % sporidesmin.

Initially dilutions of fungal extracts were added to completed monolayers of HeLa cells for toxicity tests, but it was found difficult to assess a least toxic dose. Cytopathological effects at higher dilutions were mainly observed in the peripheral cells. The technique was therefore altered so that the cells were in groups when the doses were added. Reproducible results were then obtained. Toxic effects down to about the same concentration of pure sporidesmin were found by visually observed changes in cell morphology and by the more objective criterion of protein formation in cultures (Oyama & Eagle, 1956). Figure 1 shows the amounts of protein formed in cultures in the presence of different amounts of sporidesmin. The dose of sporidesmin was added in 1 ml. medium to 24 hr. cultures, and the protein estimated 5 days later. Results are expressed in terms of the amount of cell protein formed, that in untreated cultures being taken as 100. A sharp increase in protein formation occurred when doses were less than 3 mμg. A least toxic dose of 3 mμg. was repeatedly observed when using the visual method of assessment; this value has been used in computations of the amounts of sporidesmin in extracts of fungal material. Six fungal extracts were tested using both HeLa and transformed pig kidney cell cultures. The least toxic dose was found to be the same in four cases; in two cases the pig kidney cells dose required was twice the HeLa cell dose in order to produce a marked effect. The general appearance of the pig kidney cells at the least toxic dose concentration was characterized by shrinkage to spindle-shaped cells. HeLa cells by contrast, shrank into irregular shapes. Untreated cells of both lines were of like appearance.

Excellent agreement was usually observed (Table 1) when parallel estimations were made with the iodometric procedure and the tissue culture toxicity test. Exceptions to this were interesting and point a moral, for in these cases the biological method indicated that no, or very little, sporidesmin was present. The iodometric procedure must therefore be treated with reserve.

Sporidesmolides were determined by a quantitative adaptation of the normal isolation procedure. The recovery of sporidesmolides was determined by adding known weights of the total sporidesmolide fraction to measured quantities of a

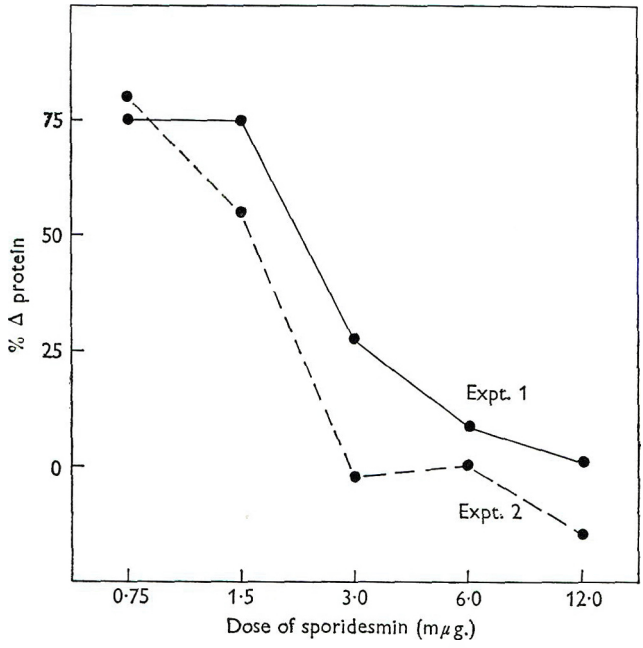

Fig. 1. Response of HeLa cells to sporidesmin doses, by colorimetric measurements of cell protein formed. Doses were added in 1 ml. amounts of medium to 24 hr. cultures which contained initially 50 μg. cell protein/ml. medium. Measurements of protein formed were made on the sixth day. Control (no dose) cultures contained 178 μg. (Expt. 1) and 68 μg. (Expt. 2) cell protein. % △ protein = protein formed as a percentage of that of controls.

methanol extract of mycelium. Quantitative recoveries of the added material were obtained. The assay provided a solution containing only sporidesmolides which current work has shown to consist essentially of two depsipeptides, closely related (Russell, 1960). The solutions were determined polarimetrically using the value obtained for the total sporidesmolide fraction of $[\alpha]_D^{20} = -205°$. The accuracy of the method depended on the constancy of composition of the mixture of sporidesmolides, thus confirmatory data were obtained by determining the weight of sporidesmolides in the solutions. Significant differences were not observed between the gravimetric and polarimetric methods.

The results shown in Table 1 were selected from about 120 batches of surface cultures because the data in these batches were fairly complete and the vessel to vessel variations were small. The sporidesmin results are based on extracts from

whole batches (usually 50–100 l.). Analyses of medium dry weight were made and also nitrogen, sugar and pH determinations both on bulked filtered medium at the end of the incubation and on averages of 5–10 sample vessels selected at random. The two series of results agreed well. The mycelium dry weights given were obtained by taking the arithmetical average of the results from 5–10 sample vessels selected at random. In Table 1 the results are expressed as differences between the values obtained before and after incubation; in this way better correlation between the analyses of medium constituents and the amounts of sporidesmin isolated were obtained. In most cases, however, the figure referring to the change in medium dry weight was proportional to the concentration of the starting medium.

Table 1. *Utilization of medium constituents and sporidesmin production by* Pithomyces chartarum

Batch no.	Filtered broth Δ dry wt. (mg./ml.)	ΔN (mg./ml.)	ΔSugar (mg./ml.)	Mycelium dry wt. (mg./ml.)	Sporidesmin HeLa	Sporidesmin R.E. (mg./l.)	Sporidesmin I_2
73A	—	0·12	4·7	4·0	0·3	—	0·3
63B	10·2	0·15	5·7	3·2	0·4	0·4	0·4
62	18·2	0·20	5·8	—	0·4	0·5	—
44	10·2	0·28	5·0	4·3	0·5	0·4	—
50	21·7	0·32	7·9	6·5	0·5	0·5	—
63A	18·5	0·34	6·7	5·9	0·7	—	—
111	15·7	0·34	7·0	6·1	0·7	—	0·8
68	19·1	0·55	11·8	7·9	1·0	—	1·1

The terms: 'Δ' refers to difference between analyses on starting media before inoculation and those on the final filtered broth; 'N' to nitrogen analyses; 'Mycelium dry wt.' to the total weight of fungal material; 'HeLa' to results obtained by the tissue culture method; 'R.E.' to the corneal opacity test; 'I_2' to results arising from iodometric estimations.

Table 2. *Utilization of sugars by* Pithomyces chartarum

Sugar added	Filtered broth Δ dry wt. (mg./ml.)	Mycelium dry wt. (mg./ml.)	ΔSugar (mg./ml.)	Final [sugar]* (mg./ml.)	ΔN (mg./ml.)
None	7·3	3·1	2·14	1·10	0·22
Glucose	9·0	4·6	5·60	1·60	0·22
Lactose	8·2	4·2	4·99	3·25	0·19
Galactose	7·3	3·2	2·10	6·10	0·20
Maltose	9·1	4·5	5·62	1·75	0·21

* The square brackets in column 5 indicate concentration; other symbols as in Table 1.

In Table 2 the results of adding 0·5% (w/v) of various sugars to the culture before inoculation are summarized. These results are based on repeated experiments which compared groups of three sugars with a control. The addition of the different sugars did not increase the yield of sporidesmin though all sugars except galactose increased the amount of fungal growth during a 7-day period of incubation. Only about half the added lactose was utilized; the effect of adding this sugar on the growth of the fungus was about half that of adding glucose. Since the addition of maltose had a similar effect to the addition of glucose the partial utilization of lactose may be explained by the apparent inability of the organism to use galactose.

The addition of ammonium sulphate (Table 3) increased growth in a 7-day fermentation but not the yield of sporidesmin. The analysis of filtrates of spent broth indicated that most of the added nitrogen remained in solution, but it is not known whether this was present as unchanged ammonium salts. It seems unlikely that the increase in growth was due to the sulphur added as other ammonium salts behaved similarly.

Table 3. *Effect of adding an inorganic and an organic nitrogen source to* Pithomyces chartarum *strain 73a fermentations on potato + carrot media*

Adjuvant	Initial N (mg./ml.)	Final N (mg./ml.)	Mycelium dry wt. (mg./ml.)	Sporidesmin HeLa (mg./l.)	Sporidesmin I_2 (mg./l.)
None	0·36	0·12	4·2	0·5	—
Ammonium sulphate	0·47	0·19	4·5	0·5	—
Ammonium sulphate	0·85	0·50	6·0	0·6	0·7
None	0·32	0·09	4·3	2·0	2·3
Glutamine	0·57	0·24	5·0	—	—
Glutamine	0·82	0·42	5·7	—	2·8
Glutamine	1·32	0·93	5·9	3·8	4·1

'Initial N' and 'Final N' refer to analytical values for nitrogen after adding adjuvants and before inoculation and after fermentation for 7 days. Other symbols have the same meaning as before.

Table 4. *Sporidesmin isolated after growth of* Pithomyces chartarum *on media from different plant sources*

Batch no.	Medium	N (% dry wt.)	Yield of sporidesmin (mg./100 g. dry medium)
63A	Potato + carrot	1·8	2·6
60	Corn steep liquor*	2·64	0·85
104	Rye corn	1·95	12·9
108	Bran	3·15	19·6

Batches 104 and 108 were 28-day fermentations. Similar results were obtained in numerous repeat experiments.

* Corn steep liquor, obtained from Messrs Clifford Love and Co., Sydney, Australia, was collected as the boat berthed and was used the same day.

Semi-quantitative, two dimensional, paper chromatography (kindly carried out by Mr G. R. Russell) of the amino acids present in lyophilically dried samples of culture filtrates taken at 2, 3, 5 and 7 days after inoculation showed a steady decrease in the quantities of these substances present. As glutamine appeared to be utilized more rapidly than the other amino acids it was chosen as a source of organic nitrogen. Again most of the nitrogen added remained in the solution at the end of the fermentation (Table 3). Increased growth was observed but also yields of sporidesmin and the sporidesmolides were increased almost in proportion to the quantity of glutamine added. The experiments relating to the addition of glutamine summarized in Table 3 were carried out with a different strain of the organism from that (strain C) used in the rest of this work. This strain (see Methods) produced

about four times as much sporidesmin when grown under exactly the same cultural conditions as did strain C. Thus a two-fold increase in sporidesmin production, on addition of 0·1 % (w/v) glutamine, was more convincing in the case of the former strain because of the limits of accuracy of the analytical methods.

These results suggested that higher yields of sporidesmin were associated with rich starting medium containing glutamine, so the growth of the fungus and its production of sporidesmin and sporidesmolides was investigated on wet grain and bran.

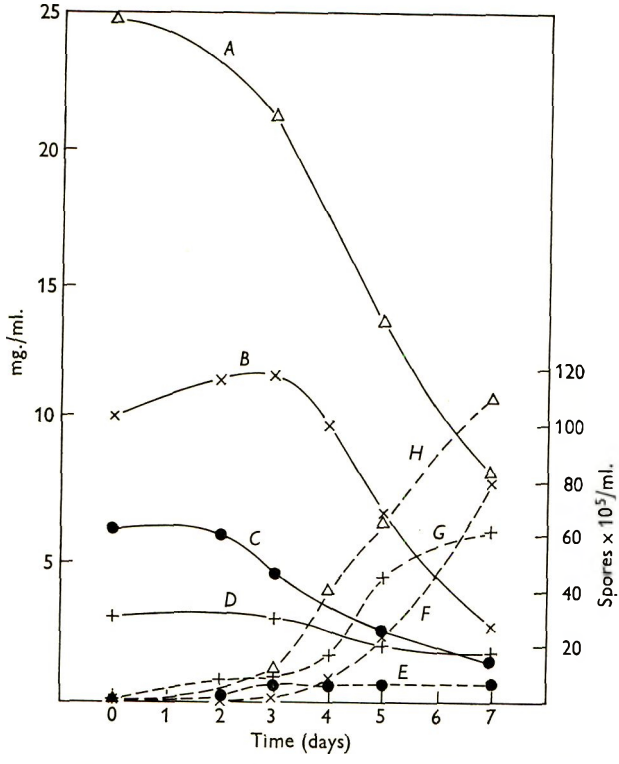

Fig. 2. Utilization of medium constituents and production of sporidesmin and sporidesmolides as a function of culture age. *A*, Dry weight of media; *B*, sugar; *C*, nitrogen (× 10); *D*, ash; *G*, mycelium dry weight; *H*, spore count; *F*, sporidesmolides (× 100); *E*, sporidesmin (× 1000).

Lloyd & Clarke (1959) showed that *Pithomyces chartarum* grows well on rye grain (*Secale cereale*) and this has been confirmed in this work. Considerable precautions were required to make sure that residual contaminants were absent after autoclaving. Table 4 compares the yield obtained after culture on rye grain and bran with that obtained on potato carrot medium and corn steep liquor.

Figure 2 summarizes results relating to the change with time of medium dry weight, medium ash values, sugars, nitrogen, fungal growth and spore, sporidesmin and sporidesmolide formation obtained partly from about 120 batches but particularly from six of these batches in which all the analytical data were obtained. The apparent rise in sugar concentration (determined iodometrically) in the first 2–3 days of the fermentation has been noted frequently. In the absence of ammonium salts

the pH value of the medium increased by more than 2 units in a 7 day fermentation. It was found that this gave a good indication of the growth of a culture. The addition of ammonium salts had a buffering effect; changes of only 0·5–1 unit were observed in this case.

Only traces of sporidesmin were isolated from cultures less than 72 hr. old. After this time the quantity of this metabolite isolated from a given batch did not vary within the limits of accuracy of the analytical methods used. Thus sporidesmin appears in the fermentation at the start of vigorous growth.

The weight of sporidesmolides isolated from unit volume of culture medium is plotted in Fig. 3 as a function of spore numbers in the same volume. Two relations are apparent. Thus the linearity of the relation is independent of the time of fermentation and of the particular experiment.

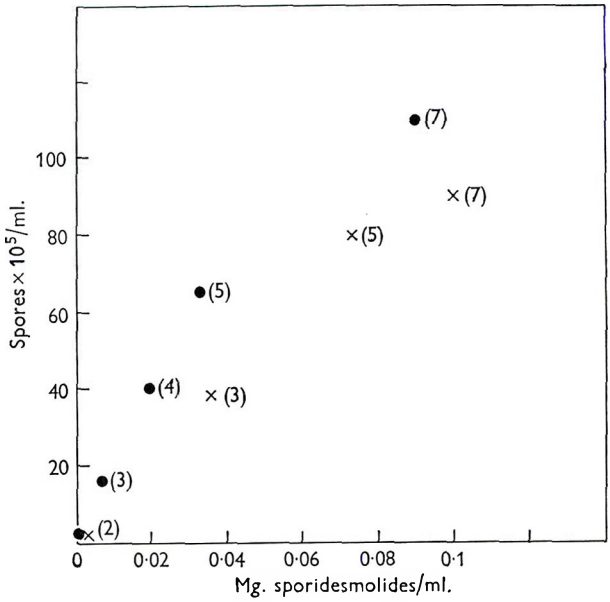

Fig. 3. Spore count/ml. medium as a function of sporidesmolide production by *Pithomyces chartarum*. ● ●, Batch no. 111; × ×, batch no. 118. Figures in parentheses indicate the age of culture (days) at which the particular analysis was made.

DISCUSSION

At the beginning of this work, it was clear that the production of sporidesmin by *Pithomyces chartarum* growing on potato carrot medium was poor. Antibacterial bioassay could not be used and other methods had to be found. A cytopathological effect of extracts of *P. chartarum* on cultures of HeLa cells was demonstrated by Murphy & Worker (1960). Evidence that this cytopathological effect was specifically due to sporidesmin was obtained by an examination of least toxic dose values on alternate fractions from the reversed phase partition chromatogram of Synge & White (1959). Perfect correlation between the highest dilution exhibiting the least toxic dose and a maximum in fraction weight was observed. Side fractions were inactive. The effective concentration of sporidesmin for HeLa cells was about

10^7 molecules/cell; this is about the lowest effective cytopathological concentration of the more cytotoxic compounds (colchicine and actinomycin D) studied by Eagle & Foley (1958).

The application of tissue culture toxicity tests to the examination of many samples from a production programme is severely restricted on a small budget. Recently (Done, Mortimer & Taylor, 1960) the inflammatory nature of the changes found in the livers of sheep showing clinical facial eczema has been emphasized. It seemed that these phenomena might be due to the presence of a toxin ingested from pasture. Further speculation associated this toxin with sporidesmin. It therefore seemed pertinent to investigate the possibility that inflammatory changes could be induced by extracts containing this material on tissues other than the liver. Such extracts were therefore instilled into rabbit's eyes and were shown to initiate the changes described in the Methods section and shown in Plate 1. Larger amounts than those described in the assay procedure cause correspondingly more severe inflammatory changes which also develop more rapidly. At the highest dose used (40 μg.) severe oedema was seen 36 hr. after the second instillation and a severe keratitis and ulceration followed with the formation of a sedimented layer of leucocytes in the anterior chamber of the eye. This procedure was used in the earliest stages of this work, before crystalline sporidesmin was available, and proved useful for comparing the biological potency of materials from different sources. It was simple to carry out, and later the results showed good agreement with those obtained by the tissue culture toxicity test and by the iodometric assay.

The possibility was considered that a limiting factor in the formation of sporidesmin by *Pithomyces chartarum* was the early exhaustion from the medium of a specific precursor of this metabolite. The fermentation displays some similarities to penicillin fermentations and the change in pH value of the medium suggested that a precursor might be acidic. However extracts of acidified starting medium did not increase the isolated yield of sporidesmin from cultures to which the extracts had been added. An explanation of this negative result might be that the postulated precursor is water soluble and not extractable under the conditions used. The effect of glutamine might be support for this view; however, the latter effect may be analogous to the effect of methionine in the production of cephalosporin N by *Cephalosporium acremonium* (C.M.I. Herbarium no. 49,137; Miller, Kelly & Newton, 1956).

The number of examples of macrocyclic peptide-like substances isolated from cultures of bacteria and fungi is rapidly increasing. In the case of bacteria the compounds are usually true cyclic peptides, e.g. polymyxin B_1 (Hausmann, 1956; Biserte & Dautrevaux, 1957). One example of this group, bacitracin, has been shown to be closely associated with the sporulation process of *Bacillus licheniformis* (Bernlohr & Novelli, 1960). Two metabolic products, valinomycin (Brockmann & Geeren, 1957) and amidomycin (Vining & Taber, 1957) have been isolated from species of *Streptomyces*; these compounds are macrocycles consisting of eight units of alternative α-hydroxy- and α-amino acids. The similarity of these compounds to the enniatins studied by Plattner and his co-workers (see, for example, Plattner & Clauson-Kaas, 1945; Plattner & Nager, 1948) was pointed out by Young (1957). This group of compounds occurs widely in *Fusarium* spp. (Lacey, 1950) and they are, structurally, closely related to the sporidesmolides (Russell, 1960). The

results in Fig. 3 support the hypothesis (Russell & Brown, 1960) that the sporidesmolides behave as an impermeable conidial coat. In view of the structural relationship of the whole of this group of substances it seems possible that they may behave generally in this way.

The practical importance of predicting the toxicity of pasture and the elucidation of the possible role of the fungus in connexion with facial eczema perhaps excuse speculation on the application of the results reported in this paper to the understanding of the growth of the organism in the field. It is widely held that pastures become toxic to grazing animals when pasture plants are growing rapidly (see, for example, Filmer, 1958). Should these conditions favour rapid growth of the fungus the results in Fig. 2 suggest that sporidesmin is produced at the onset. Thus techniques such as field counting of spores are unlikely to be of use in forecasting an outbreak of the disease since high spore counts will post-date the onset of toxicity in a pasture. The production of sporidesmin on four different undefined (Table 4) and defined (Ross, 1960) media also suggests that changes in the common species present in pasture will not affect the growth of the organism nor its ability to produce sporidesmin.

This paper was delivered in part before the New Zealand Society for General Microbiology at Dunedin, August 1960. We are indebted to Dr C. P. McMeekan for the provision of facilities. Mr J. W. Ronaldson ran the partition chromatograms; this work will be reported in detail elsewhere. We have had many helpful discussions with Miss J. M. Dingley and Dr R. H. Thornton who have also provided us with cultures. Miss H. Fountain carried out many nitrogen and sugar analyses and Mr G. R. Russell iodometric assays. We thank Miss M. E. Brown, Mrs P. R. Carnegie and Mr D. A. Lambourne for technical assistance.

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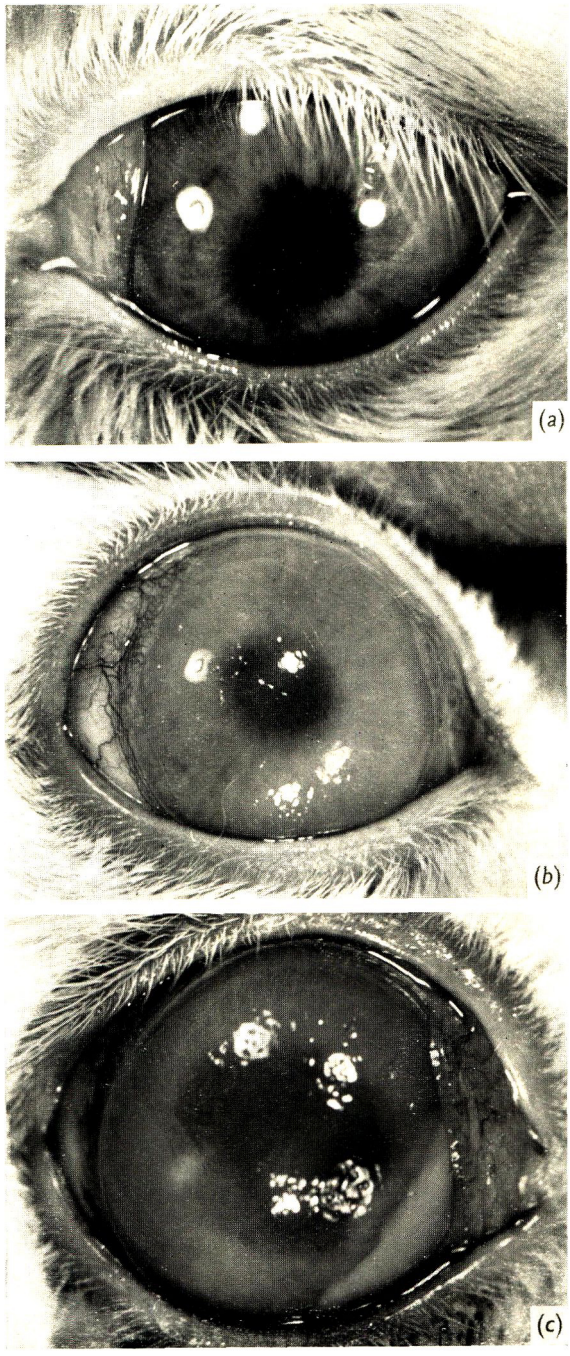
EXPLANATION OF PLATE 1

(*a*) A normal rabbit's eye.

(*b*) Eye showing congestion and proliferation of blood vessels at the corneo-scleral junction. The cornea shows obvious opacity due to migrating leucocytes in its substance. This type of lesion was produced by the instillation of 20 μg. sporidesmin into the conjunctival sac.

(*c*) Eye showing changes described in (*b*) but with more severe corneal opacity. Note especially the white layer of leucocytes which is present in the anterior chamber, having sedimented down at the inner (lower) canthus of the eye. The *membrana nictitans* is seen to be inflamed and oedematous. This severe eye lesion was produced by the instillation of 40 μg. sporidesmin. Plate 1 (*a*) and Pl. 1 (*c*) are respectively the left and right eye of the same animal.



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Growth of Leptospira in Defined Media

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SUMMARY

Serum-free chemically defined media were developed for cultivating several Leptospira strains. *Leptospira canicola*, Ruebeusch strain, was the main test strain. Esterified fatty acids (monoolein, monostearin, methyl palmitate, methyl oleate) replaced serum. Fe^{++} over a narrow range replaced haemin. Thiamine was essential. Other vitamins added to thiamine-containing medium (nicotinic acid, pantothenate, putrescine, *p*-hydroxybenzoic acid, vitamin B_{12}) speeded or increased final growth. Acetate stimulated growth moderately. Ammonium sulphate was favourable over a wide concentration range.

INTRODUCTION

Many investigators (e.g. Woratz, 1955; Fulton & Spooner, 1956; Alston & Broom, 1958; Broom, 1959) have noted that leptospires generally require serum or serum fractions for growth; nevertheless, Savino & Rennella (1944) and Woratz (1957) serially transferred *Leptospira icterohaemorrhagiae* and *L. canicola* without serum or serum fractions. Woratz's medium contained gelatin and a tryptic digest of casein. Savino & Rennella could grow only 4 of their 12 strains in a defined medium containing 'activators'—a mixture of vitamins (nicotinic acid, nicotinamide, thiamine, pyridoxine, riboflavin, pimelic acid) and aspartic acid. The present paper describes a chemically defined medium which permits continued serial growth of various leptospires.

METHODS

Leptospira canicola, Ruebeusch strain, was the initial experimental organism. This strain, originally carried in Korthof medium (Alston & Broom, 1958) containing 10 % (v/v) pooled rabbit serum, was transferred to agar-free basal medium (BM-AF, Table 1) containing 1 % (v/v) serum. This culture served as inoculum for preliminary experiments. A standard loopful (3 mm. diam.) covered with a 10 mm. $\times$ 10 mm. coverglass had about 6 leptospires/high-dry dark field ($\times$ 440) after incubation for 7–14 days at 30°.

For counts by dark field, 0·01 ml. of culture was put on a slide and covered with a 22 mm. coverglass. As determined by ocular micrometer, the area of a high-power ($\times$ 530) field was 0·0962 mm.^{2} The number of fields per coverglass was 484 mm^2. (area of the coverglass)/0·096 = 5040. The count/ml. was thus obtained by multiplying the count per high-power field by 5040 $\times$ 100. Usually 20 fields were used for

a count, and duplicate counts agreed to within 10 %. With sparse cultures counting was done at a × 100 magnification.

The same concentration of serum in BM medium (containing 0·1 % agar) permitted faint growth after 2 weeks as a faint line about 20 mm. below the surface (Dinger's phenomenon: Fletcher, 1928; Lawrence, 1951; Czekalowski, McLeod & Rodican, 1954). Serum at 0·1 % (v/v) did not give visible growth in 2–3 weeks.

Cultures of *Leptospira canicola* intended as inocula were transferred serially in BM-AF medium containing 1 % (v/v) serum. The initial nutritional experiments were undertaken in BM medium containing 1 % (v/v) serum.

The ingredients required for 10 ml. of final medium were dissolved in 9 ml.; the inoculum brought it to 10 ml. Inoculation was by means of a Cornwall side-arm syringe (Becton–Dickenson, B-D, no. 1251) delivering 1 ml. through a short 15-gauge needle passing through a Pyrex filling attachment (Corning No. 3960). Connexions were made between the syringe and needle by means of B-D adapters H 468/L and L/606 inserted in 3 mm. diam. gum-rubber tubing. The inside delivery tip of the attachment was filed back to allow the needle to project freely. Inoculation was made after the autoclaved medium had cooled to about 37°, before solidification of the agar, and was forceful enough to mix the contents of the tube.

It was convenient to prepare BM medium 5-fold concentration without agar. The pH value was adjusted with Quadrol (*N*,*N*,*N'*,*N'*-tetrakis (2-hydroxypropyl)-ethylenediamine; Wyandotte Chemicals Corp., Wyandotte, Mich., U.S.A.; Hutner, Cury & Baker, 1958), a few drops of volatile preservative added (Hutner *et al.* 1958), and the concentrate stored in the refrigerator. Test substances were prepared in ten-fold concentration and preserved similarly.

Since distillates from bleached cotton can inhibit fatty acid-sensitive organisms (Drea, 1946), the × 16 150 mm. Pyrex culture tubes were capped with inverted shell vials. Later experiments were made in 50 ml. screw-cap flasks containing 20 ml. medium. Media were autoclaved at 121° for 15 min.

RESULTS

As growth-promoters were identified, a serum-free but complex medium was assembled (Medium 283, Table 1) which supported serial growth of *Leptospira canicola*, also *L. pomona* L-9 and *L. icterohaemorrhagiae* N.I.H. strain. Dissection of medium 283 to identify its active components led to several defined agar-free media (Table 1) which supported the growth of the three original strains and 9 of 11 additional strains of *Leptospira* (Table 2). The point of departure in developing the defined medium was the observation that a supplement of 1 % (v/v) serum permitted growth of *L. canicola*, Ruebeusch strain, in BM and BM-AF media; 0·1 % (v/v) serum was ineffective.

Proteose peptone (Difco) increased growth in the presence of 1 % (v/v) serum but did not replace serum. Liver fraction 'L' (Nutritional Biochemicals Corp., Cleveland 28, Ohio, U.S.A.), soluble starch and haemin were inert or inhibitory at higher concentrations.

The Fe:Co ratio was critical; Table 3 shows a typical experiment.

The three original test strains did not grow as well in defined media as in Korthof medium containing 10 % (v/v) serum; growth however did exceed that obtained

with 1% (v/v) serum; many strains (Table 4) grew well although less than in Korthof medium with 10% (v/v) serum.

In serum-free medium, 0·5% (v/v) of a blunderbuss mixture consisting of an acid-hydrolysate of gelatin, L-tryptophan, DL-methionine, alkali-hydrolysed yeast nucleic acid, acid-hydrolysed deoxyribonucleic acid and vitamins (complete

Table 1. *Serum-free and chemically defined media for leptospires*

Media were adjusted to final pH 7·4–7·5. Sterilization was by autoclaving at 121° for 15 min.

Quantities as mg. or ml./100 ml. final medium.

	Basal medium (BM)	Transfer medium no. 283	Serum-free medium no. 198A	Chem.-def. medium no. 198B	Chem.-def. medium no. 198E	Chem.-def. medium no. 198F
KH_2PO_4	10	10	10	10	10	10
$MgSO_4.7H_2O$	40	40	40	40	40	40
Ca^{++} (as Cl^-)*	0·4	0·4	0·4	0·4	0·4	0·4
Agar, Special† (Noble, Difco)	100	100	.	.	.	.
HEDTA‡	.	2·0	2·0	2·0	2·0	2·0
TEM-4T§	.	3·5	3·5	.	.	.
Hycase SF‖	.	10	.	.	.	.
'Complete suppl. No. 9'	.	0·5 ml.	.	.	.	.
'Metals 45A'	.	1·2 ml.	.	.	.	.
Fe^{++}	.	.	0·1	0·1	0·1	0·1
Mn^{++}	.	.	0·015	0·015	0·015	0·015
Zn^{++}	.	.	0·15	0·15	0·15	0·15
Cu^{++}	.	.	0·008	0·01	0·01	0·01
Na acetate.$3H_2O$	.	.	5·0	5·0	5·0	5·0
Co^{++}	.	.	0·0015	0·01	0·01	0·01
$(NH_4)_2SO_4$	.	.	.	20	20	20
Nicotinic acid	.	.	.	0·02	0·02	0·02
Thiamine HCl	.	.	0·04	0·04	0·04	0·04
Ca pantothenate	.	.	.	0·02	0·02	0·02
Pyridoxamine.HCl	.	.	.	0·02	0·02	0·02
Vitamin B_{12}	.	.	.	0·00002	0·00002	0·00002
DL-Alanine	.	.	.	.	10	.
DL-Aspartic acid	.	.	.	.	50	.
DL-Isoleucine	.	.	.	1·0	1·0	.
DL-Leucine	.	.	.	1·0	1·0	.
DL-Methionine	.	.	.	.	0·5	.
DL-Phenylalanine	.	.	.	5·0	5·0	.
DL-Serine	.	.	.	.	1·0	.
DL-Tryptophan	.	.	.	.	2·0	.
DL-Valine	.	.	.	.	5·0	.
Glycine	.	.	.	.	1·0	.
L-Glutamic acid	.	.	.	.	10·0	.
L-Histidine HCl.H_2O	.	.	.	.	25	.
L-Proline	.	.	.	.	2·0	.
Monoolein	.	.	.	2·0	2·0	2·0

* Ca was weighed as $CaCO_3$ and dissolved in dilute HCl.

† Agar may be eliminated = BM − AF.

‡ HEDTA = hydroxyethylethylenediamine-triacetic acid.

§ Supplied by Hachmeister Inc., P.O. Box 357, Pittsburgh, 30, Pa., U.S.A. TEM-4T is a mixture of diacetyl tartaric acid esters of glycerides from tallow.

‖ Hycase SF = 'low-salt' acid-hydrolysed casein, Sheffield Chemical Co., Norwich, New York, U.S.A.

supplement No. 9; Hutner *et al.* 1957), supported growth provided that a semi-synthetic fat, TEM-4T (Shorb & Lund, 1959; a mixture of diacetyl tartaric acid esters of glycerides from tallow) was present. Addition of Hycase SF (Sheffield Chemical Co., Norwich, N.Y., U.S.A.), a 'salt-free' acid hydrolysate of casein, increased growth further (Table 5). Of the components of the 'complete supplement', the alkali-hydrolysed yeast nucleic acid had a slight effect, the vitamin mixture somewhat more.

Absolute requirements; lipids and vitamins. Thiamine was essential. Supplementation with nicotinic acid or pantothenate speeded growth. Putrescine also was

Table 2. *Strains of Leptospira investigated*

	Designation	Culture no.
L. autumnalis	Fort Bragg	5287*
L. ballum	Garcia	5303*
L. bataviae	EER (AM 2)	5304*
L. canicola	Ruebeusch	RU 43†
	Utrecht	5937*
	Undesignated	39660*
L. grippotyphosa	Andaman CH 31	5862*
L. icterohaemorrhagiae	Wijnberg	5309*
	Undesignated	39661*
	M 20	5938*
	NIH	NIH†
L. pomona	Johnson	5939
	Undesignated	L-9†

* Supplied through the courtesy of Dr A. H. Harris and Julia M. Coffey, Div. Labs., N.Y. State Department of Health. See *Annual Rep. Div. Laboratories and Research*, 1953, New York State Department of Health, Albany, N.Y., p. 149, 'Collection of Type Cultures'.

† Original strains from Communicable Disease Center, U.S. Public Health Service, Chamblee, Georgia, U.S.A.

Table 3. *Iron-cobalt relations for growth of* Leptospira canicola (*Ruebeusch strain*)

Medium: basal medium (100 ml.) + TEM-4T*, 3·5 mg. + HEDTA† 2 mg. + 'vitamin mix No. 12', 0·002 ml.; agar ('Noble', Difco, 0·1 %, w/v); pH 7·4–7·5. Fe^{++} was added as $Fe(NH_4)_2(SO_4)_26H_2O$. Growth recorded after incubation for 14 days at 30°. Arbitrary scale: 0 = no growth; + + + + = growth as in Korthof + 10 % (v/v) serum medium. Quantities of Fe^{++} and Co^{++} are in mg./100 ml. medium.

	Co^{++} (mg.)				
Fe^{++} (mg.)	No addition	0·0125	0·025	0·05	0·10
	Relative growth				
No addition	0	0	0	0	0
0·031	0	0	0	0	0
0·062	0	0	0	0	0
0·12	+ +	+ +	+ +	+ +	+ +
0·25	+ +	+ +	+	+	0
0·5	0	+	+	0	0
1·0	0	0	0	0	0

* TEM-4T = a mixture of diacetyl tartaric acid and esters of glycerides of tallow (see Table 1).
† HEDTA = hydroxyethylethylenediamine-triacetic acid.

favourable but not so clearly as was nicotinic and pantothenic acids; *p*-hydroxybenzoic acid and vitamin B_{12} also stimulated slightly.

In the presence of 1 % (v/v) serum, the optimum concentration of TEM-4T was 3·5 mg./100 ml.; Tween 80 supported growth from about 1 to 80 mg./100 ml. Tweens 60 and 40 were less satisfactory. Cholesterol at 0·5–4 mg./100 ml. and soybean lecithin from 0·6–5 mg./100 ml. were without effect in the presence of serum and did not replace serum.

In serum-free media, monacetin, methyl linoleate, and methyl linolenate at 0·5–

Table 4. *Growth responses of Leptospira strains in serum-free chemically defined media (198B, 198E, 283; see Table 1); and in Korthof medium+10% (v/v) pooled rabbit serum*

Flasks inoculated with 10^5 leptospires/ml. Most inocula made from cultures in 11–15th transfer except *L. canicola* no. 5937 (6th transfer). *L. canicola* (Ruebeusch) inoculation made from culture in 38th transfer; in medium 283; *L. pomona* (L-9) and *L. icterohaemorrhagiae* (N.I.H.) from 28th transfer.

	Defined medium						Korthof 10% (v/v) rabbit serum	
	198B		*198E*		*283*			
Leptospira spp. species and strain	Days	Max. growth (leptospires $\times 10^{-6}$/ml.)	Days	Max. growth (leptospires $\times 10^{-6}$ ml.)	Days	Max. growth (leptospires $\times 10^{-6}$ ml.)	Days	Max. growth (leptospires $\times 10^{-6}$ ml.)
L. bataviae no. 5304	14	40	10	120	.	.	10	360
L. canicola no. 39660	10	85	14	44	.	.	17	140
L. canicola no. 5937	14	65	7	35	.	.	10	220
L. canicola Ruebeusch	14	25	.	.	7	10	10	610
L. grippotyphosa no. 5862	.	.	10	100	.	.	10	600
L. icterohaemorrhagiae no. 5938	14	45	10	66	.	.	14	360
L. icterohaemorrhagiae no. 39661	10	100	10	80	.	.	10	170
L. icterohaemorrhagiae N.I.H.	7	60	.	.	7	10	10	600
L. pomona No. 5939	17	33	14	92	.	.	17	800
L. pomona L-9	.	.	.	.	14	.	17	1200

Table 5. *Hycase SF*/TEM-4T† relationships for growth of* Leptospira canicola

Basal medium: HEDTA 2 mg./100 ml.; metals 45A 1·2 mg./100 ml.; 'complete supplement no. 9', 0·5 ml./100 ml., 'Noble' agar 0·1 % (w/v); pH 7·4–7·5.

	Hycase conc. (mg./100 ml.)				
TEM-4T (mg./100 ml.)	No addition	40	20	10	5
10	+	+	±	±	±
5	±	++	++	++	+
2·5	0	0	±	+	+
1·25	0	0	0	0	±

* Hycase SF = low-salt acid hydrolysed casein (see Table 1).

† TEM-4T = mixture of diacetyl tartaric acid esters of glycerides of tallow (see Table 1).

8 mg./100 ml. were unsatisfactory fatty-acid sources. Monoolein, methyl oleate, methyl palmitate or monostearin could replace TEM-4T; monoolein was best. A mixture of 2 mg. each of monoolein, monostearin and methyl palmitate/100 ml. allowed earlier but not consistently heavier growth.

Table 6. *Serial transfer of Leptospira strains in serum-free chemically defined media*

	Media									
	198A		198B		198E		198F		283	283SS*
Inoculum size†	10%	50%	10%	50%	10%	50%	10%	50%	10%	10%
Species	No. of successful transfers									
L. autumnalis no. 5287	7‡	19	.	23	.	3‡	12‡	23	.	.
L. ballum no. 5303	.	2‡	.	2‡	.	3‡	.	1‡	.	.
L. bataviae no. 5304	21‡	21	25	24	16	.	6‡	18	.	.
L. canicola no. 39660	17	18	25	23	21	21	14‡	23	.	.
L. canicola no. 5937	22	19	1‡	7‡	10	.	1‡	10	.	.
L. canicola (Ruebeusch)	22	23	25	24	1‡	14	20	23	38	39
L. grippotyphosa no. 5862	16‡	17‡	4‡	11	19	15	22	19	.	.
L. icterohaemorrhagiae no. 5938	16‡	14‡	23	24	23	19	9‡	23	.	.
L. icterohaemorrhagiae no. 3228	17	22	.	4‡	.	4‡	.	4‡	.	.
L. icterohaemorrhagiae no. 39661	.	18	22	24	21	19	22	25	.	.
L. icterohaemorrhagiae no. 5309	.	4‡	.	3‡	.	3‡	.	5‡	.	.
L. icterohaemorrhagiae (N.I.H.)	14	25	18	.	1‡	3‡	17	18	29	.
L. pomona no. 5939	20	22	25	22	22	18	13‡	23	.	.
L. pomona (L-9)	6	23	.	3‡	.	3‡	17	13	29	.

* Semi-solid medium.
† Inoculum size; % values refer to volume of culture transferred to volume fresh medium.
‡ Died.

Amino acids. The values given in this section are mg. compound/100 ml. medium. Slight growth stimulation was obtained with glycine (0·8–3 mg.), DL-alanine or L-glutamic acid (12·5–50 mg.), L-lysine HCl or DL-methionine (1·6–6·25 mg.), L-proline or DL-valine (2·5–20 mg.), and DL-aspartic acid (50–100 mg.). Moderate stimulation was obtained by L-histidine (free base) (12·5–50 mg.), DL-isoleucine (1·2–5·0 mg.), DL-leucine (0·65–2·5 mg.), DL-serine (0·008–0·125 mg.), and DL-tryptophan (1·25–10 mg.). Marked stimulation was obtained with DL-phenylalanine (0·6–20 mg.). L-Arginine HCl (0·4–3·1 mg.), DL-threonine (0·078–2·5 mg.), and L-tyrosine (0·078–0·156 mg.) were inert.

A mixture of metals '45A' (Hutner *et al.* 1957) supported growth. Fe^{++} alone supported growth only in a narrow range (0·125–0·5 mg). Hycase became unnecessary for growth with adequate Fe^{++}. Individually added to Fe, Co (0·001–0·04 mg.), Cu (0·0015–0·24 mg.), Mn (0·015–0·02 mg), or Zn (0·005–0·34 mg.) augmented growth. Since the metal requirements were critical, metal-buffering with the chelating agent of hydroxyethylethylenediamine-triacetic acid (HEDTA;

Provasoli & Pintner, 1960) was inhibitory at low concentrations of serum or TEM-4T. HEDTA 2 mg. was optimal in the presence of low concentrations of serum or in chemically defined media containing the standard metal mixture (Table 1). Acetate was moderately stimulating. Ammonium sulphate increased growth remarkably over a very wide range (1·95–125 mg.). Table 4 shows the growth responses of various leptospiral strains in the experimental media developed. Table 6 indicates the number of successful transfers made when either an equal volume of inoculum was added to fresh media or a 1:10 ratio of inoculum to fresh medium was used.

DISCUSSION

Previous workers were unable to replace serum with amino acids and vitamins (Chang, 1947*a*, *b*; Green, Camien & Dunn, 1950), or synthetic ion-exchange resins, charcoal or polyvinylpyrrolidone (Marshall, 1949; Fulton & Spooner, 1956; Johnson & Wilson, 1960). That lipid requirements were the major obstacle had been foreshadowed; defatted fraction V of blood serum no longer stimulated respiration of *Leptospira icterohaemorrhagiae* (Helprin & Hiatt, 1957) or supported growth of treponemes (Oyama, Steinman & Eagle, 1953). Our work confirms that of Woratz (1957) and Ivler (1960) that oleic acid esters promote growth of Leptospira. As their media had complex natural materials, one could not tell whether the fatty-acid requirement was absolute. A close parallel to our results are those of Power & Pelczar (1959), who found TEM-4T an advantageous source of essential fatty acids for the Reiter treponeme; a mixture of palmitic, stearic and oleic acids permitted the same degree of growth.

That the Fe concentration is critical probably accounts for the seeming dependence of these organisms on compounds such as haemoglobin. Strains not having catalase may appear to need exogenous haeme. Fe seems adequate for strains with catalase. Faine (1960) found appreciable amounts of catalase in certain pathogenic Leptospira strains. The optimum concentration of Fe for *Leptospira canicola* agrees well with that reported by Faine (1959) for *L. icterohaemorrhagiae*.

Thiamine, found stimulatory by others, e.g. Savino & Rennella (1944), may well be an absolute requirement for all leptospires.

The results with amino acids obtained by other investigators are difficult to compare with ours; different basal media were used, the presence of serum complicates matters, and the purity of amino acids has improved in recent years. Growth of a wide range of strains in chemically defined media was achieved here without introducing new factors, merely by applying information scattered in the literature. Whether strains which will not grow in the present defined media require additional growth factors or are exceptionally sensitive to nutritional imbalances remains to be decided. Another problem is to improve defined media so that they permit growth equal to or greater than that in media which contain complex natural materials such as serum.

More specific, more conveniently prepared antigens for diagnosis of leptospiral diseases are needed as recognition grows of the gravity and economic importance of these infections in animals and man. The availability of defined media may lead to better media for diagnosis by direct cultivation and to practical antigen-free media for the preparation of diagnostic antigens.

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Distribution of Urease in *Clostridium perfringens* Types

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SUMMARY

About five hundred strains of *Clostridium perfringens* were tested for ability to produce urease. There was found to be a distinct, although not complete, correlation between urease production and antigenic type. In types B and D there was also some degree of correlation between the production of urease and of λ-antigen. The distribution of urease between the six antigenic types of *C. perfringens* was not sufficiently sharp to be of use in type differentiation.

INTRODUCTION

Clostridium perfringens has been subdivided into six types: A to E distinguished by the production of one or more of the major lethal toxins α, β, *e* and ι; and F, which on the basis of toxin production would be included in type C, distinguished mainly by heat resistance, large cell size and epidemiology (Wilsdon, 1931; Glenny *et al.* 1933; Oakley, Warrack & Warren, 1948; Oakley, 1949; Ross, Warren & Barnes, 1949; Zeissler & Rassfeld-Sternberg, 1949). Each of these types, including F, is further characterized by the production of a number of minor antigens, enzymic in nature, and distinguished by their effects on appropriate substrates and by the neutralization of these effects by the corresponding type antisera. In the course of a general survey of the cultural and biochemical characteristics of various *Clostridium* spp. a number of *C. perfringens* strains was examined for ability to produce urease. Since some of these gave positive and others negative results, a large number of strains was examined to determine whether any correlation existed between urease production and antigenic type. The results of this survey, together with the results of experiments attempting to elucidate the nature of *C. perfringens* urease, are reported in this paper.

METHODS

Strains examined. Four hundred and ninety-two strains were examined, divided between types as follows: type A, 267 strains; type B, 63; type C, 64; type D, 68; type E, 10; and type F, 20 strains. Each of the ecological subtypes recognized within types B and C was represented (Brooks, Sterne & Warrack, 1957), and the strains were drawn from a wide range of habitats, geographically widespread and including pathological and non-pathological sources. Classical and vaccine-production strains and freeze-dried and freshly isolated cultures were included in the survey.

Preparation of test material. Fifteen ml. amounts of Robertson's cooked meat medium in one ounce screw-cap bottles were inoculated from broth cultures, or

with colonies picked from the surface of blood-agar plates, and incubated at 37° for 18–24 hr. Some strains were also grown in 57 ml. amounts of a similar medium, and samples tested after 5, 24, 48 and 72 hr. incubation.

Duplicate 3·0 ml. samples from well-grown cultures were placed in small glass tubes and centrifuged. The supernatant fluids were discarded and the sediments washed once in 1·0 % (w/v) saline. The washed sediments were used for testing without any further processing or addition of preservatives. Some samples were given up to five further washings with 1·0 % saline but in no case did this alter the final result of the test, and it was therefore concluded that the single washing was adequate.

Reagents. The reagent used for routine testing (solution A) was a modified version of that used by Ferguson & Hook (1943) and by Anderson (1945), and was made up as follows: 0·1 g. KH_2PO_4; 0·1 g. K_2HPO_4; 0·5 g. NaCl; 2·0 g. urea; 0·5 g. phenol; 1·0 ml. 95 % (v/v) ethanol in water; 100 ml. distilled water; 5·0 ml. Universal Indicator (British Drug Houses Ltd., Poole); and sufficient 0·1 N-HCl to give an orange colour (about pH 6·0).

A control reagent (solution B) in which the urea was replaced by a further 2·0 g. NaCl was made up, and also both test and control reagents as above but omitting the Universal Indicator (solutions C and D, respectively).

Procedure for urease tests. For the routine test one of each pair of culture sediments was resuspended in 2·0 ml. of solution A and the other in the same volume of solution B, and both suspensions were incubated at 37° for 18 hr. The development of alkalinity, as indicated by a colour change to green or blue, in the solution A tube only, was taken to indicate the presence of urease. None of the strains examined gave a positive reaction with solution B. Although the results of the test were not recorded until 18 hr. incubation, the colour change indicating the presence of urease developed within minutes in strongly urease-positive cultures and was evident in all positive suspensions after 2–4 hr. at 37°. Some tests showing negative results were incubated for up to 72 hr. but still showed no tendency to develop alkaline reactions.

For the Nessler's reagent test the sediments were resuspended in solutions C and D, respectively, and incubated at 37°. Samples were removed after 18 hr. and tested for the presence of NH_4^+ by the addition of Nessler's reagent. The appearance of an orange-yellow precipitate, in the solution C test only, was taken to indicate urease production. No strain gave a precipitate with solution D.

Suspensions of cultures subjected to ultrasonic disintegration were tested with both the routine and the Nessler's reagents. Equal volumes of suspension and reagent were incubated together at 37° for 18 hr. and the results interpreted as described above.

Preparation of cell suspensions for ultrasonic disintegration. Seventy-five ml. amounts of a broth containing meat particles were inoculated and incubated at 37° for 18 hr. After the meat particles had been strained off, the broths were centrifuged, the supernatant fluids discarded and the sediments washed in phosphate buffer (pH 7·3) and finally resuspended in 10 ml. amounts of the same buffer. After the removal of a 2·0 ml. sample the remainder of each suspension was subjected to ultrasonic disintegration for 10 min. (Mullard 500 watt E7590-B disintegrator).

Preparation of urease suspensions for titration of anti-urease activity. Urease-

positive cultures were grown for 18–24 hr. in 75 ml. amounts of meat broth, harvested, centrifuged, and the sediments washed in 1·0 % saline and finally resuspended in 1·0 % saline. These suspensions proved to be fairly stable and showed little change in urease activity after several days storage at 4·0°.

Two dried urease preparations were obtained by drying washed bacteria harvested from 12 l. cultures of urease-positive type B and type F strains. The washed bacteria were dried in desiccators over silica gel and subsequently finely ground to give homogeneous preparations.

Sera. Antibacterial and antitoxic sera prepared by the hyperimmunization of horses, and antibacterial sera prepared by the hyperimmunization of rabbits, against urease-positive and urease-negative *Clostridium perfringens* strains, were tested for ability to inhibit the urease reaction. Normal horse and rabbit sera were used as controls. All the immune sera contained antibodies to the appropriate soluble antigens of *C. perfringens*. Two antibacterial sera prepared in rabbits against strains of the urease-producing *C. sordellii* were also tested.

Titration of sera for anti-urease activity. One ml. amounts of serial twofold dilutions of the sera under test were mixed thoroughly with 1·0 ml. amounts of suitable dilutions of the fresh or dried urease preparations, and allowed to stand at room temperature for 30 min., after which 0·5 ml. of the routine urease reagent (solution A) was added to each mixture. The results of titrations, as increase in pH value, were recorded after 18 hr. incubation at 37°. Either 1·0 % saline or distilled water was used as diluent, both giving the same results. The pH values were measured with a pH-meter.

The rabbit sera were also titrated using constant volumes of serum and doubling dilutions of the urease preparation.

Test for inhibition of urease activity by urease-negative strains. Fifteen ml. amounts of Robertson's cooked meat broth were inoculated with pairs of the strains under test and incubated at 37° for 24 hr. Smears of each culture were examined, and a culture sample was streaked on a blood agar plate and incubated anaerobically overnight, to determine the presence of growth from both inocula. In the *Clostridium bifermentans*/*C. perfringens* mixtures the examination of smears was sufficient for this purpose. In those *C. perfringens* mixtures where, even on plates, the two strains were indistinguishable, some colonies were picked into saline and tested for urease activity.

RESULTS

Distribution of urease production in Clostridium perfringens *types*

The results of the urease tests for strains of all types are shown in Table 1. Several strains were tested several times, both as replicate samples from one culture and as replicate cultures from one strain. In all cases the results for any one strain were consistent, even to the extent of giving approximately the same colour change where positive. In cultures incubated for 72 hr. the urease reactions were the same for 5, 24, 48 and 72 hr. samples. About 50 cultures were also tested with Nessler's reagent. All gave results which agreed with those for the routine test.

About one-fifth of the strains examined were found to produce urease, but this property was unevenly distributed between the various types and subtypes. All the type C strains examined were urease-negative. Type A was virtually urease-

negative, having only six urease-producing strains. The urease-positive and urease-negative type A strains were otherwise indistinguishable. Of the six urease-positive strains all were recently isolated, 5 from pathological material and the sixth from the intestines of a normal sheep. Whether or not this can be taken to indicate that type A strains tend to lose the capacity for urease production is doubtful, as numerous freshly isolated type A strains showed no tendency to produce urease. Only 10 type E strains were available for examination, and all of these were urease-positive. Of the 20 type F strains 13 produced urease, and, as in type A, the urease-positive resembled the urease-negative strains in respect of antigen production. The most interesting results were those obtained with the types B and D strains. In both these types there was a distinct correlation between the production of urease and of the λ-antigen (Table 2).

Table 1. *Distribution of urease production in* Clostridium perfringens *types*

Type		No. strains examined	Urease-positive strains	
			No.	%*
A		267	6	2
B	Classical	57	51	89
	Iranian	6	0	0
C	Classical	31	0	0
	Colorado	22	0	0
	Piglet	11	0	0
D		68	24	35
E		10	10	100
F		20	13	65

* To nearest 1·0%.

Table 2. *Urease and λ production in types B and D strains*

Type		λ production	No. strains examined	Urease-positive strains	
				No.	%*
B	Classical	Unknown	8	8	100
		+ve	40	37	92·5
		−ve	9	6	67
B	Iranian	(all −ve)	6	0	0
D		Unknown	20	4	20
		+ve	17	16	94
		−ve	31	4	13

* To nearest 0·5%.

Urease and λ production in type B and D strains

The production of λ-antigen is characteristic of the classical type B strains, and is one of the main points of difference between these and the Iranian subtype which is λ-negative. Of the 40 type B strains known to produce λ, 37 produced urease also, whilst only 6 of the 9 λ-negative classical type B strains produced urease. The λ-negative 'Iranian' strains were all urease-negative. One of the urease-negative classical strains was derived from a λ-positive urease-positive parent strain, and had lost the capacity for λ-production also. In type D the correlation

between urease and λ-production was more marked, 16 of the 17 λ-positive and only 4 of the 31 λ-negative strains producing urease.

No similar correlation was observed between urease and the production of any other of the soluble antigens in any of the *Clostridium perfringens* types, except in so far as the major lethal antigens determine type differentiation.

Ultrasonic disintegration

It was thought that the difference between the urease-positive and urease-negative strains might lie not in their ability to produce urease intracellularly, but in the release of the urease from the cells. Bacterial suspensions prepared from positive and negative strains were therefore tested for urease activity before and after ultrasonic disintegration. The urease reactions of the suspensions were in all cases the same before and after disintegration, and agreed with the results of previous tests carried out on the same strains. These results rendered untenable the theory that the differences between strains might lie in the ease of rupture or permeability of the cell walls, rather than in the actual production of urease.

Antigenicity of Clostridium perfringens *urease*

Some tests were carried out in an attempt to ascertain whether or not the urease produced by *Clostridium perfringens* was antigenic, in the hope that this would show whether the urease was type-specific, species-specific or non-specific. None of the sera tested specifically inhibited the activity of the urease preparations, although several of the sera caused apparent inhibition at low dilutions, presumably by buffering action tending to limit rise in pH value. This effect was observed with normal as well as immune sera. There was thus no evidence that *C. perfringens* urease is antigenic.

Inhibition of urease activity by urease-negative strains

In the course of a study on *Clostridium bifermentans* and *C. sordellii* it was noted that many strains of the urease-negative *C. bifermentans* were able to inhibit the positive urease reactions of *C. sordelli* strains when grown with them as mixed cultures, although this inhibition was not seen when cultures of the two species were mixed after growth (Brooks & Epps, 1959). It seemed possible that the same phenomenon might occur with urease-positive and urease-negative *C. perfringens* cultures. Accordingly 45 mixed cultures of urease-positive and urease-negative *C. perfringens* strains, and two mixed cultures of urease-positive *C. perfringens* and urease-inhibitory *C. bifermentans* were grown and tested for urease activity. In no case was there any evidence of the inhibition of urease activity.

DISCUSSION

The fact that only about 20 % of the strains examined, and only 2·0 % of the type A strains, produced urease probably accounts for the omission of *Clostridium perfringens* from Huet & Aladame's (1952) list of urease-producing anaerobes. Similarly it is not surprising that Ortali & Samarani (1955) found the one *C. perfringens* strain they examined to be urease-negative.

It is unfortunate, from the point of view of pathological investigation, that the

correlation between urease production and type differentiation is not complete. The most that can be said of the urease test as a tool in type differentiation is that since types E and F are rarely encountered, any *Clostridium perfringens* isolate producing urease can probably be assigned to types B or D. The apparent correlation between urease and λ production is similarly unhelpful as a diagnostic tool, but may, in the light of further investigations, help to elucidate the evolutionary relationships of the six *C. perfringens* types. Although the correlation is most marked in types B and D, it is interesting to note that the λ-negative type A was virtually urease-negative, and the λ-negative type C completely so, whilst in the urease-positive type E the five strains examined for λ production were all found to be positive. It is only in the type F strains, with their meagre range of soluble antigens and peculiarly limited distribution (all except one were isolated from cases of necrotic enteritis in Hamburg between 1946 and 1948, and the remaining one was isolated in the same place some years later), that the λ/urease correlation breaks down. No type F strain has been shown to produce λ, although when strains of this type were first isolated they were thought to elicit the production of small quantities of anti-λ in hyperimmune horses and were, therefore, considered to be most nearly related to *C. perfringens* type B (Oakley, 1949). When the question of the position of type F was raised during a survey of *C. perfringens* antigen production, Brooks, Sterne & Warrack (1957) considered type F to be related to type C rather than to type B, on the basis of the range of antigens produced by the three types, and Mrs I. Batty (personal communication) on retesting six of the original type F antisera, found little or no anti-λ in three and only very small amounts comparable to those found in several normal sera, in the other three.

Since it is difficult to see how there can be any direct relationship between urease and λ, particularly since one sometimes occurs without the other, and yet their occurrence together seems too consistent to be purely fortuitous, their distribution may give some indication of the relationships between the various types and subtypes, and the sequence of evolution of the types if a common ancestor is assumed. In this connexion it is of interest that the distribution of urease production between types and subtypes, although not necessarily between individual strains, parallels that of a serum-dependent non-$\alpha\theta\delta$ haemolysis, observed only in cultures grown on blood-agar containing certain antisera (Brooks *et al.* 1957), although as with the soluble λ-antigen, the correlation between the production of urease and of the serum-dependent non-$\alpha\theta\delta$ haemolysis is not complete.

I wish to thank my colleagues at the Wellcome Research Laboratories for their help in various directions: Mrs I. Batty for the determination of antibody values in sera; Dr A. J. Woiwod and Dr A. W. Phillips for the ultrasonic disintegration of cultures; Mr H. Proom and the staff of the Culture Collection for the supply of freeze-dried cultures and access to their records; Miss C. Simpson and Mrs E. Williams for technical assistance; and Dr M. Sterne for help and guidance throughout this survey and in the preparation of the manuscript.

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The Measurement of the Liberation of Penicillinase from *Bacillus subtilis*

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SUMMARY

The formation of penicillinase, induced in a growing culture of *Bacillus subtilis* 6346 by a single addition of benzylpenicillin, spontaneously ceases after about 3 hr. The enzyme continues, however, to be released into the medium, thus permitting the process of its liberation to be studied independently from its production. By using the escape into the medium of a normally intracellular, maltose-inducible α-glucosidase as a more sensitive indicator of cell damage than direct measurement of lysis, it was concluded that at least 40 % of the penicillinase is liberated from the cells without gross disorganization of their structure.

INTRODUCTION

The phenomenon in which certain enzymes are released from apparently undamaged cells of a growing bacterial culture has not so far been the subject of much systematic experimental investigation, the one notable exception being that of the extracellular α-amylase of *Bacillus subtilis*, studied by Nomura, Hosoda & Yoshikawa (1958). In a recent review (Pollock, 1961*b*) some of the principles and definitions involved in studying the problem were evaluated and the difficulties emphasized. There has been considerable scepticism about the reality of exo-enzymes as physiological entities, the view sometimes being expressed that they appear in the extracellular environment only to the extent that the cells are lysed, or damaged to a degree that is incompatible with normal growth and metabolism. However unlikely this view may appear to be in any specific instance, it is not one which can be dismissed without critical experimental evidence to the contrary.

For the work to be described here the penicillinase system of *Bacillus subtilis* NCTC 6346 was chosen for the following reasons.

(1) The enzyme is stable and can be simply and accurately assayed.

(2) It is inducible by benzylpenicillin and other closely related compounds, as with the penicillinase of *Bacillus cereus* (Pollock, 1953), and therefore its production can be brought under close control.

(3) On the reasonable assumption that its mode of production does not differ fundamentally from that of *Bacillus cereus* penicillinase, it could be concluded, on the basis of work done on the latter system (Pollock & Kramer, 1958), that appearance of enzymic activity corresponds to the biosynthesis of the relevant protein molecule itself. No substrate-accessibility factors or high molecular weight precursors appear to be involved to an extent which might complicate interpretation of results based on the direct measurement of enzymic activities.

(4) Unlike the situation in *Bacillus cereus*, however, where 85 to 95% of the penicillinase is extracellular under all conditions so far investigated (Pollock, 1956), the *B. subtilis* enzyme is mainly bound to the cells during the early stages of growth, only later being liberated into the medium. There were some grounds, therefore, for hoping that it might be possible to dissociate liberation of the enzyme from its production—an essential prerequisite for a proper study of the liberation phenomenon itself.

Most bacterial exo-enzymes, including the α-amylase of *Bacillus subtilis* 6346 itself, appear to exist almost exclusively in the medium (Pollock, 1961*b*); in other words, they are liberated as soon as they are formed. This is obviously not an ideal situation for the purpose of studying enzyme liberation distinct from enzyme formation. Although, therefore, the *B. subtilis* penicillinase system may be atypical, it seemed to offer an exceptional opportunity for studying one aspect of the phenomenon of enzyme liberation. Our objective has, correspondingly, been limited: that of attempting to understand the mechanism by which the cell-bound fraction of *B. subtilis* penicillinase is liberated into the surrounding medium. In order to do this, the problem has been divided into three parts.

(1) Measurement and control or cell lysis of gross cell damage and assessment of its possible role in the liberation process (this article). (2) Attempts to identify the location of the cell-bound fraction of the enzyme (Kushner & Pollock, 1961). (3) Observations on the 'normal' enzyme liberation process itself (Pollock, 1961*a*). It has been possible to show, by means of the studies reported in this and the following two papers, that the liberation of penicillinase occurs with little or no cellular damage, under conditions dissociated from cell growth and multiplication, and involves detachment of the enzyme from the superficial layers of the cell by a process which probably has an enzymic basis.

METHODS

Organism. *Bacillus subtilis* NCTC 6346 was chosen for reasons outlined above and because it is also a good producer of α-amylase whose production and liberation are forming the subject of a parallel study.

Medium. The basal medium (CH) for all experiments contained 1% Casamino acids (Difco), 0·02 M-KH_2PO_4 adjusted to pH 7·2 with NaOH; $1{\cdot}7 \times 10^{-3}$ M-$MgSO_4$ + 0·1 ml. 'stock iron' solution (mixture of 0·156% ferrous ammonium sulphate +0·168% citric acid per 100 ml.) +0·1 ml. trace metals mixture (= 'oligodynamic solution' as used by Pollock & Kramer, 1958) per 100 ml.

Growth conditions. Unless otherwise stated, cultures were incubated by shaking aerobically at 35°. After inoculation the previous night with a few drops of a standard spore suspension (3×10^8 viable spores/ml.), cultures were first incubated without shaking for about 16 hr. and then transferred in the morning to a shaker at 35° in a conical flask containing not more than 1/5 its volume of medium, and grown under fully aerobic conditions during the whole of the experiment.

Enzyme induction. Penicillinase was usually induced by the addition of 0·06 μg. (= 0·1 unit)/ml. benzylpenicillin (Glaxo 'Crystapen'). In some experiments, where larger amounts of penicillinase were required, 1 μg. or 10 μg./ml. of the non-metabolizable inducer, cephalosporin C, was used instead. α-Glucosidase was induced by

the addition of 0·25 mg. maltose (BDH 'Analar')/ml. Unless specifically stated otherwise, both enzymes were induced when the culture density reached the equivalent of 0·1 mg. bacterial dry wt./ml. and this point has been used as a standard zero reference time in all experiments—e.g. '3 hr. cells' means cells from a culture in CH medium 3 hr. after the point where an opacity equiv. 0·1 mg. dry wt./ml. was reached.

Enzyme assays. Samples (usually 5 ml.) were removed from the culture when required and cooled rapidly in ice. Two to three ml. were then spun at 20,000 *g* in the cold room for 10 min. and the supernatant fluid removed for assay of released enzyme. One drop of a 2 mg./ml. solution of egg-white lysozyme (Armour Labs.) was added to the remainder of the sample which was incubated at 35° until lysis was complete (2–5 min.) and then kept at 0° until assay of total enzyme. This procedure prevented any further production of enzyme and allowed full expression of α-glucosidase activity which in intact cells assayed at only about 40 % of the activity found after lysis or other methods (e.g. toluene treatment) of disrupting cell structure. Neither toluene nor lysozyme had any effect on penicillinase activity.

Penicillinase was assayed at 30° either manometrically on 1·0 ml. samples (Henry & Housewright, 1947) at pH 7·0, or iodometrically at pH 6·5 (Perret, 1954), the latter method being preferred under conditions of high buffer concentration or at pH values removed from neutrality. As used in this work, the manometric method gave approximately 25 % lower values, partly because of the CO_2 retention due to phosphate buffer in the medium. No correction was made for this, since it could be assumed that it was the same for all samples; comparisons were of course confined to results obtained by the same method, and conclusions were not in any way affected by the discrepancy. When dialysed samples of lysozyme-lysed, penicillin-induced cultures were assayed for penicillinase activity by both methods, the manometric technique still gave results 15 % lower than the iodometric method. The agreement is reasonable, and excludes the possibility of there being any significant penicillin-amidase activity present which might theoretically give misleadingly high results in the manometric assay technique for penicillinase (= penicillin β-lactamase). Penicillin-amidase is now known to be formed by certain bacterial species (Rolinson *et al.* 1960) though it has not been reported as occurring in the *Bacillus* genus. Its presence might possibly contribute to the release of CO_2 from penicillin and bicarbonate in the manometric assay for penicillinase, but would not interfere with the iodometric assay since the products of its action on benzylpenicillin (phenylacetic acid and 6-aminopenicillanic acid) do not react with iodine. If penicillin-amidase were present in significant quantity any discrepancy that might result between the iodometric and manometric assays of penicillinase would be in the opposite direction from that which was actually observed.

One ml. of 1 % (w/v) gelatin was originally included in the manometer cups for protection of the enzyme against possible loss or inactivation (see Pollock & Perret, 1951), but this was discontinued when it was discovered that *Bacillus subtilis* penicillinase is much more stable than the *B. cereus* enzyme and is not absorbed by glass to any significant extent (Kushner, 1960). All results are expressed in units of enzyme/ml. (as defined by Pollock & Torriani, 1953).

α-Glucosidase was assayed by measuring the absorption at 420 mμ (in a Unicam spectrophotometer) of the *p*-nitrophenol liberated by the enzyme after hydrolysis

of *p*-nitrophenol-α-D-glucoside, in comparison with standard concentrations of *p*-nitrophenol prepared by dissolving 0·1 g. in 500 ml. water and making suitable dilutions in 0·05 M-sodium phosphate buffer (pH 7·0) to 5·0 ml. lots of which was added 1 ml. of M-K_2CO_3. One ml. samples of enzyme (diluted, when necessary, with water) were mixed with 3·5 ml. of 0·05 M-sodium phosphate buffer (pH 7·0) in a 35° water bath and the reaction started by the addition of 0·5 ml. of a 1·0 mg./ml. solution of the substrate. The reaction was stopped by the addition of 1·0 ml. M-K_2CO_3 after sufficient colour had developed. Where necessary, the reaction mixture was clarified by centrifugation at 3000 rev./min. for a few minutes. Blanks, prepared by incubating the ingredients under similar conditions (1 ml. of water being substituted for the enzyme samples) were used for comparison in all experiments. It was shown that under these conditions the reaction was linear for at least 60 min. incubation and that the rate was proportional to the enzyme concentration, as long as no more than 80% of the substrate was hydrolysed. Activities are expressed as the quantity of *p*-nitrophenol formed/hr./ml. of original enzyme preparation. The *p*-nitrophenol-α-D-glucoside was prepared according to the method of Aizawa (1939), and gave a M.P. of 210°.

β-Galactosidase was assayed at 35° and pH 7·0 by the method of Lederberg (1950) with *o*-nitrophenol-β-D-galactoside as substrate, activities being expressed as the quantity of *o*-nitrophenol produced/hr./ml. original enzyme preparation.

Opacities of bacterial suspensions were measured in a Hilger Spekker absorptiometer with a neutral H 508 filter and expressed in terms of the equivalent bacterial dry weight as read off on a standard calibration curve prepared with suspensions of *Bacillus subtilis* of known dry weight values.

Total protein was estimated by the method of Lowry, Rosebrough, Farr & Randall (1951) against a bovine serum albumin standard, after precipitation with 5% (w/v) trichloroacetic acid (TCA) at 90° for 20 min. and subsequent washing of the precipitate twice with 5% TCA before dissolving in N-NaOH.

'260 *mμ absorption*' was measured directly in a Unicam spectrophotometer after making suitable dilutions in 10^{-2} M-KH_2PO_4 (pH 7·0) solution.

RESULTS

Kinetics of release

Figure 1 shows the production and release of penicillinase from *Bacillus subtilis* 6346 growing in CH medium after induction with 0·06 μg. benzylpenicillin/ml. at 0·1 mg. bacterial dry wt./ml. About 3 hr. after induction, formation of the enzyme practically ceased (quite unlike the situation with *B. cereus* where penicillinase synthesis continues apparently indefinitely), whereas cells went on growing and release of the enzyme persisted until, after overnight incubation, it had all been liberated into the medium. This means that, subsequent to a point 3 hr. after induction, liberation is spontaneously dissociated from production. Nearly all investigations on the liberation process were therefore confined to the first 2–3 hr. of this period. Cessation of penicillinase production at the '3 hr. point' was shown to be due to an induction failure, since on further addition of benzylpenicillin to '3 hr.' cells, production recommenced, and when an inducer, such as cephalosporin C, which is not detectably hydrolysed by the enzyme, was added at the normal induction time

(0 hr.') production persisted for as long as the organisms were growing. The liberation phase, however, could be shown to be not markedly influenced either by the absolute rate of penicillinase formation or the interval elapsed following addition of the inducer. For instance, in the experiment summarized in Table 1 there was perhaps a significantly lower proportion of penicillinase liberated at 4 and 5½ hr. by cells induced at 2½ hr. as compared with cells induced with the same concentration of penicillin at 0 hr.; but in a culture induced at 4 hr. the percentage release only 1½ hr. later was as high as that shown by cells induced 5½ hr. previously. Table 1 shows also that the proportion of extracellular penicillinase in cultures induced with cephalosporin C was not significantly different from that in penicillin-induced cultures where there might be only one-eighth as much total enzyme present.

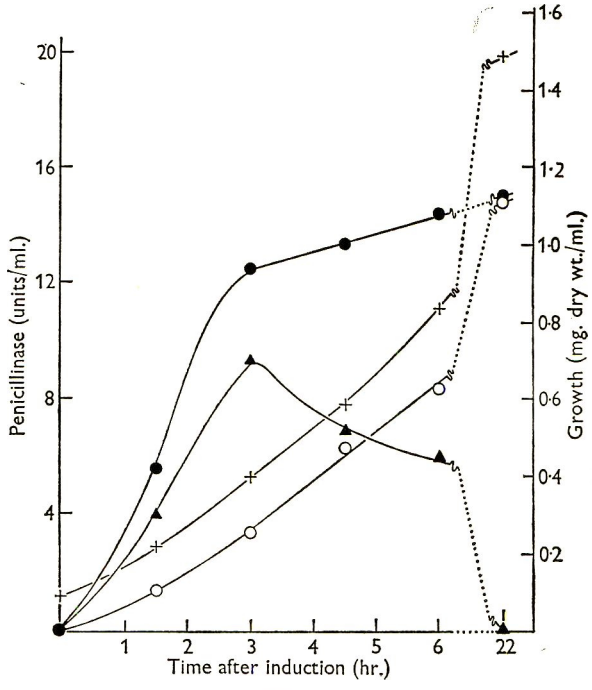

Fig. 1. Production and liberation of penicillin-induced penicillinase by cells of *Bacillus subtilis* 6346 growing in casein hydrolysate. Total penicillinase (—●—●—); penicillinase liberated from cells into medium (—○—○—); cell-bound penicillinase (—▲—▲—); growth opacity (— + —+ —).

The release of the enzyme, therefore, seemed to be related to the physiological state of the culture rather than to the rate or stage of enzyme induction. Moreover, when relatively 'young' (2 hr.) cephalosporin C-induced cells were treated with 40 μg. chloramphenicol/ml. and incubated in the supernatant fluid from an 'old' (5½ hr.) uninduced culture (after readjusting to pH 7·0 with N-HCl and supplementing with a further addition of 1 % (w/v) Casamino acids), they were found to liberate the same quantity of penicillinase into the medium as did the cells of a similarly induced and chloramphenicol-treated control culture incubated in fresh medium. It would appear, therefore, that release of the enzyme must depend upon changes in the cells themselves and not factors produced or liberated in the medium.

State of released enzyme. The penicillinase released during the 3–6 hr. period appeared to be entirely in a soluble state. The titre (3·08 units/ml.) of induced enzyme activity in the supernatant fluid of a sample from a $5\frac{1}{2}$ hr. culture centrifuged for 30 min. at 105,000 ***g*** was not significantly different from that (2·94 units/ml.) in the supernatant fluid of an sample spun for only 15 min. at 5000 ***g***. Removal of cells by an entirely different method (filtration through an 'Oxoid' membrane) left nearly the same activity (2·60 units/ml.) in the cell-free filtrate.

The control and measurement of cell lysis and gross cell damage

Cells of *Bacillus subtilis* are notoriously prone to undergo lysis (see Nomura *et al.* 1958; Nomura & Hosoda, 1956), the metabolic basis of which is not known. Gross lysis, involving obvious diminution in opacity of bacterial suspensions, was frequently observed during incubation (*a*) under conditions of suboptimal aeration, (*b*) with limiting nutrients, (*c*) after cold 'shock' at 0°, (*d*) in the presence of various growth inhibitors. It always led to an increase in the release of penicillinase. Less severe and less obvious 'interference' with cultures, insufficient to cause an obvious decrease in suspension opacity, was often found to be enough to allow leakage of enzymes otherwise found fixed to growing cells, and this, too, was usually associated with increased liberation of penicillinase. On *a priori* grounds, such enzyme release might therefore not unreasonably be regarded as an indication of cell damage: i.e. 'unphysiological'. Thus, it was first necessary to develop a method for measuring relatively small degrees of cell damage and to find a means of preventing it. The ideal tool would seem to be what we have called an intracellular 'marker', whose presence in the extracellular medium should indicate the extent of damage under all experimental conditions. To be satisfactory, such a 'marker' should have the following properties. (1) It should *normally* (i.e. under conditions of growth where there are no *a priori* grounds for believing that cell damage occurs) be entirely 'cell-bound' (i.e. associated with the deposit on centrifugation of a culture at a speed at which all the organisms are spun down). (2) It should be rapidly, and if possible completely, released into the medium by procedures known to cause cell lysis and/or disruption or disintegration of the cell 'envelope' (which, for the purposes of this work, is taken to mean the cell wall plus cytoplasmic membranes plus any other intimately associated structures or material). (3) It should be stable when present in the medium of a normally growing culture of the organism being investigated. (4) It should be capable of being easily, rapidly and accurately estimated.

The first marker tested, deoxyribonucleic acid, proved to be extremely rapidly broken down by cells in a growing culture and was therefore discarded. The second choice, a rather feeble, constitutive β-galactosidase, appeared during preliminary studies to be fairly satisfactory, although there was some loss (20–30 %) of activity during incubation with a growing culture over the 3 hr. period being studied. It was eventually discarded after the discovery that although this enzyme was almost completely released into the medium on cell disruption, less drastic damage to the cytoplasmic membrane (e.g. by incubation of cells with 4 % butanol) allowed only a relatively slow escape from the cell. It was therefore conjectured (and afterwards confirmed) that minor degrees of damage were being missed.

The marker finally chosen was α-glucosidase. This proved to be reasonably

satisfactory and its activity, in whole cultures and in the medium alone, was assayed in every experiment relating directly to the phenomenon of penicillinase liberation. The enzyme has the additional advantage of being inducible (by maltose). This meant that it was possible to adjust conditions such that its production by the cells was restricted to the period when they were forming penicillinase. Thus, unless there was a non-uniform and different distribution of the two enzymes amongst the cells of a heterogeneous population it could the more reasonably be assumed that any release of α-glucosidase after the 3 hr. point (when penicillinase production largely ceased) reflected the condition of the same cells as those which were liberating penicillinase.

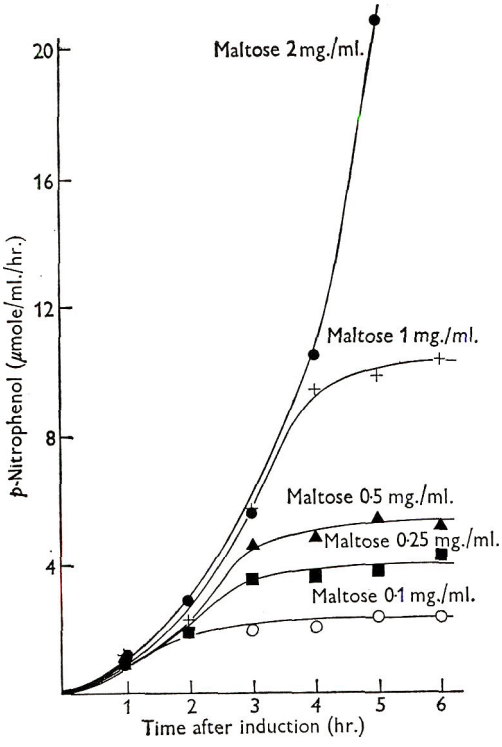

Fig. 2. The effect of different initial concentrations of maltose on the induction of α-glucosidase in a penicillin-induced culture of *Bacillus subtilis* 6346 growing in casein hydrolysate. The maltose was added at the same time as benzylpenicillin (0·06 μg./ml.), when bacterial suspension opacity reached the equivalent of 0·1 mg. dry wt./ml. Initial maltose concentration of 2 mg./ml. (—●—●—); 1 mg./ml. (—+—+—); 0·5 mg./ml. (—▲—▲—); 0·25 mg./ml. (—■—■—); 0·1 mg./ml. (—○—○—).

Properties of the α-glucosidase system

Induction. The effect of different concentrations of maltose, added at the same time as the penicillin, on α-glucosidase formation, is illustrated in Fig. 2. It can be seen that at an initial concentration of 0·5 mg. maltose/ml. and below there was little further production of the enzyme after 3 hr., due to exhaustion of the substrate. In order, therefore, to obtain reasonable α-glucosidase titres and at the same time to ensure that formation of the enzyme ceased at 3 hr. (i.e. at about the same time as penicillinase production faded out) an initial maltose concentration of

0·25 mg./ml. was chosen for all experiments. Addition of this concentration of maltose had no detectable effect either on cell growth or on penicillinase induction and liberation.

Assay. As mentioned above, the enzyme is partially cryptic, and lysis by lysozyme was used to allow full expression of activity. With a soluble preparation of the enzyme obtained from the supernatant fluid of a suspension of maltose-induced cells crushed at −30° in the Hughes's press, the pH value for optimum activity was found to be between 6·5 and 6·75 in 0·1 M-sodium phosphate buffers. The enzyme was stable during incubation for 2 hr. at 35° in 0·1 M-phosphate buffers from pH 6·0 to 8·2.

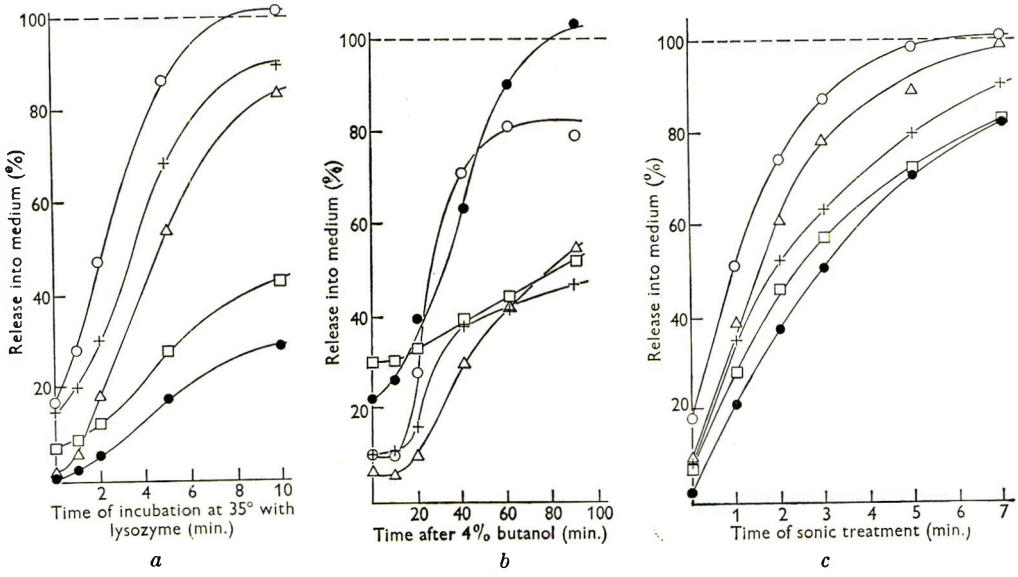

Fig. 3. Release of α-glucosidase (—○—○—), β-galactosidae (—△—△—), penicillinase (—●—●—), total protein (—+——+) and material absorbing at 260 mμ (—□—□—) from cells of suspensions of *Bacillus subtilis* 6346, previously induced with maltose and benzylpenicillin, following treatment: (*a*) with 100 μg. lysozyme/ml. + 0·05 M-$MgSO_4$ at 35°; (*b*) with 4 % butanol at 35°; (*c*) by sonication at 0–10°. In all experiments, the first samples were taken immediately after beginning of treatment. (For further technical details, see text.)

Rate and extent of release from disrupted cells. α-Glucosidase was more rapidly released from cells, after disruption or damage by a variety of procedures, than any other cell constituent measured. Figures 3*a*, *b* and *c* show the release of α-glucosidase, β-galactosidase, penicillinase, total protein and 260 mμ-absorbing substances from sedimentable material (20,000 *g*, 15 min.) after treatment of 3 hr. *Bacillus subtilis* cells (doubly induced with maltose and benzylpenicillin): (*a*) with lysozyme (100 μg./ml. + 0·05 M-$MgSO_4$ + 0·01 M-KH_2PO_4 buffer pH 7·0), after centrifugation and resuspension to ½ vol. at 35°; (*b*) with 4 % butanol added directly to the culture at 35°; (*c*) by sonication at 25 Kc/min. at 0–10° (after centrifugation of the culture and resuspension in fresh medium at 13·0 mg. dry wt./ml.). In all experiments samples were removed at the times indicated, the '0 min.' sample being taken immediately after the beginning of treatment, cooled to 0° in an iced bath and thereafter centrifuged at 2° as soon as possible.

Table 1. *Effect of time and type of induction on the release of penicillinase from cells of a* Bacillus subtilis *culture in casein hydrolysate*

Time of induction* (hr.)	Inducer	Penicillinase activities (units/ml.) after following times														
		2½ hr.			3 hr.			4 hr.			5 hr.			5½ hr.		
		Total	Sn†	% released	Total	Sn	% released	Total	Sn	% released	Total	Sn	% released	Total	Sn	% released
0	Benzylpenicillin (0·06 μg./ml.)	11·4	2·4	21	.	.	.	14·2	4·2	30	.	.	.	16·3	6·2	38
2½		.	.	.	.	.	.	8·4	1·43	17	.	.	.	14·0	4·2	30
4		.	.	.	.	.	.	.	.	.	.	.	.	5·8	2·3	40
0	Cephalosporin C (10 μg./ml.)	50·5	9·6	19	.	.	.	.	.	.	.	.	.	.	.	.
0		.	.	.	110	28·6	26	125	41	33	148	52	35	.	.	.

* Measured from point when optical density of culture reached the equivalent of 0·1 mg. dry bacterial wt./ml.
† Sn = supernatant fluid.

The '100 % release' value for comparison was obtained by assaying a measured sample of cells resuspended to the same cell density in buffer without Mg^{++}, after addition of 100 μg. lysozyme/ml. and incubation at 35° to obtain full lysis.

After crushing a 13·0 mg. dry wt./ml. suspension of cells in 0·01 M-sodium phosphate (pH 7·0) + 0·05 M-$MgSO_4$ in a Hughes's Press at −30°, 88 % of the enzyme was found to be released into the fraction unsedimented by centrifugation at 20,000 *g* at 2° for 15 min.

An analogous, dissociated release of up to 90 % of the α-glucosidase, with no more than 5 % release of penicillinase occurred when cells were treated at 0°, especially in the presence of salts (see Pollock, 1961*a*).

Stability in culture medium. An α-glucosidase preparation was obtained from the supernatant fluid of a 3 hr. maltose-induced culture of *Bacillus subtilis* lysed by subjection to 30 min. incubation at 35° without aeration. This was added at a dilution of 1/8 to a 3 hr. culture in CH medium, previously induced for penicillinase but not for α-glucosidase, growing at 35°, exactly under the conditions used for studying penicillinase release. The enzyme in the supernatant fluid was assayed at the beginning of the experiment and after 2½ hr. further incubation (see Table 2). The spontaneous release of α-glucosidase from cells of a maltose-induced sister culture, incubated under similar conditions, but without the addition of the enzyme to the medium, was followed in the same experiment.

The α-glucosidase was found to be inactivated by certain proteinases: trypsin (Armour Labs.); crystalline *Bacillus subtilis* N proteinase (Nagase and Co. Ltd.) and a concentrated supernatant fluid from older cultures of *B. subtilis* 6346 itself, known to have proteolytic activity. This proteolytic destruction was partially prevented by 10^{-4} M-diisopropylfluorophosphate (DFP) and almost completely (90 %) inhibited by 0·1 % heparin (Roche Products Ltd., 110 I.U./mg.). It seemed therefore important to know in this experiment whether the addition of heparin and other proteinase inhibitors had any effect both on the amount of α-glucosidase activity found in the supernatant fluids of cultures which were likely to be releasing the enzyme spontaneously, and on the stability of the enzyme added to cultures not forming it. None of the added substances had any significant effect on increase in bacterial suspension opacity. The results (Table 2) indicate in both experiments that, whereas soybean trypsin inhibitor (Worthington Biochem. Corporation) had no 'protective' effect, the activity in the supernatant fluid of cultures without either heparin or DFP was up to 10 % lower than in the others, therefore a very slight degree of inactivation may have occurred. Clearly, however, this was not enough to invalidate the use of the enzyme as a satisfactorily stable 'marker' in this study.

Results with α-glucosidase as 'marker'

'*Damage-release*'. By taking the release of preformed α-glucosidase into the medium as a criterion of cell damage, it was soon apparent that *Bacillus subtilis* cells were even more susceptible to harm than had previously been suspected. For instance, even removal of a culture from the shaker and allowing the flask to stand on the bench for a few minutes (such as might sometimes be necessary for sampling, etc.) could allow the subsequent release (after the culture was returned to fully aerated conditions) of up to 40 % of its α-glucosidase within 2 hr. without any significant decrease in optical density of the suspension. A similar degree of what

Table 2. *Stability of α-glucosidase in the culture medium of* Bacillus subtilis *growing in casein hydrolysate and the effect of certain proteinase inhibitors*

Culture	Additions (at 3 hr.)	α-Glucosidase activities (mμmole *p*-nitrophenol formed/ml./hr.) 3 hr. Total	3 hr. Sn*	5½ hr. Total	5½ hr. Sn
Induced with penicillin and maltose	Control (no additions)	2500	69	2660	86·5
	+ trypsin inhibitor 1 mg./ml.	.	.	.	86·5
	+ 0·1 % (w/v) heparin	.	.	.	110
	+ 3×10^{-4} M DFP	.	.	.	103
	+ Sat. soln. DFP	.	.	.	114
Induced with penicillin, but not maltose; α-glucosidase preparation added at 3 hr.	Control	353	255	.	285
	+ trypsin inhibitor, 1 mg./ml.	.	.	.	284
	+ 0·1 % heparin	.	.	.	304
	+ 3×10^{-4} M-DFP	.	.	.	297
	+ Sat. soln. DFP	.	.	.	316

For full details, see text.
Sn = supernatant fluid.

might be called 'damage-release' of α-glucosidase resulted from a single centrifugation of cells and resuspension in 0·01 M-sodium phosphate buffer, or even in the same medium.

More pronouncedly rough handling of the cells and longer periods of anaerobiosis led, as might be expected, to obvious lysis. But even the smaller degree of α-glucosidase leakage that resulted from minimal interference with standard conditions of culture was usually reflected (as long as it amounted to at least 20 % of the total) by a detectable lag in growth, although no lysis might be apparent, thus justifying the conclusion that escape of this enzyme is 'unphysiological'. Figure 4 shows the effect on α-glucosidase release of 20 min. semi-anaerobic treatment (removal from the shaking apparatus and left static at the same temperature) of a 3 hr. culture subsequently incubated with full aeration, in comparison with a control sister culture maintained under conditions of full aeration all the time. An even more striking example of cell-damage, caused by only 5 min. treatment of a 3 hr. culture at 0° (removal from 35° bath and incubation, with continued shaking in an ice bath), is illustrated in Fig. 5. This cold 'shock' led to the release of nearly 30 % of the total α-glucosidase during the first 30 min. of the ensuing incubation at 35°; and this was associated with (*a*) a parallel 'damage-release' of about the same proportion of penicillinase, over and above the normal penicillinase liberation, and (*b*) a 20 min. lag in growth, as measured by increase in optical density.

'*Physiological*' *release.* As long as proper care was taken to avoid mechanical and metabolic damage to the cells, the α-glucosidase released from cells of a growing *Bacillus subtilis* culture during the usual 0–5½ hr. period was found never to exceed 5 % of the total, whereas anything up to 50 % of the penicillinase was liberated during the same period. In a series of six similar experiments covering the 0–5½ hr. period a mean α-glucosidase release of 3·4 % was associated with a mean penicillinase release of 36 % of the total. At an earlier stage (0 to 3 hr. period) the figures were

1·2 and 20·5 %, respectively. Figure 6 illustrates a typical experiment. It was not possible to follow the subsequent release of the rest of the cell-bound penicillinase under conditions where damage-release could be excluded.

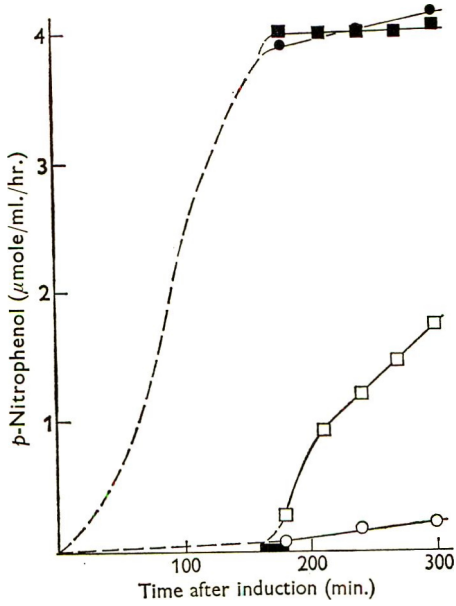

Fig. 4. The effect of transient semi-anaerobic treatment of a maltose-induced culture of *Bacillus subtilis* in casein hydrolysate on leakage of α-glucosidase from cells into the medium during subsequent aerobic incubation. Total α-glucosidase of control culture (—●—●—); total α-glucosidase of culture after 20 min. semi-anaerobic treatment (—■—■—); α-glucosidase released into medium from control culture (—○—○—); α-glucosidase released into medium from culture after 20 min. semi-anaerobic treatment (—□—□—); 20 min. period of semi-anaerobic treatment to 'test' culture (removal from shaking apparatus for 20 min.) indicated by ▬.

DISCUSSION

The fact that cell damage in *Bacillus subtilis* leading to release of α-glucosidase, associated with increased liberation of penicillinase, could take place after apparently slight interference with the normal conditions used for growing and handling the organisms, emphasized the importance of careful control of all techniques. Apart from the dangers of transient deprivation of O_2 already mentioned, it was also found that a high proportion of substances, subsequently tested for their possible effect on penicillinase liberation, promoted release of α-glucosidase (see Pollock, 1961*a*). It seemed clear that any release of α-glucosidase above the irreducible minimum of up to 5 % observed even under 'normal' conditions, would have to be regarded as indicating presumptive cell damage (probably to the cytoplasmic membrane) and that any associated increase in penicillinase liberation must be considered unphysiological and therefore, according to our criterion, an artefact. Once the conditions which caused this α-glucosidase leakage were known, the greatest care was taken to avoid them (e.g. sampling was carried out without removing culture flasks from the shaker). This 'damage-release' appears to be a

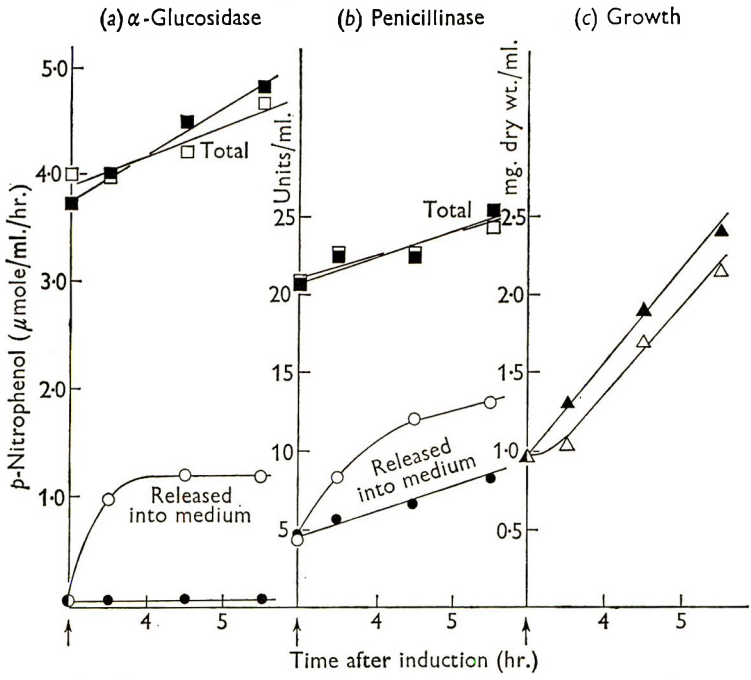

Fig. 5. Effect of transient treatment (5 min.) at 0° of a penicillin- and maltose-induced culture of *Bacillus subtilis* growing in casein hydrolysate at 35°, on leakage of α-glucosidase, and associated 'damage-release' of penicillinase from cells into the medium. Total enzyme (square symbols: ■, □); enzyme in culture supernatant (circular symbols: ●, ○); growth (triangular symbols: ▲, △); control culture (filled-in symbols: ■, ●, ▲); culture treated 5 min. at 0°, at point indicated by arrow (open symbols: □, ○, △). (*a*) α-glucosidase; (*b*) penicillinase; (*c*) growth (optical density).

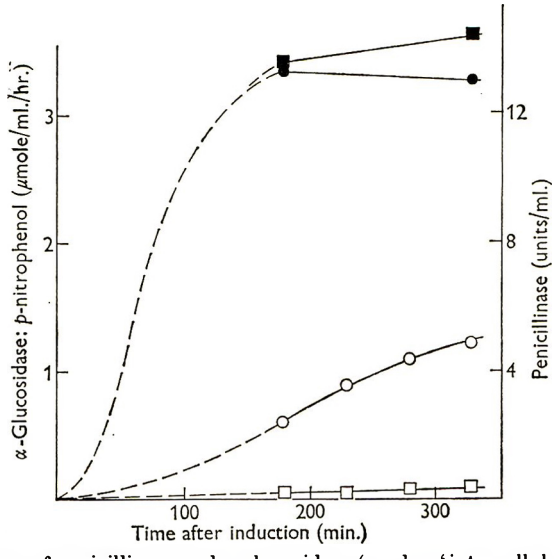

Fig. 6. Liberation of penicillinase and α-glucosidase (used as 'intracellular marker') from apparently undamaged cells of a penicillin- and maltose-induced culture of *Bacillus subtilis* growing in casein hydrolysate. Total penicillinase (—●—●—); penicillinase liberated into medium (—○—○—); total α-glucosidase (—■—■—); α-glucosidase liberated into medium (—□—□—).

secondary phenomenon, associated with autolytic processes which are initiated by cell damage and inhibited by the higher concentrations (0·05 M) of Mg^{++} (see Pollock, 1961*a*), which are known to assist in maintaining the integrity of the cytoplasmic membrane and other cell structures (Weibull, 1956). It now seems probable that the mechanism of the increased penicillinase release which occurs in association with the conditions that lead to α-glucosidase release is quite different from the normal liberation which occurs in cultures of apparently undamaged organisms.

It can, of course, be argued that, had an intracellular 'marker' more sensitive than α-glucosidase been available, some evidence of cell damage might have been disclosed during the period when what has been referred to as 'physiological' liberation of penicillinase was occurring. There is no formally satisfactory answer to this objection. A great deal must depend upon what is accepted as a definition of cell 'damage'. Clearly, organisms releasing an enzyme into the medium at a high rate must in some way be different from organisms of the same culture which liberate the same enzyme much more slowly. In this connexion, it is unfortunate that it proved to be impossible to do accurate viable counts on the *Bacillus subtilis* strain used in this study, since a comparison of these with total counts might have provided a completely independent assessment of what many people would readily accept as cell 'damage'. The ratios of viable counts to bacterial suspension opacity, however, even in samples from the same culture, were extremely variable and usually very low; no confidence whatever could be placed in their significance. Under the circumstances, however, it seems not unreasonable to assume that a good indication of cell damage is provided by the measurement of α-glucosidase release. It is therefore fair to conclude that a high proportion (up to 40 %) of the cell-bound penicillinase of *B. subtilis* is liberated from the organisms by a process not involving lysis of cells or gross interference with their physiological functions.

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The Location of Cell-bound Penicillinase in *Bacillus subtilis*

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SUMMARY

Before penicillinase is released from a penicillin-induced culture of *Bacillus subtilis* it accumulates and up to 85% is found to be bound to the cells. This cell-bound penicillinase is not free in the cytoplasm since it cannot be released in a soluble state simply by rupture of the cell envelope. It appears to be attached to the cell in a fairly superficial location since up to 40% is accessible to antiserum and up to 98% can be released, by trypsin in the presence of hypertonic sucrose, from apparently intact cells.

INTRODUCTION

Most people who have considered the problem of exoenzyme liberation from living cells appear either explicitly or implicitly to suppose that the process must involve the passage of the enzyme from 'inside' the cell (where it is assumed to be formed) across the cell membrane to the extracellular environment. With bacteria, there is no direct evidence that this is so. One essential step in the study of the liberation process is clearly to determine the location of the enzyme whilst it is still associated with the cell. Until that is known, the experimental approach to the problem of its release may be disorientated. In addition, exact knowledge of the location of the cell-bound enzyme might help to solve the question of the site of its formation, a problem which is only just becoming susceptible to experimental attack. With most bacterial extracellular enzyme systems the proportion of activity found fixed to the cells is so low as to be barely detectable (see Pollock, 1961*a*). The penicillinase of *Bacillus subtilis*, however, is a striking exception to the rule, and for this reason is particularly susceptible to analysis in relation to the problem of enzyme liberation.

METHODS

The organism, medium and general techniques used and definitions adopted were identical with those described in detail in the preceding paper (Pollock, 1961*b*). Standard 'zero' reference time (0 hr.) from which all time periods were measured, was taken to be the point when the concentration of growing cultures reached the equivalent of 0·1 mg. bacterial dry wt./ml. Unless stated otherwise, this also was the time when cells were induced to form penicillinase and α-glucosidase.

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RESULTS

Role of the cell wall

Unlike the situation with *Bacillus cereus* (Sheinin, 1959), no evidence was obtained that a significant proportion of the cell-bound penicillinase of *B. subtilis* was attached to the cell by adsorption to the cell wall. Preparations of isolated cell walls (Cummins & Harris, 1956), Mickle-disrupted cells and intact cells from an uninduced 3 hr. culture of *B. subtilis* strain 6346 grown in casein hydrolysate (CH) medium, resuspended at equiv. 2 mg. dry wt./ml. in a concentrated supernatant fluid containing 100 units penicillinase/ml. from a penicillin-induced culture, in the presence of 0·01 M-KH_2PO_4 (pH 7·0) at 35° for 1 hr., were not found to have caused more than a 15% decrease in the penicillinase activity of the supernatant fluid after centrifugation, when compared with the total activity before centrifugation (Miss E. Janczura, private communication). In another experiment, three 4·0 ml. samples of a 3 hr. uninduced culture in casein hydrolysate medium (CH) were adjusted to pH 6·0, 7·0 and 8·0, respectively, and shaken at 35° after addition of 1 ml. of a concentrated supernatant fluid from a penicillin-induced culture, giving a final concentration of 50 units/penicillinase/ml. After 2½ hr., removal of the cells by centrifugation for 20 min. at 20,000 *g* did not result in any detectable decrease in penicillinase activity, This observation that *B. subtilis* penicillinase, unlike the *B. cereus* enzyme, was neither adsorbed to intact cells nor to isolated cell walls, may be related to the fact that, again in contrast to *B. cereus* penicillinase, it is not appreciably adsorbed to powdered glass (Kogut, Pollock & Tridgell, 1956; Kushner, 1960).

Effect of repeated cell washing

A 3 hr. penicillin-induced culture was centrifuged in the cold-room at 2° and the organisms resuspended to equiv. 2 mg. bacterial dry wt./ml. in ice-cold 0·02 M-KH_2PO_4 (pH 7·0). After removing a sample for assay (27·6 units penicillinase/ml.) the suspension was re-centrifuged, and the organisms washed four times successively in 10 ml. lots of iced buffer, being finally resuspended in the same volume of buffer and reassayed (28·2 units penicillinase/ml.). It is obvious that the enzyme was not removed by repeated washing.

Effect of cell disruption

Hughes's press. A suspension of standard 3 hr. penicillin-induced organisms at a concentration equiv. 20 mg. bacterial dry wt./ml. in iced 0·01 M-KH_2PO_4 buffer, (pH 7·0) + 0·05 M-$MgSO_4$ was crushed in a Hughes's press at −30°. When the crushed preparation was thawed and homogenized to obtain an evenly dispersed suspension, 43% of the total penicillinase activity was found in the supernatant fluid after centrifugation for 10 min. at 20,000 *g*.

Lysozyme treatment. 'Spheroplasts' (see McQuillen, 1960), prepared by incubating organisms with 100 μg. lysozyme/ml. at 35° in the presence of 0·3 M-sucrose or 20% (w/v) polyethylene glycol appeared, microscopically, to be stable, but were found to leak the normally cell-bound α-glucosidase (induced in the cells as an intracellular 'marker'; see Pollock, 1961*b*) into the medium and released a variable proportion of penicillinase. The addition of high concentrations of Mg^{++} (0·05 M-$MgSO_4$) decreased the rate of enzyme release during lysozyme treatment, and the

Table 1. *Liberation of penicillinase and α-glucosidase from disrupted spheroplasts of penicillin—and maltose-induced* Bacillus subtilis 6346

		Penicillinase		α-Glucosidase	
Treatment	Description	Units/ml.	% released to medium after treatment	μmole *p*-nitrophenol ml./hr.	% released to medium after treatment
Resuspended organisms from 3 hr. culture	Whole organisms	16·80	.	5·70	.
Samples of whole organisms treated at 35° with 100 μg. lysozyme/ml. + 0·3 M-sucrose for (*a*) 2 min., (*b*) 5 min., followed by centrifugation and resuspension in 0·05 M-$MgSO_4$	Spheroplasts, prepared by lysozyme treatment for:				
	(*a*) 2 min.	17·12	(−2)	2·42	57·5
	(*b*) 5 min.	14·32	14	1·95	65
'Spheroplasts', allowed to lyse in 0·05 M-$MgSO_4$, centrifuged and supernatant fluid retained	Supernatant fluid from disrupted spheroplasts, previously prepared by lysozyme treatment for:				
	(*a*) 2 min.	0·34	2	2·24	93
	(*b*) 5 min.	0·50	3·5	1·92	96·5

For full experimental details see text.

spherical form was only slowly attained, although resuspension in dilute buffer solution (0·01 M-KH_2PO_4, pH 7·0, + 0·05 M-$MgSO_4$) without sucrose caused immediate lysis. This stabilizing effect appeared to be mainly on the cell membranes which disintegrated only very slowly as long as the Mg^{++} concentration was kept high (as found with *Bacillus megaterium* by Weibull, 1956). The use of Mg^{++} thus allowed the preparation of fairly stable 'membranes' (i.e. resuspended, lysed spheroplasts) which retained most of the penicillinase activity of the intact organisms, although nearly all the α-glucosidase had escaped into the medium during the lysozyme treatment and subsequent lysis in hypotonic buffer solution. Table 1 summarizes the results of an experiment which demonstrated this. A 3 hr. culture (90 ml.) induced with maltose and penicillin was centrifuged and the organisms resuspended at 35° in 45 ml. 0·01 M-KH_2PO_4 (pH 7·0) + 0·05 M-$MgSO_4$ and 0·3 M-sucrose. Lysozyme (100 μg./ml.) was then added and 18 ml. samples removed after incubation for 2 min. and 5 min., cooled as rapidly as possible and centrifuged at 2° for 10 min. at 20,000 *g*. The deposits were resuspended to the same volume in iced buffer + 0·05 M-$MgSO_4$ and homogenized rapidly (2 min.) in a Potter–Elvehjem homogenizer, the temperature being kept as low as possible. Samples of these 'disrupted spheroplasts' were centrifuged for 10 min. at 20,000 *g* and the penicillinase and α-glucosidase activities remaining in the supernatant fluid compared with total activities before centrifugation and with the activities of a sample of the original cell suspension treated with lysozyme in the absence of sucrose or Mg^{++}. The results showed that (*a*) practically all the α-glucosidase was liberated when a lysozyme-treated preparation

of cells was lysed by resuspension in hypotonic medium, and that more than 50% escaped even before the sucrose was removed, whereas (*b*) barely significant amounts of penicillinase were released after only 2 min. lysozyme treatment and very little even after 5 min. As shown previously (Pollock, 1961*b*), longer incubation resulted in further penicillinase liberation by processes which were considered to be secondary autolysis.

Table 2. *Liberation of penicillinase from disrupted 'spheroplasts' of* Bacillus subtilis 6346

	Total penicillinase (units/ml.)	Penicillinase released after incubation at 35° (units/ml.)	% released
Expt. 1: 90 min. incubation			
Control	13·6	7·8	58
+ $MgSO_4$ 0·05 M	13·6	2·8	21
+ *p*-Chloromercuribenzoate $2·5 \times 10^{-3}$ M	12·8	5·4	42
+ heparin 1 mg./ml.	14·4	8·0	56
+ diisopropylfluorophosphate 2×10^{-3} M	14·4	8·8	60
Expt. 2: 0·05 M-$MgSO_4$ added to all tubes; incubation for 10 min.			
Control	17·8	4·4	25
+ trypsin, 1 mg./ml.	19·0	17·6	93
Expt. 3: 0·05 M-$MgSO_4$ added to all tubes; incubation for 10 min.			
Control	32·4	7·1	22
+ trypsin, 1 mg./ml.	31·0	17·5	57
+ ribonuclease 1 mg./ml.	33·2	7·5	23
+ butanol 1%	32·4	7·8	24
+ sodium deoxycholate 1%	32·4	28·3	88

For full experimental details, see text.

This autolytic-type of penicillinase release from 'disrupted spheroplasts' (prepared as described above) was studied further in an experiment summarized in Table 2 where the effects of various reagents on the process were investigated. The following points may be seen. (*a*) Trypsin, at 1 mg./ml., rapidly liberated a large (though rather variable) proportion of the penicillinase even in the presence of 0·05 M-Mg^{++}. Sodium deoxycholate (10 mg./ml.) had a similar effect. (*b*) The 'autolytic' release was inhibited by Mg^{++} (0·05 M) and by *p*-chloromercuribenzoate ($2·5 \times 10^{-3}$ M) but not by diisopropylfluorophosphate (DFP) or heparin. The question of how closely related this 'autolytic' liberation phenomenon may be to normal (physiological) release of enzyme in growing cultures is considered later.

Combined effect of trypsin and hypertonic sucrose

Trypsin alone (at least up to 1 mg./ml.) had no detectable action on growth or enzyme liberation from cultures of *Bacillus subtilis* 6346 in CH medium, and little effect on release of penicillinase or α-glucosidase from cells of a 3 hr. penicillin- and maltose-induced culture resuspended at 35° in phosphate buffer. But (see Table 2) it rapidly liberated the enzyme from a 'membrane'/preparation (disrupted 'spheroplasts'). Sucrose alone (0·9 M) completely inhibited growth and caused a variable degree of cell damage in similarly prepared suspensions of induced cells (as indicated

by α-glucosidase release into the medium) but had no effect on penicillinase liberation, at least during incubation for 30 min. at 35°. Treatment with trypsin and sucrose together, however, led to considerable release of cell-bound penicillinase within about 20 min. The extent of this effect, however, varied from day to day, maximal penicillinase release in 30 min. varying between the extremes of 45 and 98 % in different experiments. α-Glucosidase release also varied, but not in a fashion apparently correlated with the extent of penicillinase release. In the most striking experiments, a 98 % release of penicillinase was associated with only a 5 % release of α-glucosidase. Other typical experiments are shown in Table 3 and Fig. 1.

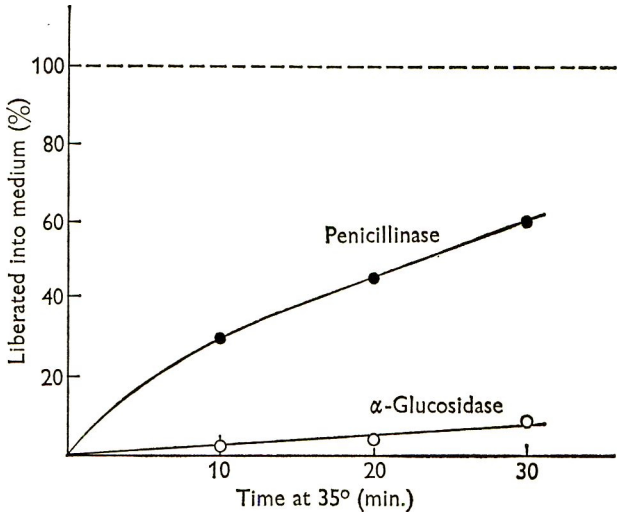

Fig. 1. The release of α-glucosidase (—○—○—) and penicillinase (—●—●—) from maltose- and penicillin-induced cells of *Bacillus subtilis* 6346 resuspended in 0·01 M-sodium phosphate (pH 8·0) at 35° in the presence of 0·9 M-sucrose + trypsin 1 mg./ml.

Table 3. *Release of penicillinase from* Bacillus subtilis 6343 *by treatment with sucrose and trypsin for* 30 *min. at* 35°

	Penicillinase units/ml.		
	Total	In supernatant fluid	Amount released into medium (%)
Control	19·8	1·8	9·1
+ sucrose 0·9 M	18·5	1·2	6·5
+ trypsin, 1 mg./ml.	19·6	3·4	17·3
+ 0·9 M-sucrose and trypsin 1 mg./ml.	18·9	13·1	69·5

For full experimental details, see text.

The full procedure for the above experiment was as follows. A 3 hr. culture of *Bacillus subtilis* 6346 induced with penicillin and with maltose was centrifuged, the organisms resuspended in different mixtures in 0·01 M-KH_2PO_4 (pH 8·0) + 0·05 M-$MgSO_4$, with or without 0·9 M-sucrose and with or without trypsin 1 mg./ml. and all mixtures incubated aerobically at 35°. Samples were cooled rapidly, a few ml.

retained for total penicillinase assay and the remainder centrifuged at 20,000 *g* for 10 min. in the cold. The supernatant fluids were assayed for penicillinase activity, and the deposits resuspended to volume in a buffer mixture containing 0·02 M-KH_2PO_4 (pH 7·0) and 0·1 % heparin (to inhibit the further action of trypsin on α-glucosidase as it is released) and 100 μg. lysozyme/ml., incubated at 35° until lysis was complete and then assayed for α-glucosidase activity as soon as possible. The proportion of α-glucosidase released was calculated from the difference between the activities of the lysed deposits and those of the sample of original cell suspension lysed directly with lysozyme under the same conditions but with Mg^{++}, sucrose or trypsin. Whatever degree of penicillinase release was achieved, it was usually maximal after 20 min. incubation (although this particular feature does not happen to be well illustrated by the example shown in Fig. 1). This indicated that the inconstant results were due to variation either in the proportion of cells completely insusceptible to the action of trypsin and sucrose or in the extent to which the cell-bound penicillinase had reached a location on the cells accessible to trypsin.

Trypsin had no effect on penicillinase, but rapidly inactivated the α-glucosidase as soon as it was liberated from the cell, though not before. The problem of assaying cell-bound α-glucosidase in the presence of trypsin was complicated by the need to lyse the cells with lysozyme in order to allow full expression of activity, after which the enzyme was rapidly destroyed by the trypsin. It had, however, been found that the inactivation of α-glucosidase by trypsin could be almost completely prevented by 0·1 % heparin (see Pollock, 1961*b*) which was therefore added as soon as samples were taken. In this way, it could be shown that as long as α-glucosidase remained within the cells, it was almost completely protected from the proteolytic action of trypsin. This suggested that the trypsin had not actually penetrated the cell membrane during the time it was releasing the penicillinase and must therefore be acting relatively superficially. At concentrations of sucrose below 0·9 M (e.g. at 0·3 M) the extent of penicillinase release by trypsin was relatively slight. It therefore seemed possible that liberation by trypsin was dependent on some degree of plasmolysis, perhaps involving separation of cell membrane and cell wall associated with surface damage, which allowed access of trypsin to certain structures (presumably fully exposed to the action of trypsin in the preparation of disrupted 'spheroplasts' described above) which were vital for the retention of penicillinase.

When 0·9 M-sucrose was replaced by 4 % (w/v) polyethylene glycol (mean M.W. 400) which should be osmotically equivalent, no release of penicillinase occurred in the presence of trypsin. It is not, however, known whether polyethylene glycol can penetrate the cell wall. If this does not occur and the cell wall is not completely rigid, the cell may simply shrink as a whole and no plasmolysis involving separation of membrane and cell wall would take place. Whatever the nature of the bonds responsible for this binding of penicillinase, it seems likely that the trypsin could not have penetrated the barrier of the cytoplasmic membrane and that the bulk of the penicillinase, therefore, is probably bound on the outer surface of the membrane, or even more superficially.

No direct evidence, however, could be obtained on the mechanism of action of sucrose; neither observation by phase contrast nor electron microscope studies revealed any convincing difference between cells treated and untreated with 0·9 M-sucrose, with or without trypsin. Amongst other enzymes tested because of their

ability to attack substrates which possibly form part of the superficial cell structure, the effect of trypsin was shared only by another proteolytic enzyme (*Bacillus subtilis* proteinase itself; Nagase and Co. Ltd., Itachibori-minamidori 1-chome, Osaka, Japan). Bovine pancreatic ribonuclease (Armour Pharmaceutical Company Ltd., Eastbourne), *Clostridium welchii* lecithinase (kindly given by Dr Marjorie Macfarlane of the Lister Institute of Preventive Medicine, Chelsea Bridge Road, S.W. 1), a pancreatic lipase preparation (Mann Research Laboratory, 136, Liberty Street, New York 6, N.Y.) and a microbial lipase preparation (Mann Research Laboratory, 136, Liberty Street, New York 6, N.Y.), all at a concentration of 1 mg./ml., tested in the presence of 0·9 M-sucrose, were without effect. The action of lysozyme has already been mentioned and discussed.

Experiments with anti-penicillinase serum

Specific neutralization by anti-enzyme serum has been used on several occasions as evidence for the localization of cell-bound enzyme on the cell surface (Sevag, Newcomb & Mill, 1954; Pollock, 1956*a*). The value of the method depends upon the assumption that rabbit γ-globulin cannot penetrate to 'within the cell' (i.e. pass through the cytoplasmic membrane), for which there is some experimental evidence in yeast (Krebs & Wright, 1951; Pasternak, Sevag & Miller, 1954). The extent to which it may or may not be able to pass through the cell wall is not known. Obviously, therefore, only positive enzyme neutralization tests can be satisfactorily interpreted. A negative result might mean that the enzyme was within the cytoplasmic membrane, or outside the membrane but protected by the cell wall from combination with antibody.

An antiserum against *Bacillus subtilis* 749 exopenicillinase prepared in rabbits was found to give 78% neutralization of the heterologous exopenicillinase from *B. subtilis* 6346 (Kushner, 1960). Cells from induced cultures of *B. subtilis* 6346 were centrifuged at times between 3 and 6 hr. after induction, resuspended to 1/3 the original volume in 0·01 M-KH_2PO_4 (pH 7·0) containing 8×10^{-4} M-8-hydroxyquinoline to prevent further enzyme synthesis. Two lots of 0·5 ml. of this suspension were put into each of two Warburg flasks, one containing 0·2 ml. water and the other 0·2 ml. of anti-749 penicillinase serum (equivalent to approximately twice the quantity necessary for maximal neutralization of the enzyme). The flasks were left at room temperature for 20 min. Then 1·0 ml. of a mixture containing 1% (w/v) gelatin $+ 8 \times 10^{-4}$ M-8-hydroxyquinoline, bicarbonate and penicillin was added as before, and the penicillinase activities measured. The results, giving '% enzyme in combination with antiserum' are calculated from the % difference in enzyme assay with and without antiserum, after allowing for the 22% residual activity of the enzyme + antibody complex consistently demonstrable, even in the presence of a gross antibody excess. It was found that the addition of an equivalent quantity of lysed or intact organisms from an uninduced culture had no significant effect on the neutralization of soluble enzyme by antisera, and that therefore the presence of cells was unlikely *per se* to affect the reaction between enzyme and antibody. Control experiments showed that negligible quantities of enzyme were liberated during the period required for allowing the antiserum to neutralize the enzyme and subsequent assay.

When the % cell-bound penicillinase combining with antiserum was measured in the period 3–5 hr. after induction, it was found, despite somewhat variable results,

that there was usually 30–40 % demonstrable at 3 hr. (after which there is little further production of enzyme in penicillin-induced cultures; Pollock, 1961*b*), but that after a further 2 hr. growth the proportion had fallen to 0–15 % (Fig. 2). By 5–7 hr., up to 60 % of the total enzyme formed had been liberated. The fact that the proportion of cell-bound enzyme which combined with antiserum tended to fall from 3 hr. onward suggested that it was this moiety which was first released. Calculations in individual experiments showed that the amount of enzyme released was sufficient to account for the decrease in antibody-combinable enzyme. These results also show, however, that enzyme release can take place from cells in which little or no neutralizable enzyme is detectable; this suggests that though enzyme molecules in the antibody-combinable state may be preferentially released, such a state is not necessarily a preliminary one to release from the cell.

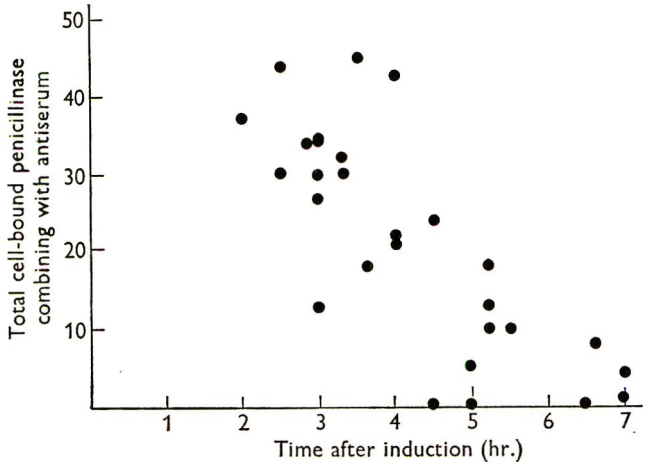

Fig. 2. A scatter diagram summarizing all results obtained in a series of experiments showing the decrease in the proportion of cell-bound *Bacillus subtilis* penicillinase combinable with anti-exopenicillinase serum, during the 2–7 hr. period following the point when the opacity of a culture growing in casein hydrolysate reached the equivalent of 0·1 mg. bacterial dry wt./ml. (being also the time when enzyme formation was induced with penicillin).

The proportion of antiserum-combinable cell-bound enzyme at 3 hr. after induction was not altered by conditions which increased the total amount of enzyme formed. In one experiment, where the culture had been induced with cephalosporin C (10 μg./ml.) and the total quantity of penicillinase produced was nearly five times as great as that of a sister culture induced with penicillin, the above proportions were not significantly different (30 and 32 %, respectively). It should be recalled that increasing the total enzyme formed, by induction with cephalosporin C, did not influence the extent to which it was liberated into the medium (Pollock, 1961*b*). After overnight incubation of the culture, all the enzyme was liberated into the medium and was then found to be neutralized by antiserum to maximal extent, as with enzyme liberated in earlier stages of growth. It is thus clear that the cell-bound penicillinase has no immunologically distinct fraction similar to the 'γ-penicillinase' possessed by *Bacillus cereus* (Pollock, 1956*b*).

When 3 hr. cells of *Bacillus subtilis* 6346 were disrupted with lysozyme, not all the penicillinase was thereby immediately made accessible to antiserum, though the proportion rose from 40 to 60 % (means of 6 experiments). A similar increase was observed when 3 hr. cells were crushed in a Hughes's press. When 5 hr.-cells, which had already released most of their antiserum-accessible enzyme to the medium, were treated with lysozyme, the proportion increased from 11·5 to 53 % (means of 2 experiments). When lysed or crushed organisms were incubated overnight at 35°, the proportion further increased to 70–90 %, possibly through an autolytic destruction of bonds which prevented access of antibody to the relevant part of the enzyme molecule.

DISCUSSION

These results show that the cell-bound fraction of *Bacillus subtilis* 6346 penicillinase is largely, if not entirely, bound to insoluble cell structures, sedimentable by 15 min. centrifugation at 20,000 *g*. This fact, and the visible appearance of the cell debris following treatment with lysozyme in the presence of high concentrations of Mg^{++}, suggest that the enzyme is probably bound to structures forming the cell envelope. At least 30 % of the cell-bound fraction is fixed superficially to the cell, being accessible to combination with antiserum. The results with antiserum also suggest that it is this superficially-bound fraction of enzyme which is selectively released during the 3–6 hr. period during which the liberation process has been studied.

The action of trypsin in liberating a high proportion of cell-bound penicillinase after treatment of the cells with hypertonic sucrose, but apparently without penetrating the cytoplasmic membrane, suggests that the penicillinase was probably released from structures outside the main permeability barrier of the cell. This effect appears to be analogous to the action of trypsin in promoting the release of the 'M' protein from streptococci (Elliott, 1945). With streptococci, no preconditioning with sucrose or other adjuvant is necessary. The M protein appears to be bound directly or indirectly to cell wall material since it is not normally liberated into the medium in significant amounts except in old cultures, whereas it is not found attached to protoplasts which, nevertheless, appear still capable of forming it (Freimer, Krause & McCarty, 1959). A proportion of this protein must likewise be superficially located since it can be shown to react with antiserum in undamaged cells. On the whole it would appear that *B. subtilis* 6346 penicillinase may be fixed to the cells in a similar manner, but perhaps rather more deeply buried than is the M protein.

The results as a whole are consistent with the hypothesis that cell-bound penicillinase is attached to the outside of the cytoplasmic membrane, and may actually be formed at its surface. It is possible that at least a portion may be bound in, or closely associated with, the cell wall. Nor is it excluded that part might be in a soluble state, sandwiched between the cell membrane and an impermeable or poorly permeable cell wall (e.g. in the so-called 'periplasm' postulated by Mitchell, 1961). But it is difficult to understand, if this be so, how most of the α-glucosidase is released from the cells under conditions (e.g. incubation at 0°; Pollock, 1961*c*; or after brief lysozyme treatment with high Mg^{++} concentrations) which do not allow escape of penicillinase. The only evidence possibly in favour of the enzyme being in a 'periplasm' is the rapid release of over 40 % of the penicillinase after crushing in a Hughes's press. However, in order to obtain evenly dispersed suspensions of

disrupted cell material by this technique (without which, of course, no comparison of particle-bound and soluble enzyme is valid) it is necessary to thaw out the frozen crushed suspension for several minutes and homogenize it thoroughly before centrifugation. It is possible that this may be time enough for secondary autolytic processes to cause a considerable amount of release of penicillinase from cell fragments. In any case, it seems at the moment reasonable to approach the problem of 'normal' release of *Bacillus subtilis* 6346 penicillinase on the basis of a working hypothesis that it may involve the detachment of the enzyme from structures on the surface of, or outside, the main permeability barrier of the cell, rather than passage through the barrier itself.

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The Mechanism of Liberation of Penicillinase from *Bacillus subtilis*

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SUMMARY

The effects of various factors on the release of penicillinase from apparently undamaged cells of a growing culture of *Bacillus subtilis* were investigated. The enzyme was not eluted from the cells by treating them with high concentrations of salt. Its liberation did not take place at all at 0°, and was nearly completely inhibited at pH values below 6·0, whereas chloramphenicol, at concentrations sufficient to cause complete cessation of growth, caused only partial inhibition of enzyme release. The penicillinase-releasing action of extracts containing heat-labile 'autolytic' factors from older cells of the same organism could not be dissociated from their damaging effect on the cell, as indicated by concomitant release of the normally intracellular α-glucosidase. It is concluded that normal penicillinase liberation is controlled by enzymic reactions, as yet unidentified, involving detachment of the enzyme from structures superficially located in the cell envelope.

INTRODUCTION

In the first two papers of this series some evidence was presented to suggest that the liberation of penicillinase from a growing culture of *Bacillus subtilis* 6346 may involve a release of the enzyme from the superficial layers of apparently intact cells. The present paper records the results of a direct investigation of the liberation process itself, with a view to characterizing it and obtaining further information on its nature. Minor degrees of cell damage, insufficient to cause measurable lysis, were detected by following the leakage into the medium of an 'intracellular' α-glucosidase whose synthesis was induced in the cells, during the period when penicillinase was being formed, by the addition of a small quantity of maltose (Pollock, 1961*b*).

'Normal' or 'physiological' penicillinase liberation is defined here as the penicillinase release which occurs in untreated cultures in the absence of cell damage sufficient to cause leakage of α-glucosidase greater than the irreducible minimum of up to 5% that was always liable to take place. On this criterion, the conditions under investigation in many experiments caused significant cell damage, and in such cases it could only be concluded that the associated increase in penicillinase liberation, which nearly always occurred, was 'unphysiological'. Unfortunately this took place all too frequently, and in most of the other experiments the factors being studied had no detectable effect. Our conclusions have therefore been limited, extending only to an indication that 'normal' penicillinase liberation is not a matter only of physico-chemical factors, but involves more complicated processes having an enzymic basis.

METHODS

The strain (6346) of *Bacillus subtilis* used, the techniques employed, the definitions adopted and the general approach to the problem have been described and discussed previously (Pollock 1961*b*). It should be particularly noted that the standard 'zero' reference time (0 hr.) from which all time periods are measured was taken as the point when the population density of growing cultures reached the equivalent of 0·1 mg. bacterial dry wt./ml. Unless stated otherwise this has also been the time when cells were induced to form penicillinase and α-glucosidase.

RESULTS

Effect of pH value

When the casein hydrolysate (CH) medium was supplemented with 2 % (w/v) glucose, the organisms were found never to release more than a small fraction of their total penicillinase. Even after 6 hr. such glucose-grown organisms had liberated only 15 % of their total penicillinase, in contrast to organisms grown in CH medium without glucose, where 40 % or more of the enzyme was released into medium during the same period (see Pollock, 1961*b*). This effect was finally shown to be due mainly, if not entirely, to the lower pH value of the culture, which usually fell from 7·0 to 5·0–5·5 in glucose-containing cultures.

It was confirmed that the low amounts of extracellular penicillinase were not due to selective inactivation of exo-enzyme under the conditions associated with glucose-grown cultures. Concentrated dialysed supernatant fluid from a penicillin-induced culture at a final concentration of 13·5 units penicillinase/ml. was added to a 3 hr. uninduced culture of *Bacillus subtilis* growing in CH medium + 2 % (w/v) glucose. Incubation continued for a further 2 hr. caused no significant loss of activity.

In the experiment summarized in Table 1 a culture grown in CH medium + 2 % (w/v) glucose was split into two portions at 3 hr., and both portions incubated in large tubes at 35°, aerated by a flow of air through a sparger at 500 ml./min. In one portion the pH value was adjusted to pH 7·8 and kept constant thereafter for the 2 hr. of the experiment by an automatic titrator (Radiometer, Titrator TTTI, V. A. Howe and Co. Ltd., 46 Pembridge Road, W. 11) delivering minute quantities of N-NaOH into the culture continuously under the control of a pH meter. The total volume of NaOH thus added never increased the volume of the cultures by more than 3·5 % and was ignored in comparing the results. A complementary experiment was also done without glucose in the medium, one of the two sister cultures being kept at pH 5·2 by automatic delivery of N-HCl while the control culture was left to drift towards alkalinity without adjustment. It can be seen that several times less enzyme was liberated in cultures at the lower pH values, regardless of whether or not glucose was present.

Effect of chloramphenicol

Addition of 40 μg. chloramphenicol/ml. to a culture of *Bacillus subtilis* 6346 inhibited growth (opacity increase) and penicillinase production almost completely. The effect on enzyme liberation appeared at first to be variable. To obtain higher enzyme titres and more reliable results, some cultures were induced with cephalosporin C (1 μg./ml.) and it was at once clear that significant enzyme release occurred

Table 1. *Effect of pH value on the liberation of penicillinase from penicillin-induced* Bacillus subtilis 6346 *growing in CH medium*

Conditions	pH value: At 3 hr. before adjustment of (B)	pH value: At 5 hr.	Penicillinase activities (units/ml.): At 3 hr. Total	At 3 hr. Sn*	At 5 hr. Total	At 5 hr. Sn	Released during 3–5 hr. period: Amount	Released during 3–5 hr. period: % of total
Exp. 1. In presence of 2 % (w/v) glucose								
(A) No special control of pH value	5·2	5·4	30·96	2·92	31·2	3·8	0·88	2·8
(B) pH Adjusted to pH 7·8 at 3 hr. and thereafter kept constant	5·2	7·8	31·60	3·4	30·0	11·2	7·8	25·5
Exp. 2. Without added glucose								
(A) No special control of pH value	6·8	7·0	28·40	6·2	30·56	10·8	4·6	15·8
(B) Adjusted to pH 5·2 at 3 hr. and thereafter kept constant	6·8	5·2	28·76	6·0	27·76	7·6	1·6	5·6

* Sn = supernatant fluid.

in the presence of chloramphenicol; but no quantitative comparison with untreated cultures was possible because the total enzyme production was so much less than in the control. Repeated experiments with benzylpenicillin-induced cultures finally showed that chloramphenicol did in fact cause a significant and consistent inhibition of liberation of 30–50 %; even in cultures where the slight difference in pH value from the control (due to growth inhibition and subsequently decreased metabolic drift towards alkalinity that is always shown by *B. subtilis* when growing in CH medium) was obliterated by the automatically titrating pH regulator, adjusted so that the pH value of the control culture was kept identical with that of the chloramphenicol-treated culture (measured independently with a second pH meter), as shown in Table 2. Neither earlier addition of chloramphenicol (namely at 2 hr.

Table 2. *Effect of chloramphenicol on the liberation of penicillinase from penicillin-induced* Bacillus subtilis 6346 *in CH medium*

Conditions	pH: At 3 hr.	pH: At 5 hr.	Penicillinase activities (units/ml.): At 3 hr. Total	At 3 hr. Sn*	At 5 hr. Total	At 5 hr. Sn	Released into medium during 3–5 hr. period: Amount	Released into medium during 3–5 hr. period: % of total
Control (no addition)	7·0	7·65	17·6	3·6	21·2	6·8	3·2	15
Chloramphenicol 40 μg./ml. added at 3 hr.	7·0	7·65	—	—	20·4	5·2	1·6	7·8

* Sn = supernatant fluid.

instead of 3 hr.) nor raising its concentration to 100 μg./ml. caused any significant change in the proportion of enzyme released.

Effect of temperature

Figure 1 shows the effect of temperature on penicillinase release from a culture previously induced with 0·25 mg. maltose/ml. and cephalosporin C (1 μg./ml.) treated with chloramphenicol (40 μg./ml.) at 3 hr. and immediately distributed in three lots in water baths maintained at 0°, 21° and 35°, respectively. The proportions of α-glucosidase released after 2½ hr. incubation at these three temperatures were found to be 36·3, 4·1 and 5·6%, respectively. The cold treatment obviously

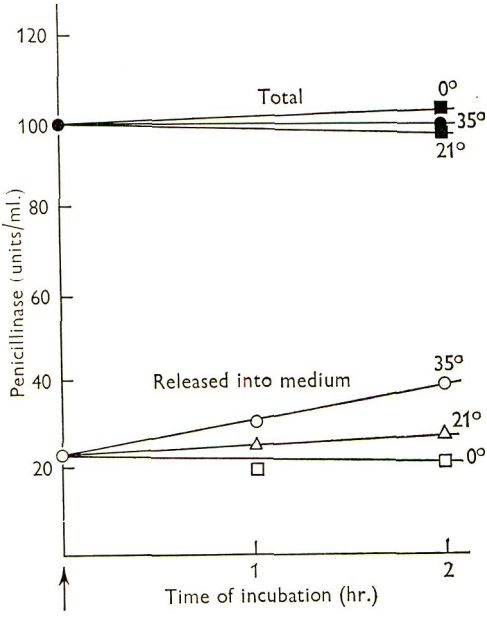

Fig. 1. The effect of temperature on the liberation in the presence of chloramphenicol of penicillinase from 3-hr. cells of *Bacillus subtilis* 6346, previously grown at 35° after induction with cephalosporin C (10 μg./ml.). 35° (circles: ●, ○); 21° (triangles: ▲, △); 0° (squares: ■, □); enzyme released into medium (open symbols: ○, △, □); total enzyme (filled-in symbols: ●, ▲, ■). Arrow indicates point of addition of chloramphenicol 40 μg./ml.

caused considerable cell damage, without, however, leading to penicillinase liberation, as long as the temperature was kept at 0°. When, however, the culture was returned to 35°, even brief cold treatment (e.g. 5 min. at 0°) resulted in substantial subsequent 'damage-release' of penicillinase, as shown in the first paper in this series (Pollock, 1961*b*; Fig. 5). In another experiment it was found that the penicillinase liberation increased further with temperatures above 35°; but at 45° this was always associated with considerable cell damage and could not therefore be regarded as physiological.

The rather striking dissociation between release of α-glucosidase and penicillinase at 0° in the experiment illustrated in Fig. 1 supports the original conclusion (Kushner & Pollock, 1961) that cell-bound penicillinase is normally fixed to particulate cell structure and does not exist 'free' in the cytoplasm (as appears to be the case with

α-glucosidase), and shows further that both the normal 'physiological' mechanism for the release of penicillinase and the secondary 'artefactual' release process, which under all other conditions so far investigated comes into effect following cell damage, are inoperative at 0° and therefore more likely to be dependent on some enzymic process in the cell rather than on diffusion or other purely physico-chemical factors.

Table 3. *Failure of high concentrations of salts to elute penicillinase from penicillin-induced* Bacillus subtilis 6346 *after* 2 *hr. treatment at* 0°

Treatment	α-Glucosidase (as mμmole *p*-nitrophenol/ml./hr.)			Penicillinase (units/ml.)		
	Total	In Sn	% in Sn*	Total	In Sn	% in Sn
Potassium phosphate (pH 7·0)						
10^{-3}M	3130	2030	65	17·0	0·48	2·8
5×10^{-2}M	3130	2500	80	17·8	0·68	3·8
2×10^{-1}M	3130	2500	80	16·1	0·76	4·7
Ammonium sulphate (pH 7·0)						
5×10^{-2}M	3130	2630	84	16·6	0·56	3·4
2×10^{-1}M	2970	2630	89	17·0	1·12	6·6

* Sn = supernatant fluid.

Effect of high salt concentrations

A 3 hr. culture of *Bacillus subtilis* 6346, previously induced with penicillin and maltose, was cooled to 0° and the organism resuspended in solutions of potassium phosphate and ammonium sulphate (pH 7·0) at concentrations of up to 0·2 M for 2 hr. (at 0°, instead of 35°, to avoid 'damage-release'). Table 3 shows that negligible quantities of penicillinase (≯7 %) were released by this treatment, which would be expected to elute a substantial fraction of enzyme, were the normal liberation process due to a desorption of protein bound to the cell by simple ionic linkages. This experiment shows the profound cell damage that occurred as a result of this treatment which led to the release of up to 90 % of the α-glucosidase. It is also a further indication of the different manner in which the penicillinase is bound to the cells of *B. subtilis* as compared with *B. cereus*, where analogous salt treatment eluted nearly all the cell-bound β-penicillinase within a few minutes (Pollock & Kramer, 1958).

Possible role of enzymes

Effect of enzyme inhibitors. With 3 hr.-cultures of *Bacillus subtilis* 6346 growing in CH medium many of the substances and factors tested ($1{\cdot}6 \times 10^{-3}$M-*p*-chloromercuribenzoate; 0·05–1·0 % (w/v) sodium deoxycholate; $8{\cdot}3 \times 10^{-4}$M-8-hydroxyquinoline) increased penicillinase liberation but rapidly led to cell damage (α-glucosidase leak) so that their action on physiological release could not be assessed. Other substances added (5×10^{-3}M-NaN_3; 10^{-3}M-2:4-dinitrophenol; $CuSO_4$, 100 μg./ml.; 0·02 M sodium succinate; mercaptoethanol, 0·5 mg./ml.; carbamylcholine, 0·1 mg./ml.; histamine phosphate, 0·1 mg./ml.) had no detectable effect. Ethylenediamine tetra-acetic acid (10^{-3}M), in the presence of chloramphenicol (40 μg/ml.), had a slight inhibitory effect on penicillinase liberation (up to 40 % from cells of a cephalosporin C-induced culture in some experiments), but this was not consistently repeatable. A similar

variable but even slighter inhibitory effect was shown by low doses of ultraviolet radiation in the absence of chloramphenicol. High concentrations of Mg^{++} (0·05 M-$MgSO_4$) which had been shown to have a very striking inhibitory effect on the release of the enzyme from membrane preparations or damaged cell suspensions (Kushner & Pollock, 1961) had no action whatever in cultures growing normally in CH medium.

Proteinases. The specific penicillinase-liberating action of proteinases on sucrose-treated suspensions of *Bacillus subtilis* 6346 (Kushner & Pollock, 1961) and the possibly enzymic nature of the liberation process suggested that this might normally involve the action of some proteolytic enzyme or esterase, either by direct detachment of the enzyme by hydrolysis of a peptide bond which linked it to some surface structure, or by destruction (solubilization) of a protein responsible for its fixation. Attempts to inhibit the hypothetical esterase involved, by addition of diisopropylfluorophosphate (DFP) and heparin (0·1 %) were unsuccessful. Concentrations of DFP up to a nearly saturated solution (addition of 1 drop of pure DFP in 10 ml. of culture, repeated twice during the 2 hr. experiment) had no significant effect on liberation of penicillinase from cephalosporin C-induced cells of a culture previously treated with chloramphenicol (40 μg./ml.).

Table 4. *The effect of crude dialysed extracts of organisms from old* Bacillus subtilis 6346 *cultures on release of α-glucosidase and penicillinase from cells of a maltose- and penicillin-induced homologous* B. subtilis *culture in casein hydrolysate (CH) medium*

Time	Volume (ml.) of extract* added (at 3 hr.) to 10 ml. culture	Bacterial suspension concentration (equiv. mg. dry wt./ml.)	α-Glucosidase (mμmole *p*-nitrophenol/ml./hr.) Total	In Sn†	% in Sn	Penicillinase units/ml.) Total	In Sn	% in Sn
3 hr. (before addition of extract)	—	0·85	4310	47	1·1	21·6	4·8	22·2
$5\frac{1}{2}$ hr.	0	2·10	4130	65	1·6	20·8	7·6	36·5
	0·023	2·25	4000	108	2·7	21·6	8·3	38·5
	0·067	2·44	4500	338	7·5	21·2	9·5	44·8
	0·2	2·10	4810	1275	26·5	22·0	12·2	55·3
	0·5	1·04	4900	3090	63	20·4	15·6	76·5
	1·8	0·37	5100	4560	89·5	21·6	13·3	61·7

* The extract was diluted so that 1·0 ml. contained material originally derived from the organisms of 10 ml. of a 24 hr. culture. Further details, see text.

† Sn = supernatant fluid.

Crude cell extracts. Extracts were prepared from organisms of an uninduced culture of *Bacillus subtilis* 6346, by resuspending the organisms at ten times their original concentration in 10^{-2} M-potassium phosphate buffer (pH 7·0) and crushing them in a Hughes press at −30°. The supernatant fluid after centrifugation of the disrupted suspension at 20,000 *g* for 20 min. was dialysed against 10^{-3} M-potassium phosphate (pH 7·0) and concentrated by freeze-drying. These extracts had enzyme-like properties which appeared to be similar to those of the 'autolysin' of *B. subtilis* strain H described by Nomura & Hosoda (1956). When added to cultures of *Bacillus subtilis* 6346 in CH medium the extracts consistently caused cell damage, the extent of

which depended upon the quantity of extract and the age of the culture from which it was prepared. Severe damage resulted in cell lysis. Less severe damage was shown by leakage of α-glucosidase and increase in penicillinase liberation; but it was not possible to dissociate these two effects by diluting the extract. A typical experiment is illustrated in Table 4 where increasing quantities of an extract prepared from a 24 hr. culture were added to 10 ml. samples of a 3 hr.-culture previously induced with maltose and penicillin. The effects on opacity increase and release of the two induced enzymes into the medium were followed during incubation for a further 2½ hr. It can be seen: (*a*) only the highest concentration of extract caused obvious cell lysis (decrease in suspension opacity) although 1/27 of this concentration caused a significant α-glucosidase leak of 7·5 %; (*b*) liberation of penicillinase was increased by addition of extract very roughly in proportion to the extent of α-glucosidase release; there was no concentration of extract which stimulated penicillinase release without significantly increasing the release of α-glucosidase above the basal value of 1·6 % shown by the control culture. Extracts from the organisms of young cultures were very much less active in causing cell damage than extracts from organisms of older cultures. For instance, the degree of cell damage, as measured by % α-glucosidase leakage, produced by extract of a given dry weight from organisms of 3 hr.- or 6 hr.-cultures, could be evoked by approximately one-fiftieth of that quantity of extract prepared from 20 hr.-cultures. The autolytic factor or factors concerned were completely inactivated by boiling for 30 min. It is clear that the factor(s) was being produced in quantity only in old cultures, where some slight activity was also demonstrable in concentrated dialysed supernatant fluid as well as in the organisms themselves.

In addition, extracts of organisms from 20 to 24 hr. cultures were found to contain proteinase activity, whose action in liquefying gelatin and destroying α-glucosidase was almost completely inhibited by 5×10^{-3} M-DFP. For this reason this proteinase activity can be almost certainly rejected as participating in normal penicillinase liberation or in the 'damage-release' effect of the cell extracts described above, because these processes are not affected by DFP. It is possible that some sort of lysozyme-like enzyme, known to be formed by certain strains of *Bacillus subtilis* (Richmond, 1959*a*, *b*) might be partly responsible for the lytic action of these extracts; but it is rather unlikely on *a priori* grounds that such an enzyme plays a part in normal penicillinase liberation. In any case, egg-white lysozyme by itself had a completely different action: at concentrations of 1 μg./ml. and above, it caused rapid lysis of 3-hr. cultures, whereas at 0·4 μg./ml. and below there was no detectable effect whatever (no growth inhibition, no leak of α-glucosidase, no stimulation of penicillinase liberation). All further attempts, by graded ammonium sulphate precipitation, zone electrophoresis and column fractionation, to isolate a specific penicillinase-liberating factor from these extracts, by separation from other material which might be interfering with its action or causing cell damage, have so far failed.

DISCUSSION

The problem of exo-enzyme liberation from bacteria has been approached in the absence of any real knowledge about the nature of the structural barriers or physical or chemical entities which may obstruct free diffusion of penicillinase from a cell after the enzyme is formed. It may well be that *Bacillus subtilis* 6346 penicillinase

is a special case and that the mechanism of its liberation is unrelated to the mechanism acting on other exo-enzymes (e.g. α-amylase from the same strain) where little or no enzymic activity is found to be associated with the cell under any conditions (Dr R. A. Darrow; personal communication) and where, therefore, the liberation process may be linked with the formation of the enzymes (either *de novo* or from an enzymically inactive high molecular weight precursor).

The classical concept of the bacterial cell as consisting of cytoplasm surrounded by a selectively impermeable membrane enveloped by a relatively permeable cell wall (see Salton, 1960; McQuillen, 1960) rather implied that the essential process involved in enzyme release must be its passage through the cytoplasmic membrane. Such a picture may be mistaken. The cytoplasmic membrane has chemical and anatomical reality and there is good reason for believing it to be mainly responsible for the selective permeability of the bacterial cell. But little is known about how exo-enzymes are formed and nothing about the site of their synthesis. It is possible that the process of specific polypeptide chain production which leads to their formation occurs outside the main permeability barrier of the cell. In any case, the classical picture of a continuous and undifferentiated cytoplasmic membrane may be a gross oversimplification. In *Bacillus subtilis* itself there are now indications of a sort of rudimentary endoplasmic reticulum (Glauert, Brieger & Allen, 1961) which might play a part in enzyme liberation. Finally, the barrier to large molecules offered by the cell wall is quite unknown, though Mitchell & Moyle (1959) and Mitchell (1961) have produced evidence which suggests that in *Staphylococcus aureus* and *Escherichia coli* it may not be negligible.

The present work permits only tentative and limited conclusions; but they may be useful in forming a basis for further studies. Assuming that the population of organisms in the culture is homogeneous with respect to enzyme release it can be accepted that at least a fair proportion of the penicillinase originally bound to cells of *Bacillus subtilis* is released into the medium by a process not involving marked cell damage. The complete inhibition of liberation at 0°, the zero order kinetics, the partial inhibition by chloramphenicol and the failure to elute enzyme from the cells by high concentrations of salts, all suggest that the process may be enzymic rather than physico-chemical. Chloramphenicol would be expected to inhibit the formation but not the functioning of such an enzyme system and would therefore cause only partial inhibition of the processes governed by the enzyme; this was what was observed. But it must be admitted that the failure to increase the inhibition of enzyme release by adding chloramphenicol earlier on during cultivation, when the cell content of the hypothetical enzymes responsible for liberation might be expected to be lower, does not support this interpretation. Conclusive evidence about the nature of the enzyme or enzymes involved has not been obtained.

Cell extracts of organisms from old cultures of the homologous strain of *Bacillus subtilis* 6346 increased the rate of penicillinase release from organisms from 3-hr. cultures. The main question is, what part, if any, substances present in these extracts may play in normal physiological liberation of the enzyme. But this stimulatory effect on penicillinase release was always associated, *pari passu*, with cell damage, as indicated by a leakage of α-glucosidase which did not occur in cultures which were spontaneously liberating penicillinase in the normal way. It did not prove possible, by a variety of techniques, to dissociate the two effects. Such

'autolytic' extracts might be expected to contain many different enzymes; but the only activity present which was specifically identified was that of a DFP-sensitive proteinase. This could be rejected as a possible agent for normal penicillinase liberation because of the latter's insusceptibility to inhibition by DFP. This negative finding does not completely exclude the possibility that a proteinase of some sort plays a part in penicillinase liberation, since DFP-insensitive examples of this kind of enzyme (e.g. cathepsin B) have been reported (see Fruton, 1960). In any case, the possibility of some specific penicillinase-liberating factor being present in these extracts cannot be excluded simply by negative results which, it might still be argued, may only reflect the difficulty of reproducing an enzymic effect which is suspected of occurring somewhere within the cell envelope, by adding the enzyme artificially from the outside. The great decrease in liberation of enzyme at a pH value (5·5) at which most cell enzymes might be expected to function poorly, is also consistent with the hypothesis that the phenomenon has an enzymic basis.

Although gross cell damage appears to be excluded, it can always be argued that minor degrees of damage (e.g. the initial steps of a chain of metabolic events finally leading to observable damage and lysis) are, nevertheless, an essential preliminary to exo-enzyme release. This possibility, which is really the essence of the view put forward by Nomura, Hosoda & Yoshikawa (1958) is made less likely by the insusceptibility to the inhibitory effect of high Mg^{++} concentration, and the absence of concomitant α-glucosidase release which characterize 'physiological' liberation of penicillinase and thus distinguish it qualitatively from 'damage-release'.

It might, perhaps, be preferable to consider the possibility that liberation of penicillinase is a reflexion of some relatively non-specific process of change in the biochemical, chemical and physical properties of the cell envelope, affecting the extents to which several other substances, as well as penicillinase, were bound to it. Many bacterial exo-enzymes appear during the later stages of batch growth of *Bacillus* sp. (see Pollock, 1961*a*). The reason for this is not yet understood. It is striking, for instance, to note that the period in a batch culture when penicillinase is being liberated corresponds closely to that during which α-amylase is appearing in the medium. It would be useful, therefore, to know whether generalized changes do occur in the cell wall and other superficial cell structures over this period.

It seems clear that in *Bacillus subtilis* 6346 cell-bound penicillinase is normally fixed to solid cell structure, possibly in part to the cytoplasmic membrane itself. Although only a proportion ($\gtrsim 50\%$) of this cell-bound penicillinase was conclusively shown by antiserum neutralization experiments (Kushner & Pollock, 1961) to be on the outside of the membrane, the results as a whole are consistent with it all being there. It is possible that the membrane itself may be the site of its formation. If this be so, the mechanism of exo-enzyme release may involve detachment of enzyme from some superficial structure (which the penicillinase-releasing effect of trypsin suggests may be protein in nature, Kushner & Pollock, 1961), followed by its passage through the cell wall. This appears, at least for the moment, to be a useful working hypothesis to guide further studies.

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The Genetic Relationship and Phenotypic Expression of Mutations Endowing Pneumococcus with Resistance to Erythromycin

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SUMMARY

Five spontaneous erythromycin resistance mutations arising independently in a pneumococcal strain have been studied. Three distinct levels of resistance are represented by these mutants (0·1, 1·0, and 10·0 μg. erythromycin per ml.). The mutations conferring resistance can be transferred to the sensitive parental strain through transforming DNA preparations. The transfer is discrete, in that the full level of resistance of the donor strain is always conferred upon the recipient. The length of time required for phenotypic expression of a mutation acquired by transformation depends on the particular marker.

A mutation in a given strain may either be replaced by or combine with a different mutation transferred from a donor DNA preparation. In the case of combination, the DNA of the recombinant is capable of transferring each of the mutations as well as the entire complex of mutations possessed by the recombinant. The frequency of transfer of the complex demonstrates the degree of linkage of the separable mutations. A group of mutations in a given recombinant strain may either display antagonistic, synergistic or non-synergistic effects on the phenotype.

Reverse mutations towards erythromycin-sensitivity generally involve alteration at the originally mutated sites, or at very closely linked sites.

INTRODUCTION

In recent years the fine structure of genetic material has been investigated in great detail in a number of micro-organisms (Pontecorvo, 1952; Pritchard, 1955; Benzer, 1955; Demerec, 1956). These investigations have shown that the particular segment of genetic material concerned with a specific function of the organism is capable of undergoing numerous mutations situated at different sites separable by genetic recombination. The order of these mutations in a linear array can generally be determined by the additivity (or near-additivity) of the frequencies of recombination observed to occur in crosses between the various pairs.

The analysis of genetic fine structure has been extended to deoxyribonucleic acid (DNA) transforming factors (Hotchkiss & Evans, 1958; Lacks & Hotchkiss, 1960; Ravin, 1960; Ephrati-Elizur, Srinivasan & Zamenhof, 1961; Rotheim & Ravin, to be published). The molecule of DNA has been found to be large and complex enough not only to bear regions concerned with different functions of the organism (Hotchkiss & Marmur, 1954; Goodgal & Herriott, 1957), but also numerous mutable sites

separable by recombination within each region (Ephrussi-Taylor, 1951; Ravin, 1960; Lacks & Hotchkiss, 1960; Ephrati-Elizur *et al.* 1961; Rotheim & Ravin, to be published). Recombinable mutations borne by the same molecule of DNA can often be arranged in a linear order (Hotchkiss & Evans, 1958; Ravin, 1960; Lacks & Hotchkiss, 1960; Ephrati-Elizur *et al.* 1961).

This report concerns the genotypic and phenotypic analysis of five independent mutations conferring resistance to erythromycin on pneumococci. It has been known (Haight & Finland, 1952*b*) that mutants arising spontaneously generally resist only a relatively low concentration of erythromycin; strains enhanced in their level of resistance can be obtained by repeated selections at gradually increasing concentrations of the antibiotic. Such step-by-step accretion of resistance is similar to that observed with penicillin (Demerec, 1945; Hotchkiss, 1951). The mutations described in this paper were found either to replace or combine with each other in transformation reactions. Some combinations led to enhanced levels of resistance, although non-synergistic and antagonistic interactions were also observed with certain groups of combined mutations. All of the mutations, however, like those affecting other functions previously studied, behaved as linked factors, that is, as factors occupying different sites of the same molecule of DNA.

Most effective against Gram-positive cocci and diphtheria bacilli (Haight & Finland, 1952*a*), erythromycin may be either bacteriostatic or bactericidal, depending upon the concentration. At the concentrations used in the present experiments, its action was principally killing. Like penicillin, its bactericidal action requires active multiplication of the treated strain (Haight & Finland, 1952*c*).

METHODS

Organisms and genetic markers. The erythromycin sensitive strain of Pneumococcus used in these experiments is Rx, a capsule-deficient mutant derived from a type III encapsulated strain (SIII-N) that had been previously produced by transformation (Ravin, 1959). The Rx strain does not form visible or microcolonies when plated on blood agar containing more than 0·01 μg. erythromycin per ml. (Table 1). Green (1957) obtained a number of spontaneous erythromycin resistant mutants by plating 10^9 Rx bacteria on a blood agar medium containing 1 μg. erythromycin per ml. Twenty large colonies appeared after 72 hr. incubation at 37°. Strains derived from three of the twenty mutant colonies isolated by Green were examined in the studies reported here; they contain, respectively, the mutations *ery-r2*, *ery-r3 and ery-r5*. The strains derived from two of the other colonies isolated by Green, originally resistant to at least 1 μg. erythromycin per ml., subsequently reverted to the wild-type erythromycin sensitive condition during the transfer of stocks. They reverted before it was possible to determine their genotypic relationship to the three mutants just mentioned. These reverted strains are referred to as E21-rev. and E24-rev.

Subsequently, two additional spontaneous mutants of independent origin were obtained. One of these was a mutant colony obtained by plating a culture of strain SIII-1b (Ephrussi-Taylor, 1951) on blood agar containing 0·1 μg. erythromycin per ml. Upon isolation, this strain was found, by the method of direct plating described below, to be incapable of growing in the presence of erythromycin at concentrations greater than 0·1 μg. per ml. The genetic factor responsible for the

erythromycin resistance of this strain was transferred to the Rx strain by exposing the latter to the DNA extracted from a culture of mutant cocci. This transformed strain of Rx proved to have the same level of resistance as that of the original SIII-1b mutant. The mutation contained in this strain is referred to as *ery-r6*. The other spontaneous mutation was obtained by plating a culture of R6R, a derivative of the capsule-deficient strain R36A (Ravin, 1960), on a blood agar medium containing 0·1 μg. erythromycin per ml. One of the colonies that developed upon incubation was found to consist of cocci capable of resisting a much higher concentration of erythromycin than that used for selecting the mutant. The mutation responsible for this high level of resistance, referred to as *ery-r7*, was subsequently transferred by means of transformation to the Rx strain. The Rx transformants thus produced possessed the same level of resistance as that of the original R6R mutant (Table 1).

Table 1. *Maximum level of resistance of mutants bearing different* ery-r *mutations, including several recombinant types, and the time required for phenotypic expression of resistance newly acquired by transformation*

Category	Marker (s)	Max. level of resistance (μg. erythromycin./ml.)	Concentration of erythromycin (μg./ml.) used to challenge transformants	Time (hr.) at which ability to resist challenging concentration is expressed in all transformants
1. Mutations of strain Rx	*ery-r6*	0·1	0·075	1
	ery-r2	1·0 ± 0·2	0·25	1
	ery-r3	1·0 ± 0·2	0·25	1
	ery-r5	1·0 ± 0·2	0·25	1
	ery-r7	10·0 ± 5·0	0·1	3
			5·0	> 5
2. Recombinations in strain Rx	*ery-r2-r6*	5·0	—	—
	ery-r3-r6	15·0	—	—
	ery-r2-r3	40·0 ± 10·0	—	—
	ery-r6-r7	10·0 ± 5·0	—	—
	ery-r5-r7	10·0 ± 5·0	—	—
	ery-r2-r3-r5	15·0 ± 5·0	—	—
3. Mutations in other strains	*ery-r6* in strain SIII-1b	0·1	—	—
	ery-r7 in strain R6R	10·0 ± 5·0	—	—
4. Wild-type (Rx)	*ery-s2-s3-s5-s6-s7*	0·01	—	—

The variability indicated for certain mutations and recombinations expresses approximately the highest and lowest maxima observed in a series of similar tests.

At various times the *ery-r* markers have been transferred into closely related strains (e.g. SIII-1, SIII-2, Rz, etc.; see Ravin, 1959) which differ from Rx only in the possession of a different mutation within the type III capsule locus (Ravin 1960). The DNAs obtained from such transformed strains have been used in some of the transformation experiments described below.

An additional genetic factor was occasionally also present in the particular donor strain from which the transforming DNA was prepared. This additional factor was

str-r1 (Ravin, 1956; Bryan, 1961), which confers the ability to resist up to 6000 μg. streptomycin per ml. Thus, DNA preparations generally contained, in addition to the *ery-r* factor being investigated, either *str-r1* or *caps*$^+$, the wild-type allele of the mutated factor (*caps*$^-$) responsible for the capsule deficiency in the Rx strain (Ravin, 1960), or sometimes both factors. Neither of these factors is linked to any of the *ery-r* factors described in these experiments (Ravin, 1960). The additional genetic factor served as a control in certain experiments; the successful transfer of this factor showed that the failure to obtain alterations in the level of erythromycin resistance of the treated recipient strain could not be ascribed either to incompetence of the recipient culture or inactivation of the transforming preparation.

The genetic factors in the sensitive Rx strain which are homologous to those in the respective erythromycin-resistant and streptomycin-resistant mutant strains are symbolized as follows: *ery-s*, *str-s*.

It should be pointed out that a factor (*dep*) linked to the streptomycin-resistance marker *str-r1*, which lowers the frequency of genetic integration of the latter into the genomes of certain recipient strains, is of no significance in the experiments to be reported here since the *dep* factor has no effect when transferred into Rx bacteria (Green, 1959; M. B. Rotheim, to be published).

Transforming preparations. The method of preparing the DNAs used in these experiments is essentially identical to that described by Ephrussi-Taylor (1951). The DNAs were preserved by solution (at concentrations from 0·01 to 0·1 %, w/v) in a physiological saline solution (0·15 M-NaCl) buffered with phosphate (pH 7) and storage at 4°. Saturating concentrations of DNA were used in all experiments.

Media. Three media, originally described by Ephrussi-Taylor (1951), were used in these experiments. Medium 1, which consists of 1 % (w/v) Neopeptone (Difco), 0·4 % (w/v) yeast extract (Difco) and 0·86 % (w/v) NaCl in demineralized water, was used for growing cultures to be used as sources of transforming DNA. High yields of bacteria were obtained by the addition of an excess of glucose (0·63 %, w/v, in final concentration) and intermittent additions of N-NaOH to neutralize the acid produced in the course of growth.

Medium 2 was the standard plating medium. In addition to the ingredients present in medium 1, it contained 1·5 % (w/v) agar (Difco or BBL), 0·033 % (w/v) glucose and 2 % (w/v) sterile defibrinated sheep blood (Cappel Labs). Glucose (sterilized separately by filtration) and sterile blood were added to the rest of the autoclaved and cooled medium just before the pouring of plates. Plates were poured and, before use, they were dried overnight by incubation in a forced air (CENCO) incubator at 37°. To media to be used for the assay of antibiotic-resistant mutants or transformants were added, at the time of addition of blood and glucose, an appropriate amount of the stock solution of the antibiotic in question. The usual concentrations of antibiotic used in the screening of resistant bacteria produced by transformation were 0·1, 0·25 and 5·0 μg. erythromycin per ml. and 100 μg. streptomycin per ml. In certain transformation experiments, other concentrations were used, and these will be specified below. The antibiotic products used were obtained as erythrocin lactobionate (Abbott) and streptomycin sulfate (Lilly).

Medium 3 consisted of inorganic salts, Neopeptone (Difco), an extract of bovine serum, charcoal-adsorbed yeast extract, and a growth-limiting concentration of glucose dissolved in glass-distilled demineralized water. Transformations and stock

transfers were carried out in this medium. For the transfer of stocks 0·1 ml. of sterile defibrinated rabbit blood (Cappel Labs.) was added per 5 ml. of medium. Cultures in any of these media were incubated at 37°.

Assay of antibiotic resistance. The maximum level of resistance of an isolated strain was determined in one of two ways: (A) Direct plating—A sample is taken from a medium 3 culture and appropriately diluted in 1 % (w/v) Neopeptone (Difco); similar samples from the dilution are spread by means of a bent glass rod over the surface of plates of previously dried medium 2, some of which are devoid of antibiotic ('plain agar') while others constitute a series of increasing concentration of antibiotic. That concentration of antibiotic above which the final number of colonies appearing is significantly less than that observed in plain agar is arbitrarily chosen as the maximum level of resistance of the strain being tested. (B) Streak testing—in the cases where one must test a large number of colonies arising from a culture treated with transforming DNA, the colonies are first transferred by isolating them individually with a needle and spreading over the surface of plain agar. Some of the growth arising on these plates is then transferred, either by streaking with a needle or by replicating a velvet impression (Lederberg & Lederberg, 1952), on to a series of plates, each containing a different concentration of antibiotic. Similar streaks are made at the same time to plain agar as a control for the viability of the growth being tested. While streak testing has certain advantages for the examination of a large number of strains, it has the disadvantage that the application of dense populations of bacteria on to a given region of an antibiotic-containing medium occasionally gives spurious results. Some physiological mechanism allows the crowded population, or part of it, to resist higher concentrations of antibiotic than the individual cocci are capable of resisting in isolation. Streak tests thus often need to be rechecked and verified by the use of the method of direct plating.

Cultures that have been exposed to transforming DNA were analysed in the following way. Appropriately diluted samples of such cultures were plated on plain agar, on agar containing various concentrations of erythromycin, and, where necessary, on agar containing 100 μg. streptomycin per ml. The titres of total viable bacteria (obtained from the plain agar plates) and of bacteria resistant to the given concentrations of antibiotic were determined from the counts of colonies formed after 48 or 72 hr. of incubation (in any case, at a time beyond which continued incubation brings forth no more colonies). The frequency of resistant transformants, expressed as the fraction of the total number of viable cocci capable of resisting a given concentration of antibiotic, can then be calculated. The maximum level of resistance of colonies growing in agar containing a certain concentration of antibiotic was determined by isolating such colonies and testing the isolates by either direct plating or streak testing.

Transformation. In a transformation experiment a recipient strain of a given genetic constitution was exposed to the DNA extracted from a donor strain of different genetic constitution. Recombinant bacteria containing some of the genetic factors of the donor and some of the recipient were sought among the progeny of the treated recipient bacteria. In a real sense, therefore, a transformation experiment is equivalent to a 'cross' between two individuals of different genotype.

The transformation was carried out in one of the following ways. (A) Long-term experiment: in this type of experiment, the recipient cells are exposed to DNA for

a relatively long period of time lasting many generations. The transforming DNA remains in contact with the cells during the time in which they are becoming competent to react with DNA, and for several generations after the effective contacts have occurred. It is a simple procedure to use when certain qualitative results are desired (such as the production of any recombinants at all from a given 'cross'). The procedure consists in transferring into medium 3 containing the appropriate DNA plus 0·2 % (w/v) bovine serum albumin (Armour Fraction V), a small number of cocci of the desired recipient strain from a medium 3 culture which has just stopped growing. The initial bacterial density is arranged to be about 500 per ml.; there is no appreciable lag following such a transfer. After 7 to 11 generations of growth ($3\frac{1}{2}$ to $5\frac{1}{2}$ hr. after inoculation), the cocci become competent and react with the DNA present in the medium (Ravin, 1956). The culture is then plated after growth is terminated by the exhaustion of glucose (10 hr. after inoculation). The time interval between effective contact of competent cocci with transforming DNA and the plating of the culture is, in every case, sufficient to permit full phenotypic expression of the newly acquired genetic determinants (Table 1). However, the time interval is large enough to permit some change in the proportion of transformant types due to differential selective action against them by the medium. This possibility has been checked by reconstruction experiments using mixtures of transformant types, and, where necessary, as will be discussed below, short-term experiments are conducted to determine the proportions of transformant types found immediately after phenotypic expression. (B) Short-term experiment: in this type of experiment, DNA is allowed to act upon competent recipient bacteria for only a brief interval of time (10–20 min. = $\frac{1}{3}-\frac{2}{3}$ generation time). Competent bacteria are usually obtained by transfer of an inoculum, similar to that described above, into medium 3 containing 0·2 % (w/v) bovine serum albumin. A volume of 0·1 ml. of the appropriate DNA is added at $4\frac{1}{2}$ to 5 hr. after inoculation, and the action of the DNA is terminated by the addition 10–20 min. later of 0·1 ml. of a Mg^{++}-activated DNase solution (Ravin, 1956). The treated culture is plated either at various hourly intervals after exposure to DNA or at a time (generally 2 hr. after exposure) when it is known that phenotypic expression is completed. In a few cases, it was necessary to perform a series of experiments over a period of time with a recipient strain using cultures of reproducible levels of competence. For this purpose, a modification of the 'freezing' technique of Fox & Hotchkiss (1957) was successfully employed. About 10^7 pneumococci previously grown in medium 3 were inoculated into a flask containing 45 ml. of medium 3 plus serum albumin (0·2 %, w/v) and incubated in a water bath at 37°. At 15 min. intervals during growth, 0·5 ml. amounts of the culture are withdrawn and added to a series of tubes containing 1·5 ml. of preserving medium, previously chilled in an ice-bath. The contents of each tube is mixed by shaking and immediately frozen in a deep-freeze at −80°. Preserving medium was prepared by mixing one part of sterile 2 % (w/v) Neopeptone (Difco) containing 10^{-3} M-$CaCl_2$ with one part of sterile 20 % (w/v) glycerol (sterilized by filtration). One tube from each series prepared in this manner was tested with an active transforming DNA preparation, to determine the level of competence of cells in each series. Those series not possessing sufficiently high levels of competence were discarded; those series containing competent cells retained their high level of competence in the frozen state for at least a few weeks. Frozen recipient cocci to be transformed are thawed in the cold (4–5°),

and then to 0·2 ml. of cell suspension are added 1·8 ml. of medium 3 containing 0·2 % (w/v) serum albumin and 0·1 ml. of the desired DNA. The mixture is incubated at 30–32° (optimal temperature for adsorption of DNA) for 20 min. The DNA is then inactivated by addition of 0·1 ml. of Mg^{++}-activated DNase. The treated suspension is then incubated at 37° (optimal temperature for growth) for a period of time sufficient to allow phenotypic expression to occur before assay of the culture.

RESULTS

Levels of resistance of various mutants and of several recombinant types. The maximum level of erythromycin resistance of each of the five spontaneous mutants was determined by the method of direct plating. The results are recorded in Table 1. It will be noted that the mutation *ery-r6* confers the lowest level of resistance, that the mutations *ery-r2*, *ery-r3* and *ery-r5* confer a similar intermediate level of resistance, and that the mutation *ery-r7* confers the highest level of resistance. The difference between the *ery-r6* level and that of *ery-r2* is about tenfold; the difference between the *ery-r2* level and that of *ery-r7* is also roughly tenfold.

Some physiological variability was observed in the maximum level of resistance obtainable by a given strain. This was found in slight differences in the resistance of a given strain using cultures grown at different times. An indication of the extent of the variability is given in Table 1. The resistances characteristic of the three levels have remained distinct, however, such that, for example, the resistance conferred by a mutation of the intermediate level has never descended as low as the level characteristic of the low level nor risen as high as the level characteristic of the high level.

Table 1 also records the maximum levels of erythromycin resistance possessed by recombinant strains obtained by transformation experiments to be described below. It will suffice to point out here certain antagonistic and synergistic actions between mutations. For example, when the *ery-r2* and *ery-r3* mutations are present in the same strain, they confer together about twenty times more resistance than the sum of their individual resistances. When the *ery-r5* mutation is combined with the *ery-r2* and *ery-r3* mutations, the level of resistance of the resulting triply marked bacterium drops by slightly more than half of the level possessed by a strain bearing only the *ery-r2* and *ery-r3* mutations. Thus, the mutations *ery-r2* and *ery-r3* are synergistic in combination, whereas the mutation *ery-r5* is antagonistic to the action of the *ery-r2-r3* combination. The *ery-r7* mutation, on the other hand, behaves neither synergistically nor antagonistically when recombined with either *ery-r5* or *ery-r6*. The recombinant (possessing both *ery-r7* and either *ery-r5* or *ery-r6*) exhibits a level of resistance which is indistinguishable from that of the strain bearing only the *ery-r7* factor.

Transfer of mutations by DNA-transforming preparations and their behaviour following transfer. The antibiotic-sensitive Rx strain was transformed in short- and long-term experiments with the DNAs obtained from strains bearing the *str-r1* mutation and one of the *ery-r* mutations. The concentration of antibiotic in the plates used to determine the presence of antibiotic-resistant transformants was always considerably less than the maximum level characteristic of the mutation in question. The reason for this procedure was twofold. Some transformants may be

lost if challenged with the maximum concentration of antibiotic they are genetically capable of withstanding due either to: (1) physiological variability, as a result of which some bacteria in a given culture always possess a lower level of resistance than the average level inherited by the strain, or (2) insufficient time allowed for the phenotypic expression of newly transformed bacteria. The amount of time required for phenotypic expression is measured by the interval between the time of exposure of the recipients to transforming DNA and the time when the fraction of viable bacteria capable of forming colonies in a given concentration of antibiotic reaches a maximum. In this respect, it was found, for example, that the time at which all of the transformants requiring *ery-r7* can be recovered depends on the concentration of antibiotic with which the bacteria are challenged. When the challenging concentration of erythromycin was 0·1 μg. per ml., 3 hr. were required to obtain all of the *ery-r7* transformants. On the other hand, when the challenging concentration was 5 μg. per ml., over 5 hr. of growth were required for all of the *ery-r7* transformants to be detected. It is entirely possible that the expression of the *ery-r7* mutation is all-or-none as Fox (1959) found for a streptomycin resistance mutation, but that the probability that an unexpressed transformant will express the phenotype corresponding to its newly acquired genotype is inversely related to the rate of killing, which is greater for higher challenging concentrations of antibiotic. In any event it is clear that, while 3 hr. were required for phenotypic expression of the resistance conferred by mutation *ery-r7* to 0·1 μg. erythromycin per ml., only 1 hr.

Table 2. *Discreteness of transfer of erythromycin resistance mutations in short-term transformation experiments*

1. *ery-r6 str-s1* × DNA *ery-r6 str-r1*; plated 1 hr. after exposure to DNA

Frequency of transformants producing colonies in presence of		Of 118 colonies tested from 0·075 μg. ery./ml.		
			μg. ery./ml.	
100 μg. strep./ml.	0·075 μg. ery./ml.	No. able to resist	0·1	0·25
$1·3 \times 10^{-3}$	$1·2 \times 10^{-3}$	118	+	−
		0	+	+

2. *ery-s7 str-s1* × DNA *ery-r7 str-r1*; plated 3 hr. after exposure to DNA

Frequency of transformants producing colonies in presence of		Of 45 colonies tested from 0·05 μg. ery./ml.			
			μg. ery./ml.		
100 μg. strep./ml.	0·05 μg. ery./ml.	No. able to resist	1	15	30
$5·9 \times 10^{-4}$	$4·5 \times 10^{-4}$	45	+	+	−
		0	+	−	−

3. *ery-s3 str-s1* × DNA *ery-r3 str-s1*; plated 2 hr. after exposure to DNA

Frequency of transformants producing colonies in presence of		Of 120 colonies tested from 5·0 μg. ery./ml.			
			μg. ery./ml.		
0·1 μg. ery./ml.	5·0 μg. ery./ml.	No. able to resist	0·1	1	5
$4·5 \times 10^{-3}$	$< 10^{-5}$	120	+	+	−
		0	+	+	+
		0	+	−	−

was needed for the completion of phenotypic expression of the resistance conferred by mutations *ery-r6* and *ery-r3* to 0·075 and 0·25 μg. erythromycin per ml. respectively. The time required for expression of the maximal level of resistance that a transformant is genetically capable of withstanding depends, therefore, on the particular *ery-r* marker being considered (Table 1).

Consequently, when the sensitive Rx strain is treated with the DNA containing the *ery-r2* marker, for example, the treated population is challenged with only 0·25 μg. erythromycin per ml., although the maximum level of resistance conferred by this marker is four times as great. Furthermore, this challenge is carried out at a time when it is known that all transformants have expressed their ability to resist the challenging concentration of erythromycin (Table 1). The same challenging concentration was found useful for the *ery-r3* and *ery-r5* markers. However, 0·075 μg. erythromycin per ml. was the challenging concentration used for detecting transformants acquiring the *ery-r6* marker, while 0·1 μg. erythromycin per ml. was the concentration used for detecting transformants acquiring the *ery-r7* marker.

In either long- or short-term experiments, it was found that each *ery-r* marker was transferred as a discrete unit. The only class of antibiotic-resistant transformants detectable was that having the same level of resistance as that of the donor strain. This conclusion is based on the fact that, when Rx cells treated with DNA bearing a given *ery-r* marker were challenged by plating in media containing a concentration of erythromycin far below that which the donor strain was capable of resisting, all of the colonies subsequently arising were successfully replicated or streaked on to agar containing the maximum concentration of erythromycin which the donor was capable of resisting, but not on to agar containing a higher concentration. Thus, transformants were not found to possess intermediate or higher levels of resistance than the donor strain. Typical results for the markers *ery-r3*, *ery-r6* and *ery-r7* are shown in Table 2.

Table 3. *Recombinations between various* ery-r *mutations*

Mutant recipient strain	Mutant donor strain				
	-r2	*-r3*	*-r5*	*-r6*	*-r7*
-r2	—	*-r2-r3* (40)	—	*-r2-r6* (5)	*s2-r7* (10)
-r3	*-r2-r3* (40)	—	—	—	*s3-r7* (10)
-r5	—	—	—	—	*r5-r7* (10)
-r6	*-r2-s6* (1) *-r2-r6* (5)	*-r3-s6* (1) *-r3-r6* (15)	*-r5-s6* (1)	—	*r6-r7* (10)

Transformants with increased level of resistance are sought by challenging on agar containing a concentration of erythromycin sufficient to prevent growth of dense populations of recipient strain (0·25 μg./ml. *-r6*; 5 μg./ml. *-r2*, *r3* and *r5*). Value indicated in parentheses is maximum level of resistance of recombinant. Negative result (—) means that number of colonies observed on agar containing the challenging concentration of erythromycin is no greater than the number arising spontaneously (i.e. in absence of DNA). Indicated genotypes of recombinants are verified in subsequent experiments.

Recombination between mutations. A given mutant may be treated with the DNA extracted from another mutant, and one can determine if transformants are produced having a higher level of resistance than that of the recipient strain. Following long-term exposure of the recipient strain to the particular DNA, one challenges by plating on agar containing a concentration of erythromycin somewhat greater than

the maximum concentration tolerated by the recipient strain. The actual challenging concentration employed is one just sufficiently high to prevent even slight or feeble growth by dense populations of untreated recipient bacteria spread over the surface of agar. Table 3 records the results of all possible 'crosses' involving the various mutants as donors and the *ery-r2*, *ery-r3*, *ery-r5* and *ery-r6* strains as recipients.

It will be recalled that the *ery-r2*, *-r3*, and *-r5* mutations confer indistinguishable levels of resistance upon a sensitive strain. Although phenotypically similar, however, the three mutations are genotypically distinct. For example, while no mutant strain can be raised in its level of resistance by autologous DNA, recombinants having increased resistance are produced in the 'cross' *ery-r2* × *ery-r3*. The outcome is identical regardless of how the 'cross' is carried out: mutant *ery-r2* + DNA *ery-r3*, or (the reciprocal 'cross') mutant *ery-r3* + DNA *ery-r2*. Thus, *ery-r2* is different from *ery-r3*. It will be noted that, while the challenging concentration for selecting recombinants in these crosses is 5 μg. erythromycin per ml., all of the selected transformants are capable of resisting as much as 40 μg. erythromycin per ml. The mutant *ery-r5*, on the other hand, when treated with either DNA *ery-r2* or DNA *ery-r3* did not give rise to cocci with an increased level of resistance to erythromycin. Similarly, neither *ery-r2* nor mutant *ery-r3* produced cocci with increased resistance when treated with DNA *ery-r5*. This is presumptive evidence that *ery-r5* is distinct from both *ery-r2* and *ery-r3*; confirmatory evidence is obtained from experiments to be described below in which *ery-5* is raised in its level of resistance by treatment with DNA containing both the *ery-r2* and *ery-r3* markers.

When the mutant *ery-r6*, which has a lower level of resistance than any of the other strains, serves as recipient, one may select for transformants capable of resisting at least the challenging concentration normally employed for detecting transformants acquiring the donor marker, and then one may determine, by subsequent replication or streak-testing, whether any of the colonies growing in the presence of the challenging concentration of erythromycin can grow in the presence of even higher concentrations. Thus, it is found that when, for example, mutant *ery-r6* is treated with DNA *ery-r2*, two classes of transformed pneumococci are obtained: those resisting up to 1 μg. erythromycin per ml., and, hence, identical to organisms containing *ery-r2*; and those resisting up to 5 μg. erythromycin per ml. The latter class is presumed to be the recombinant type *ery-r2-r6*. The presence of both markers in such bacteria can be proved by experiments described in the next section. It will also be noted that the recombinant type *ery-r2-r6* is obtained when mutant *ery-r2* is treated with DNA *ery-r6* and then challenged with 5 μg. erythromycin per ml. (Table 3). It is significant, furthermore, that when either mutant *ery-r3* or mutant *ery-r5* is treated with DNA *ery-r6*, no transformant class can be detected that is capable of resisting a higher concentration of erythromycin than the recipient strain (Table 3).

The DNA of mutant *ery-r7* has been tested on each of the other mutant strains, and in every case, regardless of the *ery-r* mutation possessed by the recipient strain, transformants enhanced in their level of resistance were detected by plating in agar containing a concentration of erythromycin just sufficiently high to block the growth of untreated recipient bacteria. By streak-testing, it was found that the transformants thus produced exhibited the same phenotype as that of the *ery-r7* strain (Table 3).

Linkage of erythromycin resistance mutations. Having obtained enhancement of resistance by treating one mutant strain with the DNA of another, it was of interest to determine the genetic relationship of the mutations involved in such a cross. When, as in the case of crosses involving *ery-r7* mutant as donor, only one transformant class was obtained, corresponding in phenotype to that of the donor, it could be supposed that *ery-r7* replaces every one of the other mutations in transformation reactions. Further investigation proved, however, that this was not the case. A DNA preparation was made from a single transformant selected in each one of the following crosses: *ery-r2* × *ery-r7*; *ery-r3* × *ery-r7*; *ery-r5* × *ery-r7*; *ery-r6* × *ery-r7*. Two of the DNAs, those prepared from transformants issuing from the former two crosses, behaved as though only the *ery-r7* mutation was contained in the transformed strain. This was determined by exposing the sensitive Rx strain to the DNA in a short-term experiment and then selecting for bacteria on agar containing 0·1 μg. erythromycin per ml. All of the selected bacteria proved to possess the *ery-r7* phenotype. However, the DNAs produced from transformants obtained in the latter two crosses produced two classes of resistant bacteria when used to treat the sensitive Rx strain.The phenotype of one of the classes corresponded to that of the *ery-r7* strain, but the phenotype of the other class corresponded to that of the other mutant involved (*ery-r5* or *ery-r6*). For example, in one experiment a transformed strain possessing the phenotype of the *ery-r7* mutant was selected following a cross between recipient *ery-r5* and donor *ery-r7*. The DNA of this selected transformant was prepared and tested in a short-term exposure of the sensitive Rx strain. Cocci capable of producing colonies in the presence of 0·1 μg. erythromycin per ml. were selected. Of 106 such colonies tested, 83 were able to resist a maximum erythromycin concentration of 1 μg./ml. while 23 were able to resist no more than 10 μg./ml. Thus two phenotypic classes were obtained; one corresponding to that of *ery-r5-s7*, the other corresponding to that of either *ery-s5-r7* or *ery-r5-r7*. The former class is by far the more abundant. The conclusion to be drawn from the ensemble of these results is that the *ery-r7* mutation is capable of replacing either the *ery-r2* or *ery-r3* mutation, but it can combine with either the *ery-r6* or *ery-r5* mutation. Whether *ery-r7* replaces or combines with another mutation, however, the recombinant possesses the *ery-r7* phenotype. It is altogether possible that the *ery-r7* mutation can combine with the *ery-r2* and *ery-r3* mutations, but the lack of synergism produced with the *ery-r7* mutation makes it difficult to determine. If, for example, *ery-r7* can replace as often as it can combine with one of these mutations, a randomly isolated transformant (obtained in the cross *ery-r2*, or *ery-r3*, × *ery-r7*) is just as likely to possess the *ery-s2-r7* genotype as the *ery-r2-r7* genotype. Since further experiments established the linkage of the *ery-r2*, *ery-r3*, *ery-r5* and *ery-r6* mutations, it is clear that the *ery-r7* mutation is linked to all of them.

It will be recalled that enhancement of resistance was obtained in a cross involving the *ery-r2* and *ery-r3* mutations, and in this case the transformed class possessed a maximum level of resistance forty times greater than that of the untreated recipient. In this case, it may be supposed that the transformed strain with the enhanced resistance possesses two mutations, the original mutation of the recipient plus that transferred from the donor via the transforming DNA preparation. Proof that this view is in fact correct may be obtained by extracting the DNA from the trans-

formant supposedly bearing both mutations (*ery-r2* and *ery-r3*) and then briefly exposing a sensitive strain to this DNA. Three genotypic classes are expected, corresponding to bacteria containing *ery-r2* alone, bacteria containing *ery-r3* alone, and bacteria containing both *ery-r2* and *ery-r3*. Furthermore, the frequency of transformed bacteria acquiring both mutations relative to the frequency of bacteria acquiring only a single mutant marker provides a means of determining how closely the two mutations are linked.

By this procedure, it has been ascertained that the two mutations are indeed linked, i.e. behave as if borne by the same molecule of transforming DNA. In Table 4 are recorded the results of a typical experiment in which the sensitive Rx strain is treated with DNA from the transformed strain *ery-r3-r2 str-r1*. It will be noted that the frequency of transformants capable of resisting 0·25 μg. erythromycin per ml. is similar to the frequency of transformants capable of resisting 100 μg.

Table 4. *Classes of transformants produced following short-term exposure of sensitive Rx strain to DNA from the presumptive Rx* ery-r2-r3 str-r1

Period of exposure of competent Rx cells to DNA: 20 min.
Period of growth following exposure to DNA: 3 hr.

1. Frequency of transformants producing colonies in presence of:

Erythromycin		Streptomycin
0·25 μg./ml.	5 μg./ml.	(100 μg./ml.)
$3{\cdot}9 \times 10^{-4}$	$5{\cdot}1 \times 10^{-5}$	$3{\cdot}3 \times 10^{-4}$

2. Tests of isolated colonies

Isolated from agar containing	Capable of growing on transfer to agar containing	Proportion
0·25 μg. ery./ml.	1·0 μg. ery./ml.	1·0
	5·0 μg. ery./ml.	$1{\cdot}5 \times 10^{-1}$
	100 μg. strep./ml.	$6{\cdot}9 \times 10^{-3}$
100 μg. strep./ml.	0·25 μg. ery./ml.	$5{\cdot}8 \times 10^{-3}$

3. Further test of 10 colonies

Isolated from agar containing 0·25 μg. ery./ml. but incapable of growing at concentrations greater than 1·0 μg. ery./ml.

Number producing transformants capable of resisting 5 μg. ery./ml. when treated with:

DNA *ery-r2*	DNA *ery-r3*	Both DNAs
4	6	0

streptomycin per ml. Of the colonies that develop in agar containing 0·25 μg. erythromycin per ml., only about one in 150 is found to be capable of resisting 100 μg. streptomycin per ml. A similar proportion of colonies that develop in agar containing 100 μg. streptomycin per ml. is found to be capable of resisting 0·25 μg. erythromycin per ml. This proportion is taken as an index of the frequency of random penetration of competent recipient cocci by two independent ('non-linked' transforming molecules. It will be noted that the frequency of transformants that develop in agar containing 5 μg. erythromycin per ml. is greater than one-tenth the frequency of transformants that develop in agar containing 0·25 μg. erythromycin per ml. (precisely, the ratio of these two frequencies is $1{\cdot}3 \times 10^{-1}$). Similarly,

if one tests a random sample of colonies that develop in agar containing 0·25 μg. erythromycin per ml., roughly one in six (precisely, $1{\cdot}5 \times 10^{-1}$) are found to be capable of resisting 5 μg. erythromycin per ml. The class of transformants capable of resisting 5 μg. erythromycin per ml., presumably possessing both the *ery-r2* and *ery-r3* factors, is obviously occurring more frequently than would be expected on the basis of the random penetration of two independent transforming factors. Furthermore, the maximum level of resistance of this class of transformants, as subsequently determined, was found to be 40 μg. erythromycin per ml., which is the phenotype of the donor strain. Similarly, it was found that the transformants that cannot resist this high concentration of erythromycin can resist at most 1 μg. erythromycin per ml., which is the phenotype of either the *ery-r2* or *ery-r3* strains. It can be shown, moreover, that these transformants are of two genetic types, one corresponding to *ery-r2*, the other to *ery-r3*. For example, in the experiment recorded in Table 4, 10 of the transformants appearing in agar containing 0·25 μg. erythromycin per ml. that subsequently were found to be unable to resist 5 μg. erythromycin per ml., were subjected to the following test. One culture of each transformant was treated with DNA *ery-r2 str-r1*, and another culture rendered competent under similar conditions was treated with DNA *ery-r3 str-r1*. Streptomycin resistance transformations were induced in all of the cultures. However, six of the transformants were enhanced in their resistance to erythromycin only when exposed to DNA containing the *ery-r3* factor, while four were enhanced only when exposed to DNA containing the *ery-r2* factor. The former corresponded, therefore, to cocci containing the *ery-r2* factor, while the latter corresponded to cocci containing the *ery-r3* factor. Thus, transformants having the *ery-r2-s3* and *ery-s2-r3* genotypes were recovered in this experiment. In summary, the *ery-r2* and *ery-r3* factors can be brought together and can be separated by genetic recombination; when together, they behave as though borne by the same molecule of transforming DNA.

Experiments of similar design were carried out to determine the linkage of other pairs of erythromycin resistance markers. For example, linkage of *ery-r2* to *ery-r6* was demonstrated in the following ways. First of all, long-term exposure of mutant *ery-r6* to DNA containing the factors *ery-r2* and *str-r1* reveals that *ery-r6* is apparently replaced by *ery-r2* most of the time (Table 5A). That the *ery-r2-r6* recombinant occurs is indicated by the existence of a class of transformant that can resist a higher concentration of erythromycin than can *ery-r2*; this class can resist 5 μg-erythromycin per ml. The existence of the recombinant is also readily demonstrable by treating mutant *ery-r2* with DNA containing the *ery-r6* and *str-r1* markers (Table 5B). For about 20 transformants acquiring the *str-r1* factor, one transformant appears that is capable of resisting as much as 5 μg. erythromycin per ml. The DNA of one such transformant was prepared and used to treat the sensitive Rx (*ery-s2-s6*) strain in a short-term experiment. The results (Table 5C) reveal that three phenotypic classes of transformants are obtainable: one corresponding to the *ery-r6* phenotype, one corresponding to the *ery-r2* phenotype, and one corresponding to the *ery-r2-r6* phenotype. The proportions of these classes were found to be approximately 0·6:3:1. Repeat experiments have confirmed the interesting finding that the *ery-r6-r2* transformant occurs more frequently than the *ery-r6-s2* transformant, which is corroborative evidence of the strong linkage of *ery-r6* to *ery-r2*.

Furthermore, *ery-r6* appears to be even more tightly linked to *ery-r3*. This is revealed by the fact that in five independent long-term experiments in which strain *ery-r3* was exposed to DNA containing the factors *ery-r6* and *str-r1*, no transformants were found capable of resisting 5 μg. erythromycin per ml. although transformants acquiring the *str-r1* factor were abundant (Table 6B). Moreover, in the reciprocal cross (strain *ery-r6* treated with DNA *ery-r3 str-r1*) the recombinant *ery-r3-r6* also occurred rarely although the replacement of *ery-r6* by *ery-r3* occurred

Table 5. *Recombination between the mutations* ery-r6 *and* ery-r2

A. Long-term exposure of mutant *ery-r6* to DNA *ery-r2 str-r1*

	Frequency of transformants producing colonies in presence of		
	0·25 μg. ery./ml.	5 μg. ery./ml.	100 μg. strep./ml.
ery-r6 + no DNA	$< 10^{-7}$	$< 10^{-7}$	$< 10^{-7}$
ery-r6 + DNA	$1{\cdot}0 \times 10^{-4}$†	$3{\cdot}1 \times 10^{-5}$	$1{\cdot}2 \times 10^{-3}$

B. Long-term exposure of mutant *ery-r2* to DNA *ery-r6 str-r1*

	Frequency of transformants producing colonies in presence of	
	5 μg. ery./ml.	100 μg. strep./ml.
ery-r2 + no DNA	$< 10^{-7}$	$< 10^{-7}$
ery-r2 + DNA	$1{\cdot}0 \times 10^{-6}$‡	$2{\cdot}0 \times 10^{-5}$

C. Short-term exposure of sensitive Rx strain to DNA *ery-r2-r6*§

	Frequency of transformants producing colonies in presence of		
	0·05 μg. ery./ml.	0·25 μg. ery./ml.	5·0 μg. ery./ml.
Rx + no DNA	$1{\cdot}0 \times 10^{-5}$	$< 10^{-5}$	$< 10^{-5}$
Rx + DNA	$3{\cdot}5 \times 10^{-4}$	$3{\cdot}5 \times 10^{-4}$	$2{\cdot}4 \times 10^{-5}$

Source of colonies: growing in presence of	Total no. tested	Maximum level of resistance (μg. ery./ml.) of isolated colonies: No. resisting			
		0·1	0·25	5	15
0·05 μg./ml.	64	9	41	14	0
0·25 μg./ml.	188	—	145	43	0
5·0 μg./ml.	15	—	—	15	0

† Of 35 colonies isolated all could grow at an erythromycin concentration of 0·25 μg./ml., while 8 could grow at a maximum erythromycin concentration of 5 μg./ml.

‡ Of 8 colonies isolated, all could grow at a maximum concentration of 5 μg. ery./ml.

§ Source of DNA being strain capable of resisting 5 μg. erythromycin per ml. obtained in experiment B.

almost as frequently as does the acquisition of the *str-r1* marker (Table 6 A,C). The rare *ery-r3-r6* recombinant obtained in the reciprocal cross was found to resist as much as 15 μg. erythromycin per ml. In accord with its presumed genotype, the DNA of the *ery-r3-r6* recombinant produces three phenotypic classes in a sensitive recipient strain: one corresponding to the *ery-r6* mutant, one corresponding to the *ery-r3* mutant, and one corresponding to the *ery-r3-r6* recombinant (Table 6C). It is interesting to note, however, that these three classes were produced, respectively, in the proportion 18:1:6. Again, as in the case of the DNA of the *ery-r2-r6* recombi-

nant, the transfer of both mutated sites into the recipient sensitive strain occurred more frequently than did the single transfer of one of the mutated sites. In the present case, however, it was the *ery-r3* mutation that was transferred least frequently, whereas in the previous case it was the *ery-r6* mutation that was transferred least frequently.

Table 6. *Recombination between the mutations* ery-r6 *and* ery-r3

A. Long-term exposure of mutant *ery-r6* to DNA *ery-r3 str-r1*

	Frequency of transformants producing colonies in presence of	
	0·25 μg. ery./ml.	100 μg. strep./ml.
ery-r6 + no DNA	$< 10^{-6}$	$< 10^{-6}$
ery-r6 + DNA	$1{\cdot}1 \times 10^{-5}$†	$1{\cdot}9 \times 10^{-5}$

B. Long-term exposure of mutant *ery-r3* to DNA *ery-r6 str-r1*

	Frequency of transformants producing colonies in presence of	
	5 μg. ery./ml.	100 μg. strep./ml.
ery-r3 + no DNA‡	$< 10^{-7}$	$< 10^{-7}$
ery-r3 + DNA‡	$< 10^{-7}$	$3{\cdot}9 \times 10^{-5}$

C. Short-term exposure of sensitive Rx strain to DNA *ery-r3-r6*§

	Frequency of transformants producing colonies in presence of		
	0·1 μg. ery./ml.	0·25 μg. ery./ml.	5·0 μg. ery./ml.
Rx + no DNA	$< 10^{-5}$	$< 10^{-5}$	$< 10^{-5}$
Rx + DNA	$1{\cdot}7 \times 10^{-4}$	$2{\cdot}9 \times 10^{-5}$	$< 10^{-5}$

		Maximum level of resistance (μg. ery./ml.) of isolated colonies				
		No. resisting				
Source of colonies: growing in presence of 0·1 μg./ml.	Total no. tested	0·1	0·25	5	15	30
	76	55	3	0	18	0

† Of 52 colonies isolated, 51 could grow at a maximum erythromycin concentration of 1 μg./ml., while 1 could grow at a maximum concentration of 15 μg./ml.

‡ Typical result of 5 independent experiments.

§ Source of DNA being strain capable of resisting 15 μg. erythromycin per ml. obtained in experiment A.

In a similar fashion, it has been determined that the site of the *ery-r5* mutation either overlaps that of *ery-r6*, or is very closely linked to it. When strain *ery-r5* was treated with DNA *ery-r6 str-r1*, no transformants were found capable of resisting 5 μg. erythromycin per ml. (Table 7B). In the reciprocal cross (strain *ery-r6* treated with DNA *ery-r5 str-r1*), transformants capable of resisting 1 μg. erythromycin per ml. occurred almost as frequently as transformants acquiring the *str-r1* factor (Table 7A). Furthermore, DNA extracted from one of the erythromycin-resistant transformants, which are identical in phenotype to *ery-r5* cocci, showed in this strain, at least, no evidence of bearing the *ery-r6* marker. On the sensitive Rx strain, only one class of transformants was produced, namely, those capable of resisting at most 1 μg. erythromycin per ml. (Table 7C). Thus, the *ery-r5* mutation was shown to be capable of replacing the *ery-r6* mutation.

The relation of ery-r5 to ery-r2 and ery-r3. On the basis of the results just discussed, it is possible to conclude:

(1) that the erythromycin resistance mutations *ery-r2*, *-r3*, *-r5*, *-r6* and *-r7* are borne by the same molecule of transforming DNA;

(2) that the sites of the following mutations are separable by genetic recombination: *ery-r2* and *ery-r3*; *ery-r2* and *ery-r6*; *ery-r3* and *ery-r6*; *ery-r6* and *ery-r7*.

(3) that the site of the *ery-r6* mutation is very closely linked to the site of the *ery-r3* mutation, more so than to the site of the *ery-r2* mutation, and it either overlaps or is closely linked to the site of the *ery-r5* mutation.

Table 7. *Recombination between mutations* ery-r6 *and* ery-r5

A. Long-term exposure of mutant *ery-r6* to DNA *ery-r5 str-r1*

	Frequency of transformants producing colonies in presence of	
	0·25 μg. ery./ml.	100 μg. strep./ml.
ery-r6 + no DNA	$< 10^{-6}$	$< 10^{-6}$
ery-r6 + DNA	$1{\cdot}4 \times 10^{-5}$†	$2{\cdot}0 \times 10^{-5}$

B. Long-term exposure of mutant *ery-r5* to DNA *ery-r6 str-r1*

	Frequency of transformants producing colonies in presence of	
	5 μg. ery./ml.	100 μg. strep./ml.
ery-r5 + no DNA‡	$< 10^{-7}$	$< 10^{-7}$
ery-r5 + DNA‡	$< 10^{-7}$	$4{\cdot}4 \times 10^{-5}$

C. Short-term exposure of sensitive Rx strain to DNA *ery-(r6)r5*§

	Frequency of transformants producing colonies in presence of 0·1 μg. ery./ml.
Rx + no DNA	$< 10^{-5}$
Rx + DNA	$5{\cdot}6 \times 10^{-3}$‖

† Of 60 colonies isolated, all were capable of growing at a maximum erythromycin concentration of 1 μg./ml.

‡ Typical result of 5 independent experiments.

§ Source of DNA is a strain capable of resisting 1 μg. ery./ml. obtained in experiment A.

‖ Of 205 colonies isolated, all could grow at a maximum erythromycin concentration of 1 μg./ml.

The relation of the *ery-r5* mutation to the *ery-r2* and *ery-r3* mutations is problematic. It was noted that the mutation *ery-r5* did not recombine with either *ery-r2* or *ery-r3* to produce a recombinant possessing a significantly greater level of resistance. This fact suggests that either the *ery-r5* mutation recombined with *ery-r2* (or *ery-r3*) to yield an *ery-r2-r5* (or *ery-r3-r5*) recombinant having no enhanced power of resistance (or else a lower level of resistance), or that the *ery-r5* mutation is allelic to the *ery-r2* and *ery-r3* mutations in overlapping both of them. The first evidence bearing on this question suggested that the former explanation is correct. When the mutant bearing the *ery-r5* mutation was treated with DNA from a strain containing the *ery-r2-r3* and *str-r1* markers, two classes of erythromycin-resistant transformants were recovered in agar containing 5 μg. erythromycin per ml.: a class the maximum level of resistance of which was 15 μg. erythromycin per ml., and a

class the maximum level of resistance of which was about 40 μg. erythromycin per ml. The former was considerably more frequent, since nine of eleven independent transformants belonged to this class. On the contrary, when the *ery-r2* or *ery-r3* mutant was treated with this DNA, the only class of transformants recovered was one capable of resisting a maximum of 40 μg. erythromycin per ml. This class corresponds to the expected *ery-r2-r3* recombinants. These findings demonstrated that the mutation *ery-r5* was indeed distinct from both *ery-r2* and *ery-r3*. They indicate, moreover, that the replacement of *ery-r5* by *ery-r2-r3* occurred less frequently than recombination. between *ery-r5* and *ery-r2-r3*. The phenotype of the presumed *ery-r2-r3-r5* recombinant has a lower level of resistance than that of the *ery-r2-r3* donor, and it is therefore suggestive that the mutation *ery-r5* in conjunction with either *ery-r2* or *ery-r3* had a lower level of resistance than a mutant bearing only one of the muta-

Table 8. *Short-term exposure of Rx* ery-s2-s3-s5 str-s1 *to DNA* ery-r2-r3-r5 str-r1

A. Frequency of transformant classes as a function of time following exposure to DNA

Incubated cells in presence of albumin for 4 hr. 10 min.; added DNA for 20 min.; added DNase; then plated at hourly intervals.

Time after addition of DNase ...	1 hr.	2 hr.	3 hr.	4 hr.
Total no. cocci per ml.	$1{\cdot}5 \times 10^{6}$	$5{\cdot}1 \times 10^{6}$	$2{\cdot}3 \times 10^{7}$	$8{\cdot}9 \times 10^{7}$
Freq. of transformants producing colonies in 100 μg. strep./ml.	$5{\cdot}2 \times 10^{-6}$	$6{\cdot}3 \times 10^{-6}$	$4{\cdot}1 \times 10^{-6}$	$4{\cdot}7 \times 10^{-6}$
Freq. of transformants producing colonies in 0·25 μg. ery./ml.	$7{\cdot}1 \times 10^{-6}$	$1{\cdot}2 \times 10^{-5}$	$5{\cdot}7 \times 10^{-6}$	$1{\cdot}0 \times 10^{-5}$
Freq. of transformants producing colonies in 5 μg. ery./ml.	$\underline{6{\cdot}6 \times 10^{-7}}$	$1{\cdot}2 \times 10^{-6}$	$\underline{1{\cdot}0 \times 10^{-7}}$	$7{\cdot}6 \times 10^{-7}$

(Underlining means frequency determination is based on colony count of < 10.)

B. Test of *str-r* transformants selected on 100 μg.strep./ml.

Total no. colonies tested:	621
Total no. colonies that can resist at least 0·25 μg. ery./ml.	3
Proportion of *str-r* transformants that can resist ery.	$4{\cdot}8 \times 10^{-3}$

C. Test of *ery-r* transformants selected on 0·25 μg. ery./ml.

Total no colonies tested:	714
Total no. colonies that can resist at least 5 μg. ery./ml.	94
Proportion of *ery-r* transformants that can resist 5 μg. ery./ml.	$1{\cdot}3 \times 10^{-1}$
Total no. of *ery-r* transformants found to resist at least 5 μg. ery./ml.	94
No. of these transformants found to resist at most 15 μg. ery./ml.	94
No. of these transformants found to resist at most 40 μg. ery./ml.	0

D. Test of *ery-r* transformants selected on 5 μg. ery./ml.

Total no. colonies tested:	15
Total no. colonies resistant to at most 15 μg. ery./ml.	14
Total no. colonies resistant to at most 40 μg. ery./ml.	1

E. Analysis of 24 *ery-r* transformants incapable of resisting 5 μg. ery./ml.

No. of transformants enhanced in erythromycin resistance:	
by exposure to DNA *ery-r2 str-r1*, but not to DNA *ery-r3 str-r1*	6*
by exposure to DNA *ery-r3 str-r1*, but not to DNA *ery-r2 str-r1*	18**
by exposure to neither DNA	0

* 2; transformants further tested; genotypes of both: *ery-r3-r5*.

** 7; transformants further tested; genotypes of all: *ery-r2-r5*.

tions. This hypothesis would account for the absence of transformants enhanced in their level of resistance when the mutant bearing the *ery-r5* marker is crossed with a mutant bearing either the *ery-r2* or *ery-r3* marker.

An investigation was undertaken of the DNA extracted from one of the presumed *ery-r2-r3-r5* recombinants (i.e. a transformant resisting at most 15 μg. erythromycin per ml.) into which the *str-r1* marker was subsequently transferred. The sensitive Rx strain was exposed to this DNA in a short-term experiment. If *ery-r5* is separable by recombination from *ery-r2* and *ery-r3*, then one should expect to recover the following genotypic products: *ery-s2-s3-r5*, *ery-r2-s3-s5*, *ery-s2-r3-s5*. In addition of course, genotypes containing two, and even three, mutant markers should be produced. The actual results obtained reveal a more complex situation (Table 8). Transformants capable of resisting at most 1 μg. erythromycin per ml. occur about six times more frequently than transformants capable of resisting a higher concentration of erythromycin (Table 8A). The vast majority of the latter are phenotypically identical to the donor strain (i.e. *ery-r2-r3-r5*), which resists at most 15 μg. erythromycin per ml. (Table 8C). Transformants capable of resisting 40 μg. erythromycin per ml. (i.e. corresponding to the genotype *ery-r2-r3-s5*) are rare, but have occasionally been detected (Table 8D). Incidentally, the relatively high frequency of joint transfer of the *ery-r2*,-*r3*, and -*r5* factors is corroborative evidence of the linkage of these three factors (Table 8 B, C). As for the transformants capable of resisting only a low concentration of erythromycin, they were subjected to further analysis (Table 8E). They were first isolated and grown in medium 3. Samples from the same competent culture of a given isolated transformant were then exposed separately to DNA *ery-r2 str-r1* and to DNA *ery-r3 str-r1*. These treated cultures were then challenged on agar containing 5μg. erythromycin per ml. and on agar containing 100 μg. streptomycin per ml. The appearance of a significant number of colonies in streptomycin-containing agar reveals that the DNAs are active and that the cultures were competent when exposed to the DNAs. In every case where these conditions were fulfilled, a significant number of colonies appeared on agar containing 5 μg. erythromycin per ml. as a result of exposure to either the DNA bearing the *ery-r2* marker or to the DNA bearing the *ery-r3*, *but not both*. Thus, among the transformants resisting a low concentration of erythromycin obtained in the original treatment of the sensitive Rx strain with DNA *ery-r2-r3-r5 str-r1*, the genotype *ery-s2-s3-r5* is rare, if it occurs at all. These transformants could have, however, one of the following genotypes: *ery-r2-s3-s5*, *ery-s2-r3-s5*, *ery-s2-r3-r5*, *ery-r2-s3-r5*. Bacteria having one of the latter two genotypes could possibly have the same level of resistance as bacteria having one of the former two genotypes, as mentioned above. However, bacteria having the genotype *ery-r2-s3-r5* could be distinguished from those having the genotype *ery-r2-s3-s5* by the fact that the latter would produce recombinants capable of resisting 40 μg. erythromycin per ml. following exposure to DNA *ery-r3*, whereas the former would produce recombinants capable of resisting at most 15 μg. erythromycin per ml. In a similar fashion, bacteria possessing the genotype *ery-s2-r3-s5* could be distinguished from those possessing the genotype *ery-s2-r3-r5* by determining the level of resistance of recombinants produced following exposure to DNA *ery-r2*. Therefore, following the exposure to *DNA ery-r2* and to *DNA ery-r3* the colonies appearing in agar containing 5 μg. erythromycin per ml. were isolated and tested for their maximum level of resistance. The outcome of such an analysis

(Table 8E) reveals that the transformants capable of resisting only a low concentration of erythromycin are of the following genotypes, in the proportions indicated: 3 *ery-r2-s3-r5*, 1 *ery-s2-r3-r5*. The *ery-r2-s3-s5* and *ery-s2-r3-s5* genotypes apparently occur infrequently.

The principal conclusion to be drawn from this study, therefore, is that the *ery-r5* mutation once linked to the *ery-r2* and *ery-r3* mutations by genetic recombination can be separated from those mutations only with great difficulty. The surprising finding is that the association of *ery-r5* with *ery-r2-r3* occurs considerably more frequently than would be expected from the rarity of dissociation of *ery-r5* from *ery-r2-r3*. A number of attempts have been made to eliminate the *ery-r5* mutation from the *ery-r2-r3-r5* strain by replacing it with its *ery-s5* allele. This was done by long-term exposure of the strain to each of the following DNAs: *ery-s2-s3-s5 str-r1*, *ery-r2-s3-s5 str-r1*, *ery-s2-r3-s5 str-r1*, *ery-r2-r3-s5 str-r1*, and by challenging samples of the treated strain with 40 μg. erythromycin per ml. or 100 μg. streptomycin per ml. Although acquisition of the *str-r1* marker could be demonstrated, no recombinants having the *ery-r2-r3-s5* genotype could be detected by this method. One may conclude either that a challenge of 40 μg. erythromycin per ml. is too great for *ery-r2-r3-s5* transformants to withstand only a few generations after transformation, or that replacement, within the *ery-r2-r3-r5* complex, of the *ery-r5* mutated site by the corresponding unmutated allele is extremely rare. The latter explanation seems more likely, since some *ery-r2-r3-s5* transformants can be detected in long-term experiments in which sensitive Rx cocci (*ery-s2-s3-s5*) are treated with DNA *ery-r2-r3-s5* and challenged with 40 μg. erythromycin per ml. It is true, however, that the recovery of such transformants is more efficient when a lower challenging concentration of erythromycin is used.

Reversions of the ery-r3 *and* ery-r5 *mutations.* In the periodic transfer of stock mutant cultures, it was noted that certain mutants often reverted to the wild-type (erythromycin-sensitive) condition. This was noted when samples of the cultures were plated on plain agar and on agar containing a concentration of erythromycin which the originally isolated mutants were able to resist. A far greater number of colonies appearing on plain agar than on erythromycin agar suggested that reversions had occurred and had been selected for. That this was indeed the case was shown by the fact that a large proportion of the colonies appearing on plain agar were identical to wild-type in their level of resistance to erythromycin, and that the cocci in such colonies (or in wild-type cultures) had a slight but definite selective advantage when mixed with the original mutant type and grown in media in the absence of erythromycin. The most commonly reverting mutant cultures were those of *ery-r3* and *ery-r5*. The mutation *ery-r2* was very stable, and indeed, no case of a reversion has been noted despite numerous transfers of several different clones bearing this marker during the more than 2 years in which this marker has been studied. Furthermore, the mutant culture *ery-r2-r3* often drops in its level of resistance from 40 to 1 μg. erythromycin per ml.; but when it does so, it is found that the mutation at the *ery-r3* site has reverted, while the mutation at the *ery-r2* site has persisted. The method of determining the site of reversion will be described below. It should be stated at this point, however, that despite the known revertibility of the *ery-r3* and *ery-r5* mutations, the recombinant *ery-r2-r3-r5* is quite stable. A search for a reverted type having the genotype *ery-r2-r3-s5*, having a higher level of resistance

than the triply mutant type, has been fruitless. Finally, it should be stated that at least in the routine inspection of transferred stocks, the *ery-r6* and *ery-r7* mutations have not been found to undergo reversions to the wild-type condition.

There are essentially four possibilities of accounting for a reversion in one of the erythromycin-resistant strains. The first possibility is that a true back-mutation occurs, in the sense that the mutated site of the DNA molecule reverts to the original physico-chemical configuration that existed in the parental wild-type erythromycin-sensitive strain. The second possibility is that, in a reversion, the mutated site undergoes a change in physico-chemical configuration, which is not to that of the parental wild-type molecule but which, nevertheless, confers the same sensitivity to erythromycin as does the wild-type configuration. For reasons to be discussed below, it is not easy to distinguish experimentally between these first two possibilities, and therefore the term 'back-mutation' will be applied to both in the sense of referring to involvement of the mutated site in reversion. The third possibility is that, in a reversion, a suppressor mutation arises linked to the site of the erythromycin resistance mutation, and as a consequence, the resistance normally conferred by the latter is suppressed. The fourth possibility is similar to the third, except that the suppressor is unlinked to the erythromycin resistance mutation. For singly marked *ery-r3* and *ery-r5* mutants, the third and fourth possibilities may be tested: (*a*) by treating one of the reverted strains with the DNA of the wild-type sensitive Rx strain, and conversely, (*b*) by treating the wild-type strain with DNA extracted from a reverted strain. In either cross, erythromycin-resistant transformants should appear having the phenotype of the original (unreverted) mutant strain, if a suppressor mutation is the cause of the phenotypic reversion to wild-type. In (*a*) the wild-type allele of the suppressor should replace its suppressing homologue in the reverted strain, whereas in (*b*) the intact erythromycin resistance mutation should be able to be transferred away from its suppressor into the recipient sensitive strain. If the suppressor is linked to the erythromycin resistance mutation, the frequency of erythromycin-resistant transformants would be expected to be lower than in the case where it is unlinked. The spontaneous rate of mutation to erythromycin resistance (10^{-8} to 10^{-9}) sets a lower limit to the frequency of separation of the suppressor from the *ery-r* mutation than would be detectable.

For doubly marked mutants (*ery-r2-r3*), the third and fourth possibilities of explaining reversions may be tested in another way. The reversions that occurred in the *ery-r2-r3* cultures were able to resist 1 μg. erythromycin per ml., although the parental culture could withstand 40 μg. erythromycin per ml. If a suppressor arose to counteract one of the mutated sites (*ery-r2* or *ery-r3*), then the level of resistance characteristic of the *ery-r2-r3* strain should be reconstituted by exposure to both the DNA of the normal (i.e. unsuppressed) *ery-r2* strain and the DNA of the normal *ery-r3* strain. For the suppressor should be replaced by its wild-type homologue in either case. On the contrary, if one of the sites had undergone a 'back-mutation', the enhanced level of resistance can be reconstituted only by exposure to *one* of these DNAs, namely, the DNA bearing the mutation lost in the reverted strain. Eleven independent reversions in *ery-r2-r3* strains were selected. Competent cultures of these reversions were tested by exposing samples separately to DNA *ery-r2 str-r1* and to DNA *ery-r3 str-r1*. Streptomycin resistance transformations were induced readily. However, transformants capable of resisting 5 μg. erythromycin per ml. were obtained only after exposure to DNA containing the

ery-r3 marker. Although the frequency of transformation to a high level of resistance induced by the *ery-r3* marker was as high as 10^{-4}, the frequency of such transformations by the *ery-r2* marker was less than 10^{-8}. These results permit the conclusion that the *ery-r2* mutation was not involved in these reversions, and that in each case the *ery-r3* mutation underwent a 'back-mutation' to the original wild-type condition.

The results observed in the reversions of the *ery-r2-r3* strains encouraged further analysis of reversions in *ery-r3* and in *ery-r5* strains in order to determine whether suppressor mutations could account for any of these reversions. Five independent reversions in strains bearing the *ery-r5* marker and five independent reversions in strains bearing the *ery-r3* marker were tested by the procedure described in the previous paragraph. Similarly, a reversion in the E21 strain and one in the E24 strain were tested. The DNA used in a given cross always contained, in addition, either the *str-r1* mutation or the *caps*$^+$ marker, so that negative findings with respect to the induction of erythromycin resistant transformants were always controlled by positive transfer of some unlinked marker. The results were uniformly negative with respect to the production of erythromycin resistant transformants: such transformants did not appear at a frequency greater than that at which spontaneous mutations arise. Consequently, it may be concluded that most of the reversions in *ery-r3* and *ery-r5* strains are due to back-mutations in the sense just described or to suppressor mutations occurring at sites so closely linked to the original mutated sites that separation by genetic recombination occurs with an undetectably low frequency.

One means exists for distinguishing between the first two possibilities for explaining reversions. If a given erythromycin resistance mutation involved a sufficiently large region of the DNA molecule, and if the reversion resulted in a diminution of the mutated region, and if different reversions caused different non-overlapping portions of the originally mutated site to persist, it may be possible to reconstitute an erythromycin-resistant strain by a 'cross' between two reverted strains. This possibility was examined by means of 'crosses' between a number of reverted strains as shown in Table 9. In no case, however, was resistance to erythromycin recon-

Table 9. *Attempts to reconstitute erythromycin resistance by crosses between reverted* ery-r *strains*

Host reverted strain	Donor reverted strain	Transformants capable of resisting 0·25 μg. ery./ml.
Rx E21-rev	Rx E24-rev	—
Rx E24-rev	Rx E21-rev	—
Rx *ery-r5-rev* no. 2		—*
3		—*
4		—*
5		—*
Rx *ery-r3-rev* no. 1	Rx *ery-r5-rev* no. 1	—*
2		—
3		—
SIII-1 *ery-r3-rev* no. 1		—*
2		—*
Rx *ery-r5-rev* no. 1	Rx *ery-r3-rev* no. 3	—*

* Control: +transfer of *str-r1* or *caps*$^+$ marker.

stituted. This finding does not rule out the second possibility of explaining reversions but it does make improbable the assumption of diminution of mutation in a reversion.

DISCUSSION

The molecule of pneumococcal DNA which regulates resistance to erythromycin is apparently distinct from other molecules of pneumococcal DNA which regulate such diverse properties as capsule synthesis, resistance to streptomycin, etc., in the sense that its transfer in transformation reactions is independent of the transfer of the others (Ravin, 1960). This finding is generally interpreted as due to a heterogeneity in the species of DNA molecules contained in a given genome (Hotchkiss, 1951). In any case, it is evident that whatever organelle organizes the DNA content of a bacterium, extraction of the DNA liberates DNA molecules of different kinds into a heterogeneous mixture. It is hoped that future biochemical investigations will reveal the chemical and/or physical differences between these molecules. In addition to the differences between molecules, however, there is evident intramolecular heterogeneity. A molecule affecting a given bacterial function is differentiable into regions having discrete quantitative influences of their own and capable of being separated from each other or brought together by genetic recombination (Ephrussi-Taylor, 1951; Hotchkiss & Evans, 1958; Ravin, 1960; Lacks & Hotchkiss, 1960). The results obtained with the molecule governing erythromycin resistance in pneumococcus supply further evidence in this regard. Current conceptions of the structure of the DNA molecule equate these different regions within the molecule to specific sequences of nucleotides in the linear DNA polymer.

Although they demonstrate the linkage of the five erythromycin resistance mutations, the present experiments are insufficient to determine the precise locations of these mutations with respect to each other. Further investigations are currently in progress with the aim of clarifying their spatial relationship. Nevertheless, the work reported at this time describes certain anomalous situations which appear to be of considerable interest for our understanding of intramolecular recombination (Ravin, 1961). These situations include the following:

(1) The frequency of recombination between given sites on the endogenous (host) and transforming (donor) DNA molecules appears to vary according to the genetic composition of the recombining molecules. When, for example, the sensitive parental strain *ery-s2-s3-s5* is exposed to the triply-marked DNA from the recombinant *ery-r2-r3-r5*, the most frequent classes of transformants produced are *ery-r2-s3-r5*, *ery-s2-r3-r5* and *ery-r2-r3-r5*. In other words, the *ery-r5* mutation tends to be transferred along with one or both of the other mutations. Such a result would be readily explained on the grounds that the *ery-r5* mutation lies between the *ery-r2* and *ery-r3* mutations, so that a double 'cross-over' within a limited segment of the DNA molecule would be necessary to free *ery-r5* from the mutations on either side of it. Such a double 'cross-over' may be expected to occur infrequently. On this hypothesis, when the *ery-s2-s3-r5* mutant is treated with DNA from the recombinant *ery-r2-r3-s5*, the transformant *ery-r2-r3-s5* should be produced more frequently than the transformant *ery-r2-r3-r5* which would require the same type of double 'cross-over' as has just been invoked. On the contrary, however, the *ery-r2-r3-r5* recombinant is the more frequent product in this cross. Similarly, to take another example, when *ery-r3* is crossed with *ery-r6*, the recombinant *ery-r3-r6* is rare. On the other hand, when *ery-s3-s6* is treated with DNA *ery-r3-r6*, the frequency of the

recombinant *ery-s3-r6* is relatively high. Yet in all respects, except for the specific nature of the sites being confronted in these crosses, the recombinations involved are similar. These results are probably similar to those of Lacks & Hotchkiss (1960), who obtained quite different recombination frequencies in reciprocal crosses between 'maltase' mutants in pneumococcus. It is possible that all of these anomalous results in transformation-mediated recombinations may be eventually explicable by a model, like that discussed by Ephrussi-Taylor (1961), in which the relative lengths of the mutated sites, as well as the distances between them, influence the frequency of recombination.

(2) When the sensitive *ery-s2-s3-s6* strain is treated either with DNA from the recombinant *ery-r2-s3-r6* or with DNA from the recombinant *ery-s2-r3-r6*, the transfer of the two resistance mutations occurs more frequently than the transfer of a specific single mutation. In the case of DNA *ery-r2-s3-r6*, it is *ery-r6* that is transferred rarely relative to the transfer of *ery-r2-r6*. In the case of DNA *ery-s2-r3-r6*, it is *ery-r3* that is transferred rarely relative to the transfer of *ery-r3-r6*. At the present time, it is premature to formulate a model to account for these results. It is interesting, nevertheless, that these two cases provide exceptions to the general rule that a linked pair of mutations is transferred less frequently than the individual mutations (for review, see Ravin, 1961).

Obviously, more crosses need to be conducted using all possible combinations of donor and recipient genotypes. Such experiments are in progress. The results of these further studies should throw light, not only upon the unusual situations described above, but also on the order of the mutated sites in the molecule of DNA governing resistance to erythromycin. It is worth remarking, however, that all five independent spontaneous mutations to erythromycin resistance have been shown to be transferred by the same molecule of DNA. This finding is in support of the possibility, outlined by Bryan (1961), that the different genetic factors known to underlie multi-step resistance to antibiotics are not unlinked polygenes, but rather closely linked members of a complex locus. The action of the latter, as in Bryan's studies on streptomycin resistance, may be affected by modifier genes, which do not confer any resistance by themselves.

Many previous genetic studies of micro-organisms and viruses have indicated the occurrence of what we have referred to above as 'back-mutations', i.e. reverse-mutations occurring at or extremely close to the original sites of mutation. Thus, it is not surprising to find further evidence of such alterations with the genetic material of pneumococci. Since, however, individual DNA molecules are the vectors of genetic transfer in transformation reactions, one has the unique opportunity of localizing the site of the 'back-mutation', not only in terms of genetic (recombination) distances, but also in particular regions of specific DNA molecules. One knows at least, from the present studies, that a 'back-mutation' may occur on the same molecule of DNA as that of the original mutation, and, indeed, may occur so close to the site of the latter as to be inseparable from it by genetic recombination. The question as to whether a 'back-mutation' always restores the original configuration of the genetic material is an interesting one, and is open to analysis. It may be possible to find at some time that a cross between two reversions restores a mutant condition, although our preliminary attempts in this regard have been uniformly negative. If such a positive case were found, evidence would be provided for the existence of different 'wild-type' states of genetic material.

The phenotypic interactions of the different mutations probably reflect the

functional differentiation of the genetic locus concerned with erythromycin resistance. It has been noted that two different mutations of the *ery* locus often interact to produce a more-than-additive, or synergistic, enhancement of resistance to erythromycin. However, this is not always the case. The *ery-r5* mutation does not add appreciably to the level of resistance of a strain already bearing the *ery-r2* or *ery-r3* mutation. Moreover, it antagonizes the action of the *ery-r2-r3* duplex. Finally, the *ery-r7* mutation does not cause any synergistic effect when coupled with either *ery-r6* or *ery-r5*. While it is altogether possible that *ery-r7* is epistatic to *ery-r6* and *ery-r5*, this cannot be readily determined from the present data since the sum of the effects of *ery-r7* and *ery-r5* (which would confer a maximum level of resistance of 11 μg. erythromycin per ml.) is indistinguishable from an epistatic effect of *ery-r7* (which would confer a maximum level of resistance of 10 μg. per ml.). New experiments are being planned to test the possible epistasis of the *ery-r7* mutation. It appears likely, in any event, that *ery-r7* acts physiologically in a different manner from that of the other mutations, because of its unique rate of phenotypic expression. Unfortunately, as yet little is known of the biochemical mechanism of erythromycin resistance. No enzymic activity capable of destroying erythromycin has been found in cultures of several different species of erythromycin resistant bacteria (Haight & Finland, 1952*a*). On the other hand, the inhibitory action of erythromycin against the diphtheria bacillus is known to be antagonized by pantothenic acid, β-alanine and L-carnosine (Brown & Emerson, 1953). This finding is suggestive of a specific metabolic block produced by the antibiotic and furnishes a means of determining the mode of resistance to erythromycin. With such information in hand, it would be of considerable interest to relate the observed epistatic, antagonistic and synergistic interactions of the erythromycin resistance mutations to the specific biochemical functions they carry out in conferring resistance upon the pneumococcus. In an elegant study by Hotchkiss & Evans (1958), the action of three closely linked mutations conferring resistance to sulphanilamide could be ascribed to specific modifications they imparted to the substrate-binding capacity of an enzyme involved in folic acid synthesis. Such studies provide a powerful means of investigating the relation of genetic fine structure to enzyme structure and activity.

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Transforming Activities and Base Contents of Deoxyribonucleate Preparations from Various Neisseriae

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SUMMARY

Genetic transformation was investigated among *Neisseria* spp. whose normal habitat is the nasopharynx of humans. Seven species, as characterized in *Bergey's Manual* (1957), were represented. Deoxyribonucleate (DNA) preparations from streptomycin-resistant mutants of *N. meningitidis*, *N. perflava*, *N. flava*, *N. subflava*, *N. sicca*, and *N. flavescens* conferred resistance upon streptomycin-susceptible parent strains of the corresponding species (intraspecific transformation) and of each other species (interspecific transformation). Ratios of interspecific to intraspecific transformation were 0·01 or higher for all possible combinations of DNA and recipient cells of the six species. On the other hand, *N. catarrhalis* cells, which exhibited high frequencies of intraspecific transformation, were not transformed at detectable frequencies by DNA from any of the six *Neisseria* species listed above. In turn, DNA from *N. catarrhalis* had little or no transforming activity for these other neisseriae.

Possible evidence of structural differences between these DNA's was sought by analysing the base contents of transforming preparations. The bases adenine, thymine, guanine and cytosine were present in about equal proportions in the DNA's of the six *Neisseria*: *meningitidis*, *perflava*, *flava*, *subflava*, *sicca* and *flavescens*. In DNA preparations from two strains of *N. catarrhalis*, however, adenine and thymine predominated. The ratio (adenine + thymine/guanine + cytosine) was higher than 1·4 compared to 1·0 for the others.

INTRODUCTION

Neisseria meningitidis undergoes genetic change (transformation) affecting capsular antigen specificities (Alexander & Redman, 1953) or response to streptomycin (Catlin, 1960*a*) following brief exposure to solutions of deoxyribonucleate (DNA). The customary source of DNA is experimentally lysed cells. However, genetically active DNA may be found also in the extracellular environment of *N. meningitidis* (Catlin, 1960*a*) and *N. sicca* (Catlin, 1960*b*), presumably owing to spontaneous cellular lysis of some proportion of the populations. DNA can be isolated from the supernatant fluids of centrifuged broth cultures and purified. Moreover, the crude DNA-containing culture slimes themselves elicit transformation. Both *N. sicca* and *N. meningitidis* culture slimes have transforming activity for *N. meningitidis* cells (Catlin, 1960*a*, *b*).

Extracellular transforming activity has been demonstrated also in cultures of pneumococcus (Ottolenghi & Hotchkiss, 1960). These findings taken together with the knowledge that transformation can occur *in vivo* (see Austrian, 1952) give substance to the idea that transformation does occur in nature, and that it may be a regular method of genetic transfer for some bacteria. Some of the consequences for bacteria of genetic recombination have been discussed by Ravin (1960).

A variety of *Neisseria* spp. inhabit the nasopharyngeal mucosa of man. They may be numerous and proximate in this niche, conditions which should have provided opportunities for recombination, if it occurs. This group appeared favourable, therefore, for investigations of genetic transfer. One or more representatives of each of seven *Neisseria* species (as characterized in *Bergey's Manual*, 1957) which inhabit the nasopharynx were examined quantitatively for their capacity to become transformed to streptomycin resistance following exposure to preparations of DNA isolated from streptomycin-resistant mutant strains derived from each of the seven species. In addition, base analyses were carried out on transforming preparations in an attempt to correlate DNA composition with affinities among the Neisseria inferred from the transformation tests; preliminary reports have appeared (Catlin, 1960*c*, 1961).

METHODS

Media. Heart infusion broth (Difco) with or without 0·3 % (w/v) yeast extract (Difco) was supplemented after sterilization with 250μg. ribonucleic acid (Nutritional Biochemicals Corporation)/ml., 0·00005 M-sodium glutamate and 0·0005 M-calcium chloride added separately as sterile solutions (Catlin, 1960*a*). In these media, designated HIY-1 or HI-1, luxuriant growth of all strains was obtained in shaken cultures. HIY-1 agar was used in all plating procedures. Concentrations of agar (Difco) employed were 1·4 % (w/v) (hard agar) or 0·7 % (w/v) (soft agar).

To eliminate aberrant responses characteristic of surface growth on dry media, agar was freshly poured on the day it was to be streaked. Hard agar bottom layers for assays were dispensed (in volumes of 20 ml. $\pm$ 0·5 ml.) 3–5 days before use, and plates were held at room temperature; when overlaid with inoculated soft agar, surface moisture was negligible.

Capacity to produce acid from carbohydrates was examined by using HIY-1 medium with 1 % (w/v) agar, phenol red 0·015 mg./ml., and 0·5 % (w/v) of either glucose, maltose, fructose, sucrose, mannitol or lactose. For the latter additions filter-sterilized 20 % (w/v) solutions were added aseptically. Media were tubed with a butt and a short slope, and were inoculated by stabbing the deep agar and streaking the surface.

Characterization of strains. Table 1 lists the *Neisseria* strains investigated, their sources and designations. Strain numbers preceded by Ne were from the culture collection of the department. All strains were Gram-negative cocci, commonly arranged in pairs with adjacent sides flattened. They grew well on HIY-1 agar, and were oxidase- and catalase-positive. Chromogenesis, if any, was essentially characteristic of the species, as given in *Bergey's Manual* (1957).

None of the strains produced acid from mannitol or lactose; the (control) medium lacking carbohydrate was invariably alkaline. Reactions of the strains in maltose, fructose and sucrose corresponded, with one exception, to descriptions in *Bergey's Manual* (1957). The exception related to reactions of *Neisseria subflava* strain 11076.

The 'species' is characterized as producing acid from glucose and maltose only. Acid was produced from maltose and fructose within 1–2 days, and usually from sucrose after 4–6 days of incubation. Reactions of this strain, as well as strains of several other species (e.g. *N. sicca* Ne 12 and *N. flava* 4), were irregular in the glucose-containing medium; acid was either not produced or was produced after a delay, as previously reported (Pelczar & Doetsch, 1949; *Topley and Wilson's Principles*, 1955).

All strains were highly susceptible to the antibacterial action of crystalline dihydrostreptomycin sulphate (Squibb). This antibiotic will be referred to as DST, and strains resistant to its action as streptomycin-resistant (*str-r*). Spontaneous single-step mutations conferring resistance to at least 500 μg. DST/ml. were obtained from all seven species, essentially as described (Catlin, 1960*a*).

Table 1. *Sources of* Neisseria *strains*

Designation			Year isolated (i) or received (r)
Strain	Species	Source	
Ne 15	*N. meningitidis*	Spinal fluid, fatal, non-epidemic meningitis	1955 (i)
Ne 16	*N. perflava*	Throat culture, outpatient	1957 (i)
Ne 20	*N. perflava*	Throat culture, outpatient	1958 (i)
4	*N. flava*	University of Maryland Collection*	1960 (r)
55	*N. flava*	University of Maryland Collection*	1960 (r)
JJ IIA	*N. flava*	Statens Seruminstitut Collection, Copenhagen†	1960 (r)
2104	*N. flava*	Sputum culture, Walter Reed Army Institute of Research, Washington	1960 (i)
2105	*N. flava*	Sputum culture, Walter Reed Army Institute of Research, Washington	1960 (i)
11076	*N. subflava*	American Type Culture Collection	1960 (r)
Ne 12	*N. sicca*	Throat culture, healthy student	1954 (i)
13120	*N. flavescens*	American Type Culture Collection, N.I.H. strain N 155*‡	1960 (r)
Ne 11	*N. catarrhalis*	University of Rochester Collection	1954 (r)
Ne 13	*N. catarrhalis*	New York State Department of Health, strain 34105	1954 (r)

* = strain examined by Hajek, Pelczar & Faber (1950); † = strain examined by Jessen (1934); ‡ = strain examined by Branham (1930).

Organisms were stored at $-60°$. A number of similar suspensions of each strain was frozen at one time for subsequent use.

DNA preparations. Streptomycin-resistant strains were subcultured several times on HIY-1 agar supplemented with 500 μg. DST/ml. The last subculture served as inoculum for antibiotic-free HIY-1 broth cultures (incubated at 37° on a shaker) or HIY-1 agar (2%, w/v) plates (incubated at 37° with 70% humidity). Cultures were harvested after 18–22 hr. Organisms were lysed with sodium dodecylsulphate, as described (Catlin, 1960*a*). The DNA-containing fibrous masses obtained by precipitation with 2 volumes of ethanol were purified by methods (Catlin & Cunningham, 1958; Catlin, 1960*a*) which included two separate steps of deproteinization with sodium dodecylsulphate; each was followed by a step involving centrifugation of the DNA solution (in M-NaCl) for 110 min. (32,000*g*; 3°), and precipitation of DNA fibres with ethanol. The final DNA solutions (in 0·14 M-NaCl) were prepared using aseptic

precautions (Catlin, 1960*a*), and were found to be sterile. Concentration of DNA was determined by the diphenylamine reaction (Dische, 1955).

Transformation tests. A procedure developed during quantitative transformation studies of *Neisseria meningitidis* (Catlin, 1960*a*) was applied uniformly to all *Neisseria* species. It involved the following steps.

(1) Preparation of suspension of recipient cells. Punctiform surface colonies were picked from HIY-1 agar which had been incubated 11·5–13 hr. in a water-jacketed incubator at 37°. A homogeneous suspension of cells was prepared in warm (36°) HI-1 broth. Occasionally the suspension was coarse, in spite of vigorous repeated expulsion from a pipette; it was centrifuged briefly, and the supernatant fluid was used. Suspensions having a barely visible turbidity contained about 10^7 colony-forming units/ml. A further dilution was made in warm broth to give the desired population size.

Absence of cellular aggregation, a condition rarely attained with cocci, is essential for an accurate determination of transformation frequencies. Among the *Neisseria* spp. examined, *N. meningitidis* was exceptional in providing suspensions having usually about 95% single or paired cocci (Catlin, 1960*a*). Other species gave suspensions having 5–30% of aggregates containing 3–8 cells, most of which numbered 3–4. (Strains of *N. sicca* and *N. catarrhalis*, which were most troublesome, produced no fewer aggregates in shaken HIY-1 broth cultures). As the same cellular suspension was used for comparisons of different transforming preparations, results were affected equally by aggregation, however, and ratios of interspecific to intraspecific transformation were found to be reproducible.

(2) Exposure to DNA. As soon as the cellular suspension was prepared, 1·5 ml. was added to 1·5 ml. of each DNA preparation (20·0 μg./ml. diluted in HI-1 broth). Each reaction mixture (in 25 × 150 mm. screw-cup tube) was incubated at 36° for 30 min., whereupon 0·03 ml. of a solution of sterile pancreatic deoxyribonuclease (Worthington) 1 mg./ml. with magnesium, was added to destroy unbound transforming DNA.

(3) Assay of number of 'cells'/ml. exposed = *E*. One or more test populations was sampled just after addition of deoxyribonuclease. Measured volumes of appropriate dilutions in HI broth were added to tubes containing 4 ml. of HIY-1 soft agar (liquefied and held at 44°). These were poured over supporting layers of HIY-1 hard agar. Plates were incubated at 37° for 2–3 days. The value E was calculated from the mean number of colonies on five plates, which usually agreed within 10%.

(4) Assay of number of transformants/ml. = T. All reaction mixtures were assayed within 90 min. of initiation of exposure. A sample of (0·1–2·0 ml.) was mixed with 40 ml. of HIY-1 soft agar (at 44°); immediately the entire volume was dispensed in 4 ml. aliquots on the surfaces of a set of 10 HIY-1 hard agar plates. Five min. later the plates were placed without stacking at 37°. Five hr. after the time of initial exposure of cells to DNA, plates were removed to room temperature, and each was overlaid with a 4-ml. top layer of HIY-1 soft agar containing DST in a quantity sufficient to give 500 μg. DST/ml. after diffusion of the antibiotic through the underlying agar. Plates were left at room temperature for a further 45 min.; then they were returned to 37° and were not stacked until each had warmed uniformly. Colonies were counted after incubation at 37° for 3–4 days. The oxidase reaction was used to check all colonies on plates having fewer than ten colonies.

A control reaction mixture containing transforming DNA which had been inactivated by addition of deoxyribonuclease 5 min. before adding recipient cells was included in all transformation tests (Catlin, 1960*a*). Streptomycin-resistant (mutant) colonies were very rarely found.

Analysis of base contents of transforming DNA preparations. RNA was removed as described by Smith & Wyatt (1951). After drying from acetone, DNA was hydrolysed as described by Wyatt & Cohen (1953), using test tubes called ignition tubes in the U.S.A. (There is danger of the tubes exploding during or after heating.) Bases were then determined as described by Wyatt (1951) except that the differential extinction method (Vischer & Chargaff, 1948) was used, the coefficients being given by Bendich (1957).

Table 2. *Transformation of neisseriae by DNA preparations (final concentration 10 μg. DNA/ml.) from streptomycin-resistant strains of the same species*

Streptomycin-susceptible recipients					
Species	Strain	No. exposed/ml. (E)	Derivation of DNA donor	*Str-r* transformants No./ml. (T)	$\frac{T}{E} \times 10^5$
N. meningitidis	Ne 15	$4{\cdot}6 \times 10^6$	Ne 15	15,700*	341·3
N. perflava	Ne 16	$1{\cdot}1 \times 10^6$	Ne 16	17,390*	1,580·9
	Ne 16	$1{\cdot}1 \times 10^6$	Ne 20	12,080	1,098·2
	Ne 20	$1{\cdot}4 \times 10^6$	Ne 16	313	22·4
	Ne 20	$1{\cdot}4 \times 10^6$	Ne 20	941	67·2
N. flava	4	$1{\cdot}7 \times 10^6$	4	17,860	1,050·6
	4	$1{\cdot}7 \times 10^6$	2104	16,070	945·3
	4	$1{\cdot}7 \times 10^6$	2105	13,260	780·0
	4	$1{\cdot}7 \times 10^6$	JJ IIA	7,470	439·4
	4	$3{\cdot}4 \times 10^6$	4	10,260*	301·8
N. subflava	11076	$6{\cdot}5 \times 10^6$	11076	78*	1·2
N. sicca	Ne 12	$3{\cdot}0 \times 10^5$	Ne 12	199*	6·6
N. flavescens	13120	$1{\cdot}1 \times 10^7$	13120	256*	2·3
N. catarrhalis	Ne 11	$8{\cdot}4 \times 10^4$	Ne 11	12,140*	14,452·4
	Ne 11	$8{\cdot}4 \times 10^4$	Ne 13	6,610	7,869·0
	Ne 13	$2{\cdot}0 \times 10^6$	Ne 11	95	4·8
	Ne 13	$2{\cdot}0 \times 10^6$	Ne 13	330	16·5

* Data used in connexion with Table 3.

RESULTS

Intraspecific transformation. Representatives of all seven species of *Neisseria* examined were transformed from streptomycin-susceptibility to streptomycin-resistance by DNA extracted from streptomycin-resistant strains of the corresponding species. Table 2 shows transformation ratios obtained for various strains. Ratios were consistently low for the single strains of *N. subflava*, *N. sicca*, and *N. flavescens* examined; between 1 and 10 transformants/100,000 treated cells (colony-forming units) were obtained in repeated tests. In comparison, high transformation ratios were regularly obtained with certain strains of the other four species. Transformation of *N. catarrhalis* strain Ne 11 varied in five independent experiments from about 5 to 15 % of the treated population. In one of these tests assays of both transformants (T) and total exposed cells (E) were carried out on fifteen plates containing an identical inoculum; ten plates were overlaid with DST-containing medium for determination of T, and five plates without overlays were used for

counts of total colonies (E). Cellular aggregation presumably gave an upward bias to T/E values, and contributed to differences between the values found in independent tests of the same recipient. One of the most extreme examples of such a difference is shown in Table 2, *N. flava* 4.

Various strains of the same species differed considerably in capacity to become transformed, as shown by T/E values for *Neisseria perflava* and *N. catarrhalis* strains (Table 2). Even greater differences were exhibited by the five *N. flava* strains (Table 1). Only strain 4 was transformed at a useful frequency. No transformants were detected in single exploratory tests of strains 55 and JJ II A ($T/E \times 10^5$ ratios were less than 0·007 and 0·06, respectively). Though a few transformants were obtained with strains 2104 and 2105, ratios were very low (0·025 and 0·017). However, DNA preparations obtained from four of these strains (DNA was not prepared from strain 55) were active in eliciting transformation of strain 4 cells (Table 2).

Interspecific transformation. Table 3 and those portions of Table 2 marked by an asterisk give results of seven representative experiments, each of which tested recipient cells of a given species with transforming DNA from all seven species. Data marked by an asterisk in Table 2 are numbers of transformants/ml. obtained in tests of each recipient with homologous DNA; these intraspecific transformation values were compared with the number of transformants/ml. elicited by heterologous DNA preparations (interspecific transformation) in the same experiment, and results are expressed in Table 3 as ratios of interspecific to intraspecific transformation.

Table 3. *Ratios of interspecific to intraspecific transformation among* Neisseria *spp.*

Recipient cells	DNA preparations from streptomycin-resistant strains of: *N. meningitidis* (Ne 15)	*N. perflava* (Ne 16)	*N. flava* (4)	*N. subflava* (11076)	*N. sicca* (Ne 12)	*N. flavescens* (13120)	*N. catarrhalis* (Ne 11)
N. meningitidis (Ne 15)	*	0·091	0·054	0·022	0·036	0·047	< 0·00003
N. perflava (Ne 16)	0·119	*	0·655	0·192	0·141	0·064	< 0·00003
N. flava (4)	0·312	0·735	*	0·188	0·342	0·142	< 0·00005
N. subflava (11076)	0·577	4·680	3·949	*	0·666	1·154	0·006†
N. sicca (Ne 12)	0·020	0·025	0·010	0·010	*	0·020	0·005‡
N. flavescens (13120)	0·180	0·262	0·094	0·070	0·051	*	< 0·002
N. catarrhalis (Ne 11)	< 0·000002§	< 0·000002§	< 0·000002§	< 0·000002§	< 0·000002§	< 0·000002§	*

* = 1·0, intraspecific transformation; see corresponding data of Table 2; † = 1 streptomycin-resistant colony/2 ml. sample; ‡ = 1 streptomycin-resistant colony/ml. sample; § = no transformants found in samples of $3·0 \times 10^6$ colony-forming units/ml., 14% transformants being elicited by homologous DNA in the same experiment (Table 2, Ne 11).

Ratios (Table 3) were 0·01 or higher for all possible combinations of recipient cells and transforming DNA involving the six *Neisseria* species: *meningitidis*, *perflava*, *flava*, *subflava*, *sicca*, and *flavescens*; each recipient was examined in three or more independent experiments. The interspecific transformation values for *N. sicca* were based on colony counts ranging from 1 to 5/ml. (as compared with 199/ml. for intra-

specific transformation (Table 2). Accordingly, their reliability is doubtful, although very similar results were obtained in two other experiments with strain Ne 12.

Transformation was not detected following treatment of *Neisseria meningitidis*, *N. perflava* (strains Ne 16 and Ne 20), *N. flava*, or *N. flavescens* recipients with DNA preparations from *N. catarrhalis*. Whether the rare streptomycin-resistant colonies found in tests of *N. subflava* and *N. sicca* represented transformants or spontaneous mutants cannot be determined from available data. One of four tests of *N. subflava* and two of three tests of *N. sicca* showed between one and three streptomycin-resistant colonies/2 ml., where corresponding numbers of cells treated with depolymerized DNA (controls) gave no resistant colonies. It is hoped that genetic tests of strains originating from such colonies will indicate their origin; we are at present inclined to regard them as spontaneous mutants, reversing the view expressed in a preliminary report (Catlin, 1960*c*).

Recipient cells of *Neisseria catarrhalis* strains Ne 11 and Ne 13, examined in five independent experiments, did not undergo interspecific transformation. In one experiment (Ne 11, Table 2) 12,140 transformants/ml. were elicited by treatment with *N. catarrhalis* DNA of a population of $8{\cdot}4 \times 10^4$ colony-forming units/ml.; in contrast, a higher concentration of the same cellular suspension ($3{\cdot}0 \times 10^6$ colony-forming units/ml.) gave no transformants following exposure to DNA preparations from each of the other six species (ratios of interspecific to intraspecific transformation less than 0·000002 (Table 3).

Base composition of DNAs. The base contents of transforming DNA preparations are given in Table 4. The ratio (adenine + thymine/guanine + cytosine) for *Neisseria meningitidis* DNA is in agreement with the value (1·00) found by Lee, Wahl & Barbu (1956) for another strain of the same species.

Table 4. *Purine and pyrimidine contents of DNA preparations obtained from streptomycin-resistant strains of* Neisseria *spp.*

Derivation of DNA donor	Mole/100 mole total bases				$\frac{A+T}{G+C}$
	Guanine	Cytosine	Adenine	Thymine	
N. meningitidis (Ne 15)	25·7	25·6	23·5	25·2	0·95
N. perflava (Ne 16)	25·4	24·9	26·0	23·8	0·99
N. perflava (Ne 20)	25·4	24·4	25·2	24·9	1·01
N. flava (JJ IIA)	25·0	24·5	25·5	25·0	1·02
N. subflava (11076)	24·1	26·4	21·9	27·6	0·98
N. sicca (Ne 12)	26·4	25·1	24·0	24·4	0·94
N. flavescens (13120)	25·6	24·5	23·6	26·2	0·99
N. catarrhalis (Ne 11)	19·9	20·8	28·6	30·6	1·45
N. catarrhalis (Ne 13)	19·1	21·0	27·9	32·1	1·50
N. catarrhalis (Ne 13 (11 *str-r*))*	19·9	21·4	28·2	30·5	1·42

* Streptomycin-resistant strain derived from Ne 13 by transformation with DNA from Ne 11 *str-r*.

DISCUSSION

The near-identity of the base ratios of DNA preparations from six different *Neisseria* spp.: *meningitidis*, *perflava*, *flava*, *subflava*, *sicca*, and *flavescens* (Table 4) is in accordance with expectation based on the results of transformation experiments. These DNA preparations elicited genetic change of recipient cells in all

thirty-six possible combinations involving the six kinds of *Neisseria* (Table 3). Thus, representatives of these six species (as defined in *Bergey's Manual*, 1957) may be regarded as members of a single group (group 1) in so far as they are related by the possibility of genetic transfer. On the other hand, a barrier to the transfer of genetic information appears to exist between group 1 strains and strains of *N. catarrhalis*. Recipient cells of the latter were not transformed by DNA preparations from group 1 strains, and conversely, DNA from *N. catarrhalis* had little or no transforming activity for recipient cells of group 1. The significant difference between the DNA base ratios of members of group 1 on the one hand, and *N. catarrhalis* on the other, indicates a possible structural basis for the barrier between the two groups.

A growing body of information suggests that genetic transfer occurs only between bacteria having DNA with similar base ratios (see discussions of this subject by Lanni, 1960, and by Ravin, 1960). Base ratios may range at least from 0·4 to 2·7, as shown by an investigation of the DNA of 60 strains of true bacteria (Lee *et al.* 1956). A significant difference between ratios found for any two strains implies remoteness of relationship; accordingly, transfer of genetic information between the two would not be expected. Although there is no agreement concerning the scope of the bacterial genus, the inclusion of *catarrhalis* strains in the genus *Neisseria* appears illogical from the evolutionary point of view.

In general, where adequate quantitative experimental design has eliminated bias due to differential selection of either the transformed or untransformed elements of the bacterial population, higher frequencies are obtained in intraspecific than in interspecific transformation tests (Schaeffer, 1958; but see Bracco, Krauss, Roe & MacLeod, 1957). Leidy, Hahn & Alexander (1956, 1959) have applied an analysis of ratios of interspecific to intraspecific transformation to the taxonomy of the genus *Haemophilus* on the premise that such ratios reflect the degree of relationship of donor and recipient cells. Thus far, however, quantitative investigations of interspecific transformations have been restricted to streptomycin resistance; in view of findings by Green (1959) interpretation of interspecific transformation data involving this single characteristic should be made with caution. He showed that heterogeneity of recipient pneumococcus strains may have an unequal influence on transformation frequencies determined for two different characteristics. The frequency of transformation to streptomycin resistance was consistently lower with one recipient than with a second, whereas these two pneumococcal strains exhibited equal frequencies of transformation to erythromycin resistance following exposure to a single doubly-marked transforming preparation.

The status of several species of *Neisseria* described in *Bergey's Manual* (1957) is doubtful. The Subcommittee on the family Neisseriaceae (1954) recognized the need to clarify the classification of all members of the Neisseriaceae. *Topley and Wilson's Principles* (1955) prefers to recognize as separate species among the Gram-negative cocci of the human nasopharynx only *N. meningitidis*, *N. flavescens* and *N. catarrhalis*; the remaining types, with the possible exception of *N. sicca*, would be combined into a single species, *N. pharyngis*. This suggestion that *N. perflava*, *N. flava*, and *N. subflava* are not sufficiently distinct to warrant separate species designations is in harmony with the present evidence from transformation tests.

DNA preparations from strains of *Neisseria flava* isolated in areas of the world as distant as the United States and Denmark (Table 1) and at times separated by

more than 25 years were capable of conveying genetic information to *N. flava* strain 4 (Table 2). However, the latter was transformed by DNA from strain JJ IIA *str-r* at frequencies lower than those elicited in the same experiment by DNA preparations from *N. perflava* (both strains Ne 16 *str-r* and Ne 20 *str-r*).

The response of *Neisseria subflava* strain 11076 was unique among the *Neisseria* spp. investigated. Recipient cells were transformed at higher frequencies by DNA preparations from streptomycin-resistant strains of *N. perflava* and *N. flava* than by DNA from *N. subflava str-r*. Further study may show that the response of this strain to homologous DNA is an example of 'depressed' transfer of the streptomycin resistance marker (Green, 1959). The atypical fermentation reactions (see 'Methods') suggest that this strain is not a representative of *N. subflava*. Strains which do correspond to the description of *N. subflava* (as given in *Bergey's Manual*, 1957) are rarely encountered (report of the Subcommittee on the family Neisseriaceae, 1954). A genetic study of available strains, together with further investigations of strain 11076 and representatives of *N. flava* and *N. perflava*, may clarify their taxonomic relations. Further discussion of possible taxonomic implications of ratios of interspecific to intraspecific transformation is better postponed until results of tests with other markers are available.

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The Role of Magnesium Ions in the Growth of Salmonella Phage Anti-R

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SUMMARY

Magnesium or certain other divalent metal ions are needed for the adsorption of phage anti-R to its host, a 'rough' strain of *Salmonella typhi*. In medium containing magnesium ions the infected organisms burst after a latent period of about 20 min. at 37°, but when infected organisms are diluted into medium without added divalent metal ions phage growth is considerably inhibited. A rapid increase in phage titre occurs when magnesium ions are added late, i.e. at some time after the end of the normal latent period, to dilute cultures of phage-infected organisms. During the interval between infection and this late addition of magnesium ions no appreciable numbers of infective intracellular phage were detected after ultrasonic disruption of the organisms. It is concluded that in addition to being an adsorption cofactor, magnesium functions at some late stage in phage development. Phage anti-R is probably related to ΦX 174; it consists of particles approximately 30 mμ in diameter and the intact phage reacts similarly with formaldehyde.

INTRODUCTION

Many phages require divalent metal ions for growth or fail to act on their specific bacterial hosts in the presence of substances such as sodium citrate and sodium oxalate which bind these ions (see Fildes, Kay & Joklik, 1952). Phage reproduction may be dependent upon divalent metals in a variety of ways. Some phages are unstable in the free state unless metal ions, particularly calcium, are present (Adams, 1949); some are unable to attach to the host in the absence of the ions (Gratia, 1940), and others need the ions to facilitate the entry of phage nucleic acid (Luria & Steiner, 1954). Fildes (1954) established that calcium or magnesium ions are needed for the activity of salmonella phage anti-R (A. 59/6SR Felix). The following paper describes further experiments on the divalent metal requirement of this phage, with special reference to the action of Mg^{++}.

METHOD

Host organism. Phage anti-R is active on 'rough' strains of *Salmonella typhi* and on some strains of *Escherichia coli*. In the work to be described, *S. typhi* R 4 derived from NCTC no. 3390 (Kay & Fildes, 1950), was used as the host organism,

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stock cultures being maintained at 4° on slopes of tryptic meat agar and subcultured monthly.

Media. Medium A contained 1 % (w/v) peptone (Evans), $7{\cdot}5 \times 10^{-2}$ M-NaCl and 2×10^{-3}M-$MgSO_4$. Medium B was the chemically defined medium of Kay & Fildes (1950) used at half strength. Before use it was supplemented with 2×10^{-2}M-glucose and 0·05 % (w/v) decalcified peptone (Fildes, 1954).

Phage. There is some doubt about the precise origin of phage anti-R but it is thought to have been isolated from a patient with paratyphoid A fever. The large size of the plaques formed by the phage suggests that it is very small (Elford & Andrewes, 1932) and this is confirmed by electron micrographs which show that it consists of apparently tailless particles about 30 mμ in diameter.

Phage assay. The standard double-layer plate method was used (Adams, 1950). The layers consisted of medium A solidified with 2 % (w/v) agar (Difco) over which a mixture of 2 ml. melted agar medium (medium A containing 1·25 %, w/v, agar) and 1 ml. host + phage mixture was poured. All phage estimations were done in duplicate. After overnight incubation at 37° plaques of 5 mm. average diameter were formed. This size limited the number of discrete plaques that could be accommodated on a 9 cm. diameter Petri dish and the usual procedure was to incubate the assay plates at 37° for 3 hr. and then to remove them to room temperature (about 20°) overnight. In this way plaques of 2 mm. average diameter, easily discernible against the bacterial background, were formed. There was a linear relation between numbers of plaques and concentration of phage suspension plated up to about 300 plaques/Petri dish.

Preparation of phage suspensions. An initial stock of phage was prepared by three successive single-plaque isolations and inoculation of the last of them into an actively growing culture of *Salmonella typhi* R 4 in medium A which was aerated in shaken ⊥-shaped tubes (Monod, Cohen-Bazire & Cohn, 1951) at 37°. When lysis was completed the cell debris was removed by centrifugation and the supernatant fluid decanted into a screw-capped bottle containing a drop of chloroform. Larger volumes of phage suspension were made by inoculating actively growing cultures of *S. typhi* R 4 at about 10^8 organisms/ml. in medium A with one-tenth the number of phage. The cultures were aerated vigorously for 3 hr. either in conical flasks attached to a reciprocating mechanical shaker or in rotating flasks (Mitchell, 1949). The cell debris sedimented by centrifugation at 2000 ***g*** retained a considerable amount of phage and this could be partially recovered by suspending the debris in $3{\cdot}3 \times 10^{-2}$M-phosphate buffer (10·1 g. $Na_2HPO_4.12H_2O$ + 0·68 g. KH_2PO_4/l.; pH 7·6) and shaking for a further hour before again centrifuging at 2000 ***g***. The supernatant fluids from both centrifugations were pooled and gave phage titres that varied from 2×10^9 to 1×10^{10} plaque forming units (pfu)/ml. Higher titre phage stocks (up to 2×10^{11} pfu/ml.) were made by alternate cycles of low-speed (2000 ***g***) centrifugation and high-speed (105,000 ***g***) centrifugation in a Spinco model L ultracentrifuge using $3{\cdot}3 \times 10^{-2}$M-phosphate buffer (pH 7·6) containing 0·01 % (w/v) serum albumin as the suspending medium.

Measurement of phage adsorption. Fresh cultures of *Salmonella typhi* R 4 grown to 10^9 organisms/ml. in medium A at 37° were centrifuged and the bacteria washed once with $3{\cdot}3 \times 10^{-2}$M-phosphate buffer (pH 7·6). Before use the organisms were resuspended in the original culture volume of buffer and further diluted into buffer with or without added divalent metals. After equilibrating 9 ml. bacterial suspen-

sion at 37° for 10 min., 1 ml. phage suspension containing about 10^5 pfu/ml. was added and rapidly mixed. Samples taken at intervals were diluted 10-fold into ice-cold $3{\cdot}3 \times 10^{-2}$M-phosphate buffer (pH 7·6) and quickly assayed for phage before and after centrifugation at 2000 *g* to sediment bacteria and adsorbed phage.

One-step growth experiments. Nine ml. medium B inoculated with 1 ml. of an overnight culture of *Salmonella typhi* R 4 in the same medium were aerated in a ⊥-tube at 37° to give about $1{\cdot}5 \times 10^8$ organisms/ml. The culture was concentrated by centrifugation and resuspension of the organisms in 1 ml. of the supernatant fluid. Magnesium sulphate (0·1 ml., 4×10^{-2}M-$MgSO_4$) and phage anti-R (0·1 ml. of a suspension containing about 2×10^{10} pfu/ml.) were added and the mixture incubated in stationary culture at 37° for 10 min. At the end of this adsorption period 9 ml. ice-cold medium B were added and the organisms sedimented in a cooled centrifuge tube. The bacteria were washed once with 10 ml. ice-cold medium, centrifuged, and after resuspension, diluted into medium B at 37°. The final dilution was aerated in a ⊥-tube at 37° and assayed for phage at intervals. Compared with one-step growth experiments in which the infected culture was not washed, the latent period was not greatly lengthened by this procedure, provided the time between the addition of ice-cold medium and the final dilution into warmed medium was discounted. The method decreased the free phage to less than 1 % of the initial count.

Determination of intracellular phage. The organisms in samples taken during one-step growth experiments were disrupted by one of two methods. The first involved incubation for 90 min. at 37° in a lysing mixture containing 2·5 M-glycine and 10^{-2}M-KCN (Kay, 1952). Preliminary experiments showed that the turbidity of either uninfected or phage anti-R-infected *Salmonella typhi* R 4 was decreased more than 85 % after 2–3 hr. incubation in the lysing mixture at 37°, and that the decrease in titre of a phage suspension maintained for 3 hr. in the mixture at 37° was no greater than that of a control suspension in buffer incubated similarly.

The second method used ultrasonic vibration (Anderson & Doermann, 1952). Potassium cyanide to a final concentration of 10^{-2}M was added to prevent further phage growth and the samples were then treated for 5 min. in a Mullard Ultrasonic Generator, Type E 7590B (Mullard Ltd., London). This period of ultrasonic vibration decreased the viability of a dilute suspension of *Salmonella typhi* R 4 to 2 % of the initial count, but periods of ultrasonic treatment as long as 20 min. of dilute phage suspensions did not decrease their titre.

Bacterial counts. The method of Miles & Misra (1938) was used. For routine purposes the number of organisms in a culture was assessed turbidimetrically by using a curve relating counts to the opacities of cultures in medium B in a Hilger 'Spekker' Absorptiometer (Hilger & Watts Ltd., London, N.W. 1).

Spectrophotometry. A Unicam SP 500 Spectrophotometer (Unicam Instruments Ltd., Cambridge) was used with cuvettes of 1 cm. light path.

RESULTS

The stability of phage anti-R

Phage lysates which had been diluted into $3{\cdot}3 \times 10^{-2}$M-phosphate buffer (pH 7·6) and maintained at 37° in unshaken test tubes lost about 20 % of their initial titre in 90 min. This inactivation was not appreciably diminished by the addition to the

diluent of $MgSO_4$ (4×10^{-3} M), $CaCl_2$ (5×10^{-4} M) or Na citrate (10^{-2} M). Some of the diluted phage suspensions showed a delayed inactivation which was best seen at an elevated temperature (50°). This delay and the inability to obtain phage titres greater than about 10^{10} pfu/ml. in multistep lysates, in spite of average burst size of 500 in one-step growth experiments in medium A, suggested that some phage may be masked either by aggregation or by reversible inactivation by material present in the lysates. Even after several cycles of alternate low-speed and high-speed centrifugation the concentrated phage suspension still produced a precipitate when incubated with bacterial antiserum, and flagellar material derived from the host bacterium could be seen in electron micrographs of phage prepared in this way. Whenever possible, concentrated phage preparations treated with bacterial antiserum were used for the subsequent experiments, though there was evidence from the occasional variation in the titre of certain stocks that the cause of the masking was not entirely eliminated by antiserum treatment.

The effect of divalent metal ions on the attachment of phage anti-R to host bacteria

There was no adsorption of phage anti-R to washed suspensions of *Salmonella typhi* R 4 in $3{\cdot}3 \times 10^{-2}$ M-phosphate buffer (pH 7·6) at 37° unless Mg^{++} or other divalent metal ions were present. In the presence of excess bacteria about 90 % of the initial phage input was adsorbed at an exponential rate which was proportional to the concentration of bacteria over the range 2×10^8 to 8×10^8 organisms/ml. The variation of the rate of adsorption of phage with Mg^{++} and other divalent metal ions is shown in Fig. 1. The rates are calculated on the assumption that the adsorption of phage anti-R behaves as a first order reaction (Ellis & Delbrück, 1939).

The effect of divalent metal ions on the stages following attachment

The stage immediately after attachment. The study of the function of divalent metals in the penetration of phage anti-R nucleic acid and in other early stages of the growth cycle was made difficult by the requirement of the metals for adsorption. Puck, Garen & Cline (1951) found with phage T1 that there were two stages in the adsorption to *Escherichia coli* and that the second step was inhibited at low temperatures. With the usual method of measuring phage anti-R adsorption there appeared to be very little attachment at 0° even in the presence of concentrations of Mg^{++} that were effective at 37°. When, however, a comparison is made of the free phage in a host-phage mixture at 0° both before and after dilution it is evident that although attachment occurred at 0° in the presence of Mg^{++}, it was reversible, unlike that at 37° (Table 1).

Advantage was taken of the temperature sensitivity of permanent adsorption to determine whether it, like reversible attachment, is cation dependent. If it is, dilution into medium at 37° of dissociable host-phage complexes that had been formed at 0° should result in the elution of phage unless the medium contains added divalent metal ions. The results of this experiment (Table 2) show that transfer to medium not containing added Mg^{++} but otherwise favourable for phage growth caused bound phage to be released.

Later stages of the phage-growth cycle. If divalent cations function only during the initial stages of the phage-growth cycle their removal once these have been

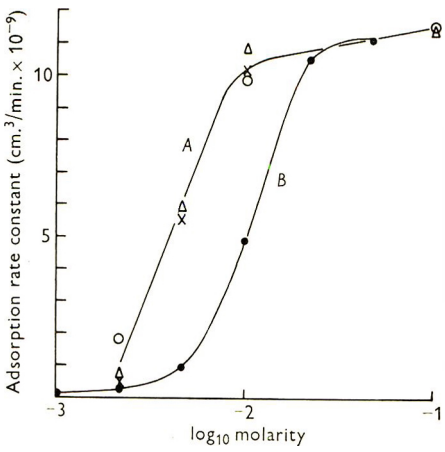

Fig. 1

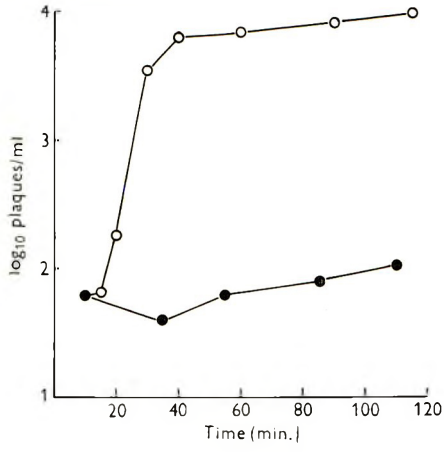

Fig. 2

Fig. 1. Effect of divalent metal ion concentration on the rate of adsorption of phage anti-R to washed suspensions of *Salmonella typhi* R 4. Curve *A*: rates of adsorption in $3{\cdot}3 \times 10^{-4}$ M-phosphate buffer (pH 7·6) containing, $MgSO_4$, ○; $CaCl_2$, △; $SrCl_2$, ×. Curve *B*: rates of adsorption in $3{\cdot}3 \times 10^{-2}$ M-phosphate buffer (pH 7·6) containing $MgSO_4$, ●. Temperature 37°.

Fig. 2. Effect of Mg^{++} on the growth of phage anti-R. *Salmonella typhi* R 4 was infected with phage anti-R in the presence of 4×10^{-3} M-$MgSO_4$. The culture was diluted $1/10^6$ in medium B after 10 min., then finally diluted 10-fold into medium B, without (●—●), and with (○—○) added $MgSO_4$ (10^{-3} M). Temperature 37°.

completed should not affect the subsequent development of the phage. In one-step growth experiments the removal of Mg^{++} after as long as 10 min. at 37° had a marked inhibitory effect on the burst size. Compared with the average burst size of about 100 in medium B containing 10^{-3} M-$MgSO_4$ there was either a decrease or at most a very slight increase in plaque-forming centres when phage anti-R infected organisms were diluted into medium B with no added Mg^{++} (Fig. 2). Under the latter conditions there was in fact an increase in free phage, probably due to the trace amounts of divalent metals in medium B. The transfer of phage-infected bacteria to medium B supplemented with Na citrate (10^{-2} M) caused a decline in the number of plaque forming centres and no increase in free phage.

Table 1. *Reversibility of adsorption of phage anti-R at* 0°

Phage anti-R was added to a suspension of *Salmonella typhi* R 4 in $3{\cdot}3 \times 10^{-2}$ M-phosphate buffer (pH 7·6) containing 6×10^{-3} M-$MgSO_4$ and distributed into two tubes; (A) for incubation at 37° (B) for incubation at 0°. After 3 min. and 15 min., respectively, two samples were removed from both tubes. One sample was centrifuged immediately and the supernatant fluid diluted in buffer before assay; the other was diluted into ice-cold buffer and assayed after centrifugation at the times indicated.

Adsorption tube	Centrifuged before dilution	Centrifuged after dilution		
		0·5 min.	5 min.	10 min.
	unadsorbed phage (%)			
A (37°)	28	39	36	33
B (0°)	46	89	94	99

Table 2. *Effect of Mg^{++} on the second stage of adsorption*

Phage-anti-R was added to a culture of *Salmonella typhi* R4 in medium B containing 4×10^{-3}M-$MgSO_4$ at 0°. After 15 min. there was 41 % free phage in a sample centrifuged prior to dilution. Two further samples were then diluted 1/200 in medium B with and without added 4×10^{-3}M-$MgSO_4$ at 37°. The adsorption was determined at intervals by assay before and after centrifugation.

Time after 1/200 dilution (min.)	No added divalent metal	With 4×10^{-3}M-$MgSO_4$
	Unadsorbed phage (%)	
0·25	81	—
0·75	—	46
1·5	90	—
3·0	100	—
3·5	—	42
7·0	—	48
8·5	100	—

The point of action of divalent metals in the post-adsorptive stage

A culture of *Salmonella typhi* R4 in medium B was infected with phage anti-R and after 10 min. was diluted into a ⊥-tube containing medium B supplemented with 10^{-3}M-$MgSO_4$. This culture was aerated at 37° and at various times samples were diluted 10-fold into tubes of medium B containing 10^{-3}M-$MgSO_4$ and into tubes of medium B containing 10^{-2}M-Na citrate. The phage content of the latter series of tubes was determined immediately and both sets of tubes were assayed after 90 min. incubation at 37°. The results (Table 3) show that however late in the growth cycle the infected organisms were transferred to medium deficient in ionized magnesium there was no further increase in phage titre. These results indicated that magnesium ions functioned at some step at the end of the phage development cycle. Further evidence for this came from experiments in which infected bacteria placed in medium B without added Mg^{++} were transferred at various times beyond the normal

Table 3. *Effect of dilution into medium containing Na citrate on the growth of phage anti-R*

A phage-infected culture of *Salmonella typhi* R4 was diluted into medium B containing 10^{-3}M-$MgSO_4$ and incubated at 37°. Samples were removed at intervals and diluted 10-fold into tubes of medium B containing 10^{-2}M-Na citrate (Series 1) and into tubes of medium B containing 10^{-3}M-$MgSO_4$ (Series 2). Series 1 was assayed immediately and both series were assayed after incubation for 90 min. at 37°.

Time after adding phage (min.)	Initial	After 90 min. incubation at 37°	
	Series 1	Series 1	Series 2
	Plaque count		
15	311	71	20,200
18	398	133	20,400
21	1,310	1,130	22,000
26	7,040	7,350	23,700
30	13,700	13,700	20,700
35	18,800	15,600	21,500

end of the latent period (20 min.) to medium B supplemented with divalent metal ions. Figure 3 shows that the late addition of Mg^{++} produces a rapid increase in phage titre. The magnitude of the burst became less the longer the addition of Mg^{++} was delayed (Fig. 4).

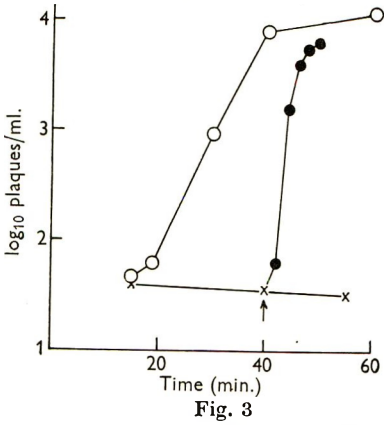

Fig. 3

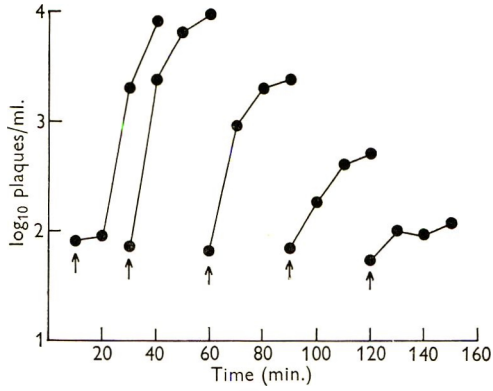

Fig. 4

Fig. 3. Effect of delayed addition of Mg^{++} on the growth of phage anti-R. A culture of *Salmonella typhi* R4 in medium B containing 4×10^{-3}M-$MgSO_4$ was infected with phage anti-R and then diluted into medium B, with $MgSO_4$, 10^{-3}M (○—○); and without added Mg^{++} (×—×). At 40 min. a sample from the latter growth tube was transferred to another tube containing added $MgSO_4$ (final concentration 10^{-3}M) (●—●). Temperature 37°.

Fig. 4. Effect of delayed addition of Mg^{++} on phage anti-R growth. A culture of *Salmonella typhi* R4 in medium B containing 4×10^{-3}M-$MgSO_4$ was infected with phage anti-R and diluted into five ⊥-tubes containing medium B only. At the times indicated by the arrows $MgSO_4$ (final concentration 10^{-3}M) was added to each of the tubes. Temperature 37°.

The nature of the late metal-dependent step in phage anti-R growth

The only well-recognized stage at the end of the phage-growth cycle is the lytic reaction whereby fully mature phage particles inside the infected host are released into the medium. To ascertain whether or not divalent metals were cofactors for lysis a concentrated culture of *Salmonella typhi* R4 was infected with a 10-fold excess of phage anti-R in the presence of 10^{-3}M-$MgSO_4$ at 37°. After 10 min. the culture was diluted with cold medium B, centrifuged, washed with cold medium and finally resuspended in the initial volume of medium B without added Mg^{++}. Nine ml. of the suspension were added to two ⊥-tubes, one of which contained 1 ml. 4×10^{-2}M-$MgSO_4$ and the other 1 ml. water. The tubes were shaken at 37° and opacity readings were taken at intervals. Phage assays were made at the beginning and at the end of the experiment. The results (Fig. 5) showed that the decrease in turbidity of the cultures was independent of Mg^{++}, though there was no rise in phage titre unless Mg^{++} was present. Similar experiments in which the degree of lysis was determined by the amount of ultraviolet-absorbing material released into the supernatant fluid of infected cultures with or without added Mg^{++} again showed that there was no difference in the degree of cell disruption.

Intracellular growth of phage anti-R

Attempts to show intracellular phage development by glycine+cyanide lysis method (Kay, 1952) were unsuccessful in spite of the good macroscopic lysis pro-

duced when either suspensions of uninfected organisms or those infected with phage anti-R were added to the lysing medium of 2·5 M-glycine + 10^{-2} M-KCN. The failure to detect intracellular phage by this method may be due to chelation of divalent metals by the high concentration of glycine (Albert, 1950). Thus intracellular phage could be shown if samples were added to a mixture of 10^{-2} M-KCN + 10^{-3} M-$MgSO_4$ but not when added to a mixture of 10^{-2} M-KCN + 10^{-2} M-Na citrate. By using ultrasonic vibration to disrupt phage-infected bacteria which had been diluted into medium B with added Mg^{++} it was possible to show that intracellular phage was formed before the bacteria lysed. In similar experiments in which infected organisms were diluted into medium B without added Mg^{++} there was little increase in the phage content of sonicated or control samples until divalent metal was added (Fig. 6).

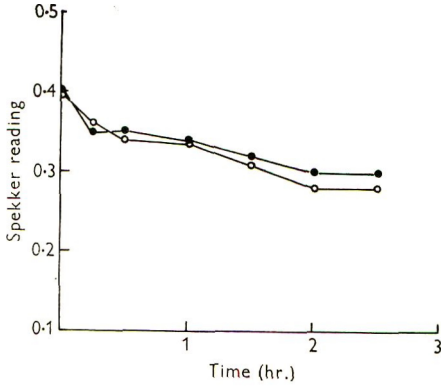

Fig. 5

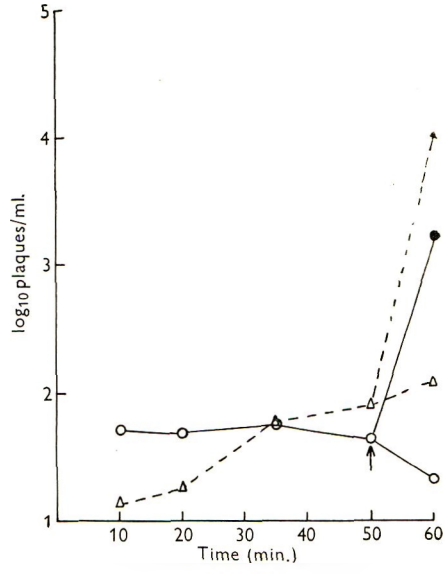

Fig. 6

Fig. 5. Effect of Mg^{++} on the lysis of *Salmonella typhi* R4 by phage anti-R. A culture of *S. typhi* R4 was infected with phage anti-R and after washing was resuspended in medium B, without added Mg^{++}, (○—○); and with added $MgSO_4$ (final concentration 4×10^{-3} M), (●—●). Both cultures were aerated at 37°. Initial phage titre = 9×10^6/ml. Final phage titre; without added Mg^{++} = 10^6/ml.; with Mg^{++} (4×10^{-3} M) = 2×10^8/ml.

Fig. 6. Effect of Mg^{++} on the intracellular development of phage anti-R. A culture of *Salmonella typhi* R4 in medium B containing 4×10^{-3} M-$MgSO_4$ was infected with phage anti-R and then diluted into medium B without added Mg^{++}. Samples were plated at intervals, before ultrasonic treatment (○—○) and after 5 min. ultrasonic treatment (△---△). At 50 min. a portion of the culture was transferred to a tube containing $MgSO_4$ (final concentration 10^{-3} M) and at 60 min. this was plated before ultrasonic treatment (-●-) and after 5 min. ultrasonic treatment (-▲-). Temperature 37°.

*The relation of phage anti-R to phages S*13 *and ΦX* 174

The small size of phage anti-R particles suggested that the phage might be related to other small phages e.g. S13 and ΦX174 and like them contain a characteristic type of nucleic acid (Sinsheimer, 1959). Concentrated preparations of phage anti-R were treated with host bacterial antiserum and then taken through a further

cycle of low-speed and high-speed centrifugation. The final suspension of the phage was made in 10^{-2}M-phosphate buffer (pH 7·0) and contained $4{\cdot}5 \times 10^{11}$ pfu/ml. The phage was diluted 10-fold into 10^{-2}M-phosphate buffer containing 1·6 % (w/v) formaldehyde and the absorption spectrum was measured immediately and after 1, 6 and 23 hr. incubation at 37°. The results (Fig. 7) show that treatment with formaldehyde caused an increase in the extinction in the 260 mμ region, with a shift in the absorption maximum to a greater wavelength. This reaction is one of the characteristics of single-stranded DNA (Sinsheimer, 1959). Other investigations (D. Kay, to be published) have shown that the nucleic acid isolated from phage anti-R has a composition which is indicative of single stranded DNA and that the intact phage differs immunologically from phage S 13.

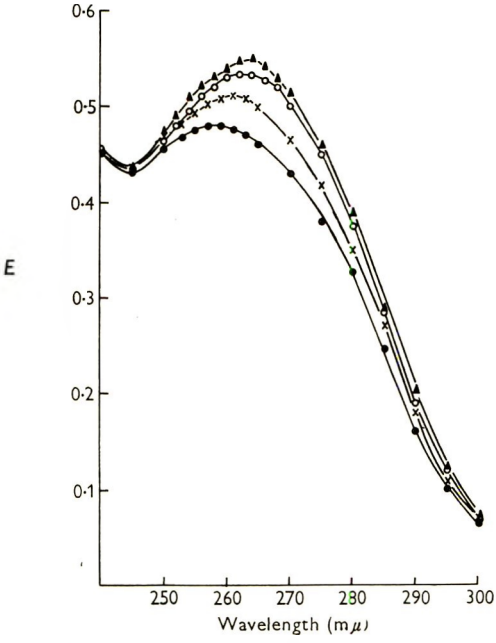

Fig. 7. Effect of formaldehyde on the absorption spectrum of phage anti-R. A suspension of phage anti-R treated with host bacterial antiserum was resuspended in 10^{-2}M-phosphate buffer (pH 7·0) and diluted into the same buffer containing 1·6 % (w/v) formaldehyde. Absorption spectrum measured at zero time (●—●); 1 hr. (×—×); 6 hr. (○—○) and 23 hr. (▲—▲). Temperature 37°.

DISCUSSION

The divalent metal requirement for the growth of phage anti-R, a small phage which may contain single-stranded DNA, can be satisfied by magnesium, calcium or strontium ions, and while the effect of Mg^{++} alone has been investigated in detail the other cations must be presumed to act in a manner that is at least qualitatively the same. The promotion of phage anti-R adsorption by Mg^{++} is similar to the action of divalent cations on many other host-phage systems. Puck, Garen & Cline (1951) measured the attachment of phage T1 to *Escherichia coli* B in the presence of different concentrations of alkaline earth metal ions and found that the optimum rate of adsorption was obtained at 5×10^{-4}M irrespective of the metal used. Mag-

nesium, calcium and strontium ions function equally well as cofactors for the adsorption of phage anti-R to suspensions of *Salmonella typhi* R 4 in buffer, and the rate of adsorption approaches a maximum at the highest concentration (10^{-1} M) investigated. The fastest rate of adsorption of phage anti-R is considerably greater than the maximum for phage T 1 (Puck *et al.* 1951; Garen, 1954) but it is not inconsistent with the small size of the phage anti-R particle.

Adsorption at 0° showed that infection by phage anti-R, like that by phage T 1, involves two early reactions, the second of which is temperature-sensitive and leads to the permanent attachment of phage to host bacterium. The need for divalent metal ions for this second step prevented examination of the metal dependence of stages which follow rapidly upon attachment.

Decreasing the concentration of Mg^{++} in the medium for a period as long as 10 min. after adding phage anti-R greatly decreased the burst size and there was evidence for some action of the metal at a late stage of the growth cycle. First, removal of divalent cations by chelation during the rising phase of the burst prevented further increase in the phage titre. Such a result would be expected if Mg^{++} or the other ions acted as cofactors for lysis. This could not be shown by direct measurement of changes in turbidity or by the estimation of ultraviolet-absorbing material released by infected organisms with or without added divalent cations; but it should be pointed out that the experimental conditions were somewhat different from those used previously and did not produce marked clearing even when divalent metals were present. Secondly, the addition of Mg^{++} to infected bacteria in low-metal medium at a time well beyond the end of the normal latent period led to a rapid appearance of infective particles. The speed with which the plaque count increased seems to exclude an action on the multiplication of the phage and the result might also be explained as an action of Mg^{++} on the lytic process. However, the inability to detect appreciable amounts of intracellular phage in organisms in low-metal medium suggests that the metals do function in some process which either leads to phage maturation or is necessary for the maintenance of intracellular phage in an infective state. Rountree (1955) described a calcium-dependent function occurring late during the growth cycle of certain staphylococcal phages. Although the nature of the function was not determined, it appeared in that case to be connected with intracellular stabilization of the phage rather than lysis, since chelation of the metals did not entirely prevent the subsequent liberation of some phage.

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Electron Microscope Observations on the Surface Structures of *Streptomyces violaceoruber*

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SUMMARY

The surface structures of *Streptomyces violaceoruber* were studied by electron microscopy of intact organisms, carbon replicas and preparations of walls from disrupted organisms. A surface layer with a fibrillar structure was observed on the aerial hyphae and spores in all three types of preparation. The fate of this layer during spore formation and germination was studied; it is a loose covering which breaks when the spores separate and when the germ tubes emerge, and it is easily lost when the organisms are disrupted. No organized surface structures were observed on the underlying hyphal or spore walls.

INTRODUCTION

We have recently studied the fine structure of the actinomycete *Streptomyces violaceoruber* (*S. coelicolor*) in electron micrographs of thin sections (Glauert & Hopwood, 1959, 1960, 1961; Hopwood & Glauert, 1960). Sectioning reveals few details of the surface structure of a specimen; in a transverse section only a small fraction of the surface is seen, while oblique sections are often difficult to interpret. Observations on thin sections have therefore been supplemented with an investigation of intact organisms, carbon replicas, and fragments of walls in preparations of disrupted organisms. The results of this investigation are described here.

METHODS

Organism. Streptomyces violaceoruber (*S. coelicolor*) strain A 3 (2) (Hopwood, 1960).

Mounting specimens on electron microscope grids. Spores and fragments of aerial hyphae were mounted on Formvar-coated grids by gently touching the Formvar film on the surface of a 4- to 6-day colony growing on complete agar medium (Hopwood, 1960). Preparations of germinating spores and hyphae of the substrate mycelium were obtained by the method developed for mycobacteria by Brieger, Cosslett & Glauert (1954). A thin layer of cottonwool soaked in liquid medium was placed in a Petri dish and covered with a disk of filter paper. Grids which had been inoculated with spores as described above were placed on the filter paper with the Formvar film uppermost, and the dish incubated at 30°. After incubation the grids were removed, floated on distilled water to remove traces of growth medium, and

placed on clean filter paper to dry. Care was taken not to allow any medium or water to get on to the upper surfaces of the grids and so contaminate them.

Organisms that were dried in air were found to be seriously distorted (Pl. 1, fig. 3; Pl. 3, fig. 12) because they collapsed on to the support film; therefore the preparations were freeze-dried. A simple freeze-drying apparatus designed by Dr M. J. P. Canny was used. Grids carrying the specimens were placed one at a time on a copper disk of 1 in. diam. (a halfpenny) which had been previously cooled to about $-195°$ by immersion in liquid nitrogen. After a few seconds the grids were rapidly transferred to a Quickfit test tube of internal diameter 34 mm., which was kept at a temperature of about $-80°$ by immersion in a mixture of acetone and solid carbon dioxide in a vacuum flask. When the grids had all been transferred, the test tube was connected by a vacuum joint to a wide L-shaped tube. The horizontal limb of the tube contained a small boat filled with dry phosphorus pentoxide and its open end was closed with a Quickfit stopper of 34 mm. diameter. A side arm from the drying tube was connected to an Edwards two-stage rotary pump, Model 2S20B, which decreased the pressure in the freeze-drying apparatus to about 0·05 mm. Hg. The specimens were dried for about 16 hr.

Some preparations of intact organisms were shadowed with gold-palladium (60:40) before examination in the electron microscope.

Carbon replicas. Replicas were made by the method described by Bradley & Williams (1957) for the examination of the spores of bacilli. Freeze-dried specimens were transferred directly from the freeze-drying apparatus to the vacuum chamber for carbon evaporation. The carbon replicas were not metal-shadowed because it was found that granulation of the shadowing metal obscured the finer details of the surface structures.

Negative staining. A suspension of spores and small fragments of aerial mycelium was prepared as described by Glauert & Hopwood (1960). The organisms were disrupted by shaking the suspension in a Mickle tissue disintegrator (Mickle, 1948) with an equal volume of grade 12 ballotini for 5 min. Unbroken organisms were removed by centrifugation at 2000 rev./min. for 5 min. and the walls were collected by centrifugation at 10,000 rev./min. for 20 min. The pellet was thoroughly dispersed in a small volume of water, and the resulting suspension was mixed with an equal volume of a 2% (w/v) solution of phosphotungstic acid adjusted to pH 7·4 with N-KOH (Brenner & Horne, 1959). Minute drops of the mixture were placed with a fine pipette on electron microscope grids coated with collodion films stabilized with a layer of carbon and allowed to dry.

Electron microscopy. Observations were made with a Siemens Elmiskop I operating at 60 or 80 kV with a 50 μ objective aperture, and photographs were taken at instrumental magnifications of $\times$ 5000 to 20,000 on Ilford Special Contrasty Lantern Plates.

RESULTS

Intact organisms

Information obtained from the study of intact hyphae and spores of *Streptomyces violaceoruber* in the electron microscope adds little to the knowledge gained by phase-contrast microscopy (Hopwood, 1960). The resolution of structures in the electron micrographs is low because the organisms are too thick to be penetrated

appreciably by the electron beam. The spores either appear uniformly opaque (Pl. 1, figs. 1, 2), or else, in air-dried preparations (Pl. 1, fig. 3), show light areas where the protoplasm has shrunk irregularly during drying; no significant internal structure is visible. The spines that are present on the spores of some other strains of Streptomyces (Flaig, Beutelspacher, Küster & Segler-Holzweissig, 1952; Baldacci & Grein, 1955) are not found in *S. violaceoruber*. Some spores have an irregular outline (Pl. 1, fig. 1), while others are smooth (Pl. 1, fig. 2); the irregular contour appears to be caused by a loose superficial layer which readily comes away, to leave the spores smooth-surfaced. Occasionally some details of the structure of this layer can be seen when it is partially separated from the spores (Pl. 1, fig. 4). In shadowed preparations it appears to consist of fibrils which are straight or slightly curved, often paired, and intersect at various angles to form a 'basket-work' pattern.

Carbon replicas

The walls of the substrate hyphae (Pl. 1, fig. 5) show no organized structure in carbon replicas; the surfaces have merely the same fine stippling, with light and dark circles, that is visible on the background. The aerial hyphae have a quite different appearance and are covered with a pattern of intersecting fibrils (Pl. 1, fig. 6), which are 5–10 mμ in diameter and 100 mμ or more long. Although often in pairs, the fibrils also occur singly, and sometimes a number of them radiate from one point; some appear beaded. Thus the aerial hyphae are covered with a fibrous layer which in thin sections (Glauert & Hopwood, 1961) can be seen to form a loose covering outside the wall of the hypha proper. The fibrous layer remains intact while the spores are forming (Pl. 2, figs. 7, 8), but as they begin to separate from one another, the outer layer of the wall of the parent hypha ruptures between adjacent spores (Glauert & Hopwood, 1961) and with it the fibrous layer, so that the underlying and apparently structureless spore wall is revealed (Pl. 2, fig. 9, SW; Pl. 3, fig. 11, SW). Each spore in a chain is surrounded by a sac consisting of the outer component of the parent hyphal wall, overlaid by the fibrous layer. Sometimes this sac is loose-fitting (Pl. 2, fig. 8) and sometimes it closely invests the mature spore (Pl. 2, fig. 10). As the spore germinates, the germ tubes, with apparently structureless walls, emerge through the sac (Pl. 3, fig. 13; Pl. 4, fig. 14).

Negative staining

Observations on fragments of cell walls in preparations of disrupted organisms examined by the negative staining technique of Brenner & Horne (1959) confirmed the results obtained from studies of carbon replicas. The surfaces of the aerial hyphae and the spores showed no defined structural pattern except in the regions where fragments of the fibrous layer were still present (Pl. 4, fig. 15). The fragility of the superficial fibrous layer is evident in these preparations; it is usually lost during the disruption of the organisms in the Mickle tissue disintegrator.

DISCUSSION

The negative staining technique of Brenner & Horne (1959) did not demonstrate fine structure within the walls of *Streptomyces violaceoruber*, although the technique is capable of very high resolution, and has revealed an ordered array of minute subunits in the walls of certain large cocci (Drs M. J. Thornley and R. W. Horne,

personal communication; Professor R. G. E. Murray, personal communication). Spherical subunits have been seen in electron micrographs of the walls of some bacteria (Houwink, 1953, 1956; Labaw & Mosley, 1954, Salton & Williams, 1954; Van Iterson, 1954) even without the use of the negative staining technique. Thus it seems that the surfaces of the walls of *S. violaceoruber* are smooth, and that the subunits seen in electron micrographs of thin sections (Glauert & Hopwood, 1961) do not give rise to irregularities on the surface. It remains to be seen whether they can be made visible in unsectioned preparations by controlled degradation of the wall.

In electron micrographs of intact organisms (Pl. 1, figs. 1, 2), the spores appear smooth or slightly irregular, and occasionally it can be seen that the irregularity is due to a detachable fibrous layer which covers the smooth spore wall. Vernon (1955) described the fine structure of a similar layer in a metal-shadowed preparation of an aerial hypha of an unnamed streptomycete, and stated that it consisted of narrow pointed plates lying side by side in groups at various angles, but he did not recognize the distinction between this fragile superficial layer and the structureless wall of the hypha underneath. The fibrous layer can be seen in electron micrographs of thin sections (Glauert & Hopwood, 1961) as a coat covering the double-layered wall of the aerial hyphae. During sporulation only the inner component of the wall gives rise to the wall of the spores; the outer component remains as a sac partially surrounding the mature spores, and is still covered by the fibrous layer.

The comparatively simple technique of carbon replicas (Bradley & Williams, 1957), particularly when combined with freeze-drying, greatly increases the amount of structure observable on the surfaces of bacteria. In electron micrographs of carbon replicas of *Streptomyces violaceoruber*, details of the structure of the fibrous coat and its behaviour during sporulation are clearly seen. The nature of the surfaces of streptomycete spores as seen in electron micrographs (that is whether they are smooth or bear projections of various forms) has recently been used as a character for the classification of the group (see Tresner, Davies & Backus, 1961, for references). Perhaps carbon replicas would enable differences to be detected amongst the many strains which have smooth spores, and might also reveal more clearly the structure of the spiny or hairy processes present in certain strains. It is probable that many of these processes are part of a superficial layer (Welch & Lechevalier, 1960) comparable to the fibrous coat of *S. violaceoruber*, rather than projections of the spore wall proper. The significance of the superficial layer, whether it bears processes or not, is unknown; possibly it is responsible for the difficulty with which streptomycete spores are wetted by water, a property that may enable them to be dispersed on air-water interfaces in the upper layers of the soil, rather than sink to lower regions. No structures comparable to the fibrils of the coat of *S. violaceoruber* have been reported on the surfaces of other bacteria; the 'paired fibrous structure' found in lysed mycobacteria by Takeya, Mori, Koike & Toda (1958) had dimensions very similar to those of the fibrils in *S. violaceoruber*, but this component was stated to be on the inside of the walls.

One of the authors (A.M.G.) is a Sir Halley Stewart Research Fellow.

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Note added in proof. In a recent paper J. Yamaguchi (*Rep. Res. Inst. Tub. & Leprosy*, **9**, 125, 1960) describes a similar fibrous layer which she considers to be on the outer surface of mycobacterial cell walls.

EXPLANATION OF PLATES

The scale marks represent 0·5 μ.

PLATE 1

Figs. 1 to 4. Electron micrographs of intact spores of *Streptomyces violaceoruber* shadowed with gold/palladium.

Fig. 1. Freeze-dried spores with rough outlines. × 36,000.

Fig. 2. Freeze-dried spores with smooth outlines. × 36,000.

Fig. 3. Air-dried spores which are distorted. × 30,000.

Fig. 4. The fibrous layer separated from the surface of a spore. × 36,000.

Figs. 5 and 6. Electron micrographs of carbon replicas of freeze-dried hyphae of *Streptomyces violaceoruber*.

Fig. 5. Part of a hypha of the substrate mycelium with a structureless surface. × 66,000.

Fig. 6. Part of a hypha of the aerial mycelium with an outer fibrous layer. Some of the fibrils appear beaded. × 66,000.

PLATE 2

Figs. 7 to 10. Electron micrographs of carbon replicas of freeze-dried spores of *Streptomyces violaceoruber*.

Fig. 7. The spores in a chain are covered with the fibrous layer. × 66,000.

Fig. 8. The spores in a chain are surrounded by a loose sac derived from the parent hyphal wall. The spores and sac are covered with the fibrous layer. × 40,000.

Fig. 9. The spores in a chain are beginning to separate from one another and the fibrous layer has broken between the spores, revealing the structureless spore wall (SW) underneath. × 30,000.

Fig. 10. An isolated mature spore is closely surrounded by the fibrous layer. × 47,000.

PLATE 3

Figs. 11 to 13. Electron micrographs of carbon replicas of *Streptomyces violaceoruber*.

Fig. 11. Two freeze-dried spores are separating from one another and the fibrous layer has broken between them revealing the structureless spore wall (SW) underneath. × 55,000.

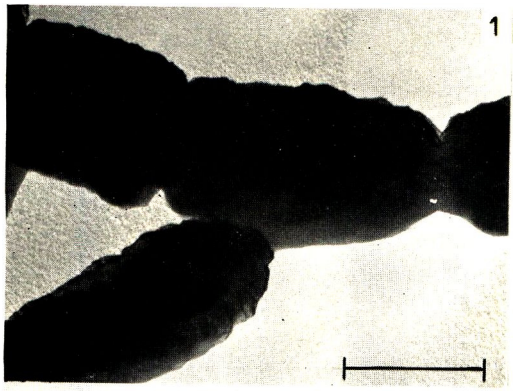
Fig. 12. An air-dried spore which is distorted. × 35,000.

Fig. 13. A spore has just started to germinate and the short germ tube has emerged through the fibrous layer; freeze-dried. × 41,000.

PLATE 4

Fig. 14. Electron micrograph of a carbon replica of *Streptomyces violaceoruber*. A group of spores has germinated and each has produced one or two germ tubes. The surfaces of the walls of the germ tubes show no structure, although the fibrous layer is still present on the spores. × 18,000.

Fig. 15. Electron micrograph of a fragment of the wall of an aerial hypha of *Streptomyces violaceoruber* stained with potassium phosphotungstate. The hyphal wall has no visible structure. Part of the fibrous layer still covers one branch of the hypha. × 30,000.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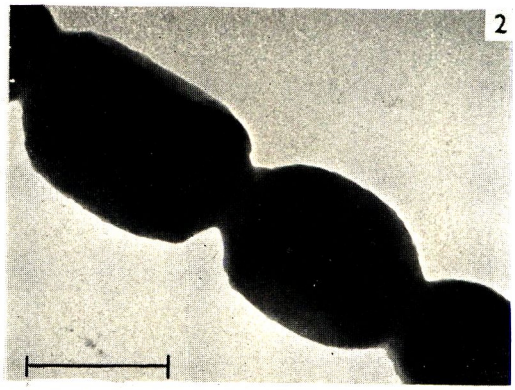
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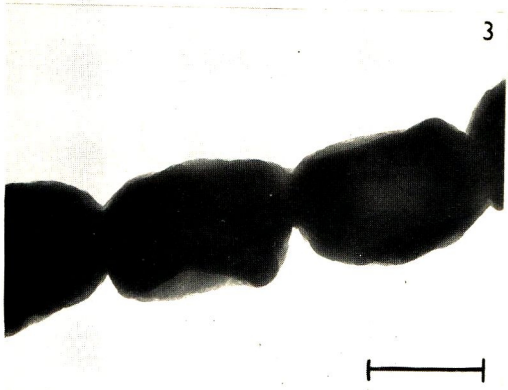
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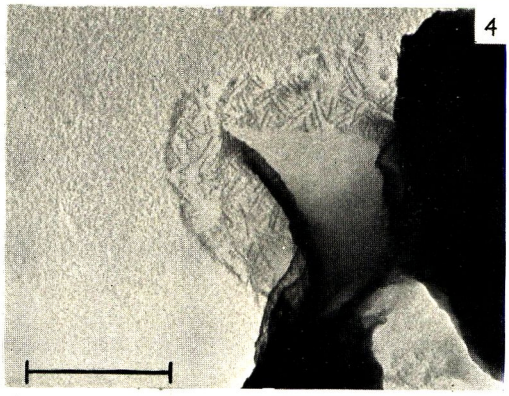
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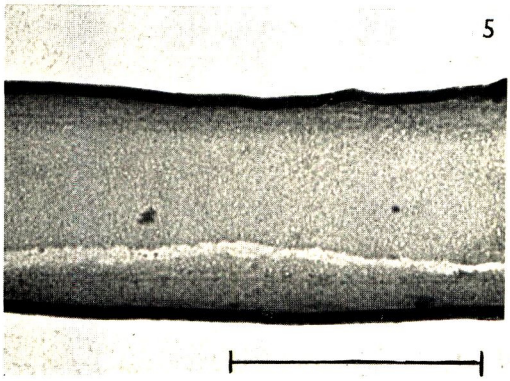
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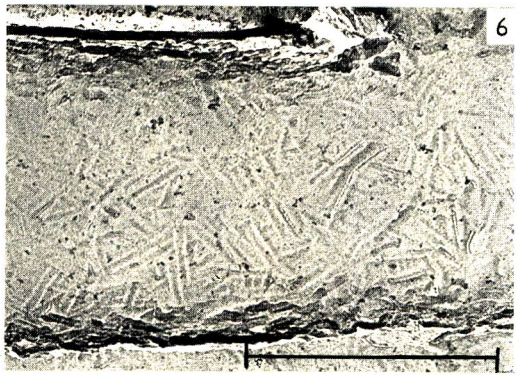
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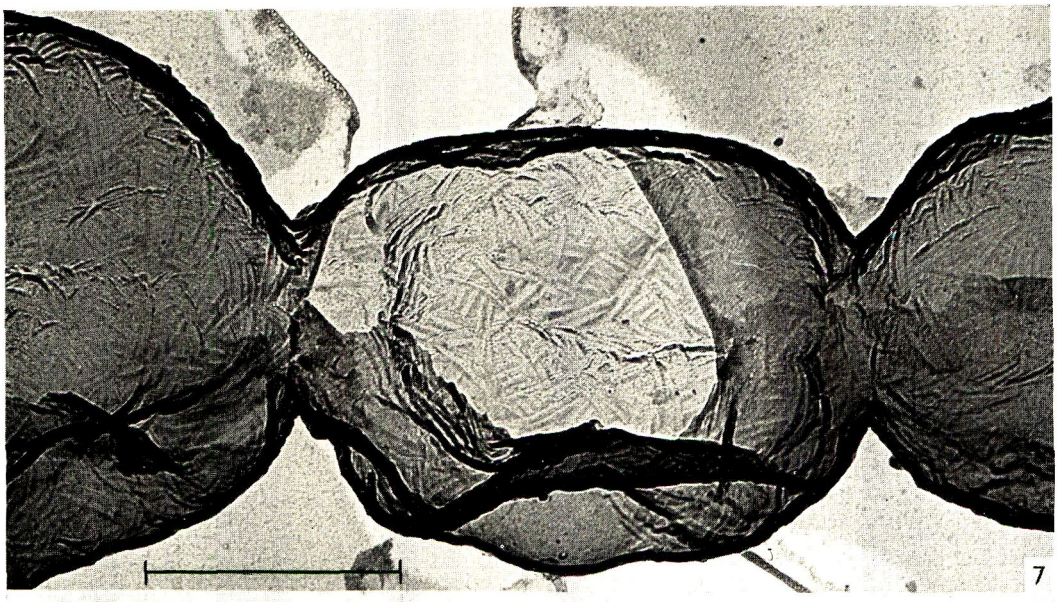
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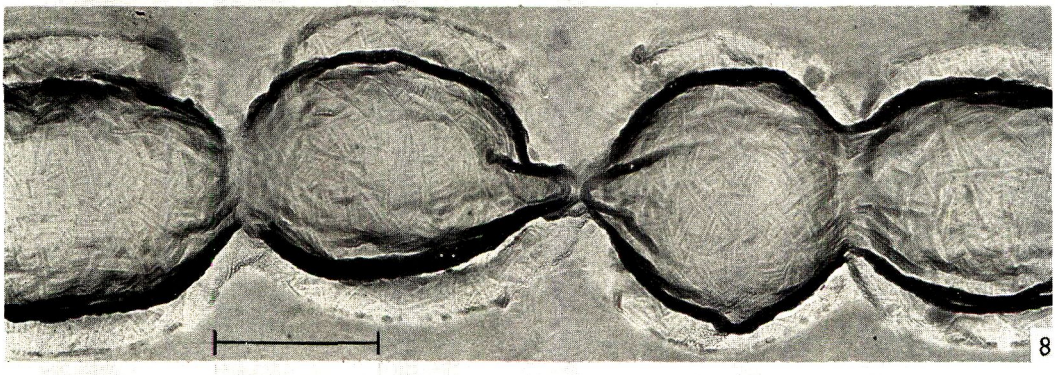
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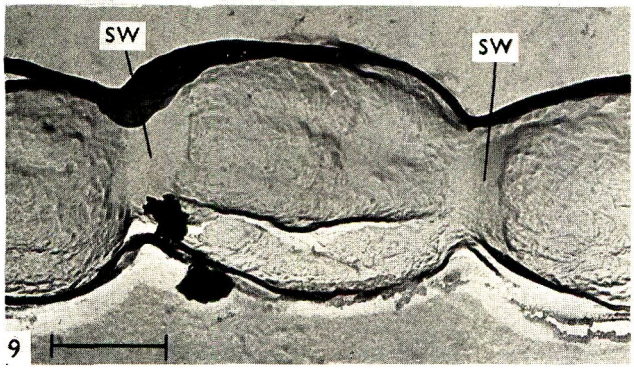
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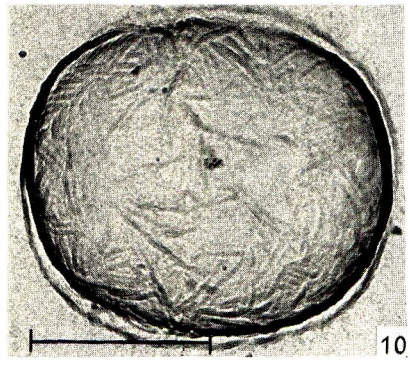
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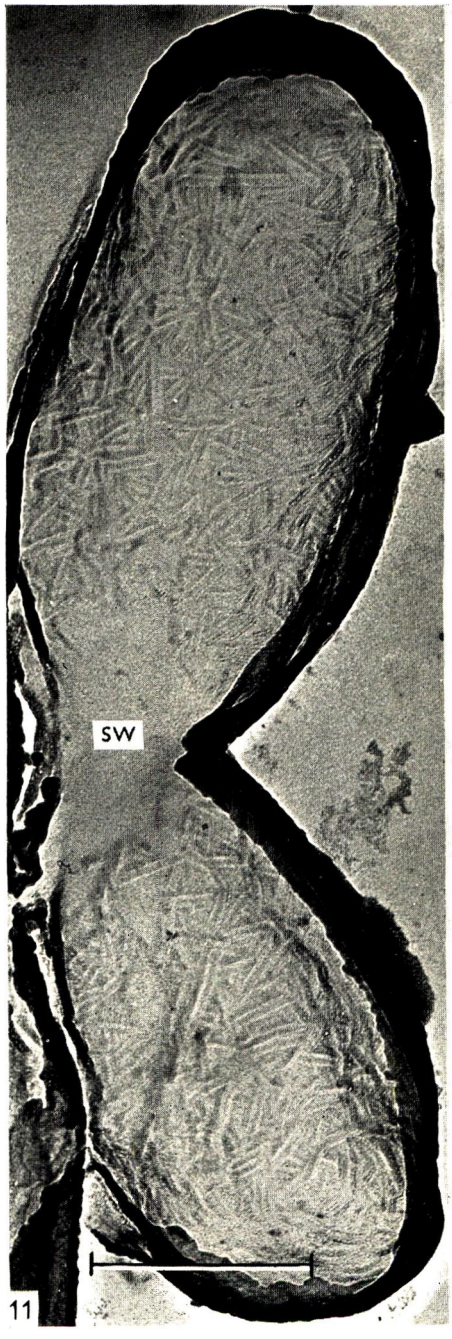
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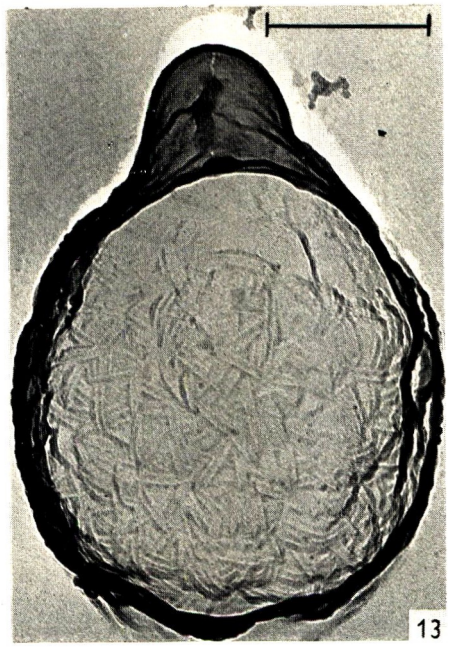
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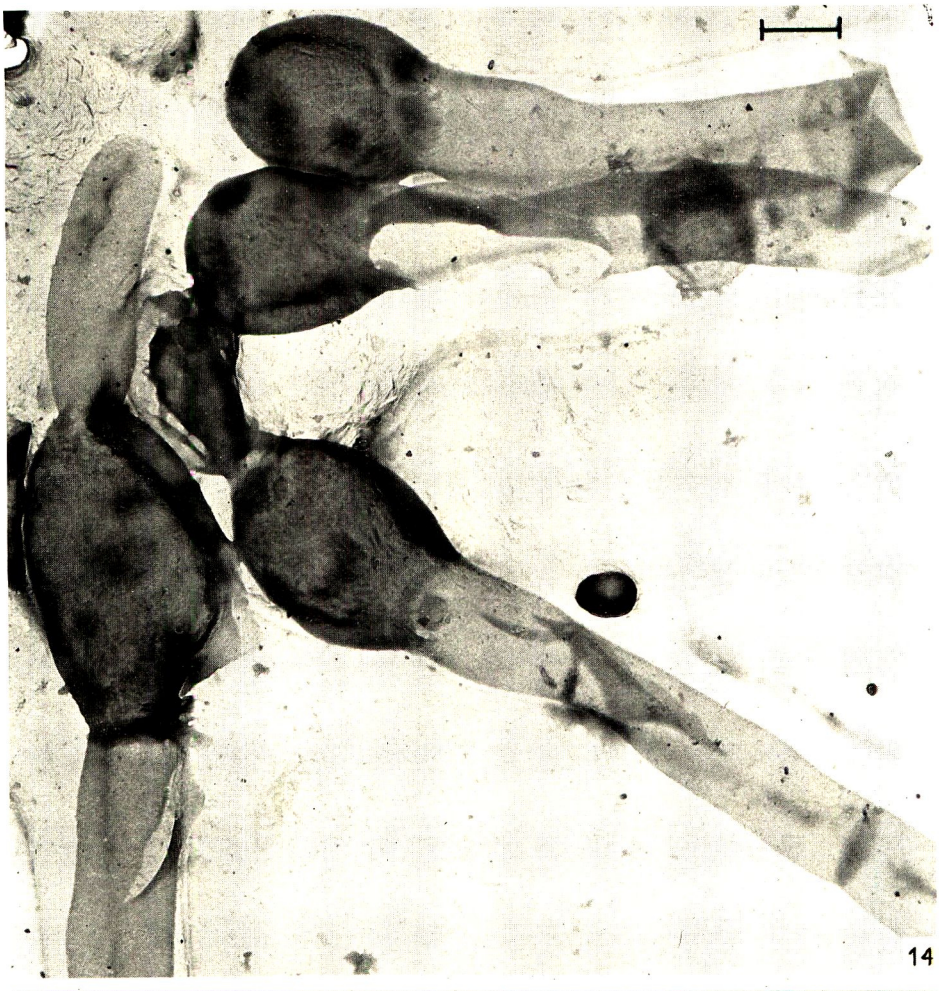
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A Study of Two Marine Agar-Decomposing, Facultatively Anaerobic Myxobacteria

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SUMMARY

Two facultatively anaerobic agar-decomposing myxobacteria were isolated from marine mud and described as *Cytophaga fermentans* Bachmann var. *agarovorans* var. nov. and *C. salmonicolor* var. *agarovorans* var. nov. A third organism which does not attack agar was isolated from the same source; it has been included in the present study for comparative purposes. The specific name *Cytophaga salmonicolor* has been given to this organism. All organisms are characterized by exhibiting flexing and gliding motility and by absence of microcysts and fruiting bodies. The organisms can be grown in a mineral glucose medium, although growth is accelerated and more abundant when a vitamin mixture is included in the medium. CO_2 is an absolute requirement for growth. Growth of *C. fermentans* var. *agarovorans* and *C. salmonicolor* var. *agarovorans* on aerobic plate cultures is markedly inhibited by 0·1 % glucose or galactose. Acetic, propionic and succinic acids are major end products of sugar fermentation of all three types. *C. salmonicolor* and *C. salmonicolor* var. *agarovorans* form in addition CO_2 and H_2, and small amounts of lactic and formic acids and ethanol.

INTRODUCTION

Agar, obtained by hot water extraction of marine Rhodophyta (Mori, 1953), contains two polysaccharides, agarose and agaropectin (Araki, 1956). The latter, a minor constituent, is a rather complex substance, comprising sulphuric and uronic acid components. The major constituent, agarose, seems to consist of alternately repeated units of 1:3-linked β-D-galactopyranose and 1:4-linked 3:6-anhydro-α-L-galactopyranose; it can be enzymically hydrolysed to neo-agarobiose, which is composed of D-galactose and 3:6-anhydro-L-galactose (Araki & Arai, 1956; Yaphe, 1957).

Although several kinds of agar-decomposing bacteria have been isolated from soil, such organisms represent only a very small fraction of the soil microflora; hence they are rarely encountered by the soil microbiologist. On the other hand, agar-digesting bacteria are common in marine environments. Undoubtedly this is due to the wide distribution and abundance of agar-containing seaweeds. Humm (1946), studying the marine agar-decomposing bacteria of the South Atlantic coast of the U.S.A., reported that the intertidal zone of the beaches contains 2–20 million agar-digesting bacteria per gram; as determined by plate counts, they represent 2–4 % of the total number of aerobic bacteria in this zone.

Among the 20 agar-decomposing bacteria Humm (1946) describes is *Cytophaga*

sensitiva, isolated directly from decaying marine algae. Stanier (1941) had previously discovered two other agar-decomposing cytophagas, *C. krzemieniewskae* and *C. diffluens*; these had been found on seawater count plates. These three cytophagas are strict aerobes. Bachmann (1955) was the first to describe a facultatively anaerobic cytophaga, *C. fermentans*. It produces craters and gelase fields on seawater agar plates with 1 % (w/v) yeast extract, but does not grow in media containing agar as sole energy source, even if they are adequate for growth when supplemented with a fermentable sugar. And, because galactose is also unsuitable as a carbon source for this bacterium, Bachmann (1955) concluded that it cannot be considered as a genuine agar-decomposer in the sense of an organism that can grow at the expense of agar. Anderson & Ordal (1961*a*) described another facultatively anaerobic cytophaga, *C. succinicans*, which was isolated from fresh water.

In 1947 and 1948, Drs S. R. Elsden and H. Larsen, respectively, isolated facultatively anaerobic cytophagas, which rapidly decompose agar and produce a pink pigment, from elective cultures of green sulphur bacteria at the Hopkins Marine Station of Stanford University, Pacific Grove, California, U.S.A. A similar organism was isolated in 1958 by Dr K. Eimhjellen in the same laboratory (Dr C. B. van Niel, personal communication). The facultatively anaerobic cytophagas described in the present paper were isolated in the same institution by Dr June Lascelles, who noticed their incidental occurrence in crude cultures of purple sulphur bacteria. She also showed, as had previously been done by Elsden, that such organisms can be more specifically enriched by using anaerobic cultures with media in which agar represents the sole carbon and energy source.

RESULTS

Isolation and maintenance

The medium used for the elective cultures contained, per 100 ml. H_2O:NaCl, 3 g.; KH_2PO_4, 0·1 g.; NH_4Cl, 0·1 g.; $MgCl_2.6H_2O$, 0·05 g.; $CaCl_2$, 0·004 g.; $NaHCO_3$, 0·5 g.; $Na_2S.9H_2O$, 0·01 g.; Fe-citrate, M/250, 0·5 ml.; trace element mixture, 0·2 ml.; powdered agar, 0·5 g.; Difco yeast extract, 0·03 g.; adjusted to pH 7·0. The trace element mixture used was that of Kohlmiller & Gest (1951).

Bottles, completely filled with this medium, were inoculated with marine mud from areas with decayed algae, stoppered, and incubated in the dark at 30°. After 3–5 days, anaerobic plate or shake cultures were prepared, using the above medium with 2 % (w/v) agar; the shake cultures were covered with paraffin to maintain anaerobiosis.

Colonies which on microscopic examination exhibited the flexing movements characteristic of myxobacteria were further purified by the shake culture method.

The isolates were maintained as paraffin-covered stab cultures in the elective culture medium with 1 % (w/v) agar, supplemented with 0·1 % (w/v) glucose for *Cytophaga salmonicolor* and as anaerobic liquid cultures in Hall tubes as modified by Barker (1936) in such a medium in which the agar was replaced by 0·1 % (w/v) glucose, galactose or starch. The cultures were incubated at 30° for 2–3 days, and thereafter stored at 4°; transfers were made monthly.

For aerobic plate and semi-anaerobic stab cultures a modified agar medium was used; it contained only 0·05 % $NaHCO_3$, no Na_2S, and 0·1 % each of yeast extract, corn steep liquor, and dehydrated nutrient broth.

Growth requirements

All three strains grew equally well anaerobically in the liquid medium when the yeast extract was replaced by a mixture of pyridoxin, riboflavin, thiamine, nicotinamide, pantothenic acid, folic acid and *p*-aminobenzoic acid, each at 0·4 mg./l.; biotin, 0·04 mg./l.; and cyanocobalamine, 0·002 mg./l. They may also grow in a vitamin-free glucose medium; but here growth is far less copious and sometimes erratic, particularly in the case of semi-anaerobic cultures of *Cytophaga fermentans* var. *agarovorans*. In contrast, aerobic cultures did not develop on chemically defined media. *C. fermentans* var. *agarovorans* and *C. salmonicolor* var. *agarovorans*, which both decomposed agar extensively under anaerobic conditions, grew at best sparsely

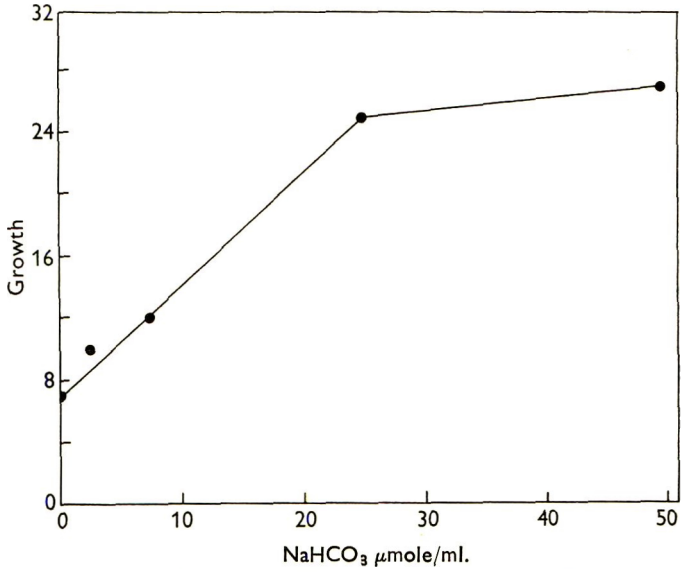

Fig. 1. Growth of *Cytophaga fermentans* var. *agarovorans* in mineral medium with 0·1 % (w/v) mannitol + 0·03 % (w/v) yeast extract at different $NaHCO_3$ concentrations. Turbidity measured after cultivation for 2 days by Eel colorimeter. Data provided by Dr June Lascelles.

on a 1 % (w/v) agar medium with 0·1 % (w/v) yeast extract. However, when this medium was supplemented with corn steep liquor and nutrient broth, these organisms grew well and formed deep craters in the agar solidified medium, showing that they digested agar aerobically also.

Bachmann (1955) observed a similar phenomenon with *Cytophaga fermentans*. Anaerobically this organism required thiamine as the only growth factor; but it did not grow aerobically unless 1 % (w/v) yeast extract was added. Then growth was accompanied by a softening of the agar and the appearance of shallow craters and gelase fields. In the absence of air this species did not grow with agar as sole C source.

Additional information about growth of our organisms on agar will be found below. A wide variety of carbohydrates is fermented by these organisms; they are listed under the respective species descriptions. The pH optimum for growth is about

7–7·5; fermentation ceased when the pH value decreased to 5·5, after which the cultures became non-viable within a week. All strains grew rapidly at temperatures between 28° and 37°. Growth under anaerobic conditions was very poor when $NaHCO_3$ was omitted from the medium; Fig. 1 shows the effect of different $NaHCO_3$ concentrations. It is evident that an optimal response was reached at a concentration of about 0·3 %. For these experiments a heavily buffered medium was used; hence it seems unlikely that the bicarbonate exerted its influence by regulating the pH value of the medium. Because our cytophagas carry out a propionic acid fermentation, which in other cases has been shown to involve carboxylation reactions, it seems far more probable that a fairly high CO_2 tension is required for metabolic activity. If CO_2 were also indispensable for aerobic growth, the need to supplement agar media with yeast extract, corn steep liquor and nutrient broth may be explained by postulating that on such enriched media an adequate CO_2 tension is produced locally. This opinion is supported by the fact that aerobic growth of the cytophagas may occur at the expense of the complex nitrogenous compounds alone; but the addition of a fermentable carbohydrate speeds up and enhances the development.

The fermentation of glucose by *Cytophaga succinicans* also appears to be CO_2-dependent (Anderson & Ordal, 1961*b*). From their experiments with this organism these authors concluded that 'CO_2 functions in the fermentation by providing, through condensation with phosphoenolpyruvate, compounds which can serve as acceptors for the available hydrogen generated in glucose degradation.'

Nitrates, ammonium salts, yeast extract, nutrient broth, and Casamino acids also can serve as nitrogen source for our cytophagas.

All strains grew well in media with 1–3 % (w/v) NaCl; very little growth occurred at lower NaCl concentrations.

Morphology

Our cytophagas could not be differentiated on the basis of their morphology. The organisms of all strains were slender with rounded ends (Pl. 1, fig. 5); they were extremely flexible and weakly refractile, and exhibited gliding movements, often alternately forwards and backwards. These movements were generally rather slow and regular, although sometimes sudden 'jumps' were observed. In wet mounts the cells were often seen to move against currents in the liquid. Organisms embedded in agar were generally much coiled. These organisms cannot be considered as involution forms since they straightened out and often appeared to be actively motile when forced into liquid channels surrounding the agar in wet mounts.

The organisms varied from 2 to 30 μ in length; occasionally very long elements, up to 50 μ long were seen. These long organisms, which also occurred in young cultures often showed flexing and gliding movements. The average length of organisms grown aerobically on agar plates was usually greater than that of organisms from stab or anaerobic liquid cultures. Short cells (about 3 μ long) did not show flexibility. They were never found to predominate, however, as they do in cultures of *Cytophaga johnsonae* (Stanier, 1947). Branched cells were never observed in our cultures. Fruiting bodies were not observed, nor were microcysts. Thus our strains are typical representatives of the genus *Cytophaga* as defined by Stanier (1942).

In cultures which had reached the stationary growth phase, spherical organisms

(Pl. 1, fig. 6) were always found. They varied in diameter from 1 to 3·5 μ, and small round bodies or rod-like structures could usually be seen within these organisms. Rod-shaped organisms with a spherical extrusion also frequently occurred in these cultures.

The fact that the spherical cells varied considerably in diameter, as well as the observation that they were formed when cultures were about to lose their viability, makes it seem unlikely that they were microcysts as formed in the genus *Sporocytophaga*. Bachmann (1955) reached similar conclusions about the round bodies she observed in aged cultures of *Cytophaga fermentans*. Coccoid involution forms were also found in cultures of *C. hutchinsonii* and *C. krzemieniewskae* (Stanier, 1942) and *C. succinicans* (Anderson & Ordal, 1961*a*). Spherical bodies reminiscent of those encountered in the *Cytophaga* cultures have also been found in *Treponema zuelzerae* (Veldkamp, 1960). Attempts to show the viability of these spherules failed. The transformation into spherical bodies in dying organisms might be a common characteristic of organisms which lack a rigid cell wall.

Growth on agar media

Anaerobic growth. Plate 1, fig. 1, shows large spherical colonies of *Cytophaga fermentans* var. *agarovorans* developing in an agar shake culture (1 % agar, 0·1 % yeast extract.) The organisms in these cream-coloured colonies were embedded in the softened agar. In the clear zone which surrounded the colonies the agar was softened by extracellular enzymic activity. *C. salmonicolor* var. *agarovorans* produced pink colonies of similar shape; the area occupied by these colonies consisted of completely liquefied agar. The shape of the colonies was not influenced by the inclusion of 0·1 % (w/v) galactose in the medium; but the colonies were then slightly more dense.

Cytophaga salmonicolor grew very poorly in the above medium unless cornsteep liquor and nutrient broth were added (0·1 %, w/v, each); spherical, pinkish colonies were then formed which never exceeded 1–2 mm. in diameter (Pl. 1, fig. 2). Softening of the agar was not observed. The addition of glucose or galactose to the medium yielded denser spherical or disk-shaped salmon-coloured colonies of the same size.

Stab cultures of *Cytophaga fermentans* var. *agarovorans* and *C. salmonicolor* var. *agarovorans* in a medium with 1 % agar and 0·1 % yeast extract are illustrated in Pl. 1, figs. 3, 4. Growth gradually spread outward from the inoculated region, as it did in the case of a colony developing in an agar shake culture (Pl. 1, fig. 1). This is attributable to the softening (*C. fermentans* var. *agarovorans*) or liquefaction (*C. salmonicolor* var. *agarovorans*) of the agar, which permits the cells to migrate. Thus the periphery of a growing colony or stab culture often appears as a diffuse area of low optical density, representing the zone of migration. In media with 2 % (w/v) agar the tendency to spread was considerably decreased. In stab cultures of *C. fermentans* (ATCC no. 12470) and *C. salmonicolor* the migration of organisms was limited to a distance of 1–2 mm. from the line of inoculation when 1–1·5 % (w/v) agar was used; decomposition of agar was not observed.

Aerobic growth

As stated above, all strains grew copiously under aerobic conditions on a mineral medium containing 1 % (w/v) agar and 0·1 % (w/v) each of yeast extract, corn steep

liquor or nutrient broth. *Cytophaga fermentans* var. *agarovorans* formed greyish to pale yellow colonies which attained a diameter of about 3 cm. on sparsely seeded plates. The colonies formed deep depressions in the agar and were surrounded by a zone in which the agar became translucent, softened, and generally slightly depressed (Pl. 2, fig. 8). On flooding the plate with a I + KI solution (a test devised by Gran, 1902) this zone remained unstained (Pl. 2, fig. 9). Colonies of *C. fermentans* var. *agarovorans* which developed on the above medium were largely subsurface, and generally had a sharp even edge. Occasionally swarming growth across the agar surface was observed, although this was generally limited to small sections of a colony. On media with 2 % (w/v) instead of 1 % agar, where the penetration of the organisms into the agar was impeded, growth occurred as flat spreading colonies, crater formation was less rapid and the depressions remained very shallow. The influence of agar concentration on the extent of penetration was reminiscent of Stanier's (1947) observations with *C. johnsonae*.

Cytophaga fermentans var. *agarovorans* tended to produce colonial variants. Two such variants were encountered: one formed relatively small bright yellow colonies of the sunken type; the other formed small raised pale yellow colonies, which hardly affected the structure of the agar. Swarming motility across an agar surface was never observed with these variant strains. The organisms in the aberrant colonies were morphologically similar to those of the parent strain. Similar variations in colony type were observed by Bachmann (1955) with *C. fermentans*. When grown on a medium containing 1 % (w/v) agar, *C. salmonicolor* var. *agarovorans* produced salmon-coloured colonies surrounded by wide gelase fields; the diameter of the colonies generally did not exceed 1 cm. The depressions formed on the agar surface were usually not so deep as those formed by *C. fermentans* var. *agarovorans*; although the organisms migrated into the agar, this tendency was less marked than with *C. fermentans* var. *agarovorans*. The agar did not become completely liquefied as in anaerobic cultures. *C. salmonicolor* var. *agarovorans* sometimes exhibited spreading growth on aerobic agar plates. Some colonies were apt to present a swarming edge, others a smooth sharply defined circumference. Either colony type yielded both forms on replating. A similar case of colony dimorphism was observed by Stanier (1942) with *Sporocytophaga myxococcoides*. Unlike *C. fermentans* var. *agarovorans*, *C. salmonicolor* var. *agarovorans* generally showed a decreased degree of swarming on a medium with 2 % (w/v) agar; crater formation was then slightly retarded.

Cytophaga salmonicolor, like *C. salmonicolor* var. *agarovorans*, formed salmon-coloured colonies with a maximum diameter of 1 cm. They were either flat and spreading (Pl. 1, fig. 7) or convex with a smooth edge. When grown on a 1 % (w/v) agar-medium, the organisms penetrated to a limited extent into the agar, and the colonies occasionally sank slightly into the agar. Gran's test was weakly positive (max. diam. of zone around colonies 0·5 mm.), but the agar around the colonies was never depressed or softened, as was the case with the two other types. Colonies never appeared sunken on a medium with 2 % (w/v) agar. Fermentation tests carried out with washed agar as a carbon source showed that *C. salmoni color* did not ferment agar.

The above-mentioned observations lead to the conclusion that *Cytophaga salmonicolor* was unable to grow with agar as sole C source. Colonies of aerobically grown *C. salmonicolor* were catalase positive. *C. salmonicolor* var. *agarovorans* and

C. fermentans var. *agarovorans* sometimes showed a negative catalase reaction when grown on a medium containing 1% (w/v) agar, even when $CaCO_3$ was included. When 2% (w/v) agar was used the tendency to penetrate into the agar was less pronounced; in this case the agar-decomposing cytophagas generally gave a positive catalase reaction.

Inhibitory effect of glucose and galactose

Under semi-anaerobic conditions (stab cultures in agar media without Na_2S and without paraffin seal), or on aerobic plates, glucose and galactose markedly inhibited growth of *Cytophaga fermentans* var. *agarovorans* and *C. salmonicolor* var. *agarovorans*. Only a minute fraction of the inoculated viable organisms produced colonies in or on the sugar-containing substrate, and these colonies appeared where the inoculum was densest. These colonies were similar to those which grow on sugar-free media, and they decomposed agar at nearly the same rate. The inhibition by glucose and galactose could not be attributed to the formation of toxic products during autoclaving; when sterilized by Seitz filtration and added to the agar after this had been cooled to 40°, solutions of these sugars exerted the same effect. The growth of *C. salmonicolor* on agar media was hardly affected by adding glucose; but the addition of 0·1% (w/v) galactose strongly inhibited the development of the organism under aerobic conditions. A similar behaviour was reported for the agar-decomposing *C. sensitiva* (Humm, 1946), which did not grow on agar media with added 0·2% (w/v) glucose or starch; and for the cellulose-decomposing *Sporocytophaga myxococcoides*, which also was prevented from growing by 0·2% (w/v) glucose (Kaars Sijpesteyn & Fåhraeus, 1949). The mechanism of this striking inhibition is still obscure.

The nature of the fermentation

Methods. Fermentations were carried out in Hall flasks or in an apparatus previously described (Veldkamp, 1960); the latter apparatus was modified to maintain a constant pH value during the fermentation by means of an automatic titrator type TTT1 (Radiometer, Copenhagen, Denmark). For this purpose the all-glass fermentation vessel was provided with ground glass sockets for the glass- and calomel electrodes and alkali inlet. During the fermentation sterile NaOH was added from a burette operated by a magnetic valve.

The electrodes were sterilized chemically using a commercial iodophor solution (JO 127; Amsterdamse Kininefabriek, Amsterdam, Netherlands) which contains 1·5% active iodine. The electrodes were immersed for 20 min. in a 1/500 dilution of the iodophor solution and then washed with sterile water.

The experiments were made at 30° in an oxygen-free nitrogen atmosphere, with a mineral medium which in addition to the usual salts contained (%, w/v) 0·1, $NaHCO_3$; 0·05, $Na_2S.9H_2O$; adjusted to pH 7.

For the fermentations with agar as carbon source, 0·3% (w/v) Difco agar was used (this had been carefully washed with distilled water for 7 days); 0·1% (w/v) each of yeast extract, corn steep liquor and nutrient broth were included. Control experiments showed that only a trace of acid was formed when the agar was omitted from the medium. For hexose fermentations 0·1% (w/v) yeast extract and either 0·1–0·2% (fermentations in Hall flasks) or 1% sugar (fermentations at constant pH) were added to the basal medium. The sugars were sterilized separately as concen-

trated aqueous solutions. The analyses of fermentation products were performed as previously described (Veldkamp, 1960).

The dry weight of the slimy cell material was determined as follows. At the end of the fermentation 3 volumes of ethanol were added to a sample of the culture liquid; the resulting stringy precipitate was centrifuged down, washed twice with 95 % (w/v) ethanol in water, then dried *in vacuo* at 45° and weighed. This material was considered to have the empirical formula (CH_2O), an approximation supported by studies of bacterial assimilation (see Clifton, 1951).

Results. The results of the fermentations with galactose as carbon and energy source are shown in Table 1 which shows that acetic, propionic and succinic acids were the major products of galactose fermentation by all the organisms tested. *Cytophaga salmonicolor* and *C. salmonicolor* var. *agarovorans* formed a considerable amount of formic acid. Fermentations with washed Difco agar as substrate showed that *C. salmonicolor* did not form acid, whereas vigorous acid-production was observed with cultures of *C. salmonicolor* var. *agarovorans* and *C. fermentans* var. *agarovorans*, with a concomitant decrease in pH value from 7·0 to 5·4. The same acids were found in agar and galactose fermentations.

Table 1. *Acids produced during fermentation of galactose in Hall flasks by various cytophagas*

Product	*C. salmonicolor*	*C. salmonicolor* var. *agarovorans*	*C. fermentans* var. *agarovorans*
	mM product/mM galactose fermented		
Formic acid	0·50	0·33	0
Acetic acid	0·42	0·26	0·27
Propionic acid	0·26	0·23	0·48
Succinic acid	0·32	0·38	0·13

A more detailed analysis of the fermentation products formed by *Cytophaga salmonicolor* var. *agarovorans* from glucose is presented in Table 2. This fermentation was run at pH 7·0; under these conditions 1 % glucose was completely consumed. From Table 2 it can be seen that acetic, propionic and succinic acids were the major fermentation products. In addition, considerable amounts of CO_2 and H_2 were formed; these were also formed by *C. salmonicolor*, but not by *C. fermentans* var. *agarovorans*. Small amounts of formic and lactic acids and of ethanol were found as end products of the glucose fermentation by *C. salmonicolor* var. *agarovorans*. As much as 21 % of the glucose consumed was converted to cell material (bacterial cells and slime). According to Bachmann (1955), 15–25 % of glucose consumed during fermentation of *C. fermentans* was converted to alcohol-precipitable cell material. The fermentation products formed by this organism are the same as those produced by *C. fermentans* var. *agarovorans*. It appears that all facultatively anaerobic cytophagas so far studied exhibit a propionic acid type of fermentation. *C. succinicans*, which Anderson & Ordal (1961*a*, *b*) showed to produce formic, acetic, and succinic acids but not propionic acid, may be considered as a variant lacking the ability to decarboxylate succinate. A similar connexion is encountered in the lactate and sugar fermentations by *Propionibacterium*, *Veillonella* and *Ruminococcus* species, respectively.

Table 2. *Fermentation of glucose at* pH 7·0 *by* Cytophaga salmonicolor *var.* agarovorans

Product	mM product/mM glucose fermented	mg. atom carbon
CO_2	0·32	0·32
H_2	0·44	—
Ethanol	0·16	0·32
Formic acid	0·05	0·05
Acetic acid	0·38	0·76
Propionic acid	0·31	0·93
Succinic acid	0·33	1·32
Lactic acid	0·09	0·27
Cell material and slime, as (CH_2O)	1·28	1·28
Total mg. atom C	—	5·25
Carbon recovery %	87·5	—
Redox index	0·96	—

Taxonomy

The following characters exhibited by our organisms indicate that they must be considered as members of the genus *Cytophaga* (Stanier, 1942): flexing and gliding motility, low-refractility, ability to swarm across an agar surface, absence of microcysts and fruiting bodies. All the strains examined were facultative anaerobes found in marine environments, characters which they have in common with *C. fermentans* (Bachmann, 1955). *C. salmonicolor* differs from *C. fermentans* by the formation of a red pigment and ability to produce formic acid, CO_2 and H_2 during fermentation of carbohydrates. Our *C. fermentans* var. *agarovorans* differs from *C. fermentans* by its ability to grow with agar as sole C source. A similar difference exists between *C. salmonicolor* and *C. salmonicolor* var. *agarovorans*. Since ability to decompose complex polysaccharides is an important characteristic in the classification of the myxobacteria, and since agar decomposition is associated with the appearrance of cultures on or in agar, our agar-decomposing cytophagas are here described as varieties of *C. fermentans* and *C. salmonicolor*.

Description of Cytophaga salmonicolor

Morphology: flexible weakly refractile, slender rods with rounded ends. Organisms vary in length from 2 to 30 μ; average about 6 μ. Width 0·3–0·5 μ. Gliding motility in young cultures. Coccoid involution forms common in old cultures. Branched organisms do not occur. Star-shaped aggregates of actively flexing organisms common in liquid cultures. Gram-negative.

Growth on agar plate: colonies on medium with 1 % agar and low nutrient concentration are salmon-coloured; may or may not show spreading growth across surface. Colonies sometimes slightly sunken into agar, but the agar around colonies never depressed or softened. On 2 % agar colonies never sunken into agar.

Agar shake culture: small, pink, spherical or disk-shaped colonies which do not attack agar. Apparently grows at expense of added nutrients to agar medium.

Agar stab culture: grows only when glucose or complex nitrogenous nutrients are added. Diameter of stab in 1 % agar does not exceed 2 mm.

Gelatin stab: very slow crateriform liquefaction.

Fermentation: the following substrates fermented: arabinose, xylose, glucose, galactose, mannose, fructose, sucrose, lactose, maltose, cellobiose, trehalose, raffinose, inulin, starch. Not fermented: rhamnose, sorbose, mannitol, sorbitol, agar. Chitin not attacked aerobically. Products of glucose fermentation: formic, acetic, propionic, succinic, lactic acids, CO_2, H_2, traces of ethanol.

Nitrogen sources: ammonium salts, nitrates, yeast extract, nutrient broth, Casamino acids.

Growth factors: grows in mineral glucose medium; heavier and more rapid growth occurs in the presence of a vitamin mixture; carbon dioxide an absolute requirement for growth.

Catalase produced.

Salt concentration range: 1·0–3·0 % (w/v) NaCl.

Facultatively anaerobic.

Optimum temperature: 28–37°.

Source: marine mud.

Habitat: probably decaying seaweeds.

Description of Cytophaga salmonicolor *var.* agarovorans

Morphology: similar to *C. salmonicolor*.

Growth on agar plate: colonies on a medium with 1 % agar and low nutrient concentration are salmon-coloured, sunken into the agar; may or may not show swarming across agar surface. Colonies surrounded by wide translucent gelase fields in which agar is depressed and softened; agar is not completely liquefied. Formation of depressions in agar slightly retarded when 2 % agar used.

Agar shake culture: in medium with 1 % agar, pink spherical colonies formed, up to 1 cm. diam.; agar in area occupied by colony completely liquefied.

Agar stab culture: in medium with 1 % agar as energy source diffuse growth with complete liquefaction of agar; diameter of stab 4–10 mm. Growth in 2 % agar considerably less diffuse.

All other characteristics are similar to those encountered with *C. salmonicolor* with the exception of fermentation test with washed agar as substrate (positive).

Source: marine mud.

Habitat: probably decaying seaweeds.

Description of Cytophaga fermentans *var.* agarovorans

Morphology: very similar to *C. fermentans* and *C. salmonicolor*.

Growth on agar plate: colonies on medium with 1 % agar and low nutrient concentration greyish; centre of colony may become cream to pale yellow. Colonies largely subsurface and form deep craters in agar; they are surrounded by wide gelase fields in which agar becomes depressed, translucent and softened. Swarming growth across agar surface may occur, although generally colonies have a sharp smooth edge. On 2 % agar media, colonies flat spreading nearly colourless translucent. In older parts of colonies organisms may tend to accumulate in drop-like masses which contain normal vegetative organisms. Depressions formed in 2 % agar very shallow.

Agar shake culture: in medium with 1 % agar, greyish to cream spherical colonies which attain a diameter of 1 cm.; agar is softened.

Agar stab culture: in medium with 1 % agar as energy source diffuse growth causing softening of agar; stabs attain diameter of 4–10 mm.; growth in 2 % agar considerably less diffuse.

Gelatin stab culture: slow stratiform liquefaction.

Fermentation: the following substrates fermented: arabinose, xylose, rhamnose, glucose, galactose, mannose, fructose, mannitol, sorbitol, sucrose, lactose, maltose, cellobiose, raffinose, inulin, starch, agar. Not fermented: sorbose, trehalose. Chitin not attacked aerobically. Products of glucose fermentation are: acetic, propionic and succinic acids.

Nitrogen sources: ammonium salts, nitrates, yeast extract, nutrient broth, Casamino acids.

Growth factors: not needed when grown anaerobically or semi-anaerobically with hexoses as carbon source. Addition of a vitamin mixture causes more rapid and abundant growth, especially when cultivated under semi-anaerobic conditions.

Carbon dioxide an absolute growth requirement.

Catalase produced.

Salt concentration range: 1·0–3·0 % (w/v) NaCl.

Facultatively anaerobic.

Optimum temperature: 28–37°.

Source: marine mud.

Habitat: probably decaying seaweeds.

Cultures of the described cytophagas have been deposited in the National Collection of Industrial bacteria, Torrey Research Station, Aberdeen, Great Britain.

I am greatly indebted to Dr June Lascelles who provided cultures of the cytophagas here described and to Dr W. Clark for supplying *C. fermentans* (ATCC no. 12470). The assistance of Miss A. van Mourik is gratefully acknowledged. My thanks are due to Professor C. B. van Niel and to Dr June Lascelles for reading and improving the text of this paper.

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EXPLANATION OF PLATES

PLATE 1

Fig. 1. *Cytophaga fermentans* var. *agarovorans*. Agar shake culture; spherical colonies in 1 % agar medium with 0·1 % each of yeast extract, corn steep liquor and nutrient broth; age 9 days.

Fig. 2. *C. salmonicolor*. Agar shake culture; small spherical colonies grown in 1 % agar medium with 0·1 % each of yeast extract, corn steep liquor, and nutrient broth; age 9 days.

Fig. 3. *C. fermentans* var. *agarovorans*. Stab culture after 5 days' cultivation at 30° in mineral medium with 1 % agar and 0·1 % yeast extract.

Fig. 4. *C. salmonicolor* var. *agarovorans*. Stab culture after 5 days' cultivation at 30° in mineral medium with 1 % agar and 0·1 % yeast extract.

Fig. 5. *C. salmonicolor* var. *agarovorans*. Phase-contrast photomicrograph of living cells from anaerobic stab culture. ×2340.

Fig. 6. *C. fermentans* var. *agarovorans*. Coccoid involution forms from agar shake culture. Phase contrast. ×1574.

Fig. 7. *C. salmonicolor*. Colonies showing flat, spreading growth on 1 % agar medium.

PLATE 2

Fig. 8. *Cytophaga fermentans* var. *agarovorans*. Aerobic plate culture with mineral medium containing 1 % agar and 0·1 % each of yeast extract, corn steep liquor, and nutrient broth. Colonies deeply sunken into agar and surrounded by zone in which agar is softened and depressed. Age one week.

Fig. 9. *C. fermentans* var. *agarovorans*. Same plate showing gelase fields after flooding with I–KI solution.

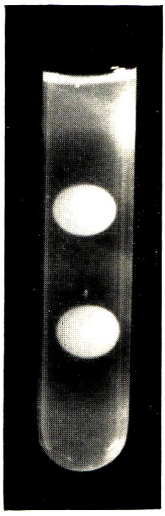


Fig. 1

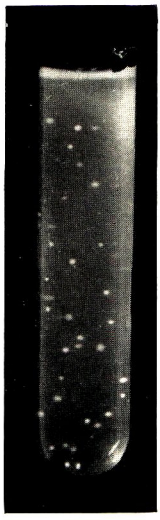
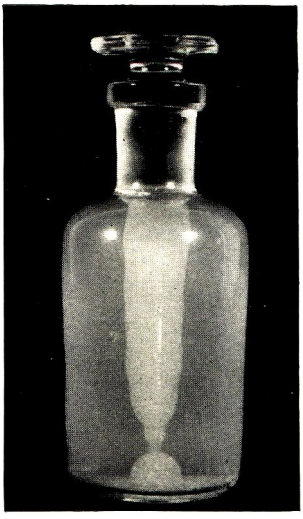


Fig. 2

Fig. 3



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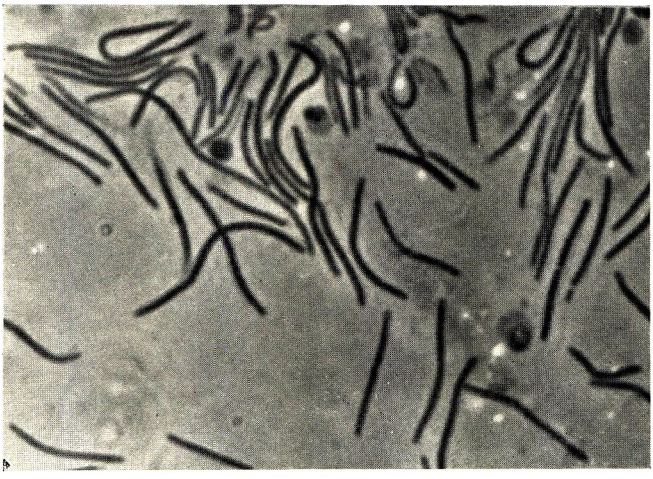

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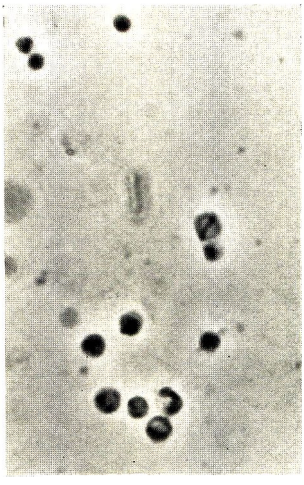

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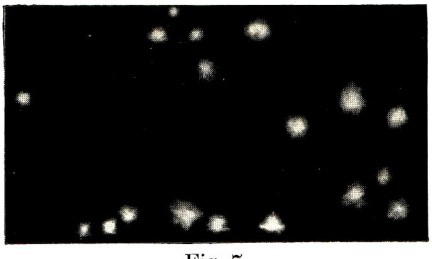

Fig. 7

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Fig. 8

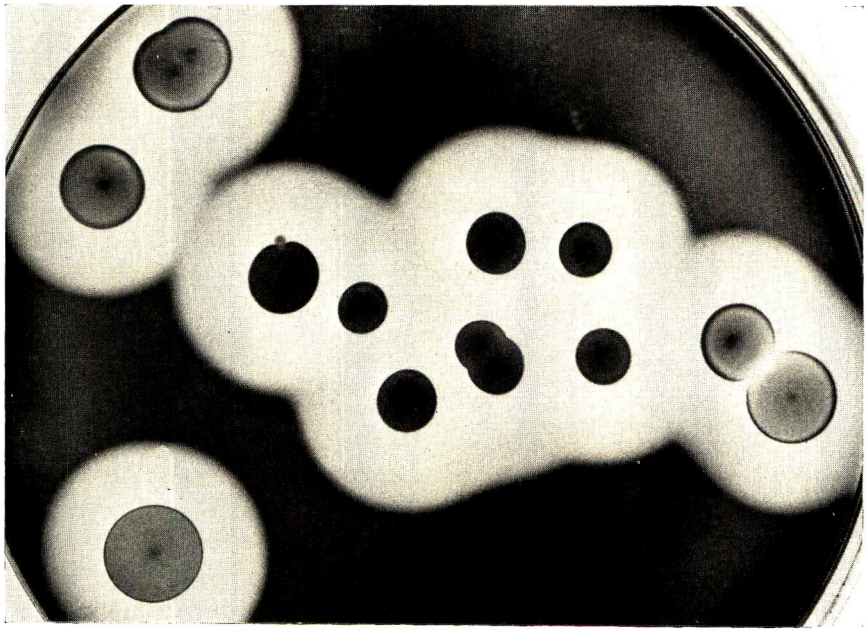

Fig. 9

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Atmospheric Content of Nigrospora Spores in Jamaican Banana Plantations

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SUMMARY

The air in three Jamaican banana plantations was sampled from 20 July 1960 to 15 April 1961 with a Hirst spore trap. Spores of Nigrospora were regular components of the air-spora. They exhibited a regular and sharply defined diurnal periodicity, rapid liberation of spores starting at about 07.00 hr. and reaching a peak between 08.00 and 10.00 hr.; very few spores were trapped during the night. This is consistent with the fact that spore discharge occurs only under conditions of decreasing vapour pressure. Rainfall and under-tree irrigation usually resulted in an appreciable increase in atmospheric content of Nigrospora spores. These high concentrations were maintained for 2–4 days before decreasing to characteristically low dry weather values. The highest daily mean concentration recorded was 1350 spores/m.^{3}, an estimate of *c.* 14,000/m.^{3} being obtained at 08.00 hr. on the same day.

INTRODUCTION

Since the introduction by Hirst (1952) of an automatic volumetric spore trap, much information about the air-spora in different parts of Britain has been collected (Hirst, 1953; Hirst, Storey, Ward & Wilcox, 1955; Gregory & Hirst, 1957; Gregory & Sreeramulu, 1958; Gregory & Stedman, 1958; Sreeramulu, 1959). In contrast, there is little information about the composition of the air-spora in or above tropical crops. Recent aerobiological studies in Jamaica (Meredith, 1961*c*, *d*) were chiefly concerned with the dispersal of *Deightoniella torulosa*, the cause of banana fruit-spot (Meredith, 1961*a*, *b*). During this investigation it was observed that spores of Nigrospora were particularly common (Meredith, 1961*e*) and their dispersal is the subject of the present paper.

METHODS

Trapping methods

A Hirst spore trap with its orifice 3 m. above ground was operated continuously from 20 July 1960 to 15 April 1961; sampling was at the rate of 14·4 m.^{3}/24 hr. Three banana plantations were selected for study, all of them being situated on the lowland plains in St Catherine where extensive banana and sugar-cane plantations are established.

Plantation A, 20 *July*–1 *September* 1960, was irrigated at fortnightly intervals by admitting water into shallow trenches (surface irrigation).

Plantation B, 4 *September* 1960–29 *January* 1961. Under-tree irrigation was carried out at intervals determined by the distribution of rainfall. In this method,

water is pumped along pipes and ejected through rotating nozzles set at intervals; pumping is usually continued for 48 hr. Since the water is sprayed several feet into the air, collapsed leaves hanging from the pseudostem are usually thoroughly wetted.

Plantation C, 15 *March–15 April* 1961. Conditions here were almost identical with those in B.

The slides, changed daily at 09.00 hr. E.S.T., were prepared and scanned according to the methods of Hirst (1953). Since no corrections for variation in the efficiency of trapping were made, all spore numbers quoted are underestimates (Hirst, 1953).

Daily records of temperature, R.H. and rainfall were taken in the vicinity of the trap.

Identity of Nigrospora spores

Conidia of Nigrospora are unlikely to be confused with those of any other genus. They are black, shiny, globose when viewed from the end and elliptical from the side. Species recorded in Jamaica include:

Nigrospora sphaerica, which is, according to Simmonds (1933), the cause of 'squirter' disease of banana fruits in Australia. It is a widespread saprophyte on banana debris and has been recorded on many other monocotyledonous hosts (Mason, 1927).

N. oryzae, a very widespread saprophyte on the banana and many other monocotyledons (Mason, 1927; Wardlaw, 1935).

N. sacchari, occurring on both banana and sugar-cane leaves (Mason 1927; Simmonds, 1933).

Mason (1927) distinguished three species of Nigrospora on the basis of spore size: *N. oryzae* 13–15·5 × 10–13 μ, *N. sphaerica* 18–21 × 14–15 μ and *N. sacchari* 18–24 μ in diameter. However, later he expressed doubt about this size criterion (Mason, 1933). In the present investigation it was found that spores measuring 13–18 μ predominated on the spore traces, suggesting that the dominant species trapped were *N. oryzae* and *N. sphaerica*, *N. sacchari* being infrequent. This is consistent with some observations on the relative abundance of these species in the plantations (Meredith, unpublished).

RESULTS

Diurnal periodicity

The diurnal periodicity curve (Fig. 1) obtained according to the method of Hirst (1953) includes data from all three plantations; each mean was from a total of 224 observations. Regular periodicity was evident, rapid liberation of spores starting after 06.00 hr. and reaching a peak between 08.00 and 10.00 hr. The concentration decreased rapidly during the afternoon and evening, very few spores being trapped during the night. On a few exceptional days the peak was not reached until about 12.00 or 14.00 hr., but on no occasion was the peak reached before 08.00 hr.

Liberation coincided with conditions of rising temperature and decreasing humidity, these usually occurring from 07.00 hr. onwards until about 14.00 hr. On damp mornings following rainfall during the previous night, humidity often showed no marked decrease until about 11.00 hr.; on these occasions the peak concentration occurred after this time. Similarly, little or no liberation was evident between 08.00 and 12.00 hr. on the few occasions when it was raining at that time.

Atmospheric content of Nigrospora spores

In Fig. 2 the daily mean spore concentration of Nigrospora is related to locality, rainfall and under-tree irrigation over the period 20 July–15 April 1961.

Plantation A

The 4 weeks prior to commencement of sampling were predominantly dry and the daily mean concentration averaged *c.* 100/m.^{3}. Traces of rain on 20 and 21 July were followed by an increase to 210/m.^{3} on 22 July. A further 0·9 in. rain on this date

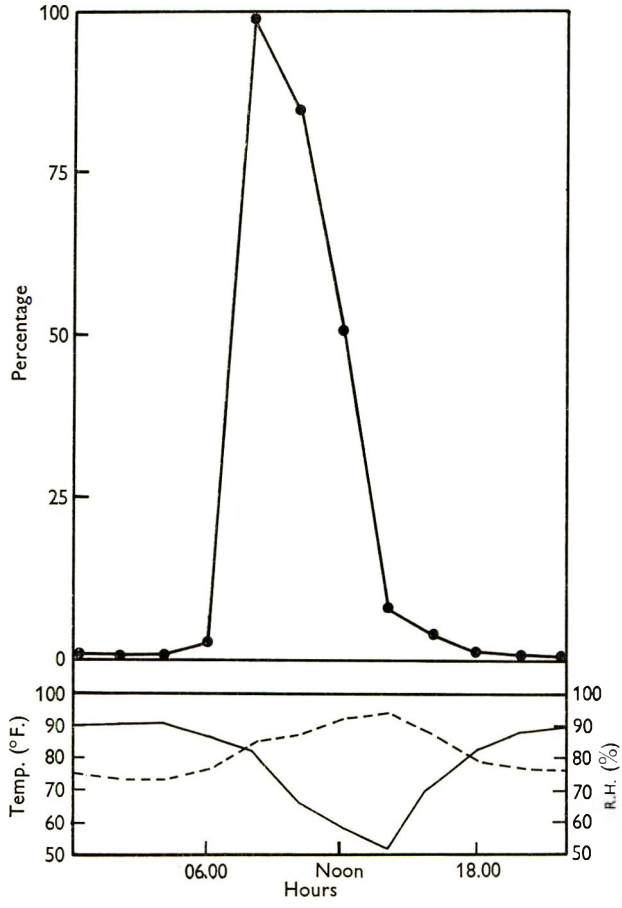

Fig. 1. Mean diurnal periodicity curve of Nigrospora expressed as a percentage of the peak arithmetic mean concentration. ----, temperature; ——, relative humidity.

and traces on the next 2 days resulted in a concentration of 450/m.^{3} on 25 July. The next 7 days were dry and there was a progressive decrease in concentration to values of *c.* 100/m.^{3}. Rainfall on various dates in August resulted in similar temporary increases in daily mean concentration. The highest 2-hourly concentration recorded in this plantation was 2400/m.^{3} at 08.00 hr. on 25 July.

The fifteenth of August was exceptional in that a concentration of only 6/m.^{3} was recorded. This was probably related to the fact that there was almost continuous

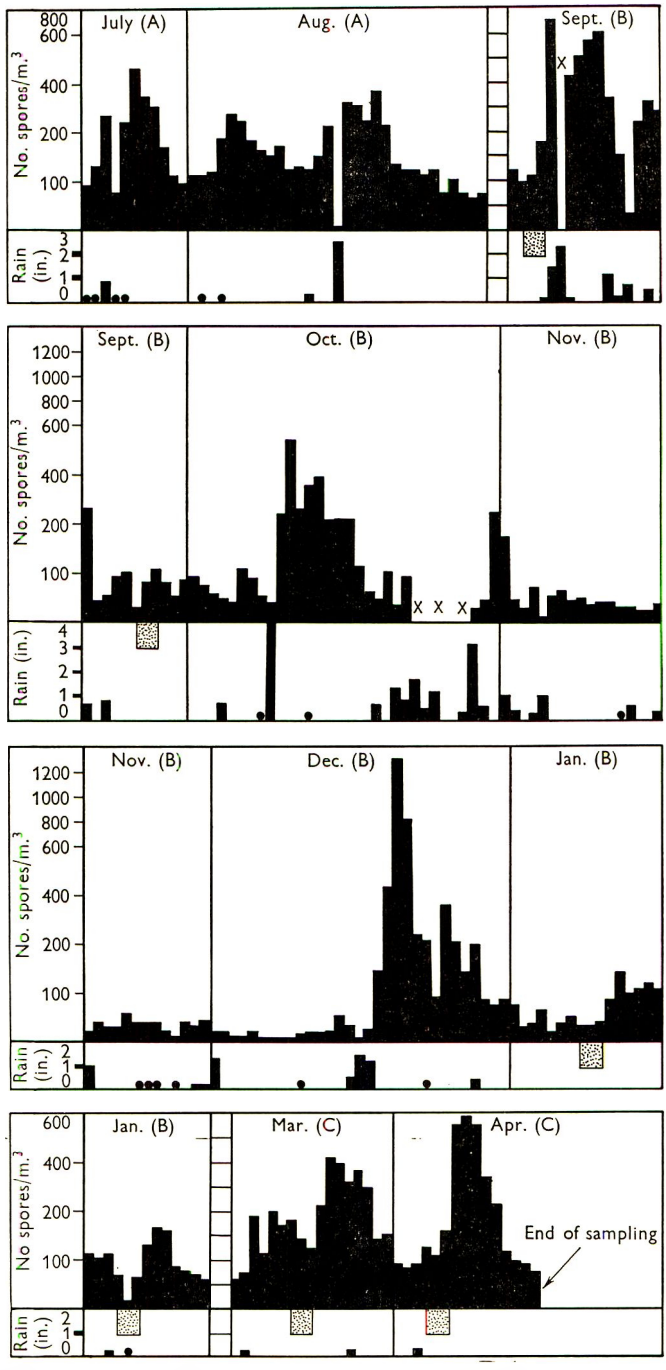

Fig. 2. Daily mean concentration of Nigrospora spores in three banana plantations (A, B and C) related to rain and under-tree irrigation over the period 20 July 1960 to 15 April 1961. ●, trace of rain; stippled areas indicate duration of under-tree irrigation periods; ×, trap not operating.

rainfall between 07.00 and 16.00 hr. on this date. Possibly rainfall occurring at the time of most rapid liberation removed many spores from the air almost immediately after their becoming air-borne. Alternatively, liberation might not have occurred due to unfavourable humidity conditions. A third possibility is that the source of spores was temporarily exhausted, but against this is the fact that relatively high concentrations occurred on both 14 and 16 August.

Plantation B

September. Initially, the concentration averaged *c.* 100/m.^{3}. Under-tree irrigation was carried out from 5 to 7 September and a total of 4·13 in. rain fell between 7 and 10 September. As a result of this, the concentration increased to 636/m.^{3} on 8 September, an estimate of 2620/m.^{3} being recorded at 08.00 hr. Daily means exceeding 300/m.^{3} were maintained until 15 September when a value of only 30/m.^{3} was obtained, possibly a result of rainfall occurring between 06.00 and 10.00 hr. The next 4 days yielded counts of more than 200/m.^{3}. Although there was more rain on 20 and 22 September and another under-tree irrigation period from 25 to 27 September, there was no appreciable increase in spore concentration.

October. The most notable feature was the greatly increased spore concentration following 4·12 in. on 9 October. The highest estimate recorded was 530/m.^{3} on 11 October, and it was not until 18 October that values fell below 200/m.^{3}.

November. The concentration rarely exceeded 50/m.^{3}, there being no apparent response to rainfall.

December. The first half of the month was predominantly dry and up to 13 December the spore concentration never exceeded 50/m.^{3}. Rain on 15, 16 and 17 December resulted in a relatively enormous increase in concentration during the subsequent 6-day period. On 20 December a concentration of 1352/m.^{3} was recorded, this being the highest value recorded in any of the three plantations studied; a count of *c.* 14,000/m.^{3} was recorded at 08.00 hr. on this date. By 29 December the concentration had decreased to 84/m.^{3}, there being no response to rain on 28 December.

January. This was a very dry month and concentrations exceeding 100/m.^{3} were recorded only after the two irrigation periods.

Plantation C

Increases in concentration were evident after rainfall and under-tree irrigation. The highest value recorded was 587/m.^{3} on 8 April, the peak 2-hourly estimate on this day being 5130/m.^{3} at 08.00 hr.

Spore projection in Nigrospora

Webster (1952) has described violent spore discharge in *Nigrospora sphaerica.* Projection occurs under conditions of decreasing vapour pressure and appears to be due to the discharge of liquid through a fine orifice at the apex of a specialized conidiophore cell.

Colonies of Nigrospora occurring on decaying banana leaves were examined to discover whether violent spore discharge occurs on this substratum. Decaying leaf material was incubated for 2 days in a damp chamber to encourage spore formation. Thin strips of epidermal tissue were then rapidly transferred to the stage of a low-

power binocular microscope. Within a few seconds, presumably as the tissues dried out, spores were shot away from the substratum; no discharge occurred when the strips of tissue were examined while inside damp Petri dishes. Light did not affect the discharge process. Thus Webster's (1952) findings were confirmed.

DISCUSSION

It is clear that Nigrospora is a common component of the air-spora in Jamaican banana plantations. The fungus exhibits regular diurnal periodicity similar to that in *Nigrospora sphaerica* in Nigeria (Cammack, 1955), *Deightoniella torulosa* in Jamaica (Meredith, 1961*c*) and *Phytophthora infestans* and *Polythrincium trifolii* in England (Hirst, 1953). Hirst (1953) suggested that this type of periodicity might be due to the existence of a definite discharge mechanism operating each day under conditions of decreasing vapour pressure. Observations reported here, and earlier ones of Webster (1952) and Cammack (1955), support this suggestion in the case of Nigrospora.

Rain and under-tree irrigation commonly resulted in large increases in concentration of Nigrospora spores. This was probably a result of increased sporulation of the fungus after wetting of the spore-bearing substratum, namely banana debris. November's data were exceptional in that there was no response to rainfall. An explanation for this must await further investigation into the ecology of Nigrospora on banana debris.

Since Nigrospora is not responsible for diseases of economic importance in Jamaica, the results presented here have little apparent practical value to the local banana industry. However in Australia, where 'squirter' disease (*Nigrospora sphaerica*) often assumes serious proportions, similar aerobiological studies may contribute to a better understanding of the epidemiology of disease.

Grateful acknowledgement is made to the United Fruit Co., Jamaica, for granting access to their plantations, and to Miss B. I. McLean, Miss V. E. Green and Mr R. R. Chen for valuable technical assistance.

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Nodule Bacteria Associated with the Indigenous Leguminosae of South-Western Australia

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SUMMARY

Root-nodule bacteria (rhizobia) isolated in pure culture from legumes indigenous to south-western Australia were examined for cultural and infective characters. All isolated strains had cultural characters consistent with the slow-growing bacteria of the lupin or soybean type. None of the strains nodulated peas, clover or medic, but hosts from the other four recognized cross-inoculation groups were nodulated. *Lupinus digitatus*, *Glycine hispida*, *Vigna sinensis* and *Phaseolus vulgaris* formed a natural grouping on the basis of susceptibility to nodulation by the native strains. Within the narrower host range of the genus *Lupinus* it was found that *L. digitatus*, *L. albus* and *L. pilosus* grouped together on susceptibility to nodulation, whereas *L. luteus*, *L. angustifolius* and *Ornithopus sativus* were not nodulated by any of the sample strains. The use of this geographically isolated sample of rhizobia has further demonstrated the weakness of the present scheme of classification of the nodule bacteria, which is based on the infective character of the bacteria. A taxonomic system based on Adansonian principles could be applied to the classification of the rhizobia, and a procedure for attempting this is outlined.

INTRODUCTION

The genus *Rhizobium* comprises a group of organisms characterized by their ability to induce the formation of nodules on the roots of legumes. Allen & Allen (1950) stated '...the ability to invade the roots of leguminous plants and stimulate the production of nodules...is the sole criterion for the existence of the genus *Rhizobium*'. Bacteria thus grouped on the feature of legume root nodulation are subgrouped on the range and type of host plants infected. *Bergey's Manual* (1957) accords six of these subgroups specific status but appends a note that some of them may be related. While this classification is generally recognized as imperfect, it persists in the absence of a better system. The present paper reports some results from a survey of the rhizobia associated with indigenous legumes of the botanical South-West Province of Western Australia (Lange, 1960). The province itself is about 100,000 square miles in area. Its climate was discussed by Gentilli (1946), its geomorphology by Jutson (1950), soils by Teakle (1938) and the indigenous vegetation by Diels (1906) and Gardner (1942). Indigenous Leguminosae total over 400 species in about 35 genera of the Caesalpiniaceae, Mimosaceae and Papilionaceae. A great many of the species are endemic to the area. Nearly half are species of

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Acacia; the remainder are mostly papilionaceous, principally in genera of the tribe Podalyrieae. The incidence of nodulation in these indigenous legumes has already been tabulated (Lange, 1959).

METHODS

Over a 2-year period, 7000 miles of traverses were made throughout the study area and the vegetation examined at intervals, usually of only a few miles. Leguminous species were collected for identification and examined for root-nodulation. Species representative of the indigenous Leguminosae were selected from those found to be nodulated. Nodules were removed from these plants in the field, and desiccated in tubes over anhydrous $CaCl_2$ (Dr D. O. Norris, personal communication). In the laboratory the nodules were reconstituted by soaking in sterile water. They were then surface-disinfected by immersion for 15 min. in a mixture of equal volumes of H_2O_2 (100 vol.) and absolute ethanol, washed and macerated. Samples were streaked on plates of yeast extract + mannitol agar and incubated at 27°.

One hundred and sixty-three strains of presumptive rhizobia were isolated in pure culture. Eighty-five of these isolates from 83 host species in 24 legume genera were selected as a fair sample of the Rhizobium population associated with indigenous Leguminosae in the area (see Table 1).

Two series of tests were applied to the sample strains.

Tests of cultural characters

Colony characters. Visible characteristics of colonies on isolation plates and in pure culture were recorded. Strains of known performance from *Glycine*, *Lathyrus Trifolium*, *Medicago*, *Phaseolus* and *Lupinus* were included in all tests to allow direct comparisons to be made.

Morphology and staining. Preparations from 6-day-old cultures were stained by Gram's method and with carbol rose bengal and examined.

Calcium glycerophosphate medium. Strains were streaked on slopes of calcium glycerophosphate agar (Hofer, 1941), incubated for 3 weeks at 27° and observed for browning of the medium.

Litmus milk. Strains were inoculated into sterile litmus milk, incubated for 4 weeks at 27° and observed for pH change and for serum zone formation.

Colony growth rate. Strains were dispersed in sterile water and dilution series made. Drops of appropriate dilutions were spread over yeast extract + mannitol agar plates to yield ten to thirty well-isolated colonies. Each plate was incubated at 27°, inspected daily, and the times for half of the colonies in sector areas to reach diameters of 1 mm. recorded.

Reactions on sugar. Cultures were inoculated on to nitrate agar medium (Baldwin & Fred, 1927) containing either arabinose, rhamnose, xylose, maltose or sucrose, and pH changes observed after incubation for 5 weeks at 27°.

Tests of infectiveness on legumes

Each of the 85 sample strains was further investigated in a series of glasshouse experiments to determine its infectiveness on the following legumes: *Pisum sativum* L., *Lathyrus cicera* L., *Lens esculenta* Moench., *Vicia sativa* L., *Lupinus angustifolius* L., *L. luteus*, L., *L. nanus* Dougl., *L. digitatus* Forsk., *L. albus* L., *L. pilosus* Murr.,

L. villosus Willd., *L. subcarnosus* Hook., *Ornithopus sativus* Brot., *O. compressus* L., *Medicago tribuloides* Desr., *Trifolium subterraneum* L., *Vigna sinensis* Engl., *Phaseolus vulgaris* L., *P. lathyroides* L., and *Glycine hispida* Maxim. These include hosts from the seven major cross-inoculation groups. The eight *Lupinus* species were included since it had been indicated earlier that lupins may be differentially susceptible to native rhizobia in the area (Adams & Riches, 1930; Lange & Parker, 1960). The following conditions were imposed during the course of the experiments.

(1) Strains known to be infective on the hosts were included. These invariably nodulated the host legume under the conditions of experiment. This ensured that experimental conditions did not restrict nodulation of the plant.

(2) Experiments were terminated when the control nodulation was fully established.

(3) Control uninoculated plants were included at a frequency of 20% of inoculated plants.

(4) Conditions for plant growth were kept as close to optimal as possible.

Experiments were conducted in closed glasshouse rooms with adequate sunlight and pressurized by air coolers delivering washed air. The rooms were sprayed down completely with insecticidal, fungicidal and bactericidal sprays, and with paraffin oil emulsion (25% paraffin oil). Dust immobilization was maintained by further spraying with paraffin oil emulsion containing antibacterial substances. Large plants were grown in open sand-filled porous drainage pots spaced on slatted bench tops over an area heavily coated with paraffin oil emulsion. Pots were dressed with a nitrogen-deficient nutrient solution providing major and minor elements. Superphosphate was mixed with the sand separately at the rate of 300 p.p.m. as the fertilizer. Trifolium and Medicago plants were grown in agar tubes after the method of Chen & Thornton (1940).

Pots were brought to field capacity, wrapped, stacked in a bin and subjected to a continuous flow of steam for 1 hr.; this raised the temperature of the sand to at least 90°. Seeds were disinfected by immersion for 10–20 min. in a mixture of equal volumes of 100 vol. H_2O_2 and absolute ethanol, followed by washing in sterile water. The seeds were then germinated and planted under sterile conditions. Inoculum was applied direct to the seed coat, and pots were watered with sterile water. The tests were completed in a total of twelve experiments, each of factorial design: host species × sample strains × a minimum of four replications.

Root systems were washed out usually about 28 days after germination and floated in water for examination. In one experiment nodulation occurred in some of the uninoculated control plants; this experiment was repeated. In the experiments reported here the control of nodulation was absolute.

RESULTS

Cultural characters

All 85 bacterial strains originated from within the tissues of sound legume root-nodules and grew as aerobic heterotrophs. Colonies after 8 days were punctiform, rarely exceeding 1 mm. diam., and were opaque or rarely translucent, whitish, and gummy. No isolated colony exceeded punctiform size within 5 days, and all strains were therefore slow-growing compared to the known strains of *Rhizobium meliloti*,

Table 1. *The infectiveness of 85 strains of nodule bacteria on 9 legume test hosts*

+, Nodulation on all plant replications; ±, nodulation on some but not all plants; −, complete absence of nodulation.

Host of isolation	Strain no.	Host nodulated: *Glycine hispida*	*Lupinus albus*	*L. digitatus*	*L. pilosus*	*L. subcarnosus*	*L. villosus*	*Phaseolus lathyroides*	*P. vulgaris*	*Vigna sinensis*
Acacia acuminata Benth.	3	±	±	±	−	−	−	+	±	+
ericifolia Benth.	54	−	−	+	−	−	−	+	±	+
stenoptera Benth.	65	+	±	+	±	−	−	+	+	+
erinacea Benth.	48	−	±	±	−	−	−	±	±	±
extensa Lindl.	121	±	±	+	±	−	−	+	±	+
Drummondii Lindl.	29	+	±	±	−	−	−	±	±	±
myrtifolia Willd.	113	−	−	+	−	−	−	+	±	+
cyanophylla Lindl.	20	−	±	+	+	−	−	±	±	+
hastulata Smith	114	+	±	+	−	−	−	±	−	+
horridula Meissn.	85	+	−	+	−	−	−	+	±	+
nervosa D.C.	115	−	±	+	±	−	−	+	+	+
volubilis F.v.M.	46	+	±	+	±	−	−	−	±	+
pulchella R.Br.	64	−	±	+	±	−	−	−	±	+
diptera Lindl.	14	+	±	+	−	−	−	+	±	+
Albizzia distachya (Vent.) Macbride	97	+	−	+	−	−	−	+	±	+
Aotus villosa Sm.	134	−	±	+	−	−	±	+	±	+
Preissii Meissn.	126	+	±	+	±	−	−	+	±	+
Tietkinsii F.v.M.	132	−	±	−	±	−	−	−	±	−
Bossiaea Presii Meissn.	92	+	−	+	±	−	−	+	±	+
Laidlawiana Tovey et Morris	96	−	±	−	−	−	−	−	−	−
Webbii F.v.M.	110	−	±	+	±	−	±	±	±	+
dentata Benth.	107	±	±	±	−	−	−	±	±	+
aquifolium Benth.	95	±	−	−	−	−	−	+	±	+
eriocarpa (R.Br.) Benth.	88	−	−	+	−	−	−	±	+	+
linophylla R.Br.	99	−	−	−	±	−	−	±	±	+
Brachysema lanceolatum Meissn.	91	±	±	+	±	±	−	+	−	+
sericeum (Sm.) Domin.	117	+	−	−	−	−	−	+	±	+
praemorsum Meissn.	124	−	−	−	−	−	−	−	−	+
Chorizema Dicksonii Grah.	90	−	±	+	±	−	−	+	+	+
ilicifolium Labill.	123	−	−	±	−	−	±	−	±	+
reticulatum Meissn.	122	+	+	+	−	−	−	+	±	+
aciculare (D.C.) C.A. Gardn.	120	+	−	±	+	−	−	−	+	+
cytisoides Turcz.	119	+	−	+	−	−	−	+	±	+
ericifolium Meissn.	52	+	+	+	±	−	−	+	+	+
Davesia hakeoides Meissn.	57	±	−	±	−	−	−	−	±	−
brevifolia Lindl.	101	±	−	±	−	−	−	−	+	+
pectinata Lindl.	68	−	±	−	±	−	−	−	±	−
sphylla (F.v.M.) Benth.	112	−	+	−	+	−	−	−	−	−
incrassata Sm.	163	−	−	−	−	−	−	±	+	±
Dillwynia uncinata (Turcz.) C.A. Gardn.	98	±	±	±	±	−	±	±	−	+
Euchilopsis linearis (Benth.) F.v.M.	62	−	−	+	−	−	−	−	+	+

Host of isolation	Strain no.	Host nodulated: *Glycine hispida*	*Lupinus albus*	*L. digitatus*	*L. pilosus*	*L. subcarnosus*	*L. villosus*	*Phaseolus lathyroides*	*P. vulgaris*	*Vigna sinensis*
Eutaxia epacridioides Meissn.	87	+	−	+	−	−	−	+	+	+
virgata Benth.	105	−	±	+	−	−	−	+	+	+
densifolia Turcz.	137	−	−	−	±	−	−	±	+	±
Gastrolobium villosum Benth.	106	−	−	+	−	−	−	±	±	+
spinosum Benth.	61	−	+	+	±	−	−	+	−	+
obovatum Benth.	32	−	±	±	−	−	−	±	±	+
trilobum Benth.	100	+	−	+	−	−	−	+	+	+
Gompholobium marginatum R.Br.	60	+	−	+	−	−	−	±	±	+
venustum R.Br.	66	−	−	+	−	−	−	−	−	+
polymorphum R.Br.	111	+	−	+	−	−	−	+	±	+
Knightianum Lindl.	108	±	+	+	+	−	−	−	+	−
tomentosum Labill.	104	±	±	+	−	−	−	+	±	+
Hardenbergia Comptoniana (Andr.) Benth.	19	+	±	+	±	−	−	−	+	+
Hovea elliptica D.C.	109	−	−	±	−	−	−	±	±	+
trisperma Benth.	15	+	±	+	−	−	−	+	+	+
chorizemifolia D.C.	26	+	±	+	±	−	−	−	−	+
pungens Benth.	118	+	±	+	±	−	−	+	±	+
Isotropis cuneifolia (Sm.) Domin.	16	+	±	+	±	−	−	−	+	+
Jacksonia hakeoides Meissn.	69	+	−	±	−	−	±	±	±	+
spinosa (Labill.) R.Br.	102	+	±	+	−	−	−	+	±	+
horrida D.C.	33	−	±	±	−	−	−	±	±	±
furcellata (Bonpl.) D.C.	125	+	±	+	±	−	−	+	±	+
Kennedya eximia Lindl.	73	+	−	+	−	−	−	+	+	+
coccinea Vent.	77	+	±	+	−	−	−	+	+	+
Stirlingii Lindl.	78	+	±	+	±	−	−	+	±	+
prostrata R.Br.	2	+	±	+	−	−	−	+	±	+
Stirlingii Lindl.	76	+	−	+	−	−	−	+	+	+
Latrobea hirtella (Turcz.)	133	+	±	±	−	−	−	+	±	+
Mirbelia floribunda Benth.	89	±	±	+	−	−	−	+	±	+
spinosa Benth.	127	+	±	±	±	−	−	+	±	+
dilatata R.Br.	86	−	−	±	−	−	−	+	±	+
Oxylobium parviflorum Benth.	27	+	±	+	−	−	−	+	±	+
lanceolatum Benth.	130	±	−	+	−	−	−	+	+	+
capitatum Benth.	17	+	−	+	±	−	−	+	+	+
atropurpureum Turcz.	141	±	−	+	−	−	−	−	+	+
reticulatum Meissn.	81	−	−	+	−	−	−	+	±	+
Pultenaea reticulata (Sm.) Benth.	84	±	−	+	−	−	−	+	+	+
strobilifera Meissn.	85	+	−	−	±	−	−	+	+	+
ochreata Meissn.	94	+	±	−	−	−	−	±	±	±
Sphaerolobium alatum Benth.	59	−	−	+	−	−	−	+	+	+
medium R.Br.	128	+	±	−	−	−	−	+	±	+
grandiflorum R.Br.	116	−	−	−	−	−	−	±	±	+
Templetonia retusa R.Br.	21	−	−	+	+	−	−	+	±	+
Viminaria denudata (Sm.)	93	+	+	+	−	−	−	+	±	+

R. trifolii and *R. leguminosarum* which were included. All strains were Gram-negative. None exhibited spores or morphologically distinctive forms, but ranged from coccobacilli to more or less straight bacilli from 0·5 to 4·5 μ by 0·5 μ. On calcium glycerophosphate agar all failed to cause browning. In litmus milk, all strains produced an alkaline reaction without appreciable dye reduction. None produced a serum zone. All 85 strains produced acid on arabinose and xylose, an acid reaction or no change of pH value on rhamnose, and alkaline reactions on maltose and sucrose.

Infectiveness

Under the conditions of the experiments, none of the 85 strains nodulated *Pisum sativum*, *Lathyrus cicera*, *Lens esculenta*, *Vicia sativa*, *Lupinus angustifolius*, *L. luteus*, *L. nanus*, *Ornithopus sativus*, *O. compressus*, *Medicago tribuloides*, or *Trifolium subterraneum*. The following plant species were nodulated by sample strains: *Vigna sinensis* (79 strains); *Phaseolus vulgaris* (76); *Lupinus digitatus* (71); *Phaseolus lathyroides* (67); *Glycine hispida* (53); *Lupinus albus* (47); *L. pilosus* (31); *L. villosus* (5) and *L. subcarnosus* (1). The details of their infective performance on these species are presented in Table 1.

DISCUSSION

Breaches of the cross-inoculation boundaries between the soybean and the cowpea (Leonard, 1923), and between lupins, soybeans and cowpea (Bushnell & Sarles, 1937) have been reported before. The results submitted here further illustrate the deficiencies of the cross-inoculation-group concept when used to delineate species of *Rhizobium*. The bacterial strains used in this study were selected as representative of the microsymbionts from indigenous legumes in the study area. When compared with recognized *Rhizobium* species on the basis of their *in vitro* characters, all of them showed the characteristics of the slow-growing species *Rhizobium japonicum*, or of the cowpea group of rhizobia.

The range of infective performance exhibited by the sample strains extended to hosts from four of the recognized cross-inoculation groups, namely, the cowpea, lupin, soybean and bean groups. However, infection across the boundaries of these four groups was prevalent to the extent of being typical rather than exceptional. Thus of the 85 strains examined

45 strains nodulated hosts in 4 cross-inoculation groups;
31 strains nodulated hosts in 3 cross-inoculation groups;
6 strains nodulated hosts in 2 cross-inoculation groups;
3 strains nodulated hosts in 1 cross-inoculation group.

On the evidence it is impossible to place these strains in specific cross-inoculation groups, or to assign species designations to them on the basis of the accepted system. Distinctly different host groupings are apparent. For example, *Lupinus digitatus*, *Glycine hispida*, *Vigna sinensis* and *Phaseolus vulgaris* group together on susceptibility to infection by the sample strains, even though these hosts are from four different cross-inoculation groups. Similarly, *L. digitatus*, *L. albus* and *L. pilosus* group with each other on susceptibility, but do not group at all with their accepted cross-inoculation associates *L. angustifolius*, *L. luteus* and *Ornithopus sativus*.

Three *Rhizobium* species were not involved at all. None of the data implicated the clover, the medic or the pea cross-inoculation groups in any way, although

evidence from other sources (Kleczkowska, Nutman & Bond, 1944; Norris, 1959) shows that the pea and clover groups are not as discrete as they were once regarded.

Past studies of root-nodule bacteria show preoccupation with symbiont infectiveness patterns because of the economic importance attached to them. For the same reason, there has been a further preoccupation with agronomic legumes. This bias has dominated microsymbiont classification. In the period around 1920 there were two conflicting approaches. On the one hand, an expedient classification of the bacteria was derived based solely on their segregations in symbiotic infectiveness patterns (Garman & Didlake, 1914). On the other hand, the practice of classifying strains on the basis of symbiotic performance alone was rejected by Löhnis & Hansen (1921). Literature published over the succeeding period recorded the continued dominance and development of the former approach (Fred, Baldwin & McCoy, 1932). A species of *Rhizobium* was recognized as containing those microsymbionts which would nodulate a particular group of legumes. Other characters of the bacteria were not accorded equivalent taxonomic weight. Subsequently, patterns of symbiotic infectiveness were demonstrated to be less discrete (Leonard, 1923; Sears & Clark, 1930; Raju, 1936; Bushnell & Sarles, 1937). Interrelationships led some authors to suggest consolidations of groups and of species (Walker & Brown, 1935), whilst others suggested retention of the old groups with provision for special cases (Allen & Allen, 1936).

A second and very emphatic rejection of the cross-inoculation basis of strain classification was made by Wilson (1939) who produced substantial data to support his contentions. Further demonstrations of the inadequacies of the cross-inoculation groups continued (Appleman & Sears, 1942; Johnson & Allen, 1952; Ishizawa, 1954; Bowen, 1960). Recently a third rejection of the cross-inoculation-group concept was made. Norris (1956) considered that there was no satisfactory known basis for the subdivision of the rhizobia into species. He suggested the use of a symbiotic rating which would indicate the relative affinities of a particular strain for hosts within the three subfamilies of the Leguminoseae.

The suggested modifications all continue to classify the nodule bacteria on the basis of their infective performance, excluding comparisons with bacteria which cannot produce nodules on the roots of a legume. This is clearly unsatisfactory. To be effective as a classification of bacteria, a system should be erected on non-biased over-all similarities. The Adansonian classification proposed by Sneath (1957) fulfils these requirements. The classification of the root nodule bacteria might thus be resolved by research within the following stages:

(1) Collection of types (or neotypes) of all recognized *Rhizobium* species.

(2) Collection from botanically representative and geographically diverse sources the widest sample of legume root-nodule bacteria possible within research resources.

(3) Collection of types and representatives of possibly related bacteria, e.g. *Agrobacterium* (Hofer, 1941; Bonnier, 1953); *Bacillus* (Bisset, 1952, 1959); *Beijerinckia* (Derx, 1953).

(4) Derivation of a range of tests by which all these bacteria may be described.

(5) Application of the tests, and classification of all strains on the results, by Sneath's (1957) method.

By thus relating a full range of root-nodule bacteria to each other and to other bacteria, their over-all similarities and groupings should become evident, and the

genus *Rhizobium* with its component species could be retained or rejected. Application of an Adansonian classification to the strains used in this study would be premature, since both the strain sample and the test range are not adequate for the derivation of a generalized classification.

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