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The Cellular Location of the Streptococcal Group D Antigen

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SUMMARY

The cellular location of the group D antigen was examined by fractionation of protoplasts prepared by digestion of cell walls with lysozyme in an osmotically protective medium. The major portion of the group D antigen appears to be closely associated with the protoplast membrane of *Streptococcus faecalis* (ATCC 9790). Some antigen was found in the supernatant fluid (lysozyme hydrolysate) after removal of the protoplasts. This was attributed to the known fragility of protoplasts and membranes.

INTRODUCTION

The development of good methods for the isolation of bacterial cell walls in relatively pure form has been followed by an accumulation of experimental evidence indicating that many of the antigens of Gram-positive bacteria are located in the cell-wall structure. The Lancefield streptococcal grouping antigens seem, in most instances, to be located in the wall. This has been demonstrated first by Salton (1953) for group A organisms, by Ottens & Winkler (1962) for those belonging to group F and by Slade & Slamp (1962*a*) for organisms of groups B, C, E, F, G, H, K, L, M, N, O and Q. The group D streptococci therefore seem to be the one notable exception, in that the group specific antigen is not in the cell wall (Jones & Shattock, 1960).

The grouping antigens of the streptococci have thus far been found to fall into two types of chemical substances. The group specific antigens of groups A and C appear to be rhamnose-containing polysaccharides (Krause & McCarty, 1961, 1962). In groups O and K the same amino sugars are present, plus glucose and galactose; however, rhamnose may or may not be present (Slade & Slamp, 1962*b*). In contrast those from groups D and N appear to be glycerol-teichoic acids (Wicken, Elliott & Baddiley, 1963; Elliott, 1962, 1963).

Recently we have reported a series of experiments which indicate that the group D antigen of an organism designated as *Streptococcus faecalis* ATCC 9790 by the American Type Culture Collection is closely associated with the protoplast membrane (Slade & Shockman, 1963*a*, *b*). The results of these experiments can be briefly summarized as follows: (1) complete disruption of either exponential phase or stationary phase cells by means of shaking with ballotini resulted in essentially all of the group D antigen remaining in the supernatant fluid after sedimentation at 10,000 *g*. Centrifugation of the 10,000 *g* supernatant fluid at 105,000 *g* for 2 hr (which will sediment fragmented membranes and, in the absence of added Mg^{2+}, some of the ribosomes)

resulted in undetectable or barely detectable amounts of antigen in the supernatant fluid and large amounts of antigen in the pellet. (2) Isolated, well-washed (10 washings in sodium chloride or phosphate buffer) protoplast membranes from stationary phase, valine or threonine depleted cultures were obtained by lysozyme treatment of cells (Shockman *et al.* 1963). These membrane preparations contained relatively large amounts of the antigen. However, similar preparations from exponential phase cultures, washed 12 or 14 times, did not give a positive test for the antigen. The absence of antigen in the latter instances was attributed to the more extensive washing procedure which has been shown to remove antigen from the membranes (see below). However, a contributory factor might be the consistently lower titre of antigen observed with exponential phase cells and fractions from exponential phase cells as compared with those from any of the preparations from stationary phase cells. (3) Protoplasts of *S. faecalis* 9790 were prepared by digestion of the cell walls with lysozyme (lysozyme to cell ratio 1:250 to 1:200 in 0·5 M-sucrose +0·05 M-phosphate buffer, pH 7·4). The development of osmotic fragility was followed by diluting samples 1 to 60 with water or the sucrose + phosphate solution. After maximum osmotic fragility had been reached, and the 3–12 % of the initial turbidity of the suspension that remained after dilution with water did not decrease any further with time, the entire protoplast suspension was lysed by dilution in 0·05 M-phosphate buffer. The membranes were sedimented and washed 8 times with sodium chloride or phosphate and for the 9th time with water on the centrifuge. All of these fractions (washed membranes, washes, and supernatant fluid) were dialysed and their content of group D antigen was determined by the capillary precipitin method. The washed membranes as well as all of the 9 washings (particularly the last water wash) gave strong precipitin reactions for the group D antigen. Additional similar experiments with both exponential phase and stationary phase cells, in which the membrane fraction was washed only once with water, gave similar results. It was clear from these experiments that a considerable portion of the group D antigen was closely associated with the membrane but could be removed, at least partially, by washing with sodium chloride, phosphate buffer and especially with water.

However, at that time we were unable to get a supernatant fluid after digestion of whole cells by lysozyme (EC 3.2.1.17) in an osmotically-protected medium that we could be certain was reasonably free from whole cells as well as membrane and cytoplasmic contents. Therefore we were unable to examine the possibility of a cellular location of the antigen, external to the membrane but not part of the cell wall. On the other hand, Hijmans (1962) clearly showed that L-forms of three strains of group D streptococci did not contain the group antigen. In addition, since the completion of our previous experiments, two groups of workers have briefly reported evidence pointing to a location of the antigen in the region between the protoplast membrane and the cell wall. Shattock & Smith (1963) examined several strains of *Streptococcus faecalis* by a variety of procedures such as (1) digestion of cell walls with lysozyme, (2) digestion of walls with a phage-associated lysin in osmotically protected media, and (3) the synthesis of the antigen by L-forms. A gel diffusion precipitin method for detection of the antigen was used. They concluded that 50–85% of the group antigen was external to the protoplast membrane and that the Hijmans L-form can, upon proper treatment, continue to produce the group D

antigen, 97% of which was found free in the culture medium. Also, Hay, Wicken & Baddiley (1963), using paper chromatography and chemical methods to identify the hydrolysis products of teichoic acids after digestion of cell walls of a group D streptococcus (NCTC 8191) with lysozyme suspended in an osmotically protective medium, showed '...that at least 85% of the glycerol teichoic acid was present in the wall hydrolysate'. These investigators interpreted these findings as indicating that the so-called intracellular glycerol-teichoic acid is 'probably located in a region between the wall and the protoplast membrane, perhaps in weak association with the latter'.

Thus an area of doubt remained. Therefore attempts were made to increase the osmotic stability of our protoplasts while still insuring essentially complete removal of the cell wall so that the supernatant fluid after digestion of the walls with lysozyme could be obtained relatively free from contamination with other cellular fractions. We were able to do this by making carefully controlled lysozyme digestions in the presence of Mg^{2+} which has been shown by Weibull (1956) to increase the stability of protoplasts. Preliminary experiments indicated that 10^{-2} M-magnesium acetate was the most suitable concentration. These protoplasts are far more stable than are those made in the absence of Mg^{2+} and in fact lyse more slowly after dilution with water or 0·05M-phosphate buffer.

METHODS

Organisms. The strain designated as *Streptococcus faecalis* ATCC 9790 was used throughout these studies. This is a group D streptococcus which is atypical of the *S. faecalis* species designation (Slade & Shockman, 1963*b*; Chesbro, 1961) and perhaps could more properly be designated as *S. faecium*. This strain has the advantage that the cell wall can be digested by lysozyme (Abrams, 1958). With appropriate conditions wall-free protoplasts can be obtained (Shockman *et al.* 1961, 1963).

Conditions for growth and harvest. Cultures were grown in a completely defined medium under conditions of valine limitation and harvested as previously described (Slade & Shockman, 1963*b*; Shockman *et al.* 1963).

Serological procedures. All serological procedures were as previously described (Slade & Shockman, 1963*b*). The capillary procedure of Swift, Wilson & Lancefield (1943) was used, starting with extracts containing 1 mg. dry weight/ml. The group D antigen was extracted from dialysed samples with 0·5 N-HCl at 100° for 10 min. and neutralized to phenol red with NaOH. The specificity of the precipitin reaction for the group D antigen has previously been determined (Slade & Shockman, 1963*b*). A single batch (lot no. 2) of antiserum obtained from the U.S. Public Health Service was used throughout.

Preparation of protoplasts and fractionation procedures. All operations, where possible, were carried out in the cold and followed by phase contrast microscopy. Cocci were grown in 1·2 l. of the defined medium containing 13μg. L-valine/ml. for 17–20 hr at 38° harvested and washed by centrifugation. Approximately 600 mg. (dry weight) of cocci were suspended in 20 ml. of 0·5 M-sucrose, 0·01 M-magnesium acetate, 0·05 M-sodium phosphate buffer (pH 6·6) and 2 mg. lysozyme (lysozyme to cell ratio 1 : 333) and incubated at 38° (Shockman *et al.* 1963). Osmotic fragility was followed by dilution of 0·1 ml. samples to 6 ml. with water. At this low ratio of lysozyme to cocci, maximum osmotic fragility was reached in about 11 hr

in Expt. 1, Table 1. (When diluted with water, 7% of the turbidity at time 0 remained.) Incubation was allowed to proceed for an additional hour to be certain that maximum osmotic fragility was obtained. Phase-contrast microscopy showed single spherical bodies which could be observed under the microscope to swell and burst upon dilution of the 0·5 M-sucrose. After dilution of the sucrose, only empty 'ghosts' could be seen. Streptococcal forms were only very rarely observed in these preparations. Whole protoplasts or streptococci would be far more easily seen in the microscope than are the membrane 'ghosts'. A sample was removed (total) and the remainder was centrifuged at 10,000 *g* for 20 min. The supernatant fluid (lysozyme hydrolysate) was decanted. It did not prove to be possible to resuspend these protoplasts without significant disruption. The protoplasts were then disrupted by dilution to 250 ml. with water containing 1 mg. DNAase. A sample was removed (protoplast lysate) and, after adequate time to insure complete disruption, the remainder was centrifuged at 25,000 *g* for 2 hr. The pelleted crude membranes were washed once with water and recentrifuged as above. A portion of the lysozyme hydrolysate was centrifuged at 120,000 *g* for 2 hr.

A subsequent batch of protoplasts (Exp. 2, Table 1) was prepared using the extremely high lysozyme to cell ratio of 1:2·5 and a 3-hr incubation period. Maximum osmotic fragility was reached in about 2 hr, at which time, when diluted with water about 30% of the turbidity at time 0 still remained. Microscopic examination showed the presence of very small spherical bodies. These protoplasts were extremely stable and, in fact, were difficult to lyse by 1 to 60 dilution with water. However, they did lyse completely in about 40 min. after centrifugation and resuspension in 250 ml. of water (about 8% of the initial turbidity remained).

The release of nucleic acids and derivatives was followed by measuring their ultraviolet absorption and comparing the 260 mμ absorption peaks of the various fractions.

RESULTS

Table 1 shows the results of two typical experiments. Clearly, the once-washed protoplast membrane fraction (line 4) reacts with the group D antiserum to a high titre. Some antigen is present in the wash (line 5). Our previous experiments (Slade & Shockman, 1963*b*) showed that subsequent washings in water, buffer, or in NaCl continued to remove antigen from the membrane. However, with similar cells considerable amounts of antigen remained with the membranes after 10 to 12 washings. It is equally clear that antigen was present in the lysozyme hydrolysate (line 6). A portion of this activity could be sedimented at 120,000 *g*. From the estimated percentage of total cell substance that each fraction represents (last column of Table 1) it is clear that the membrane fraction (27%) represents far more cell substance than does the 120,000 *g* pellet of the lysozyme hydrolysate (5%). The percentage of total cell substance represented by this fraction was directly determined from this experiment on dialysed samples. Therefore, since at least a portion of the supernatant fluid of the lysozyme hydrolysate should be dialysable (Salton 1956, 1960), the estimate of 5% for the pellet should be viewed as a maximum. These titres of antigen could easily have been derived from membrane contamination resulting from a small amount of protoplast breakage during the preparative procedure or even to the presence of a few whole cocci which, if present, would concentrate in this fraction.

Table 1. *Distribution of group D antigen in cell fractions of Streptococcus faecalis*

	Expt. no.	Dilutions of antigen 1 mg./ml. Precipitin reactions 0	1/2	1/4	1/8	1/16	1/32	1/64	Nucleic acids* (% of total)	Cell substance (estimated % of total)
1. Total cell suspension	1	4+	2+	+	±	0	.	.	(100)	100
	2	4+	2+	2+	+	0	0	.		
2. Protoplast lysate	1	3+	+	±	0	0	0	.	89	62†
	2	4+	2+	+	+	+	0	.	81	
3. 25,000 *g* supernatant fluid of protoplast lysate	1	+	±	0	0	.	.	.	86	39†
	2	2+	+	±	0	0	0	.	63	
4. Membrane fraction (25,000 *g* pellet, once washed)	1	4+	4+	4+	3+	+	0	0	.	27†
	2	4+	4+	3+	2+	2+	+	0	.	
5. Wash of membrane fraction	1	2+	+	0	0	0	.	.	4	.
	2	4+	3+	2+	+	0	0	.	5	.
6. Lysozyme hydrolysate (supernatant fluid after protoplast formation)	1	2+	2+	+	±	0	.	.	11	38†
	2	3+	2+	+	+	+	0	.	17	
7. 120,000 *g* supernatant fluid of lysozyme hydrolysate	1	+	+	±	0	.	.	.	8	33‡
	2	3+	+	+	+	0	0	.	16	36‡
8. 120,000 *g* pellet of lysozyme hydrolysate	1	2+	+	+	0	0	.	.	—	5‡
	2	4+	2+	+	+	0	0	.	—	2‡

* Estimated from the absorption peak at 260 mμ of nucleic acids and derivatives.

† Estimated from previous experiments (Shockman *et al.* 1958, 1963; Toennies *et al.* 1963).

‡ Obtained directly from this experiment, on dialysed samples. Because of the small amounts of material available these are only approximations.

The precipitin reactions are based on dilutions of antigen 1 mg/ml. We can then conclude that the antigen in the membrane fraction is largely responsible for the antigen titres extracted from the total cell suspension.

Since these results are in disagreement with those in the literature (Hay *et al.* 1963; Shattock & Smith, 1963) reasons for this disagreement were sought. Since lysozyme is known to be bound by membranes, particularly when present at high concentrations (Weibull *et al.* 1959; Shockman *et al.* 1963) and since at least one of the reports with which we are in disagreement (Hay *et al.* 1963) used a high ratio of lysozyme to cells, this point was investigated further. Two mg. of the membrane fraction from Expt. 1, Table 1, was treated with 2 mg. lysozyme in 0·05 M-phosphate buffer (pH 7·4) at 38° for 2·5 hr. No precipitin reaction was obtained with the supernatant fluid either before or after dialysis.

Since the result of this experiment was negative, an additional isolation experiment, essentially identical to the one previously described, but using a higher lysozyme to cell ratio (1:2·5) and a shorter time period for wall digestion, was performed. The results are shown in Table 1 (Expt. 2) and are in essence the same as those for the first experiment. For unknown reasons a somewhat less clean fractionation was obtained in Expt. 2 as shown by the higher percentage of total cellular nucleic acid which appeared in the lysozyme hydrolysate.

DISCUSSION

It is clear from these results that the major portion of the glycerol-teichoic acid group D antigen in our strain of enterococcus does not seem to be located between the cell wall and the protoplast membrane. The results of the experiments reported here confirm and closely correlate with our previous results (Slade & Shockman, 1963*b*) which were interpreted to indicate a close association of the antigen with the protoplast membrane. The experiments reported by both Shattock & Smith (1963) and Hay *et al.* (1963) with different strains of Group D streptococci are in disagreement with our findings. Hay *et al.* (1963) used determinations of glycerol-teichoic acid by chemical methods on paper chromatograms. No determinations of antigen by the use of antibody were made. In at least some group D organisms there may be other glycerol-teichoic acids present in addition to the group antigen. The fragility of protoplasts and protoplast membranes are definitely complicating factors for an unequivocal interpretation of the origin of the antigenic activity found in the lysozyme hydrolysate of the cell wall. We believe that adequate, although not complete, control of these factors was obtained in the experiments reported. Release of 260 mμ absorbing substances to the supernatant fluid is only a limited and relatively crude measure of damage of the permeability barrier. Control of the fragility of the membranes themselves is more difficult and was evaded in these experiments by simply washing the membrane fraction only once. Our previous series of experiments (Slade & Shockman, 1963*a*, *b*) provided evidence indicating that a large portion of the antigen remains with the membranes of these cells after extensive washings and that release of the antigen from the membrane by washing does not correlate with release of 260 mμ absorbing substances. However, during the washings the membranes do tend to fragment and it is difficult to differentiate between lack of sedimentation of membrane fragments and selective 'elution' of the antigen from the membranes.

Valine-deprived cocci were selected for specific reasons. Cocci grown in this manner seem to contain higher titres of antigen (unpublished observations) as well as more membrane substance (Shockman *et al.* 1963; Toennies *et al.* 1963) than exponentially growing cultures. Other investigators (Medrek & Barnes, 1962) have shown that nutritional and physiological conditions can influence the amount of group antigen extracted from several group D streptococci. Growth conditions resulting in decreased antigen yield might easily influence the observed cellular distribution of the antigens. Also, valine deprived cultures are not prone to autolysis (Shockman *et al.* 1961). Partial autolysis of cocci during harvesting or incubation in buffer might easily further complicate the picture.

Since the completion of these experiments an article by Smith & Shattock (1964) has appeared which presents data in support of their previous conclusion (Shattock & Smith, 1963) that the group D antigen is located between the protoplast membrane and the cell wall. On the basis of the greatest dilution of antigen extracts which showed reaction with antiserum in a slide gel-diffusion test, these investigators found that 80–90% of the group D antigen went into solution during protoplast formation as a result of treatment with a phage-associated lysin. Since neither the capillary precipitin method that we used, or the gel-diffusion test used by Smith & Shattock (1964) was quantitated (for example, on the basis of precipitated protein or nitrogen), we have taken the more conservative view and have expressed our results in a relative manner.

Smith & Shattock (1964) '...concluded that the organism received as ATCC 9790 was an unsuitable strain for investigating the location of the group D antigen'. They examined two cultures of this strain, one received from Dr A. Abrams and one from the National Collection of Industrial Bacteria, and found that they reacted only weakly with group D antiserum. These investigators therefore used other group D strains for their investigations. We were able to obtain strong precipitin reactions with the ATCC 9790 strain, and with the various cellular fractions from this strain, using group specific antisera prepared against four strains of group D streptococci (*S. liquefaciens* (strain 8175), *S. zymogenes* (strain 8176), *S. faecalis* (strain 8213) and *S. durans* (strain 8307)), which represent members of each of the four major groups of streptococci of group D. The results obtained using these four antisera correlated completely with those obtained using USPHS lot no. 2 antiserum (Slade & Shockman, 1963*b*). Perhaps the failure of Smith & Shattock to obtain satisfactory titres with the ATCC 9790 strain can be explained by their use of exponentially growing cultures as starting material. Exponential phase cultures of ATCC 9790 contain less antigen than do stationary phase cultures (Slade & Shockman, 1963*b*, unpublished observations), are prone to autolysis (Shockman *et al.* 1961) and possess membranes of extreme fragility (Slade & Shockman, 1963; Shockman *et al.* 1963; above discussion).

Smith & Shattock were also unable to obtain osmotically-fragile bodies that looked like spherical protoplasts after treatment of other group D streptococci with lysozyme. This is in contrast with our previous results and to those of Abrams (1959) with ATCC 9790 and to those of Chesbro (1961) with a variety of group D strains. Chesbro observed a spectrum of lysozyme sensitivity from resistant to complete sensitivity with a variety of group D strains. As noted above (Expt. 2) a relatively high ratio of lysozyme to cocci (1 : 2·5) can result in protoplasts that are difficult to lyse.

Smith & Shattock did not present data concerning the osmotic stability of their protoplast or spheroplast preparations. It is surprising that these investigators made no attempt to increase stability of membranes of spherical bodies such as by the addition of Mg^{2+}. The known and previously mentioned fragility of membranes plus the ease of removal of the antigen from membranes by washing procedures could easily account for the release of group D antigen to the supernatant fluid during treatment with lysozyme or phage-associated lysin. While it is true that Smith & Shattock used a different group D streptococcus from the one that we used, their experiments did not eliminate the possibility of removal of antigen from the membrane during their experimental procedures.

A hypothetical location of the glycerol-teichoic acid group D antigen on or in the external surface of the protoplast membrane would be consistent not only with our results but also with those of Smith & Shattock, and those of Hay, Wicken & Baddiley. Further resolution of this problem might be materially aided when some information concerning the biological role of glycerol-teichoic acids becomes available.

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Induction and Repression of *Pseudomonas aeruginosa* Amidase

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SUMMARY

The synthesis of an inducible amidase by *Pseudomonas aeruginosa* 8602/A was studied in cultures growing exponentially in succinate medium. Induction by both the substrate inducer acetamide, and the non-substrate inducer *N*-acetylacetamide, was repressed by cyanoacetamide. Induction by 10^{-2}M-*N*-acetylacetamide was significantly repressed by 10^{-4}M-cyanoacetamide, but repression of induction by 10^{-3}M-acetamide required a tenfold excess of cyanoacetamide. Amidase synthesis in a medium in which acetamide was the sole carbon + nitrogen source was also repressed by cyanoacetamide, which under these conditions inhibited the growth of non-induced bacteria. Several tricarboxylic acid cycle intermediates, and related compounds, repressed amidase synthesis in exponentially growing organisms. Catabolite repression by propionate in succinate medium was decreased by increasing the concentration of acetamide. These findings are discussed in relation to general theories of regulation of microbial enzyme synthesis.

INTRODUCTION

Kelly & Clarke (1962) showed that *Pseudomonas aeruginosa* 8602/A produced an aliphatic amidase which hydrolysed acetamide and propionamide. They showed that the enzyme could be induced by either of these amides, and also by certain non-substrate amides, including *N*-methylacetamide and *N*-acetylacetamide. Amides were tested for inducer activity by measuring the amount of enzyme produced after overnight growth of the organism in a medium containing succinate and ammonium chloride, to which the test amide had been added. Under these conditions amidase induction by *N*-methylacetamide was repressed by the addition of cyanoacetamide or thioacetamide to the growth medium. Estimations of enzyme activity after overnight growth of cultures can give only limited information about the course of enzyme synthesis. We have followed amidase induction in cultures of *P. aeruginosa* 8602/A in the exponential phase of growth, with succinate as carbon source. This proved a convenient technique for comparing induction by substrate and non-substrate inducers. Under these conditions it was possible to investigate the nature of amidase repression by amide analogues such as cyanoacetamide, and also repression by general cell metabolites. A brief report of part of this work has appeared previously (Brammar & Clarke, 1963).

METHODS

Organism. The strain used was *Pseudomonas aeruginosa* 8602/A, described by Kelly & Clarke (1962). The culture was maintained on slopes of Lemco agar and subcultured weekly. Stock cultures were stored at 4°.

Growth. The cultures were grown in conical flasks, of capacity 10 times that of the medium volume and shaken vigorously at 37°. To obtain exponentially growing organisms 5 ml. of an overnight culture was used to inoculate 100 ml. of the test medium. Samples (4 ml.) were removed at intervals for determinations of amount of bacteria; they were then stored at 0° and enzyme activities determined at the end of the experiment. The inducers and other test compounds were added at suitable times after the culture had reached the exponential phase of growth, and the experiments were continued over 3–4 generations.

Media. Lemco agar was prepared as described by Clarke & Meadow (1959). The minimal salt medium contained (per litre): K_2HPO_4, 12·5 g.; KH_2PO_4, 3·8 g.; $(NH_4)_2SO_4$, 1·0 g.; $MgSO_4.7H_2O$, 0·1 g.; trace element solution, 5 ml. (Kelly & Clarke, 1962).

Acetamide was sterilized by Seitz filtration and added aseptically to the minimal salt medium to a final concentration of 0·08 M. Most of the experiments with non-substrate inducers were done in minimal salt medium containing 0·05 M-succinate. A few experiments were made with other compounds as carbon source, and these were also added to a final concentration of 0·05 M.

Dry weight of bacteria. The optical extinctions of the cultures were measured at 670 mμ with a Unicam SP 600 spectrophotometer. A standard curve was used to convert the readings to dry weights.

Enzyme assays. The hydrolytic activity of the amidase was measured by Conway's microdiffusion technique (Conway, 1957) as used by Kelly & Clarke (1962). The transferase activity was measured by estimating the amount of acylhydroxamate formed from hydroxylamine and the amide substrate in a standard time. The transferase reaction became the routine method for the enzyme assay and was carried out as follows. Bacterial suspension (0·25 ml.) was added to 0·75 ml. of the substrate mixture, which was prepared by mixing equal volumes of 0·4 M-acetamide solution, 2·0 M-hydroxylamine hydrochloride freshly neutralized, and 0·1 M-tris buffer (pH 7·2). After incubation at 37° for 15 min. the reaction was stopped by adding 2 ml. of a solution of $FeCl_3$ (6%, w/v) in HCl (2%, w/v). The extinction was read at 500 mμ with a Unicam SP 600 spectrophotometer and related to the amount of acethydroxamate formed in the reaction by comparison with a standard curve. Under these conditions the enzyme activity was linear with time over at least 30 min. The substrate concentrations were such as to give complete saturation of the enzyme system. Disruption of the bacteria with acetone, toluene, ultrasonic treatment or with the French press did not increase the specific activity.

Enzyme units. The units of enzyme activity used in this paper are defined as follows. One unit of hydrolase activity corresponds to that amount of enzyme which catalyses the formation of 1 μmole ammonia/min. under the conditions used for the hydrolase assay. One unit of transferase activity corresponds to that amount of enzyme which catalyses the formation of 1 μmole acethydroxamate/min. under the conditions used for the transferase assay.

Amides. Acetamide was obtained from Hopkins & Williams Ltd. and purified by recrystallizing twice from ethanol. *N*-Acetylacetamide was prepared by refluxing acetamide with excess acetic anhydride, removing the residual acetic anhydride by distillation, and twice recrystallizing the product from methylethylketone. Cyanoacetamide was obtained from Hopkins & Williams Ltd. and purified by recrystallizing twice from methanol. The purity of these preparations was tested by melting-point determinations; occasional preparations were also examined by paper chromatography. *N*-Methylacetamide was obtained from Hopkins & Williams Ltd. and was not further purified.

Paper chromatography. Amides were analysed by ascending chromatography on Whatman No. 1 paper in toluene + ethanol (80 + 20, by vol.) solvent. Hydroxamates were produced by spraying with a saturated solution of hydroxylamine hydrochloride in methanol (80 %, v/v) in water and containing NaOH 3 % (w/v). After heating at 100° for 10 min. the papers were sprayed with a solution of $FeCl_3$ (10 %, w/v) + HCl (0·1 %, w/v) in aqueous methanol (95 %, v/v, methanol in water). The hydroxamates appeared as the red ferric complexes.

RESULTS

Comparison of hydrolase and transferase activities

During the earlier investigation of the *Pseudomonas aeruginosa* 8602/A amidase the enzyme activity was measured by estimating the ammonia produced under standard conditions, with propionamide as substrate (Kelly & Clarke, 1962). It had been shown that propionamide was hydrolysed more than twice as rapidly as acetamide, and propionamide was therefore chosen as the more sensitive substrate for enzyme assays. Some studies done with partially purified enzyme preparations suggested that the enzyme also had acyl transferase activity, and could transfer the acyl group of the substrate amides to hydroxylamine to form acylhydroxamates (Kelly, 1961). The transferase showed different substrate affinities, acetamide being more than twice as active as propionamide as a substrate in this reaction.

The two reactions can be represented as follows, with acetamide as the substrate in both reactions:

1. Hydrolase $CH_3CONH_2 + H_2O \rightleftharpoons CH_3COOH + NH_3$
2. Transferase $CH_3CONH_2 + NH_2OH \rightleftharpoons CH_3CONHOH + NH_3$

The transferase assay offered many advantages in sensitivity and speed but, before it could be adopted as a routine assay for the enzyme, it was necessary to show that both activities were due to a single enzyme protein. This was investigated in two ways. First, the relative transferase and hydrolase activities of cultures induced by either a substrate or by a non-substrate inducer, over a wide range of enzyme activities, were compared. Figure 1 shows the results obtained. Transferase activity with acetamide as substrate is plotted against hydrolase activity with propionamide as substrate. Each point represents the enzyme activity of a separate overnight culture. It can be seen that over a wide range of activity there is parallel induction of transferase and hydrolase activities. This result would be expected both for the induction of a single enzyme and also for the co-ordinate induction of two different enzymes whose synthesis was controlled by a single operon. This has been reported

for several systems, the best known being β-galactosidase and β-galactoside transacetylase (Jacob & Monod, 1961).

Secondly, we tried to separate the two activities by the usual methods for protein separation. After ammonium sulphate precipitation followed by chromatography on a column of diethylaminoethyl-cellulose, both activities came from the column with the same protein peak. When such purified preparations were subjected to starch gel electrophoresis, only one protein band was obtained, and both transferase and hydrolase activities were associated with it. Kelly & Kornberg (1962*b*, 1964) reported a constant ratio of propionamide hydrolase to propionamide trans-

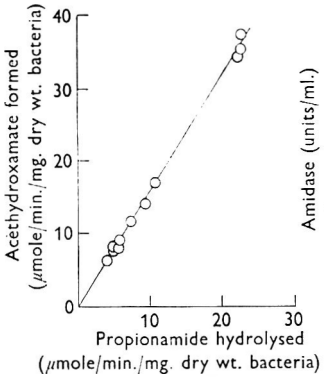

Fig. 1

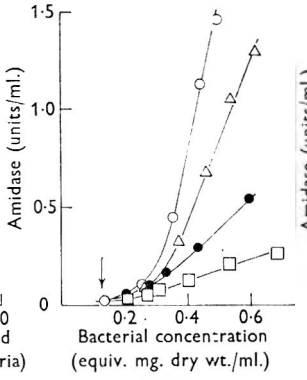

Fig. 2

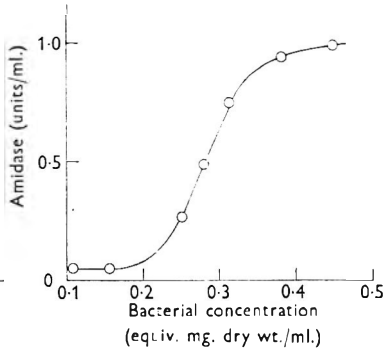

Fig. 3

Fig. 1. Correlation between hydrolase and acyl transferase activities of induced cultures of Pseudomonas aeruginosa 8602/A.

Fig. 2. Amidase induction in Pseudomonas aeruginosa 8602/A by *N*-acetylacetamide in succinate medium. Inducer added at the time indicated by the arrow to exponentially growing cultures. ○—○, 10^{-2}M; △—△, 3×10^{-3}M; ●—●, 10^{-3}M; □—□, 10^{-4}M.

Fig. 3. Amidase induction in Pseudomonas aeruginosa 8602/A by 10^{-3}M-acetamide in succinate medium.

ferase activities at several stages of enzyme purification. We therefore concluded that we were dealing with a single enzyme capable of catalysing both reactions. The term 'amidase' in this paper refers to an enzyme protein with both hydrolase and transferase activities. The transferase assay was used to measure amidase activity in all subsequent work.

Amidase induction by N-acetylacetamide

N-Acetylacetamide was chosen as the non-substrate inducer for studies of the kinetics of amidase induction under conditions of gratuity (Monod & Cohn, 1952). It could be readily prepared in a crystalline form and its purity checked. Succinate was used as the carbon source for growth. This was an arbitrary choice, but it was already known that the organism grew well under these conditions and that several non-substrate amides were effective inducers in this medium (Kelly & Clarke, 1962). Figure 2 shows the results obtained when four different concentrations of *N*-acetylacetamide were added to exponentially growing cultures. Total enzyme activity was plotted against dry weight of bacteria to give the differential rate of enzyme synthesis. Little induction occurred at 10^{-4}M-*N*-acetylacetamide. As the concentra-

tion of inducer was increased the differential rate of enzyme synthesis increased. The highest concentration of inducer shown on the graph is 10^{-2}M, and there was no increase in the differential rate of amidase synthesis when the concentration was increased beyond this value.

It was assumed that the system was saturated at 10^{-2}M-*N*-acetylamide and this was used as the standard concentration for investigating the effects of repressors on amidase synthesis. At the saturating concentration there was a lag of about one generation before enzyme synthesis started. Amidase synthesis then continued at a constant differential rate during the experimental period. There was a similar lag of about one generation before enzyme synthesis started when *N*-methylacetamide was used as the inducer under these conditions. The saturating inducer concentration for *N*-methylacetamide was also approximately 10^{-2}M.

The long lag before enzyme synthesis started might be a function of the carbon source used. The induction kinetics when the organism was growing on other carbon compounds was then examined. 10^{-2}M-*N*-acetylacetamide was added to cultures growing exponentially in minimal media containing malate, citrate, pyruvate or glycerol as carbon source for growth. The mean generation times in these media varied from 42 min. with succinate or citrate to 220 min. with glycerol. In all cases a lag occurred after the inducer had been added and before amidase synthesis started. The shortest lag was 0·25 of a generation time with pyruvate as carbon source. With all the other compounds the lag was about one generation, as with succinate.

Amidase induction by acetamide in succinate medium

Acetamide was a very effective inducer in succinate medium. Amidase synthesis could be detected in response to the addition of as little as 10^{-5}M-acetamide, although this small amount was soon metabolized. Figure 3 shows the results obtained with 10^{-3}M-acetamide as inducer. There was again a lag of about a generation, followed by a period of constant differential rate of amidase synthesis. This rate was not maintained during the experimental period, and it can be seen from Fig. 3 that it rapidly decreased. The change of rate corresponded to the time at which the acetamide would have almost disappeared from the medium.

In these experiments we were attempting to compare substrate and non-substrate inducers under conditions as similar as possible. In succinate medium, although acetamidase is not necessary for the growth of the culture, enzyme synthesis is not strictly gratuitous, since the acetamide will be hydrolysed and metabolized as soon as some amidase has been synthesized. It is therefore not possible to keep the acetamide concentration constant over a long period of time. It was concluded that if measurements were made during the early stages of enzyme induction it would be possible to compare substrate and non-substrate inducers by following amidase synthesis in succinate medium.

Repression of amidase synthesis by amide analogues

Kelly & Clarke (1962) showed that cyanoacetamide and thioacetamide repressed amidase induction by *N*-methylacetamide during overnight growth of *Pseudomonas aeruginosa*. These amide analogues also repressed amidase synthesis in exponentially growing cultures; Fig. 4 gives the results of a typical experiment. 10^{-2}M-*N*-acetylacetamide was added to a culture growing exponentially in succinate medium.

Soon after enzyme synthesis had started, the repressor amides were added to the same final concentrations as the inducer. Both cyanoacetamide and thioacetamide repressed amidase induction, and the rate of enzyme synthesis decreased almost at once; there was no effect on the growth rate. Figure 5 shows that cyanoacetamide produced a considerable repression of amidase induction by *N*-acetylacetamide even when it was present at only one hundredth of the concentration of the inducer.

Amidase induction by acetamide was also repressed by cyanoacetamide. In this case addition of cyanoacetamide to an exponentially growing culture at the same initial concentration as the inducer had no effect on the rate of amidase synthesis. It was necessary to increase the ratio of cyanoacetamide to acetamide at least tenfold to obtain significant repression. Figure 6 gives the results of an experiment in which the initial concentration of the inducer acetamide was 10^{-3} M and that of the repressor cyanoacetamide was 10^{-2} M. It can be seen that there was a marked repression of enzyme synthesis under these conditions.

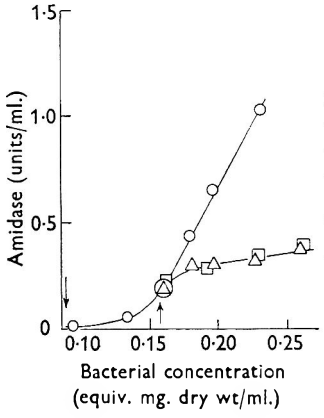

Fig. 4

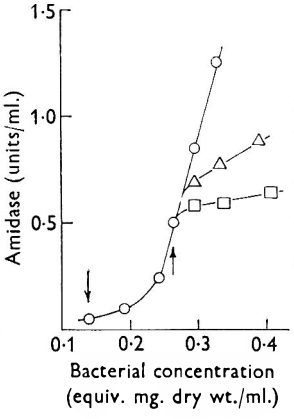

Fig. 5

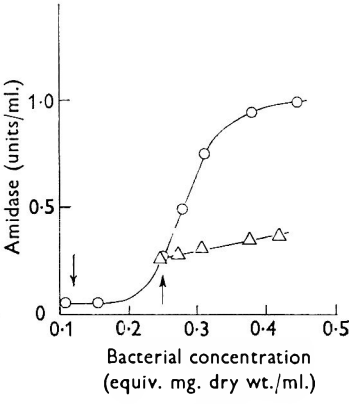

Fig. 6

Fig. 4. Effect of cyanoacetamide and thioacetamide on amidase induction in Pseudomonas aeruginosa 8602/A. 10^{-2} M-*N*-acetylacetamide added to an exponentially growing culture at time indicated by the first arrow. At the time indicated by the second arrow, the culture was split into three parts, and the following additions made: ○—○, no addition; △—△, 10^{-2} M-cyanoacetamide; □—□, 10^{-2} M-thioacetamide.

Fig. 5. Cyanoacetamide repression of amidase induction in Pseudomonas aeruginosa 8602/A by *N*-acetylacetamide. At the time indicated by the first arrow, 10^{-2} M-*N*-acetylacetamide was added to an exponentially growing culture. At the time indicated by the second arrow, the culture was divided into 3 parts, and the following additions made: ○—○, no addition; △—△, 10^{-4} M-cyanoacetamide; □—□, 10^{-2} M-cyanoacetamide.

Fig. 6. Cyanoacetamide repression of amidase induction in Pseudomonas aeruginosa 8602/A by acetamide. At the time indicated by the first arrow 10^{-3} M-acetamide was added as inducer. At the time indicated by the second arrow, the culture was split into two parts: ○—○, control culture; △—△, 10^{-2} M-cyanoacetamide added.

These experiments showed that cyanoacetamide repressed amidase synthesis in a similar way, whether acetamide or *N*-acetylacetamide was used as inducer. Because of the structural similarity between these amides, mutual competition for an amide-binding site seemed possible. If this were the case, it should be possible to annul the repression by using a sufficient concentration of inducer analogue. The

results of an experiment to test this possibility are shown in Fig. 7. An exponentially growing culture was divided into three parts, to which *N*-acetylacetamide was added to give final concentrations of 10^{-3}M, 5×10^{-3}M and 2×10^{-2}M. At the points marked on Fig. 7, cyanoacetamide was added to a final concentration of $1{\cdot}2 \times 10^{-4}$M in each case. The results (Fig. 7) showed that cyanoacetamide repression of induction was prevented by an approximately 200-fold excess of *N*-acetylacetamide.

The results shown in Figs. 5, 6 and 7 suggest that cyanoacetamide repressed amidase synthesis by competing with the inducer, acetamide or *N*-acetylacetamide for an inducer-recognizing site. The affinities of the amides for such a site would appear to be in the order acetamide > cyanoacetamide > *N*-acetylacetamide.

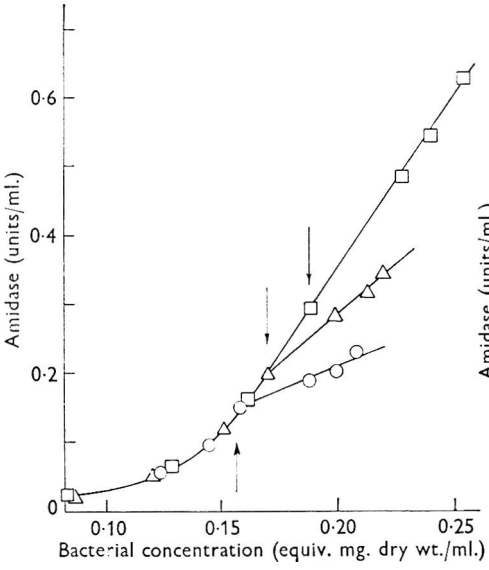

Fig. 7

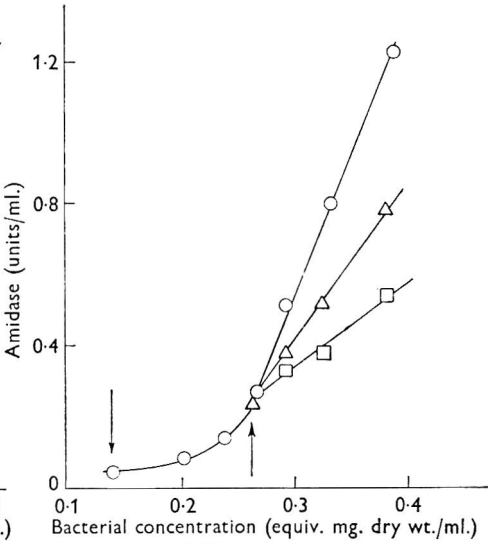

Fig. 8

Fig. 7. Effect of concentration of *N*-acetylacetamide on repression of amidase synthesis in Pseudomonas aeruginosa 8602/A by cyanoacetamide. *N*-Acetylacetamide was added to 3 flasks early in exponential growth: ○—○, 10^{-3}M; △—△, 5×10^{-3}M; □—□, 2×10^{-2}M; at the times indicated by the arrows, $1{\cdot}2 \times 10^{-4}$M-cyanoacetamide was added to each flask.

Fig. 8. Catabolic repression of amidase induction in Pseudomonas aeruginosa 8602/A. 10^{-2}M-*N*-acetylacetamide was added to an exponentially growing culture in succinate medium. At the time indicated by the second arrow, the culture was split into three parts, and the following additions made: ○—○, control culture, no additions; △—△, 10^{-2}M-acetate; □—□, 10^{-2}M-propionate.

Catabolite repression of amidase synthesis

The synthesis of many induced enzymes has been shown to be repressed by glucose or other cell metabolites. This repression was known for a long time as 'the glucose effect', but is more correctly described as catabolite repression (Magasanik, 1961). In a few cases it has been shown that the repression is specific, and that the catabolite repressor is a common intermediate in the metabolic pathways of both glucose and the normal substrate of the induced enzyme (McFall & Mandelstam, 1963). We

examined the effect of various cell metabolites on amidase induction, using either acetamide or *N*-acetylacetamide to induce enzyme synthesis.

With cultures of *Pseudomonas aeruginosa* 8602/A growing exponentially in succinate medium, and induced with 10^{-2}M-*N*-acetylacetamide, there was marked repression of amidase synthesis by tricarboxylic acid cycle intermediates and metabolically related compounds. Figure 8 shows that the most effective repressor under these conditions was propionate, which at 10^{-2}M caused 70% repression of amidase synthesis. The same concentration of citrate, pyruvate or malate resulted in 60% repression, and acetate was slightly less effective. These effects were not transient, but persisted through the experimental period, even though the concentration of the repressor compounds would be decreased by cell metabolism. Enzyme induction by acetamide was also repressed by adding these compounds shortly after amidase synthesis had been induced.

We attempted to annul catabolite repression of amidase synthesis by increasing the concentration of substrate and non-substrate inducers. With *N*-acetylacetamide we were unable to find any effect. There were technical difficulties in carrying out this experiment, since the saturating concentration of *N*-acetylacetamide required for induction was so high. Propionate was metabolized rapidly under these conditions, and concentrations lower than 10^{-2}M could not be used. When the propionate concentration was kept constant at about 10^{-2}M and the inducer concentration increased, it would have been necessary to take the *N*-acetylacetamide concentration to unreasonably high values to reach a significant excess. It was thought that acetamide might be a more suitable inducer for this purpose, since the results with amide-analogue repression had indicated that it had a much higher affinity for an inducer-binding site than had *N*-acetylacetamide. Cultures growing exponentially in succinate medium were induced with various concentrations of acetamide. When a constant differential rate of amidase synthesis had been reached, propionate was added to some of the flasks. Table 1 summarizes the results obtained. Amidase induction by 10^{-3}M and 3×10^{-3}M-acetamide was more than 90% repressed by 3×10^{-2} M-propionate; with 10^{-2}M-acetamide as inducer, repression by this concentration of propionate was only 65%. Under these conditions there would be a high concentration of metabolites in the cell, and complete de-repression by additional inducer would be unlikely. Preliminary experiments with carbon-starved bacteria showed that catabolite repression was annulled by increasing the inducer concentration.

Amidase synthesis required for growth of the culture

In all the previous experiments, amidase induction by acetamide was studied under conditions in which the enzyme was not necessary for the growth of *Pseudomonas aeruginosa*, since the medium already contained amounts of carbon and nitrogen compounds adequate to maintain growth. We also examined the kinetics of amidase synthesis under conditions in which the organism could grow only if it could synthesize this enzyme.

A culture of *Pseudomonas aeruginosa* 8602/A grown overnight in succinate medium has a very low basal amidase activity. When succinate-grown organisms are inoculated into a medium containing acetamide as the sole carbon and nitrogen source, there is an appreciable growth lag. During this time, amidase synthesis occurs and some of the acetamide is hydrolysed. When the amidase content of the organisms

has reached a significant value, the culture begins to grow and, soon after multiplication has become established, the acetamide originally added is completely hydrolysed. The amidase is then no longer synthesized and becomes diluted out, and the culture continues to grow on the acetate and ammonia.

Table 1. *Effect of acetamide concentration on catabolite repression of amidase synthesis by Pseudomonas aeruginosa 8602/A*

Acetamide concentration (mM)	Propionate concentration (mM)	Differential rate of amidase synthesis (units/ml./mg. dry wt.)	Repression (%)
1	0	17·5	—
1	30	1·2	93
3	0	17·5	—
3	30	1·5	91
10	0	17·0	—
10	30	5·8	65

The pattern of amidase synthesis in acetamide medium is, however, more complex than this, since at the end of the exponential growth phase, and long after acetamide has disappeared from the growth medium, there is a second stage of amidase synthesis. We have observed this second phase of amidase synthesis, at the same growth stage, with cultures growing with acetate as carbon source, but with no other carbon compounds. Figure 9 shows the course of amidase synthesis, growth and acetamide disappearance. Kelly & Kornberg (1962*a*) reported this phenomenon, and were able to show cyanoacetamide repression of the second phase, but not of the first phase of amidase synthesis. They showed that the enzymes produced at the two growth phases had identical properties (Kelly & Kornberg, 1964).

It had become clear from our results on amidase induction by acetamide in succinate medium that the relative concentrations of the inducer and repressor were extremely important in determining whether or not enzyme synthesis would take place. We therefore tried to repress the first phase of induction by using a higher concentration of cyanoacetamide. Figure 10 shows that amidase synthesis was completely repressed when 0·5 M-cyanoacetamide was added to a medium which contained 0·02 M-acetamide as carbon source. The concentration of cyanoacetamide required to repress the first phase of amidase induction in acetamide medium was very high. It therefore seemed possible that this was a non-specific effect, and that cyanoacetamide was a general inhibitor of cell metabolism. This possibility was examined by comparing the behaviour of pre-induced and non-induced bacteria. *Pseudomonas aeruginosa* 8602/A was grown overnight in acetamide medium, to give pre-induced bacteria, and in succinate medium to give non-induced bacteria. The pre-induced bacteria were inoculated into medium containing 0·02 M-acetamide + 0·5 M-cyanoacetamide. The bacteria already contained enough amidase to hydrolyse the acetamide in the medium and, after a short lag, normal growth occurred.

When non-induced bacteria were inoculated into this medium, growth was completely inhibited. In the absence of cyanoacetamide, the bacteria grew normally after a short lag, during which time amidase was synthesized. From these results (see Fig. 11) it is clear that cyanoacetamide is not a general growth inhibitor, even at

0·5 M, and that the inhibition of growth of the non-induced bacteria in acetamide medium was due to specific repression of amidase synthesis.

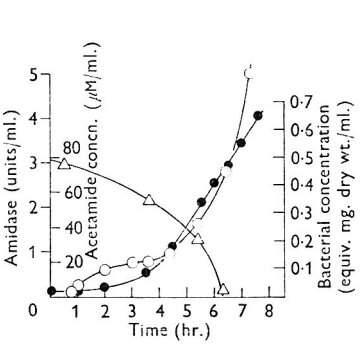

Fig. 9

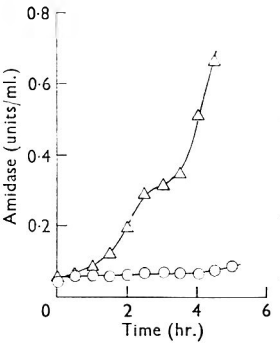

Fig. 10

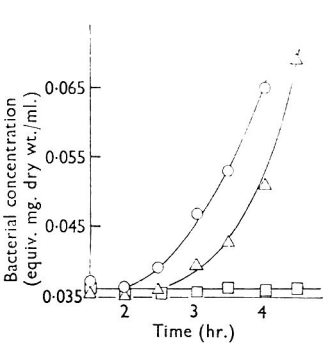

Fig. 11

Fig. 9. Growth and amidase synthesis of Pseudomonas aeruginosa 8602/A in acetamide medium. Succinate-grown organisms were inoculated into a medium containing 0·08 M-acetamide. ●—●, Growth as dry wt. bacteria/ml.; ○—○, amidase as total enzyme units/ml.; △—△, acetamide concentration.

Fig. 10. Effect of cyanoacetamide on amidase synthesis in Pseudomonas aeruginosa 8602/A in acetamide medium. Succinate-grown organisms were inoculated into acetamide medium: △—△, control culture 0·02 M-acetamide; ○—○, 0·02 M-acetamide + 0·5 M-cyanoacetamide.

Fig. 11. Effect of cyanoacetamide on growth of pre-induced and non-induced Pseudomonas aeruginosa 8602/A in acetamide medium: ○—○, pre-induced organisms + 0·02 M-acetamide + 0·5 M-cyanoacetamide; △—△, non-induced organisms + 0·02 M-acetamide; □—□, non-induced organisms + 0·02 M-acetamide + 0·5 M-cyanoacetamide.

DISCUSSION

The results obtained in this study of amidase synthesis by *Pseudomonas aeruginosa* 8602/A confirm the view that enzyme induction by substrate and non-substrate inducers follows essentially the same pattern. Acetamide and *N*-acetylacetamide induced amidase synthesis in cultures growing exponentially in succinate medium, and this induction by both amides was repressed by cyanoacetamide. The main difference between the two inducers is that acetamide is an effective inducer at a much lower concentration than is *N*-acetylacetamide. Induction of amidase by acetamide is repressed by a tenfold excess of cyanoacetamide, whereas induction by *N*-acetylacetamide is repressed by one-hundredth of its concentration of cyanoacetamide. Amidase synthesis in acetamide medium was also repressed by cyanoacetamide at high concentrations. Kelly & Kornberg (1962*a*) found that the initial phase of amidase synthesis in medium containing 5×10^{-2} M-acetamide was not affected by 5×10^{-3} M-cyanoacetamide, and we have found that it was necessary to increase the concentration to at least 20-fold excess of cyanoacetamide to show any repression. These results with acetamide and the two amide analogues suggest that they have different affinities for the same site, and that repression by cyanoacetamide is due to competition with the inducers for a site concerned with the binding of inducer and the initiation of enzyme synthesis.

We are unable to define the site at which cyanoacetamide competes with the amide inducers from these experiments, but there are at least two possibilities

which have been suggested from the results of work with other systems. Experiments on β-galactosidase synthesis in *Escherichia coli* (Cohn & Monod, 1953) and *Staphylococcus aureus* (McClatchy & Rosenblum, 1963) have shown competition between inducer-analogues and inducers. Similar competition has been shown for the induction of the galactose enzymes of *E. coli*, where methyl-β-D-thiogalactoside represses the induction of the galactose group of enzymes by competing with the non-substrate inducer fucose (Buttin, 1963).

One possible site of competition would be a specific permease. This appears to be the case in the *Escherichia coli* β-galactosidase system, when arylthiogalactosides are added to cultures induced by methyl-β-D-thiogalactoside. We think it unlikely that cyanoacetamide represses induction of the amidase of *Pseudomonas aeruginosa* 8602/A by competing with an amide permease. Cyanoacetamide has no effect on the growth of pre-induced organisms in acetamide medium, and preliminary experiments have shown that it has no effect on the entry of radioactively labelled *N*-acetylacetamide.

Current theories of enzyme induction suggest that the inducer combines with a cytoplasmic repressor molecule, thereby releasing the structural genes from repression and allowing enzyme synthesis to occur (Jacob & Monod, 1961). Another possible site of competition of inducer analogues with inducers would be the inducer-binding site of the cytoplasmic repressor. Buttin (1963) found that the thiogalactosides had no effect on galactose permease, and concluded that the site of competition was within the cell. It would appear that amide analogue competition with amidase inducers also occurs at a site within the cell, which may be the cytoplasmic repressor.

The catabolite repressor could act at the site of entry of the inducer into the cell. Kessler & Rickenberg (1963) suggested that several metabolites may compete in this way with α-glucosides. Any effect of this sort would be overcome by increasing the inducer concentration. Alternatively, the catabolite repressor could act at a site on the pathway between the structural gene and the completed enzyme protein. We are rather surprised that amidase synthesis in *Pseudomonas aeruginosa* 8602/A can be so readily induced in a medium containing succinate, which would be expected to be a good precursor of catabolite repressors. With both substrate and non-substrate inducers there is a lag of about a generation before amidase synthesis can be detected. It seems possible that this amidase is normally under repression involving a metabolite closely related to the tricarboxylic acid cycle intermediates. Part of the explanation for the second-phase amidase induction in acetate medium may be that the culture is being released from catabolite repression. This cannot be the whole story, however, since the second-phase enzyme synthesis does not occur with any other carbon source we have tested.

In discussions of the regulation of enzyme synthesis, the process of enzyme induction is usually described as being diametrically opposed to that of end-product repression. Jacob & Monod (1961) postulated that, in an inducible system such as β-galactosidase, the regulator gene produces a cytoplasmic repressor molecule which prevents gene transcription until it is removed from the operator by combination with the low molecular weight inducer. In a typically repressible system, such as the enzymes of the histidine biosynthetic pathway, they postulated that the regulator gene produces an inactive cytoplasmic repressor, which requires to be combined with the low molecular weight co-repressor before it can combine with the

operator and prevent gene transcription. McFall & Mandelstam (1963) have demonstrated the specificity of catabolite repression. Gorini (1963) has shown that some of the enzymes of the arginine biosynthetic pathway are induced by glutamic acid and repressed by arginine. We think it likely that the synthesis of amidase is controlled by induction by amide inducers, and repression by certain compounds in the metabolic pool. We would predict that all catabolite repression would be overcome by inducer, if the inducer concentration could be raised to a sufficiently high value. This does not require a particular model for inducer and metabolite repressor sites of action, but, not wishing to multiply regulatory sites unnecessarily, we see no reason why they should not both act at the level of the cytoplasmic repressor. Recent work by Nakada & Magasanik (1964) and Attardi, Naono, Rouvière, Jacob & Gros (1963) supports the view that catabolite repression of β-galactosidase synthesis acts at the same stage as inducer, that is at the level of gene transcription to produce the specific messenger RNA. Loomis & Magasanik (1964) conclude that the catabolite in glucose repression of β-galactosidase does not react with the cytoplasmic repressor produced by the *i* gene, but that another regulator molecule is involved.

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A Study of the Mallomonas, Synura and Chrysosphaerella of Northern Iceland

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SUMMARY

Species of the genera *Mallomonas*, *Synura* and *Chrysosphaerella* have been investigated in relatively few countries, and a study of their occurrence in Iceland provides additional information on their distribution. Several new species of *Mallomonas* (*M. calceolus*, sp.nov.; *M. pugio*, sp.nov.; *M. clavus*, sp.nov., *M. maculata*, sp.nov.) and one of *Chrysosphaerella* (*C. multispina* sp.nov.) were found together with numerous species already described. In this paper, observations with both light and electron microscopes are given in detail for the new species. Electron micrographs of the scales and bristles of known species are also shown and, where appropriate, compared with those of the new ones. Three out of the four new species of Mallomonas fitted into the existing taxonomic groups, one belonging to the Tripartitae, one to the Planae and one to the Torquatae. The fourth was added to a group already containing three isolated species. The new Chrysosphaerella species is of particular interest because only a few species have so far been described in this genus.

INTRODUCTION

The three genera of the Chrysophyceae investigated tend to favour rather cold climates and have been studied by workers in Central Europe, England, Denmark, the Soviet Union, Japan and Alaska. Because of its climate, Iceland seemed likely to provide a particularly suitable habitat and to add to our knowledge of these organisms. For practical reasons it was not possible to study their distribution over a wide area; the region covered was in Northern Iceland around Akureyri. Some thirty-five ponds, pools and ditches were sampled and, while the occurrence of live organisms was relatively rare, identifiable scales and bristles were found in twenty-three samples. The present paper discusses and describes the species found and how the new ones fit into the existing scheme of classification (Harris & Bradley, 1960).

METHODS

Preliminary observations with the light microscope were made in the field. Samples (about 12 ml.) were collected from ponds, pools and ditches and centrifuged at about 1500 rev./min. for 15 min. in a battery-driven centrifuge to concentrate organisms, which were resuspended in 0·2–0·5 ml. of water. A little of the suspension was transferred to a slide and examined with the light microscope. The remainder was dried down on slides for later examination in the electron microscope. In addition, organisms from each sample were disintegrated by placing a small

crystal of chloral hydrate in the drop of suspension on the slide before drying. Thus, preparations containing whole and disintegrated organisms were available for electron microscopy.

Specimens were prepared for electron microscopy as follows. A layer of carbon about 200 Å thick was deposited on the microscope slide with the organisms on it. This was then backed with a layer of Bedacryl 122 X (made by I.C.I. Ltd.) by allowing a 5% solution of the plastic in benzene to flow over the slide, which was drained vertically and allowed to dry. Small circles were then scored in the combined film in the areas containing organisms. These portions were floated on to a water surface and picked up on support grids. The Bedacryl backing was finally washed away by immersing the grids in chloroform for 15 min. This procedure transferred the organisms, scales and bristles from the microscope slides to the support grids. Replicas were obtained by floating the grids, carbon film uppermost, on dilute hydrofluoric acid for 15 min. The replicas were subsequently shadowed with platinum or gold + palladium alloy.

Particular care was taken in appropriate cases (e.g. *Mallomonas clavus*) to ensure that scales figured from the electron microscope belonged to species drawn from the light microscope.

The pH values of the water samples were taken from the water itself rather than from the subsoil.

MALLOMONAS

The species of Mallomonas are grouped and described according to the classification and nomenclature proposed by Harris & Bradley (1960).

Series I. Tripartitae

Mallomonas calceolus sp.nov. (Figs. 1, 2; Pl. 1, figs. 1–5)

Diagnosis. Cellula ovoides, post et ante rotundata. Setis contecta brevibus et gracilibus. Chromatophoros valde bilobata, viridis. Flagellum fere sesqui longius quam corpus. Squamae parvae, ovatae, tripartitae, unaquaque setam ferens. Fundus squamae paucis punctis et papillis conspicuis signatus. Setae breves, fere altero tanto longiores quam squamae, leves, bicipites.

Dimensiones. Cellula 11–18 $\mu \times 9$–12 μ, squamae 3–4 $\mu \times 1$–2 μ, setae anteriores circa 15 μ, setae corporis 4–6 μ.

English diagnosis. Cell ovoid, rounded both ends. Covered all over with short slender bristles. Chromatophore strongly bilobed, green. Flagellum about one and one-half times body length. Scales small, oval, tripartite, with one bristle attached to each. Base plate of scale has a few puncta and well-marked papillae. Bristles short, about twice scale length, smooth but with divided tip.

Occurrence. Found under ice in mid-January 1962 in a flooded pasture at Leadburn, Midlothian, Scotland; Nat. Grid 237/562; the water, dung-contaminated, was at pH 7·0. The localities in Iceland (July 1963) were Kleif, Arskogsströnd (live organisms); Hverhóll, Skidadalur and Mordruvellir provided scales and bristles only. These waters were also at pH 7·0. Mrs K. Harris (personal communication) found it at Highclere, Berkshire, England, in 1957 and 1962.

Name derivation. The name 'calceolus' refers to the slipper-like shape of the scales.

Additional characters. Mallomonas calceolus is small and insignificant in the light microscope, the bristles of the living organism being barely discernible; the deeply bilobed chromatophore is clearly visible. When dried down on to a microscope slide, the organism readily disintegrates without the need to add chloral hydrate. The scales and bristles can be discerned (Fig. 2) provided the pond water is relatively free from soluble matter, which easily obscures them on drying; little structure can be seen on the scales other than a thickening at either end.

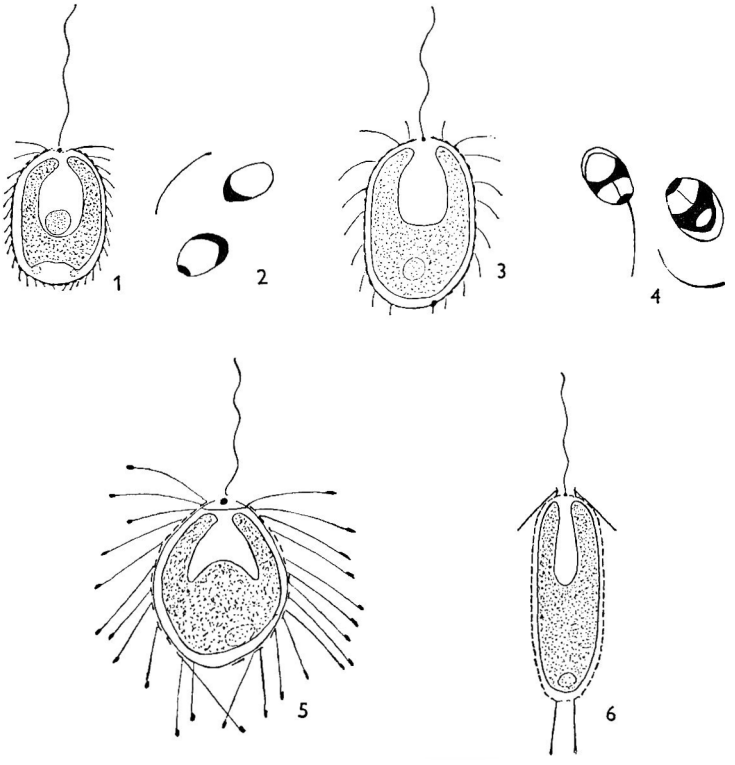

Fig. 1. Mallomonas calceolus, mature cell, × 1000.
Fig. 2. M. calceolus, scales and bristles, × 2000.
Fig. 3. M. pugio, mature cell, × 1000.
Fig. 4. M. pugio, scales and bristles, × 2000.
Fig. 5. M. heterospina, mature cell from Mordruvellir, × 1000.
Fig. 6. M. clavus, mature cell, × 1000.

Electron micrographs of shadowed and unshadowed scales and bristles clearly reveal their structure (Pl. 1, figs. 1–3). The shadowed replica of the underside of a scale in Pl. 1, fig. 4, shows that it is smooth except for a depression beneath the dome to which the bristle is attached. This species clearly belongs to the Papillosa group of the Tripartitae (Harris & Bradley, 1960). A fully developed and an immature scale are shown in Pl. 1, fig. 5.

Mallomonas acaroides Perty (Pl. 1, figs. 6, 7; Pl. 2, figs. 8–12, 15)

This species has been well studied in the electron microscope (Asmund, 1959; Harris & Bradley, 1960) and no less than three varieties have been described. These are compared in both the above works, but the pertinent differences may be mentioned here. *M. acaroides* var. *craquissima* is the most easily distinguished; its scales are readily differentiated from the other two varieties in that they are heavier and well reticulated (Pl. 1, fig. 6). The differences between the other two, *M. acaroides* var. *striatula* and var. *galeata* are slight. Their scales are identical and much more delicate than those of *M. acaroides* var. *craquissima* (Pl. 1, fig. 7). However, *M. acaroides* var. *striatula* has two types of bristle, one with and one without a 'helmet' tip, this being shown in Pl. 1, fig. 7. *M. acaroides* var. *galeata* has only helmet bristles.

Mallomonas acaroides var. *galeata* appears to be common in Northern Iceland, and as a result it has been possible to carry out a study of bristle variation. In a population from Hverhóll, Skidadalur, bristles were found with variations in the shape of their helmet tips. None of the toothed bristles typical of *M. acaroides* var. *striatula* could be found so it was concluded that the variety present was 'galeata'. A series of electron micrographs illustrating the bristle tips is shown in Pl. 2, figs. 8–11. In Pl. 2, fig. 8, there is a simple pointed tip with some thinning. Plate 2, fig. 9, shows a stage where the pointed tip has become more extended and sharper, and a widening of the shaft resembling the hood of a cobra has begun to appear just beneath it. The bristle in Pl. 2, fig. 10, is much further advanced, the typical helmet shape beginning to form. It seems, however, that this is an abnormal tip since the lower portion of the helmet has developed in the wrong way. This can be seen clearly by comparing it with the fully formed tip in Pl. 1, fig. 7. Pl. 2, fig. 11, also appears to be abnormal, the top part of the helmet having developed incorrectly.

Mallomonas acaroides var. *craquissima* bristles were found in three localities, near Kleif, Másvatn, and Myvatn, the last being the most productive of live organisms. Two kinds of helmet bristle were found here (Pl. 2, figs. 12, 15) with and without serrations. No serrated bristles without helmets could be found.

The observations described here emphasize the variation in bristle structure which may be found in all varieties of *Mallomonas acaroides*. The varieties 'striatula' and 'galeata' rely upon bristle structure for their identification; the main criterion is that the former possesses serrated bristles without helmets, whereas the latter does not. These two varieties cannot therefore be identified from scales alone.

Other species

Mallomonas papillosa (Harris & Bradley, 1957) was the third species of the Tripartitae identified. It was found in several localities: Kleif, Skidadalur and Svarfadadalur. The scales showed the characteristic wide variation in structure. Scales of *M. papillosa* forma *annulata* (Harris & Bradley, 1960) were found near Akureyri. A few scales of *M. striata* var. *serrata* (Harris & Bradley, 1960) were found at Másvatn (Pl. 2, fig. 16).

In addition, scales of two hitherto undescribed species belonging to the Tripartitae were found. The first, from Másvatn and Myvatn (Pl. 2, figs. 14, 17), has

also been found near Reading, Berkshire, and will be described in detail in a future publication (Mrs K. Harris, personal communication). The honeycomb structure of the scale is an obvious characteristic which permits its quick identification in the electron microscope. The second species (Pl. 2, fig. 13) has not previously been figured. It is considered that the illustration of isolated scales of undescribed species is of value even if there are insufficient data for a full description; information is provided about the distribution of the species if it is subsequently described in detail.

Series II. Planae

Mallomonas pugio sp.nov. (Figs. 3, 4; Pl. 3, figs. 22–25)

Diagnosis. Cellula ovoides vel paene rotundata. Chromatophoros profunde lobata. Flagellum fere duabus tertiis partibus longius quam corpus. Cellula setis brevibus, subtilibus, paullo arcuatis contecta. Squamae ovatae et subtiles, cupola satis magna et costata, proprie reticulatae ut ex costis prominentibus compositae. Pars distalis vulgo in duas, aliquando in tres regiones divisa. In cupola costae septum vel octo, axi longo squamae parallelae. Setae breves, acutissimae, uno parvo dente aptatae.

Dimensiones. Cellula 12–20 $\mu \times$ 10–15 μ, squamae 4–6 $\mu \times$ 2·5–3·5 μ, setae 5–6 μ.

English diagnosis. Cell ovoid to nearly spherical. Chromatophore deeply lobed. Flagellum about two-thirds body length. Cell covered with short, slightly curved, delicate bristles. Scales oval and delicate with fairly large ribbed dome and characteristic reticulation, consisting of raised ribs. The distal portion is usually divided into two, sometimes three areas. Ribs on dome seven or eight, parallel to the long axis of the scale. Bristles short, very sharply pointed with single small tooth.

Occurrence. Found only at Hverhóll, Skidadalur, near Dalvik, Iceland, in July 1963. It was well distributed in several stagnant pools (pH 7·0–7·2) over a large area of a valley floor.

Name derivation. The name 'pugio', meaning dagger, refers to the form of the bristles.

Additional characters. Mallomonas pugio appears in the light microscope as a delicate organism slightly larger than *M. calceolus*, the bristles being clearly visible (Fig. 3). It readily disintegrates on drying and the scales show their easily recognizable characteristic markings and the dome which appears as a thickened patch at the distal end (Fig. 4).

The appearance of the scales and bristles in the electron microscope is quite distinctive (Pl. 3, figs. 22–25). Some scales show variations such as the form of the ribs on the dome (Pl. 3, fig. 22). A very few are in the form of flat reticulated oval discs (Pl. 3, fig. 23). It is not known to which part of the cell these scales belong. One scale in Pl. 3, fig. 24, appears to have two bristles; it is believed that the second one has drifted into this position during drying and that the live organism has only one bristle per scale.

Mallomonas heterospina Lund (1942) (Fig. 5; Pl. 2, fig. 18; Pl. 3, figs. 19–21).

This was the only other species of the Planae series to be found, the locations being at Kleif, Skidadalur (Hverhóll) and Mordruvellir. The live organisms from Mordruvellir (Fig. 5) showed the spanner-like hooked bristles well in the light microscope and there was no difficulty about recognizing the species. Both hooked and

straight bristles were found at Kleif (Pl. 3, figs. 19, 21), but only hooked bristles at Skidadalur and Mordruvellir (Pl. 3, fig. 20).

Comparison of Mallomonas pugio, Mallomonas heterospina, and Mallomonas multiunca. The first species described in this group was *Mallomonas heterospina* Lund (1942), who used only the light microscope. The hooked and straight bristles were visible. *M. multiunca* was described by Asmund (1956) and compared to *M. heterospina* with both light and electron microscopes. Harris & Bradley (1957, 1960) also compared the two species, and, while they resemble one another in some characteristics such as having hooked bristles and having rather similar scales, it seems that *M. heterospina* and *M. multiunca* are taxonomically distinct. *M. pugio* has scales which are superficially similar to both the above species in some respects. The three species clearly form a group and the main features of interest are given in Table 1. The fine structure of the scales is quite characteristic for each species, and that of *M. pugio* may even be clearly recognized in the light microscope. The live organism of *M. pugio* is not distinctive in the light microscope, unlike the other two species, because of its lack of hooked bristles. It could only be confused with *M. heterospina* and *M. multiunca* when these are juvenile, in which condition they possess no hooked bristles. No hooked bristles were found in any population of *M. pugio*.

Table 1. *Features of Mallomonas heterospina, M. multiunca and M. pugio*

	M. heterospina	*M. multiunca*	*M. pugio*
Seales	Smooth dome	Ribbed dome	Ribbed dome
Bristles	Straight and hooked, hooked with no fine point	Straight and hooked, hooked has fine point	Straight only

The two better-known species appear to occur widely. *Mallomonas heterospina* has been recorded from Richmond Park (Lund, 1942), Berkshire (Harris & Bradley, 1957) and Denmark (Asmund, 1956), and in the present paper from Iceland. *Mallomonas multiunca* was recorded in Denmark and Berkshire by the same authors and has been found in Edinburgh, Scotland, by Bradley, but not at all in Iceland. *M. pugio* was only found in the type locality in Iceland and in none of the surrounding areas. The three species all seem to favour winter/spring conditions, except *M. multiunca*, which has also been recorded (at its type locality) in early autumn.

Series IV. Torquatae

Mallomonas clavus sp.nov. (Fig. 6; Pl. 4, figs. 30, 31, 34–36)

Diagnosis. Cellula ovoides, ante longa et tenuis, collari aptata squamarum quattuor vel quinque prominentium et setas ferentium. Post rotundata sed fere quattuor longis cuspidibus armata. Chromatophoros viridis, H-formis. Flagellum dimidio brevius quam corpus. Squamae collaris asymmetricales, cupola levi et apice acuto. Squamae corporis rhomboides. Squamae omnes margine prominenti. Regio interior poris ordinate dispositis et reticulationibus prominentibus insignis. Setae anteriores simplices. Squamae posteriores in cuspides longas et tenues elongatae.

Dimensiones. Cellula 20–30 $\mu \times$ 6–9 μ, squamae corporis 3·5 $\mu \times$ 2·5 μ, squamae collaris 4·5 $\mu \times$ 2 μ, setae 7 μ, clavi 2–10 μ.

English diagnosis. Cell ovoid, long and thin, anterior end with collar of four to five forward-pointing scales with bristles, posterior end rounded but with about four long spikes. Chromatophore green, H-shaped. Flagellum about one-half body length. Collar scales asymmetric, with smooth dome having a sharp point. Body scales rhomboidal. All scales have raised margin, inner region regularly perforated also with raised reticulations. Anterior bristles simple, posterior scales elongated into long spikes.

Occurrence. Found only at Hverhóll, Skidadalur, near Dalvik, Northern Iceland, in deep pit in mountain valley in July 1963, in water at pH 7·0.

Name derivation. The name 'clavus', Latin for spike or nail, refers to the posterior spikes which characterize the species.

Comparison. Mallomonas clavus has features in common with *M. doignonii* Bourelly (1951), *M. lefeuvrei* Villeret (1954), *M. pumilio* Harris & Bradley (1957), *M. pumilio* var. *silvicola* Harris & Bradley (1960) and *M. eoa* Takahashi (1963). In the light microscope it might easily be confused with *M. doignonii*, *M. lefeuvrei* or *M. eoa*, the species being similar in appearance with long posterior spikes and anterior bristles. Thus, to effect a proper identification, the electron microscope is required to show up the differences in scale structure. The cell of *M. doignonii* is the same size as that of *M. clavus*, but the scales have transverse ribs parallel to their short axis while *M. clavus* has perforations and reticulations. A comparison with unpublished electron micrographs of *M. lefeuvrei* (Mrs K. Harris, in preparation) shows that the scales and rear spines of this species are much thicker than those of *M. clavus*. The scales of *M. eoa* are also thicker than those of *M. clavus* and are ornamented with round pores instead of reticulations. In fact, the scale structure of *M. clavus* is identical with that of *M. pumilio* so that it might be confused with this species if isolated scales only were found in the electron microscope. In the light microscope, however, *M. pumilio* is much smaller and lacks the long posterior spikes, though it sometimes has very short ones. *M. pumilio* var. *silvicola* shows a somewhat closer resemblance in the light microscope, but again lacks the long spikes. In the electron microscope the scales have a different ornamentation.

There has been a good deal of confusion in the past concerning the identification of species in the Torquatae, a matter discussed by Harris & Bradley (1960). Since *M. clavus* seems virtually indistinguishable from several other species in the light microscope, it is impossible to say to which species past observations with this instrument refer. This group is obviously so confusing that a careful detailed study with both light and electron microscopes on existing and additional material is needed to clarify its taxonomy.

Undescribed species (Pl. 4, figs. 27–29)

Numerous loose scales of undescribed species belonging to the Torquatae were found in the electron microscope in water from Hverhóll, Skidadalur. Their appearance is close to that of *Mallomonas phasma* Harris & Bradley (1960) and *M. mangofera* Harris & Bradley (1960), but distinguishable by a ribbed structure round the distal edge. The anterior collar scales have a small pointed fin (Pl. 4, fig. 27) like *M. pumilio* and *M. clavus*, while the body scales are rhomboidal (Pl. 4, fig. 28).

ISOLATED SPECIES

Mallomonas maculata sp.nov. (Pl. 3, fig. 26; Pl. 5, figs. 37–41)

Diagnosis. Cellula oblonga. Ante prope cuspidata, squamis tribus vel quattuor setas ferentibus breviores, leves, paullo arcuatas. Ante squamae elongatae et oblique flexae. Squamae omnes sine cupola et crassiores, intus papillosae. Superficies magna parte levis sed papillis paucis et sine ordine sparsis signata. Labium prominens et conspicuum circa latus proximale squamae. Circa partem interiorem labii foveae circulatim dispositae. Post squamae paullo elongatae. Post et ante squamae foveam ferunt magnam sed brevum in medio et in uno latere.

Dimensiones. Cellula circa $15\mu \times 30\mu$, squamae $3{\cdot}5$–$4{\cdot}5\mu \times 2$–3μ, setae 8–10μ.

English diagnosis. Cell oblong. Anterior end more or less pointed with three or four scales bearing rather short smooth slightly curved bristles. Anterior scales elongated and curved to one side. All scales domeless and rather thick with internal papillose structure; surface generally smooth but with a few papillae irregularly distributed; a marked raised rim present round the proximal edge of the scale with a ring of pits round the inside of the rim. Posterior scales slightly elongated. Anterior and posterior scales have a large shallow pit in centre and on one edge.

Occurrence. Found only in a small man-made pit containing water at pH 7·0 at Kleif, Arskogsströnd, near Akureyri, in July 1963. Only one dried individual was found, but it was possibly to study this organism in detail in the electron microscope.

Name derivation. The name 'maculata', meaning spotted, refers to the appearance of the scales in the electron microscope.

Comparison. The scales of *Mallomonas maculata* to some extent resemble those of both *M. akrokomos* Pascher and *M. adamas* Harris & Bradley (1960). While the presence of large pits is a characteristic of the Quadratae group, to which the latter species belongs, other structural features such as the shape of the anterior and posterior scales are not; the species has thus been placed in the group of isolated species. The main diagnostic feature of the organism is the internal papillose structure. The fact that the spotting on the scales is internal can be deduced from their relatively smooth appearance when examined as replicas (Pl. 5, figs. 39–41). Direct electron micrographs of scales (Pl. 5, figs. 37, 38) show the dense array of spots.

Mallomonas akrokomos Pascher (Pl. 4, fig. 33)

A very sparse population of this species was found at Mordruvellir and scales were found at Myvatn. The species was well studied in the electron microscope by Harris (1958); no additional information has been obtained in the present work, the organism being typical in both light and electron microscopes.

SYNURA

Three species of the genus *Synura* were found in northern Iceland, two of them, *S. petersenii* Korshikov (Pl. 5, fig. 43) and *S. echinulata* Korsch. (Pl. 5, fig. 42) being common and numerous. Live organisms or scales were present in samples from almost all localities. One of the scales in Pl. 5, fig. 42, is probably in the process of developing, since it is thinner and more delicate than the other.

The third species, *Synura spinosa* Korsch., was detected by the presence of a very

small number of scales from Mordruvellir and Akureyri. One of these from near Akureyri (Pl. 4, fig. 32) can be identified as *S. spinosa* forma *spinosa* Petersen & Hansen (1956), by the presence of two teeth on the tip of the spine. No new information was obtained about these species.

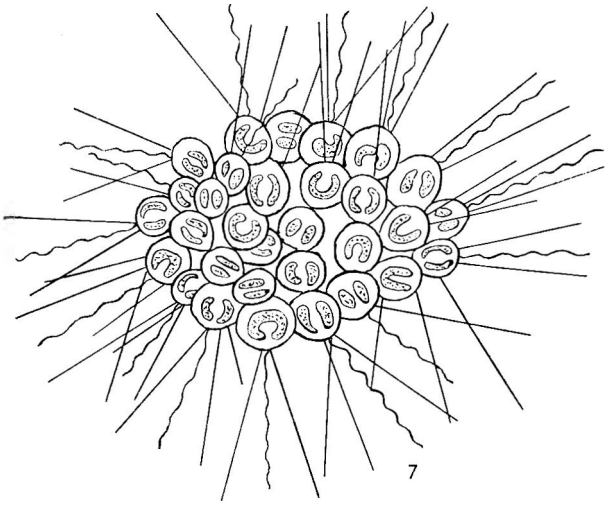

Fig. 7. Chrysosphaerella multispina, living colony, × 500.

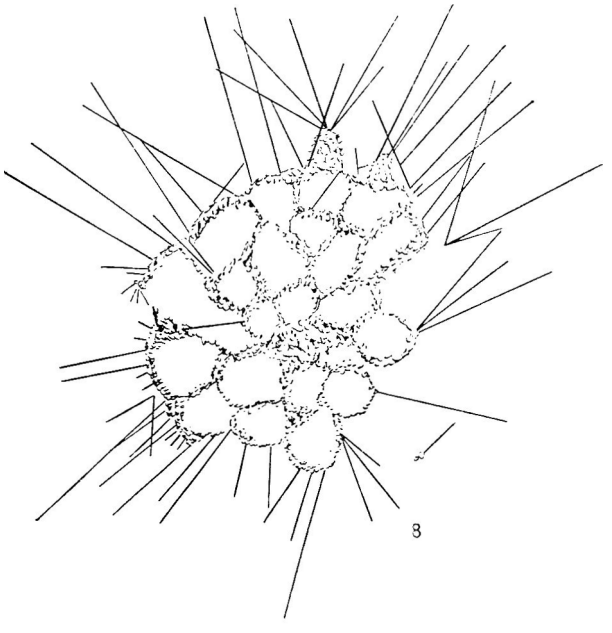

Fig. 8. C. multispina, dried colony, × 500.

CHRYSOPHAERELLA

The large colonial organisms of the genus *Chrysophaerella* Lauterborn (1896) with their numerous spines are easy to find with the light microscope. Differentiation of the species is, however, a difficult matter and can only really be achieved with an electron microscope. The taxonomy of the genus was discussed in detail by Harris & Bradley (1960). Only a few species have been described so far: *C. longispina* Lauterborn (1896, 1899), *C. brevispina* Korshikov (1941), and a third species, *C. rhodei* Skuja (1948), which Harris & Bradley (1960) believed to be identical with *C. brevispina*.

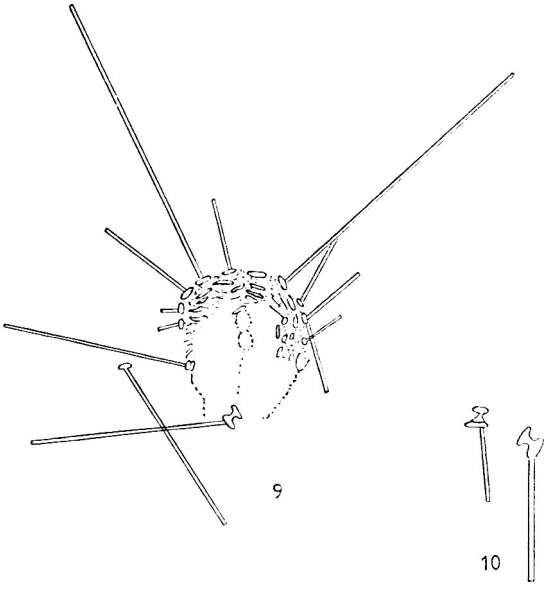

Fig. 9. Chrysosphaerella multispina, dried single cell, × 1000.
Fig. 10. C. multispina, spines, × 1700.

Chrysosphaerella multispina sp.nov. (Figs. 7–10; Pl. 6, figs. 44–50)

Diagnosis. Corpus coloniale. Unaquaque colonia cellulas continet viginti ad quadriginta. Chromatophoros profunde bilobata. Superficies cellulae spinis squamisque contecta. Unaquaque cellula fere quindecim spinas fert. Spinarum duae longae, tres vel plures mediae, breves multae. Ea pars spinae quae cellulae est coniuncta structuram fert fusiformem. Pars exterior spinae biceps, complanata. Squamae ovatae e quibus minores nullis signatae notis, maiores costis paucis et crassis, vel radiis rotae similibus vel forma reticulata.

Dimensiones. Cellula $10\mu \times 15\mu$, setae 35–40μ, 20–25μ, 5–10μ, squamae 0·6–6μ.

English diagnosis. Colonial organism, each colony with 20–40 cells. Cells ovoid. Chromatophore deeply bilobed. Cell surface covered with spines and scales. Spines about 15 in number to each cell: two long spines, three or more medium spines, and many short spines. Spines have bobbin-like structure at the end attached to the

cell. Outer end is two-pointed and flattened. Scales oval, smaller ones unsculptured, larger ones with few thickened ribs resembling spokes or in a reticulate pattern.

Occurrence. Found in a deep pit at type locality, Hverhóll, Skidadalur, near Dalvik. Apparently widespread in northern Iceland, other localities being Kleif, Arskogsströnd, near Akureyri; Ljosvatn, near Godafoss and Myvatn. The water was at pH 7·0.

Table 2. *Features of Chrysosphaerella longispina, C. brevispina and C. multispina*

	*C. longispina**	*C. brevispina*	*C. multispina*
No. of spines	Up to 5, usually 3–4	2–11	About 15
Length of spines (μ)	25–40	Up to 18	5–40
Scale morphology	Oval, thickened inner ring with spokes radiating to margin	Oval, inner ring near margin, spokes short	Oval, smooth margin, reticulate in centre

* Data from Korshikov (1941).

Name derivation. The name 'multispina' refers to the numerous spines.

Comparison. The species of Chrysosphaerella all look rather similar in the light microscope so that identifications with this instrument must be carried out with great care. The electron microscope, however, reveals clear-cut differences. The main diagnostic features are summarized in Table 2. In addition, two points may be added in comparison with *C. brevispina.* First, the cells of *C. multispina* are bigger, and secondly the shape of the bobbin is different (see Harris & Bradley, 1958).

It is pointed out that the numerous medium and long spines of *Chrysosphaerella multispina* tend to be overlooked in the live organism. They are best seen in dried preparations in the light microscope, and it seems that they provide the most obvious characteristics. Nevertheless, it may be argued that variations in the number and length of bristles might occur at different stages of the development of the organism. This is considered unlikely since the various widely spread populations in Iceland all had similar numbers of spines.

CONCLUSION

It is clear that the three genera of Chrysophyceae studied are abundant in northern Iceland. However, the noticeably sparse populations found suggest that the optimum season is probably earlier than in July, and climatic conditions indicate that it is more likely to be in May. Some eleven different species of the genus *Mallomonas* were found over a comparatively small area in the space of a few weeks in July. Thus at the optimum season Iceland could provide numerous species and much information on the taxonomy of this genus. The position regarding the genera *Synura* an *Chrysosphaerella* is less certain. The former genus, though common, possesses relatively few species at present, three of these being found in Iceland, but only two being common. The single species of Chrysosphaerella was widespread.

In view of the geographical position of Iceland, it is interesting to note that the

species of the three genera found are similar to those described from other parts of the world, and that the new species fit well with the existing scheme of classification.

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Field work was carried out with the permission of the Icelandic Government.

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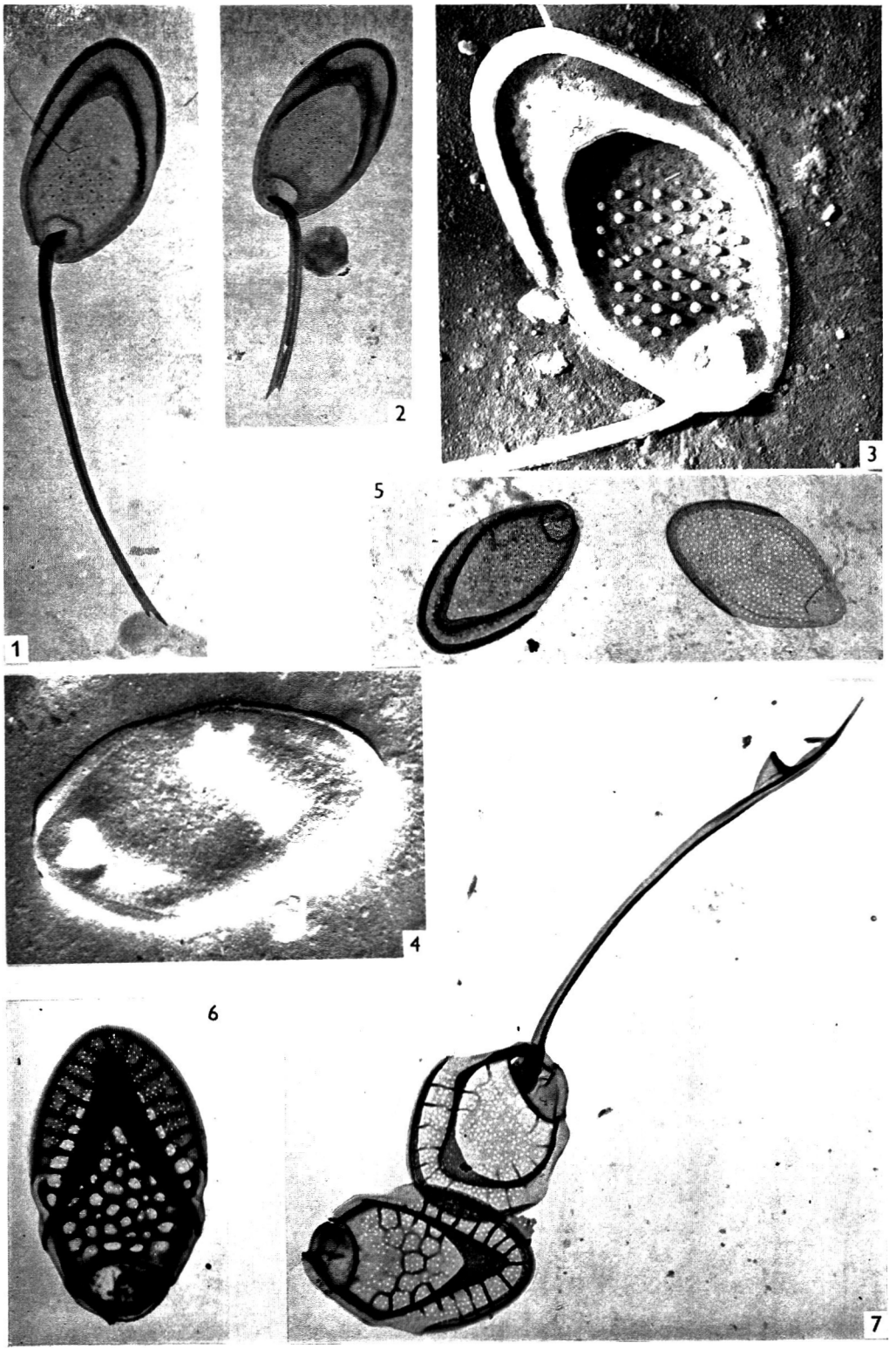

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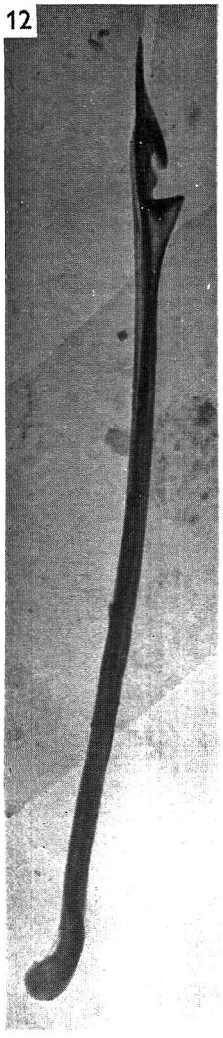
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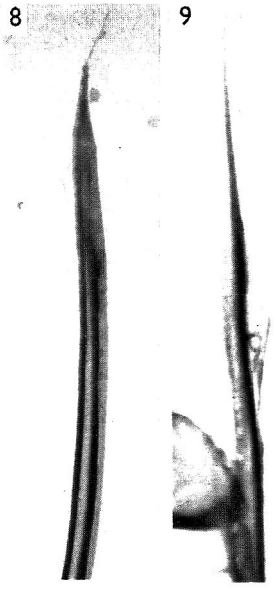
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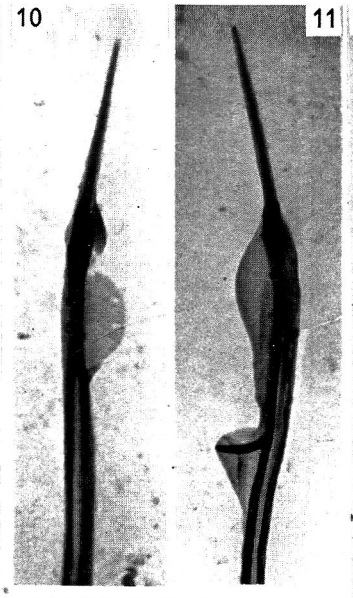
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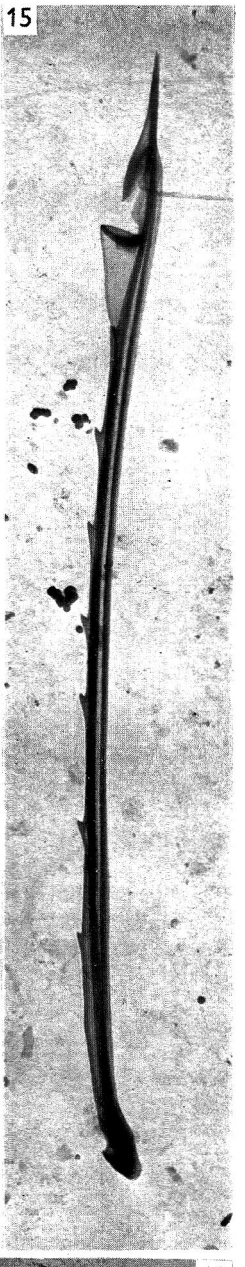
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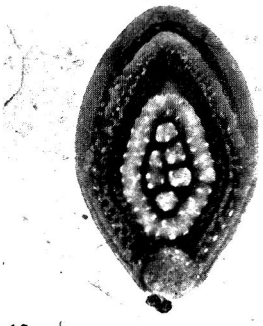
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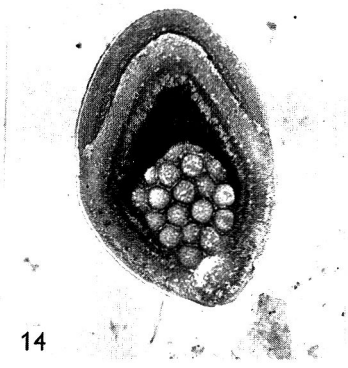
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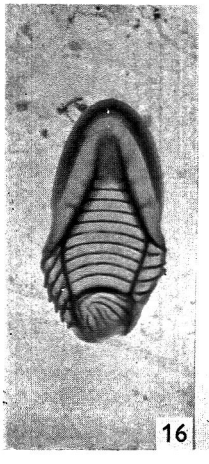
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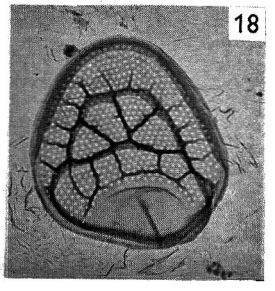
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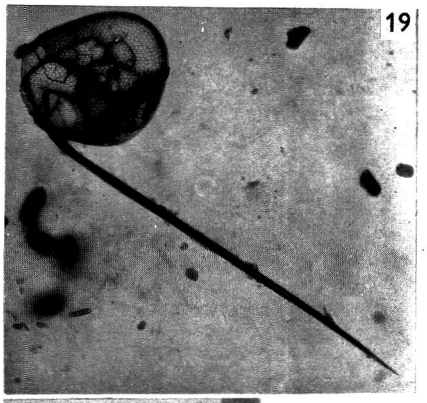
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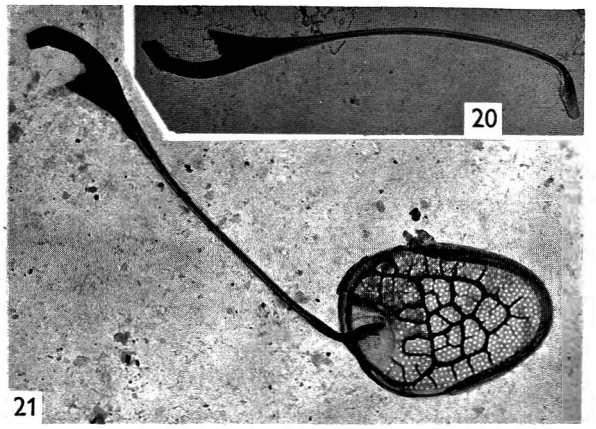
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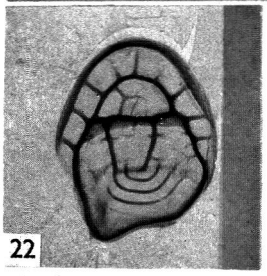
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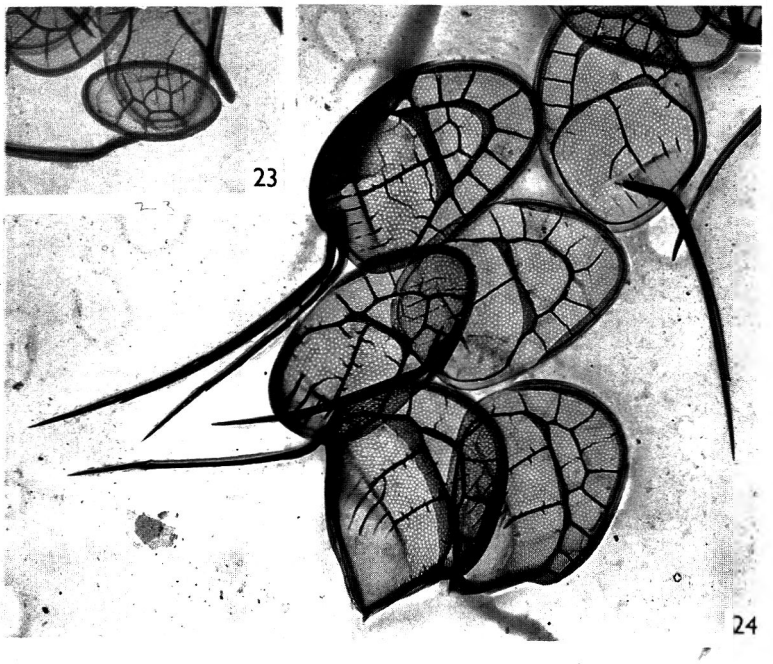
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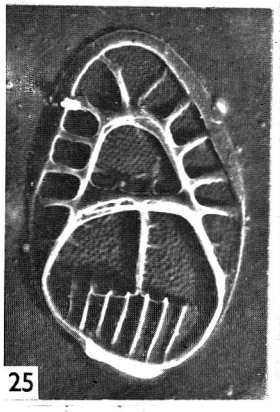
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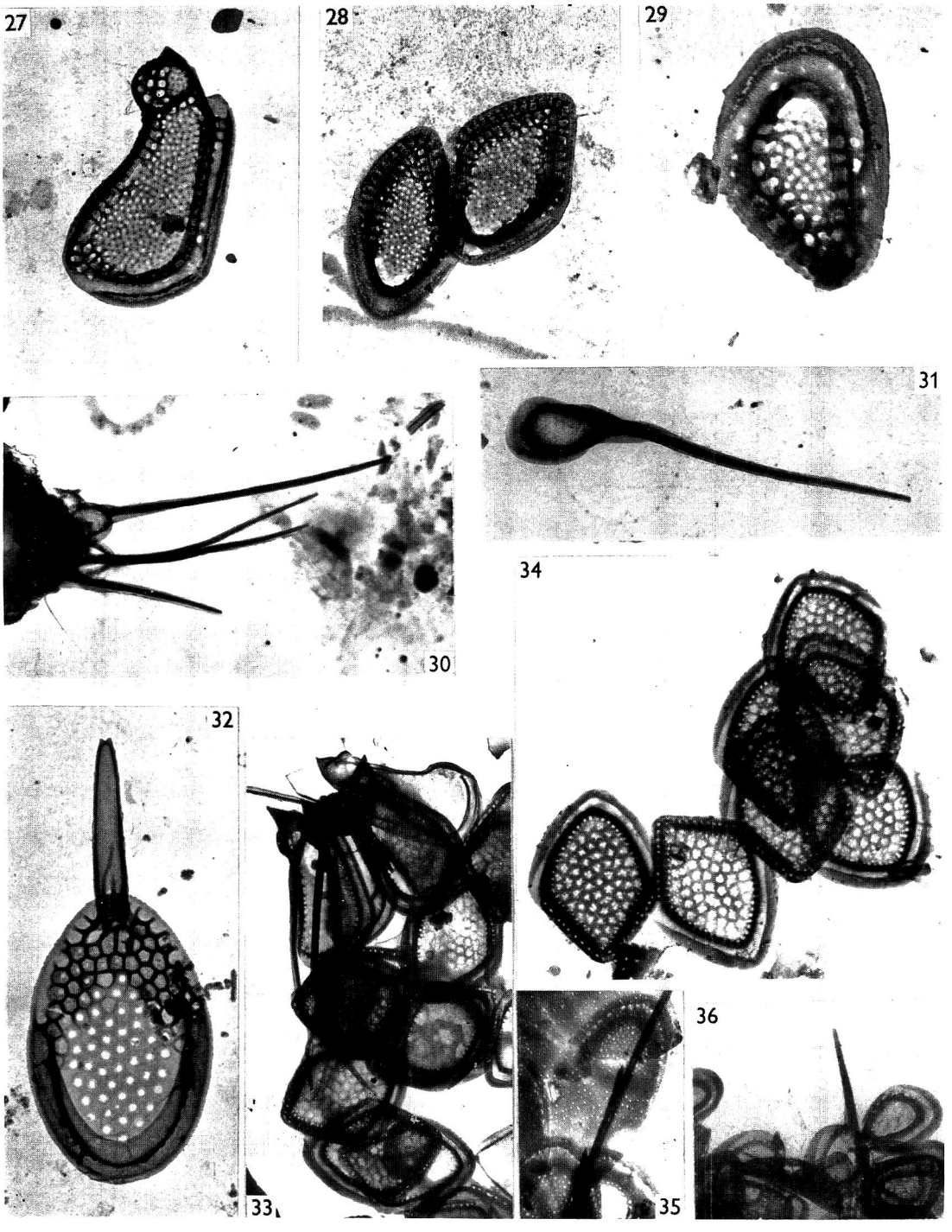
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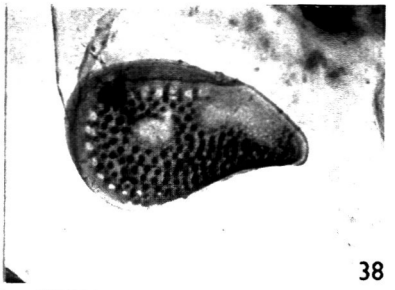

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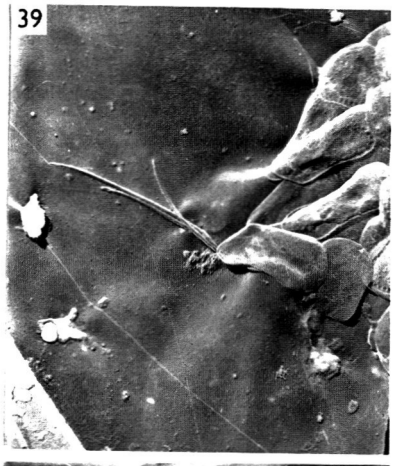

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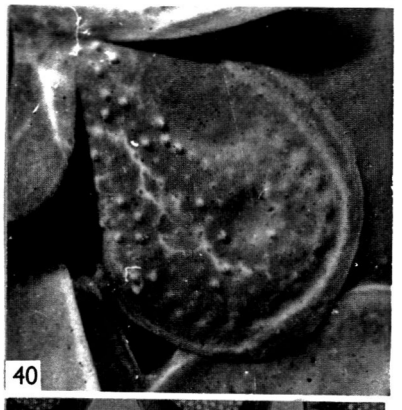

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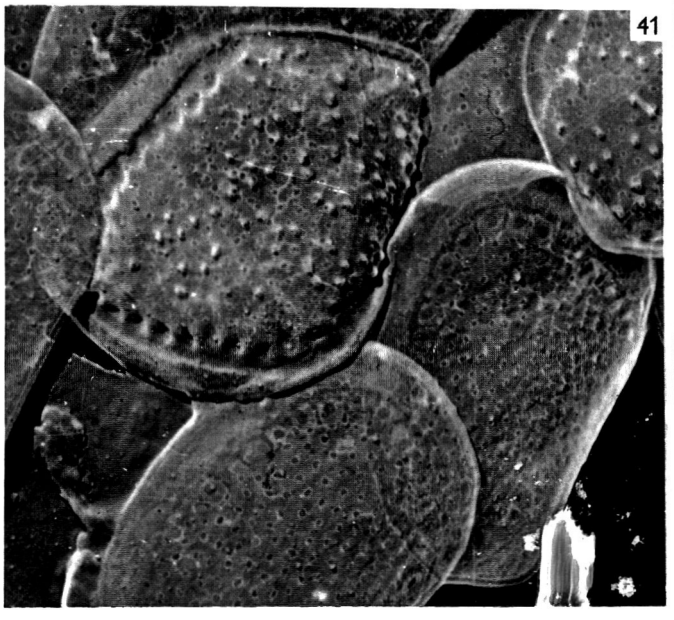

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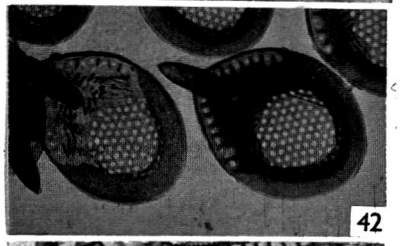

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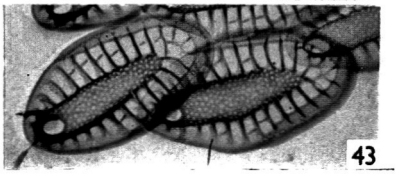

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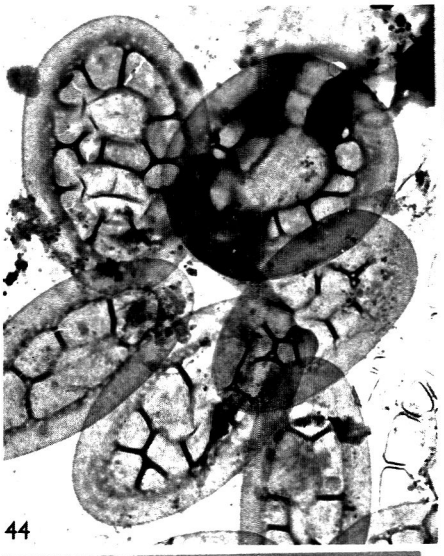
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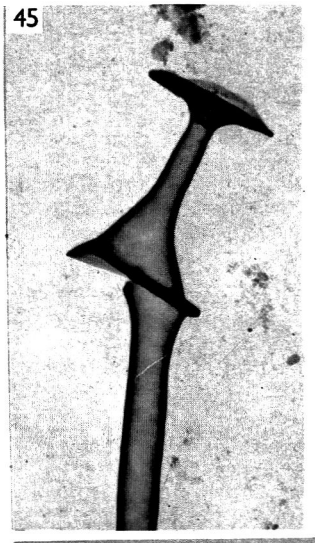
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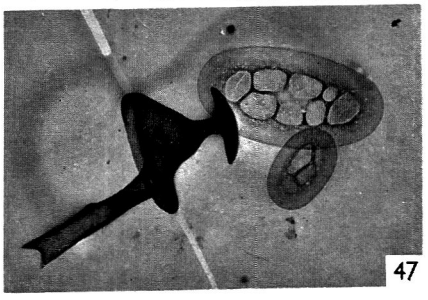
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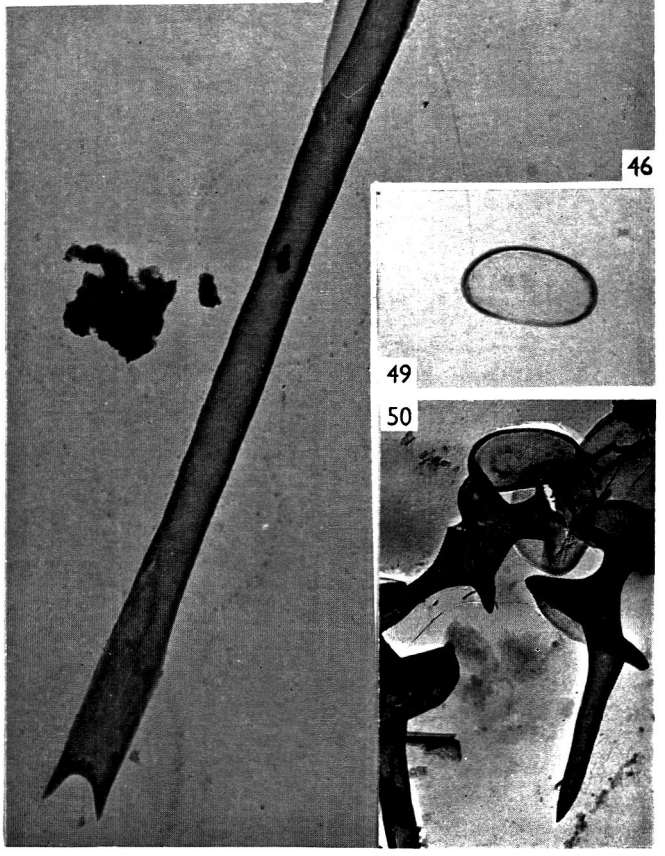
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EXPLANATION OF PLATES

Plate 1

Fig. 1. *Mallomonas calceolus*, direct micrograph of scale and bristle, × 10,000.
Fig. 2. *M. calceolus*, scale with short bristle, × 10,000.
Fig. 3. *M. calceolus*, shadowed scale showing outer surface, × 25,000.
Fig. 4. *M. calceolus*, shadowed carbon replica showing inner surface of scale, × 20,000.
Fig. 5. *M. calceolus*, mature (left) and immature (right) scales, direct, × 7500.
Fig. 6. *M. acaroides* var. *craquissima*, direct micrograph of scale, × 6000.
Fig. 7. *M. acaroides* var. *galeata*, scales and bristle, × 6000.

Plate 2

Figs. 8–11. *Mallomonas acaroides* var. *galeata*, variations in bristle tip, × 12,000.
Fig. 12. *M. acaroides* var. *craquissima*, bristle, × 6000.
Fig. 13. Undescribed species from Másvatn, scale, × 15,000.
Fig. 14. Undescribed species from Myvatn, scale, × 15,000.
Fig. 15. *M. acaroides* var. *craquissima*, serrated helmet bristle, × 6000.
Fig. 16. *M. striata* var. *striatula*, × 6000.
Fig. 17. Undescribed species (?same as in Fig. 14) from Myvatn, bristle and scales, × 9000.
Fig. 18. *M. heterospina*, scale from Mordruvellir, × 9000.

Plate 3

Fig. 19. *Mallomonas heterospina*, scale and pointed bristle from Kleif, × 6000.
Fig. 20. *M. heterospina*, 'spanner' bristle, from Mordruvellir, × 7500.
Fig. 21. *M. heterospina*, scale and 'spanner' bristle from Kleif, × 7500.
Fig. 22. *M. pugio*, scale with abnormal dome, × 7500.
Fig. 23. *M. pugio*, domeless scale, × 7500.
Fig. 24. *M. pugio*, body scales and bristles, × 7500.
Fig. 25. *M. pugio*, shadowed scale, × 9000.
Fig. 26. *M. maculata*, direct micrograph of whole organism, × 2800.

Plate 4

Fig. 27. Mallomonas (undescribed), from Skidadalur, anterior scale, × 12,000.
Fig. 28. Mallomonas (undescribed), from Skidadalur, domeless body scales, × 12,000.
Fig. 29. Mallomonas (undescribed), from Skidadalur, posterior scale, × 24,000.
Fig. 30. *M. clavus*, posterior scale elongated into spike, × 8000.
Fig. 31. *M. clavus*, posterior spikes, × 7500.
Fig. 32. *Synura spinosa* forma *spinosa*, scale × 12,000.
Fig. 33. *M. akrokomos*, scales and part of bristle, × 9000.
Fig. 34. *M. clavus*, body scales, × 7500.
Fig. 35. *M. clavus*, pointed anterior scales, × 7500
Fig. 36. *M. clavus*, posterior scales, × 7500.

Plate 5

Fig. 37. *Mallomonas maculata*, anterior scales, × 8000.
Fig. 38. *M. maculata*, posterior scale, × 8000.
Fig. 39. *M. maculata*, shadowed replica of anterior end of cell, × 2800.
Fig. 40. *M. maculata*, shadowed replica of posterior scale, × 14,000.
Fig. 41. *M. maculata*, shadowed replica of body scales, × 14,000.
Fig. 42. *Synura echinulata*, scales × 7500.
Fig. 43. *S. petersenii*, scales, × 7500.

Plate 6

Fig. 44. *Chrysosphaerella multispina*, scales, × 8000.
Fig. 45. *C. multispina*, 'bobbin' at base of spine, × 8000.
Fig. 46. *C. multispina*, entire spine, × 8000.
Fig. 47. *C. multispina*, short spine and scales, × 6000.
Fig. 48. *C. multispina*, short spine and scale in profile, × 7500.
Fig. 49. *C. multispina*, small unsculptured scale, × 12,000.
Fig. 50. *C. multispina*, short spine oriented to show flattened tip, × 8000.

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The Effect of Mucin on the Survival of Lactobacilli and Streptococci

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SUMMARY

The survival of suspensions of lactobacilli and streptococci incubated at 37° in normal saline and in 0·1% (w/v) and 1·0% (w/v) solutions of dehydrated mucin was studied by counting the number of viable organisms present at regular intervals. The survival time was markedly prolonged in the mucin solutions. In the 1·0% (w/v) mucin solution most of the strains of lactobacilli and all the strains of streptococci were still viable at the end of one year. The mucin solutions appeared to be unchanged when compared with control solutions which had been inoculated with killed bacteria and incubated at 37°.

INTRODUCTION

It has been known for many years that lactobacilli either cannot be isolated at all or are found in very small numbers in the mouths of individuals who are entirely free from dental caries, whereas in mouths where dental caries is active lactobacilli are much more numerous. There is good evidence for believing that both the dental caries and the proliferation of lactobacilli are the result of consuming a diet rich in fermentable carbohydrates. Studies of the bacteriology of the human vagina have also shown that the appearance and persistence of lactobacilli exactly parallels the availability of fermentable carbohydrate in the form of glycogen deposits in the vaginal epithelium (Cruickshank & Sharman, 1934).

It has been assumed that a few lactobacilli are always present in the mouth and vagina and they proliferate when acid conditions develop and afford them a selective advantage. But, since lactobacilli are not known to have exceptional ability to survive adverse conditions it is difficult to account for their persistence, in the absence of acid conditions and for long periods of time, in an environment containing enormous numbers of streptococci which metabolize all the nutrients required by lactobacilli and have a shorter generation time. One possible explanation, speculative, but appealing, is that lactobacilli are in fact acid-resistant mutant forms of streptococci. No experimental support for this suggestion could be obtained.

Dewar (1949) reported that saliva specimens could be kept at room temperature for up to 72 hr without the lactobacillus count falling appreciably, and this has been the experience of many other workers. Both the mouth and the vagina have a mucinous secretion and the possibility that mucin in some way preserves lactobacilli was investigated. In a preliminary experiment it was found that lactobacilli were still viable after 6 weeks incubation at 37° in whole saliva, but in saliva from which the mucin had been removed by precipitation with acetic acid they died

out within 72 hr. Clearly, mucin did prolong the time of survival of lactobacilli. In the present work the survival of strains of lactobacilli and streptococci incubated in mucin solutions was examined by doing periodic viable counts.

METHODS

Mucin. A dehydrated extract of hog stomach was used (Mucin, gastric. L. Light & Co. Ltd., Colnbrook, Bucks.). Two solutions of the mucin powder, 0·1 % (w/v) and 1·0 % (w/v), were prepared by dissolving the powder in sterile distilled water and sufficient -NaOH to give a final pH of 7·2. The solutions were dispensed in 22·0 ml. amounts in sterile Universal bottles. Sterile screw-capped $\frac{1}{4}$ oz. bijou bottles were filled with the 1·0 % mucin solution. The maximum heat treatment these solutions would withstand without a precipitate forming was autoclaving at 10 lb. for 10 min. Samples from the Universal bottles (1·0 ml.) and the bijou bottles (0·5 ml.) were cultured aerobically and anaerobically in glucose broth; all the solutions were found to be sterile. After autoclaving, some bottles were randomly selected and the pH of the mucin solutions measured; it was found to be in the range pH 6·9–7·08.

Bacteria. The mucin solutions were inoculated with either stock strains of lactobacilli and streptococci or strains of these organisms isolated from saliva specimens collected from caries-free subjects. The stock strains of lactobacilli were kindly supplied by Dr M. Elisabeth Sharpe of the National Institute for Research in Dairying, and the stock strains of streptococci were obtained from the National Collection of Type Cultures.

The strains of bacteria which were to be counted at regular intervals during the experiment were subcultured several times on blood agar, then streaked on further plates of blood agar which were incubated for 24 hr for the streptococci and 48 hr for the lactobacilli, except *Lactobacillus acidophilus*, *L. casei* and *L. fermentii* which were incubated for 72 hr. Bacterial growth was scraped from the surface of the agar and suspended in sterile saline to give a turbidity approximately equivalent to Brown's opacity tube no. 2. From each bacterial suspension 1·0 ml. amounts were transferred to each of two Universal bottles containing 1·0 % mucin and to one bottle each of 0·1 % mucin and sterile saline; the remainder of each suspension was heated at 60° for 1 hr and then 1·0 ml. transferred to a further bottle of 1·0 % mucin. All bottles were kept in the incubator at 37° throughout the experiment. Periodic viable counts were made of the bacteria in the saline, 0·1 % mucin and one of the bottles containing 1·0 % mucin; the remaining two bottles of 1·0 % mucin, one of which was inoculated with killed bacteria, were kept in the incubator until the end of the experiment when the pH and viscosity of these two solutions were compared.

Other strains of lactobacilli and streptococci were grown on blood agar and then a small amount of growth was scraped up and suspended in 1·0 % mucin solution in bijou bottles. The bottles were incubated at 37° and every 3 months a 0·3 ml. sample was cultured in either 10·0 ml. of glucose broth or 10·0 ml. of Rogosa broth (Rogosa, Mitchell & Wiseman, 1951) depending on whether the solution contained streptococci or lactobacilli. When growth occurred, a Gram-stained smear was prepared as a check.

Bacterial counts. The number of viable organisms in the solutions was estimated by the method of Miles & Misra (1938). Serial dilutions were prepared in 1·0% peptone water and from appropriate dilutions eight 0·02 ml. drops were applied to each of three plates of 1·0% glucose agar for streptococci or to plates of MRS agar (de Man, Rogosa & Sharpe, 1960) for lactobacilli. When the number of organisms remaining became too small for counting by the drop method, plate counts were made by spreading 0·1 ml. of undiluted saline or mucin solution over the surface of each of three plates of medium. If no colonies appeared on any of these plates, 0·5 ml. of saline or mucin solution was inoculated into glucose broth or MRS broth. If growth occurred, then testing for growth in broth was substituted for the bacterial count at the appropriate time intervals. Finally, when no growth occurred in broth, the bacteria were spun-down from the saline or mucin solution and the centrifuged deposit cultured in glucose broth or MRS broth.

At the end of one year the experiment was terminated and the appropriate physiological and serological tests were carried out to confirm that the organisms remaining were those which had been inoculated at the beginning.

RESULTS

The number of weeks survived by the strains of lactobacilli and streptococci, which were incubated at 37° in normal saline and mucin solutions and counted at regular intervals, are shown in Table 1. Two types of viridans streptococci are listed in Table 1; this subdivision is based on other work in which it was found that

Table 1. *The number of weeks lactobacilli and streptococci remained viable when incubated at* 37° *in saline and in* 0·1 *and* 1·0% *solutions of mucin*

The experiment was continued for 1 year. The percentage of the original inoculum still viable at the end of that time is shown in brackets.

	Saline	0·1% mucin	1·0% mucin
L. acidophilus (NCDO 4)	0	2	40
L. casei (NCDO 151)	0	1	52 (0·05%)
L. casei var. *rhamnosus* (NCDO 243)	0	6	52 (0·001%)
L. fermentii (NCDO 215)	0	2	50
L. plantarum (NCDO 352)	0	3	52 (0·0006%)
L. salivarius (NCDO 929)	0	4	52 (0·01%)
Oral enterococcus	3	24	53 (2·4%)
S. faecalis (NCTC 8213)	9	53 (0·001%)	53 (0·2%)
S. hominis (NCTC 7366)	0	41	53 (4·2%)
S. pyogenes (NCTC 8198)	0	7	53 (0·03%)
Oral viridans streptococcus type 1	0	18	53 (0·06%)
Oral viridans streptococcus type 2	0	53 (0·0001%)	53 (0·02%)

viridans streptococci which ferment raffinose but do not hydrolyse aesculin (type 2 strains) usually produce acid from glucose much more rapidly than viridans streptococci which give other combinations of reactions with these two substrates (type 1 strains). Full details of the viable counts, e.g. dilution, number of drops and number of colonies counted have been published elsewhere (Sims, 1964). In over half the counts 400 or more colonies were counted which should reduce the error to ±10% and in very few were less than 100 colonies counted which is the 20% level of accuracy (Badger & Pankhurst, 1960).

Although the streptococci survived rather better in the mucin solutions than the lactobacilli, the general trend of the viable counts over the 1-year period was similar for both groups of organisms. The results of the counts of *Lactobacillus plantarum* and *Streptococcus hominis* are illustrated in Fig. 1. The high initial death rate of *L. plantarum*, which also occurred with *L. casei* var. *rhamnosus*, *L. fermentii* and *L. salivarius*, was probably a further manifestation of the finding, clearly shown in Table 1, that survival depends on the amount of mucin available to the bacteria. *L. acidophilus* and *L. casei* which were inoculated in much smaller numbers

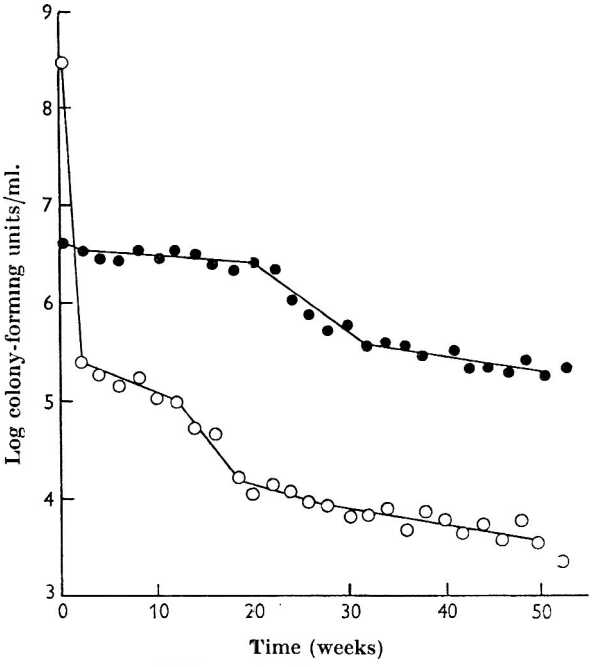

Fig. 1. Survival of Lactobacillus plantarum (○) and Streptococcus hominis (●) when incubated at 37° in 1·0 % solutions of mucin.

than the other lactobacilli (about 2×10^6) had initial death rates similar to *S. hominis*. The counts of all the organisms which survived for 1 year in the 1·0 % mucin solution show that survival was distinctly better in the second 6 months than in the first. Of the original inoculum of *L. plantarum* 0·003 % survived the first 6 months, and of these 22 % were still viable after a further 6 months incubation. The corresponding values for *S. hominis* were 15·5 and 27·1 %.

Most of the organisms which were incubated in 1·0 % mucin solution in bijou bottles and tested for viability every 3 months were still viable after 1 year. Three oral strains each of *L. acidophilus*, *L. casei*, *L. casei* var. *rhamnosus*, *L. plantarum* and *L. fermentii* were tested. Of these, one strain each of *L. casei* and *L. casei* var. *rhamnosus* failed to survive 3 months, one strain of *L. fermentii* survived for 3 months but not for 6 months, and two strains of *L. plantarum* survived for 9 months but not for 1 year. The remaining 10 strains all survived the full year. The following streptococci all survived one year: stock strains of Lancefield's group B, C, E, F,

G, H, K, L, M, O, P and Q; stock strains of *S. bovis*, *S. durans*, *S. faecalis* var. *liquefaciens*, *S. faecalis* var. *zymogenes* and *S. faecium*; 4 oral strains each of enterococci and *S. hominis*, and 10 oral strains of viridans streptococci. A stock strain of *S. pneumoniae* failed to survive 3 months.

The pH and viscosity of the control 1·0% mucin solutions were essentially the same whether they had been inoculated with living or killed bacteria. The solutions containing killed bacteria tended to be slightly more viscous and more acid than the solutions inoculated with viable organisms. None of the lactobacilli or streptococci would grow on a medium consisting of the 1·0% mucin solution solidified with agar. Thus, although the viable counts may reflect the balance between growth rate and death rate, there is no direct evidence that mucin is a nutrient for lactobacilli and streptococci, it simply seems to preserve their viability.

DISCUSSION

The results show clearly that mucin preserves the viability of lactobacilli and streptococci. Why it does so is entirely conjectural. Since the manufacturers were unable to supply a chemical analysis of the dehydrated mucin it is possible that the solutions contained some factor other than mucin which enhanced survival.

Tunnicliff (1940) has shown that bacteria acquire 'coats' of mucin when suspended in this substance. The results of the present work could be explained by assuming that the survival of an organism is dependent on its acquisition of an adequate 'coat' of mucin. Survival was better in the more concentrated mucin solution and this could be due to more organisms acquiring satisfactory coverings of mucin. If unlimited amounts of mucin were available, presumably the organisms would survive much longer or all the organisms inoculated would survive for a certain maximum time. In this respect it may be significant that the streptococcus inoculated in smallest numbers, *Streptococcus hominis* (NCTC 7366), had the highest percentage of one year survivals.

The only previous report of mucin prolonging the period of survival of an organism appears to be that of Keefer & Spink (1938) who found that gonococci survived up to 28 hr in mucin. Here again it may have been the acquisition of a mucinous capsule which facilitated survival. In the present study it was found that gonococci would occasionally remain viable for 24 hr in mucin solution at 37°, but frequently did not; the outcome was unpredictable.

There are several reports which suggest that mucin may be a source of nutrition for bacteria (Goldsworthy & Florey, 1930; McLeod, 1941; Rogers, 1948). It may be that the pH and viscosity measurements were too crude to detect small changes in the mucin and perhaps procedures such as glucosamine estimations might have revealed bacterial breakdown of the mucin. On the other hand, there are no reports of lactobacilli and streptococci surviving for such long periods even in ideal nutrient fluids.

The results provide a reasonable explanation for the continual presence of lactobacilli in the mouth and vagina, for one may suggest that if a lactobacillus acquires an adequate covering of mucin it will be able to survive for a long time, even though the competition from other bacteria for the available food is so intense that it receives no nutrients whatever. By similar reasoning it is possible, at least in some

measure, to account for the very large complex streptoccal flora of the mouth, for any streptococcus entering the oral cavity could remain viable for a prolonged period, regardless of its various attributes which would determine its survival or elimination in other non-mucinous environments. Also, any mutant streptococcus which arises may survive even though its characteristics are selectively disadvantageous in comparison with its parent form. In short, all manner of streptococci may persist, whereas in the absence of mucin they would either proliferate or die off.

Present concepts of the bacterial floras of mucous membranes may have to be revised. It seems not unlikely that many of the bacteria in such floras are simply existing rather than actively multiplying and dying. After all, many of them require complex media for their cultivation and certainly very few of them will grow in a culture medium consisting only of the mucinous secretion in which they are found. The findings of studies of the interaction of various groups of bacteria in mixed floras may be inapplicable in mucinous environments. Finally, it is conceivable that the preserving effect of mucin may be of importance in the spread of diseases by droplet infection and in the establishment of the carrier state.

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Genetic Transformation of *Neisseria catarrhalis* by Deoxyribonucleate Preparations having Different Average Base Compositions

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SUMMARY

The base contents of deoxyribonucleate (DNA) preparations from 7 strains of *Neisseria catarrhalis* were determined chromatographically. Three non-overlapping classes were distinguished by mole % guanine + cytosine. These centred about the values 41 % (2 strains), 42·3 % (4 strains, including ATCC 8193), and 44·5 % (strain NCTC 4103). Each of the 7 strains was capable of undergoing genetic transformation. DNA preparations from spontaneous streptomycin-resistant mutants of all 7 strains elicited transformation of recipient strains in all 49 possible combinations. Results with this group, therefore, do not support the hypothesis that success in transferring genetic information between 2 strains requires identity of *average* DNA base contents.

Differences of reciprocal transformation frequencies and of 4 physiological characteristics (nitrate reduction, pigment production on vancomycin-containing agar, hydrolysis of gelatin, and growth at 28°) appeared to separate 6 of the strains of *Neisseria catarrhalis* from the seventh (NCTC 4103), which may properly be named *N. cinerea*.

INTRODUCTION

The mucosa of the upper respiratory tract of humans provides microhabitats for a variety of bacteria including several potentially pathogenic species which are known to undergo genetic transformation. Dense populations are observed in nasopharyngeal or other secretions. The idea that genetic information may be transferred between such bacteria in their natural habitat has gained some support from *in vitro* demonstrations of transforming activity of extracellular deoxyribonucleate (DNA). DNA-containing cellular materials spontaneously liberated by cultures of *Neisseria meningitidis*, *Streptococcus* (*Diplococcus*) *pneumoniae*, as well as of the non-parasitic *Bacillus subtilis*, elicited transformation of recipients of the corresponding species (Catlin, 1960*a*; Ottolenghi & Hotchkiss, 1960, 1962; Takahashi, 1962), or of related species (Catlin, 1960*b*).

If unlimited transfer of genetic information were to occur between bacteria, one would expect the development ultimately of a continuous spectrum of differences. That there are relatively stable distinguishable bacterial taxa implies that barriers to gene flow exist, as Ravin (1961) has pointed out. One barrier recently recognized, which has been thought to be insurmountable, involves differences of DNA base composition. Results of an investigation of interspecific transformation of *Neisseria*

provide one example (Catlin & Cunningham, 1961). DNA preparations from representatives of six different species had transforming activity for recipient cells of all six; analyses of their base compositions showed that the content of guanine plus cytosine (G+C) was close to 50% for all. On the other hand, two transformable strains of *N. catarrhalis* did not undergo detectable transformation when exposed to transforming preparations from the other six neisserias, and their G+C content was 41%. This difference of base composition was thought to provide a possible physical basis for the genetic barrier.

Studies by Marmur and his associates of base composition, molecular hybrid formation, and transforming activities of DNA preparations from various species of *Bacillus* led them to propose that similarity of overall DNA base composition is a minimum requirement for DNA interaction and for genetic compatibility between different species (Marmur, Schildkraut & Doty, 1961; Marmur, Seaman & Levine, 1963). They noted in Haemophilus, as well as in Bacillus, that species which were genetically related had the same base composition, although they might show very different efficiencies of interspecific transformation.

Additional genetic material suitable for testing this hypothesis has been found in *Neisseria catarrhalis*. The investigation to be described here has revealed significant differences of average base composition among DNA preparations from 7 strains, 5 additional strains having been acquired since the previous study (Catlin & Cunningham, 1961). Their G+C contents ranged from 41 to 44·5%. Contrary to expectation, each of the seven *N. catarrhalis* recipients was transformed by all of these DNA preparations.

Further studies of the range of transfer of genetic information were made with selected bacteria indigenous to the mucosa which bear some resemblance to *Neisseria catarrhalis*. The following communication presents results of transformation tests of moraxellas and allied organisms, and shows the application of transformation techniques to a particular problem of classification. A third communication will describe reciprocal transformations of *N. catarrhalis* and *Moraxella nonliquefaciens*.

METHODS

Strains

Seven strains of *Neisseria catarrhalis* were investigated: Ne 11, University of Rochester collection; Ne 13, New York State Department of Health; ATCC 8193, American Type Culture Collection; Ne 23, throat culture from a healthy student; N 9, University of Maryland collection; 8313, University of Maryland collection; NCTC 4103, National Collection of Type Cultures, London. Strains Ne 11 and Ne 13 have been described previously (Catlin & Cunningham, 1961). Strains N 9 and 8313 were kindly provided by Sylvia G. Cary, Walter Reed Army Institute of Research, Washington.

When received each strain was streaked on an agar medium containing heart infusion and yeast extract (Difco) supplemented with ribonucleic acid, sodium glutamate, and calcium chloride (HIY-1 agar; Catlin & Cunningham, 1961). One or more typical colonies were isolated and separately subcultured. Thereafter, to minimize population changes, all subcultures were made by streaking out organisms selected from 10 to 20 typical colonies.

Strains were preserved at −60° in the following way. Masses of 18-hr growth were transferred with an inoculation loop from HIY-1 agar to a number of small screw-cap vials which contained HIY broth + 0·1 % (w/v) agar, and were immediately frozen. Organisms from a thawed suspension were subcultured daily on fresh HIY-1 agar over a period not exceeding 1 week. Cultures were grown, usually at 35°, in a humidified water-jacketed incubator or an incubator-shaker (Model G27, New Brunswick Scientific Co. Inc., New Jersey).

Dihydrostreptomycin sulphate (Squibb; DHS) was used for all studies involving streptomycin-resistant (*str-r*) strains. Spontaneous *str-r* mutants were obtained from each *Neisseria catarrhalis* strain by the following procedure. The parent strain was cultivated at 35° in 500 ml. flasks containing 50 ml. of heart infusion broth + 0·5 % (w/v) yeast extract (Difco). After 8–10 hr incubation with shaking, 100 ml. of additional broth was added together with DHS sufficient to give 500 μg./ml. The culture was re-incubated for 16 hr with shaking, and samples were plated in HIY-1 agar containing 500 μg. DHS/ml. A single isolated colony was picked, subcultured, and its identity established by morphological and cultural examination.

The strain designated NCTC 4103 *str-r* 11 is a transformant derived by exposing strain 4103 to DNA from Ne 11 *str-r*; a single isolated colony was picked from agar containing 500 μg. DHS/ml.

Bacteriological characteristics

Oxidase reaction. The method of Kovacs (1956) was used with 20-hr cultures. The tetramethyl-*p*-phenylenediamine dihydrochloride (Eastman) solution was prepared each day. This reagent was used, also, as a routine check of transformation test cultures, being poured sparingly on surface growth or introduced to subsurface colonies by stabbing through the semi-solid agar.

Catalase production. Vigorous evolution of gas was noted immediately after adding a drop of 3 % (v/v) solution of hydrogen peroxide to 20-hr cultures on HIY-1 agar.

Gelatin hydrolysis. The method of Frazier given in the *Manual of Microbiological Methods* (1957, p. 158) was slightly modified to increase its sensitivity. The medium was heart infusion broth (Difco) with additions of 1 % (w/v) agar (Purified, Difco) and 0·4 % (w/v) gelatin (Fisher Scientific Co.). This was dispensed in 15 ml. amounts in flat-bottom Petri dishes (molded Pyrex, 100 × 22 mm.), and cultures were spotted on the surface over an area about 1 cm. in diameter. Plates were incubated at 35° for 7 days in a covered container with moist bibulous paper to minimize drying. The reagent (15 g. $HgCl_2$ dissolved in 100 ml. distilled water + 20 ml. concentrated HCl) poured in a 10 ml. quantity on the medium precipitates unhydrolysed gelatin, resulting in a white opacity. Strong gelatinase producers were surrounded by wide clear zones (*Staphylococcus aureus* used as positive control). A test was considered to be positive, however, when the medium underlying the bacterial growth (swept off to allow the reagent to act) remained completely transparent.

Deoxyribonuclease production. This was detected by a plate method (Kay, 1954) similar to that described above for gelatin hydrolysis except that 0·2 % (w/v) sodium deoxyribonucleate (Nutritional Biochemicals Corp.) was substituted for gelatin. The reagent, which precipitates unhydrolysed DNA, was added to plate cultures

after incubation for 2–3 days. A transparent zone peripheral to bacterial growth indicated enzymic depolymerization of the DNA.

Tests of capacity to produce acid from carbohydrates. The following compounds were investigated: glucose, galactose, rhamnose, fructose, arabinose, xylose, maltose, sucrose, lactose. Sterile stock solutions (filtered through Millipore 0·45μ membranes) were added to the rich proteinaceous agar medium previously described (Catlin & Cunningham, 1961) to give concentrations of 0·5% (w/v). The possibility that slight acid production resulting from oxidative attack might be neutralized by alkali production, prompted the use of two additional media: (i) the low-peptone base of Hugh & Leifson (1953) with the concentration of agar (Purified, Difco) increased to 0·7% (w/v) to permit surface growth on agar slopes, and with aseptic addition of 1% (w/v) carbohydrate; (ii) the ammonium-free sustaining medium of Elrod & Braun (1942) with bromthymol blue indicator added, and aseptic addition of 1% (w/v) carbohydrate; this was tubed in 1 ml. volumes and inoculated with 10^{10}–10^{11} cells removed from 20 hr HIY-1 agar cultures. In addition, heart infusion agar slopes with bromthymol blue and 10% (w/v) glucose or 10% (w/v) lactose were used, as recommended for certain Gram-negative rods (Schaub & Hauber, 1948). A carbohydrate-free control tube of each basal medium was inoculated with each strain. All media were incubated at 35° for at least a week and inspected periodically for acid production.

Indole production. Heart infusion broth (Difco) was supplemented aseptically with filter-sterilized L-tryptophan to give 0·1 mM. Cultures, sampled after incubation for 2 and 7 days, were tested for indole using Kovács reagent (*Manual of Microbiological Methods*, 1957, p. 156). *Escherichia coli* was used as positive control.

Urea hydrolysis. Urea agar base concentrate (Difco) was prepared according to the manufacturer's directions. The test and control agar slopes were streaked, incubated, and examined as described below for citrate agar.

Growth on citrate agar. Simmons citrate agar (Difco) slopes, streaked with a small inoculum from a 20-hr HIY-1 agar culture, were incubated at 35° for 1 week, and examined periodically for evidence of growth and alkali production. *Klebsiella pneumoniae* was used as positive control.

Response to antibiotics. Cultures diluted to contain about 10^7 bacteria/ml. were spread on the surfaces of HIY-1 agar plates by using sterile cotton swabs. 'Sensi-discs' (Baltimore Biological Lab.) or 'Multidisks' (Consolidated Lab. Inc., Chicago Heights) containing the concentrations of antibiotics listed in Table 1 were placed on the agar, and the plates were incubated at 35° for 20 hr. A strain was regarded as susceptible to the antibacterial action of the agent when the disc was surrounded by a clear zone of any radius exceeding 1 mm., indicating inhibition of growth. Resistance was indicated by growth up to the margin of the disc.

Nitrate reduction. Nitrate broth was prepared by addition of 0·1% (w/v) KNO_3 to heart infusion broth (Difco). Periodically during incubation at 35° for 7 days, samples of the cultures were tested for nitrite with sulphanilic acid and dimethyl-alpha-naphthylamine reagents (*Manual of Microbiological Methods*, 1957, p. 153). Negative nitrite tests were examined for residual nitrate by adding zinc granules.

Nitrite disappearance. Required volumes of a 2% (w/v) KNO_2 solution were added to heart infusion broth (Difco) to give concentrations of 0·1%, 0·03, 0·01 and 0·005%. An inverted insert vial was placed in each tube of nitrite broth before

autoclaving. Cultures were inspected periodically for evidence of growth and accumulation of gas, and samples were tested for nitrite (reagents above).

Deoxyribonucleate preparations

A given *str-r* strain, subcultured on HIY-1 agar containing 500 μg. DHS/ml., was inoculated into litre quantities of HIY-1 broth + 200 μg. DHS/ml. contained in Fernbach flasks. After 16–20 hr incubation with shaking, the cultures (usually 2) were harvested in 500 ml. cups in a refrigerated centrifuge, washed once and resuspended in EDTA + NaCl solution (0·1 M ethylenediaminetetra-acetate in a solution of 0·15 M-NaCl, pH adjusted to 7·3–7·4), and frozen.

The bacterial suspension was thawed at 50°, transferred to room temperature, and stirred mechanically during lysis by 1–3% dodecyl sodium sulphate (Matheson, Coleman & Bell; twice recrystallized from hot ethanol) which was added as a 30% (w/v) aqueous solution. Lysis, essentially complete in 0·5–3 hr, resulted in a partially transparent and viscous solution. (It may be noted that N9 and some other strains of *Neisseria catarrhalis*, in contrast to *N. meningitidis* previously described (Catlin, 1960*a*), liberated a deoxyribonuclease capable of depolymerizing the transforming DNA when the process of lysis was carried out in a solution of NaCl + 0·015 M sodium citrate.) The lysate, transferred to a litre beaker, was rotated vigorously by hand in a horizontal plane while 1–2 volumes of 95% ethanol was slowly added. The resulting fibrous precipitate usually could be lifted out; occasionally centrifugation was required.

The nucleate fibres were dissolved in EDTA + NaCl solution (with added NaCl to give a final concentration of 1 M) with stirring at 5°. This solution was clarified by centrifugation (32,000*g*; 3°; 110 min.), and fibres precipitated with ethanol, as before, were lifted immediately from the fluid in which a flocculent precipitate was forming. These were washed in 75% (v/v) ethanol, drained, and redissolved in EDTA + NaCl solution. Suggestions concerning a desirable range of DNA concentration are given by Marmur (1961). The nucleate was partially deproteinized with dodecyl sodium sulphate (step 1, described in Catlin & Cunningham, 1958), and the ethanol-precipitated fibres were dissolved again in EDTA + NaCl solution, and subjected to centrifugation and reprecipitation. Digestion with crystalline ribonuclease (Worthington Biochemical Corp.) done out as described by Marmur (1961), except that the enzyme solution (5 mg./ml. in 0·15 M-NaCl) was boiled for 5 min. before use. Thereafter, the DNA preparation was subjected to two further steps of deproteinization with dodecyl sulphate, each followed by an additional step of centrifugation of the DNA solution (in M-NaCl) for 110 min. (32,000*g*; 3°), and reprecipitation of fibres with 1–2 volumes of ethanol. Sterilized for 3–4 hr in 75% (v/v) ethanol, the drained fibres finally were dissolved in sterile 0·15 M-NaCl solution. Concentration of DNA was determined by the diphenylamine reaction (Dische, 1955).

Analysis of the base composition of DNA preparations

RNA was assayed by the method of Pesez as modified for quantitative use by Zamenhof & Chargaff (Zamenhof, 1957), except that preliminary drying of the sample was omitted. Samples submitted to base analysis showed not more than 3·7% RNA.

The DNA samples were hydrolysed as described by Wyatt & Cohen (1953). Instead of an oil bath, a Slaco test-tube heater (Hallikainen Instruments, Berkeley, Cal.), set at 175°, was used to heat the sealed ampoules, which were made from ignition tubes (10 × 70 mm.). Safety shields were dispensed with, but the hydrolysates were frozen before opening to decrease the internal pressure which develops. Bases were then determined as described by Wyatt (1951), except that the differential extinction method of spectrophotometric assay was used (Vischer & Chargaff, 1948), the coefficients being given by Bendich (1957). Replicate determinations agreed within 5%.

Transformation test

A quantitative method developed for investigating transformation of *Neisseria* to streptomycin resistance has been described in detail (Catlin, 1960*a*; Catlin & Cunningham, 1961). It was used with only minor changes. A physiologically active population for use as recipient was obtained by twice subculturing the parent strain on HIY-1 agar at about 12-hr intervals. Bacteria taken from the second subculture (35° for 11–12·5 hr) were suspended in HI-1 broth (30°) to give a slight visible turbidity (*c.* $1–2 \times 10^7$ bacteria/ml.). A further dilution (in accordance with expected competence of the strain) was prepared and used immediately. Equal volumes (1·5 ml.) of recipient suspension and DNA solution (final concentration, 10 μg./ml.) were mixed and incubated at 30°. After 30 min., unbound transforming DNA was inactivated by addition of sterile crystalline deoxyribonuclease (Catlin, 1960*a*). A sample of the population taken at this time was suitably diluted and plated to determine the total number of bacteria/ml. Each reaction mixture was then assayed to determine the number of transformants/ml. For this, a sample (diluted in broth if necessary) was mixed with HIY-1 soft agar (0·7%, w/v) kept fluid at 44°; immediately the entire volume was pipetted in 4 ml. samples on the surfaces of 8 HIY-1 hard agar (1·4%, w/v) plates. The plates were promptly incubated at 35° without stacking. A soft agar overlay containing dihydrostreptomycin (a concentration sufficient to give 500 μg. DHS/ml. after diffusion) was added 5–7 hr after the time of initial exposure of bacteria to DNA: 5 hr, Ne 11; 6 hr, all other strains, routinely; 7 hr, some tests of the slow-growing strains Ne 13 and NCTC 4103. Transformant colonies were counted after incubation at 35° for 4–6 days.

Certain precautions were observed routinely. A control test with deoxyribonuclease-inactivated DNA was included in each experiment to reveal possible *str-r* mutants. These were not encountered with the population densities employed. The remnant of the broth dilution of DNA prepared for each test was incubated as a sterility control. The identity of selected transformant colonies was confirmed by microscopic examination and oxidase reaction.

Absence of aggregation cocci is essential for accurate determination of % transformation. To obtain a general impression of the reliability of tests with a given strain, a wet mount of coccal suspension was prepared soon after mixing with DNA. In all tests with strains 8313, N9, and ATCC 8193, microscopic examination revealed that more than 85% of the presumptive colony-forming units (100 counted) were single or paired cocci and aggregates numbering more than 3–4 were rare. Suspensions of Ne 13 and NCTC 4103 were somewhat less homogeneous, containing up to 5% aggregates having 5–8 cocci. Suspensions of strains Ne 11 and Ne 23

contained the most clumps: often 50% single and paired cocci, and 5–20% aggregates of more than 8 cocci. Therefore, estimates of % transformation of Ne 11 and Ne 23 were too high. As an experiment with any given recipient included tests of all transforming preparations, however, results were affected equally by aggregation.

RESULTS

Bacteriological characterization. All 7 strains were Gram-negative cocci. Cocci tended to occur in pairs with adjacent sides flattened, and their division was typical of that described for Neisseria (e.g. Murray & Truant, 1954; Piéchaud, 1961). Growing diplococci appeared to increase in width, and division occurred in a plane perpendicular to the previous plane of division.

Table 1. *Characteristics of strains of Neisseria catarrhalis*

Reaction or test*	Ne 11	Ne 13	ATCC 8193	Ne 23	N 9	8313	NCTC 4103
Oxidase reaction	+	+	+	+	+	+	+
Catalase production	+	+	+	+	+	+	+
Deoxyribonuclease production	+	+	+	+	+	+	+
Acidification of medium containing carbohydrate	−	−	−	−	−	−	−
Indole production	−	−	−	−	−	−	−
Urea hydrolysis	−	−	−	−	−	−	−
Growth on citrate agar	−	−	−	−	−	−	−
Response to antibiotics							
Penicillin (2 units) Streptomycin (2 μg.) Tetracycline (5 μg.) Oleandomycin (2 μg.) Neomycin (5 μg.) Chloramphenicol (5 μg.) Erythromycin (2 μg.) Novobiocin (5 μg.) Kanamycin (5 μg.) Vancomycin (30 μg.)	S	S	S	S	S	S	S
Vancomycin (5 μg.)	R	R	R	R	R	R	R
Nitrite disappearance	+	+	+	+	+	+	+
Nitrate reduction	+	+	+	+	+	+	−
Production of diffusible pigment	−	−	−	−	−	−	+
Gelatin hydrolysis	+	+	+	+	+	+	−

* See Methods for details and for carbohydrates tested. Symbols: +, positive results; −, negative results; S, susceptible; R, resistant.

Each strain was examined at least twice: when first received, and later when the entire group had been collected. Most of the tests (Table 1) did not reveal significant differences among the strains. Thus, all showed strong deoxyribonuclease activity. All were susceptible to the antibacterial action of 9 of the 10 antibiotics tested. Professor M. J. Pelczar, Jr. earlier noted (personal communication) that neisserias are typically resistant to low concentrations of vancomycin. In confirma-

tion of that observation, all 7 of the strains examined here were resistant to vancomycin applied in discs containing 5 μg., but were susceptible to 30 μg.

Potassium nitrite at concentrations of 0·1 or 0·03% inhibited growth of all strains, However, all tolerated 0·005% KNO_2, and growth of Ne 11 and ATCC 8193 occurred in 0·01%. Nitrite disappeared within a 5 day incubation period from all visibly turbid cultures; no gas accumulated in inverted insert vials.

Table 2. *Guanine + cytosine content of transforming DNA preparations from Neisseria catarrhalis strains*

Source of DNA	Mole % G+C from Chromatography	Tm	Density
Ne 11 *str-r*	40·7*, 41·3	41†	—
Ne 13 *str-r*	40·1*	—	42‡, 40·6§
Ne 13 *str-r* 11	41·3*	—	—
ATCC 8193 *str-r*	42·2, 42·3	—	—
Ne 23 *str-r*	42·1, 42·2	—	—
N 9 *str-r*	42·3, 43·0	—	—
8313 *str-r*	42·1, 42·4	—	—
NCTC 4103 *str-r*	44·4, 44·4, 45·1	—	43·2§
NCTC 4103 *str-r* 11	44·6	—	—

* Catlin & Cunningham, 1961.
† Marmur & Doty, 1962.
‡ Schildkraut, Marmur & Doty, 1962.
§ Determination by W. R. Guild (personal communication).

Four characteristics served to differentiate strain NCTC 4103 from the other 6 strains, which formed a homogeneous group: (i) NCTC 4103 did not reduce nitrate to nitrite during 7 days incubation; the others reduced nitrate within 20 hr; (ii) cultures of 4103 on HIY-1 agar developed a yellowish-tan coloration, presumably a water-soluble pigment; this was usually slightly visible in heavily streaked areas after incubation for 24 hr, and gradually diffused through the agar; pigment production was intense on HIY agar containing 5 μg. vancomycin/ml.; (iii) weak proteolytic activity of all strains except NCTC 4103 was detected by hydrolysis of gelatin in that part of the medium directly under the bacterial growth; (iv) on HIY-1 agar slopes streaked in duplicate with each strain, and incubated at 28° and 36°, growth-retardation at 28° was more conspicuous for NCTC 4103 than for the other strains.

Base content. Analyses of the over-all base composition of the DNA preparations showed the expected equivalence between adenine and thymine, and between guanine and cytosine. Therefore, values are given only for

$$\text{mole \% G+C} (= \text{G+C/A+T+G+C}).$$

Table 2 lists separately the results of independent determinations, and gives some analytical values obtained by other investigators for the same DNA preparations. Three distinguishable classes were found among these 7 strains. G+C values for strains Ne 11 and Ne 13 averaged 41%. Values for strain NCTC 4103 were significantly higher (about 44·5%), and the *str-r* strains were indistinguishable, although one was derived by spontaneous mutation and the second by transformation (with

DNA from Ne 11 *str-r*). The third class, the 4 remaining strains, was intermediate (average 42·3%) and showed no overlap with either of the other classes.

Transformation. Transforming activity of the DNA preparations was examined in at least three separate experiments with each recipient, in view of the cellular aggregation of some strains (Methods). Results of single experiments with 6 of the strains are given in Table 3. Transformation of strain 8313 was highly reproducible. On the other hand, up to threefold differences of % transformation were found with Ne 11 (the values given are from the experiment showing least aggregation). In general, however, the ratios of % transformation by DNA from the *str-r* strain corresponding to the recipient to % transformation by any other DNA preparation were reproducible in experiments with these 6 strains.

Table 3. *Transforming activity of DNA preparations from strains of Neisseria catarrhalis*

	Recipient strain (% transformation)					
Source of DNA	Ne 11	ATCC 8193	Ne 23	N 9	8313	NCTC 4103
Ne 11 *str-r*	5·38	1·69	8·99	1·22	2·69	0·025
Ne 13 *str-r*	3·79	0·50	6·77	0·90	1·53	0·012
ATCC 8193 *str-r*	4·90	2·00	8·77	0·98	3·58	0·011
Ne 23 *str-r*	4·99	1·26	11·08	1·21	3·18	0·011
N 9 *str-r*	4·51	1·33	6·30	1·14	3·72	0·242
8313 *str-r*	3·78	1·24	6·54	1·09	4·71	0·006
NCTC 4103 *str-r*	0·06	0·033	0·27	0·011	0·69	2·492
NCTC 4013 *str-r* 11	1·65	0·67	4·07	0·26	2·27	1·078

Erratic results were obtained in tests of strain Ne 13 recipient cocci with DNA from Ne 13 *str-r*. The portion of the treated population which attained resistance to 500 μg. DHS/ml. varied from 0·02 to 5% in separate tests. Transformation by DNA preparations from strains N 9 and 8313 frequently was too low to be detected, as previously noted (Catlin, 1963, *Microbiol. Genet. Bull.* 19, 5). However, when the challenge dose was 25 μg.DHS/ml., transformation was 0·1% or higher in tests of DNAs from all of the strains except NCTC 4103, which elicited 0·01% transformation. Clearly, then, genetic information involving the *str-r* determinant could be transferred successfully, although test conditions required for reliable quantitative assay of high-degree resistance have not been found for strain Ne 13.

Results of the transformation tests suggest that strains Ne 11, Ne 13, ATCC 8193, Ne 23, N9, and 8313 are closely related. On the other hand, this group can be differentiated from strain NCTC 4103 by the distinctly lower frequencies of reciprocal transformation. The DNA preparation from the transformant strain NCTC 4103 *str-r* 11 elicited transformation of the group of 6 strains at intermediate frequencies, indicating increased molecular homology (Marmur, Seaman & Levine, 1963; Catlin, 1964).

DISCUSSION

The hypothesis that similarity of guanine + cytosine (G + C) content is a minimum requirement for genetic compatibility among different species has predictive value (Marmur, Seaman & Levine, 1963). However, the findings with *Neisseria catarrhalis* illustrate the fact that DNA preparations may elicit genetic transformation in spite

of apparently significant differences of *average* G+C content of the donor and recipient strains. Presumably these strains possess homologies of nucleotide sequence, which are believed to be required for successful transformation, as well as some degree of heterogeneity of base composition. This possibility was recognized (e.g. Marmur *et al.* 1961), but in general the analytical results obtained with physical methods suggested that a given microbial DNA has a relatively narrow range of base composition, whereas the mean G+C content of DNAs from all microbial species varies widely (from about 25 to 75%). Moreover, intramolecular heterogeneity of base composition was thought to be relatively low (e.g. Sueoka, Marmur & Doty, 1959). These findings were the basis of the expectation that few or no base sequence homologies would occur in two DNA preparations whose average base composition differed very significantly. Recent evidence suggests that intramolecular heterogeneity in pneumococcal DNA (Guild, 1963) may be greater than earlier estimated. The *N. catarrhalis* findings presented here illustrate the difficulty of making specific inferences concerning genetic homology solely on the basis of average G+C data. With the development of new methods for detecting nucleotide sequence homologies (e.g. McCarthy & Bolton, 1963; and discussion in Marmur, Falkow & Mandel, 1963), average base composition data can be relieved of the burden of application at the fine-structure level.

Bacteriological tests adequate to reveal subgroups within the 'non-saccharolytic *Neisseria*' are rarely used. Early workers described a number of types of *N. catarrhalis* largely distinguished on the basis of colonial morphology. An organism described in 1906 by von Lingelsheim, and designated *N. pharyngis cinerea* in *Topley and Wilson's Principles* (1955), has been considered to be merely a variety of *N. catarrhalis*. The Subcommittee on the family Neisseriaceae (1954) noted that *N. catarrhalis* had not been the subject of any special study because it offered fewer complications than some of the other species to students of the genus. Berger & Paepcke (1962) investigated 30 non-saccharolytic strains from the human nasopharynx, using a variety of methods, including precipitin tests, utilization of organic acids, growth at 22°, action on nitrates and nitrites, and pigment production. They concluded that their strains could be divided into two distinct groups corresponding to *N. catarrhalis* and *N. cinerea*.

Analyses of additional *Neisseria catarrhalis* strains will be required to determine the limits of the range of DNA base content, and whether strains exist which have G+C values intermediate between the three classes (41, 42·3, and 44·5%) indicated by the data of Table 2. In spite of the reproducible differences of % G+C, strain Ne 11 and the group of 4 strains (ATCC 8193, Ne 23, N 9, and 8313) were not separable on the basis either of bacteriological characteristics (Table 1) or of transformation frequencies involving the *str-r* marker (Table 3). Differences may be revealed later, however, by investigation of other enzymic activities and additional genetic markers. On the other hand, strain NCTC 4103 is separable from the other 6 strains on the basis of differences of 4 physiological characters and of reciprocal transformation frequencies. Except that nitrite disappearance was not accompanied by gas formation, strain NCTC 4103 appears to correspond to *N. cinerea*, as characterized by Berger & Paepcke (1962); 24 of their 27 *N. cinerea* strains produced gas from nitrite. The 6 other strains are typical of *N. catarrhalis*. Interestingly, Berger & Paepcke (1962) concluded from their bacteriological study and survey of the litera-

ture that the frequency of isolation of these organisms has changed. *N. catarrhalis* which was considered to be a common inhabitant of the nasopharynx before 1928 is now comparatively rare, whereas the incidence of *N. cinerea* is believed to have increased.

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Transforming Activities and Base Composition of Deoxyribonucleates from Strains of *Moraxella* and *Mima*

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SUMMARY

Fourteen strains of *Moraxella* and possibly allied organisms were studied: *M. liquefaciens*, *M. bovis*, *M. nonliquefaciens*, oxidase-positive and oxidase-negative strains of *Mima polymorpha* and *Herellea vaginicola*. Classical bacteriological characteristics, capacity to undergo genetic transformation (resistance to 500 μg. dihydrostreptomycin/ml.), and DNA base composition were examined. *Moraxella nonliquefaciens* and *Mima polymorpha* var. *oxidans* were transformable (2 strains at frequencies of 1–4 %). Attempts to elicit transformation of the remaining organisms were unsuccessful. The ability of DNA preparations from *M. polymorpha* var. *oxidans* to elicit high frequencies of transformation of *Moraxella nonliquefaciens*, and *vice versa*, indicated their close relationship. Moreover, their DNA base contents were indistinguishable (about 44·4 % guanine + cytosine, 6 strains). The G + C contents averaged 43·1 % for 3 oxidase-negative *Mima polymorpha* strains and 39·8 % for 2 *Herellea vaginicola* strains.

INTRODUCTION

Transformation of Moraxella was described by Bövre & Henriksen (1962). Our own studies of this group were initiated earlier as a result of finding that genetic transformation of *Neisseria catarrhalis* was elicited by DNA preparations from certain strains of the so-called Mima-Herellea group. The development of a reciprocal transformation system became essential. Furthermore, elucidation of possible interrelations of these 'Neisseria-like' organisms which might be gained from transformation studies was clearly needed.

Mima polymorpha was described briefly by De Bord in 1939, and later somewhat more completely (De Bord, 1942) as a non-motile, Gram-negative pleomorphic rod which tended on solid media to exhibit diplococcal forms morphologically indistinguishable from *Neisseria gonorrhoeae*. As with *N. catarrhalis*, carbohydrates were not attacked. Though many isolates were oxidase-negative, some were oxidase-positive (*M. polymorpha* var. *oxidans*). De Bord (1942) also proposed the designation *Herellea vaginicola* for another morphologically similar form which was oxidase-negative, and produced acid without gas from glucose and from some other carbohydrates.

Henriksen (1952) and Murray & Truant (1954) considered that *Mima polymorpha* var. *oxidans* might be the same as *Moraxella nonliquefaciens*. Serological analyses provided conflicting evidence (Cary, 1961; Mitchell & Burrell, 1964). Reviewing the standing in nomenclature of subdivisions within the tribe Mimeae, Henriksen (1963)

concluded that the name *Mima polymorpha* var. *oxidans* is illegitimate because it is a later synonym of *Moraxella nonliquefaciens*, that *Herellea vaginicola* is a *nomen dubium*, and that the tribe Mimeae has no place in the taxonomic system. On the other hand, Lindberg & Moschides (1962) proposed that the species 'vaginicola' and 'polymorpha' be combined in the genus *Mima*. Problems associated with these controversial 'Neisseria-like' bacilli have been reviewed by Rosebury (1962), and various solutions of the problem of their taxonomic position have been proposed (e.g. Piéchaud, 1961; Lautrop, 1961; Stenzel & Mannheim, 1963). An *ad hoc* committee has been organized to study the nomenclature and classification of 'Bacterium anitratum', the moraxellas, the Mimeae and allied organisms (Lautrop, 1963).

When strains of *Mima polymorpha* var. *oxidans* obtained locally were found to undergo transformation, authentic strains of *Moraxella nonliquefaciens* and other possibly related organisms were obtained for comparison. Characteristics of the particular strains, their genetic competence, and the base composition of their DNA were investigated. A transformation procedure was developed which provided a genetic approach to problems concerning relationships of these enigmatic bacteria. Evidence obtained bears on two specific questions. Is there a genetic basis for the opinion that strains of *Mima polymorpha* var. *oxidans* and *Moraxella nonliquefaciens* are similar? Are strains of *Mima polymorpha* var. *oxidans* closely related to *M. polymorpha* or to *Herellea vaginicola*?

METHODS

Of the 14 strains investigated, 6 were classified as species of *Moraxella: M. liquefaciens* (NCTC 7911) obtained from the National Collection of Type Cultures, London; *M. bovis* (NCTC 9426) obtained from Professor S. D. Henriksen, Oslo; and 4 strains of *M. nonliquefaciens* also from Professor Henriksen (11865/52, 7146/51, 18522/51, 19116/51; hereafter the last two numbers will be deleted from these strain designations). Two strains designated *Mima polymorpha* var. *oxidans* were received from the Milwaukee Health Department (MHD 2/10 from a gonorrhoea-like syndrome; MHD 307 from an ulcer which developed after vein stripping). Two strains designated *M. polymorpha* were from the American Type Culture Collection, Washington, D.C. (ATCC 10973 which, although oxidase-positive, did not carry the varietal designation; and ATCC 9957). Two strains of *M. polymorpha* were obtained locally from specimens of spinal fluid (Raschig, non-fatal meningitis; MHD 12/13, fatal meningitis). Two strains designated *Herellea vaginicola* were received from the Milwaukee Health Department (MHD 11/30, throat culture; MHD 305, a stock culture obtained for reference purposes 5 yr. earlier from the Communicable Disease Center, U.S. Public Health Service).

When each strain was received, it was subcultured on heart infusion + yeast-extract agar with supplements (HIY-1 agar, Catlin & Cunningham, 1961) and on blood agar. Cultures were examined for homogeneity, and at least two typical colonies were separately subcultured. To decrease population changes thereafter, subcultures were made by picking ten to twenty young isolated typical colonies.

Storage of cultures and media have been described in the preceding communication (Catlin & Cunningham, 1964). Differential media were prepared in quantities large enough for testing all strains at one time. In addition, Loeffler medium (slopes,

Baltimore Biological Laboratory) was streaked, incubated at 35° for 4 weeks, and examined periodically for evidence of digestion of the inspissated serum.

Previously described methods (Catlin & Cunningham, 1964) were used for preparing transforming DNA, for chemical determination of base composition, and for transformation tests. Minor modifications which were found to increase transformation frequencies of the moraxellas will be described in the Results section.

Dihydrostreptomycin sulphate (Squibb) will be referred to as DHS, and strains genetically resistant to its antibacterial action as *str-r*. Spontaneous mutants resistant to at least 500 μg. DHS/ml. were obtained without difficulty from all but one of the parent strains. A resistant mutant of *Moraxella nonliquefaciens* 19116 was not recovered from any of twelve independent flask cultures. However, *str-r* strains were readily obtained by transformation. One of these, designated 19116 *str-r* 18522, was elicited by treatment of the parent strain 19116 with DNA from *M. nonliquefaciens* 18522 *str-r*, and was recovered from agar containing DHS/500 μg. ml. The identity of all *str-r* mutants and transformants from which DNA was extracted was confirmed by microscopic appearance, typical oxidase reaction, antibiotic susceptibilities, and typical reactions in selected media (glucose, gelatin). All characteristics examined were identical with those of the parent except for streptomycin resistance and, occasionally, morphology.

The temperature of circulating water baths (Bronwill and Magni Whirl) was controlled to $\pm 0{\cdot}1°$. Petri dish cultures for transformation experiments were placed in humidified air incubators (New Brunswick model G27 with circulating fans, and National water-jacketed) and were not stacked until they warmed; thereafter their temperature varied less than 0·5°. The growth response of *Moraxella nonliquefaciens* on agar medium was related in an ill-defined way to the moisture content, as noted by Henriksen (1952). Therefore, cultures of recipient organisms were incubated in jars on moist bibulous paper.

RESULTS

Bacteriological characteristics

All strains were Gram-negative rods which tended to occur in pairs and in short chains. They were non-motile and did not form spores. Some of the paired rods observed in young cultures on moist HIY-1 agar were so broad and short that they resembled Neisseria, as has been observed repeatedly (e.g. discussion in Rosebury, 1962). Three of the strains of *Moraxella nonliquefaciens* received from Professor Henriksen were examined earlier by Murray & Traunt (1954). They emphasized a useful morphological difference between the genera *Moraxella* and *Neisseria*, namely, that cellular division of moraxellas proceeds in a single plane, with resulting chain formation, whereas the division of neisseriae occurs in two planes.

The reactions of each strain were examined at least twice with similar results (Table 1). *Herellea vaginicola* strain MHD 11/30 exhibited the oxidative attack of carbohydrates typical of this disputed group. The carbohydrates tested were incorporated in the basal media of Elrod & Braun and Hugh & Leifson previously described (Catlin & Cunningham, 1964). Acid was produced from glucose, galactose, rhamnose, arabinose, xylose and lactose; whereas maltose, sucrose and fructose media remained alkaline, as did the carbohydrate-free controls.

Table 1. *Characteristics of the organisms*

Reaction or test*	*Moraxella* sp.						*Mima polymorpha*						*Herellea vaginicola*	
	liquefaciens NCTC 7911	*bovis* NCTC 9426	*nonliquefaciens* 11865	*nonliquefaciens* 7146	*nonliquefaciens* 18322	*nonliquefaciens* 19116	Var. *oxidans* MHD 2/10	Var. *oxidans* MHD 307	ATCC 10973	ATCC 9957	Raschig	MHD 12/13	MHD 11/30	MHD 305
Oxidase reaction	+	+	+	+	+	+	+	+	+	−	−	−	−	−
Proteolytic activity (against inspissated serum, gelatin)	+	+	−	−	−	−	−	−	−	−	−	−	−	−
Production of acid from one or more carbohydrates	−	−	−	−	−	−	−	−	−	−	−	−	+	−
Nitrate reduction	+	−	+	Sl	+	−	−	−	−	−	−	−	−	−
Urea hydrolysis	−	−	−	−	−	−	−	−	−	−	−	−	−	+
Growth on citrate agar	−	−	−	−	−	−	−	−	−	−	−	−	+	+
Response to antibiotics														
Penicillin (2 units)	S	S	S	S	S	S	S	S	S	S	S	S	R	R
Streptomycin (2 μg.)	S	S	S	R	R	S	S	S	S	S	S	S	R	S
Tetracycline (5 μg.)	S	S	S	S	S	S	S	S	S	S	S	S	S	S
Oleandomycin (2 μg.)	R	S	R	S	S	S	S	S	S	S	R	S	R	R
Neomycin (5 μg.)	S	S	S	S	S	S	S	S	S	S	S	S	S	S
Chloramphenicol (5 μg.)	S	S	S	S	S	S	S	S	S	S	S	S	R	R
Erythromycin (2 μg.)	S	S	S	S	S	S	S	S	S	S	S	S	R	S
Novobiocin (5 μg.)	S	S	R	S	S	S	S	S	S	R	R	R	R	R
Kanamycin (5 μg.)	S	S	S	S	S	S	S	S	S	S	S	S	S	S
Vancomycin (5 μg.)	S	S	R	S	S	S	S	S	S	S	R	S	R	R
Vancomycin (30 μg.)	S	S	S	S	S	S	S	S	S	S	S	S	R	R
Transformation test, using homologous DNA, 25 μg./ml.	−	−	−	−	+	+	+	+	+	−	−	−	−	−

* In addition, all strains gave the following reactions: catalase production +, indole production −, nitrite disappearance −. See text for details and for carbohydrates tested. Symbols: +, positive result; −, negative result; Sl, slight reduction; S, susceptible; R, resistant.

Strain MHD 305 of *Herellea vaginicola*, however, was atypical in failing to acidify any of the carbohydrate-containing media, including the supposedly decisive 10% lactose agar slope (Schaub & Hauber, 1948; Garrison & Dodd, 1962). This strain, originally a typical representative of *H. vaginicola*, had been maintained for 5 yr. at room temperature as a sealed stab culture and transferred annually. A subculture, recently obtained for the present work, showed mucoid and smooth types of colonies, which were alike in the reactions examined. The smooth type was selected for transformation experiments.

Moraxella nonliquefaciens strain 11865 on HIY-1 agar produced punctiform colonies in 48 hr. Growth was greatly improved by addition to the medium of 10% (v/v) serum (horse or calf) or 0·2% (v/v) Tween 80 (Mann Research Laboratories, New York). A loopful of Tween 80 (diluted 1/5, neutralized, autoclaved) was added to each differential medium to stimulate growth. A diffusible yellowish pigment developed in areas of luxuriant growth on HIY-1 agar containing serum or Tween. Pigment production was not detected with any of the other strains.

Transformation

Preliminary examination. All strains were examined for capacity to become transformed to high-degree streptomycin resistance. Organisms grown at 35° (and in most cases also at 30°) were exposed to preparations of DNA (final concentration 25μg./ml.) extracted from *str-r* strains corresponding to the recipient. Results obtained with each of the 14 strains are included in Table 1. Negative results signify merely that transformation was not detected under the conditions employed.

The transformation method was modified in various ways as required by special features of the strains. Horse serum (10%, v/v) was added to the culture medium for *Moraxella nonliquefaciens* strain 11865. Three of the strains, *M. nonliquefaciens* 18522 and 7146 and *Herellea vaginicola* MHD 11/30, were resistant to low concentrations of DHS (Table 1), and exhibited high frequencies of spontaneous mutation conferring resistance to DHS 500μg./ml. The parent strains were screened and, from each, a colony was selected which gave the lowest background of spontaneous *str-r* mutants. These were used as recipients in transformation tests carried out with DHS 500 and 1000μg./ml. However, evidence of transformation was obtained only for strain 18522; even in this case, only about an eightfold difference was found between numbers of *str-r* colonies on test plates and on corresponding controls. The strain appeared, therefore, to be unsatisfactory for quantitative work involving the *str-r* marker.

The 4 remaining transformable strains, *Moraxella nonliquefaciens* 19116 and all 3 oxidase-positive strains designated *Mima polymorpha*, were examined under various conditions as a basis for developing reliable transformation test methods. The temperature and time of incubation exerted profound influences on transformation to DHS resistance. Four consecutive steps of the over-all process were examined: (i) incubation of recipients to obtain the maximum proportion of competent cells; (ii) incubation of organisms with DNA; (iii) incubation required to achieve phenotypic expression, that is, the development of a physiological state of in-susceptibility to the challenge dose of the antibiotic; (iv) incubation to allow colony-formation by the genetically transformed organisms.

Characteristics of the oxidase-positive Mima polymorpha strains. Transformation reactions involving the oxidase-positive *Mima polymorpha* strains ATCC 10973 and MHD 307 were similar in most respects. Transformation frequencies, which were relatively low for both strains, were increased three- to five-fold by carrying out the first two incubation steps at 30° rather than at 36°. This is shown for ATCC 10973 in Table 2.

Table 2. *Influence of incubation temperature and DNA concentration on % transformation (resistance to DHS 500 μg./ml.) of Mima polymorpha ATCC 10973*

Incubation temperatures for		% transformation elicited by homologous DNA	
Growth of recipient organisms (14 hr)	DNA treatment (30 min.)*	50 μg./ml.	10 μg./ml.
30°	30°	0·00290	0·00087
30°	36°	0·00202	0·00053
33·5°	30°	0·00141	0·00027
33·5°	36°	0·00099	0·00028
36°	30°	0·00143	0·00031
36°	36°	0·00100	0·00023

* Reaction terminated by addition of deoxyribonuclease. Subsequent incubation, 36° for all plates (steps iii and iv, described in text).

Table 3. *Influence of temperature and time of incubation on % transformation (resistance to DHS 500 μg./ml.) of Mima polymorpha var. oxidans MHD 2/10*

Incubation Temperatures for			Time before addition of DHS (hr)	% transformation elicited by homologous DNA (25 μg./ml.)
Growth of recipient organisms (12 hr)	DNA treatment (30 min.)	Phenotypic expression*		
30°	30°	30°	5	0·98
			6	1·19
			7	1·38
			8	1·37
		36°	5	1·24
			6	1·44
			7	1·54
			8	1·52
36°	36°	36°	5	1·19
			6	1·24
			7	1·32
			8	1·40

* Subsequent incubation, 36° for all plates (step iv, text).

Transformation of MHD 2/10, which occurred at a far higher frequency, was less strongly influenced by differences of incubation temperature within this range. Nevertheless, 30° for the first two incubations, followed by 36°, gave the highest frequency. This is illustrated in Table 3, which shows in addition that a period of 7 hr intervening between initiation of exposure to DNA and application of antibiotic was adequate for maximum expression of the altered phenotype.

The following standardized modifications of transformation procedure were

adopted, therefore, for all 3 oxidase-positive strains of *Mima polymorpha*. (i) Recipient organisms were incubated at 30° for 11–12·5 hr on HIY-1 agar in a humid atmosphere. Organisms taken from agar at this time were suspended in supplemented heart infusion broth (HI-1; Catlin & Cunningham, 1961) at 30° and were immediately exposed to DNA. (ii) Reaction mixtures were incubated at 30°, usually for 30 min. before addition of deoxyribonuclease. (iii) Samples were plated promptly and incubated at 35°. A total period of 7 hr was allowed between the time of mixing recipients with DNA and time of removal of plates from 35° for overlaying DHS-containing agar. (iv) Plates were incubated thereafter at 35° for 4–6 days before counting transformant colonies.

Characteristics of transformation of Moraxella nonliquefaciens strain 19116. In exploratory tests with homologous DNA this strain appeared to be little affected by differences of incubation temperatures, and results were consistent within and between independent tests. However, equivocal results were obtained in tests of strain 19116 with DNA preparations from *Mima polymorpha* strains ATCC 10973 and MHD 307. Variations of numbers of colonies on replicate transformant plates and irregularities of distribution and size of colonies suggested that minor differences of antibiotic concentration had a marked effect. Accordingly, a study was made of the degrees of resistance conferred by various transforming DNA preparations.

Table 4. *Influence of concentration of DHS on % transformation of the oxidase-positive strains*

DNA preparation (25 μg./ml.)	DHS (μg./ml.)*	% transformation of: *Moraxella nonliquefaciens* 19116	% transformation of: *Mima polymorpha* MHD 307	% transformation of: *Mima polymorpha* ATCC 10973	% transformation of: *Mima polymorpha* MHD 2/10
(1) *Mima polymorpha*	25	0·670	0·0063	0·0011	5·31
MHD 2/10 *str-r*	600	0·605	0·0062	0·0011	5·15
(2) *Moraxella nonliquefaciens*	25	1·898	0·0075	0·0017	2·51
19116 *str-r* 18522	600	1·650	0·0070	0·0012	2·40
(3) *Mima polymorpha*	25	0·685	0·0299	0·0018	2·76
MHD 307 *str-r*	600	0·014	0·0281	0·0023	2·82
(4) *Mima polymorpha*	25	0·670	0·0161	0·0057	1·74
ATCC 10973 *str-r*	600	0·009	0·0170	0·0070	1·75
(5) *Moraxella nonliquefaciens*	25	1·260	0·0214	0·0024	(No test)
19116 *str-r* 307	600	0·019	0·0202	0·0019	(No test)
(6) *Moraxella nonliquefaciens*	25	1·400	0·0117	0·0024	1·58
19116 *str-r* 10973	600	0·035	0·0114	0·0028	1·66

* Transformant assay plates after receiving antibiotic challenge dose were incubated at 35° (step iv; other details in text).

Although all *str-r* mutants were obtained by selective growth in an environment containing 500 μg. DHS/ml., all of those examined in this connexion were resistant to at least 600 μg. DHS/ml. This higher concentration was used for the experiments reported in Tables 4 and 5 because it more clearly revealed a difference between *Moraxella nonliquefaciens* 19116 and the 3 strains of *Mima polymorpha*. A satisfactory low concentration for transformation tests with these strains was found to

be 25 μg. DHS/ml. Resistant mutants rarely appeared on control plates containing either concentration of DHS.

The results given in Table 4 were obtained using the standard transformation procedure devised for *Mima polymorpha*. The antibiotic concentration within the range examined did not significantly affect the numbers of transformants produced by 3 of the 4 recipient strains. Only *Moraxella nonliquefaciens* 19116 showed significant differences, and only in those tests where the *str-r* factor originated in strains MHD 307 or ATCC 10973 (Table 4, DNAs 3–6). Transformant colonies elicited by treatment of strain 19116 with DNA preparations 3 and 4 were far more numerous in agar containing 25 μg. DHS/ml. than 600 μg. DHS/ml. The discrepancy was about the same in the tests with DNA preparations 5 and 6, which were extracted from transformants of strain 19116 treated in a previous experiment with DNA preparations 3 and 4 respectively. The strains had been isolated at 35° as single transformant colonies in agar containing 1000 μg. DHS/ml. Microscopic observation revealed that the cells were highly pleomorphic when subcultured at 35° on medium either with or without DHS. Both morphology and growth were more typical when these transformant strains were incubated at 30°.

On the other hand, transformants of *Moraxella nonliquefaciens* 19116 elicited by treatment with DNA preparations 1 and 2 (Table 4) exhibited typical cellular morphology when cultivated at 35° (or 30°) on medium containing either 25 or 600 μg. DHS/ml. These observations of the various transformants of strain 19116 suggested that the aberrant response of some might be related to the temperature of incubation of the transformation assay plates.

In an exploratory test, strain 19116 recipient organisms were treated with DNA from *Mima polymorpha* ATCC 10973, all incubation steps (i–iv, above) being carried out at 30°. After 14 hr incubation of transformant plates (step iv), minute colonies were visible in medium containing 600 μg. DHS/ml. At this time half of these plates were transferred to 37° for a 6 day period of additional incubation; none of these colonies increased in diameter, whereas colonies on the plates retained at 30° grew to typical size.

A quantitative study was made of the influence of varying the temperature of incubation for three steps of the standard transformation test procedure. Results given in Table 5 clearly show that when the *str-r* marker of DNA from *Mima polymorpha* ATCC 10973 *str-r* was introduced into *Moraxella nonliquefaciens* 19116, the temperature of incubation of the transformant assay plates determined whether or not transformants could grow into countable colonies in the presence of DHS. (In this experiment, the incubation temperature was the same for steps iii and iv: either 29° or 36°). At 29° approximately the same number of colonies developed in medium containing 25 μg. DHS/ml. and 600 μg, DHS/ml. Incubation at 36° (steps iii and iv, Table 5) prevented the development of most of the colonies in agar containing either DHS concentration. However, in other experiments involving the same recipient and donor DNA (e.g. Table 4, DNA prep. 4) which were carried out at 35°, many more colonies developed in medium containing 25 μg. DHS/ml. than 600 μg. DHS/ml.

When DNA from *Moraxella nonliquefaciens* 19116 *str-r* 18522 served to introduce the *str-r* factor into strain 19116 recipients, the temperature at which transformant assay plates were incubated had little effect on the concentration of DHS tolerated.

Also, a higher frequency of transformation was elicited, as expected for homologous DNA. For purposes of comparison, Table 5 gives results also of a similar experiment with recipient cells of *Mima polymorpha* ATCC 10973 (used at a 200 times higher concentration). In confirmation of previous results (Table 4), the recovery of *str-r* transformants was not significantly affected by concentration of DHS.

Table 5. *Influence of incubation temperatures on the tolerance of transformants to DHS*

Temperature of incubation for				Transformation of			
				Moraxella nonliquefaciens 19116*		*Mima polymorpha* ATCC 10973†	
				By DNA preparation from		By DNA preparation from	
Transformant assay plates (steps iii, iv)	Growth of recipient organisms (step i)	Mixture: cells + DNA (25 μg./ml.) (step ii)	Conc. DHS (μg./ml.)	ATCC 10973 *str-r*	19116 *str-r* 18522	ATCC 10973 *str-r*	19116 *str-r* 18522
29°	29°	29°	25	219	458	74	7
			600	174	441	92	2
29°	29°	36°	25	296	680	28	4
			600	265	697	37	2
29°	36°	29°	25	190	397	34	6
			600	189	362	47	4
29°	36°	36°	25	151	285	21	5
			600	168	299	21	2
36°	29°	29°	25	9	495	166	24
			600	1	386	145	17
36°	29°	36°	25	3	661	48	10
			600	0	655	40	14
36°	36°	29°	25	1	426	66	12
			600	0	318	63	12
36°	36°	36°	25	5	336	28	6
			600	0	291	38	1

* Values: number of transformant colonies/$2{\cdot}5 \times 10^4$ DNA-treated recipient organisms.
† Values: number of transformant colonies/$5{\cdot}0 \times 10^8$ DNA-treated recipient organisms.

In subsequent transformation tests of *Moraxella nonliquefaciens* 19116 (Table 6) all incubation steps (i–iv above) were carried out at 30°.

Influence of DNA concentration and time of exposure. Figures 1 and 2 show the response of recipient organisms of *Mima polymorpha* var. *oxidans* MHD 2/10 (curve 1, both Figs.) and *Moraxella nonliquefaciens* 19116 (curves 2, 3) to DNA preparations from the corresponding *str-r* strains. These tests were carried out (using incubation temperatures established for *Mima polymorpha* strains: 30°–30°–35°–35°) during attempts to elucidate the aberrant response of strain 19116 (Table 4). As in previous tests of *Moraxella nonliquefaciens* strain 19116 treated with DNA from 19116 *str-r* 10973, the recovery of transformants cultivated at 35° in the presence of 600 μg. DHS/ml. (values not plotted) was low and somewhat erratic compared with % transformation obtained using the lower challenge dose (curve 2, both Figs.).

The % transformation of both recipients increased linearly with increasing concentration of DNA over a range of at least 1000-fold (Fig. 1). Notable was the finding (see also in Table 2) that a high DNA concentration (about 50 μg./ml.) was required for maximum yields of transformants. In contrast, the 'saturating' concentration of transforming DNA was only about 0·1 μg. DNA/ml. for a strain of *Streptococcus* (*Diplococcus*) *pneumoniae* (Hotchkiss, 1957), and about 5 μg. DNA/ml. for 2 strains of *Neisseria meningitidis* (Catlin, 1960).

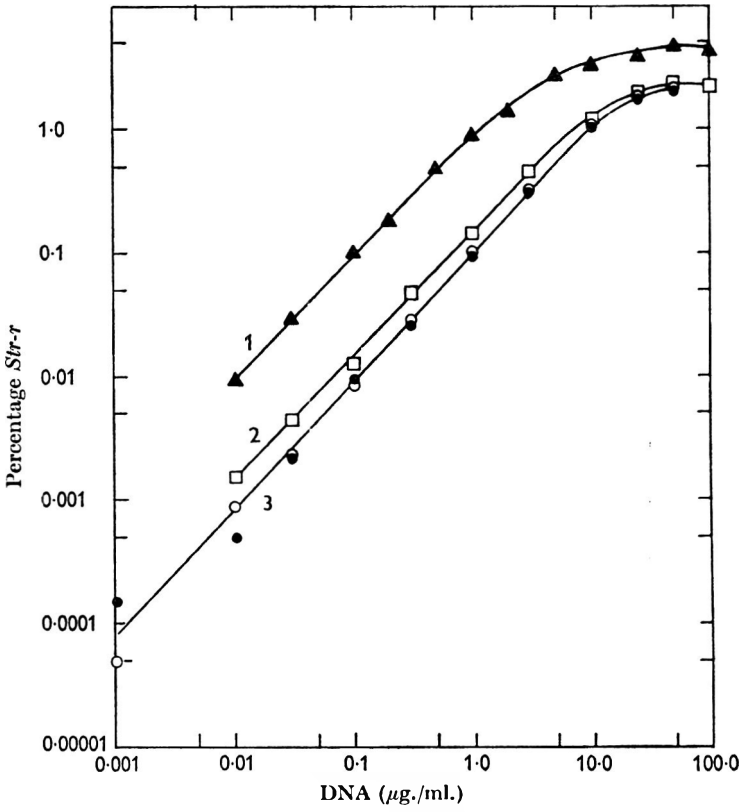

Fig. 1. Percentage transformation as a function of concentration of DNA. Exposure time, 30 min. Curve 1, Mima polymorpha var. oxidans MHD 2/10 treated with DNA from strain MHD 2/10 *str-r*; challenge dose, 600 μg. DHS/ml. Curve 2, Moraxella nonliquefaciens 19116 treated with DNA from strain 19116 *str-r* 10973; challenge dose, 25 μg. DHS/ml. Curve 3, M. nonliquefaciens 19116 treated with DNA from strain 19116 *str-r* 18522; challenge dose, 25 μg. DHS/ml. and 600 μg. DHS/ml.

Figure 2 shows that % transformation of both strains increased rapidly with time of exposure to DNA. Transformation events were initiated in about 0·5 % of the cells of the two recipient populations within the first 5 min. Continued exposure to the DNA for an additional 25 min. effected only about a fourfold increase of % transformation.

Tests of DNAs from the various organisms. DNA preparations from each of the 14 strains were examined in at least three separate transformation experiments with each of the four recipients. Table 6 gives results of a single experiment for each

DNA preparations from 3 of the strains designated *Moraxella nonliquefaciens* and from all 3 oxidase-positive *Mima polymorpha* strains exhibited high transforming activity for two of the recipients (*Moraxella nonliquefaciens* 19116 and *Mima polymorpha* MHD 2/10). On the other hand, DNAs from *Moraxella liquefaciens* and *M. bovis*, and from the oxidase-negative *Mima polymorpha* and *Herellea vaginicola* strains had practically no transforming activity for any of the four recipients in any of the tests.

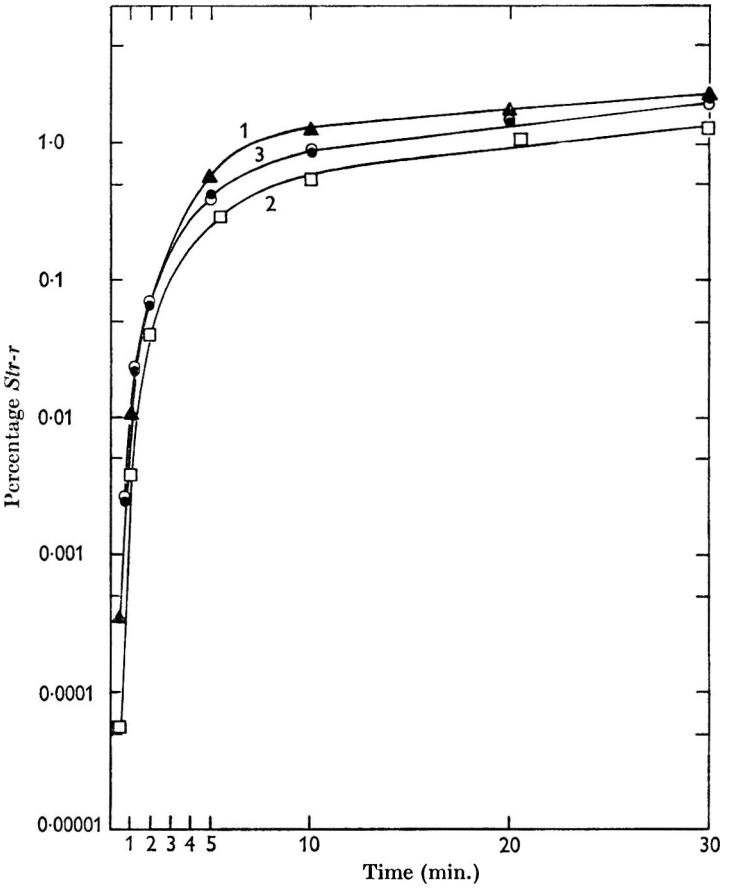

Fig. 2. Percentage transformation as a function of time of exposure of recipient organisms to 50 μg. DNA/ml. Exposure was terminated at a given time by diluting an aliquot of the reaction mixture in broth containing deoxyribonuclease. Curves 1, 2, 3 as for Fig. 1.

Results of tests of *Moraxella nonliquefaciens* 19116 and of *Mima polymorpha* var. *oxidans* MHD 2/10 were reproducible; % transformation obtained in repeated tests varied only by about a factor of 2. In contrast, uncontrollable variation by as much as 10-fold was encountered in tests of the other 2 recipients. For example, compare the selected data given in Table 4 with those of Table 6 for *M. polymorpha* strain MHD 307. In spite of differences of % transformation of these recipients, however, the relative transforming activities of the various DNA preparations were fairly similar in repeated tests.

Table 6. *Transforming activity and base composition of DNA preparations*

Source of DNA*		% transformation, Oxidase-positive recipient strain: *Moraxella nonliquefaciens* 19116	*Mima polymorpha* MHD 2/10	*Mima polymorpha* MHD 307	*Mima polymorpha* ATCC 10973	Mole % guanine + cytosine†
Moraxella						
liquefaciens	NCTC 7911	< 0·00006	0·00008	0·00001	< 0·00001	44·4, 44·2
bovis	NCTC 9426	< 0·00006	0·00004	< 0·00001	< 0·00001	44·6
nonliquefaciens	11865	< 0·00006	0·00004	< 0·00001	< 0·00001	42·5, 42·7, 42·2
	7146	1·453	2·292	0·00102	0·00040	44·7
	18522	1·041	1·784	< 0·00001	< 0·00001	44·4, 44·3
	19116	2·024	1·860	0·00155	0·00058	44·3, 44·1
Mima polymorpha						
(oxidase +)	MHD 2/10	0·870	3·550	0·00057	0·00025	44·6, 44·2
(oxidase +)	MHD 307	0·714	2·075	0·00320	0·00117	44·9, 43·8
(oxidase +)	ATCC 10973	0·775	1·388	0·00214	0·00338	44·6
(oxidase −)	ATCC 9957	< 0·00006	0·00004	< 0·00001	< 0·00001	43·6, 43·2
(oxidase −)	Raschig	< 0·00006	< 0·00004	0·00001	< 0·00001	42·1, 42·2
(oxidase −)	MHD 12/13	< 0·00006	< 0·00004	< 0·00001	< 0·00001	44·0, 43·5
Herellea vaginicola						
	MHD 11/30	< 0·00006	< 0·00004	< 0·00001	< 0·00001	39·9
	MHD 305	< 0·00006	< 0·00004	< 0·00001	< 0·00001	39·8, 39·7

* Preparations were from spontaneous *str-r* mutants of all strains, except *Moraxella nonliquefaciens* 19116 *str-r* 18522 (described in Methods). Treatment with DNA solutions (25 μg./ml.) at 30° for 30 min.; challenge dose dihydrostreptomycin 500 μg./ml.

† Values are results of separate determinations.

Base composition of the DNAs

DNA preparations which were used in the transformation experiments were analysed for base content. The results of separate determinations are given in Table 6. The values of % guanine+cytosine were practically the same for all Moraxella strains studied, except *M. nonliquefaciens* 11865. The wide divergence of values obtained for this organism suggests that it was incorrectly named. The oxidase-positive *Mima polymorpha* strains were indistinguishable from *Moraxella nonliquefaciens* by the criterion of % G+C. However, the oxidase-negative Mima strains form a second, distinct group; and the 2 Herellea strains form a third.

DISCUSSION

Our results independently confirm the finding of Bövre & Henriksen (1962) that *Moraxella nonliquefaciens* is transformable. It is of interest that transformation of different recently isolated strains can be obtained by using somewhat different techniques. Like Bövre & Henriksen, we failed to demonstrate transformation of *M. liquefaciens* strain NCTC 7911.

Genetic evidence obtained in this study corroborates the opinion of Henriksen (1952) and Murray & Truant (1954) that the organism named *Mima polymorpha* var. *oxidans* by De Bord (1942) is, in fact, *Moraxella nonliquefaciens* (Henriksen, 1960, 1963). Similarities of the base composition and transforming activity of DNA preparations from strains designated *M. nonliquefaciens* 7146, 18522 and 19116 and *Mima polymorpha* var. *oxidans* MHD 2/10, MHD 307 and ATCC 10973 establish their close genetic relationship. Accordingly, strains MHD 2/10, MHD 307, and ATCC 10973 will be designated *Moraxella nonliquefaciens* in future references to them.

On the other hand, no genetic relationship was found between *Moraxella nonliquefaciens* and either *Mima polymorpha* or *Herellea vaginicola*. The guanine+cytosine contents of DNA preparations from the 3 oxidase-negative Mima strains (43·1 %) and the 2 Herellea strains (39·8 %) were distinctly lower than the value characteristic of all the moraxellae (about 44·4 %). Furthermore, none of these DNA preparations elicited transformation of competent strains of *Moraxella nonliquefaciens*.

Incidental findings illustrate certain obstacles which might be encountered in any such genetic study. They emphasize the importance of using a number of recipients of each bacterial species, and preferably more than a single genetic marker, if the analyses are to be applied to taxonomic problems. Thus, (i) a strain selected for investigation may be named incorrectly. A DNA preparation from the strain designated *Moraxella nonliquefaciens* 11865 not only failed to elicit transformation of any of 4 transformable strains of this species, but its G+C content (42·5 %) was lower than that of any of the 8 moraxellas.

(ii) A strain may be unsuitable for use as recipient. It may not undergo detectable transformation or, as in the case of *Moraxella nonliquefaciens* strains 7146 and 18522, high background levels of spontaneous mutation of a given marker may interfere.

(iii) DNA from a given donor may elicit unequal frequencies of transformation of various recipients of the same species in respect to any given genetic marker being investigated. Two separate DNA preparations from *Moraxella nonliquefaciens* 18522

failed to elicit detectable transformation of strains ATCC 10973 and MHD 307 in any test, although they exhibited high transforming activity for strains 19116 and MHD 2/10 (Table 6).

(iv) Environmental conditions which are optimal for transformation in one instance may be deleterious in a second. Test conditions which were suitable for detecting transformation of *Moraxella nonliquefaciens* 19116 following exposure to certain DNA preparations (from *str-r* strains of 19116, 7146, 18522, and MHD 2/10) were found to be unsuitable for detecting transformation of the same recipient following treatment with other DNA preparations (from *str-r* strains of ATCC 10973 and MHD 307). In the latter case, transformants developed a temperature-sensitive condition in which, within given limits, tolerance to dihydrostreptomycin was inversely related to the temperature of incubation (Table 5). The genotype of the recipient was important, as well as that of the donor. Thus, introduced into strain 19116, the *str-r* factor of either ATCC 10973 or of MHD 307 was essentially lethal for transformants cultivated at 36–37° in a medium containing 600 μg. DHS/ml. Introduced into strain MHD 2/10, the factor gave rise to transformants capable of apparently normal growth under the same conditions (Table 4). These findings illustrate the practical necessity for investigating the effect of environmental conditions on the response of each recipient strain.

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Reciprocal Genetic Transformation Between *Neisseria catarrhalis* and *Moraxella nonliquefaciens*

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SUMMARY

Deoxyribonucleate (DNA) preparations from *Moraxella nonliquefaciens* elicited genetic transformation of *Neisseria catarrhalis* recipient cells, and *vice versa*. The frequency was low (0·0005% transformation for the most reactive of six strains), as might be expected of an interaction between two organisms as dissimilar as a rod and a coccus. Evidence that the hereditary change (attainment by susceptible cells of resistance to 500 μg. dihydrostreptomyein/ml.) was due to transformation was provided by the typical time course of the reaction, the typical linear response to decreasing concentrations of DNA below 0·1 μg./ml., and by tests of transforming activity of DNA preparations extracted from 11 dihydrostreptomycin-resistant (*str-r*) strains which arose by intergeneric transformation. These DNAs had relatively high transforming activity for recipient strains of both species. Thus, the *str-r* region of the transforming DNA molecule from a transformant strain of *M. nonliquefaciens* was recognized and genetically integrated by populations of *N. catarrhalis* recipient cells at frequencies as much as 10,000 times higher than those of DNA from strains of *M. nonliquefaciens str-r* (derived by spontaneous mutation). The results with DNA preparations from particular transformants are interpreted as indicating that the length of a DNA nucleotide sequence which is integrated by a cell during *str-r* transformation may differ for different cells of the same treated population.

INTRODUCTION

The Gram-negative diplococcus *Neisseria catarrhalis* and the Gram-negative diplobacillus *Moraxella nonliquefaciens* are both inhabitants of the mucosa of man. Both are oxidase-positive, unable to produce acid from carbohydrates, and are susceptible to many antibiotics, including penicillin and streptomycin. Recognition of various resemblances led Henriksen (1952) to suggest that the genus *Moraxella* might appropriately be placed in the family Neisseriaceae. In general, however, the similarities have been considered to be too superficial to indicate any real relationship (Murray & Truant, 1954).

Do such apparent physiological similarities of *Neisseria catarrhalis* and *Moraxella nonliquefaciens* actually indicate a significant degree of genetic relationship? This question can now be raised because methods for detecting genetic homologies have become available. As discussed by Marmur, Falkow & Mandel (1963) several methods, including genetic transformation, can be applied in experimental studies

of taxonomic and evolutionary relationships of bacteria. Such studies are in progress with several groups of bacteria (e.g. Ravin & De Sa, 1964), and many more will be required before we shall have accumulated a body of information sufficient for adequate interpretation of the genetic data and application to particular taxonomic problems.

Transformation involving streptomycin resistance is a highly sensitive method for detecting homology of a particular genetic determinant. Successful transformation of a coccus by deoxyribonucleate (DNA) from a bacterium has not been reported heretofore. In principle, however, differences relating to cellular morphology should not forbid the transfer of genetic information relating to streptomycin resistance, provided that the genetic determinants of morphological characteristics and of response to streptomycin are not associated with the same transforming DNA molecule.

Critical evaluation of the possible occurrence of genetic transformation of a member of one species by DNA extracted from cells of a different species requires a reliable test system, as well as availability of highly transformable strains of both species in order that reciprocal transformation tests can be performed. These requirements are satisfied for both *Neisseria catarrhalis* and *Moraxella nonliquefaciens* (Catlin & Cunningham, 1964*a*, *b*). Though transformation between members of the two species occurred at frequencies far lower than those characteristic of intraspecific transformation, it was amenable to study.

METHODS

The streptomycin-susceptible (*str-s*) recipient strains and the transformation methods used with each have been described in detail (Catlin & Cunningham, 1964*a*, *b*). In brief the method involved three steps: (i) A homogeneous suspension of recipient organisms was prepared from a culture on HIY-1 agar, which had been incubated for 12 hr at 30° (*Moraxella nonliquefaciens*) or at 35° (*Neisseria catarrhalis*). Microscopic examination of suspensions of *N. catarrhalis* strain 8313 showed 95–100% of the presumptive colony-forming units to be single cocci or diplococci. Suspensions of other strains were not so homogeneous, but only *N. catarrhalis* strain Ne 11 showed a pronounced tendency toward cluster-formation. (ii) Recipient organisms were added to each DNA preparation (final concentration 25 μg. DNA/ml. for *M. nonliquefaciens* recipients, 5 μg. DNA/ml. for *N. catarrhalis*). After incubation at 30° for 30 min., inactivating deoxyribonuclease was added. (iii) Appropriate dilutions were plated in HIY-1 agar to determine numbers of colony-forming units exposed, and transformants elicited. Transformant assay plates (8/sample) were incubated at 30° (*M. nonliquefaciens* strain 19116) or at 35° (all other strains). The duration of this incubation varied, depending on the time required for phenotypic expression of the particular strain: *M. nonliquefaciens* 7 hr, *N. catarrhalis* strain Ne 11 5 hr, all other *N. catarrhalis* strains 6 hr. Thereupon, the surfaces were overlaid with medium containing dihydrostreptomycin sulphate at a concentration to give 500 μg./ml. after diffusion throughout the underlying agar. Thirty min. later the plates were returned for further incubation. Colonies growing on this medium are designated *str-r*. Results are expressed as percent transformation: number of *str-r* transformants/100 *str-s* cells exposed.

Each experiment included a control, similar to other tests in all details except that the DNA (autologous) was inactivated with deoxyribonuclease 5 min. before addition of recipient organisms. It was sampled last, and always within 80 min. of the time of initial exposure of organisms to DNA. Thus, the organisms of all tests were distributed in agar within 50 min. of addition of deoxyribonuclease, a precaution to decrease possible error due to multiplication of a rare spontaneous mutant. These were not encountered with concentrations of test organisms below 10^7/ml., and only rarely at higher concentrations.

DNA preparations from *str-r* strains described previously (Catlin & Cunningham, 1964 *a*, *b*) were used in exploratory tests. Colonies appearing on various transformant assay plates were subcultured. Each strain was inspected microscopically and examined for oxidase reaction, response to antibiotics, reaction in the presence of glucose, maltose and sucrose, capacity to produce deoxyribonuclease and gelatinase, and to reduce nitrate and nitrite (Catlin & Cunningham, 1964 *a*, *b*). DNA was extracted from 12 selected strains, and was purified by methods previously described (Catlin & Cunningham, 1961, 1964 *a*).

Strains used as recipients in the transformation tests were selected on the basis of three characteristics: DNA base composition (representatives of different guanine + cytosine classes were examined), transformability, and tendency of clusters of organisms to become dispersed during preparation of suspensions. Ratios of percentage transformation by a given heterologous DNA to percentage transformation by autologous DNA, which were obtained in duplicate experiments with each recipient strain, were in close agreement.

The term 'autologous' (employed by Bracco, Krauss, Roe & MacLeod, 1957) will be used here to refer to the reaction between a given *str-s* recipient strain and DNA extracted from a *str-r* strain derived by spontaneous mutation from that *str-s* strain. The term 'homologous', normally applied to intraspecific reactions, is inappropriate here because of the differences between the strains presently classified as *Neisseria catarrhalis* (Catlin & Cunningham, 1964 *a*). Therefore, 'heterologous' will be applied to all reactions between organisms and DNA of different origins.

RESULTS

Treatment of *Neisseria catarrhalis* recipient organisms with transforming DNA from *str-r* strains of *Moraxella nonliquefaciens* repeatedly yielded a few *str-r* colonies, whereas corresponding controls were negative. For example, following treatment of *N. catarrhalis* strain 8313 ($8{\cdot}9 \times 10^5$ organisms/ml.) with DNA from *M. nonliquefaciens* 18522 *str-r*, fourteen colonies developed on a set of eight plates which had received a total inoculum of 2 ml. These colonies were picked, and were found to be similar to the parental strain in all characteristics examined except for resistance to dihydrostreptomycin. DNA preparations 2 and 3 (of Table 1) were extracted from two separate colony isolates. Similarly, in an experiment with a higher concentration ($1{\cdot}6 \times 10^7$ organisms/ml.) of the same recipient strain, the deoxyribonuclease-inactivated control showed no colonies, whereas 94 colonies/ml. were obtained from a test with DNA from *M. nonliquefaciens* MHD 2/10 *str-r*. Some of these *str-r* colonies were examined as before, and cultures of one isolate were made for extraction of DNA prep. 5.

Tests of *Moraxella nonliquefaciens* recipients against DNAs from *str-r* (mutant) strains of *Neisseria catarrhalis* yielded only a rare colony. From two of these *str-r* isolates DNA (preps. 6 and 12) was extracted. In addition, DNA preps. 7–11 were obtained from *str-r* strains of *M. nonliquefaciens* which arose in tests with DNA preps. 1–4.

Two experimental approaches were used to investigate the origin of the dihydrostreptomycin-resistant colonies arising in these intergeneric tests. The first involved examining the transforming activities of the twelve DNA preparations obtained in preliminary tests. It was reasoned that a mixed genetic background should be revealed if the *str-r* determinant had been introduced by transformation, since other studies have shown that the DNA transformants obtained in interspecific crosses may retain some genetic specificity characteristic of the donor species (Leidy, Hahn & Alexander, 1956; Schaeffer, 1958; Marmur, Seaman & Levine, 1963; Krauss & MacLeod, 1963). If this were so in our system, DNA from a *str-r* strain derived by transformation in the cross *Neisseria catarrhalis str-s* cells × *Moraxell anonliquefaciens str-r* DNA, should elicit transformation of *M. nonliquefaciens* from *str-s* to *str-r* at a higher frequency than that of DNA from spontaneous *str-r* mutants of *N. catarrhalis*. On the other hand, if the *str-r* clone of *N. catarrhalis* arose not by transformation but by mutation, its DNA should not elicit more transformants from a recipient population of *M. nonliquefaciens* than that of a proven *str-r* mutant of *N. catarrhalis*.

The results of tests with DNA preparations 1–5 and 7–12 (Table 1) are in agreement with the view that the *str-r* determinant of each was introduced by transformation. Thus, the transforming capacity of DNA prep. 5 for *Moraxella nonliquefaciens* MHD 2/10 was 480 times higher than that of a corresponding mutant strain (0·0146% transformation compared with 0·00003% elicited by prep. 14); and decrease of activity for *Neisseria catarrhalis* 8313 was less than twofold. Similarly, DNA prep. 12, obtained from *M. nonliquefaciens* MHD 2/10 *str-r*, elicited transformation of *N. catarrhalis* 8313 at a frequency 333 times higher than that of prep. 16. In this case, unexpectedly, the DNA (prep. 12; also 10 and 11) transformed *M. nonliquefaciens* MHD 2/10 recipient organisms with an efficiency even higher than that of prep. 16. A possibly analogous case was recorded for *Bacillus subtilis* by Marmur, Seaman & Levine (1963).

On the other hand, prep. 6 (as well as prep. 8, to be discussed later) exhibited low transforming activity for *Neisseria catarrhalis*, comparable to that of prep. 15. In the absence of increased homology or other evidence of transformation, the mutational origin of the *str-r* determinant of prep. 6 must be inferred.

The second approach to the problem of the origin of the *str-r* colonies involved a study of the response of *Neisseria catarrhalis* 8313 recipient organisms as related to time of exposure and concentration of DNA of *Moraxella nonliquefaciens*. A single reaction mixture (suspension + DNA prep., at 30°) was sampled repeatedly during a 30 min. period, beginning at 1 min. Each sample was added directly to broth containing deoxyribonuclease, and a suitable volume of this dilution was promptly plated.

Genetic events were rapidly initiated by the DNA preparations from *Moraxella nonliquefaciens* (Fig. 1, curves 2, 3, 4). The time course of these reactions was similar to that of the standard transforming DNA of *Neisseria catarrhalis* 8313 *str-r* (curve 1).

Table 1. *Transforming activities of various deoxyribonucleate preparations*

	Str-r DNA preparation		Transformation (*str-r*) of recipient strain (%)					
			Moraxella nonliquefaciens		*Neisseria catarrhalis*			
No.	Source	Origin of *str-r* marker	MHD 2/10	19116	NCTC 4103	8313	ATCC 8193	Ne 11
	Neisseria catarrhalis	*Moraxella nonliquefaciens*						
1	NCTC 4103	18522	0·0010	0·00003	3·333	0·555	0·025	0·0649
2	8313	18522	0·1350	0·0965	0·0029	1·947	0·620	0·9608
3	8313	18522	0·0245	0·0029	0·0004	2·343	0·525	1·0735
4	ATCC 8193	18522	0·0007	0·00003	0·0056	2·469	1·097	5·4314
5	8313	MHD 2/10	0·0146	0·0011	0·0043	2·228	1·421	2·0392
	Moraxella nonliquefaciens	*Neisseria catarrhalis*						
6	19116	NCTC 4103	1·190	2·708	0·00004	0·0006	0·0003	0·0001
7	19116	Prep. 1	0·610	2·268	0·0121	0·097	0·0082	0·0288
8	19116	Prep. 2	1·300	2·415	0·00004	0·0005	0·0003	0·0001
9	19116	Prep. 3	0·865	2·103	0·0004	0·123	0·0034	0·0291
10	MHD 2/10	Prep. 1	1·985	0·859	0·0817	0·054	0·0031	0·0198
11	MHD 2/10	Prep. 4	2·603	0·686	0·0342	0·138	0·0131	0·1839
12	MHD 2/10	Ne 23	2·522	0·765	0·0312	0·149	0·0114	0·6353
13	*N. catarrhalis* NCTC 4103	Mutation	0·00002	< 0·00001	3·531	0·637	0·0447	0·1219
14	*N. catarrhalis* 8313	Mutation	0·00003	0·00003	0·0040	3·763	1·392	10·255
15	*M. nonliquefaciens* 19116	*M. nonliquefaciens* 18522	1·047	2·224	0·00001	0·00045	0·0002	0·00006
16	*M. nonliquefaciens* MHD 2/10	Mutation	1·345	0·733	0·00001	0·00045	0·0002	0·00005
17	*N. catarrhalis* ATCC 8193	Mutation	*	*	*	*	2·956	*
18	*N. catarrhalis* Ne 11	Mutation	*	*	*	*	*	11·392

* DNA not tested in this experiment.

Moreover, it resembled transformation of other bacteria (e.g. Young & Spizizen, 1961; Goodgal, 1961). Exposure of the organisms for a period of 5 min. to autologous DNA (curve 1) and to *M. nonliquefaciens str-r* DNA (curve 4), each tested at a final concentration of 5 μg./ml., was sufficient to initiate 32 and 30 %, respectively, of the total (30 min.) genetic events. Higher yields for 5 min. samples (65 %, curve 2; 43 %, curve 3) were obtained with the DNAs at 10 μg./ml. In contrast, the time required for doubling the total population under these conditions was about 85 min. Mutant *str-r* clones were not found in the deoxyribonuclease-inactivated DNA controls that accompanied each of these experiments.

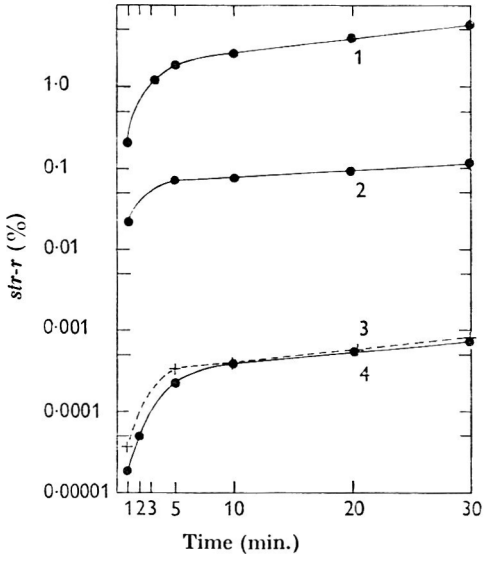

Fig. 1

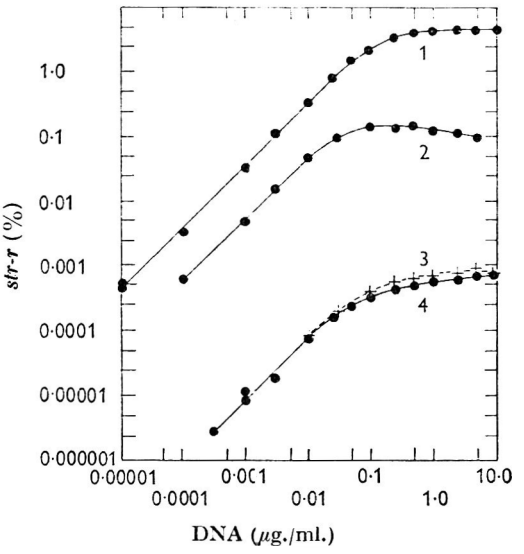

Fig. 2

Fig. 1. Effect of time of exposure to active DNA on the genetic response of Neisseria catarrhalis strain 8313. Curves correspond to 4 expts., each with a different DNA prep.: (1) N. catarrhalis 8313 *str-r* (prep. 14, Table 1), final concentration 5 μg /ml.; (2) Moraxella nonliquefaciens 19116 *str-r* (intergenus transformant, prep. 7, Table 1), 10 μg./ml.; (3) M. nonliquefaciens MHD 2/10 *str-r* (prep. 16, Table 1), 10 μg./ml.; (4) M. nonliquefaciens 19116 *str-r* (prep. 15, Table 1). 5 μg./ml. Exposure to DNA was terminated at each time indicated by adding a sample of the reaction mixture to broth containing 5 μg. deoxyribonuclease/ml.

Fig. 2. Effect of DNA concentration on the genetic response of Neisseria catarrhalis strain 8313. Time of exposure, 30 min. at 30°; DNA preps., as given for Fig. 1.

Tests were made of the effect of concentration of DNA on the genetic response of *Neisseria catarrhalis*. The results with strain 8313 recipient organisms are plotted in Fig. 2. The linear portion of each curve at concentrations below 0·1 μg. DNA/ml., and the plateau at relatively high concentrations (curves 1, 3, 4) are typical of the results obtained with other transformable bacteria (Hotchkiss, 1957; Young & Spizizen, 1961; Goodgal, 1961). The downward inflexion of the plateau region of curve 2 (possibly resembling inhibition obtained with mixtures of DNA preparations by Hotchkiss, 1957, and by Schaeffer, 1958) is a repeatable finding, which is thus far unexplained.

DISCUSSION

DNA preparations from streptomycin-resistant strains of *Moraxella nonliquefaciens* have genetic activity for streptomycin-susceptible recipient populations of *Neisseria catarrhalis*. Genetic transformation was established as the basis of this *str-s* to *str-r* change by the typical time-response and DNA concentration-response curves, and by the high reciprocal transforming activity of DNA preparations extracted from the intergeneric transformants. In addition, certain inferences can be made concerning the transfer of information between these organisms.

In order that comparisons of cellular response to various heterologous DNAs should be reliable, the tests with a given recipient strain were carried out simultaneously with samples of one cellular suspension and each of the DNA preparations (Table 1). The ratio, transformation by heterologous DNA × 1000/transformation by autologous DNA, will be referred to as the *str-r* recognition index (RI). To facilitate comparison and discussion, these ratios are given in Table 2. Use of the term *str-r* RI, rather than a term such as intergeneric transformation ratio, in discussing these results is expedient at present in view of the possibility that RI values involving genetic determinants of other characteristics may show little correlation.

Table 2. *Str-r recognition indexes**

	DNA preparation†		Recipient strain					
			Moraxella nonliquefaciens		*Neisseria catarrhalis*			
No.	Organism	Transformed by DNA of	MHD 2/10	19116	NCTC 4103	8313	ATCC 8193	Ne 11
1	*N* NCTC 4103	*M* 18522	0·8	0·01	945	147	9	6
7	*M* 19116	Prep. 1	454	837	3	26	3	3
10	*M* MHD 2/10	Prep. 1	1475	317	23	14	1	2
11	*M* MHD 2/10	Prep. 4	1935	253	10	37	4	16
12	*M* MHD 2/10	*N* Ne 23	1875	282	9	40	4	56
4	*N* ATCC 8193	*M* 18522	0·5	0·01	1·6	656	371	477
5	*N* 8313	*M* MHD 2/10	11	0·4	1·2	592	481	179
2	*N* 8313	*M* 18522	100	36	0·8	517	210	85
3	*N* 8313	*M* 18522	18	1	0·1	623	177	94
8	*M* 19116	Prep. 2	967	892	0·01	0·14	0·1	0·01
9	*M* 19116	Prep. 3	643	777	0·1	33	1	3
6	*M* 19116	—	883	1000	0·011	0·16	0·09	0·012
15	*M* 19116	*M* 18522	778	822	0·004	0·12	0·05	0·005

* Ratio: transformation by heterologous DNA × 1000/transformation by autologous DNA.
† From *str-r* strains, as given in Table 1, *N* = *Neisseria catarrhalis*, *M* = *Moraxella nonliquefaciens*.

The *str-r* RI indicates the comparative ability of a given recipient strain to recognize and integrate the *str-r* determinant of a particular DNA preparation. Prediction of RI for any given DNA may be erroneous when it is based on general knowledge of the cellular morphology of the DNA donor and recipient strains or of their DNA base average compositions. Low RI values were characteristic of the interaction between *Neisseria catarrhalis* diplococci and DNA from the rod-shaped organism *Moraxella nonliquefaciens* (preps. 6, 15, 16). A minimum requirement for genetic transformation has been thought to be similarity of the guanine + cytosine (G + C) contents of the DNAs of donor and recipient organisms (Marmur, Seaman & Levine, 1963). If this view has predictive value, the highest RI value should be found in tests involving *N. catarrhalis* NCTC 4103 and *M. nonliquefaciens*, as the G + C contents of their DNAs are practically the same (about 44·4 %, Catlin & Cunningham,

1964*a*, *b*). Of the strains examined, however, *N. catarrhalis* 8313 (42·3% G+C) was the most efficient in integrating the *str-r* marker of *M. nonliquefaciens*.

RI may be greatly affected by genetic manipulation of the DNA donor strain. Thus, an RI of 56 was found in a test of *Neisseria catarrhalis* Ne 11 (41% G+C) with a DNA preparation (12, Table 2) from *Moraxella nonliquefaciens* MHD 2/10 (44·4% G+C). The transformation event which introduced the *str-r* determinant of *N. catarrhalis* Ne 23 (42·2% G+C) into *M. nonliquefaciens*, producing the 'interbreed' strain from which prep. 12 was obtained, at the same time increased the RI by 13,000 (56 compared with 0·004, the RI for prep. 16).

Thus, recipient strains of both *Moraxella nonliquefaciens* and *Neisseria catarrhalis* recognized the *str-r* region of DNA from a first-step transformant, thereby revealing its mixed genetic background. DNAs from four strains which resulted from second-step transformations (Table 2, preps. 7, 10, 11, and 9) were recognized also by recipients of both species. Furthermore, there was evidence of 'self recognition'. In tests of *N. catarrhalis* NCTC 4103 recipient cocci with DNA from *M. nonliquefaciens* transformants, the RI value was higher when the *str-r* determinant came from strain NCTC 4103 rather than from other *N. catarrhalis* strains (compare prep. 7 with 9, and 10 with 11).

Although the homology established during the first transformation step tended to be retained by the second-step transformant DNAs (e.g. compare prep. 12 with 11), one instructive exception was found. DNA prep. 8 was obtained from a strain which arose as an isolated colony following treatment of *Moraxella nonliquefaciens* 19116 *str-s* recipient organisms with DNA prep. 2 (*Neisseria catarrhalis* first-step *str-r* transformant). RI values obtained in tests of prep. 8 against *N. catarrhalis* strains were not significantly higher than those of prep. 15 or 6. This finding, which differs markedly from results with the corresponding preps. 3 and 9, could be interpreted as indicating that the isolate was a spontaneous mutant. Arguing against this interpretation is the high transformation frequency (0·1%) calculated for the test in which the isolate was derived; the colony selected was one among 10,000. The alternative and preferred explanation, that the isolate was a transformant, is discussed below.

Studies of transformation of species of the genera *Haemophilus*, *Neisseria*, *Bacillus* and *Streptococcus* have shown in general that frequencies of transformation are high between closely related strains and lower between members of different species. This tendency has been related to the degree of homology of the DNA molecules of donor and recipient strains (Marmur, Falkow & Mandel, 1963). Attention has focused recently on molecular microhomologies, now that it is clear that genetic information carried by transforming DNA lies in the particular sequences in which the nucleotides (and their component bases) are associated. Complementary nucleotide sequences in DNAs from different though related micro-organisms have been detected by duplex strand formation by using physical methods (discussed by Marmur, Schildkraut & Doty, 1961; Marmur, Falkow & Mandel, 1963; McCarthy & Bolton, 1963). The recognition which is associated with genetic transformation likewise appears to involve interaction of corresponding nucleotide sequences of resident (recipient cell) and introduced (transforming) DNA molecules. The subsequent process of integration of the introduced genetic information remains problematic (Ravin, 1961). Nevertheless, information is accumulating on the

subject of the length of a sequence that can be integrated during a transformation event. That the integrated sequence is only a small fraction of the total genome is indicated by the fact that many genetic determinants are introduced independently. However, recent studies have revealed a number of linked markers, i.e. genetic determinants that tend to be transferred together. In *Neisseria catarrhalis*, as in *Haemophilus influenzae* (van Sluis & Stuy, 1962), the *str-r* determinant appears to be closely linked to that for kanamycin resistance (Catlin, 1963). In *Bacillus subtilis* thirteen different genetic loci are carried by a single transforming molecule (Nester, Schafer & Lederberg, 1963). The results of various analyses suggest that the integrated sequences may be of varying length, and that small regions are introduced in transformations more frequently than larger ones (Hotchkiss & Evans, 1958; Ephrussi-Taylor, 1960, 1961; Ravin, 1961).

The *str-r* RI values of Table 2 provide a basis for three inferences concerning the nucleotide sequence integrated during a transformation event. It is assumed here that the *str-r* determinants of *Neisseria catarrhalis* and *Moraxella nonliquefaciens* are similar; otherwise, the information which is meaningful for organisms of one species should be nonsense for those of the other. However, if integration involved the *str-r* determinant alone, DNA subsequently extracted from the transformant would not be expected to exhibit differences referable to the origin of the *str-r* determinant. The evidence of enhanced recognizability of DNAs from *Neisseria–Moraxella str-r* transformants thus suggests that integration involved sequences longer than the *str-r* determinant itself. Such a theory, employed by Schaeffer (1958) to explain differences between frequencies of interspecific and intraspecific transformation of Haemophilus, is supported by evidence obtained in studies of interspecific transformation of Bacillus (Marmur, Seaman & Levine, 1963). Secondly, the evidence relating to DNA preps. 2 and 3, which were from *str-r* transformants derived in the same test, suggests that they differ in respect to the length of the *M. nonliquefaciens*-specific portion which was integrated. The particular sequence of prep. 2 which bears the *str-r* determinant includes a longer stretch characteristic of *M. nonliquefaciens* than is present in prep. 3, thus accounting for the higher RI values obtained in second-step transformation tests with *M. nonliquefaciens* strains MHD 2/10 and 19116 (Table 2). Thirdly, it is inferred that DNA prep. 8 contains the *str-r* determinant of prep. 2 associated with a region which is not significantly longer than that originally taken from *M. nonliquefaciens* 18522. Thus, during the transformation event a tendency to integrate short sequences resulted in elimination of the peripheral *N. catarrhalis*-specific nucleotides required for recognition by *N. catarrhalis*. On the other hand, prep. 9 contains the shorter *M. nonliquefaciens*-specific sequence of prep. 3, together with some adjacent nucleotides which bear the specificity of *N. catarrhalis*.

Thus, unknown factors appear to affect the length of the nucleotide sequences which are integrated by different organisms of the same treated suspension. If relationships between bacteria are to be estimated on the basis of transformation frequencies, it is evident that a number of transformants should be analysed, as has been done here. Reciprocal tests clearly are useful. In addition, however, more than a single genetic determinant should be investigated. Although genetic homology involving the *str-r* determinants has been demonstrated between strains of *Moraxella nonliquefaciens* and *Neisseria catarrhalis*, the significance of this

finding remains debatable. It is hoped that further genetic studies of these species may provide a sounder basis for judgement concerning the degree of relationship between the two species.

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Immunochemistry of Group F Streptococci; Isolation of Group Specific Oligosaccharides

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SUMMARY

A disaccharide was isolated by partial hydrolysis from the group specific polysaccharide of a streptococcus strain carrying the group antigen F. The most probable structure of the substance is 3-0-β-D-glucopyranosyl-*N*-acetyl-D-galactosamine. The disaccharide was 300 times more active than cellobiose in the inhibition of the F/anti-F system. This denotes that it is a component of the determinant group of the group antigen. Two other serologically active oligosaccharides were isolated from the hydrolysate. Though their structure was not fully elucidated, evidence was obtained that a tetrasaccharide consisting of two molecules of the disaccharide is an important part of a determinant group of the F antigen.

INTRODUCTION

In previous communications it was reported (Ottens, 1961; Ottens & Winkler, 1962) that in group F streptococci one can distinguish between strains carrying only group antigen F and strains which carry one of five additional type antigens. Both the group and the type antigens are located in the cell wall and are of carbohydrate nature.

A study of the chemical composition of these antigens (Willers, Michel, Sijsma & Winkler, 1964) showed that the main components of the group antigen are glucose, galactosamine and rhamnose. The same authors showed that the precipitation of the F antigen with anti-F serum was inhibited with increasing activity by: glucose, β-methyl-glucoside, gentiobiose and cellobiose.

From this it was concluded that a glucose molecule glycosidically linked in the β-configuration constitutes an important feature of the determinant group of the group F polysaccharide. The present communication describes the isolation of three oligosaccharides from partial hydrolysates of the group F polysaccharide. All these oligosaccharides were much more inhibitory than cellobiose in the F/anti-F system. The structure of one of the oligosaccharides, a disaccharide, was fully elucidated. A related disaccharide isolated from a hydrolysate of cell walls was also used for analytical work.

METHODS

Preparation of cell wall hydrolysate. The strain used was 1 57 (Ottens & Winkler, 1962). Ten l. of Todd–Hewitt broth enriched with 0·2 % glucose were inoculated and incubated for 36 hr. at 37°. The organisms were harvested in a continuous flow

centrifuge. Cell walls were prepared as described earlier (Michel & Gooder, 1962). 300 mg. dry cell walls were hydrolysed for 2 hr at 100° with 2 N-H_2SO_4 and the hydrolysate neutralized with saturated $Ba(OH)_2$.

Preparation of the group specific polysaccharide. The same strain was grown in 80 l. of Todd–Hewitt broth with 0·2 % glucose; after harvesting the cocci were washed once with water and recentrifuged at high speed (36,000 *g*). The group polysaccharide was obtained by formamide extraction according to Fuller (1938). The amount of formamide used was 520 ml. Proteins were precipitated from the extract by the addition of acid ethanol (1·3 l.); after centrifugation the crude polysaccharide was precipitated from the supernatant fluid by the addition of acetone (2·6 l.). This material which was partially soluble in distilled water was dialysed for 48 hr against several changes of distilled water. The insoluble material was then discarded by centrifugation. The total volume at this stage was 1700 ml

Partial hydrolysis and chromatographic methods. Controlled hydrolysis was performed with polystyrene sulphonic acid (PSS; Painter, 1960); 1600 ml. formamide extract were treated in four 400 ml. portions. Each portion was brought at pH 2·3 with PSS (about 800 mg. of PSS with a monosulphonation degree of 7·3) and after addition of Dowex 50 H^+ (5 g. wet weight) heated in a dialysis bag at 60°, 70° and 86° in three successive periods of 4 hr. During the heating continuous dialysis against distilled water (1 l./hr) was applied. The volume of the dialysate was reduced to 1 l. by evaporation *in vacuo*.

The unhydrolysed polysaccharide was recovered by precipitation of PSS by the calculated amount of cetyl trimethylammonium hydroxide (Painter, 1960). The latter substance was prepared from cetyl trimethylammonium bromide by passage through a Dowex 1 OH^- column.

Separation of monosaccharides and oligosaccharides was performed on a charcoal column prepared as described by Schiffmann, Howe & Kabat (1958). The dialysate obtained after partial hydrolysis was slowly adsorbed on a column (50 × 2 cm.) containing 36 g. Darco G 60 + 36 g. Celite 535. Elution was started with water (2·5 l.) until no more reducing sugars could be detected in the eluate. After further elution with 2 % (v/v) ethanol (1 l.) an ethanol gradient using a two-flask system (Block & Ling, 1954) was imposed. The ethanol gradient range was from 2 % (v/v) to 22 % (v/v), in a total volume of 7·5 l.

Further separation of the sugars in the pooled fractions containing reducing sugars was obtained by paper chromatography on Whatman no. 3 MM paper. After elution from the paper the individual sugars were adsorbed on small Darco G 60 + Celite 535 colums and eluted with water and then with ethanol 20 % (v/v).

Quantitative analysis of the sugar composition of the formamide extract either untreated or treated with PSS was carried out as described in a previous paper (Willers *et al.* 1964). No attempt was made to separate glucosamine and galactosamine as it had been shown that the glucosamine content of the group antigen was very low.

For paper chromatography the following solvents were used: solvent A, *n*-butanol + acetic acid + water (60 + 10 + 20, by vol.); solvent B, 2,4-2,5 lutidine + water (65 + 35 by vol.). Alkaline silver nitrate reagent was used to detect reducing sugars (Trevelyan, Procter & Harrison, 1950); ninhydrin reagent for amino groups (Consden & Gordon, 1948); Elson–Morgan reagent for amino sugars (Partridge, 1948) and Morgan–Elson reagent for *N*-acetylhexosamines (Salton, 1959).

Analytical methods. Glucose was determined with the glucose oxidase reaction (Hugett & Nixon, 1957) and hexosamines by the method of Rondle & Morgan (1955). Both methods were used in micromodification (2–6 μg.) when necessary. *N*-acetylgalactosamine was determined according to Reissig, Strominger & Leloir (1956), rhamnose as described by Gibbons (1955), total sugar with phenol + sulphuric acid (Dubois, Gilles, Hamilton, Rebers & Smith, 1956) with glucose as the standard. Ethanol was determined with Anstie's reagent (Kabat & Mayer, 1961).

N-acetylation. *N*-acetylation was performed under the conditions recommended by Crumpton (1959).

Borohydride reduction. The sugar sequence of the unknown sugars was analysed by complete hydrolysis (4N-HCl for 3 hr at 100°) before and after borohydride reduction. Reduction was carried out as follows: to about 150 μg. of unknown sugar in 0·1 ml. water an equal volume of sodium borohydride in water (20 mg./ml.) was added. After standing overnight at room temperature, Dowex 50 H^+ was added to the solution until no more hydrogen escaped. After destruction of the last traces of $NaBH_4$ by the addition of a further small amount of Dowex 50 H^+, the resin was filtered off, the filtrate evaporated to dryness and boric acid volatilized by six further evaporations in the presence of small amounts of methanol.

Periodate oxidation. The amount of periodate consumed by the disaccharide (435 μg.) was determined by the spectrophotometric procedure described by Kabat & Mayer (1961). Formaldehyde liberated was estimated in the same sample with chromotropic acid (MacFadyen, 1945). The method was standardized by periodate oxidation of serine. The yield of formic acid during oxidation was estimated by the manometric method of Perlin (1954).

Enzyme sensitivity. The effect of β-glucosidase (Light's) on the disaccharide and on cellobiose and laminaribiose (which functioned as controls) was tested under the following conditions. 0·1–1 mg. of sugar in 0·2 ml. water was incubated for 2 hr at 37° with 0·4 ml. 0·2 M-acetate buffer (pH 5·5) and 0·2 ml. water with or without glucosidase (1 mg.). Glucose was estimated with the glucose oxidase reagent on pairs of appropriate samples.

Serological methods. Inhibition reactions of the quantitative precipitation were carried out as described (Willers *et al.* 1964). For a comparison of the antigenicity of the group specific polysaccharide with the material remaining unhydrolysed after partial hydrolysis, quantitative precipitations of both were performed with an anti-F serum in the same dilution.

RESULTS

Isolation and characterization of an oligosaccharide from cell walls. It was pointed out previously (Willers *et al.* 1964) that on qualitative analysis of group and type antigens the chromatograms showed one or more unidentified reducing-sugar spots. The strongest spot (compound A) was found only in hydrolysates derived from antigens containing the group antigen but was absent from those derived from type antigens.

A cell wall hydrolysate (from 300 mg. cell walls) was placed on a Dowex 50 H^+ column and monosaccharides were eluted with water. By further elution with 0·33 N-HCl on a fraction collector (Gardell, 1953) three Elson–Morgan positive

fractions were obtained. The fraction which came off in the first 30 ml., i.e. definitely earlier than glucosamine and galactosamine (Crumpton, 1959), was further purified by paper chromatography (Whatman no. 1) and eluted from the paper after location on the margin. About 3 mg. of substance was obtained. The substance had an R_g in solvent A of 0·38, and in solvent B of 0·65. As these figures correspond exactly with R_g values of compound A, it was concluded that compound A had been isolated.

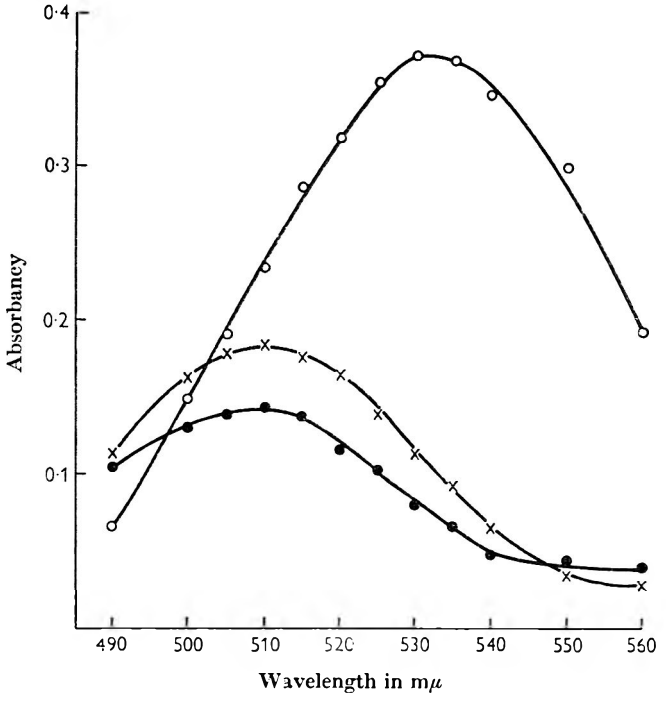

Fig. 1. Spectrum of the Elson–Morgan chromogens of glucosamine-HCl (○), compound A (×) and muramic acid (●). Compound A was isolated from a cell-wall hydrolysate of a group F streptococcus strain.

Compound A reacted positively with alkaline silver nitrate, with ninhydrin and with Elson–Morgan sprays; but not with the Morgan–Elson reagent. On complete hydrolysis (4 N-HCl for 3 hr at 100°) and chromatography the substance gave two spots, glucose and galactosamine, of about equal intensity. It was therefore thought at this stage that compound A was an oligosaccharide, probably a disaccharide, composed of these two sugars.

To obtain information on the type of linkage between the two sugars the spectra of the Elson–Morgan chromogens given by compound A, glucosamine and muramic acid were compared. From the results shown in Fig. 1 it appears that the former two substances gave a maximum at 510 mμ, whereas the maximum of the latter substance was situated at 530 mμ. Assuming that compound A is a disaccharide with a glycosidically linked glucose molecule, it was inferred from these data that the galactosamine molecule was substituted at C_3.

Confirmation of this structure was obtained by the results of periodate oxidation.

Compound A consumed 2 mole/mole periodate in the first hour and a total of 4·2 mole in 18 hr (theoretical, 4 mole). After 22 hr the formaldehyde liberated from the sample was 0·9 mole/mole (theoretical, 1 mole). The maximum formic acid production (1·7 mole/mole; theoretical, 2 mole) was obtained after 4 hr.

Table 1. *Chemical composition and precipitation of group F antigen before and after controlled hydrolysis*

	Untreated (mg.)	Treated and dialysed (mg.)
Glucose	135	55
Hexosamines	300	100
Rhamnose	910	333
	1345	488
Ratio of precipitates	3	1

The precipitation of the group specific polysaccharide and the material remaining unhydrolysed after controlled hydrolysis was assessed quantitatively with an anti-F serum in the same dilution.

Isolation of three oligosaccharides by partial hydrolysis of the formamide extract. For the production of a larger amount of compound A, partial hydrolysis by PSS was chosen since it has been reported that this method increases the yield of oligosaccharides and also because de-acetylation is prevented (Painter & Morgan, 1961) making the products directly suitable for serological use.

The effect of mild hydrolysis on the chemical composition and the antigenicity of the group polysaccharide is shown in Table 1. From the analysis of the sugar composition it appears that on hydrolysis a uniform decrease of all the components took place to about one-third of their initial values. This decrease was reflected at the serologically as the hydrolysed material formed only one-third of the precipitate obtained by a comparable amount of the original antigen. This indicates in fact that the serological activity of the polysaccharide was not affected by hydrolysis. Gradient elution of the charcoal column by ethanol produced one large peak which started at 4 % ethanol. The peak contained a shoulder in its descending part starting at 5 % ethanol. On purification of the complete fraction on Whatman no. 3 MM with solvent A, good separation was obtained. Besides a small amount of glucose, one major spot termed compound A_1 was detected with the alkaline silver nitrate spray. In addition, two weakly coloured spots with a much lower R_g value could be seen (compound A_2 and A_3).

Structure of compound A_1. The location of compound A_1 on Whatman no. 1 paper was R_g, solvent A, 0·59, solvent B, 1·0. The compound A described earlier was now *N*-acetylated. This acetylated compound had the following location: R_g, solvent A, 0·57, solvent B, 1·0. From this it was concluded that compound A_1 was the *N*-acetylated form of compound A. When investigated further, compound A_1 had the following characteristics. It had $\alpha_D^{20} + 20°$ in water. The amount of colour produced by compound A_1 in the Morgan–Elson reaction was 160 % of that obtained from an equimolar concentration of *N*-acetylgalactosamine. Glucose oxidase and galactosamine estimations of a complete hydrolysate of compound A_1 showed these sugars to be present in equimolar concentrations. However, when the substance was first reduced with sodium borohydride and then hydrolysed, the glucose content remained

unchanged, whereas the galactosamine content became negligible. From this it was inferred that compound A_1 is a disaccharide with *N*-acetylgalactosamine on its reducing end. Assuming that glucose and *N*-acetylgalactosamine are both in the D-configuration, the most probable structure of compound A_1 is 3-0-β-D-glucopyranosyl-*N*-acetyl-D-galactosamine (Fig. 2).

Glucose was not released from compound A_1 by β-glucosidase, whereas the same enzyme solution showed the expected activity towards gentiobiose and cellobiose.

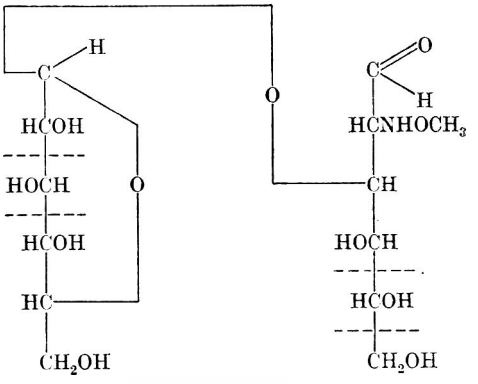

Fig. 2. 3-0-β-D-glucopyranosyl-*N*-acetyl-D-galactosamine (compound A_1) isolated from a partial hydrolysate of group F polysaccharide. The dotted lines indicate the diol linkages which are broken on periodate oxidation (theoretical 4, found 4·2). On breakage of these linkages, formic acid (theoretical 2, found 1·7) is formed and formaldehyde (theoretical 1, found 0·9) is released.

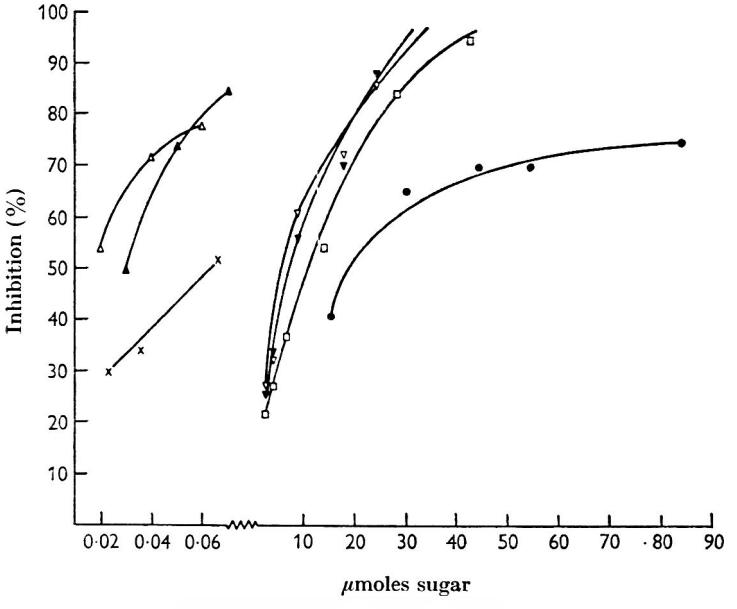

Fig. 3. Inhibitions of antigen-antibody reaction of the group F system by simple sugars and oligosaccharides isolated from the F antigen by partial hydrolysis ●—● glucose, □—□ β-methylglucoside, ▽—▽ gentiobiose, ▼—▼ cellobiose, ×—× compound A_2, ▲—▲ compound A_1, △—△ compound A_3.

Some characteristics of compound A_2 and A_3. Complete hydrolysis of compound A_2 yielded glucose, galactosamine and rhamnose. Compound A_3 on complete hydrolysis gave glucose, galactosamine and *N*-acetylgalactosamine. Estimation of glucose and galactosamine in a complete hydrolysate showed that these sugars were present in equimolar amounts. After reduction of the substance with sodium borohydride and subsequent hydrolysis the glucose content remained unchanged, whereas the galactosamine content was halved. This suggested a tetrasaccharide. On partial hydrolysis (*N*-H_2SO_4 for 1 hr) compound A_3 yielded glucose, galactosamine, compound A and compound A_1. An additional unknown spot (galactosaminyl-glucose?) was observed but not analysed further. From these data it is suggested that compound A_3 is a tetrasaccharide consisting of two molecules of A_1 in series. No information is yet available about the linkage between the two disaccharides.

Serological activity of the oligosaccharides. The inhibitory effect of the oligosaccharides isolated from the PSS dialysis residue on the F/anti-F system is shown in Fig. 3. The 50% inhibition values of compound A_1, A_2 and A_3 were obtained with 0·02, 0·05 and 0·01 μmole, respectively. All were considerably more active than cellobiose, the best inhibitor among the simple sugars tested. No inhibition of the antigen/antibody reaction of any of the type antigens was obtained with compound A_1, when used in concentrations of 0·5–0·7 μmole.

DISCUSSION

A disaccharide with the structure proposed for compound A_1 was described for the first time by Wolfrom & Juliano (1960). The substance described by this author was obtained as the crystalline dihydrate from carboxyl-reduced chondroitin. The substance had an $\alpha_D^{20} + 19°$ which agrees well with the figure found for compound A_1. As far as known the substance was isolated here for the first time from an unmodified natural source. Dorfman & Cifonelli (1958) reported that the spectra of the Elson–Morgan chromogens of the 2-,4- and 6-methyl substituents of glucosamine all showed a maximum at 530 mμ. The 3-methyl substituent however had a maximum at 510 mμ. This behaviour agrees well with the structure proposed for compound A, and has also been reported for muramic acid (Crumpton, 1959). The expected colour formation given by 3-,4- and 6-methyl-derivatives of *N*-acetylglucosamine in the Morgan–Elson reaction are 160, 3, and 100% respectively of the colour produced by the unsubstituted hexosamine (Jeanloz & Trémège, 1956). The 160% value found with compound A_1 also agrees well with the proposed C_3 substitution of its *N*-acetylgalactosamine molecule. A final argument in favour of a 1 → 3 linkage of the disaccharide was the remarkable acid insensitivity of compound A, a property which is generally found in substances linked in this way.

It is believed that the inactivity of the β-glucosidase preparation used on compound A_1 does not invalidate the assumed β-linkage of the glucose molecule. This is inferred in the first place from the low value of optical rotation of the substance and in the second place from its activity in the inhibition reaction of the F/anti-F system. It was observed earlier (Willers *et al.* 1964) that in this system inhibition was obtained by glucose derivatives in the β-configuration, whereas α-linked glucose derivatives remained without any effect.

The inhibition on the F/anti-F system by compounds A_2, A_1 and A_3 increased in that order. All were considerably more active than the simple sugars also shown in Fig. 3. It should be noted, however, that the inhibitory activity shown by compound A_3 is only slightly better than that of compound A_1. This indicates, as has been reported in another system (Kabat, 1960), that with compound A_3 the chain length giving maximal inhibition may have been attained. It is therefore believed that the tetrasaccharide termed compound A_3 is a determinant group of the group F polysaccharide. This assumption is further supported by the fact that the determinant groups of several other systems (Kabat, 1960; Schiffman, Kabat & Thompson 1964; Uchida, Robbins & Luria, 1963) are active in about the same concentrations as those used in this study.

As rhamnose has no inhibitory effect on the antigen/antibody reaction in group F streptococci, the methylpentose is believed to be situated at the reducing end of compound A_2. The substance as a whole being fairly active it probably consists of rhamnose glycosidically linked to compound A_1 (or A). The rather high serological activity which remained after controlled hydrolysis of the group specific polysaccharide might well be explained by the presence of side chains of this type.

This is the second paper of a series on 'Immunochemistry of group F streptococci'.

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The Growth of Rabbitpox Virus in Tissue Culture

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SUMMARY

The growth of rabbitpox virus has been studied in monolayer cultures of HeLa (ERK) cells. At 36°, virus was adsorbed to cells at about one-tenth of the theoretical collision rate. The adsorbed virus penetrated the cells exponentially, the time for half penetration being about 1 hr. New infective virus began to appear at 5 hr, and the final yield at 24 hr was about 100 pfu per cell. The synthesis of viral antigen began 1½ hr after infection of the cultures; this was 1 hr before the earliest appearance of viral DNA. When virus growth was complete, over 20 separate antigens could sometimes be detected in extracts of infected cells. The yield of antigens was not reduced when virus multiplication was completely inhibited by bromodeoxyuridine. It was concluded that the synthesis of viral antigens was directed by the DNA of the infecting virus, and not by the DNA formed during virus growth.

INTRODUCTION

The final maturation of pox viruses is known to be preceded by the synthesis of both viral antigen (Cairns, 1960; Loh & Riggs, 1961; Appleyard, Westwood & Zwartouw, 1962; Shatkin, 1963) and viral DNA (Cairns, 1960; Magee, Sheek & Burrous, 1960; Kato, Kameyama & Kamahora, 1960; Salzman, 1960; Loh & Riggs, 1961; Easterbrook & Davern, 1963; Shatkin, 1963). It is uncertain, however, whether these two substances appear simultaneously in the infected cells, or whether one is formed before the other. This question becomes important when considering the possible functions of viral antigen and DNA in the growth of pox viruses.

In the present study of the growth of rabbitpox virus, we paid particular attention to the times of first development of viral antigen, viral DNA and infective virus. The adsorption and penetration of virus, being essential preliminaries to the later synthetic processes, were also investigated.

METHODS

Viruses. Rabbitpox virus was the Utrecht strain, which had been passed about 25 times in HeLa (ERK) cells. Stock virus was prepared by ultrasonic disruption of infected cells in maintenance medium, and had a titre of about 10^8 plaque-forming units (p.f.u.)/ml.

Cell cultures. The ERK1 cell line (Westwood, Macpherson & Titmuss, 1957) was used. There is antigenic evidence that this probably arose from a contaminating HeLa cell (Coombs, Daniel, Gurner & Kelus, 1961). It will therefore be referred to in this and future publications as the HeLa (ERK) cell line.

The medium for cell growth consisted of Earle saline with the addition of 10 % (v/v)

calf serum, 5% (v/v) tryptic meat broth, 0·5% (v/v) yeast extract, 100 units penicillin/ml. and 100 μg. streptomycin/ml. It was equilibrated with 5% CO_2 in air. For most experiments, cell cultures were grown in 8 cm. Carrel flasks; an inoculum of 7×10^6 cells, when incubated overnight, yielded a confluent monolayer containing almost exactly the original number of cells.

Infection of cultures. In most experiments, cells were infected with a high virus multiplicity in order to obtain a one-step growth curve. Monolayer cultures in Carrel flasks were incubated for 1 hr at 36° with 2 ml. virus suspension of titre 10^8 p.f.u./ml. They were then washed three times with phosphate buffered saline, 10 ml. medium containing 5% instead of 10% (v/v) calf serum ('maintenance medium') was added, and incubation was continued at 36°. From experiments on the rate of virus adsorption, it was calculated that the amount of virus adsorbed should be about 7 p.f.u./cell and therefore almost all cells should be infected. This was confirmed by the observation that well over 90% of the cells underwent a rapid cytopathic effect and also developed inclusions of viral antigen. Virus growth times were measured from the beginning of the adsorption period.

Virus titration. Infected cultures were harvested by scraping the cells from the glass, and disrupting them when in suspension by exposure for 2 min. to ultrasonic vibration (40 kcyc./sec. at 500 W. produced by an ultrasonic transducer, type no. 1160, Dawe Instruments Ltd., London).

Virus was titrated as plaque-forming units (p.f.u.) in HeLa (ERK) cells. The disrupted cell suspensions were diluted in medium that contained 0·05% (w/v) galactose in place of glucose and lacked both calf serum and bicarbonate. Three-ml. volumes of the appropriate dilutions were added to HeLa (ERK) monolayers in 7 cm. Petri dishes, and incubated in air at 36° for 16 hr. After this adsorption period, the inoculum was replaced by 10 ml. maintenance medium containing a 1/1000 dilution of rabbitpox antiserum. The function of the antiserum was to prevent the formation of secondary plaques without interfering with the development of plaques from the primarily infected cells. The cultures were incubated at 36° in air + 5% CO_2 for 2 more days, and the plaques counted after staining with 0·04% (w/v) crystal violet. This titration method was 1·5–6 times as sensitive as the pock technique (Westwood, Phipps & Boulter, 1957) when tested with a variety of preparations of rabbitpox and vaccinia viruses. The plaque count was proportional to the virus concentration up to at least 200 plaques per culture, and the counts in replicate cultures showed a Poissonian distribution.

Staining of infected cells. Viral antigen: infected coverslip cultures were fixed in acetone at −20°, and stained with fluorescein-conjugated rabbitpox antiserum that had been adsorbed with acetone-dried human liver. The stained cells were examined microscopically by dark-ground ultraviolet illumination.

Viral DNA: cultures on coverslips were fixed in ethanol at room temperature, stained with a solution of acridine orange in methanol (Randles, 1960), and examined by dark-ground ultraviolet illumination.

Immunodiffusion tests. The infected monolayers were scraped from the glass into the culture medium, concentrated by centrifugation to 4×10^7 cells/ml. in phosphate-buffered saline, and disrupted by 5 min. exposure to ultrasonic vibration. The cell extracts produced in this way were examined for soluble precipitating antigens by micro-immunodiffusion (Crowle, 1958) against rabbitpox antiserum.

Antiserum. Rabbitpox antiserum was used for immunodiffusion tests, for neutralizing extracellular virus in some experiments, and for preventing the secondary spread of virus in plaque titrations. It was obtained from rabbits that had recovered from a rabbitpox infection and were then hyperimmunized by several intravenous injections of an extract of rabbit testes containing 5×10^8 p.f.u./ml. of rabbitpox virus. The neutralizing antibody titre of this serum against vaccinia (Boulter, 1957) was 500,000.

RESULTS

Adsorption and penetration of virus

The adsorption and penetration of vaccinia virus during the infection of cell monolayers have been studied by Postlethwaite (1960) and Allison & Valentine (1960). Our results were in general agreement, and so will be summarized only briefly.

Virus adsorption was measured by incubating cell monolayers with dilute inocula, the adsorbed virus being titrated directly by the formation of plaques on the test cultures. During the first hour at 36°, the amount of virus adsorbed per cm.^{2} of monolayer was equivalent to that contained in 0·010 ml. inoculum; for a given virus concentration, the initial rate of adsorption was not affected by the volume of inoculum. Application of the formula of Valentine & Allison (1959) to this result indicates that virus was adsorbing to cells at about 10 % of the theoretical collision rate. At 23° and 4°, virus adsorption was slower and, in contrast to that at 36°, it was incomplete.

Virus penetration was studied by investigating the rate at which virus, previously adsorbed to monolayers at 4°, became insusceptible to the neutralizing action of antiserum. At 36°, virus disappeared from the cell surface exponentially, the time for penetration of half the virus being 1 hr. At 21°, the rate of penetration was only about 5 % of that at 36°, and at 4° no virus entered the cells.

Virus growth curve

In a one-step growth curve, new infective virus was first detected at 5 hr. The virus titre then continued to rise, and the final yield at 24 hr was about 100 p.f.u./cell. Over 90% of the virus remained intracellular.

Formation of viral DNA

The synthesis of viral nucleic acid was studied by two independent methods: infected cells were stained with acridine orange to detect the presence of inclusions of DNA in the cytoplasm; and the synthesis of DNA that became incorporated into infective virus was investigated by showing how virus growth was inhibited by bromodeoxyuridine and how this inhibition was neutralized with thymidine.

Acridine orange staining. A series of cell cultures on coverslips was infected by incubation for 1 hr at 36° with a virus suspension containing 10^7 p.f.u./ml. Excess virus was removed by washing, and incubation was continued in maintenance medium at 36°. At intervals, measured from the beginning of the virus adsorption period, sample cultures were stained with acridine orange and examined microscopically under ultraviolet illumination. Cytoplasmic foci of DNA were first visible in a few cells at 3 hr, and by 8 hr about half the cells were seen to be infected.

The foci in their earliest stage of development were small, well defined, roughly circular and usually multiple. Later, at about 6 hr, many of the foci were beginning to spread irregularly through the cytoplasm. By 24 hr after infection, the cells had formed syncytial masses which contained large amounts of diffuse DNA. In some cells at the later stages of infection, newly formed virus particles could be seen as minute greenish dots; it was apparent that, even at 24 hr, only a small proportion of the total cytoplasmic DNA was incorporated into these particles. Plate 1, figs. 1 and 2, illustrate DNA foci that developed after this fairly low multiplicity of infection.

Table 1. *Effect of thymidine on inhibition of the growth of rabbitpox virus by bromodeoxyuridine (BDU)*

BDU concentration (μg./ml.)	Thymidine concentration (μg./ml.) Virus yield (p.f.u./cell)			
	0	25	100	400
0	46	—	—	60
18	0·28	6·0	41	53
54	0·20	0·33	9·4	27
162	0·26	0·26	0·31	5·4

When cells were infected with a virus suspension containing 10^8 p.f.u./ml., the inoculum used to produce a one-step growth curve, well over 90% of the cells became infected. Multiple small foci of DNA could be seen in occasional cells as early as 2½ hr, and by 3 hr about 50% of the cells contained inclusions.

Bromodeoxyuridine. A concentration of 20μg. bromodeoxyuridine (BDU)/ml. completely suppressed the formation of infective rabbitpox virus. The action of the drug was competitively inhibited by thymidine (Table 1). Staining of infected cultures with acridine orange showed that apparently typical inclusions of DNA were formed when virus growth was inhibited by BDU. These observations are consistent with the conclusion of Easterbrook & Davern (1963) that BDU competes with thymidine for incorporation into viral DNA with the result that the DNA formed in its presence gives rise to non-infective virus.

The time of viral DNA synthesis was measured by observing the effect on the final virus yield either of adding BDU to cultures at various times after infection or of annulling its action at these times. Three series of cell cultures in Carrel flasks were infected as for a one-step growth curve. Cultures from one series were harvested at intervals and titrated to show the course of infective virus production (Fig. 1, curve A). To a second series, 20μg. BDU/ml. was added at various times, and the cultures were titrated for virus after incubation for 24 hr (Fig. 1, curve B). The third series of cultures contained 20μg. BDU/ml. from the beginning of incubation, but 400μg. thymidine/ml. was added at various times after infection to annul the action of the drug; these cultures also were incubated for the full 24 hr before titrating for virus (Fig. 1, curve C). Curve B represents the viral DNA formed before the addition of BDU at the times shown, and curve C the DNA formed after these times. The two curves are mirror images of one another. They indicate that the synthesis of DNA began shortly before 3 hr, was 50% complete by about 4½ hr, and over 90% complete by 8 hr.

Similar experiments in which BDU was added to infected cultures at only ½ hr intervals, in order to determine more precisely the earliest time of DNA synthesis, showed that virus began to escape from inhibition by BDU at either 2½ hr or 3 hr; when added only 2 hr after infection, BDU was completely effective in suppressing virus multiplication. Viral DNA synthesis therefore began at about 2½ hr. This conclusion agrees well with that drawn from experiments in which infected cells were examined directly for DNA by staining with acridine orange.

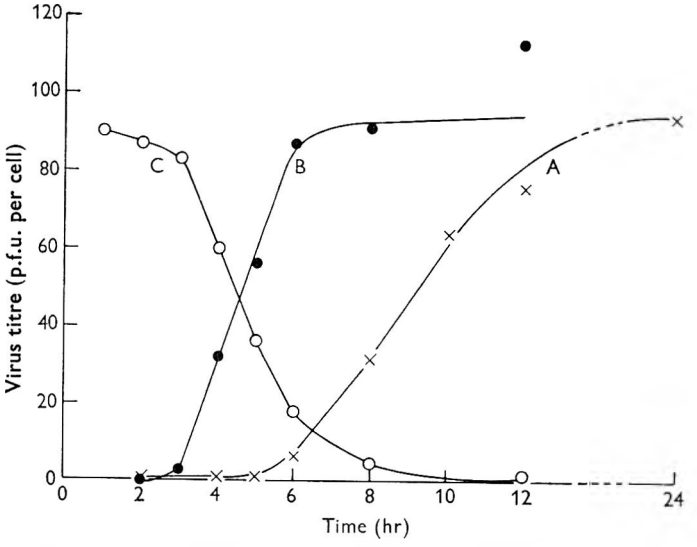

Fig. 1. Formation of viral DNA during one-step virus growth curve. ×, Virus content of cultures; ●, virus yield at 24 hr after addition of BDU at times shown; ○, virus yield at 24 hr after annulment of action of BDU at times shown.

Formation of viral antigen

The development of viral antigen, like that of viral DNA, was followed by two methods. The infected cells were stained with fluorescein-conjugated antibody; and cell extracts were examined for soluble antigens by immunodiffusion.

Fluorescent antibody staining. A series of cell cultures on coverslips was infected by exposure for 1 hr at 4° to partially purified rabbitpox virus at concentrations of 10^6, 10^7 or 10^8 p.f.u./ml. Unadsorbed virus was removed by washing and the cultures transferred to maintenance medium at 36°. At intervals, timed from the beginning of incubation at 36°, sample cultures were stained with fluorescein-conjugated rabbitpox antiserum and examined under ultraviolet illumination. Foci of viral antigen were confined to the cytoplasm. They were at first small and well-defined; later they increased in size, became irregular and fragmented, and eventually spread throughout the cytoplasm (Pl. 1, figs. 3–6). Virus particles could be seen in some cells at 8 hr, and by 24 hr they were present in most infected cells. It was obvious, as was found with viral DNA, that most of the antigen did not become incorporated into these particles. Counts were made on each coverslip of (*a*) the proportion of cells that contained viral antigen, and (*b*) the number of antigen foci in the individual infected cells (Fig. 2). After infection by the most concentrated inoculum, 22% of

the cells contained antigen as early as 2 hr, though the less heavily infected cultures did not begin to develop antigen until 3 hr or 4 hr. At the stage of growth when only a small proportion of cells contained antigen, the cells that did so showed more foci on an average than those that developed antigen later. Moreover, the foci were roughly the same size in any one cell. These observations support the conclusions of Cairns (1960) that in an individual cell all foci of virus synthesis begin development at the same time, and that the more heavily infected a cell the earlier is the growth of virus.

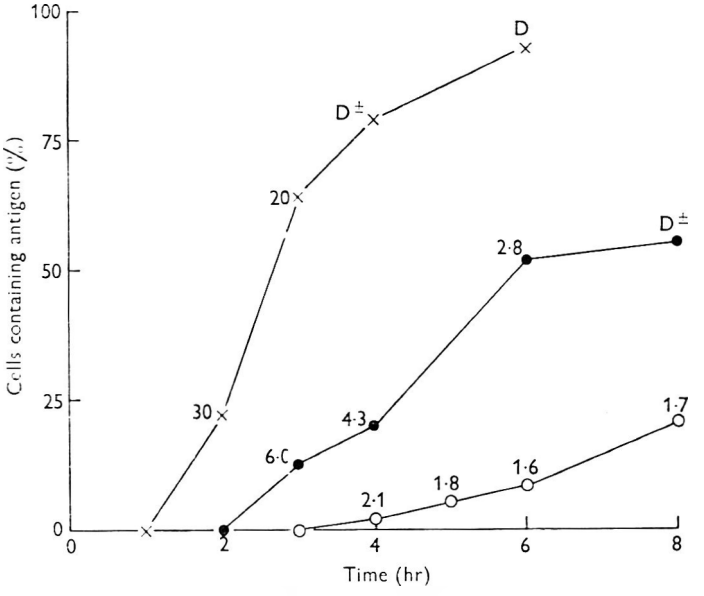

Fig. 2. Formation of inclusions of viral antigen after infection of cell monolayers with three different virus multiplicities. Titres of virus inocula: ×, 10^8 p.f.u./ml.; ●, 10^7 p.f.u./ml.; ○, 10^6 p.f.u./ml. Figures on curves indicate mean number of inclusions per infected cell; D±, diffuse antigen in many cells; D, diffuse antigen in most cells.

Further analysis of the counts of antigen foci in infected cells revealed that they fell well outside the expected Poissonian distribution. This was shown particularly well by the pooled results of five experiments in which cultures were infected by rabbitpox virus of titre 10^7 p.f.u./ml. At 8 hr, when the number of antigen-containing cells had reached a maximum, 437/750 cells (58%) were seen to be infected. Assuming random distribution of the infecting virus and equal susceptibility of all cells, this would correspond to a mean of 0·87 infective particles per cell. The expected distribution of the numbers of infective particles per cell is shown in Fig. 3, curve A. Only about 0·2% of the cells should have received more than 4 infective units, and therefore only this proportion of cells would be expected to contain more than 4 inclusions. It was not possible to count the inclusions in cells at 8 hr owing to their diffuse nature at this stage of growth. Counts could however be made in the cultures stained at 5 hr or 6 hr, at which time 217/451 (48%) of cells showed antigen. It was found that as many as 42/451 (9·3%) of the cells contained 5 or more foci (Fig. 3, curve B), and this figure excludes 8 cells in which the foci

were uncountable due to spreading. Examination of cultures stained only 3 or 4 hr after infection showed that 224/1090 (21%) of cells contained antigen; 94/1090 (8·5%) contained 5 or more foci, and 8 of these had from 10 to 14 foci (Fig. 3, curve C).

The above results could not be accounted for by variations in the number of virus particles infecting the individual cells. When cultures that had not been fixed with acetone were stained with fluorescein-conjugated antiserum, it was possible to count the virus particles adsorbed to the surface of the cells. Under the conditions already described, 143 cells adsorbed a mean of 10·1 particles per cell. The distribu-

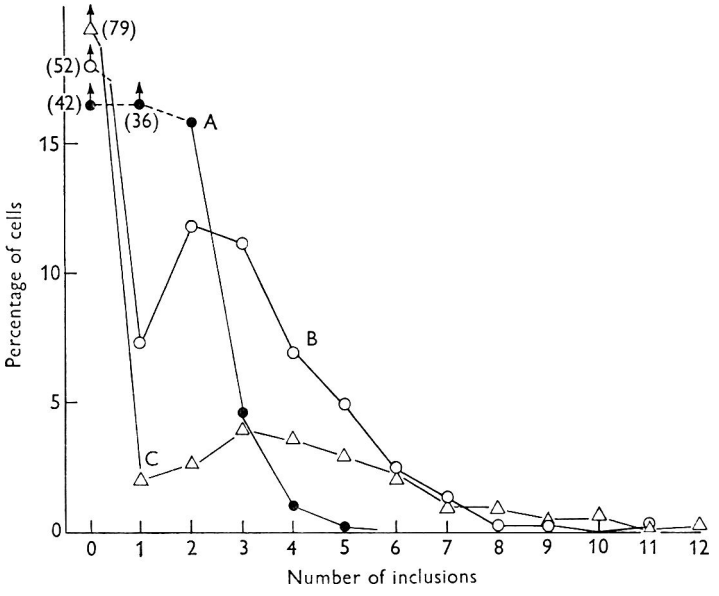

Fig. 3. Numbers of inclusions of viral antigen seen in individual cells after infection of cultures with a virus inoculum of 10^7 p.f.u./ml. ●, Theoretical counts; ○, counts made 5 hr and 6 hr after infection; △, counts made 3 hr and 4 hr after infection.

tion of counts fell only slightly outside the Poissonian expectation, and only 2 cells adsorbed more than 20 particles. These counts therefore confirmed the assumption of a random distribution of infecting virus. They also gave a particle:infectivity ratio of 10·1:0·87 or 11·6:1 for this preparation of rabbitpox virus. Another possible explanation for the distribution of counts shown in Fig. 3 would be the existence of a great variation in susceptibility of the cells to virus infection. But it is difficult to account on this basis for the unexpectedly small number of cells that developed only a single antigen focus. A more likely explanation is that some non-infective particles became 'reactivated' (Fenner, Holmes, Joklik & Woodroofe, 1959) in cells that also received fully infective virus. Since the maximum number of inclusions per cell was about 10 and the maximum number of adsorbed particles about 20, it would seem on this hypothesis that up to 50% of the virus particles might be reactivable.

Immunodiffusion analysis. Although immunodiffusion could not detect insoluble viral antigens, the method was able to reveal the great complexity of the mixture of soluble antigens. Extracts of cells harvested 24 hr after infection sometimes produced over 20 countable lines with rabbitpox antiserum. In order to follow the

course of soluble antigen synthesis, a series of cell cultures in Carrel flasks was infected as for a one-step virus growth curve. At intervals, the cells from sample cultures were concentrated by centrifugation, disrupted by ultrasonic vibration, and examined by immunodiffusion against rabbitpox antiserum. Some antigens were present in the cell extract made at 2 hr, more had appeared by 4 hr and almost the full 24 hr yield of antigens was attained by 6 hr. The examination of cell extracts harvested at ½ hr intervals during the early stages of virus growth showed that some antigens were formed only 1½ hr after infection, and a trace of antigen could sometimes be detected ½ hr earlier than this. By 2 hr after infection, when DNA synthesis had not yet begun, the cells usually contained between 5 and 10 viral antigens.

Relationship between viral DNA synthesis and the formation of viral antigens

The observation that the synthesis of viral antigens began at least 1 hr before that of viral DNA suggested that antigen synthesis was not directed by the newly formed viral DNA. This conclusion was confirmed by studying the effect of BDU on the production of viral antigens.

Cell cultures on coverslips, infected by adsorption of virus of titre 10^8 p.f.u./ml. for 1 hr at 36°, were incubated in maintenance medium with or without 20 μg. BDU/ml. The yield of virus at 24 hr was 120 p.f.u./cell in the control cultures and 0·9 p.f.u./cell in the inhibited cultures. Sample cultures were examined for intracellular antigen by staining with fluorescein-conjugated rabbitpox antiserum at 5 hr, 7 hr and 24 hr. Viral antigen developed equally well in the two groups of cultures, the foci in both being at first multiple and discrete and later becoming fragmented and diffused throughout the cytoplasm.

As an alternative test, cell cultures in Carrel flasks were infected as above and then incubated for 24 hr in normal maintenance medium or in medium containing 20 μg. or 40 μg. BDU/ml. The virus yield in the control cultures was 91 p.f.u./cell and in the inhibited cultures 0·4 and 0·3 p.f.u./cell. The concentrated cell extracts, harvested at 24 hr, were examined for soluble antigens by immunodiffusion against rabbitpox antiserum. So far as could be seen, BDU did not suppress the formation of any antigen. On the contrary, in this and in most similar experiments, the yield of antigen was rather greater from the inhibited cultures than from the controls.

DISCUSSION

The earliest time of synthesis of pox-virus DNA has been accurately measured, using the inhibitors fluorodeoxyuridine or bromodeoxyuridine, by Salzman (1960), Salzman, Shatkin & Sebring (1963), and Easterbrook & Davern (1963), who found that synthesis of DNA preceded that of infective virus by several hours. Our results with bromodeoxyuridine showed that the formation of rabbitpox virus DNA begins 2½ hr after infection, about half-way through the eclipse phase of the growth cycle. The staining of infected cells with acridine orange confirmed this, and also showed that considerably more cytoplasmic DNA was formed than ever became incorporated into virus particles. It is not yet possible to say whether this unincorporated DNA possesses some separate function, or whether its synthesis is due

merely to inefficiency of the repression mechanisms whose existence has recently been suggested by the work of McAuslan (1963*a*, *b*).

The synthesis of 'viral antigen' is a more complex process than that of DNA, since the infected cells produce a very large number of different antigens. Extracts of cells taken when virus growth was complete could produce twenty or more lines by immunodiffusion against rabbitpox antiserum, and there was no reason to suppose that each of these lines did not arise from a separate antigen. It would be expected that the various antigens would have different functions, and might therefore be formed at different stages of virus growth. There is already some indication of this from the work of Loh & Riggs (1961), who found, by staining infected cells with specific fluorescein-conjugated antisera, that LS antigen was formed before NP antigen. The method of immunodiffusion now shows that the many soluble antigens of rabbitpox virus are synthesized at very different times in the growth cycle. Some appear early in the eclipse phase, whereas others are not present in detectable amounts until virus maturation has already begun. The times of synthesis of the various antigens may be one of the pieces of information that will lead eventually to an elucidation of their functions.

Previous workers who followed the formation of both viral DNA and viral antigen in the same system found that the two types of material appeared at about the same time (Cairns, 1960; Loh & Riggs, 1961; Shatkin, 1963). In our system, some soluble viral antigens could be detected by immunodiffusion in cell extracts harvested only $1\frac{1}{2}$ hr after infection, and traces of antigen were sometimes present after only 1 hr. The synthesis of viral antigen therefore precedes that of viral DNA by at least 1 hr. The fact that antigen was not detected by staining with fluorescein-conjugated antibody until 2 hr after infection might be explained by the inability of this method to detect very small foci of antigen. Alternatively, it is possible that the earliest antigen behaved differently from that formed later, by diffusing through the cytoplasm instead of remaining localized at its site of synthesis; if this were so the antigen would be difficult to detect by staining.

The synthesis of viral antigen might be directed either by the DNA of the originally infecting virus or by the DNA formed later during virus growth. The finding that many antigens were produced in cells before any DNA synthesis had occurred indicated that the formation of some antigens at least was independent of new DNA synthesis. Moreover, Easterbrook & Davern (1963) and Shatkin (1963) have shown that some viral antigen was formed when viral DNA synthesis was interfered with by bromo- or fluorodeoxyuridine. The examination of our cell extracts by immunodiffusion indicated that bromodeoxyuridine, at a concentration sufficient to prevent all infective virus formation, did not inhibit the synthesis of any of the viral soluble antigens. It therefore seems likely that the antigens are formed solely under the influence of the DNA of the originally infecting virus, and that DNA formed subsequently plays no part in the process. On the other hand, the early antigens might themselves be essential for the synthesis of viral DNA. Some evidence on this subject will be presented later.

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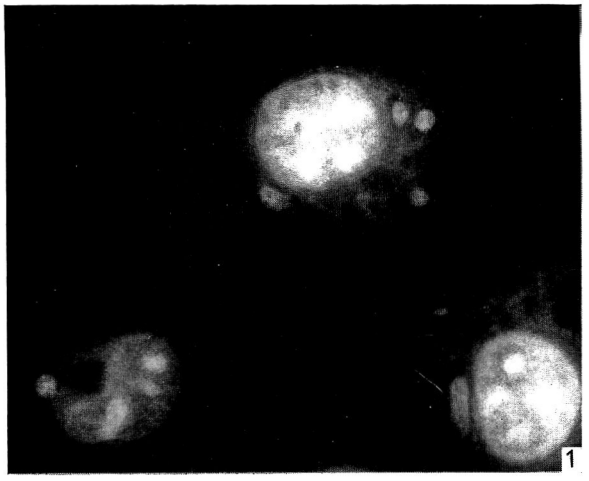
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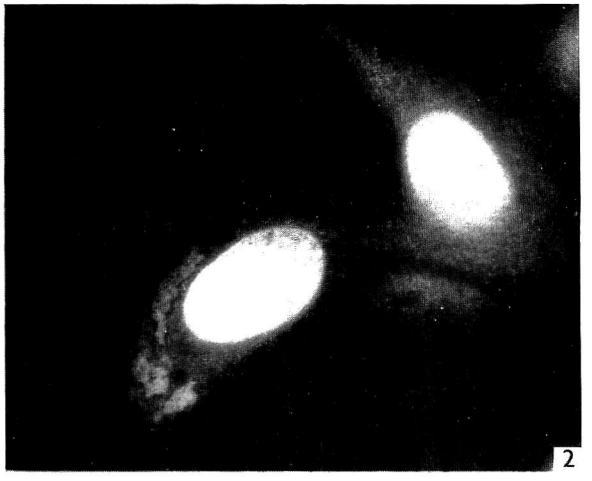
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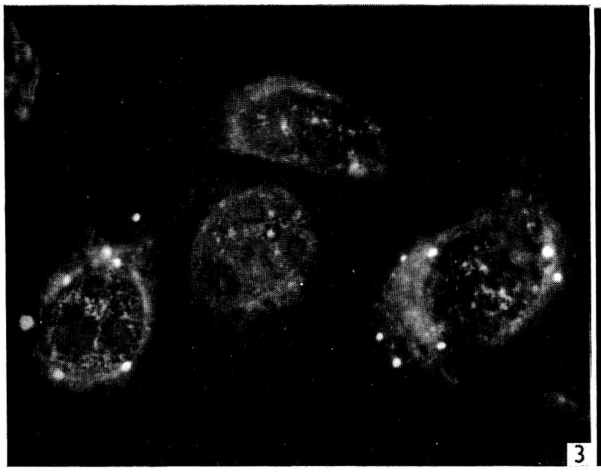
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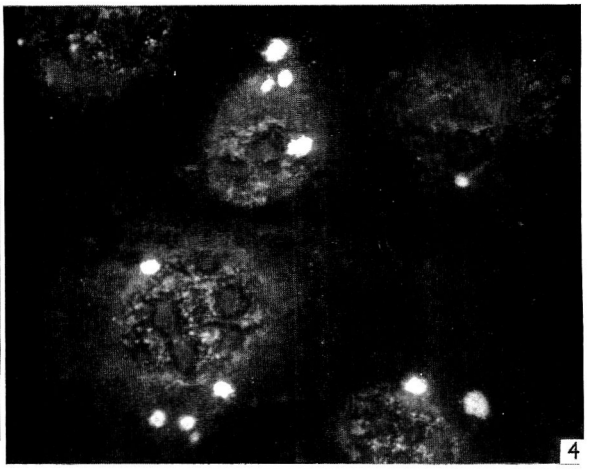
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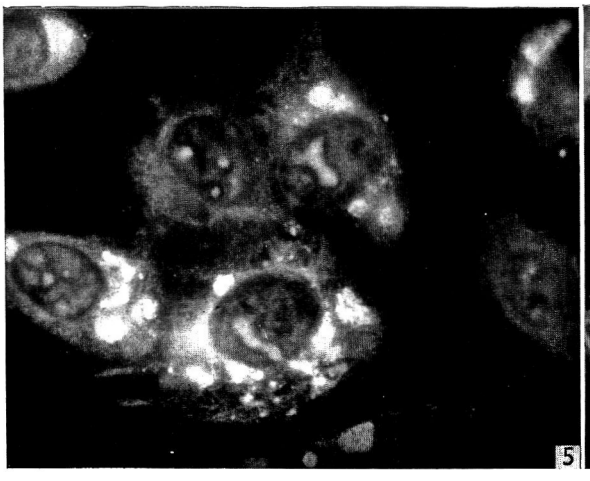
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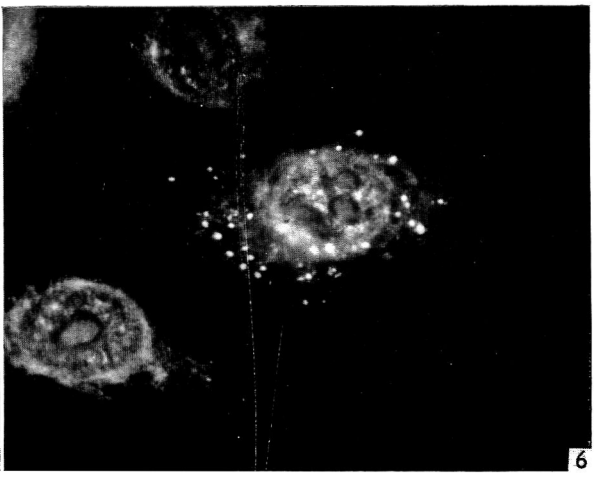
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EXPLANATION OF PLATE

PLATE 1

Fig. 1. HeLa (ERK) culture 6 hr after infection with rabbitpox virus 10^7 p.f.u./ml. Stained with acridine orange. The cytoplasmic foci of DNA are well localized.

Fig. 2. Same culture as in fig. 1. One cell is uninfected; the other contains DNA foci that are beginning to spread.

Fig. 3. HeLa (ERK) culture 3 hr after infection with rabbitpox virus 10^7 p.f.u./ml. Stained with fluorescein-conjugated rabbitpox antiserum. Two cells each contain seven small foci of viral antigen.

Fig. 4. As fig. 3, but culture stained 5 hr after infection. The foci of antigen are larger, but remain well localized.

Fig. 5. As fig. 3, but culture stained 8 hr after infection. The foci of antigen are fragmenting and spreading, and are now uncountable.

Fig. 6. HeLa (ERK) cultures 2 hr after infection with rabbitpox virus 10^8 p.f.u./ml. Stained with fluorescein-conjugated antiserum. The cytoplasm of one cell contains about thirty minute foci of antigen.

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Synergistic Effects of Salts and Carbon Dioxide on *Dermatophilus dermatonomus*

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SUMMARY

The addition of K and Na salts to cultures of *Dermatophilus dermatonomus* potentiated the effect of carbon dioxide (CO_2) in stimulating hyphal growth and delaying sporulation; the salts had virtually no effect in the absence of CO_2. The maximum production of these effects required the same minimum concentration, about 50 m-equiv. cation/l., of a comparably wide range of salts, as did maximum alteration of the chemotactic response of the zoospores to CO_2. It is suggested that there may be a common mechanism by which salts sensitize both the zoospores and the growing stages to CO_2. If these reactions are induced by the salts and CO_2 in the skin of infected sheep, they would partly account for the observed rapidity of both hyphal penetration and the emergence of zoospores.

INTRODUCTION

Carbon dioxide has been shown to affect both the growth of the actinomycete *Dermatophilus dermatonomus*, and the movement of its zoospores. Roberts (1963*a*) described a negative aerotactic response in which the zoospores moved away from the air/liquid interface in media of low salt content, but accumulated at the interface in media containing 40 or more m-equiv. monovalent salts/l. Roberts (1963*c*) showed that the movement away from the interface was migration towards an optimum concentration of endogenous CO_2. It seems likely that the salts added sensitized the zoospores to CO_2 so that they migrated towards a lower optimum concentration, which they found near the interface where CO_2 was lost by diffusion into the atmosphere. In cultures of *D. dermatonomus* Roberts (1963*d*) showed that CO_2 stimulated germination and growth but delayed development so that sporulation was inhibited and longer hyphae were formed. The present paper describes experiments which indicate that the effects of CO_2 on germination, growth and development are potentiated by concentrations of salts similar to those affecting the chemotactic response of the zoospores.

METHODS

Organism. The two strains of *Dermatophilus dermatonomus* used were strains 18 and 47 of Roberts (1963*d*).

Liquid medium. 0·5 % (w/v) Oxoid Lab-Lemco beef extract, 1·0 % (w/v) Difco Bacto or Proteose peptone, 0·1 % (w/v) Difco yeast extract, and 0·15 % (w/v) glucose,

were dissolved in distilled water; NaOH was added so that the medium was at pH 7·2 after autoclaving (120°, 20 min.).

The growth of cultures with agitation. Cultures were sown and incubated by the methods described by Roberts (1963*d*). When sporulation was studied 45 ml. lots of liquid medium in 1 l. Erlenmeyer flasks were inoculated and incubated at 37° overnight without agitation to produce a light growth of mycelium. The salts to be tested, or water in the case of controls, were then added to the medium in 5 ml. volumes, together with 1 ml. volumes of 50% (w/v) peptone to ensure a sufficiency of nutrients during sporulation.

Before the flasks were sealed, the appropriate concentration of CO_2 was added to the air space. In the case of cultures to be grown without CO_2, 5 ml. 50% NaOH was added to a test tube standing within the flask to trap CO_2 formed by the culture; 5 ml. water was added to the tubes in the flasks to which CO_2 was added, so that the tubes in all flasks would have the same weight and therefore, since they were free to move as the flasks rotated, the same tendency to disturb the swirling medium. The cultures were then incubated with agitation at 27° overnight.

When early filamentous growth was to be measured, the flasks again contained 45 ml. medium + 5 ml. of the appropriate sterile salt solutions. They were inoculated with standard volumes of suspensions of zoospores separated from the rest of the culture by passage through sterile 'Ekwip DS' filter pads (Industrial Equipment Australasia Pty. Ltd.). No extra peptone was added. The salt concentration and gaseous environment were adjusted at the time of inoculation. At the end of the incubation period growth was stopped by formalin (HCHO, 40%, w/v) added to a final concentration of 0·5% (v/v).

Growth of cultures without agitation. One-ml. volumes of solutions of K and Na salts were added to 9 ml. volumes of liquid medium in 1 oz. bottles, to give final concentrations of added cations of 0, 25, 50, or 100 m-equiv./l. Dense suspensions of filtered zoospores were added in 0·2 ml. volumes and the cultures incubated with their lids unsealed at 37° for 3 hr. Growth was then stopped by adding formalin to a final concentration of 1% (v/v). Differential counts were made to determine the proportion of the zoospores population which had budded at each salt concentration.

Reagents. The salts used were stated by the manufacturers to be of A.R. grade. The CO_2 was obtained from cylinders filled by the Commonwealth Industrial Gases Ltd., Alexandria, N.S.W.

The measurement of growth. The extinction of zoospore suspensions was measured at 670 mμ with a 1 cm. light path in a Unicam S.P. 1400 spectrophotometer, after separation by filtration as described by Roberts (1963*d*). Filamentous growth was measured with the same instrument but after treatment of the culture with a homogenizer to disperse clumped hyphae.

The determination of differences due to synergism between salts and CO_2. 'Synergism' is defined as the difference between the effect of CO_2 and the salt when supplied together, and the sum of the effects of each when provided independently. It was measured in terms of extinction (E) as follows:

Difference due to synergism

= effect of CO_2 and salt together − effect of CO_2 − effect of salt alone.

= (E CO_2 + salt − E controls) − (E CO_2 − E controls) − (E salt − E controls)

= E CO_2 + salt + E controls − E CO_2 − E salt.

Where the effect of CO_2 and salts was stimulatory, as in the case of mycelial growth, the synergism gave a positive value. In the case of sporulation the effect was inhibitory and a negative value was obtained.

The significance of the synergistic effect was determined by the application of Student's *t* test to sums and differences, as described by Mather (1949). In some experiments there were significant differences in variance between groups of flasks receiving different treatments. The application of the method of Cochran & Cox (1950) for *t* tests on samples of different variance was found in each case to give the same result as a simple halving of the total number of degrees of freedom. Thus, to simplify tabulation, a corrected number of degrees of freedom has been given in these instances.

RESULTS

The effect of salts on the germination and budding of zoospores

In these experiments the cultures were not agitated during growth. The results in Table 1 show that K and Na salts had no appreciable effect on budding at 25 m-equiv. cation/l., but usually caused a statistically significant increase in the number of budded zoospores at 50 and 100 m-equiv./l. Although CO_2 was not supplied there would have been endogenous CO_2 present since the cultures were not agitated (Roberts, 1963*c*).

Table 1. *The effect of salt concentration on the budding of zoospores of Dermatophilus dermatonomus in the presence only of endogenous CO_2*

Strain of *D. dermatonomus*	Salt	Salt concentration in m-equiv. cation/l. No. of zoospores budded over total counted: 0	25	50	100	χ^2 for comparison of pooled results at 0 and 25 with pooled results at 50 and 100 m-equiv./l.	Significance
47	NaCl	56/106	45/100	67/106	65/110	5·7	$P < 0{\cdot}02$
18	NaCl	128/257	125/256	146/253	146/250	7·46	$P < 0{\cdot}01$
47	Na_2HPO_4 + NaH_2PO_4 (pH 7·1)	56/106	57/101	69/103	69/106	5·23	$P < 0{\cdot}05$
47	Na_2SO_4	56/106	53/100	46/100	131/206	7·86*	$P < 0{\cdot}01$
47	KCl	113/214	56/102	69/116	139/219	4·24	$P < 0{\cdot}05$

* The results for 0, 25, and 50 m-equiv. cation/l. were pooled for comparison with those for 100 m-equiv./l.

The effect of salt concentration on hyphal growth in agitated cultures containing the same CO_2 concentration

It was necessary to provide a relatively low CO_2 concentration for effects of salt concentration to be demonstrable. Incubation was continued until there were measurable differences in extinction, but growth was stopped before sporulation

began. These precautions were necessary because it was difficult to obtain a satisfactorily measurable difference in extinction, even when microscopy showed that the hyphae were of different length.

NaCl or KBr was added at concentrations extending from 0 to 70 m-equiv./l. Five flasks were used for each concentration. In the five experiments which fulfilled the necessary conditions, endogenous CO_2 only was present in one, 0·1 % (v/v) CO_2 was added to the flasks in two, and 0·125 % (v/v) CO_2 to the flasks in the other two. Incubation time was from 6 to 17 hr.

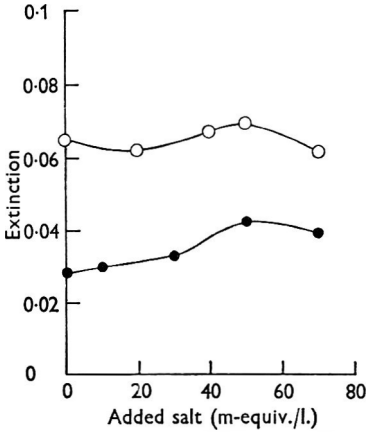

Fig. 1

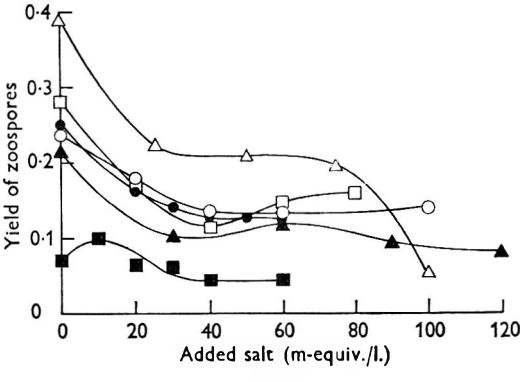

Fig. 2

Fig. 1. The effect of salt concentration on the early growth of D. dermatonomus in agitated cultures at 37°. The cultures were homogenized to disperse clumped hyphae before the extinction was measured. ○, Sown with zoospores of strain 18 and supplied with KBr and 0·125 % (v/v) CO_2 before incubation for 6 hr; ●, strain 47 with NaCl and 0·1 % (v/v) CO_2, and incubated for 1[illegible] hr.

Fig. 2. The effect of salt concentration on the sporulation of Dermatophilus dermatonomus strain 47 in agitated cultures. After an initial incubation without agitation at 37° to produce mycelium, 1 % (w/v) peptone and the appropriate salt and CO_2 concentrations were added to the flasks, which were then sealed and agitated overnight at 27°. Each culture was then divided into two portions. One was homogenized and used to measure the extinction (E) of the whole culture. The extinction of the other portion was measured after filtration to remove mycelium. The yield of zoospores (E) of filtrate/E of whole culture) was then calculated △, NaCl + 0·5 % (v/v) CO_2, ▲, KBr + 0·5 % (v/v) CO_2, ○ and ●, NaCl + 1 % (v/v) CO_2, □, KBr + 2 % (v/v) CO_2; ■, NaCl + 2 % (v/v) CO_2.

The extinction was greatest in the flasks to which 40 m-equiv. salt/l. was added in two experiments, 50 m-equiv./l. in two, and 60 m-equiv./l. in the other. Figure 1 illustrates the growth response in the two experiments which had the briefest incubation, and in which the extinction thus depended most exclusively on hyphal length.

The synergistic action of salts and CO_2 on hyphal growth in agitated cultures

To show synergistic effects of added salts and CO_2 on hyphal growth it was necessary to fulfil the conditions stated in the preceding section. In each experiment four groups of 5 or 6 flasks were incubated together. One group (controls) had neither CO_2 nor added salt in the medium; the second group had CO_2 but no added

Table 2. *The synergistic action of salts and CO_2 on hyphal growth of Dermatophilus dermatonomus*

Strain of *D. dermatonomus*	Salt added to medium (m-equiv./l.)	Initial CO_2 concentration in the air space % (v/v)	Incubation time (hr)	No CO_2 or salt added	Salt only	CO_2 only	CO_2 + salt	Difference in E due to synergism (mean and S.E.)
				Extinction (E; mean and S.E.)				
47	NaCl, 50	0·125	18	0·002 ± 0·002	0·001 ± 0·001	0·332 ± 0·013	0·383 ± 0·021	0·052 ± 0·025
18	KBr, 40	0·10	17	0·016 ± 0·002	0·014 ± 0·001	0·170 ± 0·029	0·231 ± 0·018	0·063 ± 0·035
47	NaCl, 50	0·125	16	0·004 ± 0·002	0·003 ± 0·002	0·870 ± 0·019	0·446 ± 0·016	0·077 ± 0·026

Table 3. *The synergistic action of salts and CO_2 on sporulation in Dermatophilus dermatonomus*

Strain of *D. dermatonomus*	Salt added to medium (m-equiv./l.)	Initial CO_2 concentration in the air space % (v/v)	No CO_2 or salt added	Salt only	CO_2 only	CO_2 + salt	Difference in E due to synergism (mean and S.E.)	t	Degrees of freedom	Significance
			Mean extinction (E) of filtered cultures							
47	NaCl, 100	5	0·305	0·274	0·262	0·093	−0·148 ± 0·043	3·44	20	$P < 0·01$
47	NaCl, 100	3	0·462	0·356	0·395	0·149	−0·140 ± 0·050	2·80	11	$P < 0·02$
47	NaCl, 50	2	0·347	0·349	0·230	0·106	−0·126 ± 0·034	3·71	12	$P < 0·01$
	NaCl, 100	2	—	0·359	—	—	—	—	—	—
47	NaCl, 40	1	0·418	0·427	0·291	0·209	−0·096 ± 0·027	3·56	12	$P < 0·01$
	NaCl, 80	1	—	0·419	—	0·197	−0·100 ± 0·023	4·35	13	$P < 0·001$
18	KBr, 40	1	0·062	0·069	0·101	0·050	−0·058 ± 0·014	4·14	6*	$P < 0·01$
47	KCl, 40	0·5	0·221	0·224	0·268	0·155	−0·116 ± 0·020	5·80	11	$P < 0·001$
	KCl, 80	0·5	—	0·222	—	0·110	−0·159 ± 0·022	7·23	5*	$P < 0·001$

* Number of degrees of freedom halved because of a difference in variance in the results, as discussed in Methods.

salt in the medium; the third had added salt but no CO_2; the fourth had CO_2 + salt, at the same concentrations as the flasks in the second and third groups, respectively.

In the absence of CO_2, growth was slight and gave very low extinction readings. Microscopic examination showed that virtually all of the zoospores had budded but the resulting hyphae were very short. Added salts did not increase the extinction in the absence of CO_2. The introduction of CO_2 stimulated growth significantly. In the presence of CO_2, added salts usually increased the extinction slightly. The synergism was appreciable in the three experiments illustrated in Table 2, but was statistically significant only in the third experiment shown ($t = 2{\cdot}96$, with 9 degrees of freedom after adjustment as described in Methods; $p < 0{\cdot}02$).

The effect of salt concentration on sporulation in agitated cultures containing the same CO_2 concentration

A significant effect of salt concentration on sporulation was much more easily measured than its effect on hyphal growth. It was possible to use higher CO_2 concentrations because small differences in concentration had little effect in the absence of added salts. It was not necessary to adjust the incubation time. In the absence of CO_2, sporulation was not affected by added salts. After the introduction of CO_2, however, increases in salt concentration led to a marked decrease in the production of zoospores. Usually fewest zoospores were produced after the introduction of 40 m-equiv. cation/l.

In these experiments there was sometimes considerable variation in the total amount of growth, which was reflected in the number of zoospores produced. It was found, however, that the yield of zoospores (extinction of filtrate divided by extinction of homogenized whole culture) was not greatly affected by the variation in growth and conformed to a simple curve when plotted against salt concentration. The curves in Fig. 2 show that the minimum yield, reached after the addition of about 40 m-equiv. salt/l., was maintained when 1 or 2 % (v/v) CO_2 had been supplied. With 0·5 % (v/v) CO_2 there was a plateau rather than a minimum at this concentration.

The synergistic action of salts and CO_2 on sporulation in agitated cultures

A statistically significant reduction in sporulation, due to synergism between added salts and CO_2, was produced in each of the six experiments (Table 3).

The permeability of growing forms to salts

Since Roberts (1963*a*) showed that the chemotactic response of zoospores was altered only by salts to which they were permeable, the osmotic method used in the studies on zoospores was used to determine whether the growing stages were permeable to some of the salts which affected growth. The refractive index of the cytoplasm was measured by immersion refractometry, and relative changes in protoplasmic volume were calculated, as described by Roberts (1963*a*). At a total osmolar concentration of about 0·1, the average protoplasmic solids content of hyphae and dividing stages of strains 18 and 47 was 27 % (w/v).

When the osmotic pressure was increased to 1 osmole by the addition of NaCl the average solids content of both strains was increased to 35 % (w/v), indicating a

23% loss of volume. At 1·7 osmoles, with Na_2SO_4, the average solids content of both strains was greater than 44% (w/v), indicating a reduction in volume of more than 38%. The increases in solids content were far too great to be accounted for by penetration of salt into the protoplasm. Moreover, the organisms were seen under the microscope to be smaller. There was no apparent restoration of volume or reduction in refractive index within 30 min. with either salt, showing that the organisms had remained impermeable to them both.

DISCUSSION

In liquid media zoospores of *Dermatophilus dermatonomus* accumulate where the CO_2 concentration is at an optimum (Roberts, 1963*c*). Therefore the accumulation of zoospores at the air/liquid interface at increased salt concentrations (Roberts, 1963*a*) is probably due to a decrease in the optimum CO_2 concentration so that the zoospores move to the interface, where the CO_2 content is lowest as a result of diffusion into the atmosphere. The effect of the salt could be described as an increase in the sensitivity of zoospores to CO_2 or, alternatively, as a potentiation of their response to CO_2. Roberts (1963*d*) showed that CO_2 stimulated hyphal growth but inhibited sporulation. In the experiments reported here, increased salt concentrations enhanced these effects. Thus salts seemed to sensitize the organism to CO_2, or to potentiate the action of CO_2 on the organism. This was supported by the fact that the salts had little or no effect in cultures from which all CO_2 was rapidly eliminated, whereas in the presence of added CO_2 increased salt concentration led to effects on growth significantly greater than the sum of the separate effects of CO_2 and added salt.

There was also evidence that the effects of salt on chemotaxis and growth may be mediated by the same mechanism. Both actions were produced by a similar variety of salts. With chemotaxis the effect was produced by those mono- and divalent salts of the alkali metals to which the particular suspension of zoospores was permeable (Roberts, 1963*a*). In the present experiments, germination was stimulated by the chloride, sulphate, and mono- and dihydrogen orthophosphates of Na, and by KCl. Hyphal growth was stimulated, and sporulation inhibited, by NaCl and KBr (no other salts were tested). In addition, very similar concentrations were needed to produce the maximum effect on both chemotaxis and growth. Budding was not affected by 25 m-equiv. added salts/l. but, in four of five experiments, was stimulated to a maximum degree by 50 m-equiv./l. In the case of hyphal growth, the maximum effect was at 40 m-equiv./l. in two experiments, at 50 in two, and at 60 in the other one. A minimum yield of zoospores was always produced at about 40 m-equiv./l. In all of these experiments the salt content of the medium before addition of the specific salts was about 8 m-equiv./l., due mainly to NaOH added to adjust the pH value. It may thus be stated that at each stage the maximum effect on growth was produced by a minimum salt concentration of about 50 m-equiv. cation/l. Roberts (1963*a*) observed that the chemotactic response was altered in only a few zoospores at 20 m-equiv. cation/l. but in virtually every zoospore in the suspension at 40 m-equiv./l. A slight increase in effect on raising the salt concentration above 40 m-equiv./l. was not observed, but would hardly have been recognizable under the experimental conditions. It may therefore be concluded that there was

no real difference between the minimum concentration needed to give the greatest effect on the chemotactic response, and the minimum concentration for the greatest effect on growth.

The chemotactic response of zoospores was only altered by salts to which they were freely permeable (Roberts, 1963*a*), whereas the various growing stages were found to be impermeable to salts which had been shown to potentiate the effects of CO_2 on growth. This casts doubt on the idea that there is a common synergistic mechanism in both cases. It is possible that such a mechanism operates in the interior of zoospores, but at the surface in growing stages. It seems more likely, however, that there are periods of permeability during growth which did not show under the conditions of the permeability experiment, and that the salts act within the organism in both phenomena.

According to Peters & van Slyke (1931) the total concentration of K^+ and Na^+ in animal tissue fluids is about 150 m-equiv./l. This is well above the minimum that gives the maximum synergism with CO_2 *in vitro*. If the salts and CO_2 in the lesion which develops in infected sheep had the same action on *Dermatophilus dermatonomus*, the increased hyphal growth would contribute to the rapid penetration which has been observed after artificial infection. Also, sensitization to CO_2 by salts in dried exudate on such lesions would accelerate the emigration of zoospores if, as discussed by Roberts (1963*b*, *c*), this is a response to the endogenous CO_2 in the wetted scab.

Advice on the use of *t* tests for differences due to interaction was kindly provided by Dr G. M. Tallis. I thank Mr H. H. Offord, Mrs J. Walker and Miss M. J. Crispe for their assistance with the experimental work.

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'Take-over'—an Unusual Selection Process in Steady-State Cultures of *Escherichia coli*

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SUMMARY

When the proportion of prototrophic revertants in a continuous culture of the tryptophan-requiring strain WP 2 of *Escherichia coli* was adjusted to exceed about 10^{-7}, a large increase ('take-over') often occurred after a variable delay, the proportion becoming as high as 50 % in some cases. The phenomenon appeared to be correlated with a selective advantage of revertants which became attached, with parent auxotrophs, to the wall of the culture tube. After some hours the film of growth on the wall was composed of approximately 10^7 bacteria. It was quite different from the massive 'sticky' growth sometimes encountered in continuous cultures of *E. coli*. The experimental observations of this 'take-over' are quantitatively consistent with a hypothesis of wall adsorption of rather rare and readily attached variant prototrophs.

INTRODUCTION

Turbidimetrically-controlled continuous cultures have proved useful in investigations of both the lethal and mutagenic effects of ionizing radiation (Munson & McLean, 1961; Munson & Jeffery, 1964). Success when studying mutation induction depends largely on the assumption that there exist no selection pressures operating either for or against the mutants. Such an assumption appears to be valid under most conditions in the present system (Munson & Jeffery, 1964). Occasionally, however, a rare event occurs which gives a very significant selective advantage to the mutants, resulting in an increase in their number by a factor of 10^6 or more. We have termed this phenomenon 'take-over'. It appears to arise as a result of attachment of bacteria to the walls of the culture tube, the mutant organisms adhering more firmly than non-mutant ones. It is therefore a phenomenon which is of possible importance in any continuous culture maintained for a long period in the same vessel.

METHODS

Reversions to tryptophan independence in strain WP 2 of *Escherichia coli* were studied in cultures grown at 37° in a minimal medium ('M' of Haas & Doudney, 1957) supplemented with 6 μg. tryptophan/ml. The culture vessels had volumes of approximately 9 ml. and the bacterial population was usually maintained at between 10^8 and 2×10^8 bacteria/ml. The proportion of mutants was increased artificially either by exposing the growing culture to gamma radiation, or by adding a known number of prototrophs from a culture derived from revertants which had arisen spontaneously. Viable bacteria were scored after 48 hr incubation at 37° on the surface of 'M' medium plates in the case of prototrophs and either plates of

nutrient agar or 'M' medium with 20 μg. tryptophan/ml. in the case of total counts of prototrophs and auxotrophs. Where the number of prototrophs was below about 10^3/ml., a larger volume than the 0·1 ml. used for direct plating was passed through a membrane filter and the latter placed on the surface of the agar plate. The growth rate was obtained by measuring the rate at which fresh medium was demanded by the turbidostatic control system. Automatic counting of the drops (of constant but undetermined volume) passing into the culture gave a very sensitive indication of changes in growth rate with time. Measurement of the overflow collected during a known period of time enabled the absolute growth rate to be determined once the volume of the culture vessel was accurately known. The generation time of *E. coli* WP 2 under these conditions was about 43 min. Full details of the continuous culture apparatus, radiation source and microbiological techniques are given elsewhere (Munson & Jeffery, 1964). Streptomycin-resistant varieties of *E. coli* WP 2 were obtained by growing the organism in increasing concentrations of the antibiotic. They were assayed on plates containing streptomycin sulphate (Glaxo) 15 μg./ml.

RESULTS

'*Take-over*'

In the first experiment (Fig. 1) two cultures were exposed to gamma rays from ^{137}Cs during 4 hr to a total dose of 4500 rads, during which time the frequency of prototrophs ($try^+/try^+ + try^-$) increased from 10^{-8} to 2×10^{-7}. After a short pause the try^+ frequency in one culture began to increase again and continued at a steady rate until it reached an apparent maximum of about 50 %. The other culture showed a similar increase after a longer delay. The growth rates of both cultures during the periods of 'take-over' were constant to within 1 %.

Figure 2 shows the changes in try^+ frequency in another experiment which involved two separate irradiations. The first, of 600 rads during 6 hr, increased the proportion of prototrophs to about 3×10^{-8}, at which value it remained constant for 40 hr. The second irradiation (600 rads in 6 hr) increased the value to almost 10^{-7} and, very soon after this, 'take-over' occurred, the proportion of prototrophs increasing during 30 hr to just over 10^{-2}. The constancy of the fraction of prototrophs in the interval between the two radiation exposures confirms the normal absence of selection pressures. Towards the end of the experiment there was a slight increase in growth rate, this being the only occasion in ten cases of 'take-over' when the growth rate altered perceptibly. The final value of try^+ frequency was not always the same from experiment to experiment, but in no case was 'take-over' complete in the sense that all auxotrophs were eliminated.

'Take-over' was also initiated after a variable delay when either previously isolated prototrophic revertants or the parent strain *Escherichia coli* B/r were added to a culture of auxotrophs. The smaller the number of prototrophs added, the greater was the delay observed before 'take-over' started, and when less than 100 prototrophs were added it was not observed at all. Once begun, 'take-over' continued at about the same rate in all experiments.

Cell division was necessary for 'take-over', since a culture which was allowed to become stationary by cutting-off the medium supply showed no increase in the frequency of prototrophs until fresh medium was added (Fig. 3).

Possible transfer of genetic information

It seemed possible that the phenomenon might be explained in terms of transfer of genetic information from prototrophs to auxotrophs. Filtrates through membrane filters and chloroform preparations of cultures in the process of active 'take-over' showed no transforming or transducing activity however, and the addition of acridine orange to a culture or to the plating medium did not in any way affect the normal course of 'take-over', indicating that free nucleic acid (e.g. an autonomous episome; Jacob & Wollman, 1961) was not involved.

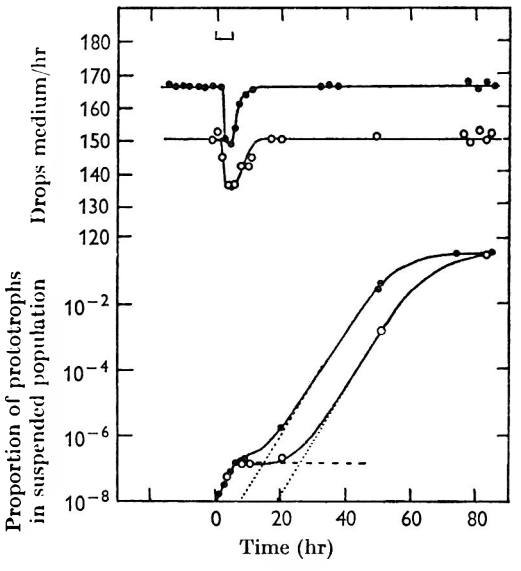

Fig. 1

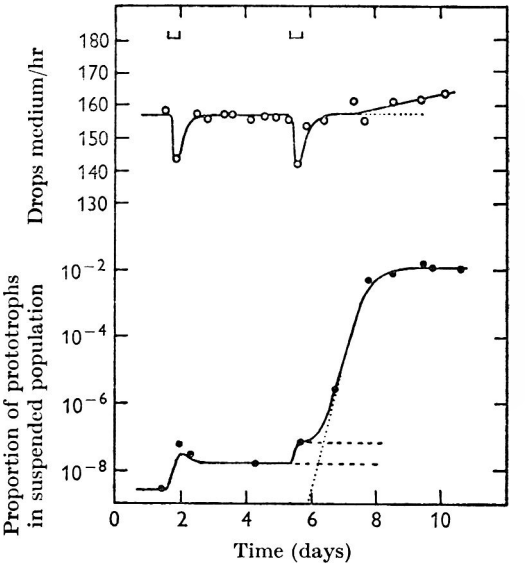

Fig. 2

Fig. 1. Increases in prototroph frequency ('take-over') in two continuous cultures of Escherichia coli WP 2. The cultures were simultaneously exposed to 4500 rads of gamma rays from ^{137}Cs during the period indicated by the heavy horizontal line (0–4 hr). The dashed line indicates the contribution from normal try$^+$ bacteria and the dotted lines the contributions from the hypothetical variant try$^+$ bacteria (see text), their slopes being 0·36 hr^{-1}. Observations of medium flow (drops/hr) showed that the growth rates were constant to $\pm 1\%$ except during and immediately after irradiation.

Fig. 2. 'Take-over' following two irradiations of a culture of Escherichia coli WP2. Two doses each of 600 rads were given during the periods indicated by the heavy horizontal lines. The dotted line, representing the contribution from hypothetical variant try$^+$ organisms, has a slope of 0·35 hr^{-1}. After 'take-over' the growth rate (indicated by medium flow, drops/hr) increased slowly by approximately 3% suggesting that there was an increase in the number of bacteria on the wall equivalent to 3% of the whole population.

Although conjugation is not known within strains of the *Escherichia coli* B family (which act as F$^-$ in crosses with some K 12 strains; de Haan, 1954) experiments were carried out to test this possibility. Normal streptomycin-sensitive prototrophs were added in the proportion of 10^{-6} to streptomycin-resistant auxotrophs. In no instance were streptomycin-resistant prototrophs recovered after 'take-over' had occurred.

The film of bacteria on the walls of the culture vessels

When a culture was divided and half of it transferred to a fresh vessel, the course of 'take-over' in the fresh vessel was delayed, indicating that something essential to 'take-over' was left behind in the original vessel (Fig. 4). Tests showed that this was not due to the continuing infection of the original culture by a build-up of prototrophs on the medium input tube or the overflow tube. After a culture in process of 'take-over' was carefully removed from its vessel by Pasteur pipette, a large number of organisms could be washed off the walls and magnetic stirrer by using sterile saline (NaCl, 0·85%, w/v) and a camel hair brush. The proportion of prototrophs among these wall-adherent organisms was 10 or more times greater than among those in suspension.

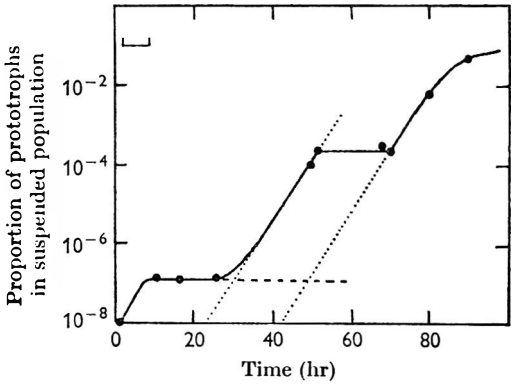

Fig. 3

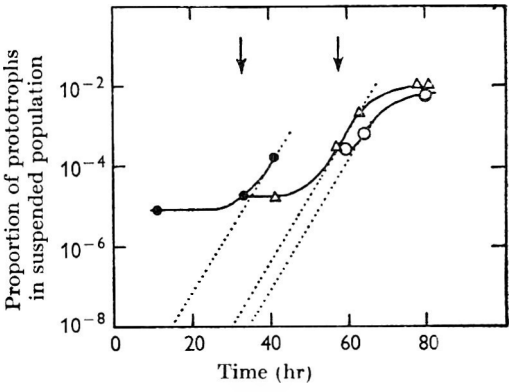

Fig. 4

Fig. 3. 'Take-over' in a culture of Escherichia coli WP2 following exposure to 4500 rads of gamma radiation: dependence upon active growth. The radiation was given during the period indicated by the heavy horizontal line. At 48 hr the supply of medium was stopped until 66 hr and then renewed. During this time of no growth the prototroph frequency did not change but subsequently 'take-over' continued as with similar cultures represented in Fig. 1.

Fig. 4. 'Take-over' in a culture of Escherichia coli WP2 after addition of a small number of prototrophs. Approximately 10^4 prototrophs of a previously isolated spontaneously revertant stock were added at 12 hr (●). At 33 hr, half of the culture was transferred to a fresh vessel (△) and, at 56 hr, half of the latter culture was put into a third vessel (○). The steepest parts of the curves all have approximately the same slope, viz. 0·35 hr^{-1}, and the delay between inoculation and 'take-over' is reduced with each transfer as would be expected on the proposed hypothesis.

Experiments were made on the rate of formation of wall films on clean culture vessels and, although the results were not quantitatively reproducible, the number of organisms in a film increased rapidly in the first few minutes and then more slowly until about 0·7% of the total population could be washed off the wall after 5 hr of growth in a culture at a population density of 10^8/ml. Thereafter, the proportion on the wall rose slowly, if at all. These wall films were invisible in the presence of the suspensions but when these were removed the wall films could be seen to cover the whole of the glass surfaces which had been in contact with the cultures. These films were very different from the massive 'sticky' wall growth which occasionally occurred and which, with turbidimetric control of the culture, was

usually manifested by an increase in apparent growth rate. When encountered, these growths have usually been entirely composed of auxotrophs.

DISCUSSION

Any postulated mechanism for 'take-over' in *Escherichia coli* as here described must account for the fact that it happens rarely and apparently randomly, except when large numbers of prototrophs are added or induced, and also that it never proceeds to a prototroph frequency of 100%. The simplest explanation of the data would be that the growth rate of prototrophs is greater than that of auxotrophs; a difference of about 30% would be necessary to explain the observed rate of 'take-over'. Growth rate of auxotrophs and prototrophs isolated from cultures in process of 'take-over' were, however, identical, whilst the overall growth rate of cultures showed no change even when the proportion of prototrophs approached 50%. Selection due to difference of growth rate is therefore ruled out.

The correlation between the delay before the onset of 'take-over' and the number of prototrophs present might indicate either that a rare spontaneous event occurs among prototrophs which renders one of them able to initiate 'take-over', or that time is required for the build-up of some essential activating substance. The experiments in which cultures were divided, one half being transferred to a fresh vessel, do not rule out the possibility of an activating substance; but they are more simply explained on the hypothesis that time is required for the establishment of try+ bacteria of a particular type on the wall. It is clear that, whatever the reason for the selective advantage of prototrophs, the phenomenon is sustained and probably initiated on the wall of the vessel.

Calculation shows that the stirring of the culture at 300 rev./min. introduces quite appreciable centrifugal forces which tend to thrust bacteria in suspension against the cylindrical wall of the culture vessel. Disregarding local turbulence in the suspension, the centrifugal force on a bacterium 2 mm. from the wall is equal to its weight; but nearer the wall its circumferential speed is decreased by viscous drag until at 1 μ from the wall the suspension is moving at only 10 μ/sec. and the centrifugal force on the bacterium is negligible. Even at this point, however, a bacterium is drifting parallel to the wall at a speed approximately 20 times greater than its mean thermal agitation velocity and a force of the order of 10^{-8} dyne would be required to hold it to the wall. Such a force could arise by electrostatic attraction between the bacterium and the wall, the magnitude of this force being critically dependent on the charge distribution on the surface of the bacterium and also on the local microstructure of the glass surface. With reference to the latter it is interesting that the wall (area about 12 cm.^{2}) could accommodate 4×10^9 bacteria in a close-packed single layer, whilst the greatest number actually found on the wall was only 1% of this.

Slow non-turbulent movement of the suspension in contact with the wall film might be expected to allow very poor mixing and so lower the growth rate of organisms there although, with only 0·7% of the population on the wall, this phenomenon would not be detected by measurements of the growth rate of the whole culture. One experiment in which macroscopic sticky wall growth eventually appeared showed that, at the time of transfer to another vessel, an increase in

apparent growth rate (5%) had taken place which corresponded almost exactly with that to be expected from a number of freely growing bacteria equal to the number brushed from the wall. It therefore seems that the rate of growth of bacteria on the vessel wall was not limited to a significant extent.

Hypothesis of formation of wall film of bacteria. By analogy with the adsorption of gas molecules to a solid surface one can regard the glass wall of the culture tube as providing a finite number s of sites for attachment of a monolayer of bacteria. If at time t a number n of these sites is occupied it may be assumed that the probability of a bacterium in suspension becoming attached between times t and $t+dt$ is $\alpha(s-n)\,dt$, where the coefficient α is approximately independent of n. The probability that in the same time interval a bacterium already attached to the wall will leave it can be written $\beta\,dt$ where β is also approximately independent of n.

Let $\bar{\nu}$ and $\bar{\nu}_a$ be the number growth rates (Powell, 1956) of bacteria in suspension and in the adsorbed wall film respectively, D the dilution rate and N the total number of bacteria in the culture suspension. The differential equations describing the changes of N and n with time are

$$\frac{dN}{dt} = \beta n - N\alpha(s-n) + (\bar{\nu}-D)\,N, \tag{1}$$

$$\frac{dn}{dt} = (\bar{\nu}_a-\beta)\,n + N\alpha(s-n). \tag{2}$$

Now the turbidostat automatically keeps $N+n$ constant at a value C say, hence

$$0 = n\bar{\nu}_a + N(\bar{\nu}-D). \tag{3}$$

When n/C is small and $n = 0$ at $t = 0$ equations (1) and (2) have the solution

$$n = C\alpha s[1-\exp(-At)]/A, \tag{4}$$

where

$$A = C\alpha+\beta-\bar{\nu}_a. \tag{5}$$

Observations on the rate of formation of a wall film of try$^-$ organisms showed that the initial rate of increase of n, viz. $C\alpha s$, was $\sim 2\times10^7$ hr^{-1}, which with C equal to 2×10^9 makes $\alpha s \sim 10^{-2}$ hr^{-1}. The final value of n as $t\to\infty$, viz. $C\alpha s/A$, was $7\times10^{-3}\,C$, or $1{\cdot}4\times10^7$, so that $A \sim 1{\cdot}4$. Since one can only assign to s an upper limit of $\sim 4\times10^9$ and a lower limit of $1{\cdot}4\times10^7$ it follows from (5) that

$$1{\cdot}4 \geqslant \beta-\bar{\nu}_a \geqslant 0. \tag{6}$$

It has been assumed above that the coefficients α and β have the same values for all sites and therefore do not change with n or with t as the sites are filled. In practice some sites will have greater energies for adsorption than others and these will be occupied preferentially so that α will diminish and β increase as n increases until eventually $\beta = \bar{\nu}_a$. After a very long time an equilibrium will be reached and at this time the right-hand side of equation (2) will become zero and $\beta-\bar{\nu}_a$ will reach its maximum positive value.

It is supposed that 'take-over' occurs when a sufficient number of bacteria, which may be one or a very few, of a rather rare try$^+$ variant become attached to the wall of the culture tube. The attachment coefficient α' of these variants is assumed to be greater than that of other try$^-$ and try$^+$ bacteria, or their detachment coefficient β' smaller. Their growth rates $\bar{\nu}$ and $\bar{\nu}_a$ are assumed to be the same as for try$^-$ bacteria.

Since the film of try$^-$ bacteria will generally be fully formed before 'take-over', the variant try$^+$ bacteria will compete for the sites of lowest binding energy already occupied by try$^-$ bacteria and for others of still lower binding energies for which α is so small and β so large that try$^-$ bacteria are held for very short times only. As a result try$^-$ bacteria will only remain firmly attached at the sites of greatest adsorption energy and since no further try$^-$ attachment can take place at these sites $\alpha = 0$ and $\bar{\nu}_a = \beta$ at an early stage of 'take-over'. Other sites occupied by try$^-$ bacteria will gradually be taken over by the variant try$^+$ bacteria since $\alpha' > \alpha$ and $\beta' < \beta$ when n is small. The initial stage of 'take-over' can be described by equations similar to (1) and (2) with the number of variant bacteria on the wall n' replacing n and N' and s' replacing N and s, respectively. When $n'/(s'-n-n')$ is small one finds

$$n' = C_1 \exp(\overline{p+q}t) + C_2 \exp(\overline{p-q}t) + C_3, \tag{7}$$

where $p = \frac{1}{2}[\bar{\nu}_a - \beta' - \alpha'\overline{s'-n} + \bar{\nu} - D]$,

$$4q^2 = [\bar{\nu}_a - \beta' - \alpha'\overline{s'-n} + \bar{\nu} - D]^2 + 4[(\bar{\nu}_a - \beta')(\alpha'\overline{s'-n} + \bar{\nu} - D - \alpha'\beta'\overline{s'-n})]$$

and C_1, C_2 and C_3 are integration constants.

If $\alpha'\overline{s'-n} \approx \alpha s$, $p+q \doteq \bar{\nu}_a - \beta'$ and $(p-q)/(p+q)$ is small and negative. Thus when t is large enough the second and third terms on the right-hand side of (7) can be neglected and

$$n' \doteq C_1 \exp(\overline{\bar{\nu}_a - \beta'}t) \tag{8}$$

so the graph of ln n' *versus* t is a straight line of slope $\bar{\nu}_a - \beta'$. During this stage one can also show that

$$N' \doteq \beta' n'/(\bar{\nu}_a - \beta'). \tag{9}$$

As n' increases β' will also increase until eventually after a very long time dn'/dt becomes zero and then

$$(\bar{\nu}_a - \beta')\, n' + (s - n - n')\, N'\alpha' = 0 \tag{10}$$

so that, if $\alpha' > 0$, $\bar{\nu}_a - \beta' \leqslant 0$.

Since the growth rates of normal and variant bacteria are assumed equal it follows from equation (3) that

$$n'/N' = n/N \tag{11}$$

and so, in the final state when n and n' become constant, the proportion of try$^+$ organisms in the suspension, namely $N'/(N+N')$, will never reach 100 % provided some try$^-$ bacteria remain attached to the wall.

In the experiments represented in Fig. 1 it is supposed that nearly all of the radiation-induced try$^+$ bacteria were of the normal type (α, β) and so had no selective advantage with respect to the try$^-$ bacteria. After the period of irradiation their contribution to the try$^+$ component remained constant. The few try$^+$ variants (α', β') present also had no selective advantage until at least one or two became attached to the wall. The time required for attachment is a matter of chance if the number of variants is very small, but since the average proportion of variants amongst the revertants is likely to be constant the time will be shorter when a large increase in number of revertants takes place. The combined frequency of both types of try$^+$ bacteria will thus rise from an initial plateau after an unpredictable latent period until eventually the slope of the graph of ln (N'/N) *versus* t becomes equal to $\bar{\nu}_a - \beta'$. In this way one can account for the random onset of 'take-over'.

All the experimental graphs of Figs. 1–4 have shapes consistent with the above analysis and maximum gradients of approximately 0·35 in all cases. Since $\bar{\nu} = 0{\cdot}97\ \text{hr}^{-1}$ and $\bar{\nu}_a \doteq \bar{\nu}$ the values of β' at this stage are approximately $0{\cdot}6\ \text{hr}^{-1}$. 'Take-over' thus seems to be a consequence of the detachment rate for variant revertants being less than their rate of multiplication, whilst for normal prototrophs on the same surface it is greater.

We thank Professor D. G. Catcheside, Dr D. A. Smith and Dr S. W. Glover for their helpful discussion of the possible involvement of genetic mechanisms. We are indebted to Mr H. Mortimer and Miss Rachel Dennis for technical assistance with some experiments.

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A Sulphate-reducing Bacterium containing Cytochrome c_3 but lacking Desulfoviridin

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SUMMARY

A sulphate-reducing bacterium (strain Norway 4) previously assigned to the species *Desulfovibrio desulfuricans* has now been found to contain cytochrome c_3 but not desulfoviridin. The organism resembles strains of *D. desulfuricans* which contain both pigments, in its morphology, nutrition, metabolism and hydrogenase activity. It is considered to be a mutant which has lost desulfoviridin. This work has not revealed the function of desulfoviridin.

INTRODUCTION

Cytochrome c_3 and desulfoviridin, a soluble porphyroprotein, have been considered (Postgate, 1956*a*, 1959) to be characteristic pigments of the anaerobic sulphate-reducing bacterium *Desulfovibrio desulfuricans*. Neither pigment was detected in six strains of thermophilic sulphate reducers (*Clostridium nigrificans*; Postgate, 1956*b*, 1959) and three strains of *D. orientis* (Postgate, 1959; Adams & Postgate, 1959) examined. Recently a new species of sulphate reducer, *D. gigas*, differing morphologically and biochemically from *D. desulfuricans* though containing cytochrome c_3 and desulfoviridin, was described by Le Gall (1963). The present communication reports the discovery of a sulphate-reducing bacterium which contains cytochrome c_3 and lacks desulfoviridin, but which nevertheless appears to belong to the species *D. desulfuricans*.

RESULTS

Isolation of the strain

The organism, a mesophilic sulphate-reducing bacterium, was originally isolated in 1949 from a sample of salt water from Oslo Harbour, designated *Desulfovibrio desulfuricans* strain Norway 4, and deposited with the National Collection of Industrial Bacteria (NCIB 8310). Until recently the only investigation performed on it was a pyruvate dismutation test (Postgate, 1952). Saleh (1964) and Saunders, Campbell & Postgate (1964) examined this and other strains of sulphate-reducing bacteria for resistance to inhibitors and for DNA base composition respectively, in the hope of detecting differences correlated with taxonomic position. The present authors subjected the 45 strains used in the inhibitor tests to routine examination for desulfoviridin by the method of Postgate (1959). All the 45 strains of *D. desulfuricans* tested gave a positive reaction except strain Norway 4. Microscopic examination of this strain showed a fair degree of pleomorphism, and though the tests for aerobic and anaerobic contaminants (Postgate, 1953) gave negative results

the culture was described by Saleh (1964) as being probably a mixed culture of sulphate reducers. A new culture was grown in Dr J. R. Postgate's (personal communication) modification of medium C (Butlin, Adams & Thomas, 1949) +0·4 M-NaCl, from a well-isolated colony in a stab culture in Baars's (1930) medium +NaCl+1 g. yeast extract/l.+agar. This isolate lacked desulfoviridin and grew anaerobically with reduction of sulphate to sulphide; it was used in the work described below. Methods of preparation of media and culture techniques were as described by Macpherson & Miller (1963).

Characterization of the strain

Morphology. The organism was a fat Gram-negative rod, sometimes slightly curved; mean dimensions about $1{\cdot}6 \times 0{\cdot}6\ \mu$, somewhat longer in old cultures. Motility was progressive and typical of *Desulfovibrio desulfuricans*. No spores were found by conventional staining techniques. Electron microscopy showed many organisms, especially in young cultures, which possessed a single polar flagellum; organisms from 8-day cultures frequently contained a number of granules of electron-dense material.

Media. Vigorous growth occurred in modified medium C+0·4 M-NaCl and in the chemically defined medium ('standard medium') of Macpherson & Miller (1963) +NaCl, in which the organism has been serially subcultured more than 30 times without noticeable decrease in rate or abundance of growth. Crops of about equiv. 700 μg. dry wt. bacteria/ml. were obtained in the latter medium. The organism has been adapted to an NaCl-free version of the standard medium. It appeared to grow equally well under an atmosphere of N_2, or H_2 +1% (v/v) CO_2. Acetate was identified chemically and chromatographically among the end-products of growth (Grossman & Postgate, 1955).

Tests of utilization of carbon sources by sulphate reducers are of limited value (see Macpherson & Miller, 1963); growth, especially in media which lack yeast extract, is often scanty or doubtful. A few tests of C sources were made: good growth occurred on pyruvate in lactate-free standard medium +NaCl; fair growth on glucose or choline; no growth on acetate.

Temperature. The organism was isolated at 30°, and grew readily at 37°. An attempt to 'train' it to grow at 42° failed; on a second attempt, with smaller temperature increments, it was trained to grow at 42·5° but would not grow at 43°.

Pigments. Thick bacterial suspensions showed a strong absorption band at about 554 mμ in the Hartridge reversion spectroscope, characteristic of cytochrome c_3, but gave a negative reaction for desulfoviridin. A spectrum of intact organisms was obtained by using a recording spectrophotometer with a reflectance head; a control of *Desulfovibrio desulfuricans* strain Canet 32 (NCIB 8392), a member of the same 'group' (Saunders *et al.* 1964) as the original strain of Norway 4, was used. Each organism showed the three absorption peaks of reduced cytochrome c_3 (419, 525 and 553 mμ; see Fig. 1), and in addition strain Canet 32 showed a strong absorption band at 632 mμ and a weaker one at 585 mμ due to desulfoviridin, both absent from the spectrum of strain Norway 4. Postgate (1956*a*) noticed a twofold decrease in desulfoviridin content in *D. desulfuricans* strain El Agheila Z grown by pyruvate dismutation in iron-deficient medium. On the assumption that the minimum iron concentration for initiation of desulfoviridin synthesis in strain Norway 4

may be abnormally high, cultures were grown in modified medium C with up to 20 times the usual iron concentration. No detectable desulfoviridin was synthesized.

Electron acceptors for hydrogenase activity. Washed organisms of strain Norway 4 suspended in non-nutrient buffer were examined at 37° for hydrogenase activity by the procedure of Littlewood & Postgate (1956) except that 15% (w/v) KOH was used as absorbent for H_2S and CO_2. Benzylviologen, thiosulphate, tetrathionate,

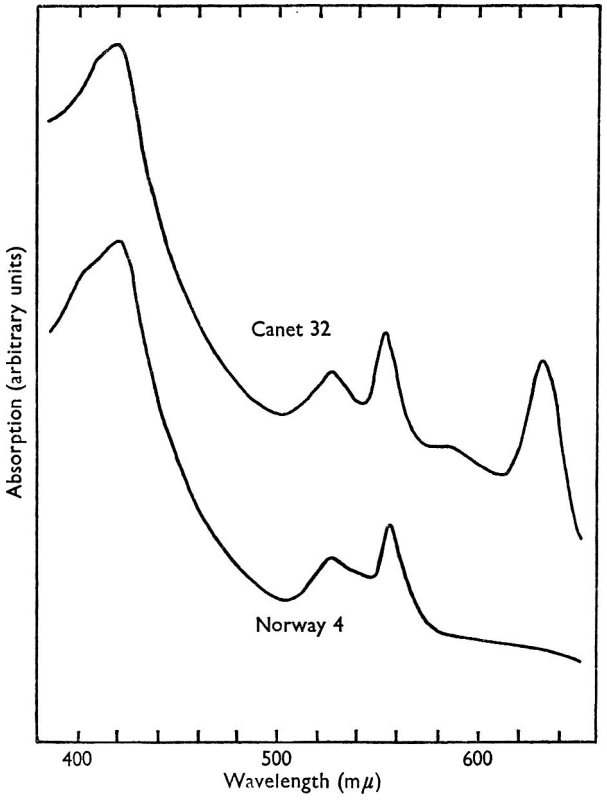

Fig. 1. Absorption spectra of Desulfovibrio desulfuricans strains Norway 4 and Canet 32, showing the absence of absorption bands corresponding to desulfoviridin in the spectrum of strain Norway 4. The absorption scales for the two spectra are different, since separate adjustments were made according to the bacterial and iron sulphide concentrations of each suspension.

sulphite and dithionite were tested as electron acceptors over the range 5–50 mM; all were utilized. The hydrogen absorption coefficient ($-Q_{H_2}$) in each case was 2–4 times that for equimolar sulphate.

Terminal electron acceptors for growth. The organism strain Norway 4 was inoculated into modified medium C + NaCl containing various electron acceptors in place of sulphate: 25 mM-$Na_2S_2O_3$ or $Na_2S_4O_6$ gave approximately the same crop, as determined by measurement of optical extinction, as equimolar sulphate; 25 mM-Na_2SO_3 was markedly inhibitory, though 10 mM-Na_2SO_3 gave about the same crop as 10 mM-Na_2SO_4. Dithionite was not tested as an electron acceptor for growth because of its instability.

Dismutation. The organism strain Norway 4 grew in a medium described by Postgate (1952) which lacked sulphate and lactate but contained sodium pyruvate, in keeping with this author's findings with the original strain of Norway 4. Choline was not dismuted.

DISCUSSION

The strain Norway 4, re-isolated by us, appears to be a normal *Desulfovibrio desulfuricans* in respect of its staining reaction, flagellation, ability to grow anaerobically on lactate or pyruvate with sulphate reduction, production of acetate, temperature tolerance and utilization of other sulphur compounds as electron acceptors for growth and hydrogenase activity. It differs from the Hildenborough strain (Postgate, 1951) in showing a considerably lower $-Q_{H_2}$ for sulphate reduction than for reduction of other sulphur compounds, though in our experience little significance can be attached to values for $-Q_{H_2}^{SO_4^{2-}}$ obtained with *D. desulfuricans* grown in batch culture. Norway 4 resembles other strains belonging to the group II of Saunders *et al.* (1964) in its adaptability to NaCl-free medium and its ability to dismute pyruvate. It contains the cytochrome c_3 unique to *D. desulfuricans* and *D. gigas*, and differs only in lacking desulfoviridin; thus it can probably be regarded as a mutant of *D. desulfuricans* which has lost this pigment. No metabolic abnormality has yet been discovered in this strain which might throw light on the function of this pigment.

We are indebted to Mrs Anita Fursey for electron microscopy, Dr D. Herbert for the reflectance spectra, Miss Janet Hughes for valuable assistance, and our colleagues in the Microbiology Group and Dr J. R. Postgate for useful discussions.

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Immunochemical Relationship between Streptococcus MG, F III and *Streptococcus salivarius*

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SUMMARY

The identity of the polysaccharide antigens of Streptococcus MG and streptococci with group antigen F and type antigen III is shown by precipitation reactions with formamide extracts, cross-absorption tests, sugar composition and by the inhibition of the quantitative precipitation reaction. On this basis Streptococcus MG can be classified as an F III strain.

The relationship between Streptococcus MG and *Streptococcus salivarius* type I is explained by their common reactions with anti-III serum. Inhibition of the quantitative precipitations with simple sugars suggest that the determinant groups of the type III and the 'salivarius' antigen are not quite identical, although both antigens contain a β-glucosidic and a galactosidic group.

A second unrelated antigen has been found in some 'salivarius' strains alone (represented by strain 51) or together with type III antigen. The quantitative precipitation of the strain 51/anti-strain 51 system was only slightly inhibited by rhamnose.

The qualitative composition of the sugar components of the formamide extracts of Streptococcus MG, F III, both 'salivarius' types of 0 III are compared.

INTRODUCTION

Mirick, Thomas, Curnen & Horsfall (1944) isolated from the lungs of a fatal case of primary atypical pneumonia a streptococcus strain which was first designated Streptococcus 344, later on Streptococcus MG. These authors described Streptococcus MG as a separate serological entity (Mirick *et al.* 1944 *a*) only related to *Streptococcus salivarius* type I (Mirick *et al.* 1944 *b*) as described by Sherman, Niven & Smiley (1943). Streptococcus MG gives capsular swelling and agglutination with homologous serum. Precipitation reactions can be done with various kinds of extracts of these streptococci.

Streptococcus MG (NCTC 8037/50) is used in several laboratories as a standard strain in testing for MG antibodies.

Many indifferent (no α- or β-haemolysis) and haemolytic streptococci isolated from dental root canals contain the group antigen F and one of the type antigens I-V observed in this group (Ottens & Winkler, 1962). The serological identification of these strains was based on the precipitin reaction with the soluble polysaccharide antigens obtained by hot formamide extraction of whole cocci. Data about the chemical constitution of these group and type antigens and about the structure of

the determinant groups of these antigens were described by Willers, Michel, Sijsma & Winkler (1964).

In the present paper data are given for the identity of the carbohydrate antigens of Streptococcus MG (NCTC 8037/50) and an F strain with type antigen III. Many *Streptococcus salivarius* strains also contain an antigen reacting with anti-III serum. The serological relationships between these strains and the chemical composition of their antigens are described.

METHODS

Strains. Streptococcus MG (NCTC 8037/50), *Streptococcus salivarius* (NCTC 8606) and 16 strains provisionally classified as MG strains were obtained from Dr C. E. de Moor (National Institute of Public Health, Utrecht, The Netherlands). Other streptococci also provisionally classified as MG strains were obtained from Professor Dr M. Seeleman (Institute of Dairying Hygiene, Kiel, Germany). Forty-two *S. salivarius* strains were isolated from dental plaque material (materia alba).

The qualitative chemical analysis was performed by the method of Michel & Gooder (1962).

The formamide extraction of the bacteria, the purification of the crude formamide extracts, the isolation of polysaccharides from the medium, the quantitative precipitin inhibition technique and the preparation of sera against group F and against the types I up to V have been described earlier (Willers *et al.* 1964). Anti-MG serum was obtained from Dr C. E. de Moor. All other anti-group sera were from Burroughs Wellcome. Anti-A variant serum was a generous gift of Mr W. R. Maxted.

Absorption of sera. Heat-killed streptococci were washed twice with saline and finally centrifuged at 36,000 ***g***. Two volumes of serum were mixed with the sedimented bacteria, incubated during 1 hr at 37° and centrifuged at 36,000 ***g***.

RESULTS

Serology. Formamide extracts of Streptococcus MG (NCTC 8037/50) gave positive reactions with anti-F and anti-III sera. Polysaccharides isolated from the medium reacted with anti-III serum only.

Anti-MG serum reacted with formamide extracts of the homologous strain and with strains containing the type III antigen.

The cross-absorption tests in Table 1 show that the anti-MG serum used is an anti-III serum. This is in accordance with our general experience that streptococci carrying group and type antigens often give rise to antibodies against the type antigen only. From these data it can be concluded that Streptococcus MG (NCTC 8037/50) is serologically identical with an F III strain.

Sixteen strains of the National Institute of Public Health (Utrecht) and 5 strains of the Institute for Dairying Hygiene (Kiel) which were classified as Streptococcus MG were tested in the capsular swelling test with anti-MG and anti-III sera. With formamide extracts of these strains capillary precipitin tests were made with all Lancefield group sera and with the type sera of group F. The correlation between capsular swelling and the precipitin reaction (Table 2) with anti-III serum is clear. Some strains gave a precipitin reaction with anti-F serum or with an anti-L serum (Burroughs Wellcome). In five strains no group antigen was detected. These

strains were designated as 0 III (zero III). The strains which gave no positive capsular swelling reaction are probably not true MG strains.

Three out of the five 0 III strains in the first group gave big slimy colonies on 5% sucrose agar. They were therefore classified as *Streptoccocus salivarius*.

Table 1. *Absorptions of anti-MG serum with different strains of bacteria*

Anti-MG serum absorbed with cells of	Formamide extract of		
	C III	F III	MG
C III	−	−	−
F III	−	−	−
MG	−	−	−
F	+	+	+
None	+	+	+

Table 2. *Capsular swelling tests and precipitin reactions of strains classified as Streptococcus* MG

No. of strains	Capsular swelling tests with antiserum against		Precipitation reactions with antiserum against		
	Type III	MG	Group L	Group F	Type III
8	+	+	−	+	+
3	+	+	+	−	+
5	+	+	−	−	+
2	+	+	−	+	+
1	+	+	−	−	+
2	−	−	−	−	−

The first group of strains was obtained from the National Institute for Public Health, Utrecht, Netherlands. The second group was obtained from the Institute for Dairying Hygiene, Kiel, Germany.

Streptococcus salivarius (NCTC 8606) and 25 out of 42 freshly isolated *S. salivarius* strains gave positive precipitin reactions with an anti-III serum. Antisera against salivarius NCTC 8606 and against one of the strains without type III antigen (strain 51) were prepared. The reactions of the formamide extracts of both 'salivarius' strains and an F III strain, with their respective antisera, are given in Table 3. It is clear that strain 51 contains an antigen different from type III antigen, whereas both antigens are present in strain 8606. These data were confirmed with cross absorption tests.

Formamide extracts of 42 'salivarius' strains were tested with anti-III, anti-8606 and anti-51 sera (Table 4). All strains react with anti-8606 serum; 5 strains contain the III antigen, 12 strains the 51 antigen and 25 strains both antigens, like strain 8606.

Chemical composition of carbohydrates in formamide extracts. Qualitative chemical analysis of various strains (Table 5) showed that in both F III and MG (NCTC 8037/50) galactosamine which represents the F antigen (Michel & Willers 1964) was the main hexosamine.

Glucosamine was present in low amounts in F III but not in MG. Glucose, galactose and rhamnose were found in large amounts in F III and MG strains.

The right-hand part of Table 5 gives a comparison of the results of the qualitative composition of *Streptococcus salivarius* (NCTC 8606), *S. salivarius* 51 and an 0 III strain. All contain glucosamine, glucose, galactose and rhamnose; *S. salivarius* 51 contains in addition mannose.

Table 3. *Cross-reactions and cross-absorptions of anti-salivarius* NCTC *8606, anti-salivarius 51, and anti-III sera*

Serum	Absorbed with	Formamide extract of Sal 8606	F III	Sal 51
Anti-sal 8606	.	+ + +	+ + +	+ +
Anti-sal 51	.	+ +	−	+ +
Anti-III	.	+ + +	+ + +	−
Anti-sal 8606	Sal 51	+ + +	+ + +	−
Anti-sal 8606	F III	±	−	+ +
Anti-sal 51	Sal 8606	−	−	−
Anti-III	Sal 8606	−	−	−

+ + + = precipitation immediately positive; + + = precipitation positive in 30 sec.; + = precipitation positive in 2 min.; ± = weak precipitation reaction within 5 min.; − = no precipitation within 5 min.

Table 4. *Reactions of salivarius strains with anti-salivarius and anti-III sera*

Number of isolated strains	Anti-8606 serum	Anti-51 serum	Anti-III serum
25	+	+	+
12	+	+	−
5	+	−	+

Table 5. *Qualitative sugar composition of formamide extracts of Streptococcus* MG, F *III, 0 III and Streptococcus salivarius* NCTC *8606 and strain 51*

	MG NCTC 8037	F III	0 III	Salivarius NCTC 8606	Salivarius 51
Galactosamine	+	+ + +	−	−	−
Glucosamine	−	+	+	+	+
Galactose	+ +	+ +	+ + +	+ + −	+ +
Glucose	+ + +	+ + +	+ + +	+ + +	+ + +
Rhamnose	+ + +	+ + +	+ + +	+ + +	+ + +
Mannose	−	−	−	−	+
N-Acetylhexosamine	±	+ +	+	+ +	+ +

Inhibition of the quantitative precipitation. The results of the inhibition reactions by simple sugars of the III/anti-III and MG/anti-MG reaction are compared (Table 6). Only the sugars giving at least 30 % inhibition are shown; for other sugars tested see Willers *et al.* (1964). In both systems the same sugars gave a measurable amount of inhibition at the sugar levels used. These results suggest that the determinant groups of the type III antigen have many properties in common with those of an antigen of Streptococcus MG. As mentioned before Streptococcus MG only gives rise to antibodies against the type III antigen. Therefore the inhibitions

of the precipitin reaction of MG antigen and anti-F serum were studied. 50% inhibition was reached by 8μM-cellobiose and by 0·01μM-3-O-β-D-glucopyranosyl-*N*-acetyl-D-galactosamine. As the latter is a disaccharide of the determinant group of the F antigen (Michel *et al.* 1964) the results of these inhibitions give further evidence for the presence of the F antigen in the formamide extracts of Streptococcus MG.

Table 6. *Inhibition by simple sugars of precipitin reactions of the type III and the* MG *systems*

	III/anti-III	MG/anti-MG
Glucose	90*	42
β-Methylglucose	46	32
Cellobiose	34	26
Gentiobiose	18	18
α-Methylglucose	41†	55†
Galactose	90	33
Melibiose	60	22†
Glucosamine	.	37
Galactosamine	.	48†

* 50% inhibition value expressed in μM-sugar.
† 30% value, where the 50% value was not reached.

Table 7. *Inhibition by simple sugars of the precipitin reactions of the type III and both salivarius systems, the cross reacting systems of type III and salivarius* NCTC *8606 and the system salivarius 8606 antigen/anti-salivarius 8606 adsorbed with salivarius 51 bacteria*

	III/ anti-III	8606/ anti-III	III anti-8606	8606/ anti-8606	8606/ anti-8606/ absorbed with 51 bacteria	51/ anti-51
Glucose	90*	30	88†	44	59	—
β-Methylglucoside	46	14	14	7	5	—
Cellobiose	34	8	48†	30	55	—
Gentiobiose	18	6	16	3	3	—
α-Methylglucoside	41†	82	110†	82	96	110†
Galactose	90	15	59†	30	44	103†
Melibiose	60	7	44†	37	60	60†
Rhamnose	—	114†	—	137†	130†	113
Glucosamine	—	100	40†	62	87	100
Galactosamine	—	50†	74†	100	99	62†

* 50% inhibition value expressed in μM-sugar.
† 30% value, where the 50% value was not reached.

The inhibition reactions of the different 'salivarius' systems, the III system and an absorbed anti-salivarius 8608/salivarius 8606 system were compared with each other (Table 7). Except for the 'salivarius' 51 system, the same sugars as in the III/anti-III system were active. In the 'salivarius' 51 system rhamnose gave a weak inhibition reaction. Because anti-A variant serum is directed against rhamnose endgroups (McCarty 1956) this serum was tested against a formamide extract of *S. salivarius* 51; it was inactive.

DISCUSSION

Soon after its discovery in 1943 Streptococcus MG was serologically examined and classified as a separate genus, which was only related to *Streptococcus salivarius* type I as described by Sherman *et al.* (1943). The precipitin reactions with formamide extracts, the cross-absorption tests and the inhibition of the quantitative precipitin reaction suggest that Streptococcus MG contains the same polysaccharide antigens as Streptococcus F III and can be classified as F III.

All strains containing the III antigen will react with an anti-MG serum. F III, I. III and 0 III strains were positive (Table 2) and as the III antigen is also found in combination with the group antigen C (Ottens & Winkler, 1962), these C III strains will also give positive reactions with anti-MG sera. Part of the strains classified as 0 III proved to be *Streptococcus salivarius*. The relationship between Streptococcus MG and *Streptococcus salivarius* type I of Sherman *et al.* (1943) as found by Mirick *et al.* (1944 *b*) can be explained by the reactions of both with anti-III serum. A second unrelated 'salivarius' antigen (strain 51) was found. The relationship between strain 51 antigen and the 'salivarius' type II antigen of Sherman could not be investigated, as the latter strain was not available.

Qualitative chemical analysis and the inhibition of the precipitin reaction gave further confirmatory information about the identity of Streptococcus MG and F III strains.

Qualitative of chemical analysis of the hydrolysates of the formamide extracts *Streptococcus salivarius* and 0 III strains showed the same sugar composition. Strain 51 however contained in addition mannose. The difference between the 'salivarius' and 0 III strains on the one hand and MG and F III on the other is shown by the difference in the hexosamines found. In the first group of strains glucosaminine is found and galactosamine is lacking, whereas in the second group galactosamine is the main hexosamine. As has been shown earlier (Willers *et al.* 1964) galactosamine is the main hexosamine in the group F antigen. The inhibition of the precipitin reactions of the 'salivarius' and III systems (Table 7) are very similar. In both a β-glucosidic and a galaetosidic group seem to act as a determinant of the antigens. Whenever anti-III serum is used cellobiose gives a better inhibition than β-methylglucoside and almost equals that given by gentiobiose. When, however, anti-salivarius 8606 serum is used the inhibiting capacity of β-methylglucoside and gentiobiose are the same and exceed that of celliobiose. From these differences it can be seen that anti-salivarius 8606 serum contains antibodies with combining sites that fit better with $1 \rightarrow 6$-β-glucosidic linkage than with a $1 \rightarrow 4$-β-glucosidic linkage. Although the type III and salivarius 8606 systems give strong cross reactions, these results suggest that the determinant groups of the antigens are not completely identical. A more detailed study with oligosaccharides obtained by partial hydrolysis of the antigens will be necessary to study possible differences between the determinant groups of the type III and salivarius 8606 antigens.

The inhibition pattern of strain 51 indicates a different antigenic structure. Only rhamnose gives a slight inhibition, a reaction found in the A variant (McCarty, 1956), C variant (Krause & McCarty, 1963) and group G (Curtis & Krause, 1964) systems.

No relationship between the variant A system and the 'salivarius' type 51 system was found.

The inhibition given by the hexosamines in several systems, in which the *N*-acetyl hexosamines were inactive is not quite clear. Good inhibitions given by arginine and lysine in the same systems suggest that anti-protein reactions are involved here.

This is the third paper of a series of 'Immunochemistry of group F streptococci'. The authors are indebted to Professor Dr K. C. Winkler for constant advice and to Dr C. E. de Moor for suggesting a relationship between the antigens of Streptococcus MG and the group and type antigens of group F. The skilful technical assistance of Miss M. J. Sijsma and Miss T. Alderkamp is gratefully acknowledged.

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The Relationship between the Capsular and Cell Wall Polysaccharides of Strains of *Lactobacillus casei* var. *rhamnosus*

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SUMMARY

The polysaccharide components of an encapsulated and a non-encapsulated strain of *Lactobacillus casei* var. *rhamnosus* were compared. The main capsular component was a polysaccharide consisting of rhamnose, glucose and galactose. This polysaccharide resembles, chemically and serologically, one of the two polysaccharide components normally found in the cell wall of *L. casei* var. *rhamnosus*. Since the cell wall of the encapsulated strain lacks the rhamnose-containing polysaccharide it seems likely that the capsule represents polysaccharide which has failed to become attached to the cell wall mucopeptide.

INTRODUCTION

The studies of Hammond (1961) and Sims (1964) have shown that some strains of *Lactobacillus casei* produce a capsule. Hammond (1961) isolated the capsular material and concluded that it contained approximately equimolar proportions of glucose and rhamnose. Subsequent reports (Hammond & Williams, 1964 *a*, *b*) have detailed further aspects of investigations on the encapsulated strain. The strain studied by Sims was defined as *L. casei* var. *rhamnosus* on the basis of physiological and serological tests. Previous studies (Knox, 1963; Glastonbury & Knox, 1963) have shown that the cell wall of *L. casei* var. *rhamnosus* contains rhamnose, glucose and galactose in addition to hexosamine and amino acids; furthermore, two serologically distinct polysaccharides are present, one containing rhamnose, glucose and galactose, and the other containing glucose, galactose and hexosamine. In the present work capsule and cell-wall preparations have been made from the strain isolated by Sims (1964), and their properties compared with those of cell-wall products previously (Knox, 1963) obtained from non-encapsulated strains of *L. casei* var. *rhamnosus*.

METHODS

Organisms. The encapsulated strain of *Lactobacillus casei* var. *rhamnosus* isolated by Sims (1964) was obtained from the National Collection of Type Cultures, Colindale, London, designated NCTC 10302. The other strains were those used in earlier studies (Knox, 1963) and were designated *L. helveticus* (*L. casei* var. *rhamnosus*) NCTC 6375, *L. casei* (var. *casei*) NIRD R094 and *L. casei* (var. *casei*) NIRD H831.

Serology. Antisera to strains NCTC 6375, NIRD R094 and NIRD H831 were prepared as described previously (Knox, 1963). Strain NIRD H831 belongs to sero-

logical group B and strains NCTC 6375 and NIRD R094 to group C (Sharpe, 1955; Sharpe & Wheater, 1957). The extent of cross-reaction between the antisera and extracts from strain NCTC 10302 were determined by qualitative and quantitative precipitin reactions (Knox, 1963; Glastonbury & Knox, 1963).

Components of cell wall from Lactobacillus casei var. rhamnosus, strain NCTC 6375. Cell wall from strain NCTC 6375 was prepared as described previously (Knox, 1963). Soluble cell wall components were obtained by incubating cell wall with a preparation of Streptomyces muralytic enzyme; the rhamnose-containing polysaccharide is the major component of the product designated Fraction I (Knox, 1963).

A preparation of Fraction I was hydrolysed for 3 hr at 100° in 0·1 N-H_2SO_4. Oligosaccharide fractions were obtained by elution of the material from a charcoal + Celite column. Fractions eluted with 7·5, 15 and 25% (v/v) ethanol in water contained mixtures of oligosaccharides which were not further resolved. The component sugars in each case were rhamnose, glucose and galactose; rhamnose was the major component (Knox, unpublished observations). For serological studies the carbohydrate content of each fraction has been expressed as the sum of the molar amounts of each of the component sugars.

Analysis of preparations. The component sugars were detected by chromatography of acid hydrolysates. Methods for the determination of nitrogen and the component sugars were as described previously (Knox & Brandsen, 1962).

RESULTS

Preparation of capsular material and cell wall from Lactobacillus casei var. rhamnosus strain NCTC *10302*

The encapsulated organism NCTC 10302 was grown under the conditions described by Sims (1964). The culture fluid (500 ml.) was centrifuged at 19,000*g* for 15 min. and the organisms washed twice with 0·9% (w/v) NaCl. The organisms were suspended in 250 ml. of 2·5% (w/v) NaCl and shaken vigorously for 2 hr at room temperature in a Dynamax Flask Shaker (Ainsworth Consolidated Industries, Sydney). The suspension was then centrifuged at 35,000*g* for 20 min.; the viscous supernatant fluid was removed and dialysed against frequent changes of distilled water. The extraction of the organisms was subsequently repeated for one 2 hr period and two 5 hr periods. After the final extraction, the organisms were washed twice with 2·5% (w/v) NaCl, and cell wall prepared by the method previously described (Knox, 1963). The cell wall and the extracts of capsular material were dried from the frozen state. The yield of cell wall was 65 mg.; the total yield of capsular material was 249 mg., made up as follows: first extract, 105 mg.; second extract, 103 mg.; third extract, 30 mg.; fourth extract, 11 mg.

The capsular material was further purified by precipitation with $(NH_4)_2SO_4$. A small amount of material was precipitated on adding an equal volume of saturated $(NH_4)_2SO_4$ at room temperature. After removing the precipitate by centrifuging, the $(NH_4)_2SO_4$ concentration of the supernatant fluid was increased to 55% saturation. A flocculent precipitate which formed on standing was dissolved in water, dialysed and dried from the frozen state. Solutions (0·5%) of the material were very viscous.

Strain NCTC 6375 was grown in the medium previously used when preparing cell wall (Knox, 1963), and also in the medium used by Sims (1964). Suspensions of

washed organisms were shaken for 4 hr, centrifuged and the rhamnose content of the supernatant fluid and of the residue determined. In each case only 0·5% of the total rhamnose of the organisms was in the supernatant fluid. It may therefore be concluded that *Lactobacillus helveticus* (*L. casei* var. *rhamnosus*) strain NCTC 6375 does not form a capsule.

Analysis of preparations from strain NCTC 10302

The cell wall preparation, capsular material (second extract) and the purified product were analysed for component sugars and total nitrogen. The results are summarized in Table 1.

Table 1. *Percentage composition of cell wall and capsule of Lactobacillus casei var. rhamnosus NCTC 10302*

	Cell wall	Capsule	
		Second extract	Purified product
	(%)		
Rhamnose	3	39	49
Glucose	19	21	25
Galactose	14	17	27
Hexosamine	23	0	0
Nitrogen	6·5	2·1	1·3

Serological examination of preparations

Acid-extracts of cell wall of *Lactobacillus casei* var. *rhamnosus* strain NCTC 10302 were examined for serological activity as described by Sharpe (1955). The extract gave a positive reaction with group C antiserum but not with group B antiserum. The serological properties of the purified capsular material were also studied; at a concentration of 10 μg./ml. the capsular material gave a strong reaction with antiserum to *L. casei* var. *rhamnosus* NCTC 6375 (serological group C) but did not react with antiserum to *L. casei* var. *casei* NIRD R094 (group C) and *L. casei* var. *casei* NIRD H831 (group B).

The cross-reaction between the capsular material of strain NCTC 10302 and the antiserum to strain NCTC 6375 was examined by the quantitative precipitin method. Antiserum (0·2 ml.) was mixed with increasing amounts of purified capsular material (5–120 μg.) in a total volume of 1·5 ml. After 1 hr at 37° and 3 days at 4° the protein content of the precipitate was determined by the method of Heidelberger & MacPherson (1943) by using Folin & Ciocalteu's phenol reagent. The results are compared in Fig. 1 with those obtained for the homologous reaction between the antiserum and Fraction I from strain NCTC 6375.

As shown previously (Knox, 1963), the precipitin reaction between Fraction I from strain NCTC 6375 and homologous antiserum is inhibited by rhamnose and, more particularly, glucose. Preparations of oligosaccharides from Fraction I contained rhamnose, glucose and galactose (see Methods) and their ability to inhibit the precipitin reaction was examined. The oligosaccharide preparations were a mixture of products eluted from a charcoal + Celite column by increasing concentrations of

ethanol in water, and the amount of carbohydrate added to the precipitin reaction could not, therefore, be expressed in terms of μmoles of oligosaccharide; instead, the amount added was expressed as the sum of the molar amounts of the component sugars. In Table 2 the inhibition of the precipitin reaction between capsular material of strain NCTC 10302 and antiserum to strain NCTC 6375 by oligosaccharides from Fraction I has been compared with the inhibition of the homologous reaction between Fraction I and antiserum to strain NCTC 6375.

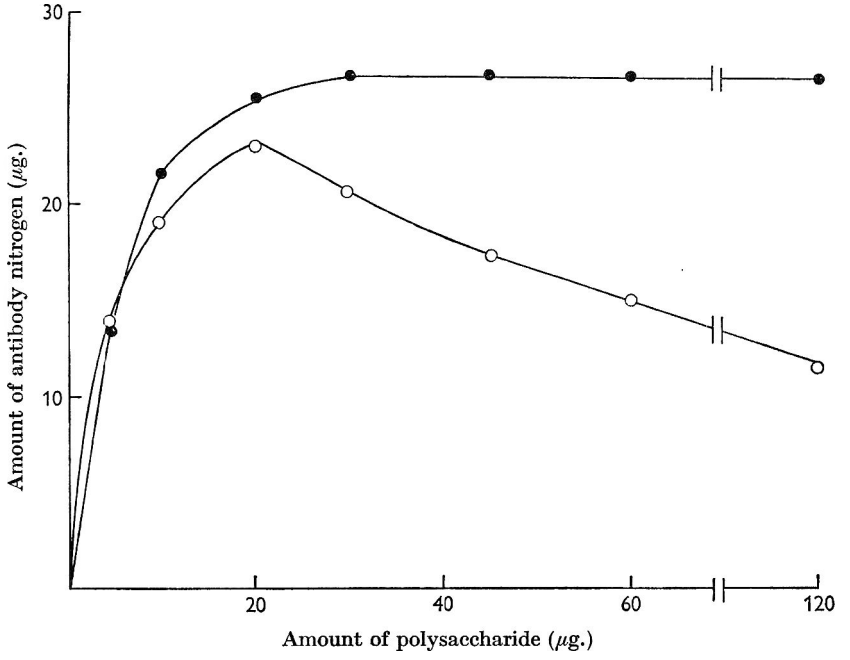

Fig. 1. Comparison of the precipitin reaction between capsular material from Lactobacillus casei var. rhamnosus strain NCTC 10302 (●) and Fraction I from L. casei var. rhamnosus strain NCTC 6375 (○), with antiserum to NCTC 6375.

Table 2. *Comparison of the ability of oligosaccharide fractions from strain Lactobacillus casei var. rhamnosus* NCTC *6375 to inhibit the precipitation of Fraction I from strain* NCTC *6375, and capsular material from L. casei var. rhamnosus strain* NCTC *10302 by antiserum to strain* NCTC *6375*

The antiserum (0·1 ml.) was mixed with the oligosaccharide preparations in a final volume of 0·8 ml., the solution of oligosaccharide containing approximately 5 μmole of component monosaccharides. After 1 hr at 37°, 0·2 ml. of solution containing 10 μg. of Fraction I or purified capsular material were added and the amount of precipitate formed on standing was estimated. The extent of inhibition was estimated by comparing the results with the amount of precipitate formed in the absence of oligosaccharides.

Carbohydrate fraction (eluted by %, v/v, ethanol in water)	Carbohydrate content (as μmole monosaccharide)	% Inhibition by	
		NCTC 6375 Fraction I	Capsular material
7·5	5·4	21	18
15	4·7	30	21
25	4·7	30	19

DISCUSSION

Sims (1964) defined the encapsulated strain of *Lactobacillus casei* var. *rhamnosus* (NCTC 10302) on the basis of physiological and serological tests. He was unable to show the presence of antibodies to the capsule nor would the encapsulated organisms absorb antibodies to a stock strain of *L. casei* var. *rhamnosus*. However, these studies were not extended to include the properties of soluble capsular material. Hammond & Williams (1964 *a*) studied the serological properties of the capsular material of a strain of *L. casei* but were unable to show any cross-reaction between the soluble capsular material and antiserum to a 'non-encapsulated variant'.

Strains of *Lactobacillus casei* var. *rhamnosus* belong to serological group C, whereas strains of *L. casei* var. *casei* belong to group B or C (Rogosa & Sharpe, 1959). Strains of *L. casei* var. *casei* contain a cell wall polysaccharide in which glucose and galactose are the predominant sugars and are responsible for serological specificity (Knox, 1963; Knox & Hall, 1965). Strains of *L. casei* var. *rhamnosus* contain an additional specific polysaccharide as a component of the wall, the predominant carbohydrate of this polysaccharide being rhamnose (Knox, 1963).

Analyses of cell wall preparations from a number of strains of group C organisms indicate that rhamnose is always a major component of the cell wall of *Lactobacillus casei* var. *rhamnosus*, whereas it is only a minor component of the wall of *L. casei* var. *casei* (Glastonbury & Knox, 1963). It would, therefore, have been expected that rhamnose would be a component of the cell wall of *L. casei* var. *rhamnosus* strain NCTC 10302; however, rhamnose is virtually absent from this, the amount of rhamnose in the wall preparation representing 2% of the amount in the capsular material. The carbohydrate components and serological properties of the wall are thus those expected for a strain of *L. casei* var. *casei*, group C, or alternatively for an atypical strain of *L. casei* var. *rhamnosus* lacking the rhamnose-containing polysaccharide.

The carbohydrate analyses of the capsular polysaccharide indicate that this product is similar in composition to the specific rhamnose-containing polysaccharide previously found to be a component of the cell wall of *Lactobacillus casei* var. *rhamnosus* (Knox, 1963); in each case the molar ratio glucose:galactose:rhamnose approximates 1:1:2, and hexosamine is absent. The results of serological studies also indicate a similarity between the capsular and cell wall polysaccharides, both reacting with antiserum to *L. casei* var. *rhamnosus* but not with antiserum to *L. casei* var. *casei* of group B or C. Further, oligosaccharide preparations from Fraction I of strain NCTC 6375 inhibit the precipitin reaction between the capsular material from strain NCTC 10302 and antiserum to strain NCTC 6375.

As shown in Fig. 1, differences in the reactivity of capsular and cell wall polysaccharides were noticeable when increasing amounts of the preparation were added to antiserum to strain NCTC 6375. With the capsular material, addition of increasing amounts did not lead to a diminution of precipitation while the cell wall product showed inhibition when twice the quantity required for maximum precipitation was used. These differences are probably due to differences in the molecular weight of the two products, as indicated by the marked differences in the viscosities of their solutions. Kabat & Berg (1953) obtained similar results in their studies on the precipitin reaction between antiserum to dextrans and dextrans of

various molecular weights. They concluded that the effect was almost certainly a function of the larger number of molecules per unit weight of the smaller molecular weight products.

The results of chemical and serological studies on the encapsulated strain of *Lactobacillus casei* var. *rhamnosus* (NCTC 10302) isolated by Sims (1964) show that the cell wall lacks the rhamnose-containing polysaccharide found in non-encapsulated strains, whereas the capsular layer contains a polysaccharide which is indistinguishable from the specific rhamnose-containing polysaccharide. This specific polysaccharide is normally joined to the cell wall mucopeptide by a covalent linkage (Knox, 1963; Hall & Knox, unpublished observations) and strain NCTC 10302 may, therefore, lack the ability to complete the synthesis of cell wall by attaching the rhamnose-containing polysaccharide to the cell wall mucopeptide. The continued production of the polysaccharide then results in its being present as a capsular layer which is readily extracted from the cells.

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