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SOME PHYSICAL INVESTIGATIONS OF THE BEHAVIOUR OF BACTERIAL SURFACES.

VIII. STUDIES ON THE CAPSULAR MATERIAL OF *STREPTOCOCCUS PYOGENES*

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SUMMARY

1. On repeated washing in phosphate buffer solution the electrophoretic mobility of young cells of M variants remained unchanged: the mobility of young G cells, however, always decreased to the value characteristic of old cells of that strain.

2. There was a correlation between the hyaluronic acid content of the cells and the electrophoretic mobility for a large number of strains during growth; both the mobility value and the hyaluronic acid content increased to a maximum at the end of the logarithmic growth phase and decreased to the original value during the stationary phase.

3. The variation of the mobility with the pH of the suspending medium of 24-h cells of the M and G variants of a number of different types confirms the presence of both amino and carboxyl groups on the surface. Further information on the nature of the surface was obtained by treatment of the cells with hyaluronidase.

INTRODUCTION

As a result of extensive chemical studies, the general structure of the cell wall of the group-A streptococcus is well established. The basic cell-wall structure consists of a mucopeptide[1], containing muramic acid, glucosamine, alanine, lysine and glutamic acid. Associated with this is the group-specific polysaccharide or C substance, containing rhamnose and *N*-acetylglucosamine, which accounts for approx. 50% by weight of the cell wall, and the type-specific protein antigens. The group-A streptococcus also produces a capsular mucopolysaccharide, hyaluronic acid. Although such chemical studies have given information concerning the nature of the components of the cell wall and capsule, there is still a lack of knowledge concerning their arrange-

Abbreviations: CTAB, cetyltrimethyl ammonium bromide; M, "matt", and G, "glossy" refer to the possession and lack of M protein antigen, respectively; the colonial appearance after 24 h growth on horse-blood agar and the presence of M antigen are related.

ment. It was with this problem in mind that the detailed study of the electrical properties of a number of strains of group-A streptococci was undertaken; in this paper the study has been limited mainly to the capsular material.

From a preliminary report[2] on the M and G variants of one strain (Type 6) of *Streptococcus pyogenes* it appeared that changes of the electrophoretic mobility during growth might be related to the variations observed by FABER AND ROSENDAL[3] in the amount of capsular hyaluronic acid present at different stages of the growth cycle. The reduction of the mobility value of young cells of the G variant on repeated washing was attributed to the loss of loosely bound capsular hyaluronic acid. In contrast, the mobility of young cells of the M variant remained constant on washing. The pH–mobility curves of the two variants indicated that a protein–hyaluronic acid complex was an important part of the streptococcal surface.

In extending this work it was necessary to correlate, without any doubt, the relationship between the mobility and the measured hyaluronic acid content of the cells of Type 6M and Type 6G, and then to screen a number of M and G variants belonging to a variety of different serological types to see if the same correlation applied. The effect of repeated washing on cells of pairs of M and G variants, each of which had been obtained by colony selection from the same strain, was also investigated to ascertain whether the loose binding of the capsular material occurs only with the G variants. A study was made of the variation of mobility with pH of the suspension liquid for a range of strains, in order to relate the variation of surface charge with the known antigenic characteristics. All the organisms studied belonged to Lancefield's group A; the group-specific carbohydrate was therefore a common feature; the organisms selected all contained a T antigen, some carrying in addition the type-specific M antigen. A further protein antigen designated R (ref. 4) was present in two strains.

EXPERIMENTAL

Cultures and culture media. All organisms were grown in a modified Todd Hewitt medium[5] at 37°, from a 2% v/v inoculum of 24-h cells in the same medium.

The strains studied, with their antigenic properties, are listed in Table I. The strains were checked serologically at all stages. The presence of T antigen was established by means of the slide agglutination test using a suitably absorbed homologous antiserum and a trypsinized cell suspension. The M antigen was identified by a precipitin test between the absorbed antiserum and the acid-extracted antigen[6]. The strength of the serological reaction varied amongst the different strains; Table I includes an indication of the strength of these reactions.

Measurement of growth. The growth of a culture was estimated by measuring the absorbancy at 600 mμ against a blank of distilled water. The dry weight (mg/ml) was obtained, when required, from the absorbancy using a standard calibration curve.

Determination of hyaluronic acid. Hyaluronic acid was removed from the cells by the method of SEASTONE[7] and estimated by the method of DI FERRANTI[8]. A 50-ml aliquot of a culture of known absorbancy was centrifuged and the cells washed twice with acetate buffer solution (pH 6.0, I 0.35) and finally resuspended in 5 ml of this solution. The suspension was heated in a boiling-water bath for 30 min and centrifuged; the sediment was discarded. 1 ml of the supernatant and 1 ml of acetate buffer solution were incubated at 37° for 10 min and then 2 ml CTAB solution (2.5 g CTAB

TABLE I

THE ANTIGENIC PROPERTIES OF STRAINS OF *Streptococcus pyogenes* USED

+++ = strong serological reaction; ++ = average serological reaction, + = weak serological reaction.

Type	Strain	NCTC* No.	Abbreviated name	Surface antigen M	Surface antigen T	Surface antigen R	Other properties
2	T2/44/Rb4	8322	2M	+	+++	−	
			2G	−	++	−	
2	7150		2M′	+	++	−	
3	D58X/11		3M	++	++	++	
	D58X		3G	−	+	++	
5	R54/2034		5M	++	++	−	
			5G	−	++	−	
6	S43	8302	6M	+++	+	−	Readily auto-agglutinated in the buffer solutions used
	S43	8709	6G	−	++	−	
12	1130		12M	+++	+	−	
			12G	−	++	−	
14	Lowe	8199	14M	++	++	−	
			14G	−	++	−	
22	63T	8330	22/63T	−	++	−	Produces large amounts of hyaluronidase
22	9RI'76		22M	++	++	−	
			22G	−	++	−	
36	C119/58	8227	36M	++	++	−	Very mucoid strain

* One NCTC number indicates that the variants were isolated by colony selection.

in 100 ml 2% NaOH solution), also at 37°, was added. The resultant turbidity was measured immediately in a Unicam SP600 spectrophotometer at 400 mμ against a blank in which 1 ml acetate buffer solution replaced the supernatant. The calibration curve, constructed using known concentrations of hyaluronic acid, was linear up to 200 μg hyaluronic acid per ml. It was thus possible to determine the hyaluronic acid content (μg) per mg dry wt. of culture.

Measurement of electrophoretic mobility. The cells from 50 ml of culture were harvested when required, washed three times in, and finally resuspended in, 50 ml of the appropriate buffer solution of known pH and ionic strength. The suspension was introduced into the observation chamber of the electrophoresis apparatus at 25° and the average mobility of 30 cells determined by the method described by GITTENS AND JAMES[9]. The technique and precautions described by these authors were rigorously followed. In general the populations were electrophoretically homogeneous and so only mean values are quoted: the confidence limit for a single mean at $P = 0.05$ was ± 3%, values differing by more than 10% are thus significant.

Buffer solutions. The "standard phosphate buffer solution" was prepared by dissolving 4.536 g KH_2PO_4 and 11.949 g $Na_2HPO_4 \cdot 12H_2O$ in 10 l glass-distilled water; pH 7.00, I 0.013. Buffer solutions, used in pH–mobility studies, were prepared from sodium barbiturate, sodium acetate, and HCl[10] with the addition of NaCl to give a constant ionic strength (0.02).

RESULTS

The change of electrophoretic mobility and hyaluronic acid content of 6-h and 24-h cells of M and G variants, after repeated washing

Following the previous observation[2] of change of mobility of young cells of the G variant of Type 6 simply by repeated washing, it was decided to study this phenomenon on variants of different types. It was necessary first to determine the number of washings required to remove all the growth medium from the cells, while at the same time leaving undisturbed as much of the capsule as possible.

The organisms from 500 ml of culture, grown under standard conditions, were harvested after 6–8 h growth. The cells were resuspended in 25 ml phosphate buffer solution and an aliquot removed for mobility determination (zero washing); the remainder was centrifuged and the cells resuspended by agitation in phosphate buffer solution. The mobility of cells of this suspension was again determined, and the residual suspension centrifuged. This process was repeated so that the mobility values of cells after 0–6 washings were obtained (Table II), all the cells being derived from the same initial population. The experiment was repeated on 24-h cells of each strain.

Cells after sedimentation from the growth medium and resuspension (*i.e.* zero washing) showed a wide range of mobility values due to adsorbed material from the growth medium. The mean value was without meaning and is not included in Table II. It was found that the mobility value after 2 washings was often greater than after 1 washing, suggesting the presence of adsorbed components of the growth medium. It was apparent that:

TABLE II

THE VARIATION OF THE ELECTROPHORETIC MOBILITY OF CELLS OF *Streptococcus pyogenes* ON REPEATED WASHING IN PHOSPHATE BUFFER SOLUTION

Strain	*Electrophoretic mobility (μ/sec/V/cm)*					
	Young cells (6–8 h)					*Old cells*
Number of washings	*1*	*2*	*3*	*4*	*6*	*3*
2M	−1.05*	−1.53*	—	−1.29*	−1.20*	−1.22
2G	−1.52	−1.50	−1.33	−1.36	−1.35	−1.35
2M′	−2.04	−2.05	−2.05	−2.01	−2.01	−1.20
3M	−1.57	−1.79	−1.81	−1.83	−1.71	−1.33
3G	−0.84	−1.36	−1.10	−0.99	−0.99	−0.99
5M	−1.58	−1.92	−1.92	−1.92	−1.92	−1.56
5G	−1.76	−1.70	−1.06	−1.54	−1.32	−1.32
6M	−1.90	−1.94	−2.07	−2.10	−1.96	−1.19
6G	−0.95	−1.30	−1.30	−1.30	—	−1.32
12M	−2.61	−2.49	−2.51	−2.48	−2.46	−1.35
12G	−2.04	−2.00	−1.84	−1.75	−1.70	−1.65
14M	−2.39	−2.39	−2.41	−2.42	−2.43	−1.57
14G	−2.78	−2.40	−2.16	−1.95	−1.85	−1.83
22M	−1.59	−1.59	−1.59	−1.59	−1.60	−1.43
22G	−1.79	−1.72	−1.65	−1.59	−1.59	−1.61
22/63T	−1.14	−1.60	−1.63	−1.50	−1.52	−1.49
36M	−4.82	−4.86	−4.82	−4.77	−4.82	−3.69

* These populations were electrophoretically heterogeneous.

(a) two washings were sufficient to remove the last traces of growth medium, leaving an electrokinetically reproducible and homogeneous surface;

(b) the mobility value of 24-h cells of both the M and G variants of all types was constant, within the limits of experimental error, after 1–6 washings (only the value after 3 washings is quoted);

(c) the mobility value of 6-h cells of the M variants of all types, except type 2M (NCTC 8322) was constant on repeated washing;

(d) in contrast, the mobility value of 6-h cells of the G variants of all types decreased with repeated washing and eventually attained the value of 24-h cells of that variant.

It was shown that the decrease in mobility of the 6-h cells of the G variants was due to the removal of hyaluronic acid on repeated washing. The previous experiment was repeated and, in addition to the mobility determination, the hyaluronic acid content of the cells was determined after each washing. Fig. 1 shows typical results

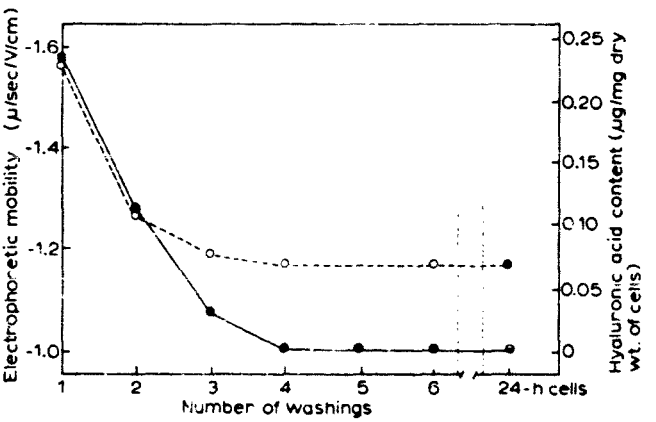

Fig. 1. The effect of washing on the mobility and hyaluronic acid content of young cells of Type 2G. O—O, mobility; ⊗, mobility value of 24-h cells; ●—●, hyaluronic acid; ◒, hyaluronic acid content of 24-h cells.

on Type-2G cells, from which the relationship of mobility and hyaluronic acid content is immediately apparent. When all the capsular material has been removed, as in the 24-h cells, the mobility becomes constant at the value characteristic of 24-h cells. In contrast, the hyaluronic acid content of M cells of the same variant remained constant on washing, again in agreement with the mobility determinations.

It is also apparent from Table II that the ease of removal of hyaluronic acid from the G variant depended on the type. Thus cells of Type 6 lost their capsular material completely after 1 washing at pH 7.0, while with cells of Types 12 and 14, 6 washings were required. Further it was observed with Type-6G cells that while 1 washing was sufficient to remove all the hyaluronic acid at pH 7.0, 2 washings were required at pH 5.5 and 4 at pH 8.0. Since the cells were more heavily sedimented in the centrifuge at pH 5.5 and 7.0, the extra mechanical agitation (which in any case was not reproducible) required for resuspension, could account for the increased efficiency of removal of capsular material at these pH values.

The variation of the electrophoretic mobility and the hyaluronic acid content of cells of Streptococcus pyogenes with culture age

Two 50-ml samples were removed aseptically from a growing culture at intervals during the growth cycle (0–24 h). The hyaluronic acid content and the mobility of the cells was determined as previously described. Smaller samples, removed periodically, were used to plot the growth curve. Extreme care was taken to standardize the washing procedure for the cells of the G variants to avoid superimposition of the change of mobility due to washing alone. Cells of Type 6G were washed twice at pH 8.0 before final suspension at pH 7.0 for mobility determinations. Typical curves for the variation of the mobility, determined in standard phosphate buffer solution, and of hyaluronic acid with age are shown in Fig. 2 for Type-12G cells. Similar shaped curves, displaced on the ordinate, were obtained with cells of the M and G variants of Types 2, 6, 12 and type 36M. The mobility and the amount of capsular hyaluronic acid always showed a parallel change, both increasing during

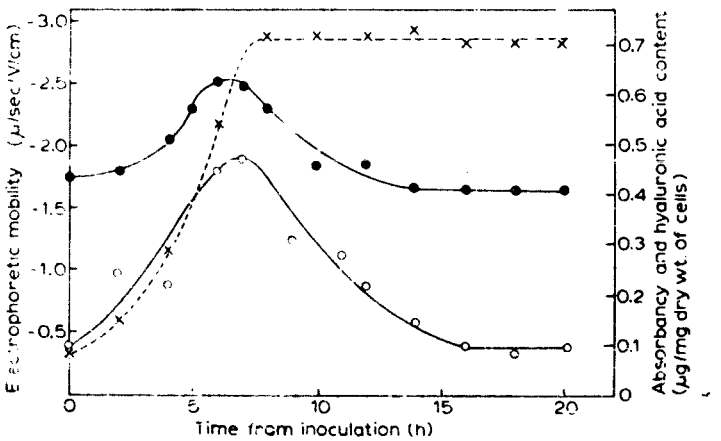

Fig. 2. The variation of the electrophoretic mobility and hyaluronic acid content with age during the growth of Type-12G organisms. ×—×, growth curve; ●—●, electrophoretic mobility; O—O, hyaluronic acid content.

the phase of active cell division to a maximum at the end of this phase, thereafter decreasing during the stationary phase to their original values. Cells of Type 36M, which were shown to produce large amounts of hyaluronic acid, showed a similar but considerably larger variation. The rate of removal of hyaluronic acid and, in consequence, the rate of decrease of mobility varied with the strain, but in general all strains attained both a constant mobility value and a constant hyaluronic acid content (usually zero) by 24 h.

When 6–8-h cells, of maximum mobility value, were subcultured into fresh growth medium the mobility did not rise further but remained constant at its original high value during the logarithmic phase of growth, and subsequently decreased in the stationary phase to the value characteristic of normal 24-h cells.

In contrast to this variation of mobility and hyaluronic acid with age, cells of a Type-22 strain (T22/63T) showed no change of mobility at all throughout the growth cycle (Fig. 3), the mobility being that of 24-h cells. In addition, no hyaluronic acid

could be detected on these cells at any stage of growth. It was known that cells of this strain produced large amounts of hyaluronidase, enough apparently to depolymerise and thus prevent the accumulation of any hyaluronic acid which might be formed, even in the early stages of growth. This suggested that hyaluronidase released into the medium might be responsible for the decrease in hyaluronic acid content with the concomitant decrease in mobility.

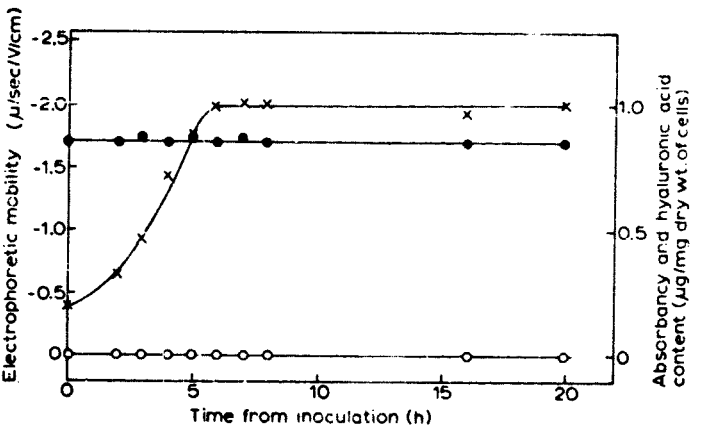

Fig. 3. Graphs showing the constancy of the electrophoretic mobility and hyaluronic acid content with age during the growth of Type-22/63T organisms. · · , growth curve; ● ●, electrophoretic mobility; O O, hyaluronic acid content.

An attempt was therefore made to detect hyaluronidase in the growth medium[8]. 1 ml of culture supernatant was incubated at 37° with 1 ml of standard hyaluronic acid solution (200 μg/ml) and the remaining substrate estimated with CTAB. Since the growth medium may contain a turbidity-producing substance under these conditions a blank consisting of 1 ml of fresh growth medium and 1 ml of acetate buffer solution was included. The difference between the supernatant value and the blank gave the amount of residual substrate. Complete destruction of 200 μg of hyaluronic acid was achieved with 1 ml of supernatant of Type 22/63T in less than 30 min. The use of this method for the estimation of small amounts of enzyme was, however, complicated by the fact that hyaluronic acid appeared to be bound by components in this growth medium and was, in consequence, difficult to estimate. It was found that after incorporating 200 μg of hyaluronic acid in 1 ml of growth medium only 100 μg could be detected. Thus the reduction due to the small amounts of hyaluronidase was masked, and it was not possible, by this method, to demonstrate with certainty the presence of hyaluronidase in the supernatants of any of the strains, other than 22/63T, even after 24-h incubation of supernatant and substrate. Since there was such a marked decrease in the hyaluronic acid content of the cells, it seemed probable that the amount of hyaluronidase present was not sufficient to account for the observed reduction.

A study was therefore made of the variation of the hyaluronic acid on the cells and in the growth medium with age. In this work the large changes in the hyaluronic acid in the medium were not so susceptible to masking by the medium as previously. Little hyaluronic acid appeared in the growth medium until the end of the logarithmic

growth phase. Then the hyaluronic acid content of the cells decreased but the amount in the supernatant increased sharply and attained a maximum value some 5 h later. This suggested that more hyaluronic acid was being formed by the cells during the stationary phase. Later the amount in the supernatant decreased very slowly in accord with a very low level of hyaluronidase activity. It would thus appear that the capsular hyaluronic acid was thrown off the cells into the medium at the onset of the stationary phase, and subsequently depolymerised by the enzyme at a very slow rate.

The variation of the mobility with the pH of the suspension liquid for 24-h cells of Streptococcus pyogenes

So far it has been established that, with one exception, hyaluronic acid is present on cells of all the strains tested, and that the variation of mobility can be correlated with hyaluronic acid content of cells grown under standard conditions and tested at constant pH and ionic strength. Under constant conditions of age and growth, a study of the effect of variation of the physical properties (*e.g.* pH) of the suspension liquid used for the mobility determinations will give information about the nature of surface ionogenic groups. It should further reveal similarities or differences between M and G variants of the same strain and also between strains belonging to different types. Because of the loss of capsular material on washing of cells of the G variants only 24-h cells were studied. These were harvested by centrifugation and washed twice in the standard phosphate buffer solution (pH 7.0) and divided into a number of portions. The cells in each portion were subsequently washed twice in the appropriate buffer solution of known pH (I 0.02) and resuspended in this solution for mobility determination. Two general types of curve were obtained (Fig. 4), in one of these there was a mobility plateau in the pH region 6.0 to 9.5 whilst in the other there was an additional small but significant increase in mobility at pH 7.0. It was found that strains exhibiting the long plateau were those which still had residual hyaluronic acid on the surface, whilst strains possessing no surface hyaluronic acid gave the second type of curve.

The experiment was repeated, but now the cells were divided into two fractions. Cells of the first fraction were used to determine the normal pH–mobility curve. The cells in the second fraction were treated with hyaluronidase (L. Light and Co.)

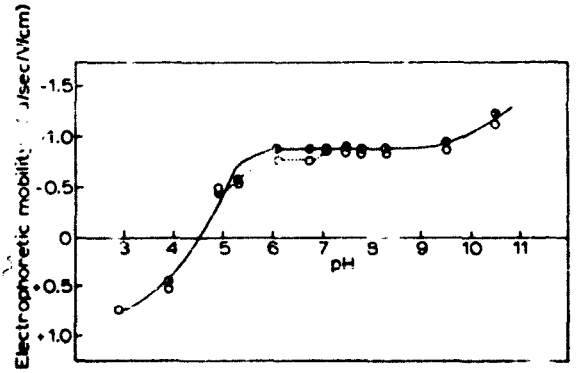

Fig. 4. pH–mobility curves for 24-h cells of Type 2G. ●—●, normal cells; ○—○, cells after treatment with hyaluronidase.

at a final concentration of 5 I.U./ml in standard phosphate buffer solution (pH 7.0) for 1 h at 37°. These cells were then washed twice in the appropriate buffer solutions and the pH–mobility curves determined. It was found, irrespective of the previous curve obtained, that all cells showed a common curve, *i.e.* the one with the additional increase at pH 7.0. Apart from the region pH 5.5 to 7.0 the two curves were coincident, showing that the hyaluronidase is not irreversibly adsorbed on the surface.

To ensure that no irreversible surface changes had occurred, cells after suspension in solutions of extreme pH values were washed and resuspended in standard buffer solution at pH 7.0. If the mobility of these cells was not the same as that of normal cells then it was assumed that surface disorganization had occurred. Mobility values were only accepted and plotted (Fig. 4) when the cells were reversible to pH 7.0; the pH range for reversibility for all strains was 3 to 11.

The curves for the M and G variants of Types 2, 3, 6 and 12 were all similar, they showed a positive mobility value in solution of low pH, indicating the presence of surface amino groups, an isoelectric point between pH 4.3 and 4.7 (except Type 12G at pH 3.5), followed by an increase in the negative mobility to a plateau region indicative of carboxyl. The increase in mobility at pH 7.0 suggested the presence of a component which ionises at this pH and which in some strains was masked with hyaluronic acid.

DISCUSSION

The surface components of *Aerobacter aerogenes* previously studied[11] were simple macromolecular polysaccharides possessing acidic groupings. The surface was stable to repeated washing and independent of the nature of the growth medium, so that the mobility changes observed were easily explained. In contrast, the streptococci studied here present a more complex problem. The growth medium, prepared from meat extract, is not chemically defined and reproducible, and consequently slight variations in the electrical properties of the cells occurred from batch to batch. All mobility measurements, *e.g.* variation with washing, age and pH were therefore made on cells of the same culture, thus avoiding effects produced by using different growth media or inocula. The nature of the surfaces of these organisms presents a more complex picture; the outermost components undoubtedly include capsular material, which may or may not be firmly bound, and protein antigens and macromolecular acidic polysaccharide which appear to be firmly bound to the cell wall. Since these latter components are type- and group-specific, respectively, valid comparisons can only be made between M and G variants of the same strain, although some general conclusions may be drawn for cells of all types. Hyaluronic acid was present, at some stage in the growth cycle on cells of all strains except those of the Type-22 strain 22/63T. The antigenic structure appears to control the strength of bonding of, but not the amount of, capsular material.

All the organisms possess a large negative charge at neutral pH values and for a given type there is no relationship between the magnitude of the charge and the antigenic structure (Table II).

Repeated washing of some other organisms has no significant effect on their mobility[12,13] suggesting that a stable surface is immediately formed. Whether this surface is the capsule or the cell wall will to a large extent depend on the constitution of the medium. The Todd Hewitt medium used in this work was sufficiently nutrient

to allow capsular material to be formed. The M and G variants differed in the strength of binding of this capsular material. In general (Table II, Fig. 1) the capsular hyaluronic acid was removed from the young cells of G variants merely by repeated washing in phosphate buffer solution at pH 7.0. The removal was purely mechanical, for if stronger agitation was used in resuspension of the cells then fewer washings were necessary. In contrast, young cells of the M variants showed no loss of hyaluronic acid and no change in mobility on repeated washing, even with strong agitation. It was thus a general property of the M-variant strains studied that the capsular hyaluronic acid is strongly bound to the surface of young cells and cannot be removed merely by washing. M cells of Strain NCTC 8322 designated Type 2M were an exception to this rule. These cells were anomalous in their behaviour in that, firstly, they produced the only electrokinetically heterogeneous populations observed in this work and secondly, on repeated washing the mobility approached a value characteristic of 24-h cells. A negative serological test indicates that the number of organisms carrying the test antigen is small, but a positive test only indicates that a certain proportion, not necessarily a majority, of the cells carry the antigen. Thus, although the cells of Type 2M were serologically M^+ it is possible that most of them did not carry the M antigen. The anomalous behaviour of Type 2M could be explained by such a serologically heterogeneous population. Another strain, 7150, also classified serologically as Type 2M, but giving electrophoretically homogeneous populations, exhibited the washing characteristics of other M-variant strains. The 24-h cells of all variants, however, showed a constant mobility on repeated washing, despite the possession by cells of some strains of residual hyaluronic acid removable only by treatment with hyaluronidase. Thus it would appear that hyaluronic acid exists on the cell surfaces of all strains, but the strength of binding to the surface is dependent on the antigenic structure. Possibly the hyaluronic acid is chemically bonded to the M antigen and, in the absence of this antigen, as with G variants, the capsule is only loosely adsorbed.

Variation of mobility with age has been reported for several organisms[11,14,15]. These changes have not been adequately correlated with any reported change in biological properties of the cells or with change of the properties of the growth medium. The results reported here show an excellent correlation between electrophoretic mobility and the amount of capsular hyaluronic acid. With the exception of cells of Type 22/63T, which produce large amounts of hyaluronidase, there was always an increase in the amount of hyaluronic acid on the organisms during the logarithmic growth phase followed by a decrease during the stationary phase. This was accompanied by a parallel variation in the negative charge carried by the organisms. If it is assumed (a) that the difference in mobility between 6- and 24-h cells is entirely due to the loss of hyaluronic acid, *i.e.* the hyaluronic acid is not covering other ionogenic groups which will then contribute to the charge and, (b), that the hyaluronic acid is of no more than molecular thickness, then it is possible to calculate this loss from mobility data and compare it with that obtained by direct measurement. For cells of Type 36M the change in charge density, calculated using the Gouy equation was $1.86 \cdot 10^4$ esu/cm^2. From the direct measurement of hyaluronic acid, assuming that the cells are spheres of diameter 1 μ and that all of the carboxyl groups are oriented to the aqueous phase the corresponding change in charge density was $3.95 \cdot 10^4$ esu/cm^2. Despite the facts that the estimation of hyaluronic acid is of

limited accuracy, and that the Gouy equation is not strictly applicable to a surface penetrable by ions, underestimating the charge density by a factor of two (ref. 16), the agreement between the two values is good. Similar results obtained from other strains are sufficient to indicate that the variation of capsular hyaluronic acid was largely responsible for the variation of electrophoretic mobility.

Hyaluronidase was present in sufficient amount in the culture supernatant of Type 22/63T to prevent the formation of capsular material (Fig. 3), but could not be detected in the supernatant of the other strains tested. This is in agreement with the observations of ROSENDAL AND FABER[17] that it may be necessary to incubate substrate and supernatant of group-A streptococci for up to 5 days to demonstrate depolymerisation of substrate. The results indicate that this small amount of hyaluronidase was not responsible for the decrease in mobility at the onset of the stationary phase, but that the capsular material was lost to the growth medium where it was slowly depolymerised.

After treatment with hyaluronidase, 24-h cells of all the strains studied, irrespective of their antigenic structure, all have similar surfaces (Fig. 4). These pH–mobility curves are characteristic of an ionogenic surface containing amino groups (pK about 10), carboxyl groups (pK 3.5–4.0) and an unidentified component (pK 7). The amounts of these ionogenic groups vary with the strain, and this results in a displacement of the curves along the ordinate. Since the pH–mobility curves bear no resemblance to those of acidic polysaccharides, hyaluronic acid[18] and proteins[19], none of these components can completely cover the surface. However, the presence of both amino and carboxyl groups on the surface of cells treated with hyaluronidase suggests that the outer layers of the wall are composed of a polysaccharide–protein complex.

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