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SOME PHYSICAL INVESTIGATIONS OF THE BEHAVIOUR OF BACTERIAL SURFACES

IX. STUDIES ON THE STREPTOCOCCAL CELL WALL

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SUMMARY

1. The T antigen is more readily removed from the surface of matt than from glossy variants of *Streptococcus pyogenes* on treatment with trypsin (EC 3.4.4.4).

2. Electrokinetic studies of cells treated with proteolytic enzymes indicate that, irrespective of their original surface antigens, all strains have a common basic surface structure.

3. Studies on cells treated with FDNB show that the amino groups contributing to the bacterial surface charge are those of ε-lysine and alanine. The same amino acids provide the N-terminal groups on the surface after treatment with trypsin.

4. Although the amino acid composition of the trypsin-sensitive material is the same for the matt and glossy variants of a given strain, the charge densities due to both the amino and carboxyl groups are greater on the matt than on the glossy variant. It appears that the essential difference between the protein antigens of the matt and glossy variants lies in the extent of cross-linking of the proteins.

INTRODUCTION

The principal components of the cell wall of *Streptococcus pyogenes*, after treatment with trypsin, pepsin and RNAase (EC 2.7.7.16), are rhamnose, glucosamine, muramic acid, alanine, glutamic acid and lysine[1-3]. The cell wall may be separated by formamide extraction into two fractions, the formamide-soluble group polysaccharide, containing 60% rhamnose and 30% glucosamine[4], and the formamide-insoluble mucopeptide containing muramic acid, glucosamine, alanine, lysine, and glutamic acid in the molar ratio 1:1:4:1:1, respectively[5].

In addition to this "basal cell wall", a number of protein antigens, including the M, T, R (ref. 6) and B (ref. 7) antigens, and a hyaluronic acid capsule[8] may be present. The protein antigens may be extracted in a condition suitable for serological examination, but little is known about their chemical composition or their structure. The M antigen is readily removed from the cells and destroyed both by trypsin and by pepsin. The T antigen is destroyed by pepsin but not by trypsin, although trypsin at

50° will remove it from the cells. The R antigen of type 3, and the B antigen, resemble the T antigen in their sensitivity to pepsin and trypsin, but their removal from the cells by trypsin at 50° takes place much more slowly. The R antigen of type 28, on the other hand, is resistant both to trypsin and to pepsin[6]. TEPPER, HAYASHI AND BARKULIS[9] analysed the amino acids of the protein removed by trypsin treatment from a type-14 strain, but did not differentiate between components present in the M and T proteins. The strain used was subsequently shown to be of an unusual type which may be serologically active against two M antisera, types 14 and 51. However, these workers could find no difference between the composition of the trypsin-sensitive protein of the type-14 virulent strain and an untyped avirulent strain. This suggested that, although glossy variants do not carry the M antigen, they might carry the M protein in a nonantigenic form.

The micro-electrophoretic technique, previously used in the study of the streptococcal surface and the formation of the hyaluronic acid capsule[10], has now been applied to cells after various enzymic and chemical treatments in a study of the surface components of the cell wall itself. The results obtained are discussed in the light of chemical analyses of the M and T protein fractions.

EXPERIMENTAL

Growth media

All cultures were grown in a modified Todd–Hewitt broth[11] at 37° from a 2% (v/v) inoculum of 24-h cells in the same medium.

Cultures

The strains used are listed in Table I, together with their antigenic composition. All strains were tested serologically at each stage for the presence of the antigen under study.

Enzyme treatment

Before the specific enzymic treatment was applied the cells from a 24-h culture

TABLE I

ANTIGENIC COMPOSITIONS OF STRAINS USED

Strain	*Abbreviated name used*	*Antigen present*			
		M	*T*	*R*	*B*
NCTC 8330	2M	2	2	—	—
	2G	—	2	—	—
D 58X/11	3M	3	3	3	—
D 58X	3G	—	3	3	—
B 3264	B 3264	—	3/12/B 3264	—	B 3264
T5/R54/2034	5M	5	5	—	—
NCTC 8302	6M	6	6	—	—
NCTC 8709	6G	—	6	—	—
NCTC 1130	12M	12	12	—	—
	12G	—	12	—	—
Lowe	14M	14	14	—	—
T 22/76	22G	—	22	—	—
NCTC 8312	26M	26	4	—	—
NCTC 8227	36M	36	36	—	—

were washed and suspended in phosphate buffer solution at pH 7.0, and then incubated with hyaluronidase (EC 4.2.99.1) (1 I.U./ml) at 37° for 1 h to remove residual capsular material. The cells were then washed three times in, and resuspended in, the appropriate buffer solution. The enzymes used were crystalline products with high activity.

Trypsin (EC 3.4.4.4). The cells were suspended in phosphate buffer solution (pH 7.8, I 0.12) containing 0.005 % trypsin (Armour Pharmaceutical Co.), and incubated either at 37° for 16 h, or at 50° for successive periods of 2 h until the required antigenic state had been attained.

Pepsin (EC 3.4.4.1). The cells were suspended in barbiturate buffer solution (pH 2.5, I 0.02) containing 0.25% pepsin (Armour Pharmaceutical Co.) and incubated at 37° until no more protein antigen could be detected (usually 24 h).

Alkaline phosphatase (EC 3.1.3.1). The cells were suspended in barbiturate buffer solution (pH 7.9, I 0.02) containing $5 \cdot 10^{-5}$% alkaline phosphatase (B.D.H.) at 37° for 1 h.

FDNB treatment of cells

A suspension of 50 mg of washed hyaluronidase-treated cells in 5 ml of sodium bicarbonate solution (10 mg/ml) was shaken in the dark and at room temperature with 10 ml of a 5% (v/v) solution of FDNB in absolute ethanol for 5 h (ref. 12). The suspension was centrifuged and the yellow deposit washed 4 times in water, 4 times in ethanol and 4 times in ether. The sediment was then washed 3 times in the appropriate buffer solution and finally resuspended in this buffer solution. Controls, in which the FDNB was omitted, were always included. Since the mobility of these control cells was always the same as that of untreated cells at all pH values, it was concluded that the ethanol and ether treatment did not produce any serious surface disorganization.

Measurement of the electrophoretic mobility

The electrophoretic mobility was measured by the method of GITTENS AND JAMES[13]. 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 $\pm$ 3%, values differing by more than 10% are thus significant. The washing procedure and the barbiturate buffer solutions used for measurement were as described previously[10].

Since buffer solutions of extreme pH were used, it was necessary to check that the surface was unaffected by this treatment. Cells after suspension in buffer solutions of extreme pH were washed twice and resuspended in buffer solution of pH 7.1. If the mobility differed significantly from that of control cells at pH 7.1 it was assumed that this was due to the effect of the acid or alkaline conditions, and the results obtained at that pH were discarded.

RESULTS

The mobility of cells of types 6M and 6G after the removal of the M protein by trypsin

Matt cells of *Strep. pyogenes* lose their M antigen when treated with trypsin at 37°. Under these conditions, however, the cells retain the T antigen. It is thus possible to investigate, electrokinetically, the effect of removal of the M antigen alone. Two

methods of enzyme treatment are available: (a) the trypsin can be included in the growth medium, since, at a concentration of 0.005%, it does not seriously inhibit the rate of growth of *Strep. pyogenes*, or (b) the organism can be grown in the normal medium and then treated with trypsin until no reaction with M antiserum can be detected by the precipitin test[14].

Cells from a 24-h culture of type 6M grown in medium which contained 0.005% trypsin were serologically M-negative. The cells were sedimented by centrifugation, washed twice in phosphate buffer solution (pH 7.0, I 0.012) and then divided into a number of portions. The cells in each portion were washed twice in the appropriate

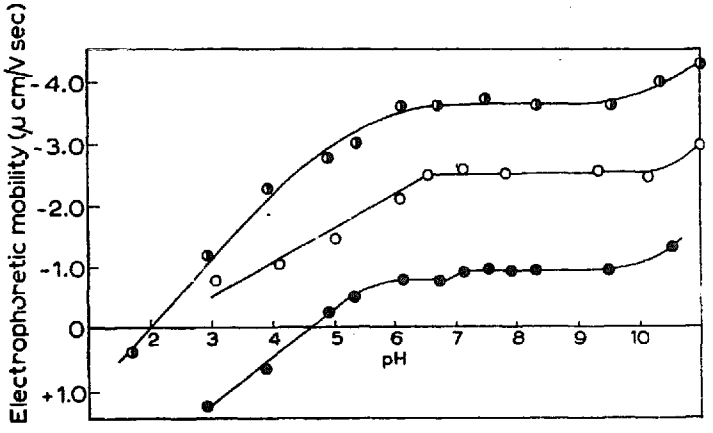

Fig. 1. pH–mobility curves (I 0.02) of type-6M cells after removal of the M antigen by treatment with trypsin at 37°. ●—●, normal cells; ○—○, cells grown in broth which contained trypsin; ◑—◑, cells treated with trypsin at 37° for 16 h.

barbiturate buffer solution of known pH (I 0.02) and resuspended in this solution. Mobility determinations were made over the range of pH 1.7 to 11. The treated cells possessed a much higher negative charge at all pH values than those grown in normal medium (Fig. 1); further, the group of pK 7.0 was no longer present.

24-h cells of type 6 M, grown in normal medium were harvested and treated with trypsin at 37° for 16 h, after which time no M antigen was detectable by serological tests. The pH–mobility curve (Fig. 1) showed that, at all pH values, these cells possessed a greater negative charge than either the untreated control cells or cells grown in the presence of trypsin.

When cells of type 6G were treated with trypsin in the same way the resultant surface was electrophoretically indistinguishable from that of trypsin-treated type-6M cells.

Analysis of the protein fraction removed from cells of types 6M and 6G by trypsin at 37°

Cells of types 6M and 6G were treated with 0.005% trypsin at 37° and pH 7.8 for 16 h. The cells were removed by centrifugation and the supernatant, after desalting, was evaporated to dryness. The residue was hydrolysed with 6 N HCl at 110° for 16 h, the HCl removed by evaporation and the hydrolysate analysed by paper partition chromatography. The solvents used were *n*-butanol–acetone–water (2:2:1, v/v)[15]. A beaker of cyclohexylamine was placed in the chromatography tank[16] to increase the separation of the amino acids. The amino acids identified in this fraction from both type 6M and 6G cells were alanine, arginine, aspartic acid, glutamic acid, glycine, histidine, isoleucine, leucine, lysine, methionine, phenylalanine, proline, serine,

threonine, tyrosine and valine; histidine and tyrosine were further confirmed using a modification of the Pauly reaction[17]. Of all these amino acids histidine is the only one with a pK of 7, suggesting that the group present on the normal bacterial surface with a pK of 7 is the imidazole group of histidine.

The mobility of cells of types 6 and 12 after the removal of the M and T proteins by tryptic action

Two methods of removing the T antigen are available, either (a) the cell suspension may be heated to 60–70° for 10 min and then treated with trypsin at 37°, or (b) the cells may be treated with trypsin at 50°. During both processes the M protein is removed from the cells and is destroyed. In general, method (b) is preferable as it is more rapid. Both methods were used in this work.

The T antigen is heat-labile and, with cells of type 6, was denatured if the cells were heated at 80° or above. The cells were therefore heated at 70°, a temperature which had no effect on the mobility of normal cells. 24-h cells of the matt and glossy variants of types 6 and 12 in standard phosphate buffer solution were heated at 70° for 10 min, sedimented by centrifugation, washed twice in phosphate buffer solution (pH 7.8) and then treated with trypsin at 37°. Samples of the cells were tested serologically at 2-h intervals for the presence of T antigen by the slide agglutination test[18]. The matt variants lost their T antigen after 4–6 h treatment, but, the glossy variants still gave very strong T-serological reactions even after 16 h. The glossy variants were, therefore, not studied further by this method. The pH–mobility curves (Fig. 2) of the resultant surfaces of the matt variants of types 6 and 12 were coincident, demonstrating that the two surfaces were electrokinetically indistinguishable.

Cells of both matt and glossy variants, when treated with trypsin at 50°, will eventually fail to react with T antisera. This method was therefore applied to cells of types 2M, 2G, 3M, 5M, 6M, 14M, 22M, 26M and 36M, and B 3264. Serological tests

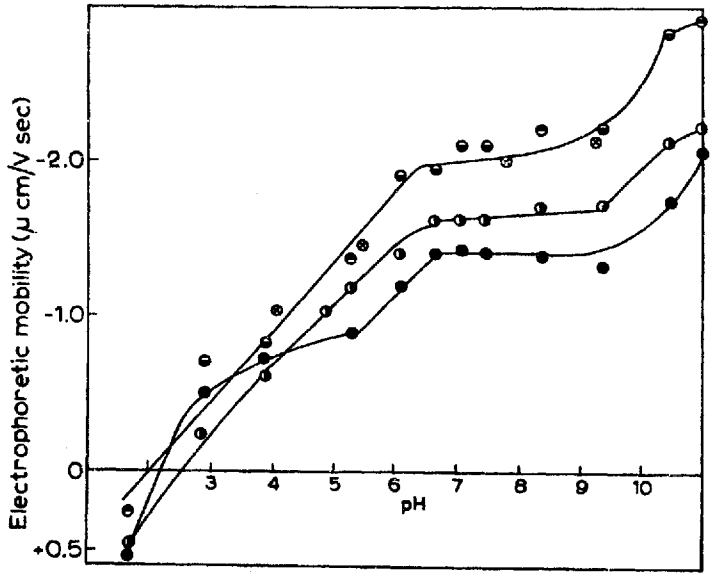

Fig. 2. pH–mobility curves (I 0.02) after removal of the M and T antigens with trypsin. O—O, type 6M after trypsin treatment of heated cells; ×—×, type 12M after trypsin treatment of heated cells; ⊖—⊖, mean values (Table II) of types 2M, 2G, 5M, 6M, 12M, 14M, 22M, 26M, and 36M after treatment with trypsin for 4 h at 50°; ⊖—⊖, representative strains, types 6M and 12M, after treatment with trypsin for 4 h at 50°; ●—●, type 3M after treatment with trypsin for 4 h at 50°; ◑—◑, B 3264 after treatment with trypsin for 4 h at 50°.

after 4-h treatment indicated that both M and T antigens had been completely removed but that cells of type 3M and B 3264 still retained their R and B antigens, respectively. The cells, after this treatment, were washed and suspended in the appropriate buffer solutions and the mobility values were determined at pH 1.7, 5.3, 8.4 and 10.9 (Table II). The surfaces were indistinguishable, within the limits of experimental error (± 3%), for all the strains studied with the exception of cells of type 3M and B3264. Full pH–mobility curves for cells of type 3M and B 3264 and the common curve for the remaining strains are shown in Fig. 2. This common curve was coincident with that obtained for the matt variants of types 6 and 12 using the heat-treatment method for removal of T antigen.

TABLE II

THE ELECTROPHORETIC MOBILITY OF CELLS OF *Strep. pyogenes* AT VARIOUS pH VALUES AFTER TREATMENT WITH TRYPSIN AT pH 7.8 AT 50°

Strain	*pH of measurement*	*Electrophoretic mobility* ($cm^2 \cdot V^{-1} \cdot sec^{-1} \times 10^5$)			
		1.7	*5.3*	*8.4*	*10.9*
2M		+0.41	−1.45	−2.33	−2.91
2G		0	−1.34	−2.22	−2.81
5M		0	−1.43	−2.20	−2.98
6M		+0.51	−1.39	−2.20	−2.88
12M		+0.47	—	−2.18	−2.86
14M		+0.51	−1.39	−2.28	−3.01
22M		0	−1.37	−2.18	−2.82
26M		+0.40	−1.40	−2.24	−2.97
36M		+0.59	−1.32	−2.16	−2.95
Mean value		—	−1.39	−2.22	−2.91
Mean value ± 3%		—	−1.34 to −1.44	−2.15 to −2.29	−2.81 to −3.01
3M		+0.53	−0.90	−1.41	−2.06
B 3264		+0.46	−1.20	−1.73	−2.22

Analysis of the T protein

The "T protein" refers to that material which is not removed from the cells by trypsin at 37° but is removed by similar treatment at 50°. This fraction contains the T antigen, as shown by the reaction obtained with the appropriate specific antiserum; it undoubtedly includes other material as well.

The protein was extracted by treatment with trypsin, at 50°, of cells previously extracted with trypsin at 37° for 16 h. The cells were removed by centrifugation and discarded, and the supernatant adjusted to pH 2 by the addition of HCl and then stored at 0° for 48 h. The precipitate which formed was washed twice with 0.01 N HCl dissolved in phosphate buffer solution at pH 7 and reprecipitated with acid. The product after three such precipitations was checked serologically by the precipitin test and then hydrolysed with 6 N HCl at 110° for 16 h. The excess acid was evaporated off and the hydrolysate analysed by paper partition chromatography, the solvents used were *n*-butanol–acetic acid–water (6:1:2, v/v) and lutidine–water (65:35, v/v). The six principal amino acids, obtained from the T proteins of the glossy variants of types 2, 6 and 12, were identified as alanine, isoleucine, lysine, serine, valine and

phenylalanine; the molar ratio[19] of these acids obtained from the analysis of type-2G cells was 3:2:2:2:2:3 and from type-12G cells was 3:2:1.6:2:2:3, respectively. Traces of a few other amino acids were occasionally observed, but they were not consistently present.

In a subsequent experiment the purified protein fractions were treated with FDNB before hydrolysis. Paper chromatographic analysis revealed the presence of the DNP-derivatives of alanine and ε-lysine in all three protein fractions, showing that these amino acids are N-terminal groups.

The mobility of cells after the removal of all the protein antigens by treatment with pepsin

The removal of the type-specific trypsin-sensitive protein antigens from a number of streptococci revealed a common electrokinetic surface. Strains with trypsin-resistant R or B antigens, however, did not conform to this pattern after treatment with trypsin. It was therefore of interest to see if treatment with pepsin, which removes R and B antigens as well as M and T antigens, resulted in cells with common surface characters.

TABLE III

THE ELECTROPHORETIC MOBILITY OF CELLS OF *Strep. pyogenes* AT VARIOUS pH VALUES AFTER TREATMENT WITH PEPSIN AT pH 2.5 AT 37°

Strain	*pH of measurement*	*Electrophoretic mobility* ($cm^2 \cdot V^{-1} \cdot sec^{-1} \times 10^4$)			
		1.7	*5.3*	*8.4*	*10.9*
2M		+1.03	−1.19	−1.92	−2.12
2G		+1.03	−1.17	−1.97	−2.13
3G		+1.11	−1.17	−1.88	−2.07
B 3264		+1.00	−1.18	−1.98	−2.12
5G		+1.01	−1.20	−1.93	−2.13
6M		+0.77	−1.16	−2.00	−2.20
12M		+1.00	−1.20	−1.96	−2.12
14M		+1.05	−1.18	−1.95	−2.18
22G		+0.99	−1.20	−1.97	−2.08
26M		+1.02	−1.20	−1.98	−2.17
Mean value		+1.03	−1.18	−1.95	−2.13
Mean value ± 3%		+1.00 to +1.06	−1.14 to −1.22	−1.89 to −2.01	−2.07 to −2.19

Suspensions of 24-h cells of 10 strains (2M, 2G, 3G, B 3264, 5G, 6M, 12M, 14M, 22G, 26M) were treated with 0.25% pepsin in buffer solution (pH 2.5) at 37° until the corresponding antiserum failed to show the presence of T, M, R and B antigens. The cells were washed twice in the appropriate buffer solution and the mobility values determined at pH 1.7, 5.3, 8.4 and 10.9. The resultant surfaces were all electrophoretically identical (Table III); the mobility values at each pH were the same, within the limits of experimental error (± 3%), with the exception of types 6M and 3G at pH 1.7. The common basic surface is not identical with that produced by trypsin treatment, because of the difference in the sites of attack of trypsin and pepsin.

The mobility of cells of type 6M after treatment with alkaline phosphatase

Normal 24-h cells of type 6M, suspended in barbiturate buffer solution (pH 7.9), were treated with alkaline phosphatase. The cells were then washed three times in the

appropriate buffer solution, and the mobility values determined at a number of pH values. Phosphatase had no effect on the mobility at any pH (Table IV). The isopotential point was unchanged, indicating that phosphatase-sensitive material was not present on the surface in appreciable amount. The increase in mobility at pH 7, still apparent, is probably not due to the presence of a hexose or pentose phosphate.

TABLE IV

THE ELECTROPHORETIC MOBILITY OF CELLS OF TYPE 6M AT DIFFERENT pH VALUES AFTER TREATMENT WITH ALKALINE PHOSPHATASE

pH	Electrophoretic mobility ($cm^2 \cdot V^{-1} \cdot sec^{-1} \times 10^5$)	
	Control cells	Cells after treatment with phosphatase
2.9	+1.51	+1.53
3.9	+1.27	+1.27
4.9	−0.67	−0.67
6.1	−0.73	−0.72
6.7	−0.74	−0.74
7.1	−0.82	−0.82
7.5	−0.82	−0.82

Chemical modification of the cell surface by treatment with FDNB

Cells of type 6M harvested after 24 h were treated with hyaluronidase, and then with FDNB. The pH–mobility curve of the resultant surface (Fig. 3) still showed the presence of a group of pK 7, indicating that this group does not react with FDNB.

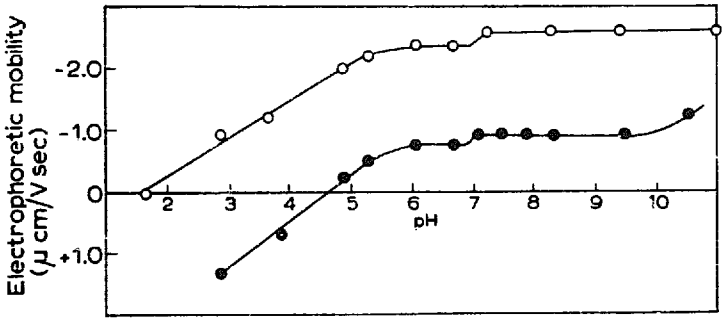

Fig. 3. pH–mobility curves (I 0.02) of cells of type 6M after treatment with FDNB. ●—●, normal cells; ○—○, FDNB-treated cells.

If it is assumed that there is no anion adsorption on to an ionogenic surface[20] then the charge density of FDNB-treated cells at pH 7–9 is due entirely to carboxyl ions, whereas that of the normal cells is the difference between the charge densities of the ionised carboxyl and amino groups. The mobility values of normal and FDNB-treated cells of a number of strains were measured at pH 8.3, a pH value at which both the amino and carboxyl groups are ionised. The charge densities (Table V) were calculated from the mobility using the Gouy equation. Since both groups are univalent the ratio of the numerical values of the charge densities (which are of opposite sign) is a measure of the ratio of the number of carboxyl to amino groups on the normal cell surface. There were always more carboxyl and amino groups on the matt variant than on the

glossy variant of the same strain, but the ratio carboxyl:amino was always greater for the glossy variant.

Samples of FDNB-treated cells from all the strains tested in Table V were hydrolysed and analysed by paper partition chromatography to identify the N-terminal groups. Only two spots corresponding to the DNP derivatives of ε-lysine and alanine, were obtained for each strain.

TABLE V

THE CHARGE DENSITIES OF NORMAL AND FDNB-TREATED CELLS AT pH 8.3

Strain	Charge density × 10^{-8} (esu/cm^2)				Ratio
	Normal cells $COO^- + NH_3^+$ (a)	FDNB treated cells: COO^- (b)	NH_3^+ (a) − (b)		COO^-/NH_3^+
2M	−1.09	−3.72	+2.63		1.4
2G	−1.26	−3.03	+1.77		1.7
6M	−1.01	−3.90	+2.89		1.3
6G	−1.08	−3.05	+1.97		1.6
12M	−1.17	−3.75	+2.58		1.4
12G	−1.42	−3.13	+1.71		1.8

Cells of type 6M and 12M, previously treated with trypsin at 50° until they showed no trace of T antigen, were also treated with FDNB. The pH–mobility curves of these cells (Fig. 4) were characteristic of a simple carboxyl surface and indicated that there were no free surface amino groups. The mobility value of FDNB-treated, trypsin-treated cells at pH 11 was the same as that of the control trypsin-treated cells

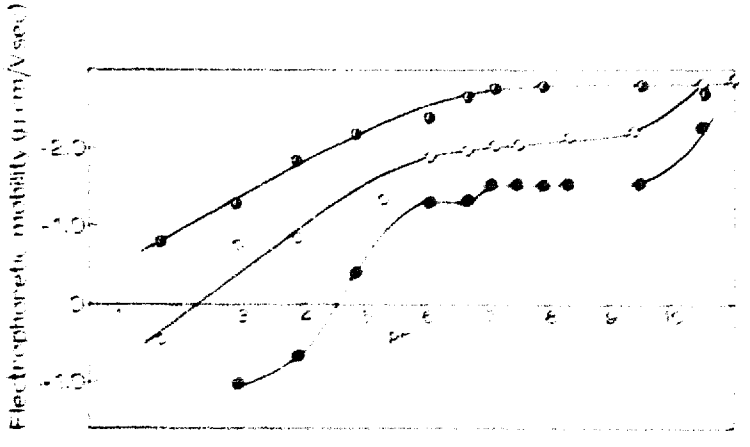

Fig. 4. pH–mobility curves (I 0.02) of type 12M cells after treatment with trypsin and FDNB. ●—●, normal cells; ○—○, cells after treatment with trypsin at 50°; ◑—◑, cells treated with trypsin at 50° and subsequently reacted with FDNB.

at this pH. The mobility of normal cells should theoretically attain the value of FDNB-treated cells at high pH values; however, this was not attained experimentally[20], owing to the excessive surface disorganisation above pH 11.

Chemical modification of the cell surface by treatment with diazomethane

Since the pH below which surface destruction occurred was 2.9, the standard treatment with diazomethane[22] to esterify the carboxylgroups of pK 2.2 (ref. 21) was

unsuccessful. In a modification of this method, cells of the matt variant of type 6 were alternately washed at pH 2.9 and treated with a solution of diazomethane in ethanol. The mobility, measured at pH 6.0, decreased rapidly during the first one or two treatments, and became positive due to the complete esterification of the γ-carboxyl group of glutamic acid (pK 4.1). The remaining carboxyl groups of α-glutamic acid and alanine (pK 2.2) were only slowly esterified, since diazomethane reacts only with unionised carboxyl groups, and at pH 2.9 the acids will be mainly in the ionised form. Eventually, after many treatments, the mobility attained a constant positive value, in agreement with that predicted from the mobility values of normal and FDNB-treated organisms at the same pH. Control cells, treated repeatedly with ethanol under the same conditions, had the characteristic mobility value at pH 6. This indicates that there is little if any anion adsorption[20] on to the mixed amino-carboxyl surface of the normal organism at pH values exceeding 6. The charge densities (Table V) need not, therefore, be corrected for such adsorption.

DISCUSSION

The study of cell walls by normal chemical methods provides much valuable information but suffers from some disadvantages. To obtain a clean preparation, the material obtained by the standard techniques of cell disruption must be treated with trypsin and RNAase to remove the protoplasmic material[3]. This treatment also removes the M and T antigens[3]. Consequently, although a reproducible fraction is obtained, it contains only the trypsin-resistant part of the cell wall. The microelectrophoretic method does not suffer from this disadvantage and, when used in conjunction with chemical methods, can provide valuable additional information.

In a previous paper[10] the pH–mobility curves of cells treated with hyaluronidase were reported; these curves were characteristic of the capsule-free cell surface and indicated the presence of surface amino and carboxyl groups, together with a group of pK 7. The amino groups have now been identified as those of alanine (pK_a 9.9) and ε-lysine (pK_a 10.5), in good agreement with the pK of the surface (10–10.5) obtained from the pH–mobility curves after applying the HARTLEY–ROE correction[23]. The group of pK 7 has been identified as the imidazole group of histidine. The group was removed by trypsin at 37° indicating that it must be associated with the trypsin-sensitive protein material. Analysis of this protein material revealed the presence of histidine. The carboxyl groups contributing to the surface charge have been identified as alanine and the α- and γ-carboxyl groups of glutamic acid[21].

The M-protein fraction removed from the cell wall by trypsin contains 16 amino acids; only 6 of these are present in the T-protein fraction. It has been shown[24] that, for a type-14 strain, 8 amino acids: alanine, arginine, glutamic acid, histidine, isoleucine, proline, threonine and valine must be present in the growth medium for the synthesis of the M antigen. All 8 were found in the M-protein fraction of type-6 cells. The other amino acids reported here, aspartic acid, glycine, leucine, methionine and tyrosine, were present only in small quantities. A study of the trypsin-sensitive protein material of a type-14 virulent strain[9] and of an untyped avirulent strain showed the same amino acid composition as that described above, except that histidine was

not identified. Quantitatively, the amino acid composition of the material from the virulent and from the avirulent strain was the same. Preliminary observations suggest that the quantitative amino acid compositions of the material removed from cells of the matt and glossy variants of type 6 are also the same.

Since the capsular material is removed more readily from the cells of the glossy than of matt variants[10] it was of interest to observe the ease of removal of the T antigen from the two variants. In general, glossy variants give stronger serological reactions for the T antigen than do matt variants of the same type, suggesting that the difference in the rate of removal of T antigen might merely reflect a difference in the amount present. If this were so, the T antigen of type 6G should be removed as rapidly as that of type 12M, and the order of the rate of removal should be 6M $>$ 12M $\simeq$ 6G $>$ 12G. In fact, the sequence is 6M $\simeq$ 12M $\gg$ 6G $\simeq$ 12G.

The use, in electrophoretic studies, of bacteria that have been treated with FDNB to block the amino groups suffers from the possible disadvantage that ethanol and ether might be injurious to the cells. The surface of cells of streptococci is, however, resistant to ethanol and ether treatment, since there is no change of mobility after suspension in these solvents. The charge densities of the amino and carboxyl groups, calculated from the mobility values of normal and FDNB-treated cells, are of significance for an understanding of the differences between the matt and glossy variants. The amino acid composition of the trypsin-sensitive material is the same in both matt and glossy variants, but the charge densities of both the amino and the carboxyl groups are much higher for matt than for glossy variants of the same strain. The simplest explanation of this is that the protein material is more highly cross-linked on the glossy variants; this would result in fewer free ionogenic groups in material of the same composition. In consequence, the protein material on the matt strains would be more easily attacked by proteolytic enzymes than in its more highly cross-linked form on the glossy strains. This explains the anomalous ease of removal of T antigen from heated cells. Further, it is highly probable that the capsular material would be bound strongly to the more highly charged protein on the matt variants, thus accounting for the relative ease with which capsular material is removed by washing[10] from young glossy cells.

The results suggest that the action of trypsin on organisms with protein antigens belonging only to the M and T series is to produce cells with common surface characters. When all the protein antigens are removed with pepsin, all streptococci, including those with trypsin-resistant R and B antigens, have a common electrokinetic surface. Since the sites of attack of trypsin and pepsin on the protein molecule are different, however, it was to be expected that the basal surface structure revealed by treatment with the two enzymes would not be the same.

The presence of a common surface beneath the type-specific protein antigens is in agreement with the observations of MICHEL AND GOODER[3] that a large number of Group-A streptococci contained the same amino acids in their cell walls and that the relative proportion of these amino acids was also constant.

It is of interest, also, that the same N-terminal groups (ε-lysine and alanine) are present on the normal cell surface, on the surface after the removal of the M and T antigen, and in the T antigen itself. This suggests that the protein may be linked to the surface by peptide bonds through one or both of these amino acids.

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REFERENCES

1 C. S. CUMMINS AND H. HARRIS, *J. Gen. Microbiol.*, 14 (1956) 583.
2 W. S. L. ROBERTS AND F. S. STEWART, *J. Gen. Microbiol.*, 24 (1961) 253.
3 M. F. MICHEL AND H. GOODER, *J. Gen. Microbiol.*, 29 (1962) 199.
4 R. M. KRAUSE AND M. MCCARTHY, *J. Exptl. Med.*, 114 (1961) 127.
5 H. HEYMANN, L. D. ZELEZNICK AND J. A. MANNIELLO, *J. Am. Chem. Soc.*, 83 (1961) 4859.
6 R. C. LANCEFIELD, *J. Exptl. Med.*, 108 (1958) 329.
7 J. S. HAMBLY, *J. Gen. Microbiol.*, 18 (1958) 285.
8 J. E. MORISON, *J. Pathol. Bacteriol.*, 51 (1940) 401.
9 B. S. TEPPER, J. R. HAYASHI AND S. S. BARKULIS, *J. Bacteriol.*, 79 (1960) 33.
10 M. J. HILL, A. M. JAMES AND W. R. MAXTED, *Biochim. Biophys. Acta*, 66 (1963) 264.
11 R. E. O. WILLIAMS, *Bull. World Health Organ.*, 19 (1958) 153.
12 V. M. INGRAM AND M. R. J. SALTON, *Biochim. Biophys. Acta*, 24 (1957) 9.
13 G. J. GITTENS AND A. M. JAMES, *Anal. Biochem.*, 1 (1960) 478.
14 H. F. SWIFT, R. T. WILSON AND R. C. LANCEFIELD, *J. Exptl. Med.*, 78 (1943) 127.
15 T. L. HARDY, D. O. HOLLAND AND J. H. C. NAYLOR, *Anal. Chem.*, 27 (1955) 971.
16 M. MIZELL AND S. B. SIMPSON, *J. Chromatog.*, 5 (1961) 157.
17 L. A. A. SLUYTERMAN, *Biochim. Biophys. Acta*, 38 (1960) 218.
18 F. GRIFFITHS, *J. Hyg.*, 34 (1934) 542.
19 R. E. KAY, D. C. HARRIS AND C. E. ENTENMAN, *Arch. Biochem. Biophys.*, 63 (1956) 14.
20 G. J. GITTENS AND A. M. JAMES, *Biochim. Biophys. Acta*, 66 (1963) 250.
21 M. J. HILL, A. M. JAMES AND W. R. MAXTED, *Biochim. Biophys. Acta*, 71 (1963) 740.
22 G. J. GITTENS AND A. M. JAMES, *Biochim. Biophys. Acta*, 66 (1963) 237.
23 G. S. HARTLEY AND J. W. ROE, *Trans. Faraday Soc.*, 36 (1940) 101.
24 E. N. FOX AND L. O. KRAMPITZ, *J. Bacteriol.*, 71 (1956) 454.