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SOME PHYSICAL INVESTIGATIONS OF THE BEHAVIOUR OF BACTERIAL SURFACES

VI. CHEMICAL MODIFICATION OF SURFACE COMPONENTS

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SUMMARY

1. Cells of *Aerobacter aerogenes* were chemically modified using *p*-toluenesulphonyl chloride, fluoro-2,4-dinitrobenzene, diazomethane, methanolic HCl, dicyclohexylcarbodiimide, *N* ethyl-5-phenylisoxazolium-3'-sulphonate, ethylene and propylene oxides and ethyleneimine.

2. The electrophoretic mobility of the modified cells was determined on suspensions in solution at various pH values.

3. The infrared spectra of the modified cells was plotted to confirm the type of modification which had occurred.

4. The results indicate that for cells of *Aerobacter aerogenes* there is no adsorption of ions from the suspension media onto the basic surface matrix, and that their mobility value is largely determined by the surface ionogenic groups, modified by gegenion association.

5. Diazomethane and methanolic HCl were the most suitable reagents for the modification of the carboxyl groups on alcohol-stable surfaces, and fluoro-2,4-dinitrobenzene and *p*-toluenesulphonyl chloride for amino groups.

INTRODUCTION

The aim of the work was to establish a simple method for completely modifying the carboxylic acid groups and others, if present, on bacterial surfaces with a specific reagent in, if possible, aqueous solution. From a study of the variation of the electrophoretic mobility of the modified cells with pH it should be possible to identify the nature and quantity of such groups.

Cells of *Bacillus proteus* modified with benzenesulphonyl chloride at pH 8.4 were electrophoretically more heterogeneous than originally[1]. The amino groups of *Bacillus*

Abbreviations: CHMC, 1-cyclohexyl-3-(4-morpholinyl-(4)-ethyl)carbodiimide; DAM, diazomethane; EI, ethyleneimine; EO, ethylene oxide; DCHC, dicyclohexylcarbodiimide; NEPIS, *N*-ethyl-5-phenyl-isoxazolium-3'-sulphonate; PO, propylene oxide; PTSC, *p*-toluenesulphonyl chloride; MHC, methanolic HCl.

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subtilis spores were modified with PTSC at pH 7.5 leaving a simple carboxylic surface[2]. The treatment of cells with nitrous acid, ninhydrin and *N*-bromosuccinimide gave electrophoretic results which were difficult to interpret[2]. Similar results were obtained[2] with DCHC in alcoholic solution, a reagent specific for carboxyl groups[3]. Treatment of *Escherichia coli* K 12 (ref. 4) with DAM in aqueous solution at pH 4 resulted in the partial esterification of the carboxyl groups.

EO and PO have been used for the esterification of protein carboxyl groups[5] and polygalacturonic acid[6] in aqueous solutions; in the latter case 100% esterification was achieved with 12% EO. EI has been used for the alkylation of amino groups[7], although it will also esterify acids forming a β-aminoethyl ester[8]. NEPIS forms enol esters with acids in aqueous or non-aqueous solutions[9]. FDNB used to identify the amino end groups in bacterial cells should be a useful reagent for modification since the actual group blocked can be subsequently identified by paper chromatography[10].

Carboxyl groups on reaction with carbodiimides form acyl derivatives, the charge characteristics of which largely depend on the carbodiimide used, *e.g.* a neutral group results from treatment with DCHC and a positive one with CHMC, a water-soluble reagent. Both groups, however, contain secondary nitrogen atoms which may assume a positive charge in acid solution. Treatment with NEPIS replaces the carboxyl group with a sulphonic acid group.

Chemical modifications to the bacterial surface were followed by studying the pH–mobility curves of the treated cells at constant ionic strength. Further confirmation of the type of modification was obtained from infrared spectra of the cells dispersed in KBr discs. Complete modification of the specific surface group was never achieved in aqueous solution, except after treatment with PTSC.

EXPERIMENTAL

Cultures and media

Two organisms were used: *Aerobacter aerogenes* N.C.T.C. 418; and *Escherichia coli* K 12 on occasion to test the further applicability of the techniques described. This latter organism, kindly supplied by Dr. W. HAYES, is the same as that used by MACCACARO AND JAMES[4]. *A. aerogenes* was maintained by monthly sub-culture in Lab-lemco broth and grown for routine purposes in a chemically defined medium of final composition: glucose, 20 g/l; potassium dihydrogen phosphate, 3.6 g/l; ammonium sulphate, 1.0 g/l and magnesium sulphate, 0.04 g/l, adjusted to pH 7.12 with NaOH solution. The strain was sub-cultured daily in this medium and grown at 40° with aeration. *E. coli* was always grown in Nutrient Broth (Oxoid) at 37° in stoppered bottles or flasks filled to 40% of their volume.

Reagents

All chemicals used were of the highest quality available. NEPIS was obtained from Pilot Chemicals, Inc., Watertown 72, Mass. (U.S.A.); DCHC from Lights and Co., Ltd., Colnbrook, Bucks.; and CHMC from Aldrich Chemical Co., Inc., Milwaukee 10, Wisc (U.S.A.).

The following reagents were further purified: PTSC was recrystallized from petroleum ether (40–60°). Dioxan was refluxed for 24 h with KOH pellets and finally distilled from them. The distillate was refluxed with Na for 2 h and then distilled

from the Na, the fraction boiling in the range 101–102° was collected. This treatment removed all the peroxides and the greater proportion of water present in the original material.

DAM was prepared as an alcoholic solution[11], standardized by the method of MARSHALL AND ACREE[12] and used as a 0.1 N solution. MHC was prepared by passing HCl into methanol. The solution obtained was adjusted by dilution with methanol to give a 0.2 N solution. DNP derivatives of amino acids used as markers in paper chromatography were prepared by the standard methods[13,14]; these ran as single spots when subjected to paper partition chromatography.

Electrophoretic measurements

The organisms were always harvested from cultures in the stationary phase[15], and after washing three times and resuspending in the appropriate buffer solution mobility values were reproducible to ± 3%. Mobility measurements were made at the upper stationary level in a horizontally mounted rectangular closed cell, thermostatted at 25.00° ± 0.05°, using the apparatus and technique previously described[16]. A minimum of 30 individual timings were made for the determination of the mean mobility value. Re-suspension of heavily agglutinated cells, which resulted from some chemical treatments, was achieved with a loosely fitting glass homogenizer.

Cells from a 16-h culture of *A. aerogenes* had a mobility of 1.30 ± 0.05 μ/sec/V/cm in barbiturate buffer solution (pH 7.00, *I* 0.05), using human erythrocytes in phosphate buffer solution (*I* 0.15, pH 7.35) as standard[17].

The following series of buffer solutions were used: pH 1.5–2.6: HCl and NaCl; pH 2.6–9.6: NaCl, sodium acetate, sodium barbiturate and HCl (prepared by adding 1 N HCl to the stock solution to the required pH followed by dilution with water to the required ionic strength); and pH 9.6–11.0: NaCl, sodium acetate, sodium barbiturate and NaOH (prepared by adding NaOH solution of the correct ionic strength to the diluted stock solution). The stock solution contained sodium barbiturate, 30.927 g/l; hydrated sodium acetate, 20.413 g/l and NaCl, 11.69 g/l; *I* 0.5. All solutions were prepared in glass-distilled water. pH–mobility curves were always determined at *I* 0.05 unless otherwise stated.

Methods of treatment

Cells were treated with reagents after two types of pretreatment, (a) cells washed three times in buffer solution of pH 7.0: "neutral washed cells"; and (b) cells washed once in buffer solution of pH 7.0 and then twice in 0.05 M HCl: "acid washed cells". This means that carboxyl groups on the surface were (a) in the ionized and (b) the unionized state. Where organic solvents were used for suspension of cells they were washed twice in the solvent before reagents were added.

The amounts of reagent (except where stated) are for approx. $7 \cdot 10^{10}$ organisms (determined by direct counting) or 20 mg dry wt.

PTSC: Suspensions of cells in phosphate buffer solution (*I* 0.05) at pH 7.0 and 11.0 were shaken with 50 mg PTSC for periods of up to 24 h. The suspensions were then decanted from excess PTSC, the cells washed and the mobility determined.

FDNB: Neutral washed cells (phosphate buffer solution) were treated with various amounts of ethanolic 0.2% FDNB solution made up to 20 ml with alcohol and mixed with 5 ml half-saturated aq. sodium bicarbonate solution for periods up

to 5 h. Cells were washed twice with absolute alcohol and then with the buffer solution required for mobility measurements.

Normal cells of *E. coli* were treated in 4 ml of water with 200 mg FDNB and 200 mg sodium carbonate at 40° for up to 24 h.

EO and PO: Treatment under aqueous and non-aqueous conditions was investigated on both the acid and neutral washed cells. The most complete reaction was achieved when acid washed cells were treated with a 12% (v/v) solution of the epoxides in water at 20° for 72 h.

DAM: This reacted only with acid washed cells suspended in alcohol; 5 ml of DAM solution in 25 ml alcohol for 15 min was sufficient for complete modification. Excess reagent was indicated by the yellow colour of the supernatant.

MHC: Treatment of acid washed cells for 3 h at 40° was sufficient for complete modification.

EI: Neutral and acid washed cells were treated with EI in various solvents, *viz.* water, ethanol and dioxan, at several temperatures. Complete modification was achieved when acid washed cells, suspended in 10 ml dioxan, were treated with 0.2 ml EI for 7 days at 40°.

DCHC and CHMC: Acid washed cells were suspended in 10 ml dioxan with 0.1 ml of DCHC or CHMC for 3 h at 20°. CHMC was also used under aqueous conditions.

NEPIS: Experiments under various conditions indicated that 0.03 g NEPIS for 3 h at 20° in nitroethane produced the most complete modification.

Controls, using identical conditions to the chemical treatments, in which the specific reagent was omitted were always included.

After treatment all cells were washed once in the solvent used for treatment, and finally in the required buffer solution.

Infrared spectra

Samples of normal and chemically modified cells, obtained by normal growth and modification methods, were washed with absolute alcohol, and dried over concd. sulphuric acid. 2-mg portions were ground with 200 mg of pure dry KBr and compressed into a 13-mm-diameter disc. Spectra were recorded on a Perkin Elmer Model 137 spectrometer over the region 5–15 μ using a pure KBr disc in the reference beam.

RESULTS

Variation of the mobility of normal cells of A. aerogenes with pH of the suspension medium

The pH–mobility curve was determined in buffer solutions at I 0.05 (Fig. 1). Since the distribution of all samples was homogeneous (as were all the results, except where stated) only the mean values are plotted. To check that no irreversible processes occurred at the extreme pH values, the cells after suspension at these values were washed three times in and resuspended in buffer solution of pH 7.0. The mobility values obtained in these solutions showed that at pH values above 11 surface disorganization occurred, since more heterogeneous mobility distributions were obtained.

Variation of the mobility of chemically treated cells of A. aerogenes with the pH of the suspension medium

(a) Effect of solvents. Suspension of acid and neutral washed cells in ethanol, methanol, ether, dioxan, pyridine, nitroethane, methyl cyanide and diglyme for

periods up to 24 h had no effect on the electrophoretic mobility determined in buffer solution. Dioxan had no effect on the mobility of acid washed cells even after suspension for 7 days.

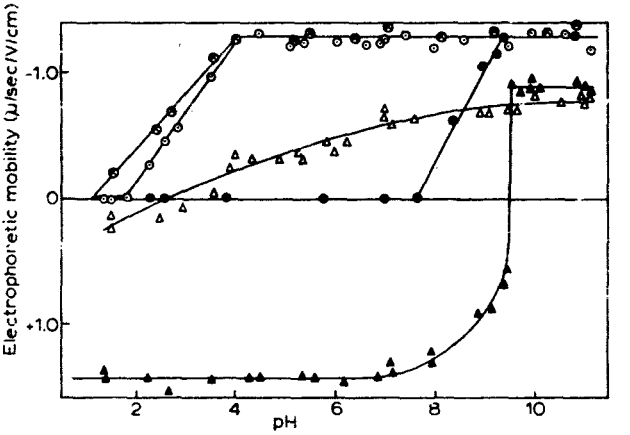

Fig. 1. pH–mobility curves for normal and chemically modified *A. aerogenes* at *I* 0.05. ⊙—⊙, normal cells; ●—●, DAM-treated; ⊗—⊗, NEPIS-treated; △--△, DCHC-treated; ▲—▲, EI-treated.

(*b*) *PTSC and FDNB.* Neither reagent had any effect on the mobility of cells, although the FDNB-treated cells were yellow. Subsequent acid hydrolysis of the FDNB-treated cells and paper chromatography of the hydrolysates revealed that FDNB had combined with the free ε-amino group of lysine; no other spots were found on the chromatograms. The ε-amino lysine groups are probably present within the cell wall, thus amino groups so situated have no effect on the electrophoretic mobility.

(*c*) *EO and PO.* Cells modified with these reagents had pH–mobility curves characteristic of a simple carboxyl surface although the mobility plateau (−0.64 μ/sec/V/cm) was less than that of normal cells. This value could not be reduced further by any number of resuspensions in fresh epoxide solution. Suspension of the modified cells for 1 h at 40° in buffer solution at pH 11.0 resulted in the complete hydrolysis of the ester group to give cells of normal mobility; in contrast similar suspension in solution at pH 1.5 had no effect on the mobility.

The presence of carboxyl groups on the modified surface was confirmed by subsequent treatment of the epoxide-treated cells (after washing once with 0.05 N HCl) with alcoholic DAM when a zero mobility was obtained at pH 7.0. The extent of epoxide modification, assuming that it was proportional to the decrease in mobility, was approx. 50%.

(*d*) *DAM and MHC.* Treatment with either of these reagents produced cells with zero mobility at pH 7.0. The mobility (Fig. 1) of the DAM-treated cells, after 2.5-h suspension in buffer solutions, was zero between pH 2.0 and 7.5. The increase above pH 7.5 was due to hydrolysis of the ester group. Suspension for 1 h at pH 11.0 at 40° gave cells electrokinetically identical to the untreated organisms, suspension at pH 1.5 caused much slower hydrolysis.

Zero mobility values were obtained on suspension of DAM treated cells at pH 7.0

at all ionic strengths from 0.005 to 0.1. Measurements on bacteria with low or zero mobility were difficult due to aggregation. In all cases of zero mobility recorded no systematic movement of cells was observed at the stationary level in any one direction on the application of an electric field.

The amount of DAM used to esterify the cells was determined by measuring the excess when modification was complete. The suspension was added to a known amount of benzoic acid in ether and the excess back titrated with caustic soda. The DAM used by $6 \cdot 10^{10}$ bacteria was equivalent to 0.14 ml 0.1 N NaOH solution. From these results the calculated surface-charge density was $2.15 \cdot 10^5$ e.s.u./cm^2 for cells of surface area $31.24 \cdot 10^{-8}$ cm^2 (determined by direct measurement of the cell size).

(*e*) *EI*. The pH–mobility curve of EI-treated cells (Fig. 1) indicates that the carboxyl groups have been replaced by β-aminoethyl ester groups; the plateau in the acid region is characteristic of an amine-type surface. Mobility values between pH 9.0 and 9.5 were difficult to measure. Suspension of the treated cells for up to 1 day at pH 1.5 or pH 11.0 had no effect on the shape of this curve and demonstrated, possibly because of the presence of the amine group, that this ester is very stable. Confirmation of the presence of surface amine group was obtained by subsequent treatment of the cells with PTSC. The results (Table I) indicate that whatever produces the negative charge above pH 9.5 on the EI-treated cells is also present after PTSC treatment.

Modification was complete when the PTSC treatment was carried out at pH 11.0 for 3 h, although some modification occurred at pH 7.0. Measurements were difficult due to the aggregation of the doubly modified cells.

TABLE I

THE MOBILITY OF EI-TREATED CELLS AFTER TREATMENT WITH PTSC

pH of PTSC-treatment	*Length of treatment (h)*	*pH of mobility measurement*	*Mobility (μ/sec/V/cm)*	
			Sample	*Control (EI-treated cells)*
7.0	2	5.1	+0.58	+1.44
	3	5.1	+0.37	+1.44
	2	10.5	−0.89	−0.89
	3	10.5	−0.83	−0.89
11.0	2	5.1	−0.88	+1.44
	3	5.1	−0.87	+1.44
	2	10.5	−0.67	−0.89
	3	10.5	−0.99	−0.89

TABLE II

THE MOBILITY OF NEPIS-TREATED CELLS AFTER TREATMENT WITH DAM

	Mobility (μ/sec/V/cm)	
	pH 7.0	*pH 3.13*
NEPIS-treated	−1.30	−1.06
NEPIS + DAM-treated	−0.78	−0.75
Untreated control	−1.30	−0.74

(*f*) *DCHC and CHMC*. CHMC had no effect on the mobility of the cells in any of the solvents examined. Treatment with DCHC produced cells which had a pH–mobility curve (Fig. 1) indicative of some modification. If an acyl derivative had been formed with all the surface carboxyl groups then a low negative or positive mobility would have resulted over the whole pH range, provided there was no hydrolysis. Suspension of DCHC-treated cells for 1 day at 40° in solutions of pH 11.0 or 1.5 had no effect on the mobility of the cells at pH 7.0, indicating the absence of hydrolysis. Subsequent treatment of the modified cells with DAM reduced the mobility at pH 7.0 from −0.5 to −0.18 μ/sec/V/cm, indicating that about 68% of the carboxyl groups had been modified with DCHC. The positive charge at low pH of the DCHC-treated cells is probably due to adsorption of hydrogen ions onto the secondary amine group of the acyl derivative of the carbodiimide.

(*g*) *NEPIS*. The pH–mobility curve of NEPIS-treated cells (Fig. 1) together with the results of subsequent DAM treatment (Table II) demonstrated that only about 60% of the carboxyl groups had reacted forming an enol ester.

Suspension of the NEPIS-treated cells at pH 11.0 and pH 1.5 for 3 h at 20° produced no significant change in the mobility at pH 3.13, the enol ester thus formed was stable. The mobility at pH 7.0 was unaltered after NEPIS treatment due to the 1:1 replacement of carboxyl for sulphonic acid groups. In support of this the apparent pK of the NEPIS-treated cells was less than that of the normal cells (Fig. 1)

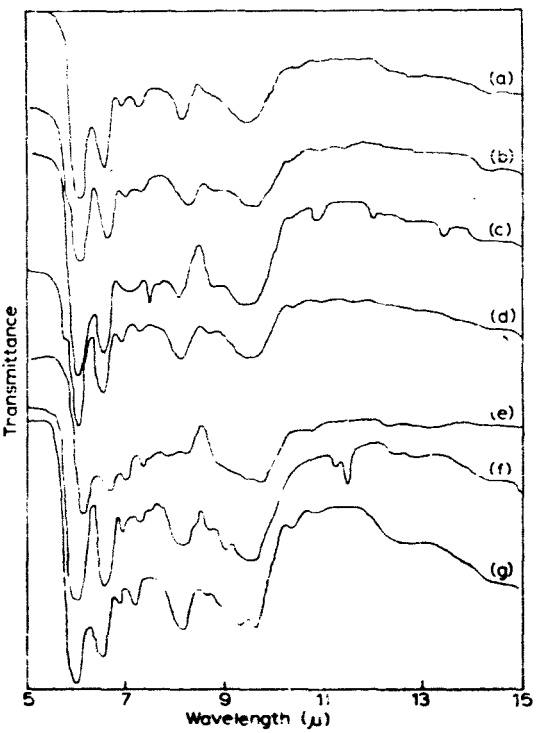

Fig. 2. The infrared spectra of (a) neutral and (b) acid washed, (c) FDNB-, (d) DAM-, (e) EI-, (f) DCHC-, (g) NEPIS-treated cells of *A. aerogenes*.

due to the simultaneous dissociation of both the sulphonic (pK about 1) and the carboxyl (pK about 3) groups.

(*h*) *Reduction of the carboxyl group.* An attempt, using diborane in bis(2-methoxyethyl)ether, (diglyme)[18], to reduce the carboxyl groups was unsuccessful, even after vigorous drying of the reagents.

Infrared spectra of normal and chemical modified cells of A. aerogenes

The infrared spectra of the acid and neutral washed normal cells, and the FDNB-, DAM-, EI-, DCHC- and NEPIS-treated cells (Fig. 2) contained absorptions common to all cells, the groups responsible for these are listed[19] in Table III.

TABLE III

REGIONS OF ABSORPTION COMMON TO SPECTRA OF ALL CELLS

Wavelength (μ)	*Probable origin*
5.8–6.2	Carbonyl stretch of amides and carboxylic acids, etc.
6.4–6.6	–NH-deformation of amides etc.
6.9	Methylene-group stretch
7.3	Symmetrical stretch of carboxylate ion or C–O stretching and O–H deformation in alcohols
7.8–8.3	Methylene vibrations of fatty acids and P–O stretch of phospholipids
9.2–9.8	C–H stretch of aliphatic amines and phosphate ions

The only significant difference between the spectra of the neutral (Fig. 2a) and acid washed cells (Fig. 2b) was the presence of a shoulder at 5.8 μ due to the unionized carboxyl group. The spectrum of the DAM-treated cells (Fig. 2d) has an even more pronounced shoulder at 5.73 μ due to the carbonyl stretch of the ester. The spectrum of the FDNB-treated cells (Fig. 2c) possessed absorptions at 8.70, 10.85, 11.95 and 13.35 μ assigned to aromatic-ring substitution of the DNP group. That at 7.47 μ is characteristic of the C–NO_2 group and those at 6.02 and 7.08 μ of the aromatic C=C group. The spectrum of the EI-treated cells (Fig. 2e) showed increased absorption at 6.35, 6.90 and 7.70 μ due to the presence of $-NH_2$, $-NH_3^+$ and methylene groups, respectively. The loss of absorption at 5.73 μ was due to the change in character of the ester group. The spectrum of DCHC-treated cells (Fig. 2f) showed a marked increase in absorption at 6.90, 8.00, 8.90, 11.20 and 11.45 μ; the latter two bands were very strong. All these bands by comparison with the spectra of the pure carbodiimide and dicyclohexyl urea were assigned to bands present in the expected derivative. Apart from an absorption band at 9.60 μ, probably due to the sulphonic acid group, there was no marked change in the spectrum of cells after NEPIS treatment (Fig. 2g).

The variation of the mobility of normal and treated cells of E. coli with pH of the suspension medium

To test the applicability of some of the methods described to more complex cell surfaces, a study was made with cells of *E. coli* K 12. The surface of these cells was sensitive to alcohol and so experiments were made on two types of cell; normal and

alcohol-extracted. The alcohol-extracted cells were prepared by suspending acid washed cells in absolute alcohol for 2 h.

(*a*) *Normal cells.* The pH–mobility curve of normal cells (Fig. 3) indicated the presence of some hydrogen-ion-adsorbing material but no primary amino groups with a pK value less than 11. The mobility of cells at pH 7 after suspension in solutions of extreme pH was the same at the control.

The pH–mobility curve (Fig. 3) of cells treated with PTSC for 1 h at pH 11.0 provided evidence of the absence of surface basic groups (pK > 5). There was no change in the position of the plateau mobility value; probably due to the combination of PTSC with secondary amine groups on the surface, this will also eliminate the positive charge at low pH. The mobility was not zero at the lowest pH measured suggesting that a phosphatidic group may be present. FDNB treatment in purely aqueous solution produced no change in the mobility of normal cells and confirmed

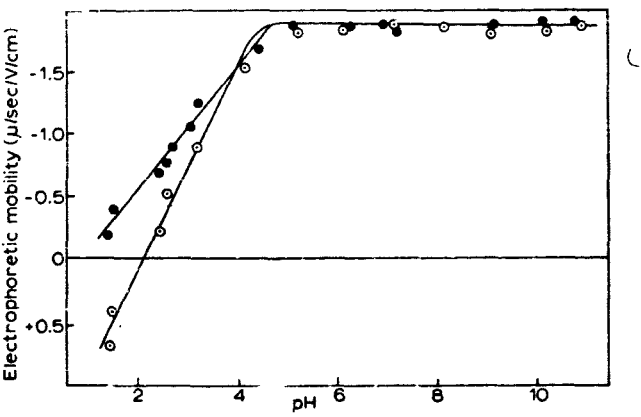

Fig. 3. The pH–mobility curves for normal and PTSC-treated cells of *E. coli* at *I* 0.05. ⊙—⊙, normal cells; ●—●, PTSC-treated cells.

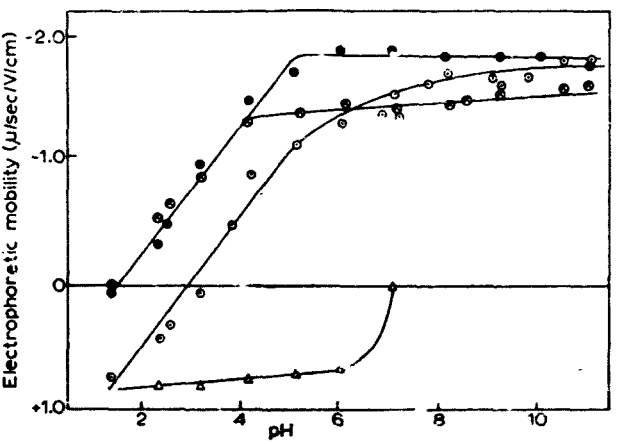

Fig. 4. The pH–mobility curves for alcohol-extracted *E. coli* treated as indicated (*I* 0.05). ⊙—⊙, alcohol-extracted; ●—●, PTSC-treated; ⊗—⊗, FDNB-treated; ∧—∧, DAM-treated. (Only results for the DAM-treated cells constant after 2 h are recorded.)

the absence of primary amino groups. Diazomethane treatment was not possible in the absence of alcohol.

(*b*) *Alcohol-extracted cells.* The pH–mobility curve of these cells (Fig. 4), markedly different from normal (Fig. 3), indicated that a strongly acidic component had been (partially) removed leaving a protein-like surface with more basic groups than the original.

PTSC-treatment, for 3 h at pH 11.0, gave cells with a pH–mobility curve (Fig. 4) indicating that the difference in shape of the pH–mobility curve of normal and alcohol-treated cells (Figs. 3 and 4) was primarily due to the presence of amino groups of pK between 7 and 11. The corresponding curve for FDNB-treated cells (Fig. 4) further confirmed this conclusion and illustrated the similarity of the results obtained by these two techniques. The mobility of both FDNB- and PTSC-treated cells was reversible from extremes of pH to pH 7. DAM-treatment was possible on the alcohol-extracted cells and mobility values were measured after 1 and 2 h suspension in buffer solutions following treatment. The results Table IV and Fig. 4 indicated that the ester group on DAM-treated cells was not hydrolysed significantly in suspension in the range pH 2.3 to 7.0 during a period of 2 h.

Mobilities measured above pH 7.0, like those on DAM-treated *A. aerogenes*, were time variable indicating that hydrolysis was occurring. At pH 10.0 hydrolysis was complete, *i.e.* the cells had the normal mobility when resuspended at pH 7.0 (Table IV). The results further confirmed the belief that the steady increase of mobility above pH 6.0 for alcohol-extracted cells (Fig. 4) was due to dissociation of basic groups of p$K > 7.0$. Further, it is believed that some negative charge would be present on the DAM-treated surface above pH 7, had it been stable enough for study, in agreement with the observations on EI-treated *A. aerogenes*.

TABLE IV

THE MOBILITY OF ALCOHOL-EXTRACTED *E. coli* AFTER DAM TREATMENT AND SUBSEQUENT SUSPENSION IN BUFFER SOLUTIONS

pH	*Mobility (μ/sec/V/cm)*		
	After 1 h	*After 2 h*	*Control (alcohol washed)*
2.32	+0.80	+0.78	+0.51
3.18	+0.82	+0.81	+0.05
4.17	+0.75	+0.70	−0.89
5.05	+0.71	+0.72	−1.15
6.00	+0.66	+0.60	−1.37
7.04	0	−0.05	−1.56
8.50	−1.04	−1.36	−1.70
9.20	−1.50	−1.68	−1.76
10.00	−1.76	−1.74	−1.77
Reversibility			
10.0–7.04	−1.50		−1.56

DISCUSSION

The pH–mobility curve for normal cells of *A. aerogenes* and unchanged mobility value on treatment with PTSC and FDNB, confirmed the conclusion of PLUMMER AND JAMES[18] that the only ionogenic groups on the surface of this organism are carboxyl.

The zero mobility obtained below pH 2.0 at I 0.05 demonstrates that there is no hydrogen or other cation adsorption onto the surface, similarly the mobility plateau from pH 4.5 to 11.0 indicates no hydroxyl-ion adsorption, since either or both of these phenomena would be accompanied by further changes in mobility. The pK value of the carboxyl group on the surface, calculated using the Hartley–Roe equation, is 2.81 compared with the value of 3.19 for glucuronic acid determined electrometrically at the same ionic strength ($pK_a = 3.23$ at 25°; *cf.* ref. 20: 3.18 at 20°). The difference is most probably due to the fact that glucuronic acid does not exist in the free state on the surface but is combined in a matrix with other monosaccharides, which may include mannuronic acid[21].

The original aim to obtain specific reagents for modification of surface carboxyl groups was not achieved with any of the reagents examined. The most satisfactory reagents for this purpose were DAM and MHC; but for maximum effect alcoholic or ethereal solutions were required. This is a disadvantage with many organisms, *e.g. E. coli* K 12, which contain solvent-extractable surface components. The use of these reagents in aqueous media leads to negligible or incomplete reaction because of the competitive reaction with water. For amino groups FDNB is a very suitable reagent since the interpretation of the electrophoretic measurements on the modified organism is relatively simple. PTSC although reacting with amino groups under relatively mild aqueous conditions has the disadvantage that it can react with both primary and secondary amines.

FDNB combined with the ε-amino groups of lysine, present in the cell-wall mucopeptide[22], PTSC probably combined at this site below the electrokinetic surface as well. The cell walls of *E. coli* contain alanine as the major component[22] with free amino groups, and it is most likely these are the groups which have been blocked by PTSC- and FDNB-treatment of the alcohol-extracted cells. The secondary amine group on normal cell surfaces with which the PTSC has combined is probably present at a peptide linkage. This together with the pH–mobility curves of the normal and alcohol-extracted cells (Figs. 3 and 4) suggests that the material extracted by the alcohol is lipoprotein in nature and that its removal reveals a further layer (mucopeptide). This would be in harmony with the structure of the surface layers of *E. coli*[23,24].

The reaction of EO and PO with the surface of *A. aerogenes* was inconclusive and incomplete. The reason for the incompleteness of the reaction is not clear, it may be that only the truly surface carboxyl groups (*i.e.* the outermost layer) have been esterified while those in lower layers, although electrokinetically active, are sterically protected from attack by these reagents. This would then be evidence for a number of electrokinetically involved layers as suggested by HAYDON[25]. This theory is supported by the incomplete modifications achieved with DCHC and NEPIS; since the group to be introduced is larger the steric inhibition to the approach of further reacting molecules is even more likely. These reagents are, therefore, of little use for the identification of the type and number of electrokinetically involved carboxyl groups; they do, however, give some information concerning the structure of the double layer.

DAM- and MHC-treated cells of *A. aerogenes* had a zero-mobility value independent of the ionic strength and pH, over the range where no ester hydrolysis occurred. The zero mobility is a direct result of the removal of the surface charge

due to the carboxyl groups. This leads to two important conclusions regarding the structure of the electrical double layer at the surface of *A. aerogenes*:

(1) No adsorption of anions or cations occurs onto the basic surface matrix, unless adsorption is initiated by the presence of carboxyl groups; this seems unlikely; and

(2) the mobility and consequently the zeta potential[26] is a direct function of the ionogenic surface charge, modified by the presence of the ions in the solution.

These points are considered more relevantly in connection with the surface conductance of the organisms in the following paper[26].

DAM-treatment of the alcohol-extracted *E. coli* gave cells with a positive mobility of the same value as that obtained with normal cells in suspension at pH 1.5 (Fig. 4). This suggests that the positive charge in acid solutions is due to $-NH_3^+$ and that the steady increase of mobility above pH 6 of the untreated cells (Fig. 4) is due to the dissociation of this group, the commencement of which is apparent (Fig. 4). Alanine the suspected N-terminal compound has a pK of 9.87. The hydrolysis of the ester group above pH 7 detracts from the usefulness of DAM-treatment of surfaces containing both amino and carboxyl groups.

EI esterified the carboxyl groups with the formation of a β-aminoethyl ester. The mobilities at pH 5 and pH 10 after subsequent PTSC-treatment were similar indicating that the amino group had now been blocked. This result is similar to that obtained by DOUGLAS[2] who found that PTSC-treated *Bacillus subtilis* spores had a mobility plateau of similar value to the mobility of normal cells at high pH values. It was assumed that this plateau was due to the presence of unionized and uncharged amino groups on the normal cell surface and that the introduction of an uncharged radical caused little change of the mobility at high pH. EI-treated cells have a negative charge at high pH and a similar negative charge after PTSC-treatment. This negative charge is not due to hydrolysis of the ester bond and exposure of carboxyl groups but probably arises from adsorption of anions onto the primary amino group and the tosylated amino group. The adsorption of such ions onto the unionized amino groups would also explain the rapid change of mobility with pH about 9.5; the dissociation of the charged amino groups and the adsorption of the negative ion occurring simultaneously. Thus the deduction of the number of amino groups present from change produced on PTSC- or FDNB-treatment should be modified to allow for the charge due to anion adsorption.

The effect of chemical modification on the infrared spectra of *A. aerogenes* gives some indication of the information which can be obtained about the chemical composition of bacteria. The spectra generally contained absorption maxima which were expected for the particular type of modification. Some ambiguity was possible in the interpretation of the spectra of EI-treated cells since the maxima could be due to the presence of either a primary amino or alcoholic group; the electrophoretic results indicated it to be amino. The spectra of acid washed, neutral washed and DAM-treated cells showed only minor differences, similar to those observed in different strains of a given species of organism by other workers, who found that because of the complexity of the material under examination little differentiation was possible. Only when major changes in the type of group present occur, *e.g.* when cells were modified with FDNB or DCHC, did any strong new absorption peaks arise, and even then, unless these were in regions of the weak absorption for normal cells they were masked.

The surface-charge density obtained from measurements on the esterification of the surface with DAM ($2.15 \cdot 10^5$ e.s.u./cm^2) was much greater than that calculated from the mobility using the equation for the charge density in the diffuse layer[26] ($2.78 \cdot 10^3$ e.s.u./cm^2) for cells in buffer solution at pH 7.0 and I 0.05. Since the direct methods give the total carboxyl content of the cells in contrast to the surface carboxyl content measured by the electrokinetic method the large discrepancy was not unexpected. These values of charge density would correspond to areas 22.3 and 1730 Å^2 per carboxyl group, respectively. The area of a carboxyl group is approx. 20 Å^2, thus for surface-charge densities of the order of the former to be possible successive layers of charge must contribute to the electrokinetic charge. The electrokinetic results obtained after some of the specific chemical modifications support this. The electrokinetic value (for the area) does not require any such condition although the value of the absolute surface charge is undoubtedly greater since the electrokinetic value includes the decrease in charge due to gegenion adsorption[26].

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