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CHARACTERISATION OF *ESCHERICHIA COLI* CELL SURFACE BY ISOELECTRIC EQUILIBRIUM ANALYSIS

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SUMMARY

A characterisation of the lipopolysaccharide (outermost) layer of *Escherichia coli* cells has been made by isoelectric equilibrium analysis. Unmodified *E. coli* cells show a surface isoelectric point (pI) of 5.6. Cells treated with ethyleneimine in order to esterify the carboxyl groups are isoelectric at pH 8.55. When amino groups are blocked the bacterial surface has a pI of 3.85. An analysis of these results suggests that the ionisable groups occurring in the isoelectric zone *i.e.* the zone amenable to investigation by the isoelectric equilibrium method are: carboxyl groups and amino groups of polysaccharide and protein components. The carboxyl groups have a pK between 3.2 and 4.5 and the amino groups have a pK of 7.5. ε-Amino groups, phenolic hydroxyl groups and guanidyl groups do not occur, and phosphate and amino groups of the phospholipid complex are not detected. The number of thiol groups in the isoelectric zone has been determined using 6,6′-dithiodinicotinic acid. The number of anionogenic and cationogenic groups has been determined. From the density of the negative charges on the surface it is estimated that the isoelectric zone might extend up to 60 Å below the cell surface. The data discussed in this paper relate to the outermost layer of the bacterial cell wall composed of lipopolysaccharide–phospholipid–protein complex. Since reactive groups of the phospholipid component of the complex have not been detected in the isoelectric zone, it is suggested that the arrangement of lipopolysaccharide–phospholipid–protein complex is such that the phospholipids are located at a depth of more than 60 Å from the bacterial surface.

INTRODUCTION

The chemical composition of bacterial surfaces has been extensively studied[1,2]. The surface properties of cells whether bacterial or of animal origin are attributable to a large extent to the kind of ionisable groups present on the cell surface and to their spatial distribution. Such a characterisation until recently was based mainly on studies of the electrophoretic behaviour of cells. Much work has been done using this technique on the characterisation of certain bacterial cells[3–5] in conjunction with methods devised to modify the surface groups by specific chemical treatments. The present report deals with an investigation of the surface of *Escherichia coli* by

isoelectric equilibrium analysis, which, as earlier work in our laboratory has shown, is as efficient as cell electrophoresis in providing information about cell surfaces[6].

MATERIALS AND METHODS

Cells

E. coli cells were used in the present experiments, either in the unmodified state or after treatment with specific chemicals which block or modify surface ionisable groups. The cells made available for our use by Dr E. Rowatt of the Department of Bacteriology, were supplied by the Microbiological Research Establishment at Porton Down, Salisbury, England, who have also provided us with the following information. The cells had been grown in 400-l batch culture in tryptone glycerol medium, 150 l/min air rate, glycerol-feed and pH between 7.4 and 7.7. The medium contained (per l) L42 Tryptone, 20 g; light grade Yeatex, 1.0 g; NaCl, 2.9 g; $MgSO_4 \cdot 7H_2O$, 1.0 g; Na_2HPO_4, 5.76 g; $NaH_2PO_4 \cdot 2H_2O$, 1.48 g; K_2HPO_4, 0.87 g; $MnCl_2$, 0.005 g; ferric citrate, 0.336 g and $CaCl_2$, 11.1 g. The cells were washed in buffer containing 10 mM Tris–HCl *plus* 10 mM magnesium acetate, pH 7.0.

Chemical modification of surfaces

Modification of amino groups. The amino groups present on the surface were modified by treatment with 1.5% (w/v) formaldehyde in phosphate-buffered saline (NaCl 0.137 M, KCl 0.0027 M, Na_2PO_4 0.0065 M and KH_2PO_4 0.0015 M, pH 7.3) prepared as described by Ward and Ambrose[7] by depolymerising paraformaldehyde (British Drug Houses). The cells (approx. $9 \cdot 10^{10}$) were suspended in 10 ml of the solution for 4–12 weeks at room temperature (HCHO-cells).

Modification of carboxyl groups. The carboxyl groups were modified by treating the cells with ethyleneimine as described by Gittens and James[3]. The cells were treated with ethyleneimine after they had been pretreated in two ways (a) neutral cells *i.e.* washed twice in phosphate-buffered saline, pH 7.0, and (b) acid-washed cells, *i.e.* washed twice in phosphate-buffered saline, pH 7.0, and then washed twice in 0.05 M HCl. Both neutral and acid-washed cells were washed twice with dioxan (B.D.H., Analar). The cells (approx. $9 \cdot 10^{10}$) were then suspended in 10 ml dioxan containing 0.4 ml ethyleneimine. The treatment was carried out at 40 °C. There are thus three types of treated cells in the ethyleneimine series *viz.* (a) ethyleneimine-treated neutral cells, designated as Neu-EI cells, (b) ethyleneimine-treated acid-washed cells designated as AW-EI cells, and (c) acid-washed cells not treated with ethyleneimine but left in dioxan, designated as AW-D cells.

Modification of sulphydryl groups. The surface thiol groups were blocked using 6,6′-dithiodinicotinic acid (from Newcell Biochemicals)[8] at $1.5 \cdot 10^{-4}$ M in phosphate-buffered saline, pH 7.3. The cells (approx. $9 \cdot 10^{10}$) were suspended in 10 ml of this solution for 30 mins, then washed in phosphate-buffered saline. The effect of dithiodinicotinic acid treatment was examined on AW-EI cells (after treatment designated as AW-EI-CPDS cells) as well as on unmodified cells (known after treatment as *E. coli*-CPDS cells).

Isoelectric focusing of cells

The methods employed for isoelectric focusing of the cells are generally similar to those used in earlier studies on animal cells[6]. Some of the important features of

the method are discussed below. All the experiments were performed in a 110-ml LKB 8101 column fitted with platinum electrodes.

Supporting gradient. Ficoll (30–5%) was found to be most suitable for experiments with unmodified *E. coli*. For AW-EI and HCHO-cells glycerol gradients (75–25%) were used. In post-pH equilibrium loading (described in a later section) of cells glycerol gradients were routinely used since the cells remain in the column only for 2–3 h. The isoelectric points of cells are independent of the supporting medium[6].

Ampholines. Ampholines were used at a concentration of 1%. All preliminary experiments were performed using pH 3–10 range. Depending upon the pI of the cells other appropriate ranges such as 3–6 (for formaldehyde modified cells and *E. coli*-CPDS cells) and pH 7–10 (for AW-EI cells) were used in subsequent experiments. The pI value in each experimental series was also confirmed by reversing of polarity.

Number of cells loaded. The number of cells loaded is not critical but is subject to a maximum of $9 \cdot 10^{10}$ organisms. About $6 \cdot 10^{10}$ cells were usually employed.

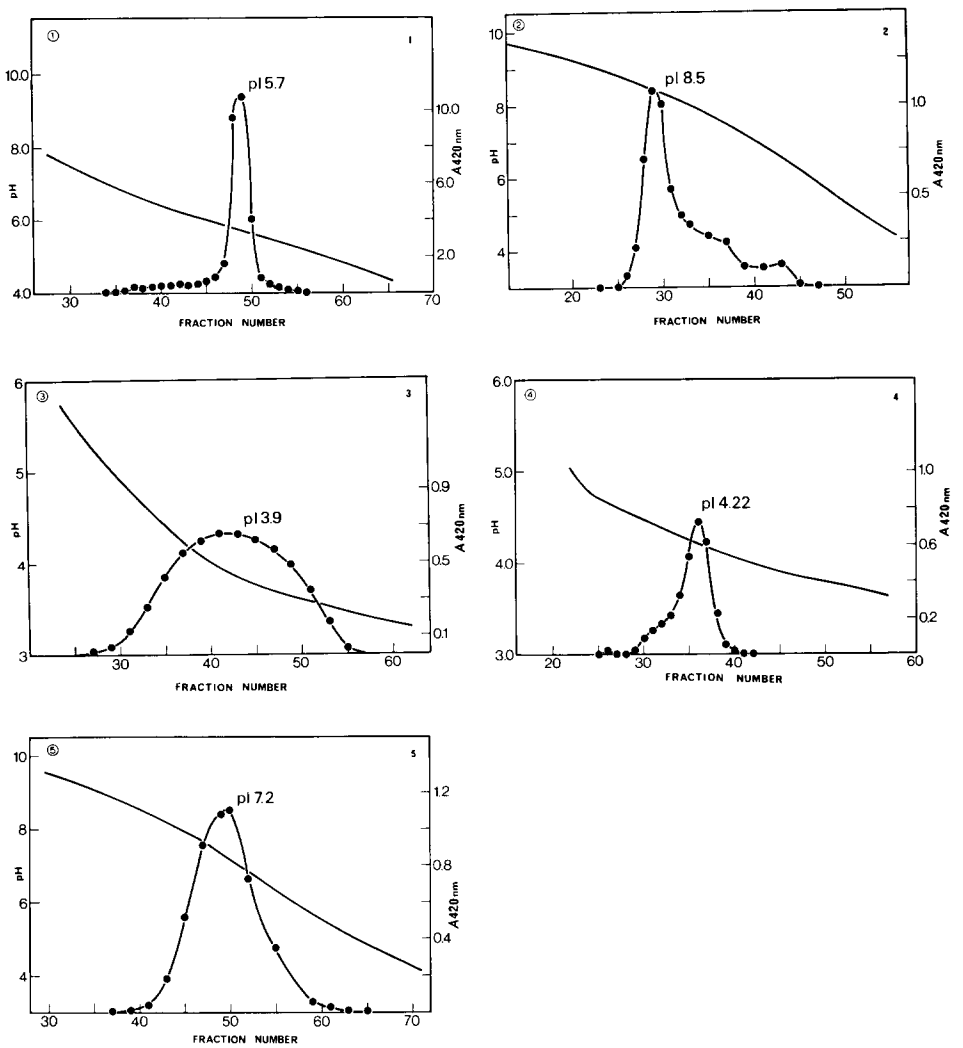
Loading of cells. In the early stages of this investigation cells were loaded during the building up of the density gradient as described previously[6]. In order to improve the viability of cells following electrofocusing, and in order to avoid the agglutination and resultant sedimentation, the cells were loaded, in more recent experiments, after the pH gradient had formed. This mode of loading of cells has been described here as post-pH equilibrium method. In order to be able to do this a modification was made in the LKB 8101 column. A side arm was fused on to the middle of the column. The end of the side arm was sealed with a special septum (Hamilton, Calif. part No. 760-07).

When the pH gradient was formed *i.e.* after about 24 h of the start of the experiment, a size 0 stainless-steel serum needle was inserted into the column by piercing the septum, and about 3 ml of the density gradient were withdrawn from the column, and in this the cells were suspended. The suspension was reintroduced at the same position in the column and the needle withdrawn. The puncture in the septum seals off effectively.

In post-pH equilibrium loading the cells focused in 2–3 h. The advantages of this method of loading the cells are obvious. Experiments are now underway in order to assess the improvement in the viability of cells as a result of this modified method of loading of the cells.

RESULTS AND DISCUSSION

Electron microscopy of *E. coli* and other gram-negative bacterial cells has revealed the presence of three distinct layers in the cell wall[9–11]. The innermost layer is the cell membrane which is surrounded by the rigid layer made up of peptidoglycans. The outermost layer contains lipopolysaccharides, phospholipid, and lipoprotein and is about 80 Å thick. It has been suggested that the lipopolysaccharide is present in the outer layer in the form of a complex with phospholipid and protein, the protein in turn being covalently bound to the rigid peptidoglycan layer[12,13]. The data obtained in the present series of experiments relate to this outermost layer composed of the lipopolysaccharide–phospholipid–protein complex.



Figs. 1–5. Elution profiles of isoelectric equilibrium experiments.

Fig. 1. Unmodified *E. coli* cells.

Fig. 2. Ethyleneimine-treated acid-washed *E. coli* cells (AW-EI).

Fig. 3. Formaldehyde-treated *E. coli* (HCHO cells).

Fig. 4. *E. coli* cells treated with 6,6′-dithiodinicotinic acid (*E. coli*-CPDS cells).

Fig. 5. Acid-washed ethyleneimine-treated *E. coli* subsequently treated with 6,6′-dithiodinicotinic acid (AW-EI-CPDS cells). Curve starting at left top corner and falling steadily represents pH gradient; ●—●, cell density ($A_{420\ nm}$).

The pI of unmodified E. coli cells

The isoelectric point (pI) of cells as determined by the isoelectric focusing method provides information about the ionogenic groups present on the surface[6].

The pI of unmodified *E. coli* cells has been determined in the present experiments to be 5.6±0.1 (Fig. 1). Using the method of cell electrophoresis Gittens and James[3] found that these cells had a pI of approximately 2.2 at an ionic strength of 0.05 M. The discrepancy between the pI determined by cell electrophoresis (the isoelectrophoretic point) and the pI as determined in the present experiments is large. But it may be recalled here that in the electrophoresis of proteins it has been observed that the point of zero charge determined by titration methods does not correspond with the isoelectrophoretic point. The isoelectrophoretic point decreases with increasing ionic concentrations. This divergence between the isoelectrophoretic point and the point of zero charge is attributed to the fact that at low ionic concentrations the particle behaves as though it had a smooth surface, while at higher ionic strengths the surface behaves as a rugged surface consisting of "numerous hills and valleys" with the charged groups in the valleys not being able to exert their electrophoretic effect[14]. It appears possible that under isoelectric focusing conditions with ionic environment afforded by the low-mol.-wt ampholines, the pI would again not coincide either with the isoelectrophoretic point or the point of zero charge.

The surfaces of *E. coli* and *Aerobacter aerogenes* have been shown to contain predominantly carboxyl and amino groups[3]. The intrinsic pK of carboxyl groups of the glucuronic acid component of the bacterial surface is 3.2 at 20 °C[3], while that of α-amino groups in proteins varies between 7.0 and 8.4[15,16].

The pI value calculated on the assumption that carboxyl and amino groups of proteins or polysaccharide components of the surface are the only ionogenic groups present on the bacterial surface is 5.38 from which the observed pI differs by about 4%. The pI value was also calculated from the pI 3.85 of HCHO-cells with their purely carboxyl surface and from that of AW-EI-CPDS cells. By this method of calculation a value of 5.3 was obtained.

The high isoelectric point of these cells excludes the occurrence of phosphate groups of the phospholipid complex in the isoelectric zone, *i.e.* the region of the surface which is amenable to isoelectric equilibrium analysis which is estimated to be up to a depth of 60 Å below the surface (see later discussion).

The pI of AW-EI cells

Ethyleneimine reacts with carboxyl groups to form β-aminoethyl esters. It could be expected, therefore, that the ethyleneimine-treated cells should possess a purely amino surface. The pI of these cells was in fact found to be 8.55 (Table I; Fig. 2).

Chemical analyses of cell walls of gram-negative bacteria have indicated that a variety of amino acids including aromatic aminoacids, arginine, lysine, and sulphur-containing amino acids occur in the cell wall material. However, the pI value of 8.55 of AW-EI cells is not compatible with the data regarding the occurrence of these amino acids (Table II). The range of intrinsic pK of these aminoacids is between 9.1 to 12.5 (pK of ε-NH_3^+ of lysine side chain 10.4; –SH group of cysteine side chain approx. 9.1; phenolic –OH groups of aromatic side chains of ca 10.0; guanidyl group of arginine side chain approx. 12.5[16]). In other words the pI of 8.55 of AW-EI cells indicates that groups with intrinsic pK values greater than 8.55 do not occur on the bacterial surface in the isoelectric zone. The possible exception may be sulphur-containing amino acids. A pI of 8.68 was calculated for these cells assuming that

TABLE I

SURFACE CHARGE OF UNMODIFIED AND CHEMICALLY TREATED *E. COLI* CELLS

*Cell designation**	*Surface pI*	***Number of effective charges/cell* $\times 10^{-6}$	*Number of thiol groups per cell* $\times 10^{-6}$	*Total charges/cell* $\times 10^{-6}$	*Net negative charge/cell* $\times 10^{-6}$
E. coli unmodified	5.6 ±0.1	−0.3723		−0.3723	−0.3723
AW-EI	8.55 ± 0.04	+0.4121	0.0109***	+0.4230	−0.4145
HCHO	3.85 ± 0.06	−0.8375		−0.8375	
E. coli-CPDS	4.28 ± 0.05	−0.7233	0.3510§		
AW-EI-CPDS	7.47 ± 0.18	+0.1250	0.2871		

* AW-EI cells: acid-washed ethyleneimine-treated *E. coli* cells; HCHO-cells: formaldehyde-modified *E. coli* cells; AW-EI-CPDS: acid-washed ethyleneimine-treated cells subsequently treated with 6,6′-dithiodinicotinic acid; *E. coli*-CPDS: *E. coli* cells treated with dithiodinicotinic acid.

** The number of charges on the cell surface were calculated as described by Sherbet *et al.*[6] using the equation $Q = (PDr^2K \cdot 3.3 \cdot 10^{-3})/e$ (electrons per cell at pH 7) where Q is the net charge per cell. The potential P on the surface of the cells is estimated as the potential difference between a solution at neutral pH and a solution isoelectric with the cell surface and is given by $(7 - \text{pI}) \cdot 2.303\ RT/F$ V where R is the gas constant (8.315 Joules per degree), T the absolute temperature, and F the Faraday (96500 C). The value of $2.303RT/F = 0.0592$ at 25 °C. D is the dielectric constant of water (78.54 at 25 °C), e the electronic charge $4.8 \cdot 10^{-10}$ e.s.u., K the Debye–Hückel function $0.327 \cdot 10^8 \sqrt{I}$ cm at 25 °C where I is the ionic concentration due to ampholines assumed to be 0.01 M, r the radius of the cell in cm.

*** This figure represents the number of thiol groups dissociated at the cell pI of 8.55 which is 3.1% of the total number of surface thiol groups.

§ Total –SH groups equivalent to the number of carboxyl groups introduced by dithiodinicotinic acid.

both amino groups and thiols were present on their surface, which closely approximates the observed pI 8.55 of AW-EI cells. It was therefore felt necessary to examine the effects on the cell pI of specific-SH-blocking reagents.

TABLE II

IONOGENIC GROUPS ON THE SURFACE OF *E. COLI* CELLS

Cell designation	*pI*	*Nature of groups in isoelectric zone**		*Calculated pI***
		Present	*Excluded*	
E. coli unmodified	5.6	Carboxyl; amino group pK approx. 7.5	Acidic group with pK <3.2; ε-amino groups	5.38
AW-EI	8.55	Amino groups pK approx. 7.5; thiols	Amino groups of phospholipids; phenolic –OH, guanidyl	8.7
HCHO	3.85	Carboxyl groups of glucuronic and neuraminic acids, β-, and γ-carboxyl	Acidic groups with pK <3.2	3.5

* Isoelectric zone is estimated to extend to a depth of 60 Å below the cell surface.

** pI values calculated from surface pK of ionogenic groups corrected using Hartley–Roe equation. $pI = -\log\sqrt{\Sigma k'a\ k'w/\Sigma k'b}$ where $k'a$ and $k'b$ are the corrected dissociation constants of the strongest anionogenic and cationogenic groups respectively, $k'w = 6.81 \cdot 10^{-15}$ at 20 °C.

The pI of HCHO-cells

Treatment with formaldehyde has been used as a means to block the amino groups on the cell surface by Heard and Seaman[17] and Ward and Ambrose[7]. This method of surface alteration using prolonged formaldehyde treatments may be subject to the criticism that the information so obtained may not be fully relevant to the living state of the cells. Ward and Ambrose found that after 30 days of fixation in formaldehyde the electrophoretic mobility (m) of CL3 murine leukemia cells rose from -1.06 to -1.28 $\mu m \cdot s^{-1} \cdot V^{-1} \cdot cm^{-1}$. However, in bacterial cells the modification seemed to be slow and inefficient (Table III). That the treatment was modifying the groups was clear enough, but all the cells in a sample did not appear to be modified until after about 15 weeks of treatment. Even after such a prolonged treatment the cells did not focus into a sharp band but showed much scatter between pH 3.6 and 4.2. A majority of the cells, however, showed a consistent pI of 3.85 (± 0.06) from 10 weeks treatment onwards (Fig. 3), which compares favourably with the pI of 3.5 calculated for these cells.

The pI of the HCHO-cells confirms the exclusion of phosphate groups of the phospholipid complex, which was suggested earlier in the context of unmodified cells. If phosphate groups had been present the pI of HCHO-cells would have been 3.1 or less and that of the unmodified cells approx. 4.4.

It may be recalled here that a mosaic structure has been suggested for the outermost layer of bacterial cell walls in which the lipopolysaccharide lies beneath a discontinuous layer of lipoprotein. Ionisable groups occurring in the 60 Å isoelectric

TABLE III

MODIFICATION OF *E. COLI* SURFACE WITH FORMALDEHYDE

Duration of treatment (weeks)	*Percentage*		*pI of modified cells*
	Unmodified cells	*Modified cells*	
3	50	50	4.9
7	30	70	4.41
10	30	70	3.85
15	—	100	3.9

zone are detected in the present analysis and these appear to include groups belonging to the polysaccharide-phospholipid-protein complex, but reactive groups of the phospholipids have not been detected. It appears, therefore, that the arrangement of the complex is such that the phospholipids are located at a depth more than 60 Å from the bacterial surface.

The CPDS-cells (E. coli-CPDS and AW-EI-CPDS)

Mehrishi and Grasetti[8] found that 6,6′-dithiodinicotinic acid specifically blocked thiol groups of the cell surface and in doing so introduced one carboxyl group for each thiol group blocked. The increase in the charge could be assessed by measuring the increase in the electrophoretic mobility of the cells treated with dithiodinicotinic acid.

The thiol groups present on the surface of *E. coli* have similarly been estimated using dithiodinicotinic acid. When *E. coli* cells were treated with this compound the pI decreased by 1.32 pH units (Fig. 4) which corresponds to $0.351 \cdot 10^6$ thiol groups per cell. However only $0.287 \cdot 10^6$ thiols were estimated to be present on the AW-EI cells (Table I). The difference in these estimates may be attributed to the scatter of pI values in the case of AW-EI cells (S.D. ± 0.18) (Fig. 5). This could be due to changes in the reactivity of the thiol groups produced by dioxan and ethyleneimine treatments. The estimate of thiols using unmodified *E. coli* cells may, therefore, be considered as more accurate.

The dermination of total anionic and cationic charges

HCHO-cells. The calculation of charges on the surfaces of unmodified *E. coli* cells, AW-EI cells, and HCHO-*E. coli* cells was made according to Sherbet *et al.*[6]. Formaldehyde-modified cells had a pI of 3.85 ± 0.06 which gave a value of $0.8375 \cdot 10^6$ negative charges due to carboxyl groups. This value represents the total negative charges present on the cell surface. The surface area of *E. coli* is $3 \cdot 10^8$ Å^2 (ref. 1). In other words, in these cells, one carboxyl group occurs per about 350 Å^2. The corresponding value calculated from the *m* data on *E. coli*[3] is one carboxyl group per approximately 2000 Å^2. The density of carboxyl groups as determined by the isoelectric focusing method is nearly 6 times their density as assessed by the electrophoretic method. At physiological ionic strength the electrophoretic effective zone has a depth of about 10 Å[18]. On this basis therefore it might be suggested that the isoelectric zone is nearly up to 60 Å below the cell surface.

AW-EI cells. The number of positive charges present on the surface of AW-EI cells was estimated to be $0.4121 \cdot 10^6$. This also represents the total number of cationogenic groups on the *E. coli* surface. The number of net negative charges calculated from the unmodified *E. coli* cells is $0.3723 \cdot 10^6$ per cell. But the net negative charge calculated from the total negative charges (from HCHO-*E. coli* cells) and the total positive charges (from AW-EI cells) is $0.4254 \cdot 10^6$ which differs by 14.2% from the net negative charge estimated from the unmodified cells.

This discrepancy could be due to non-specific adsorption of formaldehyde molecules to the cell surface. But this was not considered probable on account of the fact that a gradual blocking of the amino groups was achieved over a period of several weeks (Table III). If unspecific adsorption of formaldehyde molecules had occurred the change in the pI as a result of the treatment would have been abrupt and rapid. The second possibility that was envisaged was that there were negative charges still present on the AW-EI cell surface, that did not react with ethyleneimine This was considered likely because Gittens and James[3] found that there were groups on *Aerobacter aerogenes* surface which contributed negative charges at pH above 9.5. Experiments described earlier show that *E. coli* surface possesses $0.351 \cdot 10^6$ thiol groups. The surface pK of the thiols was calculated from the pI values of AW-EI cells bearing both amino and thiol groups, HCHO cells and unmodified *E. coli* cells. A pK of 10.04 was obtained for the surface thiol groups. In AW-EI cells with pI 8.55 3.1% of the thiols will be ionised, which partly accounts for the discrepancy in the net negative charges calculated by the two methods, *viz.* from unmodified *E. coli* cells and from AW-EI cells and the HCHO cells.

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REFERENCES

1 Lamanna, C. and Mallette, M. F. (1959) *Basic Bacteriology*, pp. 55, 185–203, Bailliere, Tindal and Cox, London
2 Salton, M. R. J. (1966) *Symp. Soc. Gen. Microbiol.* 6, 81
3 Gittens, G. J. and James, A. M. (1963) *Biochim. Biophys. Acta* 66, 237–249
4 Gittens, G. J. and James, A. M. (1963) *Biochim. Biophys. Acta* 66, 250–263
5 James, A. M. (1965) in *Cell Electrophoresis* (Ambrose, E. J., ed) pp 154–170, Churchill, London
6 Sherbet, G. V., Lakshmi, M. S. and Rao, K. V. (1972) *Exp. Cell Res.* 70, 113–123
7 Ward, P. D. and Ambrose, E. J. (1969) *J. Cell Sci.* 4, 289–298
8 Mehrishi, J. N. and Grasetti, D. R. (1969) *Nature* 224, 563–564
9 Kellenberger, E. and Ryter, A. (1958) *J. Biophys. Biochem. Cytol.* 4, 323–326
10 Murray, R. G. E., Steed, P. and Elson, H. E. (1965) *Can. J. Microbiol.* 11, 547–560
11 Ogura, M. (1963) *J. Ultrastr. Res.* 8, 251–263
12 Tomasz, A. (1971) *Nature* 234, 389–392
13 Wright, A. and Kanegasaki, S. (1971) *Physiol. Rev.* 51, 748–784
14 Bull, H. B. (1964) *An Introduction to Physical Biochemistry*, p. 319, F. A. Davis Co., Philadelphia, Pa.
15 Dawes, E. A. (1965) *Quantative Problems in Biochemistry*, p. 61, Livingstone, Edinburgh
16 Tanford, C. (1962) *Adv. Protein Chem.* 17, 73
17 Heard, D. H. and Seaman, G. V. F. (1961) *Biochim. Biophys. Acta* 53, 366–374
18 Ambrose, E. J. (1966) *Prog. Biophys. Mol. Biol.* 16, 243–265