Hydrogen-Ion Titration Studies on Erythrocyte Membranes

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1. H^+ titration was used to detect the presence of ionizable groups on human erythrocyte plasma membranes. Between pH 2.9 and 11.3, two significant peaks of H^+ association/dissociation occur in the differential form of the titration curve, one at pH 3.1 and the other at pH 10.3. 2. After disruption of membrane structure by exposure to high pH or by the addition of sodium dodecyl sulphate, maxima of H^+ association/dissociation were seen at pH 3.1, 4.3, 6.5, 10.3 and 10.7. 3. Spectrophotometric assay and selective chemical treatments were used to identify several of the titratable residues. 4. The degree of electrostatic interaction between titratable charged groups was investigated by comparing the titration characteristics of the membranes before and after modification of membrane structure.

Charge interactions within the plasma membrane appear to play a significant role in the response of the cell to its environment. Many ions and charged molecules act primarily on the plasma membrane and strongly affect cellular activities. Examples include lectins (Sharon & Lis, 1972), various hormones (Perry, 1974), several toxins (Kryzhanovsky, 1973; Holmgren *et al.*, 1974) and, in certain cases, Ca^{2+} (Rasmussen, 1970). The passive permeability of several ions across the erythrocyte membrane is strongly influenced by pH, ionic strength and chemicals that react with amino and thiol groups in the membrane (Knauf & Rothstein, 1971). Charge interactions also appear to influence membrane structure. An extreme example is the membrane of *Halobacterium halobium*, which requires high cation concentrations for stabilization against electrostatic disaggregation (Brown, 1965). Changes in pH and ionic strength have also been shown to induce lateral movement of proteins in the plane of the erythrocyte membrane (Pinto da Silva, 1972). These alterations in topography are similar in nature to many of the aggregation changes seen in membrane proteins after antibody interaction (Nicolson & Painter, 1973).

Some information about the nature and structural arrangement of membrane charge has come from electrophoretic studies. Negatively charged sialic acids on membrane glycoproteins in the erythrocyte have been shown to be exclusively located on the outer surface of the plasma membrane and to contribute a major part of the surface charge (Eylar *et al.*,

1962). Protein carboxyl groups also appear to contribute to the electrophoretic surface charge of the erythrocyte (Tenforde, 1970). One disadvantage of electrophoretic studies, however, is that they are limited to an evaluation of the net charge density at the surface of shear and do not give information on individual, deeper-lying, charged groups. For example, there is no indication that basic groups on membrane components affect the electrophoretic mobility of the erythrocyte (Tenforde, 1970) in spite of the detection of basic groups on the external surface of the membrane by specific labelling techniques (Gordesky *et al.*, 1975). On the other hand, H^+ -titration techniques have long been used on proteins to gain information on the nature, number and interactions of ionizable groups on the molecules (Tanford, 1962) and there seems no reason why H^+ -titration studies should not yield equally valuable information on the surface-charge structure of plasma membranes. Sanui *et al.* (1962) have used H^+ titration to monitor the charged groups on rat liver-cell microsomal fractions and erythrocyte membranes responsible for binding Na^+ and K^+ ions at pH 7. Passow (1969) has demonstrated the presence of basic groups titrating at alkaline pH on the erythrocyte membrane and has suggested that amino groups could act as the main source of fixed positive charge within the membrane structure. We report here the results of H^+ titration of erythrocyte plasma membranes under various conditions. Several charged groups titrating between pH 3 and 11 have been identified. Modification of membrane structure by detergents and selective chemical treatment has been found to alter the degree of electrostatic coupling between these groups in the membrane.

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Materials and Methods

Chemicals

KOH solutions were standardized against 1 M-HCl obtained from BDH Chemicals Ltd., Poole, Dorset, U.K. Glutaraldehyde was obtained in aq. 8% (v/v) solution sealed under N₂ from Polaron Equipment Ltd., Watford, Herts., U.K. All other chemicals were analytical-grade reagents of commercial origin.

Preparation of erythrocyte 'ghosts'

Outdated (6 weeks) blood-bank human blood was washed several times in 0.13 M-sodium phosphate, pH 7.4. Any leucocytes were removed by aspiration after each centrifugation. The erythrocyte 'ghosts' were prepared at 0–4°C by the method of Dodge *et al.* (1963), in 7 mM-sodium phosphate buffer, pH 7.4. After the final wash in buffer, the 'ghosts' were washed three times in, and finally suspended in, 120 mM-KCl. Residual P_i (Wootton, 1974) in the final preparation was less than 40 nmol/mg of membrane protein. The haemoglobin content of the final preparation was less than 20 nmol/mg of membrane protein as determined by spectrophotometric measurement (Dodge *et al.*, 1963).

Titration measurements

H⁺ titrations were carried out at 32°C under N₂ as described (Hallam & Wrigglesworth, 1973). All readings were taken at equilibrium, which was usually attained within 1 min of adding the titrant, except between pH 5 and 3, where several minutes were needed to obtain a constant reading. The titration range was usually confined to pH 3–11. Outside this range, ionic-strength variations make the subtraction of appropriate blank titrations subject to large error. Equilibrium readings were unaffected by the presence of carbonyl cyanide trifluoromethoxyphenylhydrazone, a proton porter, indicating equilibration of protons across the membrane under the present conditions.

Determinations

Thiol groups were assayed by the method of Butterworth *et al.* (1967). Ionized tyrosine residues were detected by absorption measurements at 295 nm as described by Wetlaufer (1962). Sialic acid was determined as described by Warren (1959). Histidine residues were determined by titration before and after photo-oxidation in the presence of 0.01% Rose Bengal (Bellin & Yankus, 1968). For photo-oxidation, the aerated suspension, at pH 7 and 25°C, was illuminated for 2 h from a distance of 20 cm with a tungsten lamp (150 W) focused through a heat filter. Protein was assayed by the method of Lowry *et al.* (1951) in the presence of 0.1% Triton X-100, and also by the biuret method (Gornall *et al.*, 1949) in the presence of 1% (w/v) deoxycholate to solubilize

the membrane. Bovine plasma albumin was used as protein standard. Extinction measurements at 600 nm (Wrigglesworth & Packer, 1970) were used to monitor solubilization and pH-dependent, light-scattering changes of membrane suspensions.

Results

Titration of erythrocyte 'ghosts'

Fig. 1(a) shows the titration curve of erythrocyte 'ghost' suspension after subtraction of the appropriate blank titration of 120 mM-KCl, pH 7.0 being taken as the point of reference. In Fig. 1(b) the curve has been converted into the differential form, change in H⁺ bound/unit of pH (in this case points were calculated every 0.2 pH unit), as this method of presentation more clearly indicates where association and dissociation of H⁺ ions occur. The main features of the differential-titration curve are two significant peaks of H⁺ association/dissociation, one at pH 3.1 ± 0.3 and one at pH 10.3 ± 0.2 , and a gradual increase in H⁺ binding as the pH is lowered from pH 7 to 3. No significant difference was noted between different blood groups used (in both ABO and Rhesus systems) and the results shown in Fig. 1 are therefore a compilation of all types.

The titration curve was reversible between pH 5 and 8.5. Outside this range, changes in the titration curve were seen on reversal. The maximum change

Fig. 1. Normal (a) and differential (b) titration curve of human erythrocyte plasma membranes in 120 mM-KCl at a final concentration of approx. 2 mg of membrane protein/ml

Each half of the titration was started from pH 7. For clarity, only the S.E.M. of the two main peaks are shown by vertical bars (13 observations). The horizontal bars indicate S.E.M. of pH position.

Fig. 2. Differential titration curve of human erythrocyte plasma membranes exposed to alkaline pH

The membranes were suspended in 120mM-KCl and the suspension was adjusted to pH11.5 with KOH. After 5min, titration with HCl was started from pH11.5. Bars indicate s.e.m. (six observations).

Table 1. Changes in amount of H⁺ ions bound to erythrocyte membranes between pH2.8 and 11.5

Membranes were suspended in 120mM-KCl to a concentration of approx. 2mg/ml. Separate samples were titrated over the pH ranges 7.0-2.8 and 7.0-11.5 starting from pH7.0 in each case. Single samples adjusted to pH11.5 were used for the back titrations. The values are given as means $\pm$ 1 s.e.m. and the number of observations are shown in parentheses.

	pH range	Change in H ⁺ bound (μmol/g)
Titrations from pH7.0	7.0- 2.8	1160 $\pm$ 70 (15)
	7.0-11.5	910 $\pm$ 40 (13)
Back-titration from pH11.5	11.5- 7.0	1150 $\pm$ 50 (5)
	7.0- 2.8	1470 $\pm$ 140 (5)

occurred on reversal from pH11.5 (Fig. 2). In this case, two peaks can be seen in the alkaline range, one at pH10.7 $\pm$ 0.2 and one at pH10.3 $\pm$ 0.2. Between pH8 and pH3, three peaks of H⁺ association/dissociation are found, one at pH6.5 $\pm$ 0.2, one at pH4.3 $\pm$ 0.3 and one at pH3.1 $\pm$ 0.2. The total number of titratable groups increased after denaturation with alkali (Table 1).

Detergent effects

Exposure of the membranes to high pH was accompanied by a distinct clarification of the membrane suspension. It was decided to compare the titration results obtained after this alkaline denaturation with those obtained after exposure of the membrane to solubilizing agents at neutral pH. Fig. 3 illustrates the effect of two detergents, Triton X-100 and sodium dodecyl sulphate, at solubilizing concentrations, on the titration curve of erythrocyte membranes. The titration results for the membranes in the presence of sodium dodecyl sulphate (Fig. 3a)

are very similar to those found after alkaline denaturation; three major peaks can be seen between pH8 and 3. In contrast, Triton X-100 caused little change (Fig. 3b), the membranes titrating in a manner similar to normal, non-denatured membranes, even though the concentration of Triton X-100 used (4μmol/mg of membrane protein) did cause full clarification of the suspension as monitored by absorption at 600nm. It appears that the structural interactions responsible for the shape of the titration curve between pH3 and 11 are unaffected by solubilization of the membranes by this non-ionic detergent.

Identification of charged groups

Of the many groups available for titration in the range pH3-11, several are amenable to direct identification.

Spectrophotometric titration of tyrosine residues (Fig. 4) indicates the presence of 200nmol of tyrosine/mg of membrane protein, titrating with pK_a 10.7 $\pm$ 0.2. After exposure to high pH, the pK_a of the residues falls to 10.2 $\pm$ 0.2. The presence of this number of titratable tyrosine residues could account for approx. 17% of the groups titratable at alkaline pH.

Measurement of sialic acid residues and membrane thiol groups gave values of 42 $\pm$ 10nmol/mg of membrane protein and 130 $\pm$ 20nmol/mg of membrane protein respectively. The value for sialic acid is lower than reported values of 80nmol/mg (Eylar *et al.*, 1962; Forstner & Manery, 1971) and 102nmol/

Fig. 3. Effect of detergents on the titration characteristics of erythrocyte plasma membranes

Membranes were suspended to approx. 2mg of protein/ml in 120mM-KCl and 1% sodium dodecyl sulphate (a) or 120mM-KCl and 1% Triton X-100 (b). Each half of the titration was started from pH7. Bars indicate s.d. (three observations).

Fig. 4. Spectrophotometric titration of tyrosine residues in erythrocyte plasma membranes in 120 mM-KCl

Phenolic hydroxyl groups were measured by difference spectroscopy at 295 nm by using as reference a membrane suspension at neutral pH. Both forward (●) and reverse (○) titrations are shown. Bars indicate S.D. (three observations).

Fig. 5. Effect of glutaraldehyde treatment on the titration characteristics of erythrocyte plasma membranes

(a) Membranes were suspended in 0.25% glutaraldehyde in 7 mM-sodium phosphate buffer, pH 7.4, for 5 min, centrifuged at 20000g for 40 min, washed once in 7 mM-sodium phosphate buffer, pH 7.4, and twice in 120 mM-KCl, and finally suspended in 120 mM-KCl for titration. Each half of the titration was started from pH 7. (b) Glutaraldehyde was directly added at a final concentration of 0.25% to a suspension of erythrocyte membranes at pH 11.5. Titration was started from pH 11.5. In this case, glutaraldehyde was also added at the same concentration to the blank titration of 120 mM-KCl. Bars indicate S.D. (three observations).

mg (Fairbanks *et al.*, 1971) for fresh blood. However, the sialic acid content of erythrocyte 'ghosts' depends on the age of the blood (Devenuto *et al.*, 1969) and falls on storage under blood-banking conditions. None of the groups titratable down to pH 2.9 were found to be sensitive to neuraminidase (1 h incubation at 37°C with neuraminidase from *Clostridium perfringens*, Sigma type V, at a concentration of approx. 1 mg of enzyme/6 mg of 'ghost' protein). This was not unexpected, since the reported range for the pK_a of sialic acid residues is pH 2.5–3.0 (Tenforde, 1970).

An estimation of the number of titratable amino groups was obtained by titration measurements on the membranes before and after glutaraldehyde treatment (Habeeb & Hiramoto, 1968). The effect of 1% glutaraldehyde on erythrocyte membranes (Fig. 5a) is to decrease the numbers of groups titratable at alkaline pH by approx. 50%. No significant change was found in the number of groups titratable at acid pH, but the shape of the curve was altered with a general increase in the number of groups titratable between pH 3.5 and 7.0. The effect of glutaraldehyde is more clearly seen on alkali-denatured membranes (Fig. 5b), with a decrease of 78% in the numbers of groups titratable between pH 8 and 11. The titration curve remains essentially unaltered in the acid pH range except for a slight but significant shift in numbers of groups titratable in the pH range 3–3.5 to the pH range 4–5.

The presence of titratable histidine residues was detected by titration over the range pH 9–4 before and after photo-oxidation in the presence of Rose Bengal. Fig. 6 shows that a decrease in titratable residues (140 nmol/mg of membrane protein) occurs between pH 5.5 and pH 7.5 after photo-oxidation. A decrease in the number of titratable groups was only detectable when samples of alkali-denatured membranes were used, indicating the inaccessibility of histidine to the dye in the intact membrane.

Charge modification and membrane structure

The pH-dependence of extinction at 600 nm is shown in Fig. 7. All absorbance values are expressed as percentages of the absorbance at pH 7.0. An approximately linear fall in absorbance can be seen from pH 6 to 9. In this range, the extinction changes were reversible. Above pH 9, absorbance falls sharply, to a minimum at pH 10.5. Formaldehyde abolishes the extinction changes in the alkaline region. Below pH 6, extinction rises to a maximum at pH 3.8 in both control and formaldehyde-treated membranes. In samples of control membranes, flocculation occurred between pH 4.0 and 5.5. This was a concentration-dependent effect and could be decreased by lowering the concentration of the membranes in suspension. Triton X-100 abolished all pH-dependent

Fig. 6. Titration characteristics of erythrocyte plasma membranes before (a) and after (b) photo-oxidation in the presence of Rose Bengal

The pH of the membrane suspension was adjusted to 11.5. After 5 min the pH was re-adjusted to 7.0 for photo-oxidation.

Fig. 7. pH-dependence of extinction at 600 nm of a suspension of erythrocyte plasma membranes

Membranes were suspended in 120 mM-KCl (●) or 120 mM-KCl+0.25% formaldehyde (○). ----, change in extinction, corrected for dilutions, on addition of Triton X-100 to a final concentration of 1%.

extinction changes in suspensions of both control and formaldehyde-treated membranes.

Discussion

One characteristic of H⁺ titrations of macromolecules is that strong electrostatic interaction between dissociating sites causes considerable flattening of the titration curves (Tanford, 1962).

For example, the interaction between charged sites in a protein causes each segment of the titration curve corresponding to a particular set of groups to be more spread out compared with the titration of a mixture of the corresponding simple acids and bases in free solution (Steinhardt & Beychok, 1964). A similar effect appears to be present in the titration characteristics of erythrocyte membranes. The differential-titration curve shows only two significant peaks between pH 3 and 11, but when membrane structure is disrupted, either by exposure to alkaline pH or by solubilization by an ionic detergent, the resulting titration curve shows several distinct regions of H⁺ association/dissociation. It appears that, in the intact membrane, electrostatic interaction between neighbouring sites is sufficiently strong to influence the H⁺ titration characteristics of the individual groups. It is notable that a non-ionic detergent such as Triton X-100 does not affect these interactions. The addition of Triton X-100 clarified the membrane suspension as monitored by extinction at 600 nm and abolished all pH-dependent changes in extinction without significantly affecting the shape of the titration curve. Bilayer structure would not therefore appear to be necessary for maintenance of electrostatic interactions between membrane components. These appear to be confined within individual lipoprotein complexes and are unaffected by a non-ionic detergent.

Not all groups are available for titration in the intact membrane. There is a slight increase in the number of titratable groups over the pH range 3–11 after membrane structure is disrupted by exposure to alkaline pH or by treatment with sodium dodecyl sulphate. It is unlikely that hydrolysis of membrane components contributes to the increased titration capacity of the suspension. Hydrolysis of phospholipids does occur in alkaline conditions and subsequent acidification can free the base from the glycerophosphate, but the conditions needed are much harsher than those used here (Ansell & Hawthorne, 1964). In addition, treatment with sodium dodecyl sulphate at neutral pH gives results similar to those obtained by exposure of the membranes to alkaline pH. The results with Triton X-100 and the fact that protein porter, carbonyl cyanide trifluoromethoxyphenylhydrazone, did not affect the titration curves also suggest that the interior of the 'ghosts' is in equilibrium with the external pH. The increase in numbers of titratable groups therefore appears to result from an unmasking of inaccessible groups by conformational changes in membrane components, rather than from groups on the interior surface of the 'ghosts' becoming accessible to protons by simple permeability changes.

The greater number of groups titrating in the acid region of the titration curve compared with the alkaline region agrees with the amino acid analysis of

erythrocyte membrane protein, which shows an excess of acidic over basic residues (Rosenberg & Guidotti, 1968). The negative surface charge of erythrocytes has been attributed solely to acidic groups near the surface of shear (Tenforde, 1970). The anionic groups involved are the carboxyl groups of sialic acid and cell-surface proteins. The nature of the negative charge remaining after the removal of sialic acid by neuraminidase has been investigated by Haydon & Seaman (1967), who reported the presence of anionic groups with pK_a 3.35 in acetaldehyde-fixed human erythrocytes. This could correspond to the titratable group with pK_a 3.1 which can be distinguished in the titration curve of 'ghost' membranes. The position of this peak does not change on denaturation of membrane structure, suggesting a lack of involvement in strong electrostatic interactions, in accord with its possible surface role in the membrane. The remaining acidic residues can be titrated after membrane denaturation, with pK_a 4.3, characteristic of side-chain carboxyl groups in proteins, and appear to be involved in tight electrostatic interactions within the membrane. The very low pK_a values reported for the primary ionization of the phosphate group in phospholipids (Abramson *et al.*, 1964; Wills *et al.*, 1971) would preclude any contribution from this source to the titration characteristics of the membranes over the pH range used in the present study, but a small contribution (approx. 10%) will occur from phosphatidylserine, which has a reported pK_a value for the carboxyl group of between 3.8 and 4.6 (Garvin & Karnovsky, 1956; Abramson *et al.*, 1964).

There was a 50% decrease in groups titratable in the alkaline range after treatment of the membranes with glutaraldehyde. In denatured membranes, this value increased to almost 80%. As a bifunctional cross-linking reagent, glutaraldehyde reacts with lysine, tyrosine, cysteine and, to a lesser extent, histidine residues (Habeeb & Hiramoto, 1968). In addition, phosphatidylethanolamine and phosphatidylserine can be affected (Roozemund, 1969). From the present measurements of the numbers of tyrosine, cysteine and histidine residues in the preparations it is clear that the major contribution to the titration characteristics in the alkaline region comes from amino groups. These would presumably arise from protein and phospholipid, since most carbohydrate amino groups in the erythrocyte membrane are acylated (Kathan & Adamany, 1967).

The present results give a value of $720 \mu\text{mol}$ of basic residues/g of membrane protein. This closely agrees with a calculated contribution of $830 \mu\text{mol}$ of basic residues/g of membrane protein from erythrocyte membrane protein and the two phospholipids phosphatidylethanolamine and phosphatidylserine, assuming that one erythrocyte membrane contains 0.66 pg of protein (Dodge *et al.*, 1963). Approx. 60%

of the titratable residues would therefore arise from ϵ -amino groups on membrane protein (Tenforde, 1970). Passow (1969) has suggested that many features of the passive permeability of the erythrocyte membrane to anions can be explained by the presence within the membrane of fixed positive charges. The present results show that the erythrocyte membrane contains several populations of positively charged groups with up to 35% of the amino and most of the imidazole residues being inaccessible to protons in the intact 'ghost' membrane. This latter finding is consistent with the observations of Knauf & Rothstein (1971) and Poensgen & Passow (1971) that several amino-reactive reagents inhibit anion permeability, but not all the groups involved are accessible or reactive to the reagents.

The number and characteristics of charged groups that contribute to the titration curve of erythrocyte membranes is evidently very different from those of the groups that contribute to the negative electrophoretic mobility of erythrocytes. This suggests that the majority of titratable charged groups are not accessible to the external surface of the membrane. This is consistent with present views on the asymmetric distribution of proteins and phospholipids in the erythrocyte membrane (Steck, 1974; Zwaal *et al.*, 1973). Outer-surface charged groups will strongly influence cell-cell interactions and changes in proton association of these groups are probably responsible for flocculation of the membrane suspension under acidic conditions. However, the extinction changes in suspensions of erythrocyte membranes after alterations in pH closely relate to changes in proton association as detected by titration. It is likely therefore that changes in proton association of components within the membrane or on the inner surface are contributing to the extinction changes. The lateral distribution of membrane components may be affected. Changes in the organization of lipoprotein complexes within the plane of the membrane can be induced by alterations in pH or ionic strength of the suspending medium (Wrigglesworth & Packer, 1970; Pinto da Silva, 1972). Wrigglesworth (1975) suggested that disturbances in the electrostatic balance between charged groups on membrane proteins and glycoproteins, for example by certain bivalent cations or by the close approach of another charged surface as in cell-cell contact, could cause a reorganization of inner-surface charged groups and may be important in the regulatory mechanisms controlling the response of the cell to its environment.

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