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# The Ionogenic Nature of the Secretory-Granule Membrane

## ELECTROKINETIC PROPERTIES OF ISOLATED CHROMAFFIN GRANULES

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1. Chromaffin granules isolated from the bovine adrenal medulla possess an electrophoretic mobility of $-1.12\mu m \cdot s^{-1} \cdot cm \cdot V^{-1}$, corresponding to a surface $\zeta$ potential of $-14.4$mV and surface charge density of $1.38 \times 10^{-6} C \cdot cm^{-2}$. 2. The mobility of chromaffin granules is pH-dependent, indicating an amphoteric surface with an isoelectric point at pH3.0 and acidic groups with a $pK_a$ of 3.11. 3. Addition of bi- and ter-valent cations decreased the mobility of chromaffin granules in a dose-dependent fashion with a relative potency of $La^{3+} \gg Mn^{2+} > Ca^{2+} > Sr^{2+} > Mg^{2+} > Ba^{2+}$. 4. Treatment with neuraminidase decreased the mobility of erythrocytes by 84%, whereas chromaffin-granule mobility was decreased by only 14%. This correlates well with the small complement of neuraminic acid present in the granule membrane. 5. The nature, origin and significance of the anionic surface charge of the chromaffin granule is discussed. It is concluded that the net negative charge at the surface of shear derives chiefly from a single type of chemical group, namely $-CO_2^-$, contributed by the $\alpha$-carboxyl group of constituent proteins, the phospholipid phosphatidylserine and, to a lesser extent, the sialic acid component of glycoproteins.

The catecholamines of the adrenal-medullary chromaffin cell are packaged in discrete membrane-bound granules and stored intracellularly before release (Blaschko & Welch, 1953; Hillarp *et al.*, 1953). In these and many other secretory cells for which the chromaffin cell may form a useful model, the process of exocytosis involving apposition, adhesion and fusion of the storage granule with the cell membrane is believed to constitute the principal release mechanism (Douglas, 1968; Matthews, 1970; Smith, 1971). Thus the molecular constraints to granule-cell-membrane interaction are, in the first instance, likely to be closely dependent on the nature of the granule surface and its electrostatic charge. Secondly, the chemical groups located on the granule membrane will be a major determinant of ligand formation in the process of adhesion and subsequent fusion of the granule with the cell membrane. Characterization of the surface ionogenic groups of the granule membrane and their topographical disposition is therefore vital for a full understanding of the interfacial dynamics of exocytosis. One particularly useful way in which the nature and magnitude of the secretory granule surface charge can be explored and characterized is by particle micro-electrophoresis.

In the present study the electrokinetic properties of isolated chromaffin granules have been established by ultramicroscopic electrophoresis. To gain more information about the biochemical nature of the ionogenic groups contributing to the electrostatic surface charge, the effects on granule electrophoretic mobility of a change in pH of the bulk solution, the specific removal of membrane components by enzymic attack, and the adsorption of polyvalent counterions, were determined. From the particle-mobility results described in the present paper, the surface potential, surface charge and charge density of the secretory granules were derived. These parameters determine the magnitude of any potential energy barrier arising from purely electrostatic forces which may have to be overcome for the granule to interact with the cell membrane at close approach (Matthews, 1970). A preliminary report of some of these results has been published (Matthews, 1971).

## Methods

### *Isolation of chromaffin granules*

The medullae were dissected from bovine adrenal glands, blotted dry and weighed. The tissue was minced and homogenized in an all-glass homogenizer with ice-cold sucrose (0.3M; 5ml/g of tissue).

A purified chromaffin-granule fraction was isolated from the homogenate by sucrose centrifugation (Smith & Winkler, 1967), sucrose–Ficoll–$^2H_2O$ centrifugation (Trifaró & Dworkind, 1970), or Millipore filtration (Poisner & Trifaró, 1967). Granule preparations were analysed for satisfactory catecholamine yield (Bertler *et al.*, 1958; von Euler & Lishajko, 1961;

Haggendal, 1962; Iversen, 1963), monoamine oxidase activity (Jarrott, 1971) as an index of mitochondrial contamination, and acid phosphatase content (Bessey *et al.*, 1946) to indicate microsomal contamination.

Lysed granules were prepared by mixing 1ml of the purified granule fraction with 25ml of water and centrifuging at 30000*g* for 60–120min. The pellet was resuspended in 1ml of 0.3M-sucrose or 0.16M-KCl.

*Preparation of erythrocytes and erythrocyte 'ghosts'*

Human erythrocytes were obtained from whole blood containing approximately 0.17M-disodium hydrogen citrate as anticoagulant. The cells were washed three times in iso-osmotic $Ca^{2+}$-free tris buffer (NaCl, 0.145M; tris–HCl, 5mM; EDTA, 1mM) at 4°C, the supernatants and buffy-coat layer being removed by aspiration after centrifugation at 1000*g* for 10min. Erythrocyte 'ghosts' were prepared from the washed cells by lysing 0.5ml of packed cells with 29.5ml of tris–HCl buffer (5mM) containing EDTA (1mM), pH7.0; after 15min of incubation at 4°C the cells were centrifuged (MSE 25 centrifuge; 27000*g* for 10min), the supernatant was removed, and the 'ghosts' were washed and centrifuged three times in the $Ca^{2+}$-free tris buffer.

*Neuraminic acid*

Neuraminic acid, liberated from lysed granules or erythrocyte 'ghosts' by incubation with *Vibrio cholerae* neuraminidase (50munits/ml in NaCl, 0.145M; $CaCl_2$, 5mM; $NaHCO_3$, 2mM) for 60min at 37°C, or by hydrolysis in 0.5M-$H_2SO_4$ for 60min at 80°C, was determined by the thiobarbituric acid method of Warren (1959). Corrections were applied for incidental interference from a D-ribose chromophore, and *N*-acetylneuraminic acid was used as reference standard.

*Protein*

Protein was determined by the modified Folin reaction of Lowry *et al.* (1951), with bovine serum albumin (10mg/ml) as reference standard.

*Microelectrophoresis*

The electrophoretic properties of the chromaffin granules were measured in a micro-electrophoresis unit (Mark II; Rank Bros., Bottisham, Cambs., U.K.) as described by Bangham *et al.* (1958), with modifications to allow dark-field illumination. This is essential to make the ultramicroscopic granules visible. The cell was of the Van Gils thin-walled cylindrical type, which allows good temperature regulation. Optical distortion and optical corrections are also minimal with this type of cell (Shaw, 1969).

Electrophoretic studies were undertaken in 0.16M-KCl to which concentrated (1M) solutions of bivalent or tervalent chloride salts were added to give the required final concentrations. A dispersion of 25–200$\mu$l of granule fraction in 8–10ml of medium was used, the volume being chosen to give an adequate number of particles in the field while minimizing consumption of granules. The pH of the medium was adjusted with 0.16M-KOH or 0.16M-HCl immediately before addition of the granules. All measurements were made at 25°C. Mean mobilities were determined from ten readings, and for each granule with forward and reverse polarity. This procedure minimizes errors caused by convectional drift of fluid in the cell and by electrode polarization. All observations were made in the near-stationary layer, the position of which was determined from measurement of the wall thickness and cell internal diameter. These measurements were made *in situ* with the micrometers of the microscope assembly.

The effective electrical length of the cell was determined with the cell filled with 0.1M-KCl (specific conductance 0.012886mhos$\cdot$cm$^{-1}$). Plots of current versus the voltage applied across the cell were obtained; from Ohm's law cell resistance was calculated. From this value, together with the cross-sectional area of the cell, the effective cell length was obtained by application of the equation: $l = K_c \cdot A \cdot R$, where $l$ is the effective cell length, $K_c$ the specific conductance of the medium, $A$ the cell cross-sectional area and $R$ the cell resistance.

The eyepiece micrometer was calibrated, before the electrophoresis cell was fitted, against a stage micrometer suspended in the water bath of the unit. Mobility studies were done with an applied potential of 15–30V. This gave transit times, for a distance of 58$\mu$m, of about 30s. Under conditions of lowered mobility, potential differences of up to 40V were employed to decrease transit times.

The mobility of human erythrocytes in 0.145M-NaCl was measured as a direct check on the calibration of the instrument. The result, $-1.03 \pm 0.02$ (S.E.M.) $\mu m \cdot s^{-1} \cdot cm \cdot V^{-1}$ was in close agreement with published values, $-1.08 \pm 0.03$ (S.D.) $\mu m \cdot s^{-1} \cdot cm \cdot V^{-1}$ (Ambrose, 1966).

*Theoretical*

*$\zeta$ potential.* In particle electrophoresis the mobile or electrokinetic unit comprises the particle plus a region of the ionic double layer close to its surface. The potential measured by electrophoresis is that at the surface of shear between the electrokinetic unit and the stationary part of the double layer. This is designated the $\zeta$ potential and, at low potential, values may be obtained by using the Smoluchowski equation (Shaw, 1969).

Thus for an aqueous medium at 25°C: $\zeta = 12.85 u_e$

(mV), where $u_e$ = electrophoretic mobility ($\mu m \cdot s^{-1} \cdot cm \cdot V^{-1}$).

*pK values.* The determination of surface p$K$ and its relationship to electrophoretic measurements have been described by Ottewill & Shaw (1967). An indication of the p$K$ of the ionogenic groups giving rise to surface charge (p$K_s$) may be obtained as follows:

$$pK_s = pH_b - \log\frac{[A_s^-]}{[HA_s]} + \frac{e\zeta}{2.303kT}$$

since at the bulk pH where:

$$\zeta = \tfrac{1}{2}\zeta_0, \quad \log\frac{[A_s^-]}{[HA_s]} = 0$$

$$\text{then} \quad pK_s = pH_b + \frac{e\zeta}{2.303kT}$$

where $pH_b$ is the bulk solution pH; $A_s$, the anion concentration at the shear plane; $e$, the electron charge; $\zeta$, the zeta potential; $k$, Boltzmann's constant; and $T$, the absolute temperature, °K.

Significance tests were carried out by using Student's $t$ test.

### *Materials*

The following substances were used: sucrose (Mann, special density-gradient grade ultra-pure), Ficoll [Pharmacia (G.B.) Ltd., London W.5, U.K.], $^2H_2O$ (Bio-Rad Laboratories, St. Albans, Herts., U.K.), neuraminidase (500 units/ml, from *Vibrio cholerae*, Behringwerke A. G.; supplied by Hoechst Pharmaceuticals, Hounslow, Middx., U.K.), Millipore filters [Millipore (U.K.) Ltd., Wembley, Middx., U.K.], bovine serum albumin (fraction V), tris, *N*-acetylneuraminic acid and reagents for acid phosphatase determination [Sigma (London) Chemical Co. Ltd., London S.W.6, U.K.]. All other salts and reagents were the best grade commercially available (BDH Chemicals Ltd., Poole, Dorset, U.K.).

## Results

### *Micro-electrophoresis*

Chromaffin granules from purified granule fractions were detected in the micro-electrophoretic field and identified as discrete particles for mobility measurement by their distinctive light-scattering properties. The chromaffin granules migrated towards the anode and therefore bear a net negative charge. The mobilities of granules, whether prepared by centrifugation or by Millipore filtration, were similar at all pH values; they were therefore treated throughout as equivalent and the results were pooled. Fig. 1 illustrates the pH-dependence of mobility; at lower pH values the curve is sigmoid (Fig. 1*a*). The mobility

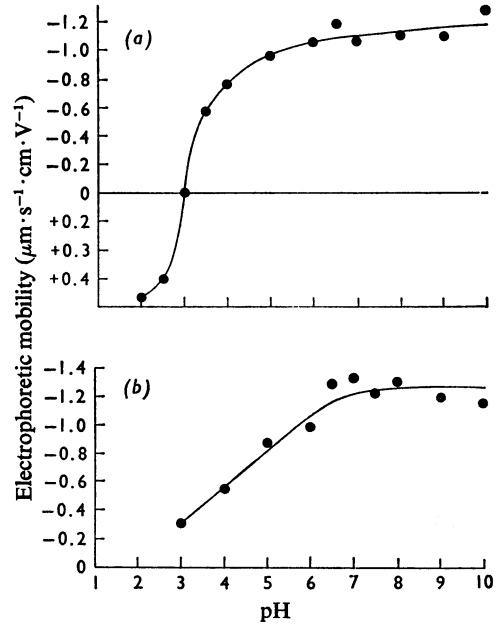


Fig. 1. *Effect of pH on the electrophoretic mobility of unlysed (a) or lysed (b) chromaffin granules*

All measurements of mobility were made at 25°C in 0.16M-KCl; the pH was adjusted with 0.16M-NaOH or 0.16M-HCl immediately before electrophoresis.

is zero at pH3.0 and shows a marked variation with pH on either side of this point. Above pH5.0 the curve becomes linear, with a slight positive gradient. At pH7.0 unlysed granules had a mobility of $-1.05 \pm 0.02$ (S.E.M.) $\mu m \cdot s^{-1} \cdot cm \cdot V^{-1}$ in 0.16M-KCl.

Granules lysed in water and investigated under the same conditions show a mobility of $-1.34 \pm 0.04$ (S.E.M.) $\mu m \cdot s^{-1} \cdot cm \cdot V^{-1}$ at pH7.0 and of $-0.31 \pm 0.05$ (S.E.M.) $\mu m \cdot s^{-1} \cdot cm \cdot V^{-1}$ at pH3.0. The mobility of lysed granules increases less rapidly with increasing pH than that of untreated granules (Fig. 1*b*).

Granules maintained in medium at either pH5.0 or pH9.0 for 10min and restored to pH7.0 before mobility determinations had mobilities of $-1.29 \pm 0.05$ (S.E.M.) $\mu m \cdot s^{-1} \cdot cm \cdot V^{-1}$ and $-1.27 \pm 0.05$ (S.E.M.) $\mu m \cdot s^{-1} \cdot cm \cdot V^{-1}$ respectively.

The addition of bivalent counterions decreased the mobility of both untreated and lysed granule preparations (Figs. 2 and 3). Of the bivalent cations, $Mn^{2+}$ was the most effective and $Mg^{2+}$ and $Ba^{2+}$ the least effective in decreasing granule mobility, but the concentration required for a 50% decrease in mobility was of a similar order (Table 1). No charge reversal was produced by bivalent cations in concentrations

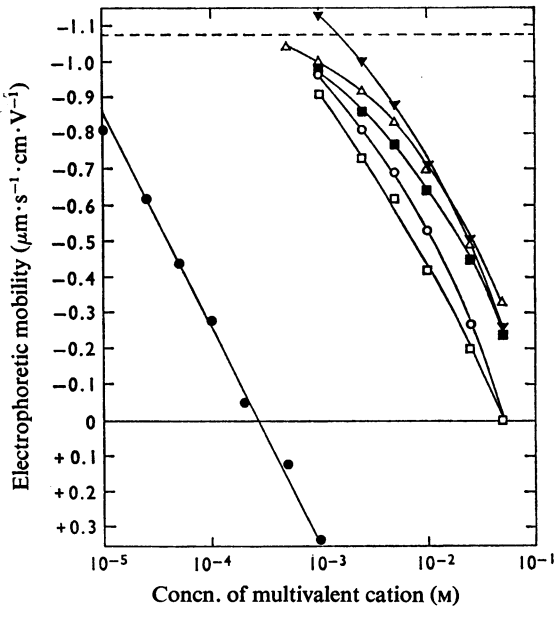


Fig. 2. *Effects of bivalent and tervalent cations on the electrophoretic mobility of chromaffin granules (unlysed)*

$La^{3+}$, (●); $Mn^{2+}$, (□); $Ca^{2+}$, (○); $Sr^{2+}$, (■); $Ba^{2+}$, (△); $Mg^{2+}$, (▼). ---, Normal mobility value in the absence of added multivalent cations. All measurements of electrophoretic mobility were made at 25°C in 0.16M-KCl, pH7.0; concentrated (1M) solutions of bi- or ter-valent chloride salts were added to give the required final cation concentrations.

Table 1. *Decrease of the electrophoretic mobility of chromaffin granules by multivalent cations*

All mobility measurements were made at 25°C in 0.16M-KCl, pH7.0; concentrated (1M) solutions of bi- or ter-valent chloride salts were added to give the required final concentrations, as in Fig. 2. The second column gives the concentration of cation required for a 50% decrease in mobility.

| | Concentration (mM) |
|---|---|
| $La^{3+}$ | 0.034 |
| $Mn^{2+}$ | 6 |
| $Ca^{2+}$ | 10 |
| $Sr^{2+}$ | 16 |
| $Mg^{2+}$ | 20 |
| $Ba^{2+}$ | 21 |

as high as 50mM (Fig. 2). The tervalent cation $La^{3+}$ was very much more effective than the bivalent cations in decreasing surface charge. Not only was the concentration required for a 50% decrease in mobility only one-three-hundredth that with the bivalent cations (Table 1), but charge reversal was clearly seen at low concentrations, i.e. 270μM (Fig. 2). Although the concentration of cation required for a given decrease in mobility differed in order of magnitude, the gradient of the linear part of the mobility-concentration regression for bivalent and tervalent cations was nevertheless similar (Fig. 2).

The effect of increasing the concentration of $Ca^{2+}$ ions was also similar for both lysed and unlysed granules (Fig. 3). At pH9.0 the mobility at any concentration of $Ca^{2+}$ was higher than at pH7.0, but the curves are parallel (Fig. 3).

*Neuraminic acid*

The mobility of intact granules after enzymic treatment with neuraminidase is summarized in Table 2. Results for erythrocytes treated under similar conditions are also included for comparison. Incubation with inactivated neuraminidase produced no significant change in the mobility of the intact granule or erythrocyte. In contrast, the active enzyme decreased erythrocyte mobility by 84% whereas granule mobility was decreased by only 14% (Table 2). To see whether this difference in effect arose from a comparative lack of glycoprotein in the granule compared with the erythrocyte membrane, the amount of free *N*-acetylneuraminic acid liberated by hydrolysis, or more specifically by neuraminidase action, was determined (Table 3). These experiments were done with lysed granules and erythrocyte 'ghosts', respectively, to avoid possible interfering effects of granule contents with the spectroscopic analysis of the neuraminic acid. The use of the membrane fractions also allowed direct comparison of the amount of *N*-acetylneuraminic acid liberated per mg of membrane protein from both erythrocyte and granule membranes. The neuraminidase concentration used (50 units/ml) was clearly completely effective in removing the available *N*-acetylneuraminic acid from either chromaffin-granule or erythrocyte-membrane glycoprotein, since prolonged acid hydrolysis produced no significant increment ($P>0.05$) in the total amount of free *N*-acetylneuraminic acid released beyond that produced by neuraminidase action.

The mobility of unlysed granules tended to increase on storage for 48–96h (at 4°C). This effect might be due to an 'aging' process expressed as some structural membrane change or to the progressive leakage of the granule content, e.g. of catecholamines. To test this latter possibility granule mobility (in 0.16M-KCl) was observed in the presence and absence of added adrenaline (1mM). Adrenaline caused a definite increase ($P<0.01$) in granule mobility, from $-1.11\pm0.02$ (S.E.M.) to $-1.28\pm0.02$ (S.E.M.) $\mu m \cdot s^{-1} \cdot cm \cdot V^{-1}$. It is therefore likely that the

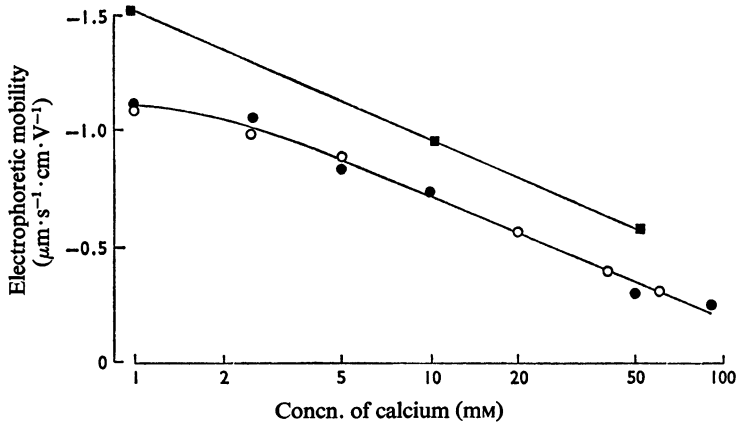


Fig. 3. *Effects of calcium and pH on the electrophoretic mobility of chromaffin granules*

The granules were: lysed, (●) and unlysed, (○) at pH7.0; unlysed, (■) at pH9.0. Measurements of electrophoretic mobility were made at 25°C in 0.16M-KCl.

Table 2. *Effect of neuraminidase on the electrophoretic mobility of erythrocytes and chromaffin granules*

Erythrocytes or chromaffin granules were incubated with neuraminidase for 60min at 37°C before measurement of electrophoretic mobility at 25°C in 0.16M-NaCl (erythrocytes) or 0.16M-KCl (granules) at pH7.0.

| | Neuraminidase concentration (munits/ml) | Mobility ($\mu m \cdot s^{-1} \cdot cm \cdot V^{-1}$) (±S.E.M.) | | | |
|---|---|---|---|---|---|
| | | Untreated | Inactive enzyme | Active enzyme | Decrease* (%) |
| Erythrocytes | 25 | −0.93±0.03 | −1.01±0.02 | −0.16±0.02 | 84 |
| Chromaffin granules | 100 | −1.00±0.01 | −1.02±0.02 | −0.88±0.02 | 14 |

* Percentage decrease in mobility caused by active compared with heat-inactivated enzyme.

Table 3. *Neuraminic acid liberated by neuraminidase or hydrolysis from erythrocyte 'ghosts' and lysed chromaffin granules*

Erythrocyte 'ghosts' or lysed chromaffin granules were incubated with *Vibrio cholerae* neuraminidase for 60min at 37°C, or hydrolysed in 0.5M-$H_2SO_4$ for 60min at 80°C. Results are means±S.E.M. for the numbers of experiments given in parentheses.

| | | Neuraminic acid liberated (μmol/mg of protein) |
|---|---|---|
| Erythrocyte ghosts | (i) Neuraminidase (50 units/ml) | 0.031±0.005 (6) |
| | (ii) Hydrolysis | 0.033±0.001 (22) |
| Chromaffin granules (lysed) | (i) Neuraminidase (50 units/ml) | 0.020±0.003 (21) |
| | (ii) Hydrolysis | 0.023±0.002 (50) |

increased electrophoretic mobility observed on granule storage is due, at least in part, to the release and subsequent surface adsorption of catecholamines.

## Discussion

In the preparation of purified fractions of chromaffin granules for micro-electrophoretic study the

most important particulate contaminants are mitochondria and lysosomes. The methods used for the isolation of chromaffin granules in the present study (see the Methods section) were chosen because they allowed recovery of granules in high yield with comparatively little mitochondrial or lysosomal contamination, the sucrose–Ficoll–$^2H_2O$ method being particularly useful in this respect.

Additionally an important advantage of both the Millipore-filtration and sucrose–Ficoll–$^2H_2O$ centrifugation methods is that, unlike sucrose-gradient centrifugation, the granules are not exposed to a hyperosmotic medium at any stage in their preparation; for this reason most experiments were carried out by using the $^2H_2O$–Ficoll–sucrose method of granule isolation.

*Microelectrophoresis of chromaffin granules*

The results show that the chromaffin-granule membrane possesses a net negative surface charge at physiological pH values. This confirms the finding made by Banks (1966) using a moving-boundary technique. Above pH6.5 the mobility of both lysed ($-1.25\mu m \cdot s^{-1} \cdot cm \cdot V^{-1}$) and unlysed granules ($-1.12\mu m \cdot s^{-1} \cdot cm \cdot V^{-1}$) is almost constant. These mobility values correspond to surface potentials of $-14.4$mV for unlysed and $-16.1$mV for lysed granules. The maximum mobilities obtained correspond to potentials of $-16.2$mV (pH10.0) for unlysed granules and $-17.0$mV (pH7.0) for lysed granules. The application of the Smoluchowski equation to the mobility values for derivation of surface potentials is therefore justified, since all potential values are considerably less than 25mV. Hence, the surface potential closely approximates to the potential at the Stern plane, and no correction proved necessary for force relaxation effects, surface conductance, particle conductivity, or difference in dielectric constant and viscosity between the double-layer and bulk solution (see Tenforde, 1970).

Variation of mobility with pH at constant ionic strength reflects the ionogenic character of the granule surface. The mobility versus pH curve of the intact granules shows charge reversal at low pH values, indicating that the surface has an amphoteric nature (Bangham & Pethica, 1960), with an isoelectric point at pH3.0. The simple shape of the curve suggests the involvement of only one major type of chemical group, with a p$K$ value in the range 2.0–10.0. The calculated p$K_a$ of the unlysed granule membrane surface is 3.11. Comparison with other systems suggests that this p$K$ may reflect the ionization of carboxyl groups (Shaw, 1969). The ionization of carboxyl groups is affected by the electronegativity of adjacent atoms of the molecule (inductive effect and bond polarization) which may lead to some differences between the theoretical and measured p$K$ values. Values reported for p$K$ include 4.0 for the surface carboxyl groups of rat liver cells (Bangham & Pethica, 1960), 3.0 for the carboxyl groups of pectic acid adsorbed on to hydrocarbon carrier particles (Shaw, 1969) and 2.8 for the sialic acid carboxyl groups of the erythrocyte membrane (Cook *et al.*, 1961). The p$K$ of phosphate groups is, at least in model systems, lower, i.e. 1.0–2.0.

If the net negative surface charge of the chromaffin-granule membrane is due predominantly to carboxyl groups it could arise from ionization of the constituent carboxyl groups of surface proteins, phospholipids (e.g. phosphatidylserine), or acid polysaccharides (e.g. sialic acid).

These possibilities will now be considered in detail.

Neuraminidase cleaves the 2→3- or 2→6-glycosidic linkage between terminal sialic acid (e.g. *N*-acetylneuraminic acid) residues and cell-surface mucopolysaccharides to liberate free sialic acid (Gottschalk, 1960). On this basis it has been used to assess the contribution of sialic acid carboxyl groups (p$K_a$ 2.6) to the net negative surface charge of the erythrocyte membrane (Cook, 1968). In our control experiments, neuraminidase decreased the electrophoretic mobility of human erythrocytes by 84%, well within the range of the 65 and 94% decreases observed by Cook *et al.* (1961) and Eylar *et al.* (1962) respectively. On the other hand, whereas chromaffin-granule membranes possess approximately two-thirds of the sialic acid complement of erythrocytes (Table 3), their electrophoretic mobility was decreased by only 14% on treatment with neuraminidase in concentrations in excess of those giving a maximal effect on erythrocytes. However, it is important to recall that there is a relative paucity of protein in the chromaffin-granule membrane, the protein/lipid ratio (w/w) of 0.45 for the granule membrane (Winkler *et al.*, 1970) being much lower than that of 1.2 for erythrocyte 'ghosts' (Dodge *et al.*, 1963). Thus, taking total membrane protein and phospholipid into account, the sialic acid component of the granule membrane then becomes only 25% of that of the erythrocyte membrane. In contrast with the erythrocyte, therefore, chemical groups other than sialic acid appear to endow the chromaffin-granule membrane with a significant net negative charge. The existence of glycolipid-bound sialic acid residues contributing to the surface charge but not susceptible to neuraminidase attack (see Tenforde, 1970) can probably be ruled out because no additional sialic acid was liberated from either lysed granules or erythrocytes on acid hydrolysis.

Membrane proteins are characteristically high in acidic amino acids, e.g. glutamic acid, and show a lack of cysteine cross-linking (Tria & Barnabei, 1969). Amino acid analysis of the total insoluble protein of the chromaffin-granule membrane by Winkler *et al.* (1970) indicates the presence of acidic

amino acids (glutamic acid, 15%, w/w, and aspartic acid, 10% w/w), a lesser proportion of basic amino acids (16%, w/w), little cysteine (0.5%, w/w), and the remainder non-polar or neutral amino acids. The $\alpha$-carboxyl groups ($pK_a = 3.0$–$3.2$) of the protein *C*-terminal amino acids may therefore make an important contribution to the net negative charge of the granule membrane. Some contribution may also stem from the $\beta$- and $\gamma$-carboxyl groups of the acidic amino acid residues, but since these groups generally have higher $pK_a$ values (3.0–4.7) they are probably of less significance.

Of the phospholipids of the chromaffin granule (Blaschko *et al.*, 1967) phosphatidylserine is the only major component that would carry a net negative charge at pH7, due to ionization of its carboxyl group ($pK_a$ 3.0).

On balance therefore it appears that the net negative charge of the granule membrane at the surface of shear derives chiefly from a single type of chemical group, i.e. $-CO_2^-$, contributed by the $\alpha$-carboxyl group of constituent proteins, the phospholipid phosphatidylserine and, to a lesser extent, the sialic acid of glycoprotein. In this context it is noteworthy that at least three classes of surface anionic sites are indicated by analysis of the cation-binding characteristics of the chromaffin-granule membrane (P. M. Dean & E. K. Matthews, unpublished work).

There is evidence from mobility measurement in the presence of various anions that the chromaffin-granule membrane possesses only a very limited proportion of ionizable positive charges at its surface (P. M. Dean & E. K. Matthews, unpublished work). The absence of any marked inflexion in the mobility–pH curve at high pH values certainly suggests the presence of relatively few free amino groups. Any basic groups which may be inferred to be present from the reversal of charge at low pH values may be the amino groups of amino acid side chains in protein or the amino and quaternary nitrogen atoms of phospholipids. Alternatively, a positive charge density at low pH values could arise from a non-specific proton adsorption on to non-ionogenic regions of the granule membrane surface; such can occur with the erythrocyte membrane (Tenforde, 1970).

With lysed granules the increased mobility at high pH values suggests the unmasking of acid groups or the removal or occlusion, by this treatment (lysis), of basic groups. The removal or occlusion of basic groups seems the less likely suggestion. The decreased gradient of the curve at low pH values, compared with that of unlysed granules, may represent a change in the population of different types of carboxyl group contributing to the surface charge after conformational rearrangement of the membrane. The state of membrane integrity of lysed granules is unknown, but even if a proportion of the lysed granules are unsealed, groups other than those on the external surface are unlikely to make any major contribution to the surface charge and thus affect mobility. In human erythrocyte 'ghosts' the electrophoretic mobility is actually identical with that of the unlysed cells (Furchgott & Ponder, 1941).

The mean $\zeta$ potential of unlysed granules, at pH6.0 and above, corresponds to a surface charge density of $1.38 \times 10^{-6}\,C \cdot cm^{-2}$, which is equivalent to one electronic charge per 11.67nm² (1167Å²), with a charge separation of 3.85nm (38.5Å). This is close to the value of one electronic charge per 10nm² (1000Å²) and charge separation of 3nm (30Å) reported by Bangham & Pethica (1960) for other biological membranes. On this basis one granule 200nm in diameter would bear 10800 negative groups. The results of a more comprehensive analysis of particle surface charge density and its significance will be of interest.

Theoretically the electrophoretic mobility of particles with a net negative charge should be progressively decreased by the surface adsorption of counterions at the Stern plane (Davies & Rideal, 1963). In this respect polyvalent cations should be particularly effective in decreasing the particle $\zeta$ potential. Bivalent cations were clearly able to decrease the mobility of chromaffin granules in a dose-dependent fashion (Fig. 2). The tervalent $La^{3+}$ ion was even more effective in decreasing the granule charge, but the concentration/mobility regression for $La^{3+}$ and the bivalent ions is essentially parallel, suggesting a similar mode of action, i.e. surface adsorption and progressive neutralization of the net negative charge. The inhibitory effect of high pH values on a $Ca^{2+}$-induced charge decrease (Fig. 3) may indicate competition between hydroxyl groups of the bulk solution and granule binding sites for $Ca^{2+}$ ions, or, since the curves at pH7 and 9 are parallel, it may represent an increased ionization of surface anionic groups at pH9 causing a higher granule mobility at any $Ca^{2+}$ concentration. Both lysed and unlysed granules appear to possess similar $Ca^{2+}$-binding characteristics at pH7 (Fig. 3).

Banks (1966) found that the net negative charge of chromaffin granules was progressively decreased by $Ca^{2+}$ ions, but over a lower range of $Ca^{2+}$ concentration than that indicated by our experiments, i.e. 1–5mM compared with 1–50mM. However, Banks (1966) was using a different method, that of moving-boundary electrophoresis with granules suspended in an iso-osmotic solution of sucrose containing KCl, rather than particle micro-electrophoresis. The quantitative difference between our results is therefore most probably explained by the fact that in the highly concentrated dispersions required for boundary electrophoresis the effect of $Ca^{2+}$ is influenced by particle aggregation; this would lead to an underestimate of the concentration required for charge neutralization.

The neutralization of granule surface charge similarly by $Mg^{2+}$, $Ca^{2+}$ and other bivalent cations might suggest that the ionic specificity of the exocytotic granule release process is not dependent on the biophysical properties of the granule membrane, since release from intact secretory cells requires $Ca^{2+}$ but is blocked by $Mg^{2+}$. However, the inhibitory effect of $Mg^{2+}$ is observed in systems both *in vivo* and *in vitro* only with increases in the extracellular concentration of $Mg^{2+}$. It is therefore quite probable that ionic discrimination between $Mg^{2+}$ and $Ca^{2+}$ ions occurs initially at the level of the cell rather than the granule membrane. In addition, it is apparent that the intracellular concentration of ionized $Ca^{2+}$ in non-stimulated cells is of a low order of magnitude, e.g. 10$\mu$M in nerve cells (Hodgkin & Keynes, 1957), when compared with the total intracellular calcium content. The binding sites of granule and cell membrane, with the adsorption and desorption of bivalent cations, may thus present a multiequilibrium system in a dynamic state, the concentration of bound against free $Ca^{2+}$ ions being critically poised for participation in the secretory process.

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