

CONTRIBUTIONS OF LIPIDS AND PROTEINS TO THE SURFACE CHARGE OF MEMBRANES

An Electron Microscopy Study with Cationized and Anionized Ferritin

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ABSTRACT

The surface charge of cultured neurons was investigated with the electron microscope markers anionized ferritin (AF) and cationized ferritin (CF). To determine which membrane components could react with the markers, model reactions were used. Both protein-coated Sepharose beads and lipid vesicles were reacted at physiological pH. Results with these model reactions indicate that the following groups may contribute to the surface charge: acidic groups—the sialic acid of both glycoproteins and gangliosides, the carboxyl group of proteins, and the phosphates of phospholipids; basic groups—the amines of proteins.

The effect of chemical fixation on the surface charge was investigated. Glutaraldehyde fixation was shown to increase the charge of neutral proteins but not by a mechanism involving unbound aldehydes. Glutaraldehyde fixation of phospholipid vesicles in the presence of CF showed that amine-containing phospholipids were cross-linked to CF. This cross-linking was seen with the electron microscope as the clumping of CF and the burying of CF in the membrane. Paraformaldehyde fixation had a lesser effect on the charge of proteins but did react with phospholipids as did glutaraldehyde.

It is concluded that at physiological pH: (a) most of the charged proteins and lipids on cell surface can contribute to the membrane surface charge, and (b) the membrane surface charge of cells can be greatly changed by chemical fixation.

KEY WORDS cationized ferritin · anionized ferritin · membrane · surface charge · glutaraldehyde fixation

The exact composition and distribution of the molecules responsible for the net negative charge over the surfaces of cells (37, 38, 56, 63) has been the subject of increasing interest. In early studies, the charge on the surfaces of cells was determined by whole cell electrophoresis (5). With this tech-

nique a current was passed through a solution containing suspended cells, and the velocity of movement toward an electrode was used as a measure of the surface charge. Results of these studies indicated that the phosphate groups of the phospholipid might be responsible for the acidic surface charge (20, 62). Later, pretreatment of cells with neuraminidase, an enzyme that removes sialic acid, showed that the surface charge of erythrocytes was almost entirely eliminated (17, 23, 50,

51, 63). Thus, sialic acid and not phosphate appeared to be the major contributor to the surface charge. It was further suggested that the erythrocyte was a pure anion which possessed no basic groups such as amines (31, 50, 58).

Cell electrophoresis methods have several drawbacks which limit usefulness in the study of the composition of cell surface charge. First, the mobility studies measure surface charge not at the surface of the lipid bilayer but at a shear plane some distance out from the bilayer (5, 24, 55). The charged groups below the shear plane may contribute to a net surface charge but cannot be measured with electrophoretic mobility method (51). Second, determinations of the velocity at which cells move in the electrophoretic apparatus represent an average for the entire cell and do not give information about the contribution of individual groups to the net charge (19, 38, 57). Recent evidence has also shown that the surfaces of cultured cells in an electric field do not remain unaffected but rather that some of the surface groups collect on the sides of the cell toward specific electrodes (32, 45, 46). The effect of this type of redistribution of surface groups in cell electrophoresis has not been evaluated.

Cytochemical techniques were developed to give more specific information about the distribution of charged groups and their relative contribution to total membrane charge. Gasic et al. (21) used colloidal iron, both positively charged (CI pos) and negatively charged (CI neg), on fixed cells. It has been shown that neuraminidase pretreatment removes most of the CI pos binding (20, 21, 39, 40, 59, 60). These results confirmed the electrophoretic mobility studies which indicate that sialic acid contributes a major part of the surface charge of most cells. In addition, the binding of CI neg was reduced when cells were deaminated before treatment (18, 21). These results showed that some cultured cells and synaptosomes have exposed amines which may also contribute to the surface charge.

Because cell surface labeling with CI pos could only be done on cells at pH 1.8, the development of cationized ferritin (CF) by Danon et al. (15) allowed, for the first time, the localization of charged groups on living cells (7, 25-27, 36, 44, 53, 61) and cell fractions (1, 9). In most studies, the binding of CF correlated well with the presence of neuraminidase-sensitive sialic acid groups as determined with either CI pos binding or cell electrophoretic mobility. Recently, however, Ack-

erman (4) questioned the exact nature of CF binding to bone marrow cells based on inconsistencies of CF labeling and electrophoretic mobility. In addition, Wessells et al. (61) and King and Preston (33) were unable to obtain removal of CF binding with neuraminidase pretreatment.

Our preliminary results with cultures of developing neurons have indicated that CF particles on the membrane form a small ball or clump (13, 14), not the expected uniform layer of particles. The clumps of CF particles may not then accurately reflect the distribution of charged groups in living cells. The previously mentioned studies have indicated that sialic acid can bind polybasic compounds such as CF, but it could be that other acidic groups are involved. For instance, although it is known that polybasic peptides can bind electrostatically to phospholipids (28, 54), phospholipids have not seriously been considered as contributing to the surface charge.

While it has been reported that sialic acid-containing groups can bind CF, a characterization of the potential binding of CF to other charged groups has not been reported. We have studied several of the potential CF binding groups and the effect of fixation on several model systems.

MATERIALS AND METHODS

Anionized Ferritin (AP) and CF

AF was prepared by a modified procedure from Klotz (34) and Renneke et al. (48). Ferritin (Ft), 34.0 mg/ml, 2 × crystallized with trace cadmium, was obtained from Miles Laboratories Inc. (Miles Research Products, Elkhart, Ind.). In 25 ml of distilled H₂O, 25 mg of Ft was treated with 0.25 g of succinic anhydride. The succinic anhydride was added slowly to the solution at room temperature with constant stirring over 30 min while the pH of the solution was maintained above 7.0 with 1 N NaOH. If the pH dropped below 7.0, rapid and irreversible precipitation of the Ft occurred. It should also be stressed that the ratios of the amounts of H₂O, Ft, and succinic anhydride must be maintained or the Ft will precipitate before all of the succinic anhydride has been added. The reaction was complete when all of the succinic anhydride was added and the pH remained stable for 30 min. The reaction mixture was concentrated in a vacuum-colloid-bag-type protein concentrator to ~3 ml. The mixture was then dialyzed against 0.133 M NaCl and 50 μM NaOH in the cold with three changes of 1 liter each. The protein content, as determined by the Hartree (29) method, of the batch used here was 11.4 mg/ml.

Isoelectric focusing (47, 48) of AF gave a pI of between 3.4 and 3.7 as compared to a pI for Ft of between 3.8

and 4.2. The lower pI for AF confirms that succinylation reduces the number of primary amines contributing to the surface charge and increases the number of carboxyl groups (Table I).

CF, prepared by the method of Danon et al. (15), was obtained from Miles Laboratories Inc. The procedure used to prepare CF adds positively charged tertiary amines to the surface of the Ft molecule, which replace the carboxyl groups, giving the molecule a net basic charge. The isoelectric point of CF has been reported to be between 8 and 9 (15, 47) (Table I).

Both AF and Ft were spread on a coated grid and examined in an electron microscope without fixation or staining (Fig. 1). The Ft particles were distributed irregularly throughout the grid (Fig. 1A). Most of the AF particles (Fig. 1B) were distributed irregularly but many of the AF particles were aggregated into clumps (Fig. 1B, arrows). Isoelectric focusing showed that some of AF did not enter the gel, confirming the presence of some aggregated AF particles.

Cultures

The cultures of 2-d-old rat cerebellums were prepared as described by Lasher (35), and used at 7–10 d in vitro when neuronal processes had begun to form synapses (11). Unfixed cultures to be exposed to the Ft probes were rinsed twice in phosphate-buffered saline (PBS) (16) and then exposed for 5 min to the Ft probe in PBS. The culture was rinsed twice in PBS and fixed as described in the following section.

Cultures to be incubated in the Ft probes after fixation were prepared by rinsing the cultures twice in PBS and then adding the fixative. After fixation for 30 min and a buffer rinse, the cultures were rinsed three times in PBS and incubated in the Ft probe for 5 min.

Protein-Coated Beads

Cyanogen bromide-activated Sepharose-4B beads (Pharmacia Fine Chemicals, Div. of Pharmacia, Inc., Piscataway, N.J.) were coated with the following compounds: bovine serum albumin fraction V (BSA) from Miles Laboratories, histones Type II-S from Sigma

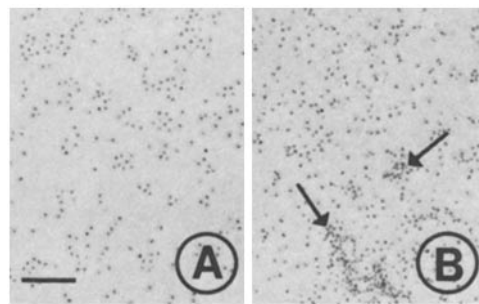


FIGURE 1 (A) Ft spread on a coated grid and examined unstained. The Ft particles distributed irregularly over the field. $\times 75,000$. Bar, 100 nm. (B) AF spread on coated grid and examined unstained. The majority of the AF particles were single; the arrows indicate presence of occasional aggregates. $\times 75,000$.

Chemical Co. (St. Louis, Mo.), and polyglutamate, mol wt 50,000, from Miles Laboratories Inc. The suspended beads were treated with the Ft probes and fixed as described below.

For quantitative analysis, micrographs were taken of beads at their largest diameters and printed at $\times 50,000$. A line was drawn 0.5 μm from the surface, and the number of Ft particles was determined over a distance of at least 1.5 μm , with 3–6 beads sampled for each mean. SEM were determined for each group.

Lipid Vesicles

The isolation of the lipids from rat brain was based on a procedure of Wood and Dawson (65). An entire adult rat brain was homogenized in chloroform-methanol (C:M) 2:1, and proteins were extracted with 1/5 vol of 0.9 NaCl. The lipids were suspended in 0.05 M Tris (pH 7.2) by shaking for 10 min, then removed. The lipid preparation was sonicated on ice with a probe sonicator for 5 min. The total phosphorus was determined according to Bartlett (6).

Lipid vesicles to be freeze-fractured were incubated in 25% glycerol for 20–30 min before freezing in Freon 12. The lipid vesicles prepared for freeze-fracture were not fixed unless indicated. Deep etching of CF-treated lipid vesicles was not possible because etching of nonglycerinated buffered vesicles gave precipitation of salts on exposed surfaces and faces. In addition, incubation of CF-aggregated vesicles in H_2O changed the binding of CF as seen by negative stain and spectrophotometry. After fracturing in a Balzers 360 (Balzers Corp., Nashua, N.H.), the replicas were floated on chlorine bleach (Purex) for 1 h, then on two changes of 20% ethanol for 5 min each and three changes of distilled water for 10 min each.

Reaction of lipid vesicles with the Ft probes was read in a spectrophotometer at 700 nm, 3–5 min after mixing. The coalescence of lipid vesicles into multilayer aggre-

TABLE I
Characterization of CF and AF

Ferritin probe	pI	Net change from native ferritin		
		Carboxyls	Primary amines	Tertiary amines
Ft (native ferritin)	3.8–4.2	—	—	—
AF	3.4–3.8	↑	↓	no change
CF	8.0–9.0*	↓	no change	↑

* From Danon et al. (15) and Rennke et al. (47).

gates can be measured by increasing the turbidity of the lipid solution (2, 3).

The following lipids were obtained from the Sigma Chemical Co.: gangliosides, Type III; phosphatidyl choline, Type V-E; phosphatidyl ethanolamine, Type III; phosphatidyl D-L-glycerol, Type I; phosphatidyl inositol, Type III; and phosphatidyl serine. The lipids were dried down with nitrogen gas and suspended in 0.05 M Tris by sonication on ice with a probe sonicator for 5 min.

Electron Microscopy

The standard glutaraldehyde fixation procedure used to prepare either the culture or the Sepharose beads was that of Burry and Lasher (11). The paraformaldehyde fixation procedure used was the same as that described above, but the glutaraldehyde was replaced with 4% paraformaldehyde. Sections were stained with uranyl acetate and lead citrate, and were carbon coated before examination with a Philips 201 electron microscope.

Freeze-Substitution of Cultures

Cultures were grown directly on 9-mm circles of 5 mil Aclar 33C (Allied Chemical Corp., Specialty Chemicals Div., Morristown, N.J.) and incubated in 0.1 mg/ml CF for time from 5 s to 5 min. After incubation, the cultures were rapidly brought into contact with the polished surface of a large copper block in liquid nitrogen (12). The cultures were transferred to 4% OsO₄ in acetone at -70°C. After 4 d the cultures were warmed, rinsed in acetone without OsO₄, and embedded in Epon 812. The sections were examined without further staining.

RESULTS

Localization of CF, AF, and Ft on Neuronal Membranes

CF and AF were bound to the surfaces of neuronal processes at physiological pH in different patterns, depending on the incubation and fixation procedures used.

CF was found primarily in spaces between adjacent cells or processes in incubations of live cultures (Fig. 2A). The CF particles were seen clearly as a line of electron-dense particles spaced equidistant between adjacent membranes. Rarely, clumps of CF particles were seen on exposed surfaces of neuronal processes (Fig. 2A, inset). These clumps varied in size from just a few to many CF particles. Isolated CF particles on exposed surfaces were rarely seen (Fig. 2A), and most of the membranes of neuronal process were free of CF particles.

Prefixed cultures incubated in CF had a pattern of CF labeling very different from that of unfixed cultures. The CF particles in these glutaraldehyde-

prefixed cultures were found to bind to exposed surfaces in a continuous, irregular layer from 1 to 5 CF particles deep (Fig. 2B). The CF particles penetrated very poorly into spaces between cells of <5 nm. Pretreatment of fixed cultures with 0.5 M NH₄Cl to cross-link unreacted aldehyde groups had no effect, and the binding pattern was the same as in glutaraldehyde-fixed cultures.

AF bound in lumps to both exposed surfaces and appositional membranes of unfixed cultures (Fig. 3). Large lumps of AF particles were seen on exposed surfaces, while in appositional spaces small lumps and single particles were found (with particles in these spaces seen more frequently than on exposed membranes). In glutaraldehyde-prefixed cultures, incubation with AF did not show binding on any of the membranes.

In unfixed cultures, Ft bound very sparsely and particles were seen too infrequently to attempt a characterization of the binding sites.

Freeze-Substitution of CF-Treated Cultures

In an attempt to determine whether fixation affected the distribution of CF bound to unfixed membranes, freeze-substitution was employed. In cultures prepared this way, sporadic but monolayer labeling of neuronal processes was seen (Fig. 4A and B). Although CF was rarely stacked two deep, no clumps were found. Thus, the appearance of clumps of CF particles on the cell membranes was found only after chemical fixation and dehydration.

Model Reactions

The results with cultured cells indicate that areas of both acidic and basic groups can be localized on the surfaces of neuronal processes. In addition, the fixation of membranes appears to change the CF-binding pattern of membranes from a monolayer to clumped pattern. Two model systems were used to investigate the character of the membrane surface groups: protein-coated Sepharose beads and lipid vesicles. By selecting a single protein or lipid, experiments could be done to determine the binding for CF and AF of the specific compound. As the investigations of CF and AF binding to cells were performed at physiological pH, the reactions with the beads and vesicles were run at pH 7.2.

AF and CF Binding to Protein and Glycoprotein

Cyanogen bromide-activated Sepharose 4B

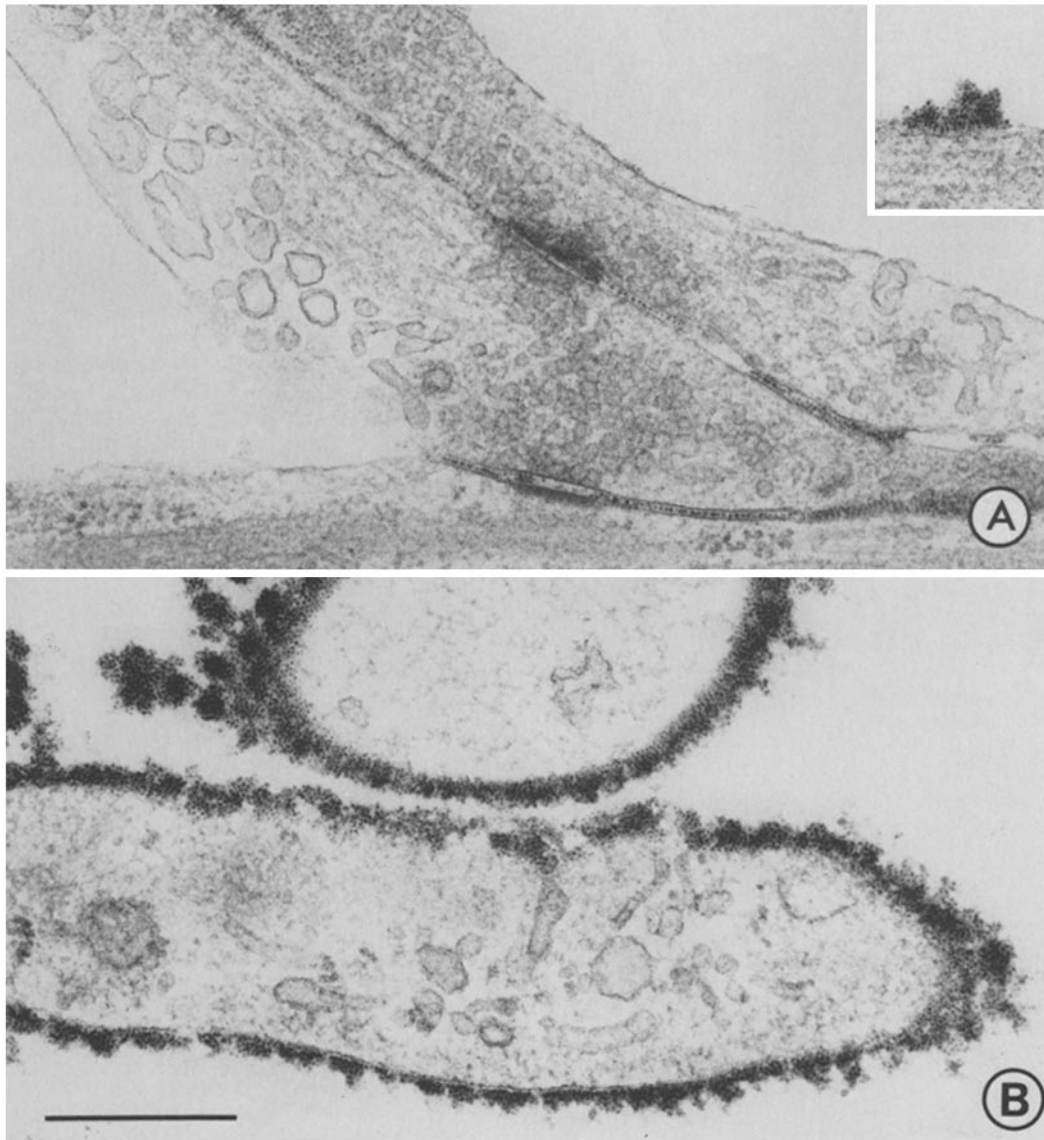


FIGURE 2 (A) Neuronal processes from a culture incubated for 5 min in 0.10 mg/ml CF and fixed for electron microscopy. The exposed surfaces were unlabeled but the CF particles were seen lined up between the neuronal processes. *Inset*: neuronal process selected to show the appearance of the rarely found clumps of CF on exposed surfaces of cell processes. $\times 50,000$. (B) Neuronal processes from a culture fixed in 1.6% glutaraldehyde and then incubated for 5 min in 0.11 mg/ml CF. All exposed surfaces were labeled with an irregular layer of CF particles. $\times 50,000$. Bar, 500 nm.

beads were used to investigate binding of AF and CF to surfaces composed of a known protein, because they can be easily prepared for electron microscopy. Beads were coated with an acidic protein (polyglutamate), a neutral protein (BSA), or a basic protein (histone).

More CF particles bound to acidic protein-coated beads than to basic protein-coated beads, and the neutral protein-coated beads bound an intermediate number of particles (Table II and Fig. 5). CF, therefore, bound to the carboxyl groups of the amino acids on exposed polygluta-

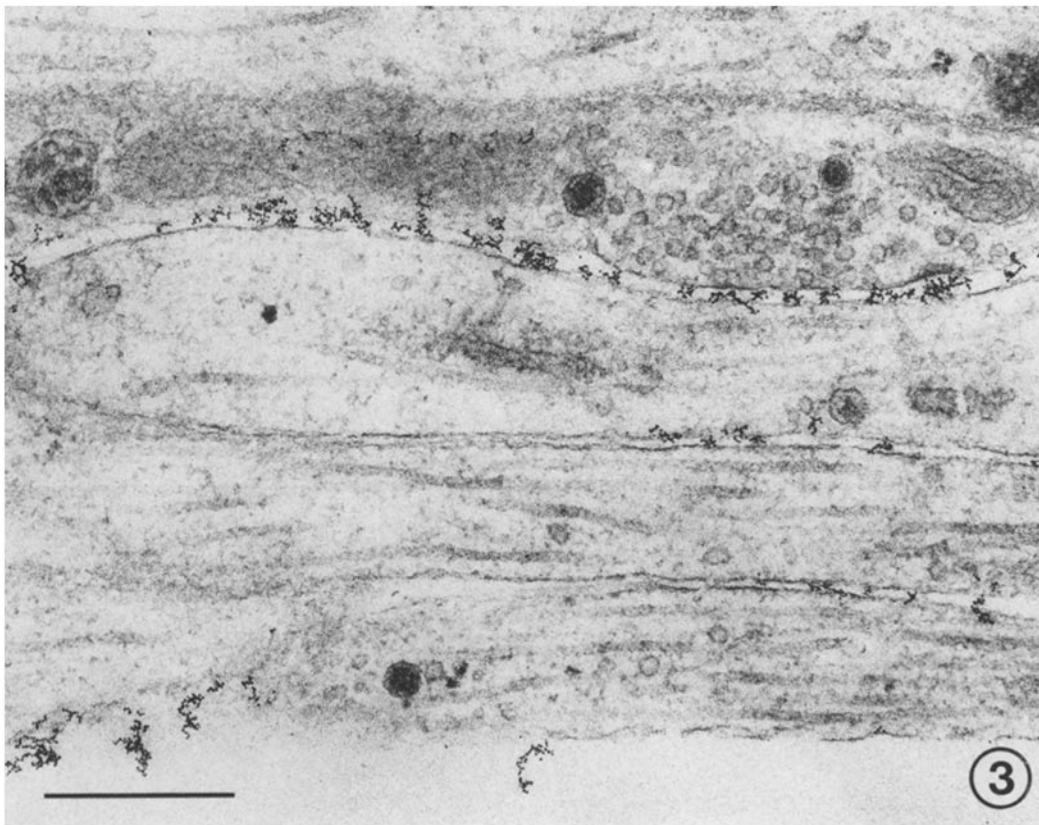


FIGURE 3 Neuronal processes from a culture incubated for 5 min in 0.10 mg/ml AF and fixed for electron microscopy. Most of AF particles were found in spaces between adjacent processes. $\times 50,000$. Bar, 500 nm.

mate molecules.

The AF particles bound to the protein-coated beads in a pattern opposite that for CF (Table II). The acidic protein-coated beads bound no AF while basic protein-coated beads bound substantial amounts of AF, because of the amine groups of the basic protein histone.

CF particles also bound to beads coated with fetuin, a sialic acid-containing protein. In this case, 271.3 ± 0.3 particles/ μm were found, suggesting that, in addition to binding to carboxyl groups of proteins, CF can bind also to carboxyls of sialic acid.

AF, CF, and Ft Binding to Lipid Vesicles

For investigation of the binding of these probes to membrane lipids, lipid vesicles were prepared from rat brain. When examined by negative stain or by freeze-fracture (Fig. 6A and B) the vesicles were seen to be lamellar bodies ranging in diam-

eter from 0.04 to 1.0 μm , with 2 mean diameter of $\sim 0.2 \mu\text{m}$. When CF was reacted with lipid vesicles, the solution became increasingly cloudy and the lipid finally formed a flocculent precipitate. Freeze-fracture of these CF-aggregated lipid vesicles (unfixed) showed no CF particles on the fracture faces (Fig. 7). With cross-fractured outer leaflets, CF particles can be seen (Fig. 7, arrowheads). The drawing (Fig. 7C) shows how CF particles (arrowheads) attached to the surface of the outer leaflet can be seen in the freeze-fracture replicas. The rim of CF particles seen where the fracture plane enters vesicles consists of CF particles attached to the outside surface of the lipid vesicle. Note that only a single layer of CF particles is seen on the outer surface of the unfixed lipid vesicles similar to the thin layer seen on unfixed freeze-substituted cells (Fig. 4).

In addition to the CF particles seen on outer exposed surfaces, CF particles were also seen be-

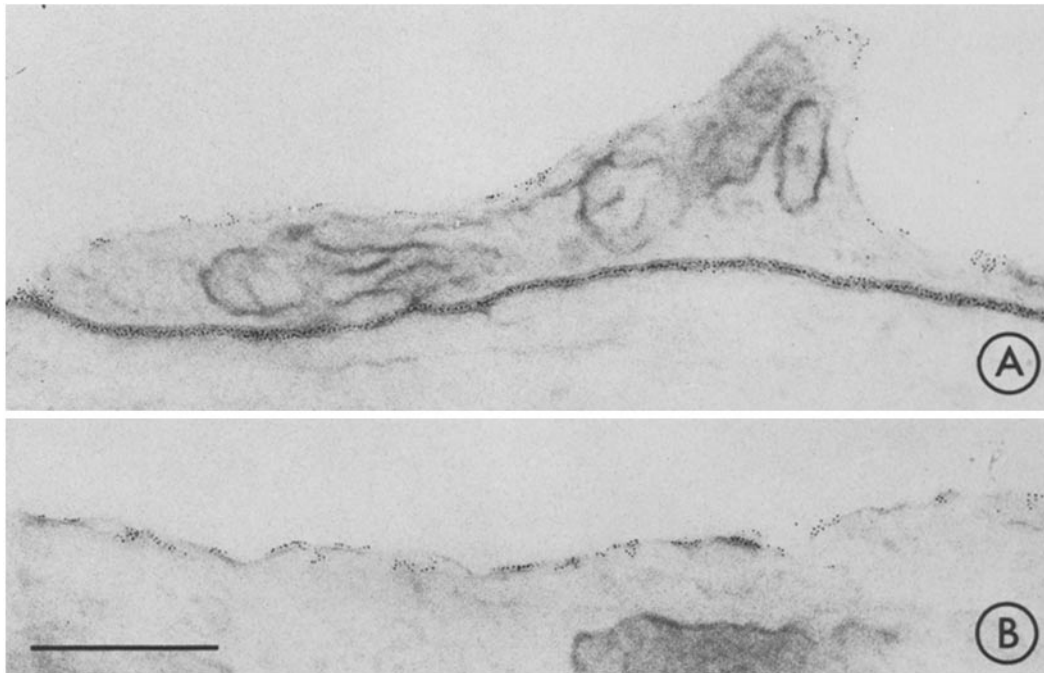


FIGURE 4 (A) Neuronal processes incubated in 0.11 $\mu\text{g}/\text{ml}$ of CF for 5 min before freeze-substitution; no glutaraldehyde fixation was used. The CF particles are distributed on exposed surfaces irregularly in a layer one or two particles thick. No clumps of CF particles were found. In spaces between adjacent cells, the CF particles are bound in a uniform row. $\times 50,000$. (B) Neuronal process incubated and prepared as described in Fig. 4A. $\times 50,000$. Bar, 500 nm.

TABLE II
Labeling of Coated Sepharose Beads

Type of ferritin*	Preparation	Beads coated by			
		Polyglutamate	BSA	Histone	Uncoated
CF	Unfixed	187.5 \pm 13.6	120.3 \pm 18.3	14.4 \pm 2.1	20.1 \pm 6.6
AF	Unfixed	0	1.8 \pm 1.0	364.2 \pm 92.0	ND
CF	Glutaraldehyde fixed	192.5 \pm 25.3	517.3 \pm 55.6	24.0 \pm 6.8	21.2 \pm 2.7
CF	Paraformaldehyde fixed	173.3 \pm 25.3	243.0 \pm 45.7	42.5 \pm 14.7	ND
CF	Osmium tetroxide fixed	274.8 \pm 26.0	406.5 \pm 10.5	306.0 \pm 30.4	130.7 \pm 10.2

Values represent the number of ferritin particles per micrometer of linear surface, counted 0.5 μm into the bead. The numbers are means of 3–8 beads \pm SEM. ND indicates reaction not done.

* CF applied at 0.11 $\mu\text{g}/\text{ml}$ for 5 min. AF applied at 0.34 $\mu\text{g}/\text{ml}$ for 5 min.

tween adjacent bilayers of lipid (Fig. 7, arrows). These CF particles bound to the adjacent bilayer surfaces were also seen only in cross-fracture. The drawing (Fig. 7C) shows how CF particles (arrows) between lipid bilayers can be seen.

When lipid vesicles were treated with either AF or Ft, no changes were found either in the turbidity of the solution or in the electron microscope ap-

pearance of the lipid vesicles from untreated lipid vesicles.

As seen in Fig. 8A, the OD of the lipid solution increased as more CF was added. When lipid vesicles were titrated with HCl (Fig. 8B) an increase in OD occurred only when the pH was below 3.0. Precipitation occurred at a pH of 1.9, suggesting that the titrated acid groups had a pK

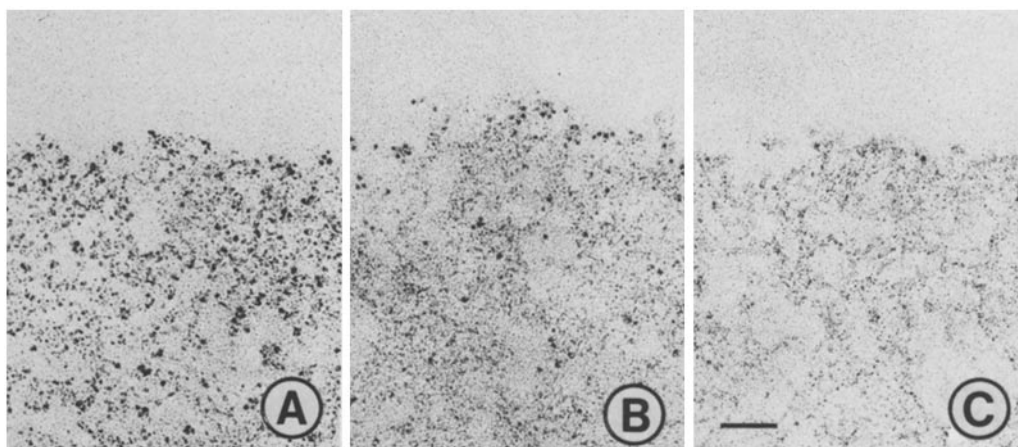


FIGURE 5 (A) Polyglutamate-coated Sepharose bead reacted for 5 min with 0.10 mg/ml CF and fixed for electron microscopy. Beads prepared in this manner had a mean of 223 CF particles/ μm of length. $\times 75,000$. (B) BSA-coated Sepharose bead reacted for 5 min with 0.10 mg/ml CF and fixed for electron microscopy. Beads prepared in this manner had a mean of 85 CF particles/ μm of length. $\times 75,000$. (C) Histone-coated Sepharose bead reacted for 5 min with 0.11 mg/ml CF and fixed for electron microscopy. Beads prepared in this manner had a mean of 18 CF particles/ μm of length. $\times 75,000$. Bar, 100 nm.

of ~ 2.0 . After adjustment of the pH of the lipid vesicles to 3, the addition of even 3 μg of CF caused precipitation of the lipid vesicles. The lipid vesicles were also reacted with Ca^{++} (Fig. 8C). Addition of Ca^{++} caused a sharp rise in OD with aggregation of the vesicles. Freeze-fracture showed that the Ca^{++} -aggregated vesicles had no particles.

Fixation of Protein-Coated Beads

When glutaraldehyde-fixed beads were incubated with CF, a sixfold increase was found in the binding of CF particles to neutral beads, while the acidic and basic protein-coated beads showed no significant change (Table II). To determine whether this increase resulted from free aldehyde cross-linking CF to the BSA-coated bead, three different blocking amines were reacted with glutaraldehyde-fixed beads before incubation in CF. As seen in Table III, these compounds had no effect on the number of CF particles. Fixation with paraformaldehyde before incubation with CF produced a slight increase in labeling of neutral protein-coated beads (Table II). These results confirm those found with fixed cultures that were pretreated with NH_4Cl .

Osmium tetroxide (OsO_4) fixation changed the pattern of CF binding to the beads (Table II). In control uncoated beads, an increased amount of CF binding occurred, indicating that some component of the Sepharose bead bound the OsO_4 and

increased the acidic charge of the bead. One mechanism of OsO_4 adding across double bonds leaves an exposed oxygen which may act as an acidic group (30). Both neutral and basic protein-coated beads showed strong increases in CF binding over uncoated beads, indicating an additional reaction of OsO_4 with these proteins.

These results suggest that (a) glutaraldehyde fixation may change the tertiary structure of at least neutral proteins, exposing more acid groups; (b) paraformaldehyde fixation had slight effect on the charge of the proteins; and (c) OsO_4 fixation added acid charges to some of the proteins.

Fixation of Lipid Vesicles in the Presence of AF, CF, or Ft

To determine the effect of fixation on the interaction of phospholipids with the Ft probes, lipid vesicles from the rat brain were reacted, in the presence of glutaraldehyde, with AF, CF, or Ft. For reference, Fig. 9A shows the reaction of these three Ft probes with rat brain lipid vesicles without glutaraldehyde. When glutaraldehyde (1.6% final concentration) was added to the lipid vesicles for 30 min before the reaction, all of the probes reacted with the vesicles (Fig. 9B). Glutaraldehyde up to a concentration of 10% had no effect on the turbidity of the lipid vesicle solution alone. The AF particles reacted as strongly as the CF particles, and even the Ft particles showed some in-

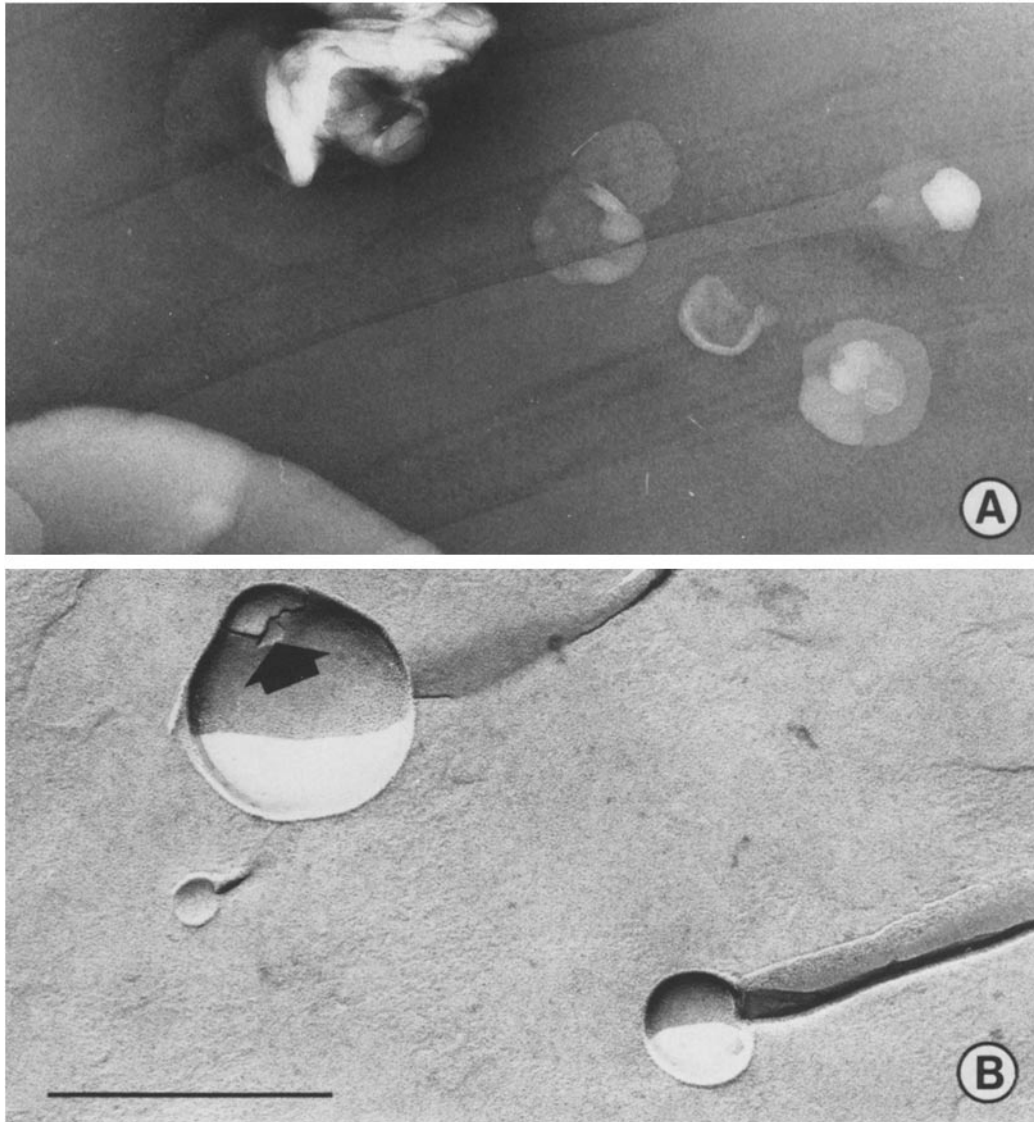


FIGURE 6 (A) Lipid vesicles prepared from the lipid fraction of adult rat brain. The lipid vesicles were negatively stained with 2% phosphotungstic acid. The vesicles ranged in diameter from 0.05 to 1 μm with a medium diameter of $\sim 0.2 \mu\text{m}$. Some multilayered vesicles were present. $\times 75,000$. (B) Lipid vesicles prepared as in Fig. 6A but examined with freeze-fracture. The shadowing was from the bottom of this and all freeze-fracture micrographs. Arrow indicates cross-fracture of membrane layers. $\times 75,000$. Bar, 500 nm.

crease in turbidity. This order of reactivity does not correlate with the charge of the Ft probes (i.e., Fig. 10A; Table I) but does correlate with the number of primary amines available to react with glutaraldehyde (Table I).

Freeze-fracture of glutaraldehyde-CF-precipitated lipid vesicles (Fig. 10) revealed several

changes from unfixed lipid vesicles precipitated with CF (Fig. 7). No CF particles were seen on cross-fractured membrane surfaces in the glutaraldehyde- and CF-treated lipid vesicles. The rim of CF particles observed around the fractured edge of lipid vesicles was never seen. Many CF particles were seen in clumps between aggregated lipid

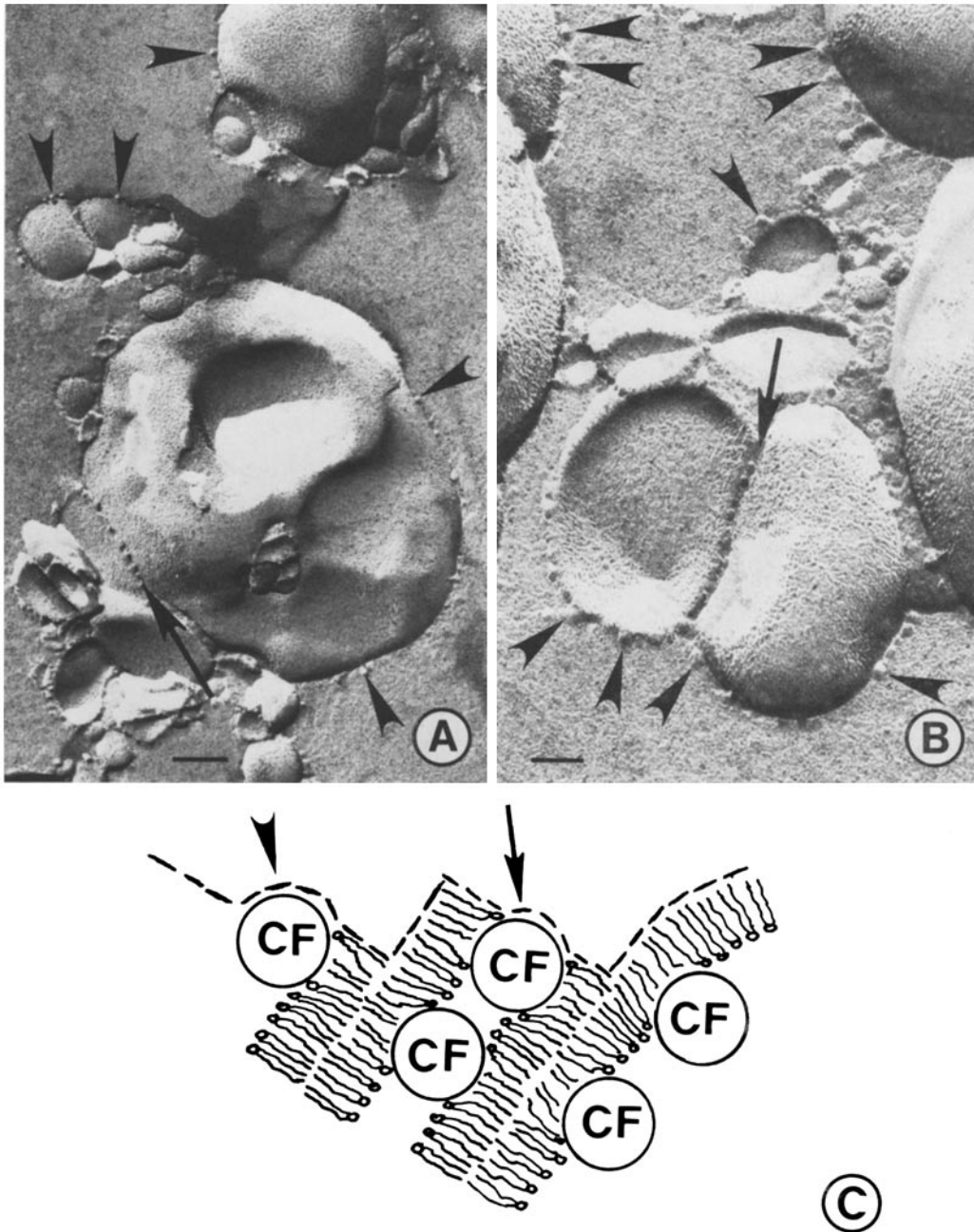


FIGURE 7 (A) CF-aggregated lipid vesicles treated with 25% glycerol but not fixed in preparation for freeze-fracture. The arrowheads indicate CF particles present on surfaces of the aggregate. The arrows indicate CF particles present between layers of the lipid bilayer. Shadowing was from the bottom of the micrograph. $\times 75,000$. Bar, 100 nm. (B) CF-aggregated lipid vesicles. $\times 150,000$. Bar, 50 nm. (C) Schematic drawing of the proposed relationship between the unfixed lipid vesicle membrane and CF. A fracture plane is indicated by the dashed line. The arrowhead indicates a CF particle attached to the outer surface of the membrane and seen where the outer leaflet of the bilayer was cross-fractured. The arrow shows a CF particle bound by two adjacent bilayers. The lipids in the bilayer are indicated by the circles (lipid head groups) and the wavy lines (lipid tails).

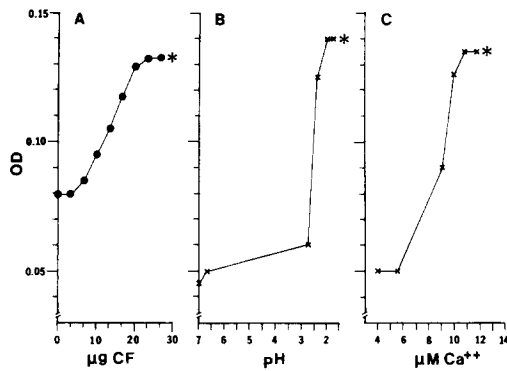


FIGURE 8 (A) Graph of the reaction of 0.15 $\mu\text{mol}/\text{ml}$ of phospholipid vesicles with CF. The star (*) indicates formation of a flocculent precipitate. (B) Graph of the change in OD with decrease in pH with 0.15 $\mu\text{mol}/\text{ml}$ of phospholipid vesicles in 0.05 M Tris buffer, pH 7.3. The pH was reduced with 0.5 M HCl. The star (*) indicates formation of a flocculent precipitate. (C) Graph of change in OD with increase in Ca^{++} . The lipid vesicles were present with 0.15 $\mu\text{mol}/\text{ml}$ of phospholipid in 0.05 M Tris buffer pH 7.3. The Ca^{++} ion was added as CaCl_2 . The star (*) indicated formation of a flocculent precipitate.

TABLE III
Effect of Postfixation Treatment on CF Binding to BSA-Coated Beads

Incubation	Ferritin labeling
None	478.3 \pm 36.3
1 M NH_4Cl	465.0 \pm 27.7
1 M lysine	433.3 \pm 76.5
1 M ethanolamine	515.0 \pm 5.6

BSA-coated beads were fixed in glutaraldehyde and incubated as indicated above. The ferritin labeling is the mean of 3–6 beads as determined in Table I.

vesicles (Fig. 10, open arrows). These clumps of CF particles were seen only in glutaraldehyde-treated lipid vesicles and not in unfixed lipid vesicles. These results correspond to those obtained with the fixed and unfixed (freeze-substituted) cultures.

In addition, CF particles were now seen as particles in the fracture face of the lipid vesicles (Fig. 10, arrowheads). In freeze-fracture replicas of the lipid vesicles, particles were not seen on the fracture faces except for glutaraldehyde-CF-treated lipid vesicles. As seen in the drawing (Fig. 10D, arrowhead), the CF particles were embedded in the lipid bilayer after the glutaraldehyde treatment.

Individual purified phospholipids and ganglio-

sides were also tested, in the presence of glutaraldehyde, for reaction with the probes. Glutaraldehyde by itself had no effect on the turbidity of any of the lipids. As seen in Fig. 11A–C, gangliosides and the phospholipids with no primary amine did not react with the probes in the presence of glutaraldehyde. The reaction of phosphatidyl serine vesicles (Fig. 11D) with CF were similar with or without glutaraldehyde.

Phosphatidyl ethanolamine did not react with AF or Ft in the presence of glutaraldehyde (Fig. 11E), but the reaction of this lipid with CF in the presence of glutaraldehyde was greatly increased. Phosphatidyl choline, while reacting with CF without glutaraldehyde, did not react with CF, AF, or Ft in the presence of glutaraldehyde (Fig. 11F).

Paraformaldehyde (4%) fixation was also tested, and the reaction pattern with the rat brain lipid vesicles was the same as that for glutaraldehyde (Fig. 9B).

DISCUSSION

Membrane Components Contributing to the Surface Charge

The results presented here show that, besides the carboxyl and the amine groups, the phosphate groups on cell membranes should be considered

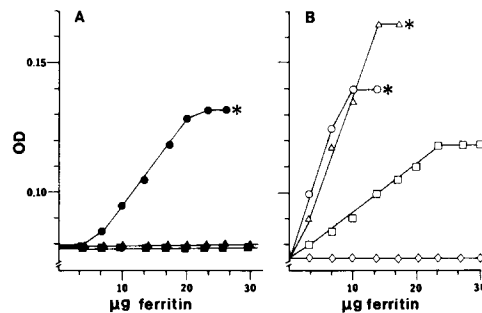


FIGURE 9 (A) Graph of the reaction of rat brain lipid vesicles with three ferritin probes. The lipid vesicles were present at 0.15 μmol of phospholipid/ml. AF (\blacktriangle), CF (\bullet), and Ft (\blacksquare) were added and the change in OD was followed. The star (*) indicates formation of a flocculent precipitate. (B) Graph of the reaction of lipid vesicles with the three ferritin probes in the presence of 1.6% glutaraldehyde. The lipid vesicles were present at 0.15 μmol of phospholipid/ml. AF (\triangle), CF (\circ), and Ft (\square) were added and the change in OD was followed. The star (*) indicates formation of a flocculent precipitate. Phospholipid and glutaraldehyde without the ferritin probes showed no reaction (\diamond).

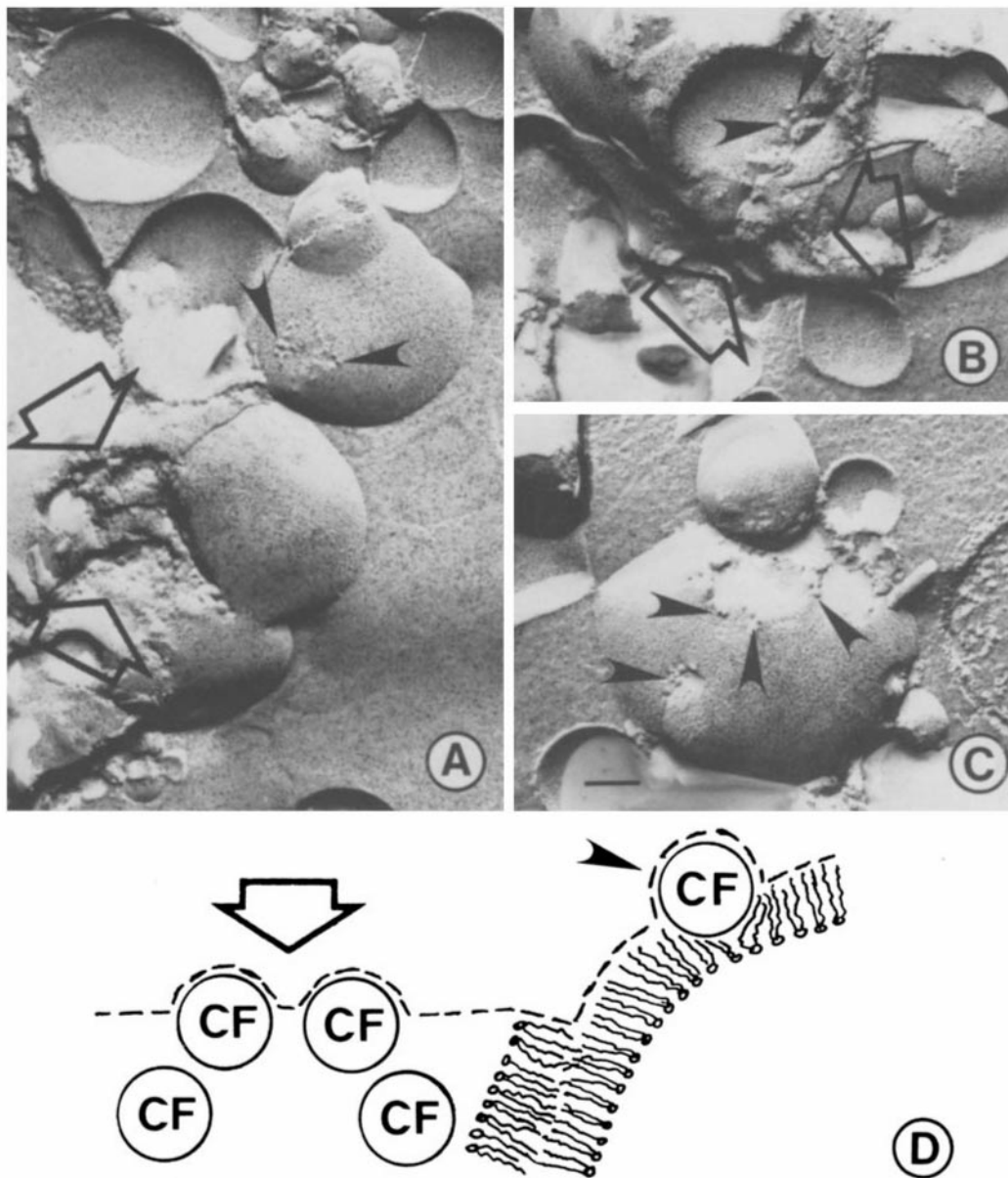


FIGURE 10 (A) Glutaraldehyde-fixed CF-aggregated lipid vesicles treated with 25% glycerol for freeze-fracture. The open arrows indicate CF particles precipitated between lipid vesicles. The arrowheads indicate CF particles that were found in the fracture plane. No CF particles were seen on exposed surfaces. $\times 75,000$. (B) Glutaraldehyde-fixed CF-aggregated lipid vesicles as in Fig. 10A. $\times 75,000$. (C) Glutaraldehyde-fixed CF-aggregated lipid vesicles as in Fig. 10A. $\times 75,000$. Bar, 100 nm. (D) Schematic drawing of the relationship between the glutaraldehyde-treated lipid vesicle membranes and CF. A fracture plane is indicated by the dashed line. The arrowhead indicates a CF particle seen in the fracture face of the lipid bilayer. The open arrow indicates CF particles aggregated into a clump between lipid bilayers. The lipids in the bilayer are indicated by the circles (lipid head groups) and the wavy lines (lipid tails).

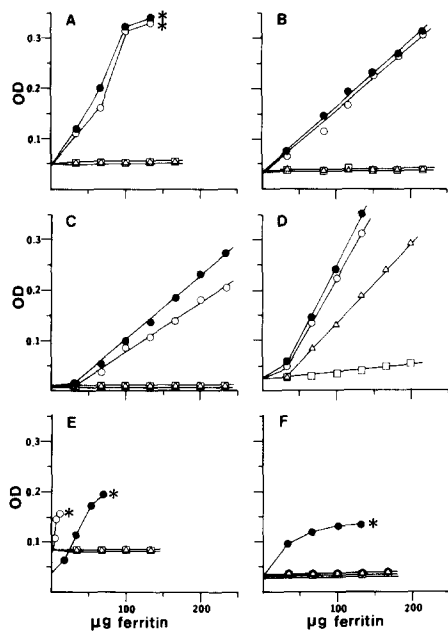


FIGURE 11 Graph of reactions of individual lipids with the three ferritin probes. The following symbols apply to all graphs. ●-CF no glutaraldehyde, ○-CF plus glutaraldehyde, △-AF plus glutaraldehyde, □-Ft plus glutaraldehyde, *-formation of a flocculent precipitate. (A) Phosphatidyl glycerol vesicles, 0.33 µg/ml. The presence of glutaraldehyde did not affect the reaction of CF with the vesicles. (B) Phosphatidyl inositol vesicles, 0.17 µg/ml. The presence of glutaraldehyde did not affect the reaction of CF with the vesicles. (C) Ganglioside vesicles, 0.33 µg/ml. The presence of glutaraldehyde did not affect the reaction of CF with the vesicles. (D) Phosphatidyl serine, 0.33 µg/ml. In the presence of glutaraldehyde, both AF and Ft reacted with the vesicles. The reaction with CF was unchanged by glutaraldehyde. (E) Phosphatidyl ethanolamine, 0.08 µg/ml. In the presence of glutaraldehyde, the reaction of CF was greatly increased. (F) Phosphatidyl choline, 0.17 µg/ml. In the presence of glutaraldehyde, CF no longer reacted with the vesicles.

as potentially contributing to the surface charge of cells at physiological pH.

Reactions of lipid vesicles with CF showed that individual phospholipids can bind these basically charged particles. In the lipid vesicles made with either phosphatidyl glycerol or phosphatidyl inositol, the phosphate group represents the only charged molecule in the head groups that could react with CF. With phosphatidyl ethanolamine, despite the presence of both a charged amine and phosphate in the head group, the lipid binds basically charged CF. Stollery and Vail (54) found

that phosphatidyl ethanolamine vesicles were weakly acidic and electrostatically bound polybasic proteins. The study of Phillips et al. (43) has shown that the terminal amine portion of the head group lies parallel to the plane of the membrane. This orientation could expose the phosphate group to extracellular ions and other charged groups. Phosphatidyl choline reacts in a limited manner with CF, indicating that its phosphate group is only partially accessible. In spite of the perpendicular orientation of the choline head groups with respect to the lipid bilayer, several authors (10, 43, 52) have shown that positively charged ions can induce changes in the orientation of the choline. The choline head group is not rigid, but is capable of bending to expose the phosphate groups. Phosphatidyl serine possesses, in addition to the phosphate group, a carboxyl and a primary amine. The binding of CF to this lipid could be via either the carboxyl group of the serine or the phosphate.

Effect of Glutaraldehyde on the Surface Charge Contribution by Proteins

In electron microscope analysis of surface charge using glutaraldehyde-fixed cells, the effect of the fixation procedure has been minimized by the use of free amines to inactivate unreacted aldehyde groups (25, 61). The effect of glutaraldehyde fixation on membrane protein surface charge has been assumed to be only that of adding free aldehydes.

Results presented in Tables II and III show that this assumption is not true. The number of CF particles that bound glutaraldehyde-fixed BSA-coated beads was four times that for unfixed BSA-coated beads. To determine whether this increase was a result of unreacted aldehydes binding CF to the fixed BSA-coated bead, the beads were treated with several different amines after fixation. As seen in Table III, this treatment had no effect on the number of CF particles that were bound to the fixed BSA-coated beads. These results are similar to those found for CF binding to fixed cells. Thus, the enhanced CF binding to fixed protein was not caused by aldehyde cross-linking of CF.

A second explanation might be that the glutaraldehyde cross-linked amines on the BSA were no longer contributing to the surface charge, and thus that the acid groups present were predominant. This explanation also is probably not valid. Bishop and Richards (8) found that glutaraldehyde bound to 86% of the alpha-lactoglobulin lysine residues

but did not affect the apparent pK of the molecule. Thus, although glutaraldehyde reacts with primary amines, it has a minimal effect on the pK of these charged groups and reduces the pK by a maximum of one or two pH units. Evidence for this conclusion from the present study can be seen in Table II where glutaraldehyde increased the binding of CF to BSA-coated beads but did not affect the binding of CF to histone-coated beads.

The most likely explanation for the increased binding of CF to BSA-coated beads after glutaraldehyde fixation involves the addition of new acid groups to the exposed surface of the molecule. In cross-linking protein, glutaraldehyde may alter the tertiary structure, exposing more acidic groups than were present before fixation. Thus, it is not possible to assume that the surface charges both of the unfixed membranes and of the glutaraldehyde-fixed membranes are the same. Glutaraldehyde has the potential for changing the surface charge of membranes.

In paraformaldehyde-fixed beads, only small differences with BSA-coated beads were seen in the CF binding patterns with either fixed or unfixed beads. Thus, paraformaldehyde fixation probably has a less drastic effect on protein molecules and alters their tertiary structure minimally. This explanation is supported by current immunocytochemical methods which employ paraformaldehyde fixation because it has a lesser effect on the antigenicity of proteins than does glutaraldehyde.

OsO₄ fixation involves addition of the molecule across a double bond, with a single oxygen molecule remaining unreacted (30). Thus, it is possible that the increased number of acidic groups observed in OsO₄-fixed beads (Table II) may result from the presence of the unreacted oxide which can bind CF.

Effect of Glutaraldehyde on the Surface Charge Contribution by Phospholipids

Several authors (22, 41, 49, 64) have shown that some phospholipids cannot be extracted from glutaraldehyde-fixed nervous tissue. The phospholipids that were retained in the fixed tissue were those with primary amines, phosphatidyl ethanolamine, and phosphatidyl serine. Our results show that both of these phospholipids had increased reactivity with the Ft probes in the presence of glutaraldehyde.

Phosphatidyl serine reacted with both AF and Ft, but only in the presence of glutaraldehyde,

while the CF reaction with these lipid vesicles was unaffected by glutaraldehyde. The phosphatidyl ethanolamine reaction with CF was greatly enhanced in the presence of glutaraldehyde, while AF and Ft showed no reaction. Phosphatidyl choline reacted with CF without glutaraldehyde but failed to react when glutaraldehyde was present. This pattern of reactivity of glutaraldehyde with the different lipids and Ft probes confirms the multiplicity of reactions available with glutaraldehyde (42). Despite the lack of a simple explanation for these results, they do show that glutaraldehyde can change the way that CF interacts with membrane lipids during fixation. Thus, even if CF were bound electrostatically to the lipids before addition of glutaraldehyde and paraformaldehyde, it is possible that aldehyde fixation after CF incubation would alter the distribution of particles.

With rat brain lipid vesicles incubated in CF, single particles were seen on outer membrane surfaces (Fig. 7). When the lipid vesicles were treated with glutaraldehyde in the presence of CF, no CF particles were found on outer membrane surfaces (Fig. 10). In these fixed vesicles some of the CF particles were found in the fracture faces of the lipid vesicle membranes, indicating that the CF particles were now seen as particles within the membrane bilayer (Fig. 10D). While CF particles may have reacted electrostatically with the lipid head groups before fixation, the addition of glutaraldehyde can evidently change the reaction mechanism. Because CF particles were found in the fracture faces, the CF particles were now probably covalently cross-linked to some of the lipids by the glutaraldehyde.

The rough irregular regions seen in freeze-fracture of glutaraldehyde-fixed CF lipid may represent regions of lipids cross-linked to CF. The clumps of CF particles seen on exposed surfaces of CF-incubated fixed cells may also represent clumps of lipid cross-linked to CF. Additional evidence for this interpretation comes from the freeze-substitution results. In cultures incubated and frozen but with no glutaraldehyde fixation, clumps of CF particles were not found on the exposed surface.

The results support the conclusion that CF particles bound to unfixed cell membranes were aggregated into clumps by subsequent glutaraldehyde fixation.

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