THE DISTRIBUTION OF ANIONIC SITES ON THE SURFACES OF MITOCHONDRIAL MEMBRANES

Visual Probing with Polycationic Ferritin

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ABSTRACT

Polycationic ferritin, a multivalent ligand, was used as a visual probe to determine the distribution and density of anionic sites on the surfaces of rat liver mitochondrial membranes. Both the distribution of bound polycationic ferritin and the topography of the outer surface of the inner mitochondrial membrane were studied in depth by utilizing thin sections and critical-point dried, whole mount preparations for transmission electron microscopy and by scanning electron microscopy. Based on its relative affinity for polycationic ferritin, the surface of the inner membrane contains discrete regions of high density and low density anionic sites. Whereas the surface of the *cristal membrane* contains a low density of anionic sites, the surface of the *inner boundary membrane* contains patches of high density anionic sites. The high density anionic sites on the inner boundary membrane were found to persist as stable patches and did not dissociate or randomize freely when the membrane was converted osmotically to a spherical configuration. The observations suggest that the inner mitochondrial membrane is composed of two major regions of anionic macromolecular distinction. It is well-known that an intermembrane space exists between the two membranes of the intact mitochondrion; however, a number of *contact sites* occur between the two membranes. We determined that the outer membrane, partially disrupted by treatment with digitonin, remains attached to the inner membrane at these contact sites as inverted vesicles. Such attached vesicles show that the inner surface of the outer membrane contains anionic sites, but of decreased density, surrounding the contact sites. Thus, the intermembrane space in the intact mitochondrion may be maintained by electrostatic repulsion between the apposed electronegative surfaces of the two mitochondrial membranes. The distribution of anionic sites on the outer surface of the outer membrane is random. The nature and function of fixed anionic surface charges and membrane contact sites are discussed with regard to recent reports relating to calcium transport, protein assembly into mitochondrial membranes, and membrane fluidity.

Isolated rat liver mitochondria as well as preparations of inner and outer mitochondrial membranes have previously been shown to migrate toward the anode in an electrical field (2, 14). Such electro-

phoretic mobility of biological membranes is due primarily to their net electronegative or anionic fixed surface charges. Electrophoretic mobility provides important data from which the relative net surface charge can be estimated but does not indicate the arrangement or distribution of charges on membrane surfaces (17, 22).

The density distribution of anionic charges on the surfaces of mitochondrial membranes is unknown, i.e., whether the anionic charges are random or nonrandom, single or clustered. Indeed, such charges may be confined to specific regions on the membrane surface in an anisotropic distribution. Further, high density anionic regions may be interspersed with low density anionic regions on the surfaces of the two mitochondrial membranes.

Determination of the density distribution of anionic surface charges may help to resolve the function of such charges. Depending on their relative distributions, affinities, and densities, fixed anionic surface charges may play a role in contributing to mitochondrial transmembrane potential during oxidative phosphorylation, in cation binding during ion transport, and in the recognition or interaction between the two mitochondrial membranes. Such functional possibilities depend also on the nature of the anionic charges. At physiological pH, the surface anionic charge of mitochondrial membranes can be accounted for, at least in part, by the free carboxyl groups of sialic acid of membrane sialoglycoproteins (2, 6, 20). Surface anionogenic groups may also be represented by exposed free carboxyls of glutamic and aspartic acids of membrane proteins and glycoproteins as well as free carboxyls of glycolipids.

In the present study we have used polycationic ferritin as a multivalent ligand to visually probe for membrane surface anionic sites by electron microscopy using thin sections and critical-point dried, whole mount preparations of mitochondrial membranes. Polycationic ferritin binds electrostatically to available anionic charges on membrane surfaces (5). An advantage of this probe over other electron-opaque probes is that it binds to anionic sites on surfaces of either glutaraldehyde-fixed or unfixed membranes at physiological pH. The ability of the probe to resolve membrane anionic sites is enhanced by the constancy of its size, its monomeric form below pH 9, and the constancy of charge density on its surface for any given pH. At physiological pH, polycationic ferritin has a high electropositive charge density of approximately 250 per molecule; thus regions on membrane surfaces containing the highest density of anionic sites will show the highest affinity for the probe.

We have focused our attention on an analysis of the density distribution of electronegative sites on both the inner and outer mitochondrial membranes at physiological pH. Priority has been given to the distribution of anionic sites on the apposing surfaces of the inner and outer membranes. This question is of interest in light of the intermembrane space normally found in the intact mitochondrion and the observation that *contact sites*, or points of contact, occur between the two mitochondrial membranes (10).

In addition, it has been suggested that a molecular and functional distinction may exist between the two major morphological regions of the inner mitochondrial membrane (1, 3, 23). These two regions are the morphologically distinct *cristal membrane* and the *inner boundary membrane*, the latter in parallel with and often in contact with the outer membrane at contact sites. Thus, we have also undertaken to compare the distribution of anionic sites on the cristal membrane with the distribution of anionic sites on the inner boundary membrane. Any differences in the relative density distributions of fixed anionic sites between these two regions of the inner membrane would represent a difference in the macromolecular structure of the two regions.

A subsequent communication will deal with the metabolic effects of binding polycationic ferritin to anionic sites on the inner mitochondrial membrane.

MATERIALS AND METHODS

Membrane Preparations

Liver mitochondria were isolated from male Sprague-Dawley rats by a sucrose-mannitol-HEPES-bovine serum albumin (BSA) method, and the subsequent removal of the outer mitochondrial membrane and purification of the mitoplast fraction was carried out by use of a controlled digitonin incubation (12). For purified mitoplasts 0.75% digitonin was used for 15 min in order to remove most of the outer membrane.

Mitochondrial preparations showing only partial disruption, but not removal, of the outer membrane were prepared as were mitoplasts except that a lower concentration of digitonin (0.3%) was used in the 15-min incubation. Swollen, purified mitoplasts were prepared by titrating the mitoplasts with water to a 20 mosM concentration (12).

Metabolic intactness of mitochondria and mitoplasts was determined by monitoring electron transport as a

function of oxygen disappearance (8). Purified mitoplast fractions contained approximately 10% of the total monoamine oxidase with a specific activity of 1.8 (nanomoles benzylamine oxidized/minute per milligram protein). Purified mitoplasts showed adenoside diphosphate: oxygen ratios of 1.7 to 2 with succinate as respiratory substrate and acceptor control ratios of 1.5 to 1.8. Protein was determined by a biuret reaction (16).

Binding Procedures

Polycationic ferritin (5) was added to unfixed mitochondrial membranes at 4°C while mixing rapidly for 5 or 15 s. The reaction was then either stopped by a 30-fold dilution or left to incubate for an additional 10 or 15 min and then stopped by dilution. The reaction medium and dilution medium consisted of 225 mM mannitol, 70 mM sucrose, and 2 mM HEPES buffer pH 7.4. After stopping the reaction, the mixture was centrifuged at 9,800 *g* for 10 min, resuspended, and centrifuged again. Microsamples of 2–5 μl were then fixed by centrifuging through 2% glutaraldehyde, pH 7.4, into micropellets (8).

Binding with polycationic ferritin was also carried out on prefixed mitochondrial membranes. The membranes were first fixed in 1% glutaraldehyde, pH 7.4, in suspension for 30 min at 4°C. Ammonium chloride was then added to a final concentration of 0.1 M to block any unreacted aldehyde groups on the membrane-bound glutaraldehyde. The membranes were washed by centrifuging and resuspending twice. The binding with polycationic ferritin was then carried out for short or long periods as with unfixed membranes. Native ferritin and polycationic ferritin were purchased from Miles Laboratories Inc., Miles Research Div., Elkhart, Ind.

Electron Microscopy

For thin sections, the glutaraldehyde-fixed preparations were postfixed for 1 h in phosphate-buffered 2% osmium tetroxide, pH 7.4, as described previously (12). Gray sections of Epon-embedded micropellets were cut on diamond knives and stained for 5 min in saturated uranyl acetate in 50% ethanol and then stained for 4 min in lead hydroxide. Thin sections were photographed with a JEOL 100B electron microscope operated at 60 kV.

Glutaraldehyde-fixed mitoplasts, bound or unbound with polycationic ferritin, were critical-point dried in preparation for scanning and transmission electron microscopy. A drop of fixed mitoplasts was placed on Formvar-coated copper grids and left at 4°C for approximately 1 h, during which time the mitoplasts became anchored to the Formvar. The preparations were then serially dehydrated through 100% acetone, and critical-point dried in a Sorvall apparatus (DuPont Instruments, Sorvall Operations, Newtown, Conn.) (18). Critical-point dried mitoplasts were then viewed and photographed, without further treatment, at 100 kV with a JEOL 100B transmission electron microscope. Other specimens of critical-point dried mitoplasts were coated with palladium-gold and photographed with a JEOL JSM-U3 scanning electron microscope operated at 25 kV with a 200-μm objective aperture.

RESULTS

Anionic Sites on the Outer Surface of the Outer Mitochondrial Membrane

Various concentrations of polycationic ferritin were used to determine the distribution of anionic sites on the outer surface of the outer mitochondrial membrane. For this analysis, glutaraldehyde-fixed, freshly isolated, intact mitochondria were used. Fig. 1 shows the typical condensed configuration of isolated rat liver mitochondria (8, 9). Contact sites between the inner boundary membrane and outer membrane were a typical occurrence. Such points of contact average 150 per mitochondrion and are found in all mitochondria after glutaraldehyde or osmium tetroxide fixation as well as in unfixed freeze-cleaved preparations (10, 12). The inner boundary membrane and cristal membrane were clearly distinguishable (Fig. 1).

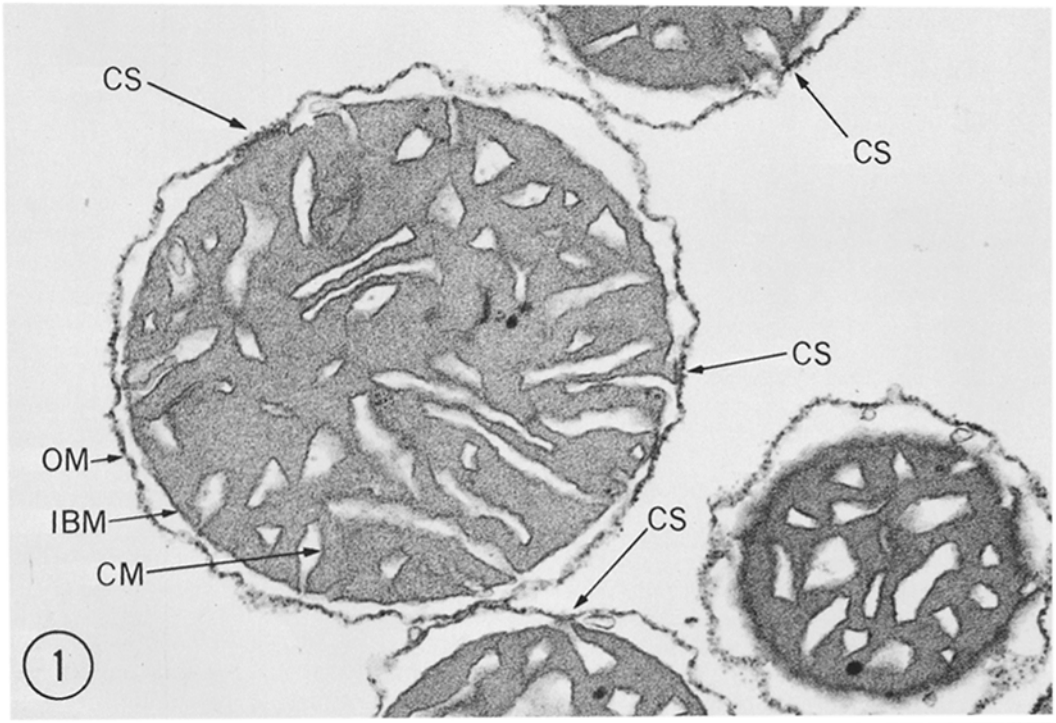
Polycationic ferritin was observed on the outer membrane surface after 5 s of rapid mixing (Fig. 1). The concentration of ligand used in these experiments was 60 μg/mg mitochondrial protein. This concentration led to the binding of the available anionic sites on the surface of the outer membrane and showed such sites to have a random distribution (Fig. 1). Higher concentrations of polycationic ferritin or longer reaction times gave similar results with respect to distribution and density, while lower concentrations gave a random distribution of lower density.

These observations indicate that the outer surface of the outer mitochondrial membrane contains anionic surface charges in a random distribution.

Comparative Distribution of Anionic Sites on the Apposed Surfaces of Mitochondrial Membranes

Controlled removal of the outer mitochondrial membrane may be complete or partial depending on the concentration of digitonin used in the procedure. Mitochondria showed a typically discontinuous outer membrane after treatment with 0.3% digitonin for 15 min (Fig. 2).

Polycationic ferritin at 60 μg/ml mitochondrial protein was permitted to bind for 5 s and again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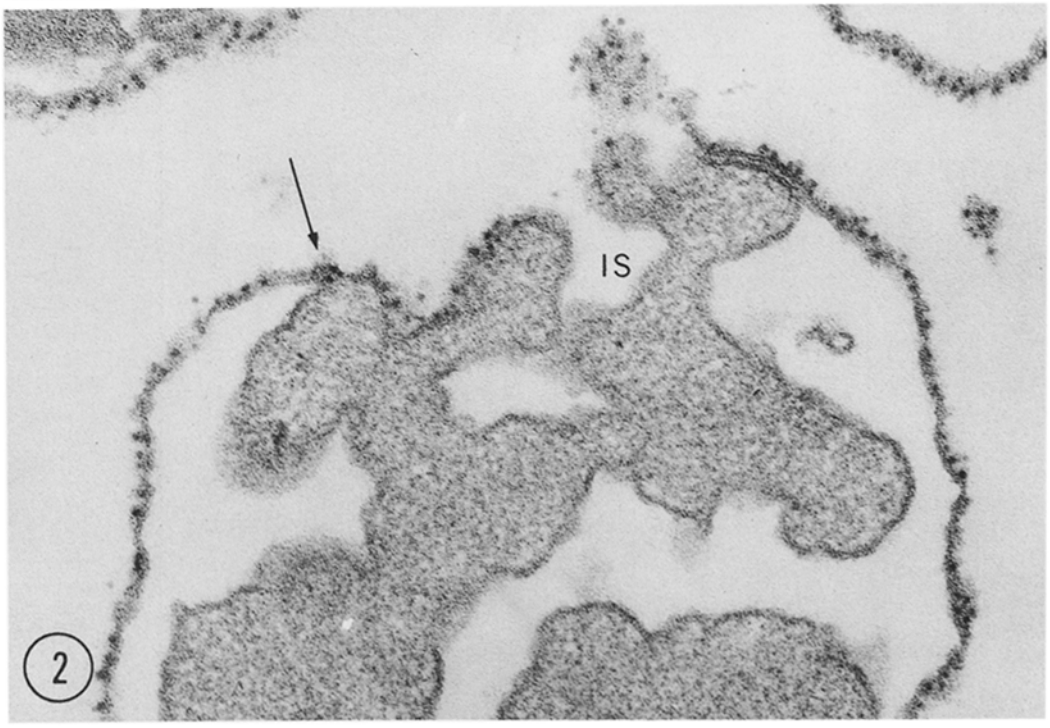
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showed the anionic sites on the surface of the outer membrane to be random (Fig. 2). Digitonin treatment, therefore, did not affect the distribution or density of membrane anionic sites. It could be observed that in those mitochondria, where the inner membrane matrix protruded through discontinuities in the outer membrane, the ligand appeared to favor binding to inner boundary membrane extensions. Very little ligand was observed bound to cristal membrane, i.e., membrane which bordered the enlarged intracristal spaces (Fig. 2). Contact sites between inner boundary membrane and outer membrane persisted in such mitochondrial preparations (Fig. 2).

At higher digitonin concentrations (0.65%–0.75%) most of the outer membrane is removed as inverted vesicles which contain most, if not all, of the mitochondrial monoamine oxidase (19). The purified inner membrane-matrix preparation is referred to as the mitoplast fraction (7, 12). The inner membrane surface topography of the mitoplast is quite complex as shown by transmission and scanning electron microscopy (Fig. 3). The mitoplast membrane surface is composed of many finger-like extensions which are often observed in the process of forming from inner boundary membrane (as observed in Fig. 2). Membrane which makes up the proximal ends of the finger-like extensions and continues down to cover the body of the mitoplast matrix is the cristal membrane. By definition, the cristal membrane borders the enlarged intracristal spaces (Figs. 3, 4).

Under the influence of 0.65% digitonin for 15 min, inverted outer membrane vesicles often persisted in attachment with the inner boundary membrane at contact sites and thus sedimented with the more dense mitoplast fraction. The contact sites, with outer membrane vesicles attached, most often were found at or near the distal ends of the inner boundary membrane extensions (Fig. 4).

The distribution of polycationic ferritin bound to anionic sites on the inner membrane during a 5-s period appeared to be nonrandom (Fig. 4). At low concentrations (30 μg/mg mitoplast protein) the ligand appeared to preferentially bind to distal ends of inner boundary membrane extensions, with less binding occurring on the cristal membrane. The outer surface of the inverted outer membrane vesicle (which is the inner surface of the intact outer membrane) also bound the ligand. We observed that small areas on the surface of the vesicles, near the contact site, often appeared to be free of bound ligand (Fig. 4).

To analyze this apparently polar distribution of anionic surface sites more critically, we studied the distribution and density of the bound polycationic ferritin on the surface of outer membrane vesicles in thin sections in which the membrane contact sites were located just below or above the plane of sectioning (Fig. 5). In most cases there were less anionic sites revealed on the surface area of the vesicle which was nearest the contact site (Fig. 5). Usually the binding of polycationic ferritin showed an anisotropic distribution on the outer membrane vesicle, with the highest density found on the vesicle surface which faced away from the inner boundary membrane extensions. In addition, where the distal ends of the inner boundary membrane occurred in the plane of sectioning, polycationic ferritin showed a higher density of binding than it showed on cristal membrane surfaces bordering typically enlarged intracristal spaces.

These observations reveal that the majority of

FIGURE 1 Isolated intact rat liver mitochondria showing the condensed configuration. Typical contact sites (*CS*) between the outer membrane (*OM*) and inner boundary membrane (*IBM*) are observed. The inner boundary membrane and cristal membrane (*CM*) are clearly distinguishable. After fixation, mitochondria were rapidly mixed for 5 s with 60 μg polycationic ferritin/mg mitochondrial protein. The ligand is observed bound to the outer surface in a random distribution. × 55,200.

FIGURE 2 Rat liver mitochondrion showing a discontinuity in, and partial removal of, the outer membrane after treatment for 15 min with 0.3% digitonin. Contact sites between the inner boundary membrane and outer membrane persist after such treatment (arrow). Fixed mitochondria were rapidly mixed with polycationic ferritin as in Fig. 1. The ligand is bound to the outer surface of the outer membrane in a random distribution. Polycationic ferritin is also bound to the inner boundary membrane observed as inner membrane extensions projecting through the discontinuous outer membrane. Very little ligand is bound to inner membrane cristae bordering the enlarged intracristal spaces (*IS*). × 129,000.

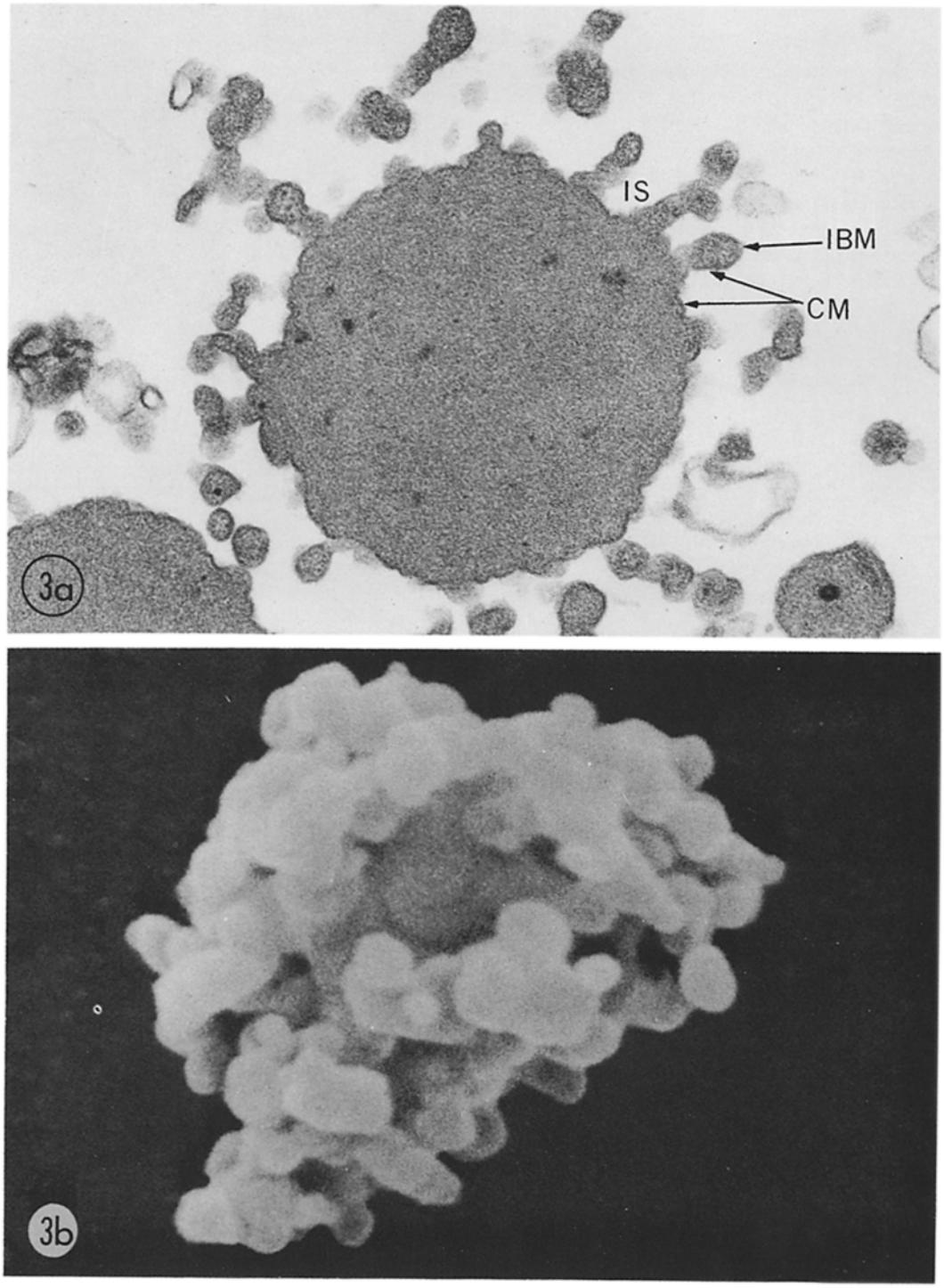

FIGURE 3 Rat liver mitoplast (intact inner membrane and matrix) after removal of outer mitochondrial membrane by treatment with 0.65% digitonin for 15 min. The surface contains numerous finger-like extensions, the distal ends of which represent regions of inner boundary membrane (*IBM*). The proximal ends of the extensions border enlarged intracristal spaces (*IS*) as the cristal membrane (*CM*). (3 *a*) Thin section transmission image. × 78,000. (3 *b*) Scanning image. × 78,000.

the apposing surfaces of the inner and outer mitochondrial membranes contain high density anionic sites. However, a small region of the inner surface of the outer membrane, which more or less surrounds the contact site, contains a lower density of anionic sites.

High Density and Low Density Regions of Anionic Sites on the Surface of the Inner Mitochondrial Membrane

Several variables were examined in studies designed to determine the distribution of high density and low density anionic regions on the inner membrane. These variables included fixation, concentration of the probe, and length of the mixing and incubation time with the probe (Table I).

Concentrations of from 4.5 μg to 450 μg of polycationic ferritin/mg mitoplast protein were used. The ligand was rapidly mixed with mitoplast membranes for 5 or 15 s, or rapidly mixed for 15 s followed by an additional 10- or 15-min incubation. We reasoned that the lower concentrations of the multivalent ligand would bind with highest affinity, and perhaps be restricted, to areas on the membrane surface which possessed the highest density of anionic sites.

Concentrations of 9–60 μg polycationic ferritin/mg mitoplast protein resulted in preferential binding to small patches of anionic sites located on the distal ends of most inner boundary membrane extensions, and little or no binding to cristal membrane surfaces. This difference in affinity of the two membrane regions for polycationic ferritin was observed on unfixed and fixed inner membranes as well as after short (Fig. 6 *a*) and long (Fig. 6 *b*) incubation periods with the probe.

Mitoplasts were critical-point dried after binding of the ligand at low concentrations and examined as whole mount preparations by transmission electron microscopy at 100 kV for maximal electron penetration (Fig. 8). Unlike thin-section electron microscopy, this method afforded observation of the entire circumferential surface of the inner-boundary membrane extensions and clearly verified the distribution of high density anionic regions on the distal ends of the extensions. This method demonstrated conclusively that cristal membrane areas observed to be free of bound polycationic ferritin were clearly accessible to the ligand (Fig. 8). Accessibility was implied in scanning images (Fig. 3 *b*).

Higher concentrations of polycationic ferritin (above 60 μg/mg mitoplast protein) demonstrated consistently that the ligand bound to anionic sites over the entire membrane surface, i.e. not only to the high affinity anionic regions on inner boundary membrane extensions but also to lower affinity anionic regions on the cristal membrane (Fig. 7). Table I summarizes the results of experiments in which the concentration of polycationic ferritin and the incubation time of mitoplasts with the probe were varied.

Most concentrations of polycationic ferritin used were in excess of that required to bind all available anionic sites. After binding and centrifugation of the membranes, excess polycationic ferritin remained in the supernate when used at initial concentrations above 9 μg/mg mitoplast protein.

Neither chelators of divalent cations nor uncouplers of oxidative phosphorylation influenced the binding distribution of the probe to the membrane. Under identical conditions of binding and fixation, native ferritin was not found to bind to the inner membrane.

We conclude from these experiments that the inner mitochondrial membrane contains small regions of anionic sites distributed anisotropically on its outer surface. The inner boundary membrane which is closely apposed to the outer membrane in the intact mitochondrion contains a high density of anionic sites. The inner membrane comprising the mitochondrial cristae contains a lower density of anionic sites.

The Stability of the Patched Distribution of High Density Anionic Regions on the Inner Membrane

The inner mitochondrial membrane of the mitoplast can be converted to a near perfect sphere by osmotic swelling (12). It was of interest to determine if the high density anionic sites of the inner boundary membrane would persist as stable patches in an anisotropic distribution after the membrane was converted to a spherical configuration. The alternative probability was that during swelling the high density anionic sites making up the patches on the distal ends of inner boundary membrane extensions would dissociate and randomize. It will be recalled that the intact outer mitochondrial membrane, a near perfect sphere, showed a random distribution of anionic sites on its outer surface without evidence of a patched distribution (Figs. 1, 2).

Polycationic ferritin was added at 45 μg/mg protein to osmotically swollen, fixed mitoplasts

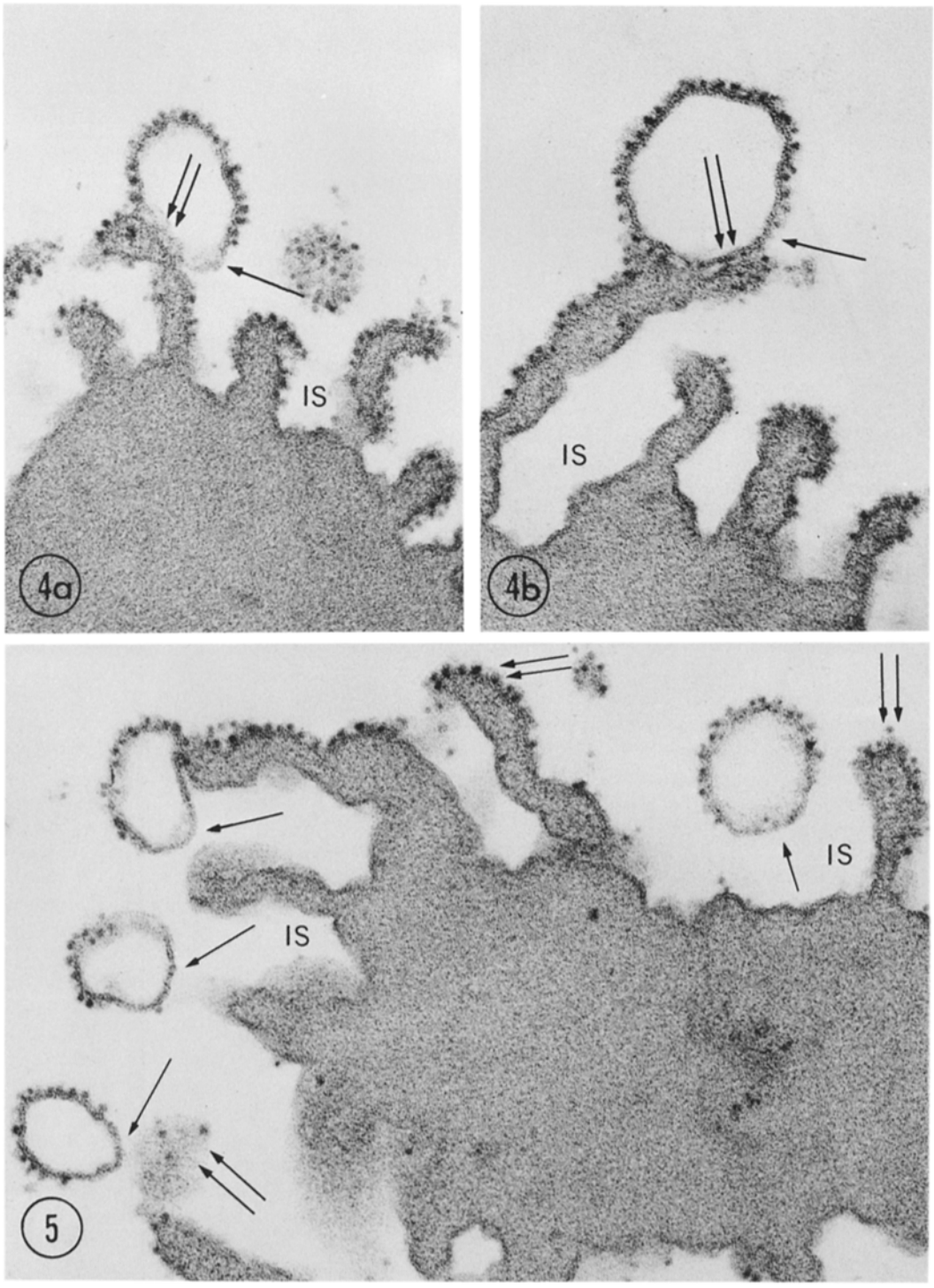
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TABLE I

Polycationic Ferritin Bound to Inner Mitochondrial Membrane

Polycationic ferritin/protein	Binding time		Location of bound polycationic ferritin	
	Mixing	Incubation	IBM	CM
μg/mg	*s*	*min*		
4.5	15	10	0	0
45	15	10	++	0
90	15	10	+++	+++
450	15	10	+++	+++
9	15	None	+	0
45	15	None	+	0
90	15	None	+++	+++
45	15	15	+	0
60	15	15	+++	0
75	15	15	+++	+++
30	5	None	+	0
45	5	None	++	0
60	5	None	++	0
90	5	None	+++	+++

Binding of polycationic ferritin was carried out with both glutaraldehyde-fixed and unfixed membranes as outlined in Materials and Methods. IBM = inner boundary membrane; CM = cristal membrane; 0 = no significant binding; +++ = greatest degree of binding.

and rapidly mixed for 15 s. Fig. 9 shows the typical distribution of anionic sites on the inner mitochondrial membrane of the swollen mitoplast. High density anionic sites were found to persist in a patched, anisotropic distribution on the nearly spherical membrane. Large regions of the membrane surface, presumably originating from areas of inner membrane cristae possessing low density anionic sites, essentially lacked ligand binding (Fig. 9).

These results suggest that high density anionic sites persist in small domains of anisotropic distribution on the outer surface of the inner mitochondrial membrane irrespective of changes in the configuration of the membrane.

DISCUSSION

Fig. 10 diagrams the morphological distinction between the inner boundary membrane and the cristal membrane in the preparations used in this study; freshly isolated intact rat liver mitochondria, mitochondria with disrupted outer membranes, mitoplasts, and swollen mitoplasts. Fig. 11 is a model which details the distribution and density of membrane surface fixed anionic sites, based on the relative affinities of the membrane surfaces for polycationic ferritin.

The identification of inner boundary membrane vs. cristal membrane as distinct regions on the mitoplast surface has been previously ascertained by utilizing thin-section and freeze-cleave electron microscopy (12) and, in addition, in the present study, by scanning and transmission electron microscopy of critical-point dried, whole mount preparations of mitoplasts. Utilizing these techniques, we have carefully monitored changes in the condensed configuration of the inner membrane in intact mitochondria during the preparation of mitoplasts by digitonin treatment. The visual observation of the binding of polycationic ferritin to high-affinity anionic regions on the inner membrane, especially in mitochondria prepared with partially disrupted outer membranes, has further

FIGURE 4 *a* and *b* Mitoplast surface. Inverted outer membrane vesicles persist in attachment with the inner membrane at contact sites (double arrows) which are most often located at the distal ends of inner boundary membrane extensions. Cristal membrane borders typically enlarged intracristal spaces (*IS*). Fixed mitoplasts were rapidly mixed for 5 s with 30 μg polycationic ferritin/mg mitoplast protein. The ligand is bound heavily to the outer membrane vesicles and distal regions of the inner boundary membrane extensions. Less ligand is bound to cristal membrane areas. In addition, small areas on outer membrane vesicles (single arrows) in the vicinity of contact sites appear to bind less ligand. × 129,000.

FIGURE 5 Mitoplast showing persistence of attachment of four inverted outer membrane vesicles at contact sites located just above or below the plane of sectioning. Fixed mitoplasts were rapidly mixed with polycationic ferritin as in Fig. 4. The ligand shows an anisotropic distribution. The highest density is located on the distal ends of inner boundary membrane extensions in the plane of sectioning (double arrows) and on those regions of outer membrane vesicles facing *away* from the inner boundary membrane. Polycationic ferritin is noticeably lacking in areas of membrane cristae bordering enlarged intracristal spaces (*IS*) and on outer membrane vesicle surfaces in the vicinity of contact sites (single arrows). × 129,000.

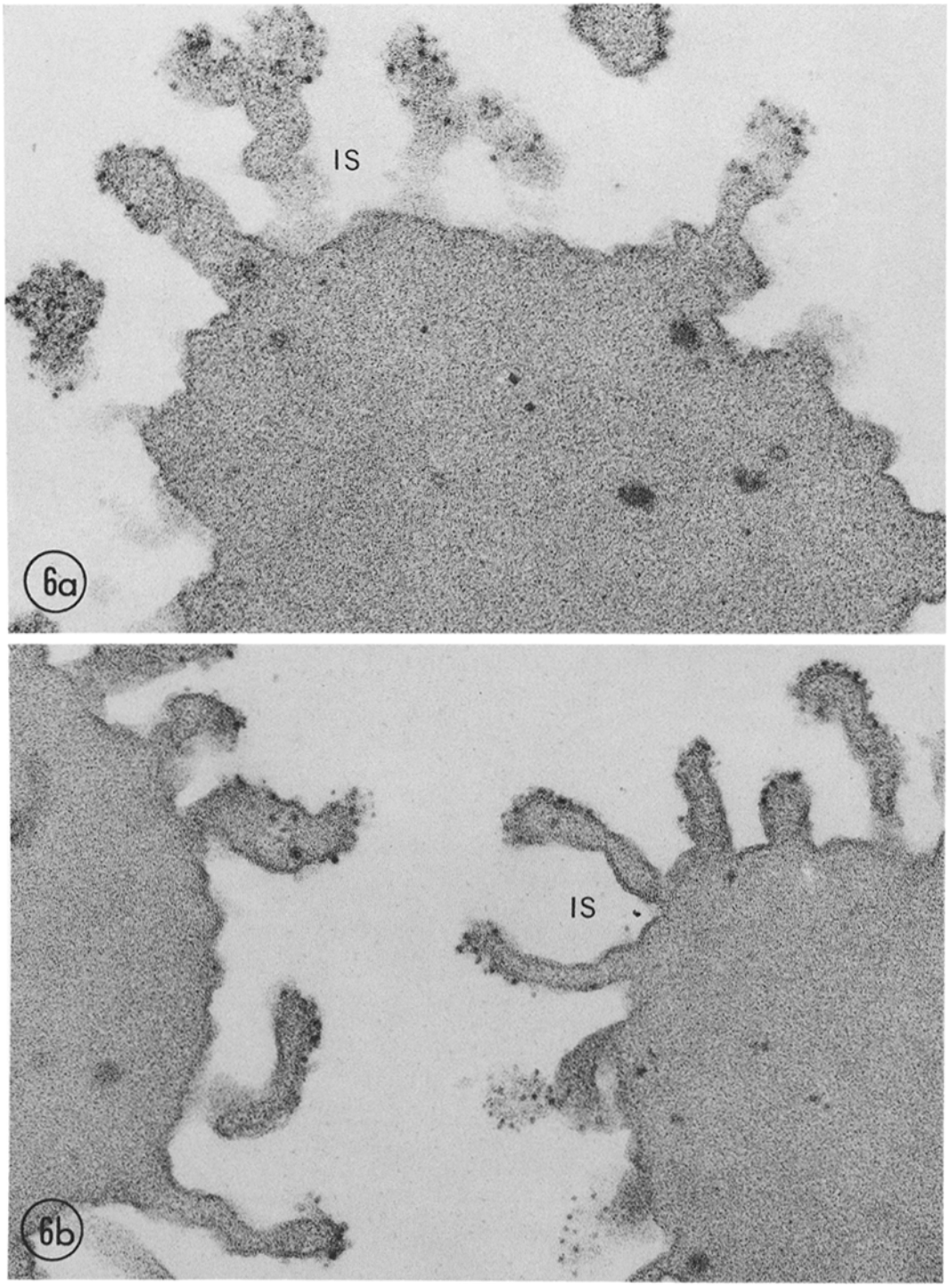


FIGURE 6 Mitoplast surfaces. Mitoplasts were rapidly mixed with 45 μg polycationic ferritin/mg mitoplast protein. The probe shows an anisotropic distribution with preferential binding occurring at the distal ends of inner boundary membrane extensions. The cristal membrane bordering typically enlarged intracristal spaces (*IS*) is relatively free of polycationic ferritin. (6 *a*) Unfixed mitoplasts mixed with the probe for 15 s. × 127,000. (6 *b*) Fixed mitoplasts mixed with the probe for 15 s and incubated for an additional 15 min. × 120,000.

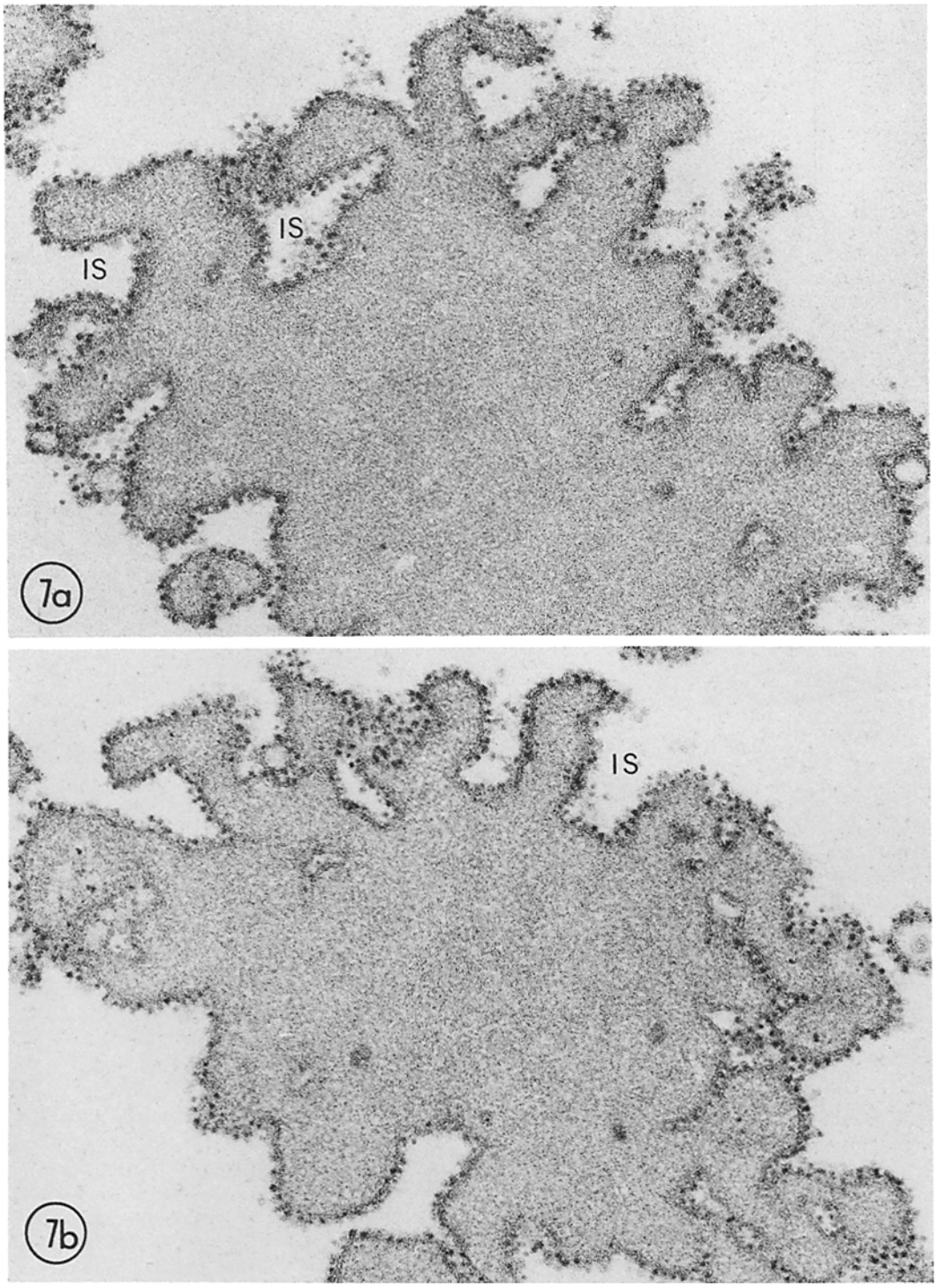
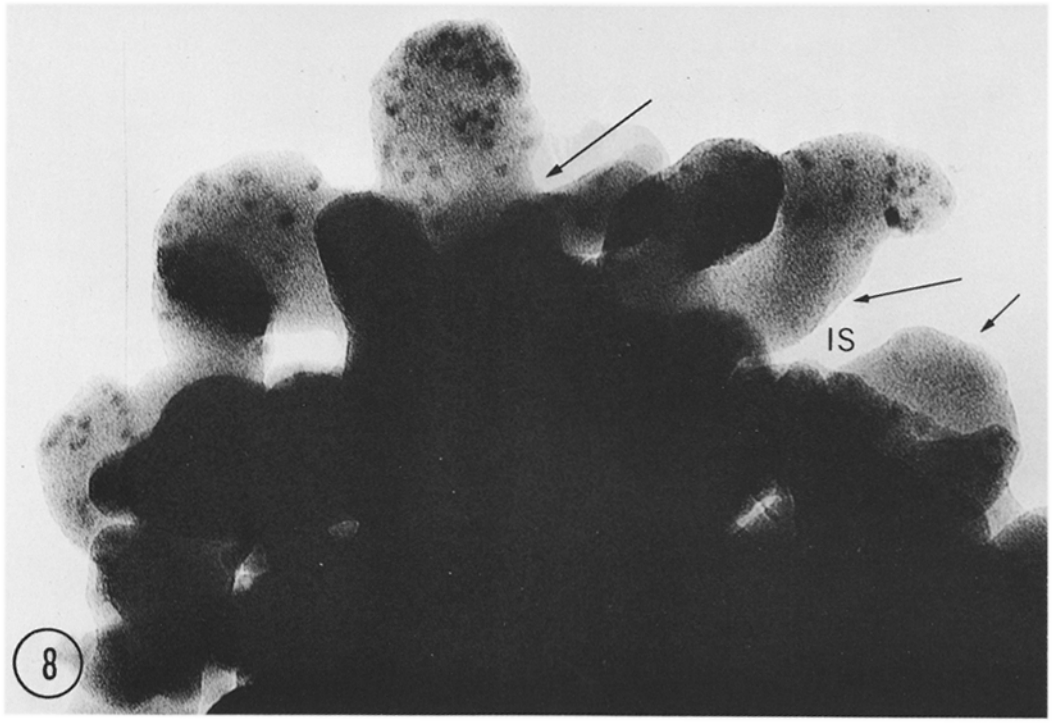


FIGURE 7 Mitoplast surfaces. Mitoplasts were rapidly mixed with 90 μg polycationic ferritin/mg mitoplast protein. The ligand is bound to the entire surface of the membrane including inner boundary membrane extensions and the cristal membrane bordering intracristal spaces (*IS*). (7 *a*) Unfixed mitoplasts mixed with the probe for 15 s. × 127,000. (7 *b*) Fixed mitoplasts mixed with the probe for 15 s and incubated for an additional 15 min. × 120,000.



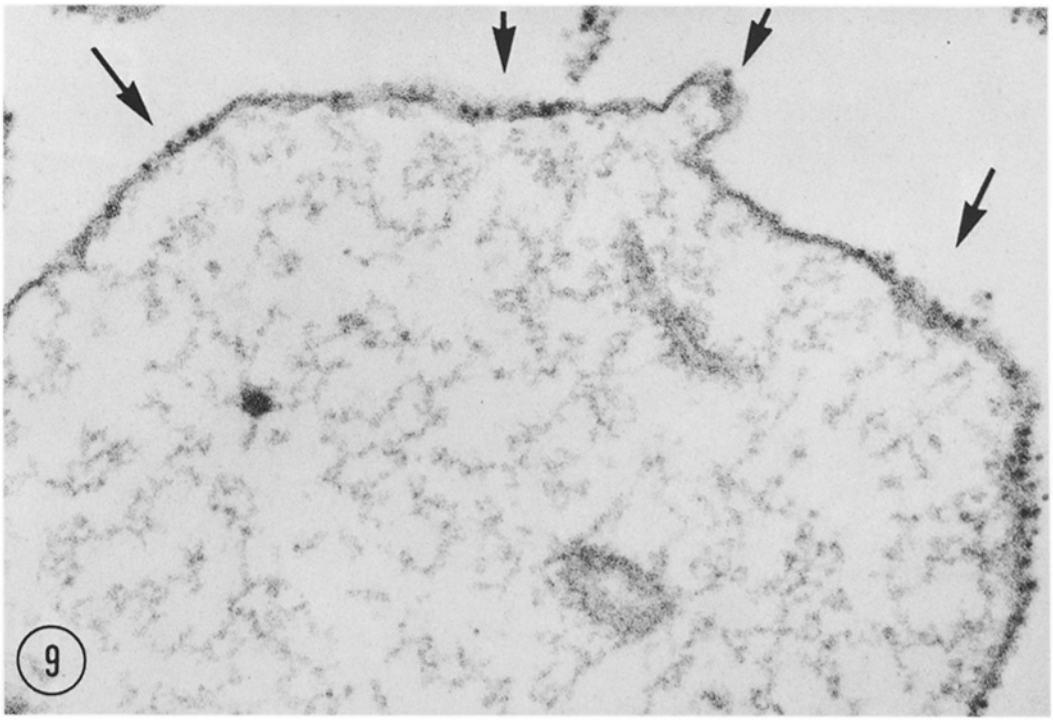


FIGURE 8 Mitoplast surface. Unfixed mitoplasts were rapidly mixed with polycationic ferritin as in Figure 6 *a*. Mitoplasts were then fixed, critical-point dried, and the whole mount preparations observed and photographed at 100 kV. This method allowed observation of polycationic ferritin-binding sites over the entire circumferential surface area of the inner boundary membrane extensions. Highest density of ligand binding occurs on the distal ends of the inner boundary membrane extensions. Arrows point to cristal membrane regions relatively free of bound polycationic ferritin although definitely accessible to the ligand. Intracristal space (*IS*). × 318,000.

FIGURE 9 Surface of a near spherical inner mitochondrial membrane of a swollen mitoplast. Fixed swollen mitoplasts were rapidly mixed for 15 s with 45 μg polycationic ferritin/mg mitoplast protein. The ligand is observed bound to the membrane surface in a patched anisotropic distribution (arrows). × 127,000.

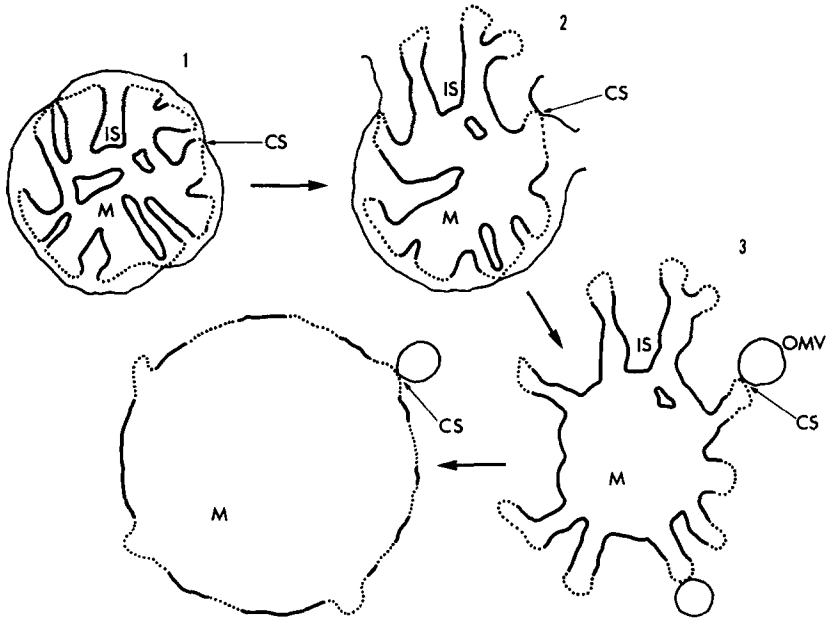


FIGURE 10 Diagrammatic representation of the intact rat liver mitochondrion (1), removal of the outer mitochondrial membrane by digitonin treatment (2, 3), and subsequent induced osmotic swelling of the mitoplast (4). The inner membrane composed of dots represents high density anionic regions of the inner boundary membrane; inner membrane made up of heavy continuous lines represents low density anionic regions of the cristal membrane; outer membrane is represented by a thin continuous line. Contact sites (*CS*); intracristal space (*IS*); matrix (*M*); outer membrane vesicle (*OMV*).

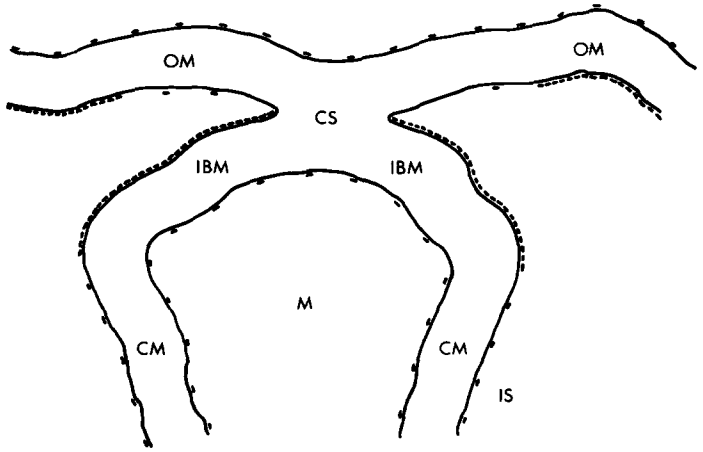


FIGURE 11 Diagrammatic representation of the distribution and density of anionic sites on the surface of mitochondrial membranes in the region of the contact site. Outer membrane (*OM*); contact site (*CS*); inner boundary membrane (*IBM*); cristal membrane (*CM*); matrix (*M*); intracristal space (*IS*).

permitted us to identify the inner boundary membrane and cristal membrane of the mitoplast with reasonably good accuracy. The data presented in this report have been compiled from approximately 50 such monitored preparations. To quantitate the content of outer membrane remaining attached to the inner membrane after treatment of mitochondria with different concentrations of digitonin, we determined the percent of monoamine oxidase, an outer membrane marker enzyme (19), which sedimented with the mitoplasts. Clearly, outer membrane which sedimented with

the mitoplasts remained attached at contact sites as *inverted* vesicles.

At a pH of 7.4, the pH at which the experiments of this report were carried out, most of the anionogenic sites on membrane surfaces are free carboxyl groups of various membrane macromolecules (17). The membrane carboxyl groups, whether of sialic, aspartic, or glutamic acid, have pK's in the range of approximately 3–4. Therefore, at a pH of 7.4, the binding constants of these groups for polycationic ferritin should be very nearly equal. Thus, under the conditions of our experiments, the relative affinity of the membranes for polycationic ferritin depended primarily on the density of the fixed anionic sites on the membrane surfaces, i.e., the degree of packing of anionic sites per unit surface area.

Our results show that the distribution of fixed anionic sites on the outer surface of the inner mitochondrial membrane is anisotropic. Patches of high density fixed anionic sites occur on the inner boundary membrane while the cristal membrane contains significantly fewer anionic sites. The inner boundary membrane showed a high affinity for polycationic ferritin, with binding occurring at concentrations of between 9 and 60 μg of polycationic ferritin/mg mitoplast protein. The cristal membrane, however, showed binding of polycationic ferritin only when the concentration of the probe exceeded 60 μg/mg mitoplast protein. These different binding affinities for the probe by the inner boundary and cristal membranes were revealed to be independent of the length of time incubated with the probe. In addition, fixed and unfixed inner membranes produced the same results.

It was of interest to determine the degree of stability of the patches of high density anionic sites on the surface of the inner boundary membrane. By swelling the mitoplast to a near perfect spherical configuration, the inner membrane loses its morphologically distinct regions of inner boundary membrane and cristal membrane. Such an osmotic perturbation to the inner membrane can be expected to require considerable molecular rearrangement since the membrane is converted to a near spherical form. However, it was determined that the spherical membrane of the swollen mitoplast also showed a patched distribution of high density anionic sites. This finding is to be compared with the observation that the outer surface of the outer membrane, also of spherical configuration, contains a random distribution of fixed anionic sites. We would add here that preliminary results with fragmented inner membranes show a random distribution of anionic sites on the inner surface of the inner membrane.

It is unlikely that the multivalent ligand induced the patching by bridging anionic sites which were randomly moving in a fluid membrane, since both prefixed and unfixed membranes were found to bind polycationic ferritin to the same degree and showed the same patched distribution of anionic sites.

Our observations suggest that the patches of high density anionic sites on the spherical membrane of swollen mitoplasts represent the areas of high density anionic sites of the inner boundary membrane. These patches of high density anionic sites could not be dissociated or randomized by changing the gross configuration of the membrane. Such changes in configuration are indicative of a highly plastic membrane; however, the stability of the patches of high density anionic sites on the membrane surface is not suggestive of a highly fluid membrane. Studies to test the possible lateral fluid translocation of anionic sites and of specific cytochromes in this membrane are in progress in our laboratory. We observed previously that the inner mitochondrial membrane displayed a limited degree of fluidity, at least as determined by the degree of redistribution of intramembrane particles during changes in metabolic state (11, 13). It is assumed that the inner mitochondrial membrane will display only limited fluidity, since it is a membrane containing a high content of hydrophobic proteins which stabilizes a large percentage of the total membrane lipid as nonfluid, protein-bound lipid (15). Related to these considerations is our previous finding that the outer mitochondrial membrane, which is composed of a lower protein to lipid ratio, shows considerable fluidity as determined by dramatic redistributions of intramembrane particles (11, 13).

Except for a small area near the contact site, the inner surface of the outer membrane contains a high degree of anionic sites. Since the apposing surfaces of the outer membrane and inner boundary membrane both contain a relatively high density of anionic sites, the electron-lucid space normally observed between the two membranes of the intact mitochondrion *in situ* may be maintained by electrostatic repulsion.

Fixed anionic sites on most biomembranes represent various macromolecules: proteins, glycoproteins, glycolipids, but most often, sialoglyco-

proteins. The high density electronegative charge distribution on the inner boundary membrane may be due to sialoglycoproteins which are known to occur on or in the inner mitochondrial membrane (2, 20). The inner membrane contains 1.1 nmol of sialic acid/mg mitoplast protein (2).

The function of sialoglycoproteins in mitochondria has not been determined. Recently, however, a high affinity calcium-binding sialoglycoprotein has been isolated from liver mitochondria (6, 20). The high density anionic sites on the inner boundary membrane revealed by the present study may indicate the location of such an anionic glycoprotein, and it may be the high density anionic sites which coordinate with the calcium during transport. Consistent with this view are recent findings which suggest that most of the carbohydrate-containing proteins of the inner membrane appear to be confined to the inner boundary membrane and that such proteins are nearly absent in cristal membranes (3). If mitochondrial contact sites represent sites of membrane-membrane recognition, sialoglycoproteins in the inner boundary membrane would make good candidates as informational molecules required for such recognition. However, the nature of the contact site remains elusive. Studies in progress are designed to remove sialic acid from the surface of the inner mitochondrial membrane and then to treat the preparation with polycationic ferritin.

It should be mentioned here that the function or functions of the mitochondrial contact sites are unknown. It is noteworthy that recent studies suggest that newly synthesized polypeptide chains, transcribed by the mitochondrial genome and destined for the inner membrane, appear first in the region of the inner boundary membrane rather than in the cristal membrane (23). Cytoplasmic ribosomes, which translate nuclear encoded polypeptides destined for mitochondria, have recently been observed to anchor to the outer mitochondrial membrane in the vicinity of contact sites (4). Related to these observations is the very interesting fact that certain inner membrane proteins, e.g. cytochrome oxidase, are assembled from several polypeptide chains, some chains synthesized exclusively on cytoplasmic ribosomes and others synthesized on mitochondrial ribosomes (21). Since cytoplasmic ribosomes associate with mitochondria in the region of contact sites, an effective area for the cooperative assembly of such complex mitochondrial membrane proteins would be the membrane-membrane contact site. We would speculate that once a particular protein was assembled in the membrane at the contact site, it could undergo lateral fluid translocation into either the outer or inner membrane, depending on the protein and its ultimate functional location.

In conclusion, the observations presented in this report provide visual evidence for the existence and density distribution of anionic sites on the surfaces of mitochondrial membranes. Based on the use of polycationic ferritin as a visual probe for the location of anionic sites, it was observed that high density anionic sites occur as patches on the inner boundary membrane, while lower density anionic sites are located on the cristal membrane. The outer surface of the outer membrane contains anionic sites in a random distribution. The inner surface of the outer membrane contains anionic sites at relatively high density, except in small areas surrounding membrane-membrane contact sites.

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