# SYNAPTIC VESICLES: STRUCTURE OF CHROMAFFIN GRANULE MEMBRANES

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## INTRODUCTION

Synaptic vesicles are pre-synaptic subcellular membraneous organelles which mediate synthesis, storage, and release of neurotransmitters. In spite of their apparently central role in nervous function, little is known about them in molecular terms. One of the most easily obtained adrenergic synaptic vesicle preparations is the chromaffin granule from the adrenal medulla (1). Chromaffin granules contain adrenalin and nonadrenalin, and are chemically and immunochemically identical to adrenergic synaptic vesicles from conventional nerves; these similarities also cross species lines (2–4). The adrenal medulla can rightly be considered nervous tissue since it is derived from neur-ectoderm, and is essentially a displace sympathetic ganglion (5).

In this presentation we discuss some of the reasons for our experimental interest in this material, outline our newly developed method for membrane purification, and give a progress report on the status of our X-ray and electron microscopic studies on the chromaffin granule membrane.

### General Properties of Chromaffin Granules

The chromaffin granule, a 2000 Å diameter sperical organelle, contains an enzyme, dopamine-B-hydroxylase (DBH) which catalyzes the biosynthesis of the neurotransmitter noradrenaline (6, 7). Dopamine, the substrate of DBH, is synthesized in the cytoplasm and is transported into the granule by an ATP-dependent active transport system (8). Release of catecholamines from the cell occurs after a cholinergic stimulus, and proceeds by an exycytosis process involving fusion of the granule membrane with the plasma membrane (1, 9). The release process, as for most synaptic vesicles, is inhibited by microtubule inhibitors, such as colchioine (10).

Many of these processes are mediated in part by enzymes associated with the chromaffin granule membrane. For example, the membranes contain the DBH, and a $Mg^{2\pm}$ ATPase, which is presumably responsible for transport. The granule membrane is able to bind microtubule proteins under certain conditions. In addition, the membrane contains an electron transport system comprising approximately 20% of the protein mass (11), al-


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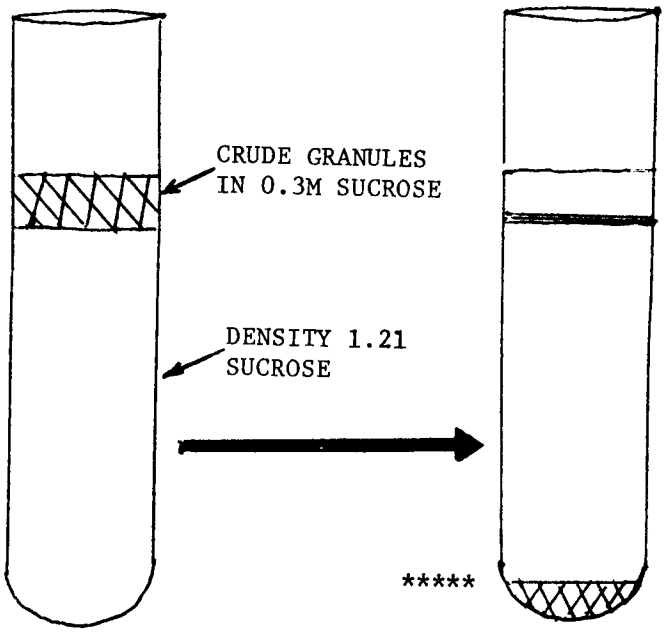


Fig. 1. Initial purification of chromaffin granules from adrenal medulla. A large granule fraction is centrifuged over a density 1.21 g $cm^{-3}$ sucrose solution. Granules sediment to the bottom, while less dense membranes remain at the origina. (From Smith and Winkler, 1967).

though its function is unknown. On the basis of these few facts it is clear that the granule is involved in many fundamental life processes, and that the membrane is the main focus of granule function.

### Chemistry of Chromaffin Granule Membranes

As indicated in Fig. 1, chromaffin granules can be conveniently prepared by centrifugation as described by Smith and Winkler (12); an electron micrograph of the pellet is shown in Fig. 2. The isolated granules can then be subjected to cycles of hypotonic and hypertonic shock in sucrose in order to remove contents and leave membrane ghosts. The resulting membranes can be purified by equilibrium flotation on a sucrose gradient (11). The membranes, having a hydrated density of 1.12 in sucrose, can be prepared in bulk by flotation to an interface of density 1.15/1.05 gm $cm^{-3}$. These membranes are vesicular as defined by negative stained preparations and have a thickness of approximately 70 Å. They are essentially free from microsomal and mitchondrial contaminants as defined by enzymatic assays of marker enzymes (Table I). As indicated in Table I, the purified membrane possesses enzymes for transport (ATPase) and biosynthesis of noradrenalin (DBH), and also contains cytochrome $b_{562}$ reductase. On the basis of a reduced–minus – oxidized difference spectrum (Fig. 3) it can be estimated that the electron tansport system may constitute up to 20% of the membrane protein (11). Disc electrophoresis in SDS reveals that the membrane has only 8 to 10 major protein size classes, all with molecular weights of less than 65,000.

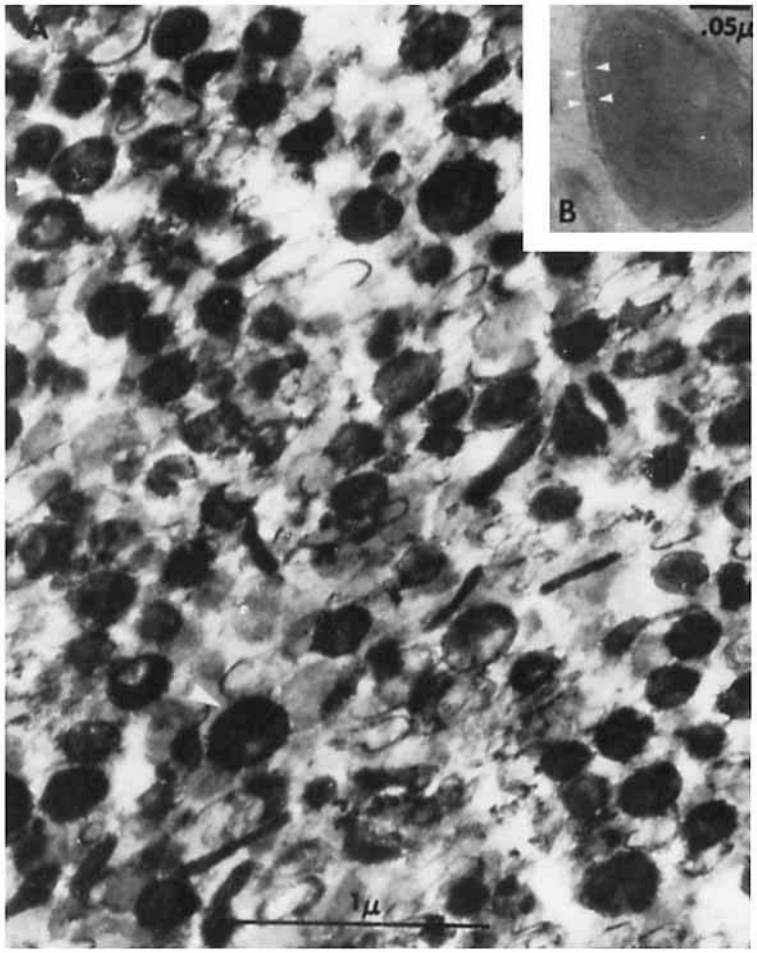


Fig. 2. Thin section electron micrograph of a pellet of chromaffin granules. A. Lower power (12,000 X) field of granules. B. High magnification view of a 70 Å unit membrane surrounding an intact granule (216,000 X).

## X–ray Diffraction of Oriented Membranes

In order to prepare oriented membranes suitable for X-ray diffraction analysis, the membranes were sedimented onto an X-ray transparent plastic plate attached to the bottom of a single sector cell of the analytical ultracentrifuge. Thin sections of the resulting pellet (see Fig. 4), parallel to the axis of centrifugation, revealed that the membrane vesicles had stacked up in ordered arrays. This stack of membrane vesicles if formally analogous to the naturally occurring pile of retinal discs in the rod outer segment and to myelin membranes in nerves. This preparation is essentially a fiber of membranes oriented with regard to an axis perpendicular to the plastic plate, but rotationally symmetric with regard to other areas.

An X-ray beam was directed edge-on to the membrane stack in order to examine the possible unit cell along the fiber axis. The data, shown in Table II, are consistent with a

**Table I. Enzyme Activities and Proteins of Purified Chromaffin Granule Membranes**

| Enzyme or Protein | Nanomoles / mg Protein[e] |
|---|---|
| Dopamine–β–hydroxylase[a] | 9.7 ± 4/min. |
| $Mg^{2+}$–ATPase[b] | 250 ±5 min |
| Cytochrome $b_{562}$[c] | 7.7 ± .08 |
| Flavoprotein[c] | 2.1 ± .05 |
| Cytochrome $b_{562}$ reductase[b] | 7.9 ± .8/min |
| Monoamine oxidase[d] | 0 (<0.05/min) |
| Glucose–6–phosphatase[d] | 0 (<0.05/min) |

[a]Protein concentration between 0.005 and 0.04 mg/ml.

[b]Protein concentration between 0.1 and 0.3 mg/ml; concentration of ATP and $MgCl_2$ were 2.5 mM.

[c]Protein concentration between 0.1 and 0.3 mg/ml.

[d]Protein concentration between 0.1 and 1.0 mg/ml.

[e]Average of determinations from three independent preparations.

**Table II. Diffraction Parameters for Hydrated Membranes Oriented "Edge-On" to the Beam**

| Observed Spacing, Å | Order | Calculated Spacing, Å 1 = 143 Å |
|---|---|---|
| 71.5 | 2 | 71.5 |
| 48.7 | 3 | 47.6 |
| 34.5 | 4 | 35.8 |
| 30.5 | 5 | 28.6 |
| 23.1 | 6 | 23.8 |
| 20.6 | 7 | 20.4 |
| 17.8 | 8 | 17.8 |
| 14.1 | 10 | 14.3 |
| 12.15 | 12 | 11.92 |
| 9.60 | 15 | 9.53 |

repeat along the fiber axis of 143 Å for 100% hydrated specimens. This value was noted to be approximately two membrane thicknesses, as defined by thin-section electron microscopy of intact granules and purified membranes. All odd orders, except for the seventh, were bery weak. Upon drying in air, the repeat distance decreased to 134 Å. When lipids were extracted from oriented membranes using ethanol: either (3:1, v/v), the repeat was reduced to 65 Å and only 6 orders were detected. In the latter experiment, approximately 95% of the phospholipids (as defined by phosphate analysis) were removed.

The second experiment involved turning the membrane stack face-on to the X-ray beam. This would allow us to search for any order that might exist in the membrane plane. As indicated in Table III, a series of rings were obtained which were different in spacing from those observed in the edge-on view. The face-on spacings were essentially the same regardless of the state of hydration. Following extraction of lipids, as described above, the same set of spacings were found. In addition, the spacings were unaffected by treatment of the membrane with phospholipase C (resulting in quantitative hydrolysis of all lecithin).

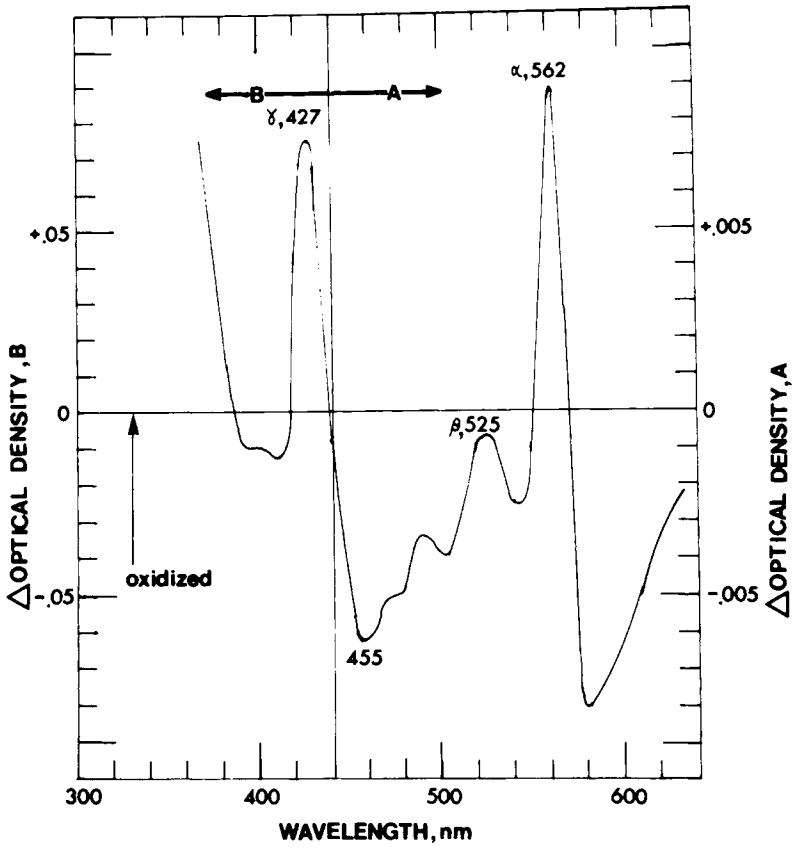


Fig. 3. Reduced-minus-oxidized difference spectrum of purified membranes. Peaks at 427, 525, and 562nm are indicative of a b-type cytochrome. The trough at 455nm may represent flavin.

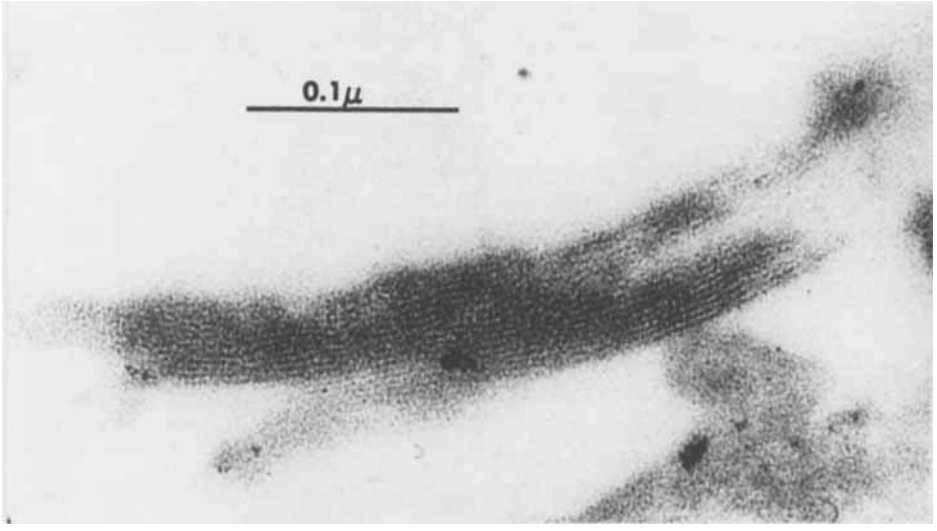


Fig. 4. Thin section of oriented membranes. The section is parallel to the centrifugation field. The bar represents 1000 Å.

Table III. Diffraction Parameters of Membranes Oriented "Face-On" to the Beam

| Observed Spacing, Å | Index | Half-Width, Å | Calc. Spacing a = 131Å |
|---|---|---|---|
| 114 | (1,0) | | 114 |
| 64.2 | (1,1) | 500 | 65.4 |
| | (2,0) | | 56.6 |
| 47.6 | | | |
| 41.3 | (2,1) | 500 | 42.8 |
| | (3,0) | | 37.9 |
| 29.9 | (2,2) | | 32.7 |
| | (3,1) | | 31.5 |
| 26.3 | (3,2) | | 26.1 |
| | (4,0) | | 28.3 |

However, treatment of the membrane prior to centrifugation with trypsin resulted in complete loss of these reflections.

The spacings obtained from the face-on orientation could be indexed on a hexagonal lattice, as indicated in Table III, with a center-to-center distance of 131 Å. At this point we regard this specific indexing as tentative since the symmetry on a spherical structure is most likely not perfectly hexagonal. For example., one would expect the lattice to break down at "bends" in the curvature of the membrane.

We concluded from these studies on oriented membranes that the 143 Å repeat along the fiber axis might represent two membrane thicknesses. By analogy with the case of myelin, this might suggest that the individual membrane thickness was asymmetric. The set of reflections obtained from the face-on view of the membrane stack was different from those obtained from the edge-on view and we concluded that some unit different from just membrane layers was responsible. It is possible that the unit may be arranged with a special symmetry, perhaps hexagonal in part. It could also be concluded that the units were most likely protein, since the organization in the pane of the membrane was abolished by trypsin treatment, but not lipid extraction, or phospholipase C treatment.

### X–ray Diffraction of Dispersions of Intact Granules and Membranes

X-ray diffraction of dispersions (solutions) of biological membranes has been used by Wilkins and his co-workers to study membrane structure (13). By this approach evidence for phospholipid bilayers has been obtained for many membrane systems. This method was applied to intact chromaffin granules and purified membranes in an effort to detect phospholipid bilayers and compare the structure of the purified membrane with that of the intact granule. Figure 5 shows a densitometric scan of diffraction from dispersions of intact chromaffin granules at 4°C and purified membranes at 18°C. Several peaks at low angles are observed which have been associated with organization in the place of the membrane. At higher angles, two broad peaks are observed (~30 Å and ~10 Å), the envelopes of which are not superimposable. As indicated in Table IV, the low angle peaks can be indexed on a hexagonal lattice similar to that noted in face-on-views of oriented membranes. The high angle peaks are from an evidently different phase. According to the analysis by Wilkins (13), these high angle peaks are most likely evidence of phospholipid bilayers. The movement of the envelopes of these peaks to lower angles at higher temperatures is consistent

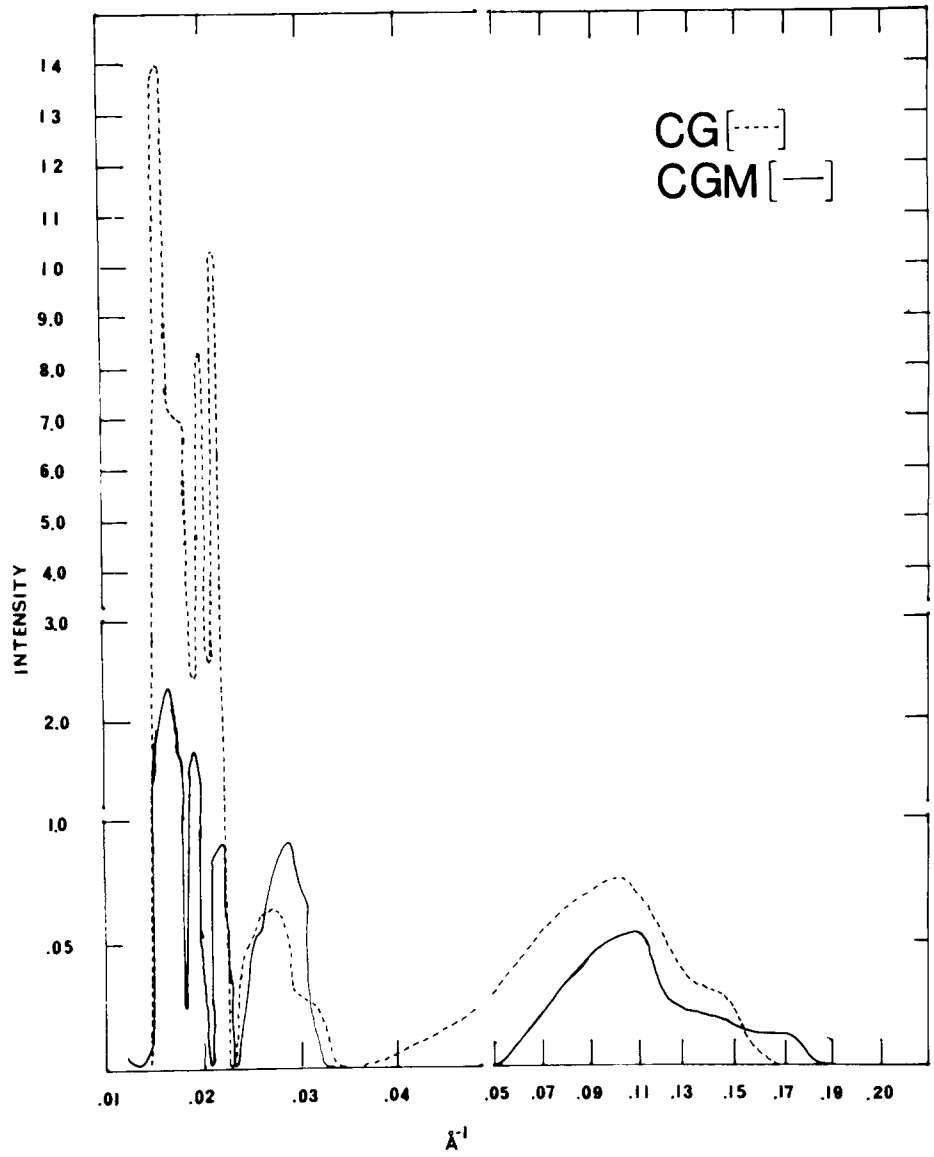


Fig. 5. X-ray diffraction of a dispersion of intact chromaffin granules (5°C) and purified chromaffin granule membranes (18°C). The tracing is a densitometer output from a film.

with the negative thermal coefficient of expansion of pure phospholipid bilayers. In addition, one can calculate from the position of the first obvious zero in the diffraction pattern that if a spherical object were responsible for diffraction it would have a diameter of 60–70Å.

We conclude from the study of dispersions of granules and membranes that the membrane is primarily responsible for diffraction by intact granules, and that therefore purification of the membrane does not materially change the membrane structure. In addition, reflections from the surface organization of the membranes appear to coexist with reflections from phospholipid bilayers. Since both structures occur in the same material, and since evidence for only one phase in the fiber axis was obtained from studies

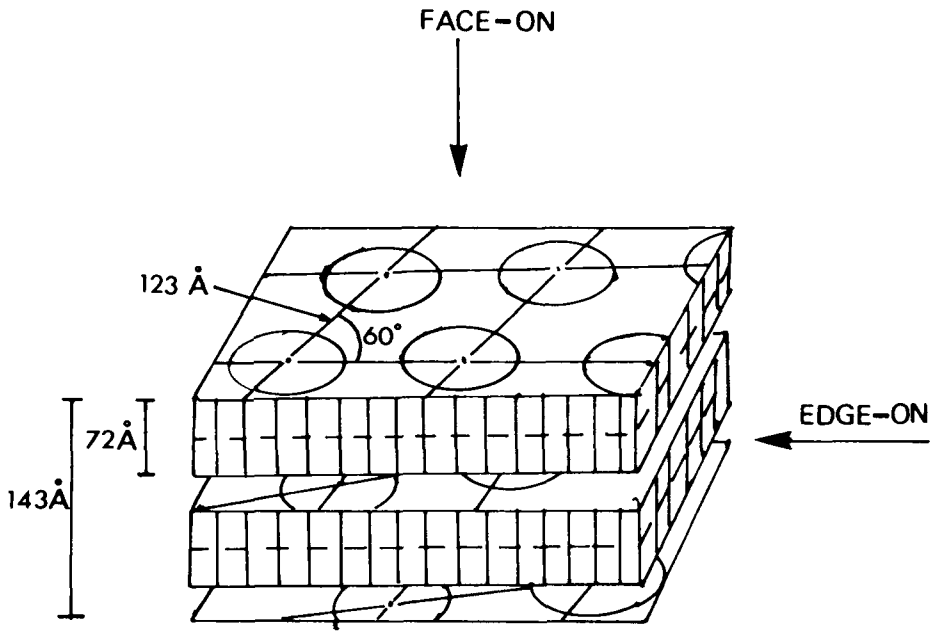


Fig. 6. Model of chromaffin granule membrane in three dimensions as visualized in a membrane array.

**Table IV. Diffraction Parameters for Dispersions of Chromaff in Granules and Membranes**

| Observed Spacing, Å | | Index | Half-Width, Å | | Calculated spacing, Å a = 123 Å |
|---|---|---|---|---|---|
| CG | CGM | | CG | CGM | |
| 61.0 | 61.0 | (1,1) | 620 | 500 | 61.5 |
| 52.0 | 52.0 | (2,0) | 1000 | 620 | 53.3 |
| 48.0 | 45.0 | – | – | – | – |
| 36.0 | 38.0 | (2,1) | 220 | 400 | 40.0 |
| | 34.0 | (3,0) | | | 35.5 |
| | 32.0 | (2,2) | | | 30.7 |
| 9.6 | 9.0 | – | | | – |
| 7.0 | 6.0 | – | Very Broad | | – |

of oriented membranes, it is apparent that both the phospholipid bilayer and surface organization in the plane of the membrane are located in the same phase. This is suggested by a three-dimensional schematic model of the membrane, shown in Fig. 6, which summarizes the X-ray data in a simple fashion. It is evident that in this model the objects in the plane of the membrane apparently do not touch. However, the apparent diameter of the particle is that of a hard sphere and tenuous connections between the particles could exist.

### Freeze–Fracture Electron Microscopy

Freeze–fracture studies of intact chromaffin granules were performed in an effort to visualize the structure suggested by X-ray diffraction studies. Figure 7A is a low-power

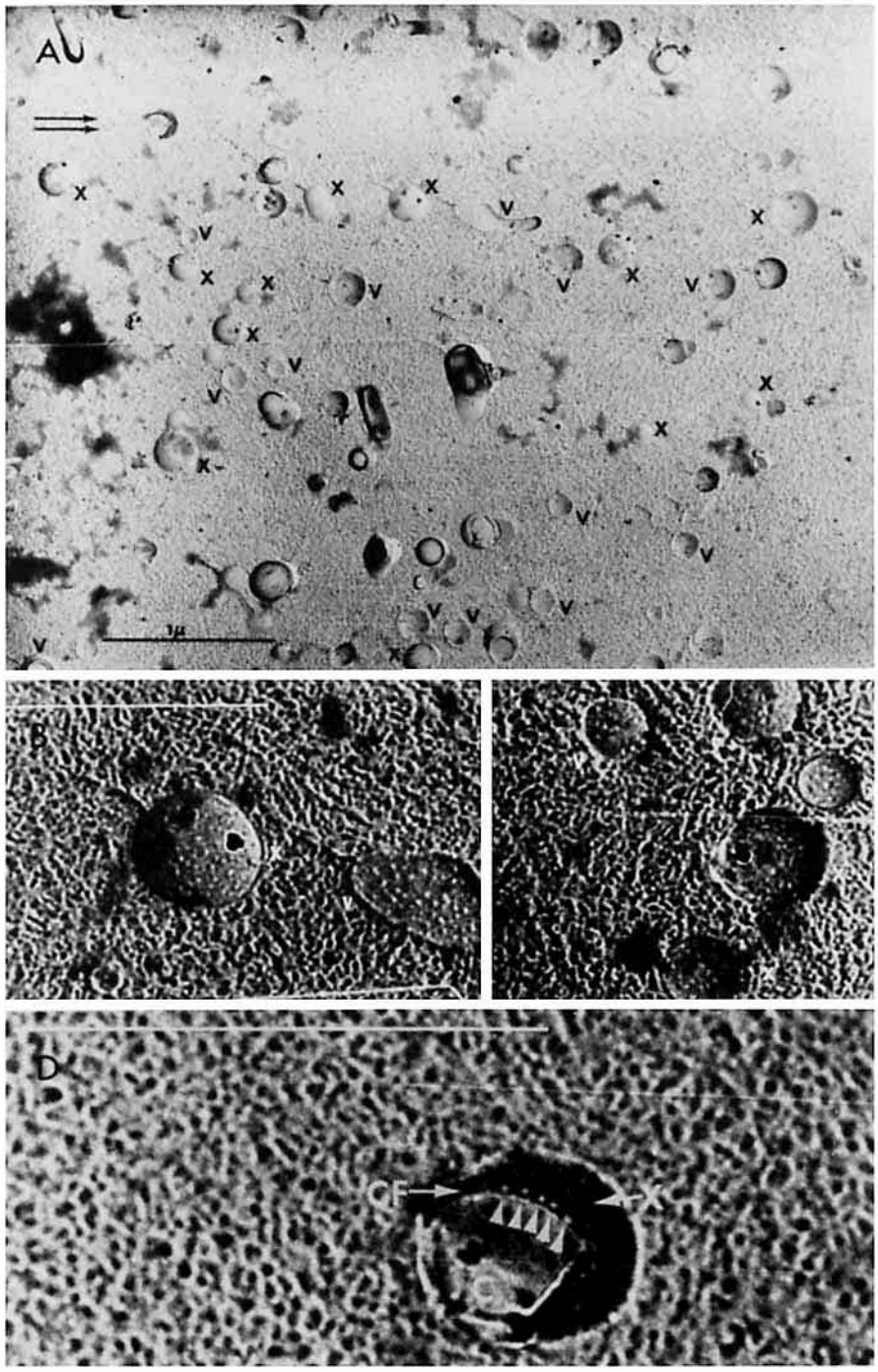


Fig. 7. Freeze–fracture electron micrograph of intact chromaffin granules (12,000 × ). X and V represent convex (X) and concave (V) fracture faces of membranes; B, C. High power view of convex (X) and concave (V) fracture faces showing submembrane particles (135,000 × ); D. Freeze–fracture of membrane showing both X and V surfaces on the same profile. Arrows indicate subunits in the cross-fracture or concave fracture surface. (135,000 ×).

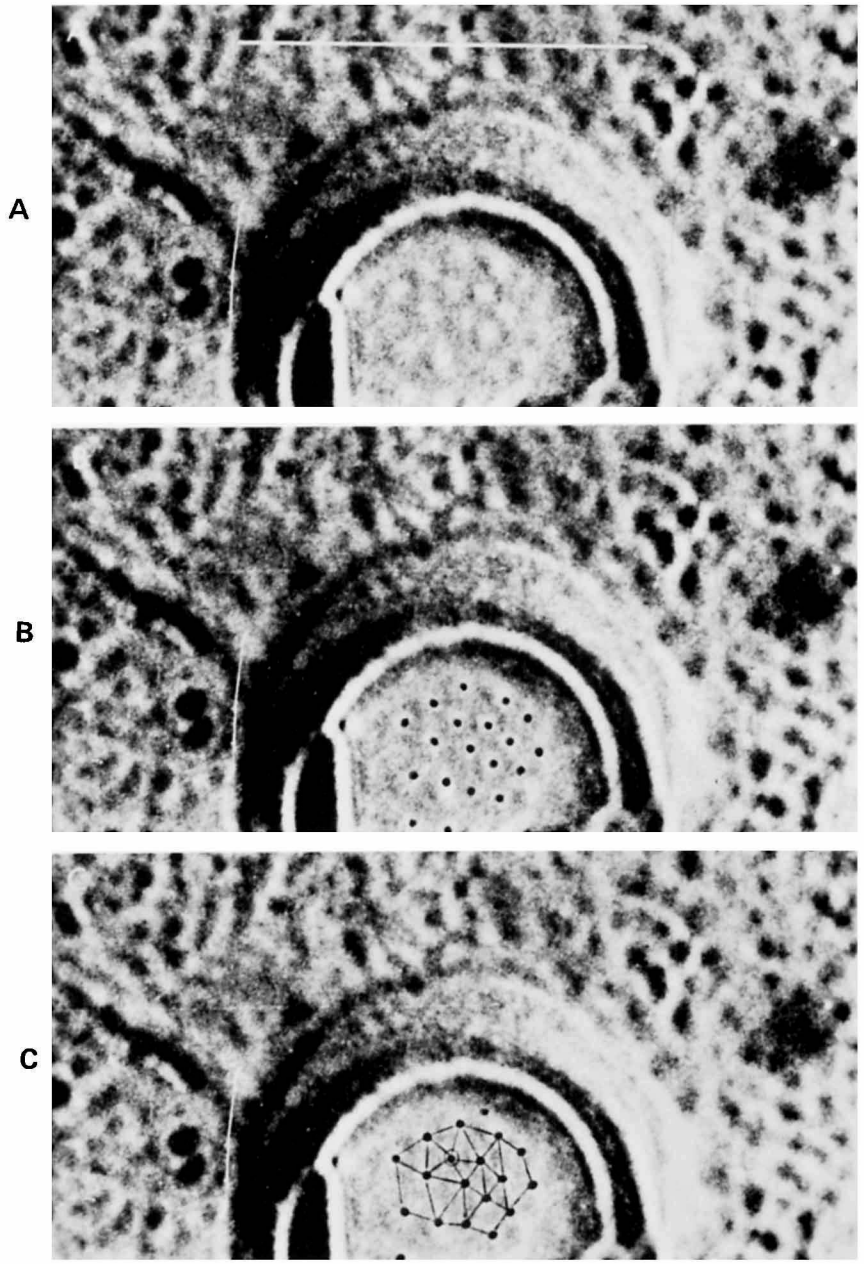


Fig. 8. A. Freeze–fracture of membrane showing submembrane particles in apparently symmetric array (320,000 ×). B. Submembrane particles are denoted by black dots. C. Dots are connected by lines to accentuate possible symmetry. Circled dot represents a five-fold axis. Bar is 2000 Å.

view of a typical field of chromaffin granules. Round profiles are observed which have an average diameter of 2250 Å; both convex (X) and concave (V) profiles are seen. A higher magnification view is seen in Fig. 7B, C where particles, approximately 70 Å (±5 Å) are observed on the fracture surfaces of the membrane. Quantitative particle counts reveal

that convex surfaces have 5000 to ± 250 particles/$u^2$ while concave surfaces have 2500 ± 400 particles /$u^2$ (n = 200). On convex surfaces the average distance between membrane particles is 120 Å ± 10 Å, while on concave surfaces the particles are essentially randomly distributed.

The profile in Fig. 7D apparently has both concave and convex surfaces on the same membrane. The arrows indicate particles of similar size to those noted in fracture profiles in Fig. 7B, C. These particles are most likely located either on a cross-fracture or concave fracture surface. This would suggest that the particles were located within the membrane's hydrophobic center, in accord with proposals of Branton (15) and others.

Finally, in Fig. 8 is a concave fracture profile which appears to have particles forming a five-fold axis juxtaposed to several six-fold axes. The particles are of the same size as those in other views and some are separated by approximately 120 Å.

On the basis of the freeze–fracture studies we can conclude that the intact chromaffin granule membrane is constructed of particles of approximately 70 Å in diameter, and that their separation is approximately 120 Å on the convex fracture surface. The particles appear be be localized to the interior of the hydrophobic region of the membrane and have a statistical affinity for the anticytoplasmic face of the membrane (roughly 2:1). Finally, occasional regions can be detected that appear highly ordered, possibly with partial hexagonal symmetry. However, the detection of a five-fold axis juxtaposed to a six-fold axis suggests that the spherical membrane may actually possess icosahedral symmetry.

## CONCLUSION

The chromaffin granule membrane appears to be a multi-enzyme assembly composed of 45% protein containing comparatively few protein components. It has several properties that make it ideal for X-ray diffraction analysis: the ability to form oriented arrays when centrifuged, and an apparent order in the membrane plane. The membrane vesicles apparently retain their inside–outside orientation, and it is likely that the individual membrane thickness is asymmetric. More work is yet to be done on the problem of surface symmetry and on the nature of the units constituting the surface. The interpretation of "hexagonal symmetry" may prove to be too simplistic, in view of the problem of such a symmetry on a spherical surface. In addition it is not clear whether the unit deduced from X-ray and the sperical unit observed by electron microscopy are the same, or what their compositions might be. At present we have only circumstantial evidence linking the two. For example, it is likely that the units responsible for the X-ray diffraction are protein while freeze–fracture membrane particles are, in general, also believed to be protein. In addition, it may be computed from the composition data of the membrane that the protein (distributed in 70 Å diameter spheres on a phospholipid bilayer) takes up 31% of the available membrane surface area. Coincidently, one can compute from freeze–fracture data that the membranes have approximately 7500 particles of /$\mu^2$ of 70 Å diameter, also taking up 31% of the surface area. The particles would appear capable of accommodating all of the protein in the membrane.

Work on the structure of the chromaffin granule membrane is presently continuing at a rapid pace in both Oxford and Bethesda. Therefore we can anticipate ongoing changes in our understanding of the structure of this membrane.

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