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# The Membrane of the Chromaffin Granule

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# The membrane of the chromaffin granule

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[Plate 51]

Chromaffin granules of the adrenal medulla are surrounded by a single unit membrane. So far no special morphological characteristics of these membranes have been described. However, biochemical analyses have revealed the special properties of these membranes.

The lipids are characterized by a high content of lysolecithin. It has been suggested that this specifically localized phospholipid is essential for the secretion of catecholamines, which involves membrane fusion.

The proteins of the granule membrane have also been investigated. Two major components appear to be specific for chromaffin granules of several species. Three enzymes, namely an $Mg^{2+}$-activated ATPase, dopamine $\beta$-hydroxylase and cytochrome *b*-559, are also known to be present in the granule membranes. The membranes of these organelles have no common structural backbone with microsomal membranes.

## Introduction

The discovery that the catecholamines in the adrenal medulla are stored in a specific subcellular particle, the chromaffin granule (Blaschko & Welch 1953; Hillarp, Lagerstedt & Nilson 1953; Blaschko, Hagen & Hagen 1957), has triggered off numerous biochemical and morphological investigations. Today we are in a position to discuss a specialized topic such as the membrane of chromaffin granules (for a wider perspective see Smith 1968). This discussion will concentrate on morphological and biochemical data concerning the membranes of the granules,

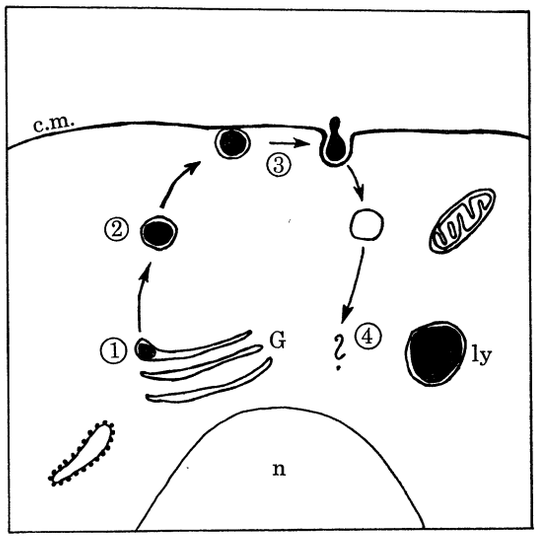


Figure 1. Schematic drawing of part of a chromaffin cell. Four aspects of the functional role of the membranes of chromaffin granules are indicated by numbers. (1) Are chromaffin granules surrounded by microsomal membranes or are their membranes specially assembled? (2) What role does the membrane play in the accumulation of a high concentration of catecholamines within the granules? (3) What is the mechanism of the fusion process preceding the secretion of catecholamines? (4) What is the fate of the empty granule membranes following exocytosis? Abbreviations: n, nucleus; ly, lysosomes; c.m., cell membrane; G, Golgi region.

but by relating these results to functional questions, we will be immediately involved in fundamental problems of the whole chromaffin cell. Four aspects of these questions are indicated in figure 1 which gives a schematic drawing of part of a chromaffin cell.

## The morphology of the chromaffin granule membranes

Let us first turn to morphological studies. These have provided direct evidence that chromaffin granules are membrane-limited structures. The first electron micrographs of sections of adrenal medulla were published by Lever (1955) and his results have been confirmed and elaborated in subsequent studies on various species (Wetzstein 1957; De Robertis & Vaz Ferreira 1957; Eränkö & Hänninen 1960; Yates 1964; Coupland 1965; Elfvin 1965; Benedeczky, Puppi, Tigyi & Lissak 1965; Moppert 1966; Diner 1967; Bloodworth & Powers 1968; Al-Lami 1969; see also Grynszpan–Winograd, this volume, p. 291). Chromaffin granules, which vary in size from 50 to 400 nm, are surrounded by a single unit membrane. Apparently no detailed investigation of the thickness of this membrane and of the presence of any substructures has been undertaken. Such a study might be worth while, since the various membranes of a cell can be differentiated by morphological details of this kind (see Sjöstrand 1968).

Recently we were able to investigate the morphological characteristics of the chromaffin granules of cat adrenal medulla by a special method of electron microscopy, namely, the freeze-etching technique (Plattner, Winkler, Hörtnagl & Pfaller 1969). A survey picture of chromaffin cells is given in figure 2, plate 51. Freeze-etching, which allows one to obtain electron micrographs from tissues without preceding chemical fixation, reveals the various membranes of the cell in a striking way (see Moor 1969). The membrane surfaces of chromaffin granules as exposed by this technique were covered with minute granules and plaques of varying size. Similar structures were also seen on plasma membranes of the adrenal medullary cells (Plattner *et al.* 1969) and they have also been described for membranes from other sources (see Staehelin 1968). Thus, neither conventional electron microscopy nor the freeze-etching technique has yet revealed any special characteristics of the chromaffin granule membrane. However, from the biochemical results reported below it becomes apparent that these membranes have indeed distinctive properties.

## Isolation and composition of chromaffin granule membranes

Isolated chromaffin granules are lysed when treated with hypotonic buffer, and the soluble contents, i.e. catecholamines, ATP and chromogranins, are dissolved in the medium (Hillarp 1958*a*). Since there is no morphological evidence for the presence of any insoluble material within chromaffin granules (Diner 1967) the insoluble constituents must be all part of the membrane. These membranes can be recovered as a sediment by centrifugation. A further purification can be achieved by several washing steps, a method which has recently been investigated in some detail (Winkler, Hörtnagl, Hörtnagl & Smith 1970*b*). The composition of membranes purified in this way is given in table 1. The lipids of chromaffin granules were recovered quantitatively in the membranes and so were cytochrome *b*-559 and the enzyme ATPase. The activity of dopamine $\beta$-hydroxylase, however, was equally divided between the soluble contents and the washed membranes, a finding that has already been described (Viveros, Arqueros & Kirshner 1968; Belpaire & Laduron 1968). Only traces of chromogranin A, the

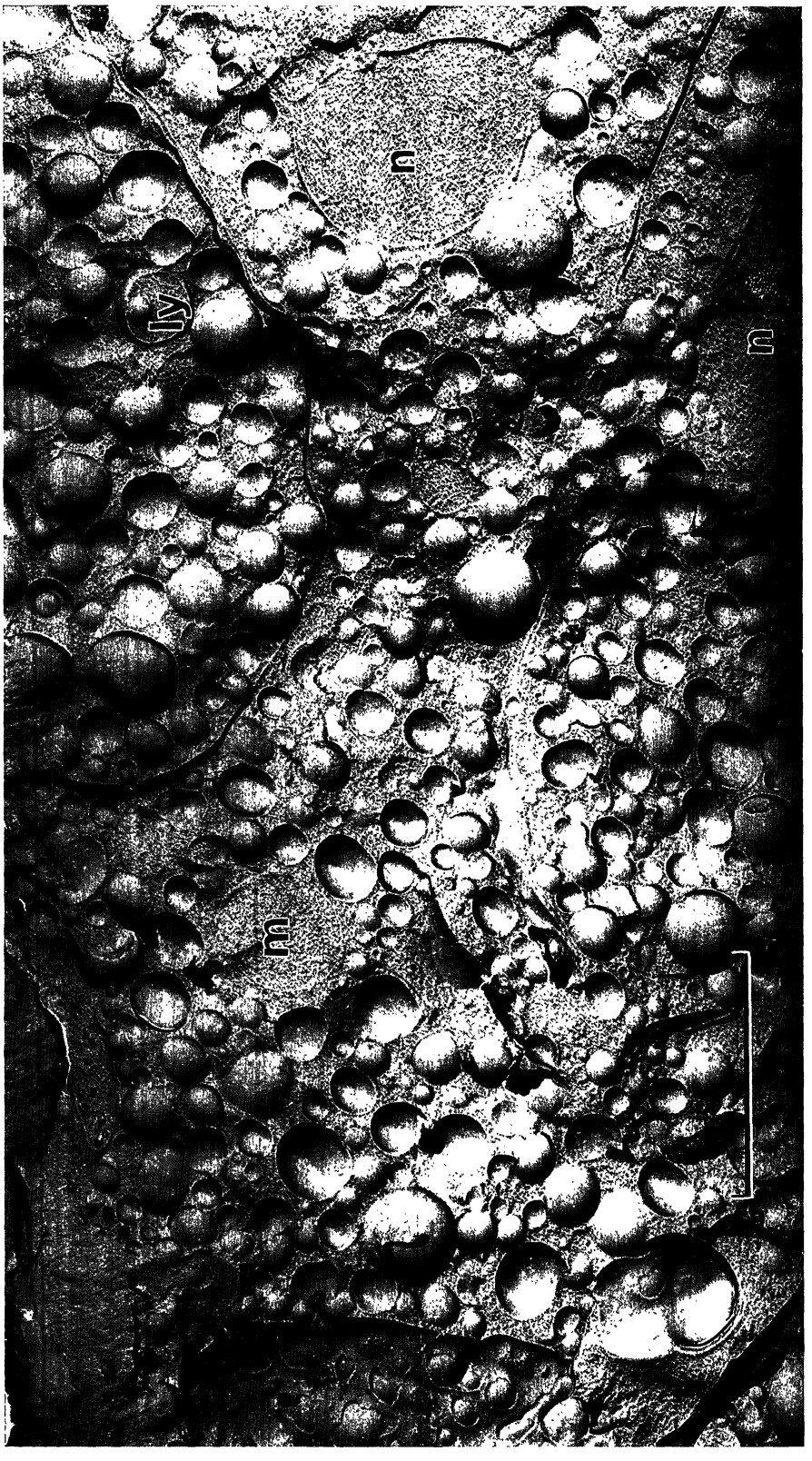


FIGURE 2. Electron micrograph obtained by freeze-etching of cat adrenal medulla (see Plattner *et al.* 1969). Survey picture of adjacent chromaffin cells. Two nuclei (n) are seen in cross-section. The globular particles are mostly chromaffin granules, although some might present lysosomes (ly) and mitochondria (m). The scale indicates 1 $\mu$m.

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main soluble protein, were found to be present in the membrane fraction when measured by micro-complement fixation.

Let us now make the first attempt of several to relate the biochemical characteristics of these membranes to the role they have to perform during the life span of the chromaffin granules. What is the significance of the presence of the three enzymes mentioned above?

The function of cytochrome *b*-559 (Spiro & Ball, 1958; Ichikawa & Yamano 1965; Banks 1965) is completely unknown. This cytochrome is common to both the chromaffin granules and microsomes. The function of dopamine $\beta$-hydroxylase is obvious since this enzyme is essential for the conversion of dopamine to noradrenaline. Its localization in chromaffin granules has

TABLE 1. COMPOSITION OF MEMBRANES OF BOVINE CHROMAFFIN GRANULES

The data are taken from the paper by Winkler *et al.* (1970*b*). $Mg^{2+}$-ATPase was measured at the optimal pH of 6.4; dopamine $\beta$-hydroxylase was determined at pH 5.5. Chromogranin A was assayed by micro-complement fixation.

| | |
|---|---|
| protein | 1 mg |
| lipid-phosphorus | 2.4 $\mu$mol |
| cholesterol | 1.66 $\mu$mol |
| $Mg^{2+}$-ATPase | 1.8 $\mu$mol/h |
| dopamine $\beta$-hydroxylase (substrate 5 $\mu$mol/l) | 0.012 $\mu$mol/h |
| cytochrome *b*-559 | 0.46 ($E_{425}$) |
| chromogranin A | 0.04 mg |

been repeatedly demonstrated (Kirshner 1959; Oka *et al.* 1967*a*; Laduron & Belpaire 1968). However, we do not yet know why it is partly bound to the membrane and partly confined to the soluble content, with which it is released from the cell during the secretion of catecholamines (Viveros *et al.* 1968). It is even not clear whether the bound and the soluble enzyme are the same molecular species. Is therefore the enzyme which has been purified from bovine adrenal medulla (Kaufman & Friedman 1965) derived from the membranes or the soluble contents?

The functional implications of the enzyme ATPase, first described by Hillarp (1958*b*) and later by Banks (1965) and by Kirshner, Kirshner & Kamin (1966*b*) are even more complicated. It has been suggested that this enzyme is involved in two apparently opposed functions, in the uptake of catecholamines and in their release. First, it has been proposed that ATPase might be essential for an active uptake of the amines into the chromaffin granules (see Banks 1965; Kirshner *et al.* 1966*b*; Taugner & Hasselbach 1966). Such an uptake has been demonstrated with isolated chromaffin granules incubated in isotonic sucrose at 37 °C in the presence of ATP and $Mg^{2+}$ (Carlsson, Hillarp & Waldeck 1962; Kirshner 1962). Amine uptake and the ATPase activity are both inhibited by *N*-ethylmaleimide, and both are activated by $Mg^{2+}$. This may indicate that the two processes are interconnected. On the other hand, it is not yet clear how an ATPase activity localized within the membranes may provide an active uptake through these membranes which are said to be permeable to catecholamines *in vitro*. However, the evidence for their permeability is convincing only for granules incubated at 0 °C (Carlsson & Hillarp 1958; Hillarp 1959; Kirshner, Holloway & Kamin 1966*a*). An additional complication arises from the recent demonstration of a stable catecholamine/ATP complex *in vitro*, which is furthered by alkaline-earth ions including $Mg^{2+}$. The presence of this complex in granules may account for the high concentration of catecholamines in these organelles and may even offer an explanation for an apparent uptake mechanism (Berneis, Pletscher & Da Prada 1969). Therefore, the mechanism of the uptake of catecholamines seems to depend upon several factors. Further results are needed before any unifying hypothesis can be advanced.


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The second suggestion as to the function of ATPase is related to the release of catecholamines. Isolated granules incubated in an electrolyte solution containing ATP, $Mg^{2+}$ and $Cl^{-}$ release their total soluble contents (Oka, Ohuchi, Yoshida & Imaizumi 1967*b*; Poisner & Trifaró 1967; Lishajko 1969). It has been suggested that an activation of ATPase is triggering off this release *in vitro* and that a similar process is involved in the secretion of catecholamines *in vivo* (Poisner & Trifaró 1967; Douglas 1968; Ferris, Viveros & Kirshner 1970). However, *in vivo* the process of catecholamine release is not confined to granules alone but involves an interaction between two membranes, that of the granule and that of the cell. This interaction leads to a fusion of these two membranes and an expulsion of the total soluble contents (see figure 1 (3); Banks & Helle 1965; Diner 1967; Sage, Smith & Kirshner 1967; Schneider, Smith & Winkler 1967; Poisner, Trifaró & Douglas 1967). Therefore, even if ATPase is triggering off some change in the granule membrane, the actual fusion process must involve two membranes and in these membranes both the main lipid and protein components must interact. It is necessary, therefore, first to discuss the characterization of these two membrane components before we can return again to functional problems.

## The lipids of granule membranes

The main lipids of chromaffin granules are cholesterol and phospholipid (Winkler, Strieder & Ziegler 1967*b*). The molar ratio of cholesterol to lipid-phosphorus is 0.58 for the granules, whereas for mitochondria and microsomes this ratio is lower (Blaschko, Firemark, Smith & Winkler 1967). However, a much more striking difference between these organelles was found when the various phospholipids were quantitatively determined. Ox chromaffin granules

Table 2. Lysolecithin content of chromaffin granules from the adrenal medulla of various species

| adrenal gland | lysolecithin (% of total lipid-P) | references |
|---|---|---|
| ox | 16.8 | Blaschko *et al.* (1967*a*)<br>Winkler *et al.* (1967*b*) |
| ox | 12.9 | Trifaró (1969) |
| horse | 7.1 | Winkler *et al.* (1967*b*) |
| rat | 15.4 | Winkler *et al.* (1967*b*) |
| pig (total chr. gran.) | 11.3 | Winkler *et al.* (1967*b*) |
| pig (mainly noradrenaline-containing) | 19.7 | Winkler (1969) |
| human phaeochromocytoma (3) | 11.7, 17.8, 23.8 | Blaschko *et al.* (1968) |

contained lysolecithin in a concentration of 16.8% of the total lipid phosphorus, whereas mitochondria and microsomes contained only traces of lysolecithin (Blaschko *et al.* 1967*a*; Winkler *et al.* 1967*b*). Further studies (see table 2) revealed that the lysolecithin content was high not only in chromaffin granules of the ox, but also in granules from various other species including those from human phaeochromocytoma (Winkler *et al.* 1967*b*; Winkler, Ziegler & Strieder 1967*c*; Blaschko *et al.* 1968). It is noteworthy that already in 1957, Hajdu, Weiss & Titus had found that the adrenal medulla was a rich source for lysolecithin. No doubt this was due to the lysolecithin in chromaffin granules, since it has been calculated that practically all of the lysolecithin found in the adrenal medulla is confined to these cell organelles (Winkler 1969).


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The finding of lysolecithin in chromaffin granules initiated a study on the metabolism of this compound. Lysolecithin can be formed from lecithin by phospholipase A and in fact two such phospholipases could be demonstrated in the adrenal medulla, a phospholipase $A_1$ and a phospholipase $A_2$ (Blaschko *et al.* 1967*b*; Winkler, Smith, Dubois & van den Bosch 1967*a*; Smith & Winkler 1968). Since the lysolecithin of chromaffin granules has the fatty acid in the 1 position of the molecule (Winkler & Smith 1968), it can be formed by a phospholipase $A_2$, which removes the fatty acid in the 2 position of the lecithin molecule. It is, therefore, the phospholipase $A_2$ that has to be considered as responsible for the formation of lysolecithin.

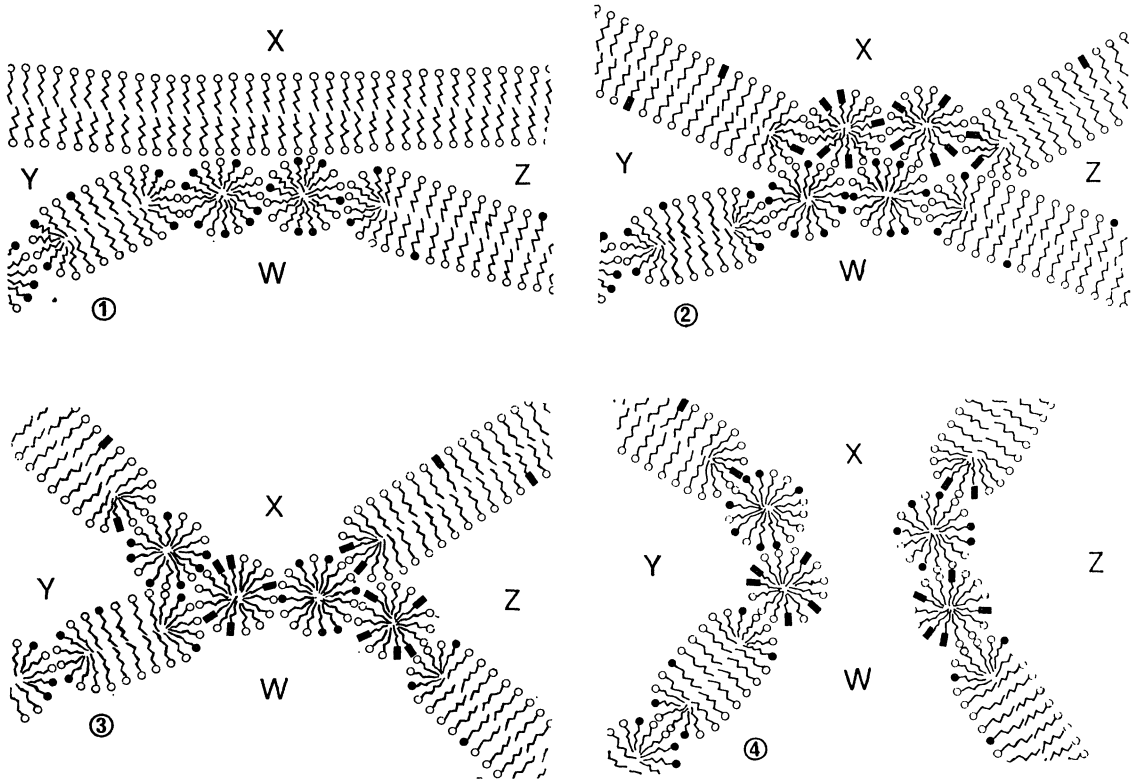


FIGURE 3. Diagram illustrating the possible involvement of lysolecithin in fusion processes (according to Lucy 1969). In the adrenal medulla the upper membrane in each diagram represents the plasma membrane, whereas the lower membrane corresponds to the membrane of a chromaffin granule. This latter membrane is organized partly (see 1) in globular micelles which might be due to the wedge-shaped molecules of lysolecithin. Fusion of these two membranes (see 3 and 4) can only occur if globular micelles are formed (see 2) in the plasma membrane. This may be caused, e.g. by the stimulus for secretion activating either lysolecithin molecules already present in the cell membrane or by a phospholipase A which might form lysolecithin.

However, the subcellular localization of this enzyme, which was found to be confined to lysosomes, could not easily be correlated with lysolecithin in chromaffin granules. Two explanations have been offered (Winkler & Smith 1968): either the phospholipase $A_2$ responsible for the lysolecithin in granules has not yet been discovered or the lysolecithin is produced in the Golgi region, from where both lysosomes and chromaffin granules originate.

Just as there is no enzyme that can be found in granules which is responsible for the formation of lysolecithin, there also is no enzyme in these granules to effect its breakdown since lysophospholipase activity in adrenal medulla is confined to microsomal elements (Hörtnagl, Winkler & Hörtnagl 1969). In this connexion, there is no evidence for a high turnover of

lysolecithin in the ox adrenal medulla either when stimulated or when unstimulated (Trifaró 1969).

The discovery of lysolecithin happened at a time when evidence for the occurrence of exocytosis, and hence of membrane fusion, in the adrenal medulla was rapidly accumulating. Lysolecithin, which, as the name already implies, can lyse erythrocytes and other membrane-limited structures, is a membrane-active compound; it was, therefore, an obvious suggestion that this specifically localized phospholipid might be involved in the fusion process between the membranes of chromaffin granules and the plasma membrane. The molecular mechanism of membrane lysis and fusion due to lysolecithin probably depends on the fact that this molecule has a wedge shape (Haydon & Taylor 1963), as a result of the hydrophilic portion being broader than the hydrophobic part. A molecular model (see figure 3) of how the wedge-shaped molecule of lysolecithin might be involved in fusion processes has recently been drawn up by Lucy (1969). If a membrane whose lipids are partly in the form of globular micelles, e.g. due to the presence of lysolecithin, is approaching another one, whose lipids are in the form of a bimolecular leaflet, then these two membranes can fuse only if the bimolecular leaflet in the second membrane is partly transformed into globular micelles. If we adopt this model for the process of fusion between chromaffin granules and the cell membrane, then the granule membrane is already partly in the form of globular micelles, but fusion can only occur if the cell membrane is also partly in a globular micellar configuration. Unfortunately, plasma membranes of the chromaffin cell have not been isolated, and so we do not know whether this membrane contains lysolecithin or a phospholipase A which might produce it when triggered off by the secretion stimulus. But it is noteworthy that Howell & Lucy (1969) have not only presented a hypothetical model but also provided experimental evidence for the fusing capacity of lysolecithin. When erythrocytes were treated with low concentrations of lysolecithin then the membranes of these cells fused with each other forming giant cells. Maybe this phospholipid is, if we consider its physiological role, not so much a lysolecithin but a fusolecithin.

Already in the previous section we discussed the possibility that ATPase might be involved in the secretion of catecholamines and in this section we have presented a likely candidate for the actual fusion process. Thus, we might now ask the specific question how an activation of ATPase might trigger off a fusion process involving lysolecithin. However, as already pointed out, it is only when we know what all membrane components are, and how they are arranged in the membrane, that such questions become meaningful. We, therefore, turn now to the characterization of further membrane constituents, the main protein components.

## The proteins of granule membranes

Three membrane proteins, namely ATPase, cytochrome *b*-559 and dopamine $\beta$-hydroxylase have already been mentioned. This section will discuss the characterization of the total membrane proteins which has been achieved during the past year (Winkler *et al.* 1970*b*).

The proteins of the membranes of bovine chromaffin granules were solublized by either detergents or phenol–acetic acid–urea and then subjected to polyacrylamide gel electrophoresis in two different buffer systems. A separation of the proteins into several components could thus be achieved (see figure 4). One of the proteins corresponded to chromogranin A, the main soluble protein; however, it was only present in small amounts (see table 1) which probably represented traces of the soluble proteins still adhering to the membranes. Therefore,


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we cannot support the suggestion made by Helle & Serck-Hanssen (1969) that chromogranin A is a major component of both the content and the membranes of these granules. The membranes contain in fact two major proteins (A and B in figure 4), which are not found in the soluble content. These two proteins were present not only in membranes of bovine granules but also in granules from horse and pig adrenal medulla and also in those isolated from a human phaeochromocytoma (H. Hörtnagl & H. Winkler, unpublished observations). It is noteworthy that a common soluble component was detected only in ox, horse and pig granules (Winkler,

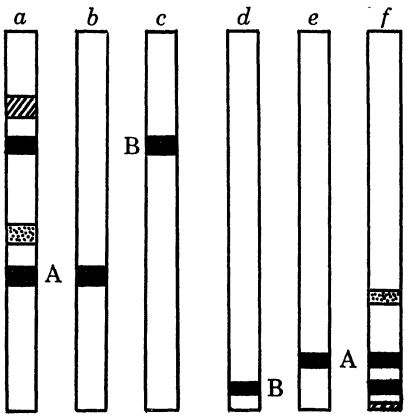


FIGURE 4. Polyacrylamide gel electrophoresis of membrane proteins from bovine chromaffin granules in two different gel systems. Only the main components (compare Winkler *et al.* 1970*b*), which were visible after staining, were drawn. *Alkaline gel system*: (*a*) total membrane proteins, (*b*) chromomembrin A, (*c*) chromomembrin B; *acid gel system*: (*d*) chromomembrin B; (*e*) chromomembrin A; (*f*) total membrane proteins.

TABLE 3. AMINO ACID COMPOSITION OF INSOLUBLE PROTEINS FROM BOVINE CHROMAFFIN GRANULES (IN % BY MASS)

The data from the first columns of numbers are taken from Winkler *et al.* (1970*b*), those from the second columns from Helle & Serck-Hanssen (1969).

| | | | | | |
|---|---|---|---|---|---|
| Lys | 6.1 | 8.0 | Gly | 4.2 | 5.0 |
| His | 2.8 | 2.7 | Ala | 5.3 | 5.8 |
| $NH_3$ | 2.0 | 4.2 | Cys | 0.5 | 1.4 |
| Arg | 7.1 | 8.1 | Val | 5.0 | 6.3 |
| Asp | 9.6 | 8.5 | Met | 2.9 | 1.6 |
| Thr | 4.6 | 3.3 | Ile | 3.7 | 3.9 |
| Ser | 7.6 | 5.0 | Leu | 9.9 | 9.9 |
| Glu | 15.4 | 13.9 | Tyr | 4.1 | 3.5 |
| Pro | 5.4 | 4.4 | Phe | 5.5 | 4.1 |

Ziegler & Strieder 1966; Helle 1966; Hopwood 1968), but not in those from a human phaeochromocytoma (Strieder, Ziegler, Winkler & Smith 1968).

Recently, a preparative separation of the two major membrane proteins has been achieved by chromatography with Sephadex G-200 in the presence of detergents (H. Hörtnagl & H. Winkler, unpublished observations). By this procedure these two proteins could be purified. Work is now in progress to obtain an amino acid analysis of these proteins. Such an analysis (see table 3) has hitherto been obtained only for the total membrane proteins (Winkler 1969; Helle & Serck-Hanssen 1969; Winkler *et al.* 1970*b*).

When the purified protein components became available we were able to correlate the electrophoretic behaviour of the main proteins in two different gel systems (see figure 4). The component B, which moves faster in the alkaline system, migrates very slowly in the acid

system, whereas component A moves similarly in both systems. Since these two membrane components have been isolated and are now defined by electrophoresis we suggest to call them chromomembrin A and B in order to distinguish them from chromogranin A, the major soluble protein.

The proposal to call these proteins chromomembrin A and B already implies that these proteins can be considered as specific for the chromaffin granules. A comparison of the membrane proteins of granules with those from mitochondria and microsomes revealed that in these organelles the two granule components are present in insignificant amounts, if at all (Winkler *et al.* 1970*b*).

We now consider the question of the origin of the granule membranes (see figure 1 (1)). We have already pointed out that there are clear differences between the lipids of the granule membranes and those from microsomes. The characterization of the membrane proteins exemplifies the differences further. Chromaffin granules are surrounded by membranes which are different from microsomal membranes and which have no common structural backbone with them. These experiments allow us to distinguish between two modes of origin of the chromaffin granule membrane. First, the membranes could be replenished by vesiculation of *specific* regions of the membranes of the endoplasmic reticulum or, secondly, the granule membranes could be formed continuously by a non-selective flow of complete membranes from the endoplasmic reticulum. The second possibility seems to be excluded. A still unresolved question is whether the membranes of the Golgi apparatus, lysosomes and chromaffin granules contain the same main protein components. Morphological studies have led to suggestions that the chromaffin granules and the lysosomes of the adrenal medulla originate in the Golgi region (De Robertis & Sabatini 1960; Coupland 1965; Elfvin 1967; Ratzenhofer & Müller 1967; Holtzman & Dominitz 1968). Nothing is known at present about the composition of the membranes of lysosomes and of the membranes in the Golgi region.

We now know something about all the main constituents of the membranes and, therefore, of the components which must be involved in membrane fusion during secretion. Nevertheless, for a further understanding we must know a good deal about the substructure of these membranes. One approach to this problem is to treat the granule membranes with various hydrolytic enzymes. When isolated membranes are incubated with pronase, up to 50 % of the protein can be digested. The ATPase activity disappears completely, whereas the two main protein components are still observable in disk electrophoresis (H. Hörtnagl, H. Winkler & A. D. Smith, unpublished observation). These results demonstrate that ATPase must be localized in a position accessible to pronase, i.e. somewhere on the surface of these membranes (see also Taugner & Hasselbach 1967), and furthermore that chromomembrin A or B are unlikely to possess ATPase activity. It is hoped that further studies will lead to an understanding of the substructure of the membrane and finally, when we have also learned something about the plasma membrane, to a more precise knowledge of the fusion mechanism.

A final point which should be discussed concerns the fate of the granule membrane after secretion (see figure 1 (4)). According to recent studies (Malamed, Poisner, Trifaro & Douglas 1968; Viveros, Arqueros & Kirshner 1969) it seems likely that the empty membranes return to the cytoplasm. There they could be taken up by lysosomes and become digested or they could return to the Golgi region in order to be re-used. To distinguish between these alternatives a study on the turnover of the proteins of chromaffin granules in bovine adrenal medulla is in progress. It is likely that the turnover of membrane and soluble proteins would be similar if the


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membranes are not re-used, since both components are lost, the soluble ones by secretion, the insoluble ones by digestion; both components must then be replenished by synthesis. However, if the membranes are re-used, their proteins should have a low turnover. Preliminary studies (Winkler, Hörtnagl, Hörtnagl & zur Nedden 1970*a*) indicate that this latter possibility may be more likely.

## Conclusions

The components of the membranes of chromaffin granules, namely lipids, proteins and enzymes, have been investigated in some detail. Current attempts aim at correlating these analytical results with functional problems. It may well be that such a correction is more readily achieved in this particularly specialized membrane than in the more complex membranes derived from other sources.

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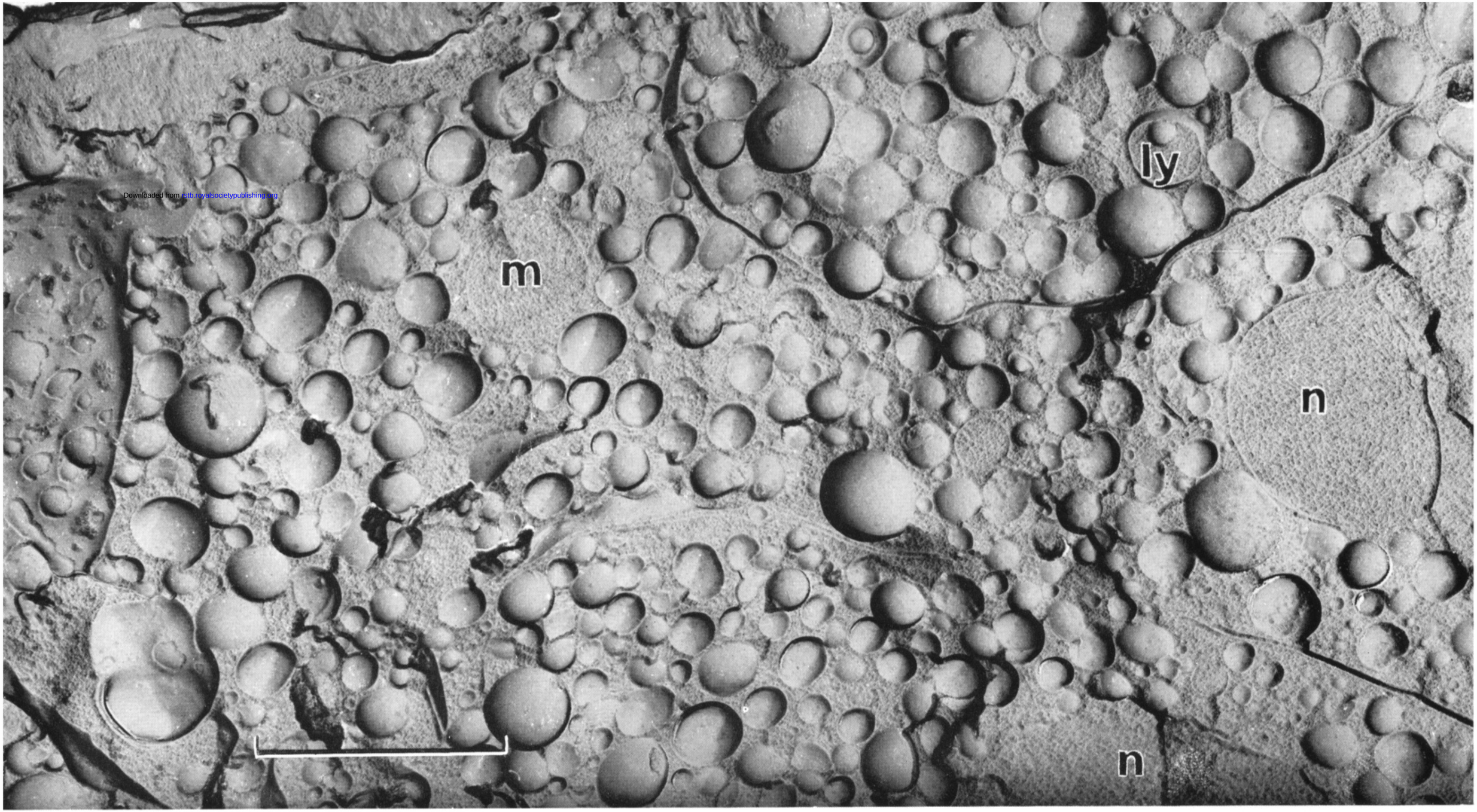


FIGURE 2. Electron micrograph obtained by freeze-etching of cat adrenal medulla (see Plattner *et al.* 1969). Survey picture of adjacent chromaffin cells. Two nuclei (n) are seen in cross-section. The globular particles are mostly chromaffin granules, although some might present lysosomes (ly) and mitochondria (m). The scale indicates 1 $\mu$m.


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