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BIOCHEMICAL STUDIES OF THE CHROMAFFIN GRANULE

III. REDISTRIBUTION OF LIPID PHOSPHATE, DOPAMINE-β-HYDROXYLASE AND CHROMOGRANIN A AFTER FREEZING AND THAWING OF THE ISOLATED GRANULE MEMBRANES

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SUMMARY

Freezing and thawing a dialysed suspension of lysed chromaffin granules and sedimented membrane preparations resulted in redistribution of lipid phosphate and protein. By this treatment the high ratios of lipid phosphate/protein in the membrane fragments, isolated on sucrose density gradient from the dialysed suspension and the sedimented membrane preparation, were reduced from 1.56 to 1.03 μmoles/mg and from 1.97 to 0.83 μmoles/mg, respectively.

Multilamellar, liposomal structures could be isolated from the frozen and thawed membrane preparations and were found to sediment in the 0.4 M sucrose layer by density gradient centrifugation. This fraction was without morphological resemblance to the intact chromaffin granules or their membranes and was found to account for 53% of the lipid phosphate, 35% of chromogranin A, 21% of dopamine-β-hydroxylase activity and 12% of the protein of the total preparation. The specific activities of chromogranin A and dopamine-β-hydroxylase in the artificially formed liposomal structures closely resembled that of the solubilized protein and was significantly higher than in the lipid phosphate-depleted membrane fragments recovered in the 1.1 M sucrose layers.

It is concluded that freezing and thawing as a means of purifying the isolated granule membranes lead not only to the solubilization of chromogranin A, but also to removal of dopamine-β-hydroxylase activity and lipid phosphate from the labile membrane fragments.

INTRODUCTION

Substantial amounts of experimental work have been carried out in the last decade in order to elucidate the molecular architecture of the storage complexes for catecholamines in adrenergic tissue[1]. The available evidence indicates that the integrity of the membrane and matrix phases is essential for the storage of catecholamines in the chromaffin granules and the axonal vesicles of the splenic nerves, tissue organelles which serve as experimental models for adrenergic mechanisms[2–4].

Chromogranin A and dopamine-β-hydroxylase have been taken as "markers" of the matrix and membrane phases of storage granules, respectively, although the distribution of these two constituents in the soluble and sedimentable granule fractions has remained a matter of some dispute[5].

The apparent disagreements over the distribution patterns of these two constituents may stem from differences in methods employed for the lysis of the granules and the washing of the sedimentable fractions. Recent findings[6] have shown that sedimentable aggregates, with a 4-fold increase in dopamine-β-hydroxylase activity, were formed when the soluble chromogranins were sonicated in the presence of phospholipids. Furthermore, the artificial aggregates sedimented into 0.4–0.6 M sucrose and would thus, if formed during the separation of the granule phases, be expected to contaminate the membrane-containing fraction and lead to artifactual distribution patterns of chromogranin A, dopamine-β-hydroxylase and phospholipids. Since these constituents are so frequently used as "markers" of the granule fractions, it has been necessary to reinvestigate the distribution patterns of these "marker" constituents in the soluble and sedimentable fractions of the bovine adrenomedullary chromaffin granules.

The present study deals with the effects of the methods for the separation of the soluble and sedimentable granule fractions recently proposed, *e.g.* lysis by dialysis[7] and repeated freezing and thawing of the isolated membrane-containing fraction[8]. The results obtained indicate that particularly the latter method leads to artifactual redistributions of membrane-bound lipid phosphate, dopamine-β-hydroxylase and chromogranin A into a heterogeneous suspension of solubilized protein, phospholipid-rich particles and membrane fragments depleted in lipid phosphate, dopamine-β-hydroxylase and chromogran in A.

METHODS

Chromaffin granules were prepared from bovine adrenals by density centrifugation[9]. In some experiments the highly purified granules were lysed by suspension in 10 vol. of the hypotonic buffer, 5 mM sodium succinate pH 6.0 (S1). For the other experiments the granules were suspended in 2 vol. of the hypotonic buffer and dialysed against 2 changes of 500 vol. over a period of 48 h in the cold. The matrix and membrane phases of the dialysed granules were separated by density gradient fractionation (see below) or by centrifugation at $72 \cdot 10^5 \times g_{av} \cdot$ min (MSE Superspeed 40, angle rotor 25 ml $\times$ 10) as described earlier[7].

The first supernatant was taken as the matrix protein fraction (SN1) and the remaining water-insoluble sediment was further washed twice in 10 vol. of buffer. The final, twice-washed pellet was resuspended in 1 vol. of buffer and used as source of the dialysed, isolated membrane phase (S4).

Density gradient fractionations were carried out in a Model L 50 Spinco, rotor SW 39. Discontinous gradients of sucrose were used after 20 h equilibration in the cold. After centrifugation at $78 \cdot 10^5 \times g_{av} \cdot$ min the fractions were collected by careful suction with calibrated pipettes from the top of the tubes.

Dopamine-β-hydroxylase (EC 1.14.2.1) activity was assayed with 10 mM tyramine as the substrate according to the spectroscopic method of Gibb *et al.*[10]. The enzyme activity was determined by the amounts of oxidized product formed after

10 min of incubation at 37 °C at three concentrations of sample protein. The unit of activity is expressed as nmoles/min (=10 nmoles/10 min[11]).

Chromogranin A was determined immunologically[12] by the double diffusion technique. The liposomal and membrane fractions were assayed in presence of Triton-X-100 (2%, v/v).

Protein was assayed by the Folin method of Lowry *et al.*[19].

Lipid-*P* was determined in 0.1-ml samples by extraction with 20 vol. of chloroform–methanol (2:1, v/v) and the organic phase was washed with 10 vol. of 0.17 M NaCl. Aliquots of the chloroform phase were assayed for total extracted phosphate by the Fiske-SubbaRow method after digestion in 5 M H_2SO_4 and 30% H_2O_2, as previously described[11].

Copper was assayed in the protein fractions by the standard procedure of Perkin–Elmer, using their model 303 atomic absorption spectrophotometer.

Electron microscopy of the membrane fractions was performed on material fixed as pellets in 2.5% glutaraldehyde, buffered at pH 6.3 with 30 mM potassium phosphate and made isotonic with the samples by addition of sucrose. The material was post-osmicated and embedded in Durcupan[9,11]. Lead citrate was used as a positive stain and the morphology of the fixed fractions was studied in the Siemens electron microscope.

Reduction of pore size in the dialysis tubing was achieved by treatment with hot $NaHCO_3$, according to the method of Nelbach *et al.*[14]. By this method the dialysis tubing was rendered 100% impermeable to lysozyme (mol. wt 14600).

RESULTS

Separation of the matrix and membrane phases

The suspension of lysed chromaffin granules (S1) was fractionated on a density gradient ranging from 0.4–1.6 M sucrose and the distribution patterns of Folin-reactive material and lipid-*P* obtained in a representative experiment, are given in Fig. 1. Dialysis of the lysed suspension prior to gradient fractionation ($S1_D$, Fig. 1), resulted in loss of Folin-reacting material, particularly from the supernatant fraction while the amount of material recovered in the 1.1 M sucrose layer, *i.e.* the membrane fraction, seemed largely unaffected by the dialysis procedure. The loss probably mainly reflects the loss of catecholamines, since mM concentrations of catecholamines interfere with the Folin reagent. The estimations of lipid-*P* in the gradient fractions (Table I) suggested that dialysis had also caused a removal of phospholipids from the supernatant fraction, *i.e.* the matrix protein (Fraction 1, $S1_D$). Analysis of the membrane (Fraction 4, $S1_D$) showed that after dialysis this fraction was markedly enriched in lipid-*P*, suggesting that the phospholipids lost from the supernatant fraction might partly have been co-sedimented with the membrane fragments. The latter was supported by the observation that freezing and thawing of the dialysed suspension prior to fractionation abolished the apparent loss of phospholipids from the supernatant (Fraction 1, $S1_{D-20}$, Table I). The ratio of lipid-*P*/protein in the membranes (Fraction 4, $S1_{D-20}$) was lower than that of the unfrozen (Fraction 4, $S1_D$) and only slightly higher than that of the undialysed, unfrozen suspension (Fraction 4, S1), indicating that freezing and thawing affected the structural organization of the phospholipid-rich membranes obtained after dialysis.

Analyses of the distribution patterns of protein and lipid-*P* in purified mem-

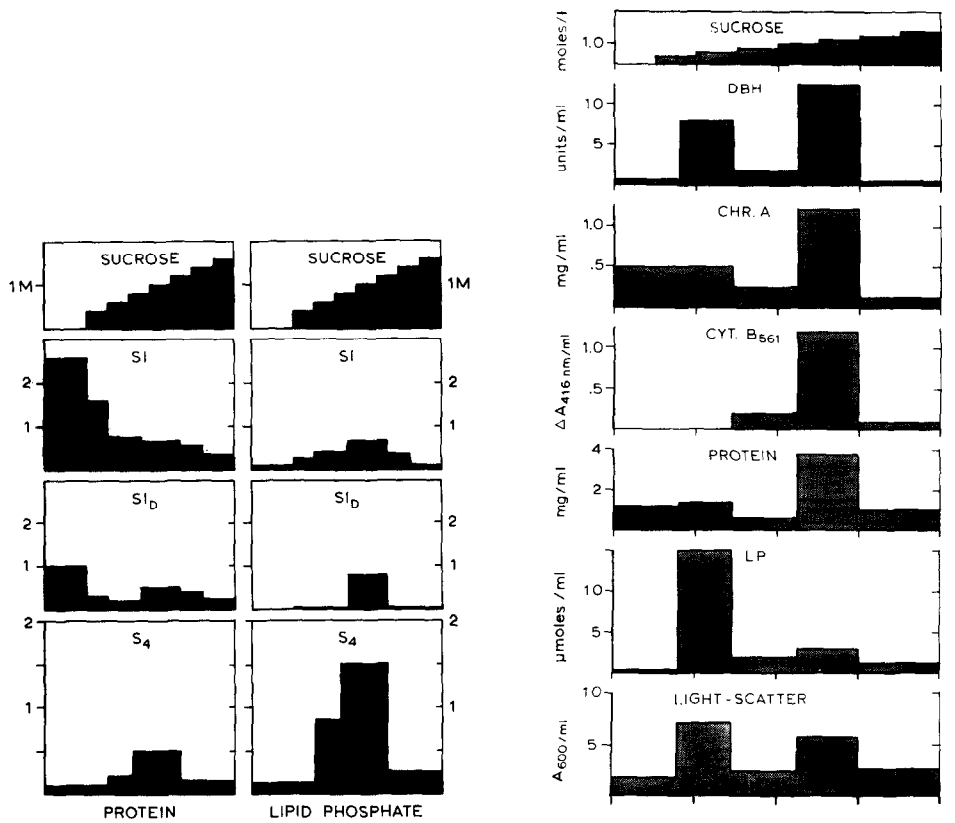

Fig. 1. Distribution of constituents in fractions of lysed granules. Chromaffin granules were lysed by suspension in hypotonic buffer at pH 6.0 and samples were subjected to isopycnic centrifugation on a gradient ranging from 0.4 to 1.6 M sucrose before (S1) and after dialysis (S1$_D$) against large volumes of buffer. Isolated membrane preparations (S4) were suspended in 1 vol. of buffer before application to the gradient. Left abcissa: mg Folin protein/ml; right abcissa: μmoles lipid-*P*/ml.

Fig. 2. Distribution of constituents in subfractions of isolated membrane preparations subjected to freezing and thawing. Isolated membrane preparations (S4) were frozen at -20 °C at a protein concentration of approximately 5 mg/ml in the hypotonic buffer and thawed prior to fractionation on the sucrose gradient ranging from 0.4–1.6 M. The total gradient volume was 4 ml and the developed gradients were collected in 5 fractions (*cf.* Methods). DBH, dopamine-β-hydroxylase; CHR. A, chromogranin A; CYT. B$_{561}$, cytochrome b_{561}; LP, phospholipid.

brane preparations (S4, see Methods) revealed that both constituents were largely recovered in the 1.1 M sucrose layer (Fig. 1). The value of lipid-*P*/protein in Fraction 4 (Table I) suggested that the extensive washing procedures employed as means of purification had resulted in an enrichment in phospholipids compared with Fraction 4 of the unwashed suspension (S1$_D$, Table I).

The effects of the freezing and thawing procedure on the distribution patterns of the membrane constituents are shown in Fig. 2; the patterns were markedly different from those obtained for the untreated membrane preparation (S4, Fig. 1). Two peaks were apparent in the distribution patterns shown in Fig. 2, one in the 1.1 M sucrose

TABLE I

LIPID PHOSPHATE IN MATRIX AND MEMBRANE FRACTIONS OF LYSED CHROMAFFIN GRANULES

The values are given as means ± S.D. Lysis in these experiments were complete, as 79% of the total protein was solubilized and recovered in Fraction 1. The total granule suspension contained after dialysis ($S1_D$) 0.51 ± 0.01 μmole lipid-*P*/mg protein ($n=3$). The recovery of solubilized protein in Fraction 1, S4, was 5%. The recovery of protein in the fractions of S4 after freezing and thawing ($S4_{-20}$) is given in Table II. The total amount of lipid-*P*/mg protein in S4 was 2.4 ± 0.1 ($n=3$), the Cu content was 6.9 nmoles/mg protein and the specific dopamine-β-hydroxylase activity was 3.2 units/mg.

Gradient fractions	*μmoles lipid-P/mg protein*				
	Suspensions of lysed granules			*Isolated membrane preparations*	
	S1	$S1_D$	$S1_{D-20}$	*S4*	$S4_{-20}$
Fraction 1					
(Supernatant = matrix protein)	0.028 ± 0.004	0.000*	0.020 ± 0.006	0.25 ($n=1$)	0.11 ± 0.09
Fraction 4					
(1.1 M sucrose layer = membranes)	0.81 ± 0.07	1.56 ± 0.19	1.03 ± 0.10	1.97 ± 0.08	0.83 ± 0.06
n	6	6	6	3	4

* The Cu content was 5.24 nmoles/mg protein and the specific dopamine-β-hydroxylase activity was 2.8 units/mg protein.

TABLE II

PROTEIN, LIPID-*P*, CHROMOGRANIN A AND DOPAMINE-β-HYDROXYLASE ACTIVITIES IN GRADIENT FRACTIONS OF FROZEN MEMBRANE PREPARATIONS

The values for each fraction represent the mean ± S.D. of three gradient fractionations. The distribution patterns of $S4_{-20}$ in Fig. 2 represented one of the three experiments included in this table. The specific activities given are related to mg protein in the fractions. The percent of the total activities in the gradient of each of the constituents have been calculated from the mean values. The ratios of Cu/protein were 34 ± 13, 16.8 ± 6.8 and 11.4 ± 3.3 nmoles/mg protein in Fractions 1, 2 and 4, respectively.

$S4_{-20}$ fractions	*Total protein*		*Lipid-P*		*Chromogranin A*		*Dopamine-β-hydroxylase*	
	(mg)	(%)	*(μmoles/mg protein)*	(%)	*(mg/mg protein)*	(%)	*(units/mg protein)*	(%)
(1) (Supernatant)	0.83 ± 0.17	10.8	0.13 ± 0.09	0.8	0.36 ± 0.04	31	4.1 ± 2.8	14.6
(2) (Liposomes)	0.96 ± 0.26	12.5	6.46 ± 2.3	53.4	0.36 ± 0.01	35	5.1 ± 0.9	21.0
(3)	0.66 ± 0.16	8.6	1.98 ± 0.06	11.2	0.18 ± 0.10	11	4.1 ± 1.4	11.6
(4) (Membrane fragments)	4.27 ± 0.87	55.9	0.75 ± 0.09	27.6	0.03 ± 0.01	14	2.7 ± 0.3	49.6
(5)	0.92 ± 0.30	12.0	0.87 ± 0.09	6.8	0.10 ± 0.01	9	0.8 ± 0.5	3.1

layer and another at the level of 0.4 M sucrose. Light-scattering material was associated with both peaks, indicative of particulate matter in both. The profile of light absorption at 416 nm suggested that the membrane-specific cytochrome b_{561} was associated with the 1.1 M sucrose layer only, and the electron microscopic study of this fraction (Fig. 3B) indicated the presence of membrane fragments, possibly in dispersed and broken-up forms but not forming liposomes (as seen in Fig. 3A).

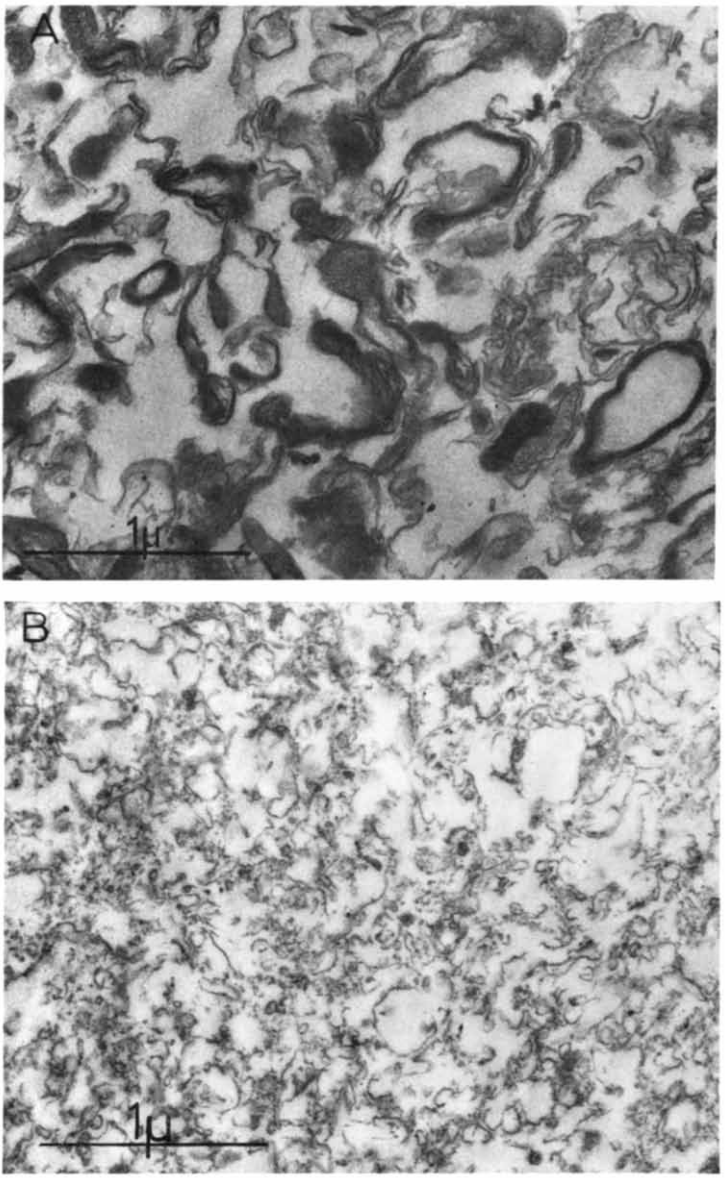

Fig. 3. Electron micrographs of fractions of frozen membranes. (A) Material of the 0.4 M sucrose layer (Fraction 2), lipid-*P*/protein = 6 μmoles/mg. (B) Material of the 1.0–1.2 M sucrose layer (Fraction 4), (0.8 μmole lipid-*P*/mg protein).

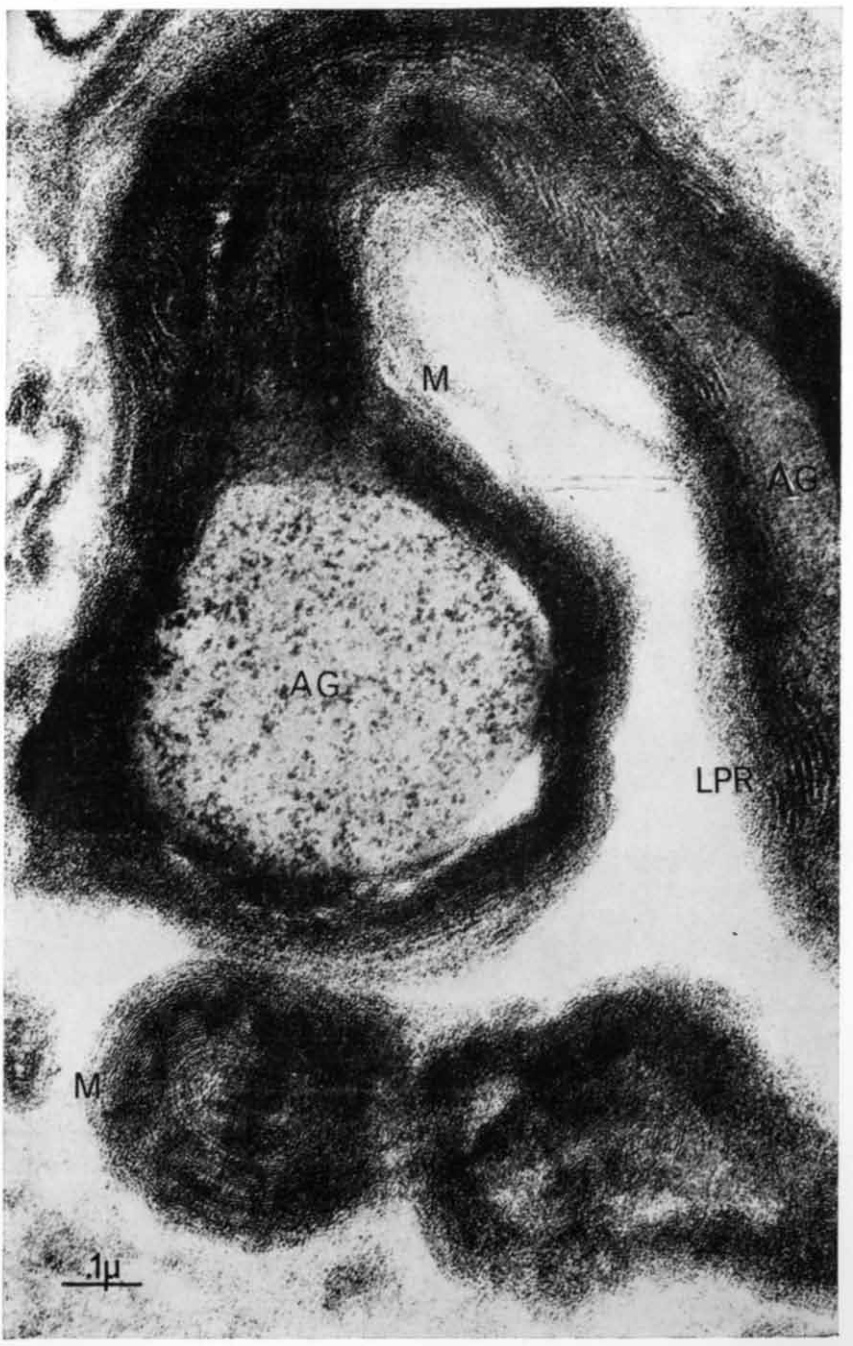

Fig. 4. Electron micrograph of the phospholipid-rich fraction (Fraction 2) of frozen membranes (S4). Enlargement of a typical liposomal structure, with myelinoidic (M) figures with repeating units 42–50 Å width, enveloping amorphous granulated material (AG) in many structures. At the interphase between the fine lamellae of M and that of AG, lamellae of repeating units of 125 Å (LPR) may be seen.

As indicated in Table I, the ratio of lipid-*P*/protein in the membrane fragments was reduced from 1.97 to 0.83 μmoles/mg as a result of the freezing and thawing procedure. The lipid-*P* thus removed was recovered mainly in the fraction of low buoyant density (Fraction 2, Table II) for which a ratio of 6.46 μmoles lipid-*P*/mg protein was obtained. This fraction appeared unique to the isolated membrane preparations which were subjected to the procedure of freezing and thawing, and the properties of the phospholipid-rich fraction were therefore studied in detail.

Electron microscopic studies revealed that the phospholipid-rich fraction (Fraction 2, Table II) consisted of large, irregular vesicles (Fig. 3A) which at a higher magnification (Fig. 4) exhibited multilayered, lamellar substructure. The periodicity of the lamellae (42–50 Å) suggested the presence of smectic mesophases of phospholipids, commonly referred to as multilayered liposomes[15]. In some parts of the liposomal structures a different periodicity in the lamellar arrangement was noted (Fig. 4, LPR, 125 Å) suggestive of protein incorporated in the phospholipid lamellae. Enclosed in the multilayered liposomes, granular cores of less organized substructure (AG) could also be seen, indicative of protein material trapped within the liposomes. The morphological appearance of the material equilibrating in the 1.1 M sucrose layer (Fig. 3B) bore little resemblance to the liposomes. Since cytochrome absorbance could only be detected in the 1.1 M sucrose layer (Fig. 2) it was assumed that this material accounted for the purified membrane fragments with the characteristic cytochrome b_{561} (ref. 4).

Dopamine-β-hydroxylase activity was present in all fractions of the gradient. The specific enzyme activity was highest in the liposomal fraction and significantly higher than that of the membrane fraction (Fractions 2 and 4, Table II). Immunological titration of chromogranin A revealed its presence throughout the gradient. Furthermore, the distribution pattern resembled that of dopamine-β-hydroxylase, and the specific activities of chromogranin A and the enzyme were the same in Fractions 1 and 2 of the gradient (Table II).

Only a third of the total chromogranin A of the isolated membrane preparation $S4_{-20}$ could be rendered soluble by freezing and thawing, and another third was found to be recovered within the liposomal structures. The presence of Triton X-100 was needed to unmask the titratable chromogranin A in the liposomal fraction, indicative of a close association of this protein with the phospholipid-rich particles. Of the remaining portion 14% of the titratable chromogranin A were still detectable in the membrane fragments (Fraction 4, Table II). The specific activity in the latter fraction (0.03 mg chromogranin A/mg protein) indicated that the freezing and thawing procedure had markedly depleted the membrane fragments of bound chromogranin A, parallel to the removal of phospholipids and dopamine-β-hydroxylase activity.

When the specific dopamine-β-hydroxylase activities of the isolated fractions of $S4_{-20}$ were plotted against the ratio of Cu/protein of these fractions, a straight line relationship was obtained (lower line, Fig. 5) with one exception: the liposomal particles ($\blacktriangle_{II}$) exhibited a specific enzyme activity up to twice the value expected from the respective Cu contents. These observations pointed to an enhancement of β-hydroxylation by the association of the enzyme protein with the phospholipids within the multilamellar vesicular structures, as further studied in the accompanying paper.

The upper line in Fig. 5 represents the specific dopamine-β-hydroxylase activi-

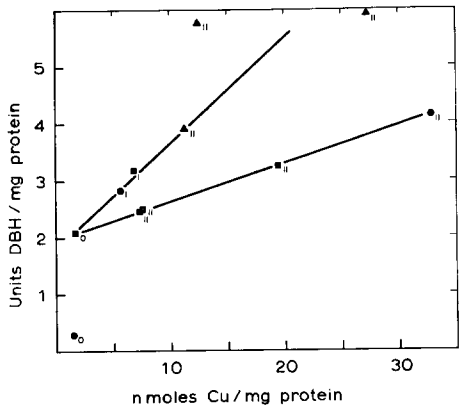

Fig. 5. Dopamine-β-hydroxylase activities and Cu content of granule fractions. The specific dopamine-β-hydroxylase (DBH) activity has been plotted against the ratio of Cu/protein in the respective fractions of lysed granules dialysed in tubings of restricted porosity: (I) Unfrozen preparations (*cf.* Table I): $\bullet_I$, Fraction 1, $S1_D$; $\blacksquare_I$, Fraction 4, $S1_D$. (II) Frozen and thawed preparations of isolated membranes (*cf.* Table II): $\bullet_{II}$, Fraction 1 (solubilized protein); $\blacktriangle_{II}$, Fraction 2 (liposomal protein); $\blacksquare_{II}$, Fraction 4 (membrane fragment protein). The earlier reported values[7] of matrix ($\bullet_O$) and membrane ($\blacksquare_O$) dopamine-β-hydroxylase activities, obtained after dialysis in untreated dialysis tubing have been included.

tive Cu contents (*cf.* Table I); and the lower slope relates to the frozen and thawed preparations and thus suggests an inactivating effect of this method on the enzymic activity, which appeared to be without relationship to the content of Cu retained in the fractions.

The retention of high concentrations of Cu in all preparations studied in the present work was found to be directly related to the restricted porosity of the dialysis tubing used in the preparation of $S1_D$. It may be seen from Fig. 5 that the low Cu content of the matrix protein reported in earlier work[7,11] coincided with an apparently artificially low specific dopamine-β-hydroxylase activity ($\bullet_O$) while the specific activity of dopamine-β-hydroxylase in the membrane preparations ($S4_{-20}$) earlier described[7,11] appeared to be related to the Cu content in a manner similar to that of the more Cu-enriched preparations obtained in the present study.

Taken together these results indicated that the specific dopamine-β-hydroxylase activity of the matrix and membrane preparations could be directly related to the ratio of Cu/protein retained in the dialysed fractions provided that the porosity of dialysis tubings had been reduced to give 100% retention of proteins of mol. wts $\geq$ 14600 (*cf.* Methods).

DISCUSSION

The present studies of suspensions of highly purified chromaffin granules and of isolated membrane preparations have clearly shown that the ratio of phospholipid/protein in the membrane fragments varies with the methods employed for their isolation.

Firstly, the recovery of high ratios of lipid-*P*/protein in the membrane fragments (1.56–1.97 μmoles/mg) depended on the exposure of the lysed granules to large

volumes of hypotonic buffer, *e.g.* by dialysis ($S1_D$) or by extensive washing of the isolated membrane preparation (S4). In this respect the present results confirm earlier reports by other groups[8,16,28] as well as our own[7].

Secondly, the absence of phospholipid in the supernatant fraction of lysed chromaffin granules depended on extensive dialysis of the suspension against the hypotonic buffer (Fraction 1, $S1_D$). In all other preparations studied, a small amount of phospholipid was always detected as a constituent of the supernatant fraction, *i.e.* the matrix protein fraction containing the soluble chromogranins. These findings thus support our earlier observations of phospholipid as a constituent of the matrix phase in association with the soluble chromogranins[17,18,11] and agree with reports of Mylroie and Koenig[19]. The present results also offer an explanation for the fact that Winkler *et al.*[4,8] have not been able to confirm our observations in their extensively dialysed and washed preparations. Evidently phospholipids from the matrix phase have become associated with the membrane protein as a result of the rapid introduction of the aqueous phase in the course of the lysis procedure, and thereby escaped detection as a constituent of the matrix fraction[20].

In view of the efficiency of the dialysis procedure in the dissociation of the phospholipid from the matrix protein, weak ionic bondings must be responsible for the phospholipid–chromogranin interactions in the matrix phase.

Unless freezing and thawing were applied, the high lipid-*P*/protein ratios were confined to particles in the 1.1 M sucrose layers. At low magnifications of such membrane preparations in the electron microscope, membrane fragments and small, irregular, vesicles may be seen[7] and it is not possible to separate between small, unilayered, liposomes of mainly phospholipids and closed vesicles of the fragments of the chromaffin granule membranes. The fact that freezing and thawing of the isolated membrane preparations resulted in the formation of the "enormous" multilayered liposomal structures (Figs 3A and 4) does provide us with some indication of a fusion of small liposomes into larger structures. It is not unlikely that a disruption of interactions between dopamine-β-hydroxylase and phospholipids by the formation of ice crystals has disturbed the protein–lipid equilibrium in part of the membrane fragments and thus led to a further saturation of the small liposomes with lipid phosphate, with the resulting change in size as well as in buoyant densities.

The dopamine-β-hydroxylase activities obtained for the fractions of the isolated membrane preparation ($S4_{-20}$, Table II) compared well with the results reported by Schneider[16] (212 nmoles product/h per mg = 3.5 nmoles product/min per mg for a membrane fraction containing 2.06 μmoles lipid-*P*/mg). Freezing and thawing were omitted in the latter work which furthermore described the usefulness of sucrose gradient fractionation as a means of membrane purification, in accordance with the present findings.

The purified dopamine-β-hydroxylase is a Cu enzyme[21,22] which has been reported to contain up to 7 moles of Cu/mole of enzyme (Mol. wt 290000). The Cu content was, however, found to vary with length of dialysis[23], a finding which we have been able to confirm in the studies of the effect of dialysis on the Cu content in relation to the specific enzyme activity of the matrix and membrane fractions (*cf.* Fig. 5). The ratio of Cu/protein reported for the highly purified enzyme[21,22] is equivalent to a minimum mol. wt of 41000/mole Cu. In the solubilized protein fraction (Fraction 1, Table II) obtained from the frozen and thawed membrane preparation a minimum

mol. wt of 31000/mole Cu was obtained as an average of three experiments. Furthermore, the Cu content of the solubilized protein corresponded to the high specific dopamine-β-hydroxylase activity of this fraction (Fig. 5), suggestive of a relatively pure form of the enzyme released from the isolated membrane preparation by the freezing and thawing procedure. This purification step was introduced as a means of removing the last traces of chromogranin A from the isolated membrane preparation[8,20]. In the present study not more than 31% of the total titratable chromogranin A of the isolated membrane preparation could be rendered soluble by this procedure. The titre suggested the presence of 0.36 mg chromogranin A/mg protein in both Fractions 1 and 2 (Table II), a value that closely corresponds with that of the matrix phase[7,18,24]. Thus, the high specific dopamine-β-hydroxylase activities released by the freezing and thawing coincided with the release of chromogranin A in amounts common to the matrix phase. Furthermore, the electrophoretic mobility pattern of the solubilized protein (Fraction 1, $S4_{-20}$) could not be distinguished from that of the matrix phase, *i.e.* Fraction 1 obtained in experiments with the lysed granules (Table I, $S1_D$). These findings, taken together, make it reasonable to assume that chromogranin A and dopamine-β-hydroxylase have subunits in common, as earlier concluded[11] but recently contested by Hörtnagl *et al.*[5].

The latency of dopamine-β-hydroxylase activity by immunological studies of isolated membrane preparations[5] may be explained by an enclosure of the latent activity within multilayered liposomal structures which occur as a result of the freezing and thawing procedure. A similar latency of chromogranin A has been repeatedly recorded in preparations of the isolated membranes (S4) in our earlier[7,11,12,18] studies. In a recent report[26] it was further shown that this latency was different from that of the water-insoluble fraction of the splenic nerve vesicles containing 0.79 μmole lipid-*P*/mg protein. In the nerve vesicles the titratable amounts of chromogranin A appeared much more firmly embedded in the lipids[25] together with 80% of the dopamine-β-hydroxylase activity of the vesicles[27].

Membrane material equilibrating in 0.4 M sucrose was recently found to account for higher storage capacity for noradrenaline than the membrane fragments in the 1.0–1.1 M sucrose layers[28]. The present results suggest that not only chromogranin A but also dopamine-β-hydroxylase activity of high degree of purity may be displaced from the membrane fragment or from liposomes, contaminating the membrane preparations, into the 0.4 M sucrose layers. This suggestion implies that not only catecholamines and chromogranin A but also dopamine-β-hydroxylase are mainly constituents of the granule matrix. The estimation of the amount of this enzyme in the latter phase strongly depends on the specific enzyme activity recorded. As is clearly shown in Fig. 5, the specific enzyme activity could be directly related to the amounts of Cu/mg protein remaining in the fractions after dialysis. Retention of high [Cu] in the matrix and membrane fractions has been found to depend on the use of dialysis tubings of restricted porosity, indicative of Cu associated with low mol. wt subunits in the dopamine-β-hydroxylase complexes. When the loss of these Cu-containing subunits was prevented, *e.g.* by use of dialysis tubings of restricted porosity, the specific enzyme activity of the matrix phase protein was restored to the same order as that of the membrane preparations, *i.e.* 2–4 units per mg for Cu/protein ratios above 5 nmoles/mg. At Cu/protein ratios below this value the enzyme activity of the matrix protein was greatly impaired. As previously reported[7], the addition of

Cu^{2+} to the inactivated enzyme did not effect the enzyme activity of the matrix protein depleted in Cu by the dialysis step. This finding thus strengthens the assumption that the lost Cu have been bound to a macromolecular component, *i.e.* a subunit, which is essential for the complete enzyme and which has been removed from the purified chromogranin A of low activity.

These observations have implications not only on the distribution patterns of the two specific "markers" of the chromaffin granule, but also on the use of dopamine-β-hydroxylase as a specific "marker" of the membrane phase in studies which aim at a discrimination between the two granule phases. It is evident from the present observations that dopamine-β-hydroxylase and chromogranin A occur as "double markers" of the granule protein expected to be secreted from the stimulated gland by the exocytotic mechanism of secretion[4] as well as of the remaining lysis-resistant phases[18,7,11,12,25] believed to be retained within the stimulated cells[4].

In view of the artifactual redistribution of granule constituents which results from freezing and thawing of the dialysed lysate as well as of the isolated membrane preparations, the method of isopycnic gradient centrifugation, recently also suggested by Schneider[16] and by Agostini and Taugner[28] emerges as the most suitable method for the separation of matrix and membrane phases of the chromaffin granule.

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