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FORMATION *IN VITRO* OF MATRIX-LIKE AGGREGATES OF CHROMOGRANINS AND PHOSPHOLIPIDS DERIVED FROM CHROMAFFIN GRANULES OF THE BOVINE ADRENAL MEDULLA

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SUMMARY

Matrix-like particles were formed in sonicated suspensions of soluble chromogranins and phospholipids extracted from the isolated membrane phase of chromaffin granules. The artificial particles which sedimented into 0.4–0.6 M sucrose layers during isopycnic centrifugation, consisted of liposomal vesicles and granular aggregates indicative of lamellar and possibly hexagonal organization patterns of phospholipids and the aqueous phase containing the soluble chromogranins. The ultrastructure of the granular aggregates was strikingly similar to the matrix phase of the intact chromaffin granule.

The dopamine-β-hydroxylase activity (EC 1.14.2.1) of the soluble chromogranin preparation was potentiated by the addition of the phospholipids and further enhanced by the sonication procedure. The enzymic activity was highest in the 0.4–0.6 M sucrose layers, rich in the artifical matrix-like particles.

The artificially formed particles incorporated ATP by further sonication and the presence of ATP in the particle fractions did not inhibit the specific dopamine-β-hydroxylase activity, even at ratios of 4 and 2 μmoles ATP/mg protein in the 0.4 and 0.6 M sucrose layers, respectively. Noradrenaline was incorporated into the particle fractions in presence of ATP. However, the presence of noradrenaline above $3 \cdot 10^{-6}$ M resulted in a 50–75% inhibition of the dopamine-β-hydroxylase activity of the artificial matrices.

INTRODUCTION

The chromaffin granules of the adrenal medulla and the axonal vesicles of the splenic nerve serve as convenient models of storage and release mechanisms in adrenergic tissue. The apparent equivalence between positive and negative charges provided by the catecholamines and ATP in the vesicular stores, has led to the postulation of complexes of these constituents in molar ratios of 4 to 1 (ref. 1). In order to account for the great stability of the catecholamine store in the intact organelles, the hypothetical complexes of catecholamine and ATP have been assumed to involve a protein component[2]. The water-soluble chromogranin A of the adrenomedullary granules has been examined for its postulated role as the storage protein. Although the early work was

suggestive of aggregate formation between chromogranin A, adrenaline, ATP and Mg^{2+} (ref. 3), the purified protein failed to exhibit any significant capacity for catecholamine storage[4].

For the axonal vesicles, which are richer in lipid-*P* than the adrenomedullary granules, a lipoprotein has been postulated to serve as the macromolecular component in the storage complex[5]. Chromogranin A as well as dopamine-β-hydroxylase occur as common constituents of the two types of adrenergic storage organelles although the solubility characteristics of these two macromolecular constituents differ in the two types of organelles, probably as a function of different ratios of lipid-*P* to protein in the matrix and membrane phases[6,7].

The aim of the present study has been to elucidate the role of phospholipids in the aggregation of the soluble chromogranins derived from the adrenomedullary granules. By use of a method[8] primarily designed for the formation of artificial phospholipid membranes in vesicular form, *i.e.* liposomes[9], suspensions of soluble chromogranins and phospholipids were allowed to interact in an aqueous phase. In the sonicated suspensions not only liposomes but also matrix-like aggregates were formed, and the heterogenous population was found to retain ATP as well as noradrenaline. The implication of these observations with respect to a function of lipoprotein matrices as the template for the storage of catacholamines and ATP in the intact organelles will be discussed.

METHODS

Chromaffin granules were isolated from bovine adrenal medullae as described by Helle *et al.*[13] and suspensions of the highly purified granules at a concentration of $\approx$10 mg/ml were dialysed in tubings of restricted porosity against large volumes of the hypotonic buffer, 5 mM sodium succinate, pH 6.0.

Soluble chromogranins were obtained as the supernatant fraction of the dialysed suspension of the lysed granules after centrifugation at $78 \cdot 10^5 \times g_{av} \cdot$ min. Analysis for lipid-*P* was negative, *i.e.* <0.001 μmole/mg protein[11].

Phospholipids were extracted from a liposomal fraction (6.5 μmoles lipid-*P*/mg protein) derived from the isolated membrane preparation of chromaffin granules by isopycnic gradient centrifugation after freezing and thawing[11]. The phospholipids and cholesterol were removed from the protein by extraction with 20 vol. of chloroform–methanol (2:1, v/v) and the organic phase was isolated by washing with changes of large volumes of 0.17 M NaCl. The chloroform extract was filtered to remove traces of denatured protein and freed of dissolved water by addition of dry $CaCl_2$.

Suspensions of soluble chromogranins and phospholipids were prepared essentially as described by Bangham[9] for liposome formation, but with some modifications. The chloroform extract, containing $\approx$4 μmoles lipid-*P*, was allowed to dry as a coat on the inner side of a small test tube under a stream of N_2. The aqueous phase, added to the dried phospholipids, contained 2–4 mg soluble chromogranins in 5 mM sodium succinate, pH 6.0, in presence of 60 mM KCl in a total volume of 1 ml. Suspension was achieved by vortex shaking and after 24 h at 0 °C followed by sonication for 3 min on ice.

Isopycnic gradient centrifugation of the suspension was carried out on gradients of sucrose ranging from 0.1 to 0.8 M at $72 \cdot 10^5 \times g_{av} \cdot$ min in the Spinco Model L50,

with the SW 39 rotor. Fractions were collected from the top of the tubes by careful suction with calibrated pipettes (500 μl).

Protein was determined according to Lowry *et al.*[12].

Lipid-*P* was assayed in chloroform extracts of the fractions as earlier described[11,15].

Dopamine-β-hydroxylase (EC 1.14.2.1) activity was estimated by the method of Gibb *et al.*[13]. The activity of each fraction was obtained as the mean of three assays with three different protein concentrations. The unit of enzyme activity is expressed as nmoles/min of the oxidized product formed in the 1-ml assay volume during the 10-min incubation period at 37 °C.

Electron microscopy of sedimented material in the gradient fractions was performed on ultrathin sections of pellets fixed in 2.5% glutaraldehyde in sucrose buffered at pH 6.5 and of density equivalent to that of the gradient fraction in question[10]. The sections were stained with lead acetate.

ATP was determined by the firefly method as described by Holmsen *et al.*[14].

[γ-^{32}P]ATP (ammonium salt) and DL-noradrenaline-([^{14}C]carbinol)-DL-bitartrate were purchased from Amersham, England. The isotopically labelled fractions were counted in 50-μl aliquots dried on filters and counted by liquid scintillation in 5 ml toluene–POPOP (4 g PPO *plus* 0.1 g POPOP/l toluene) using the Beckman Model LS100.

RESULTS

The effect of adding phospholipids to the soluble chromogranin preparation was followed by using the specific dopamine-β-hydroxylase activity as a "marker" for protein incorporation into the phospholipid particles. The results presented in Table I show that the particles, which had been formed by vortex mixing of the dry phospholipids and the aqueous phase containing the soluble chromogranins, revealed a 4-fold activation of the dopamine-β-hydroxylase activity compared to that of the phospholipid-free, soluble chromogranin preparation alone (Expt A). Particles were separated from the soluble phase of the suspension by isopycnic centrifugation in a gradient ranging from 0.3–1.6 M sucrose, and the phospholipid-rich particles were recovered mainly in the 0.4 M sucrose layer. The relative amount of protein material recovered in this layer accounted for 21% of the total soluble chromogranins in the suspension. Sonication of the suspension was performed in order to reduce the particle size, analogous to the effect of sonication on pure phospholipid particles (liposomes). As indicated by the results (Table I, Expt A) the amounts of protein recovered in the particle fraction increased with prolongation of the sonication period. After 2 min of sonication a floating layer of lipids could no longer be seen in the supernatant layer. The result also revealed that the specific dopamine-β-hydroxylase activity was further potentiated by the increased amount of protein in the particle fraction. Control studies of the effect of sonication of liposomal structures obtained from purified granule membranes (Expt B, Table I) showed, on the other hand, that a reduction in particle size of the liposomes resulted in an inactivation of their specific enzyme activity. Similar results were obtained when the particles (Expt AIII, Table I) were sonicated for 3 min in the presence of ATP and noradrenaline. As shown in Table II, the specific dopamine-β-hydroxylase activity was reduced by 75% concomittantly

TABLE I

EFFECT OF PHOSPHOLIPID AND SONICATION ON SPECIFIC DOPAMINE-β-HYDROXYLASE ACTIVITY OF SOLUBLE CHROMOGRANINS

	Particle material in 0.4 M sucrose layer		
	Units dopamine-β hydroxylase/mg protein	*% of total protein*	*μmoles phospholipid/mg protein*
Expt A			
Soluble chromogranins	3.4*	—	—
I. Soluble chromogranins + phospholipid	12.8	21	1.7
II. Soluble chromogranins + phospholipid + 1-min sonication	15.0	25	1.7
III. Soluble chromogranins + phospholipid + 2-min sonication	18.0	28	1.7
Expt B			
Liposomal fraction of isolated membranes**	9.8		3.9
+ 1-min sonication	6.8		3.9
+ 2-min sonication	5.2		3.9
+ 3-min sonication	3.6		3.9

* Specific dopamine-β-hydroxylase activity of the soluble chromogranin preparation prior to addition of phospholipid and subsequent fractionation on isopycnic gradient (0.3–1.6 M sucrose). Sonication did not affect the specific dopamine-β-hydroxylase activity of soluble chromogranin.

** The phospholipid-rich fraction recovered in the 0.4 M sucrose layer after isopycnic fractionation of an isolated membrane preparation subjected to freezing and thawing prior to fractionation (*cf.* ref. 11, Table II, $S4_{-20}$).

with a fall in the buoyant density of the major part of the sedimentable particles, and only 6–7% of the protein could be recovered in the 0.4 M sucrose layer after the second sonication period. On the other hand it was found that the particles which still exhibited the same buoyant density as the source material (0.4 M sucrose), was still highly potentiated with respect to dopamine-β-hydroxylase activity and the high enzyme activity appeared unaffected by the ATP present in the particle fraction.

As shown in Fig. 1A, ATP was found throughout the gradient and the sedimentation pattern suggested that the ATP associated with the particles in the 0.4 M sucrose layer might in part have dissociated from the ATP-containing particles in the 0.6 M layer. Noradrenaline was found to be inhibitory to the dopamine-β-hydroxylase activity of the particles (Table II) and the effect was related to the concentration of noradrenaline in the fractions (Fig. 2). Like ATP noradrenaline was also found in all gradient fractions, and the sedimentation pattern (Fig. 1B) suggested that the amine was associated with the particles which had equilibrated in the bottom (0.6 M sucrose) layer. The association of ATP with this fraction (2.2 and 2.0 μmoles ATP/mg protein in Expts 2 and 4 (Table II), respectively), appeared to be independent of the presence of noradrenaline in the particle fractions. A ratio of 0.3 μmole noradrenaline/mg protein was on the other hand obtained for the particles in the 0.6 M sucrose layer

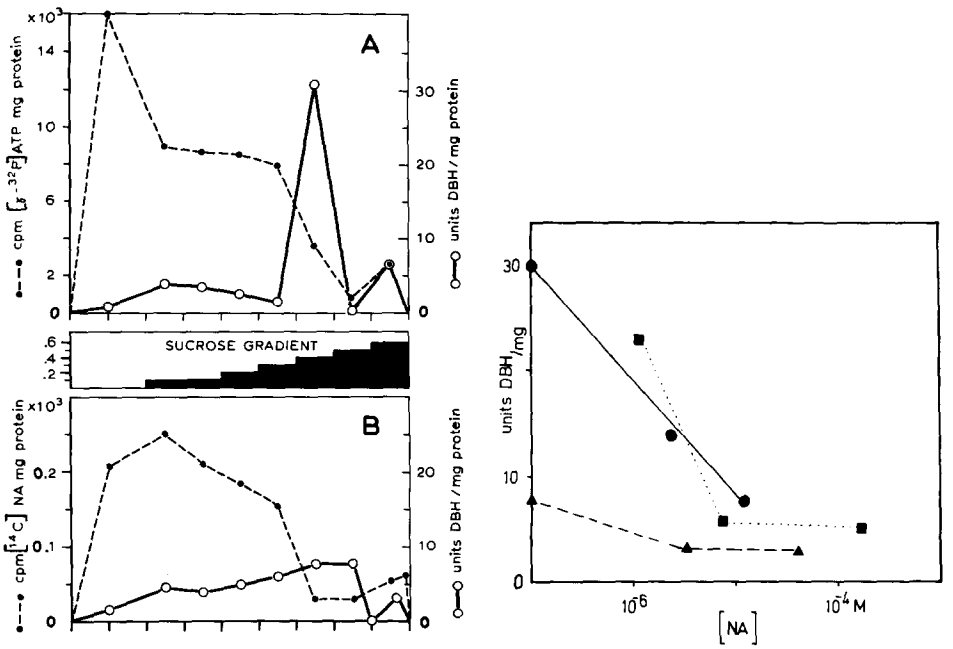


Fig. 1. Sedimentation patterns of ATP, noradrenaline and enzyme activity. A. The particles (Expt AIII, Table I) were sonicated in the presence of 10 mM [γ-^{32}P]ATP (spec. act. 17400 cpm/μmole), 5 mM sodium succinate in 0.002% NaN_3, pH 6.0 prior to fractionation through the sucrose gradient ranging from 0.1–0.6 M sucrose (Table II, (2)). DBH, dopamine-β-hydroxylase. B. The particles Table II(4) were sonicated in presence of 1 mM noradrenaline ([^{14}C]NA) (spec. act. 40480 cpm/μmole), 10 mM ATP, 5 mM sodium succinate in 0.002% NaN_3, pH 6.0, prior to sedimentation through the sucrose gradient (as in A). The bottom fractions (0.6 M sucrose layer) contained 2.2 and 2.0 μmoles ATP/mg protein determined by the firefly method, in A and B, respectively.

Fig. 2. Effect of noradrenaline on the dopamine-β-hydroxylase activity of the particles. The concentration of noradrenaline (NA) in the 0.4 M sucrose layers was plotted against the specific dopamine-β-hydroxylase (DBH) activity of the material in these fractions (●—●); *cf.* Table II. For comparison the corresponding values of these parameters obtained for the material in the 0.2 M (■—■) and the 0.6 M (▲—▲) layers of the same experiments were also plotted.

(Expt 4, Table II) at a total concentration of bound and free noradrenaline of $4 \cdot 10^{-5}$ M. Control experiments with thin-layer chromatography confirmed that [^{14}C]noradrenaline and [^{32}P]ATP were intact after the sonication and isopycnic fractionation procedures.

The effect of ATP and adrenaline on the initial formation of the particles was also investigated. The results presented in Fig 3 and Table III show that the specific dopamine-β-hydroxylase activity of the sonicated suspension was of the same order as that obtained in the absence of ATP and the amine (*cf.* Table I). The particles which equilibrated in the 0.3–0.8 M sucrose layers accounted for about 27% of the total protein, 60% of the total phospholipids and 51% of the total enzymic activity of the gradient fractions. The ratio of lipid-*P*/protein observed for the sedimented particles indicated that enrichment in phospholipids increased the sedimentation rate of the protein-containing particles. The specific dopamine-β-hydroxylase activity also appear-

TABLE II

EFFECT OF ATP AND NORADRENALINE ON THE DOPAMINE-β-HYDROXYLASE ACTIVITY OF THE CHROMOGRANIN PARTICLES

Experiment AIII	*Units dopamine-β-hydro-xylase/mg protein*	*In 0.4 M sucrose layer*				*In 0.6 M sucrose layer*			
		% of total protein	*Units dopamine-β-hydro-xylase/mg protein*	*[ATP] (10^{-6} M)*	*[noradrenaline] (10^{-6} M)*	*% of total protein*	*Units dopamine-β-hydro-xylase/mg protein*	*[ATP] (10^{-6} M)*	*[noradrenaline] (10^{-6} M)*
Control	18.0*								
(1) +0.1 mM ATP	4.2	6.9	29.0	5		8.0	6.2	0	
(2) +10 mM ATP	4.6	5.9	30.8	445		9.2	6.5	271	
(3) +10 mM ATP +0.1 mM noradrenaline	3.7	3.2	14.8		2.3	7.9	3.3	80	3.0
(4) +10 mM ATP +1.0 mM noradrenaline	2.9	3.2	7.7		12.4		2.9	220	40.0

* This material was recovered as particles (1.7 μmoles/mg protein) in the 0.4 M sucrose layer of an isopycnic gradient (0.3–1.8 M sucrose) and accounted for 28% of the total protein of the suspension applied to that gradient (Table I). Aliquots of the particle suspension were sonicated for 3 min in presence of 5 mM sodium succinate containing 0.002% NaN_3, buffered at pH 6.0. [γ-^{32}P]ATP (1 mM) were also added (Expts I and II) while 1 μM [^{14}C]noradrenaline was added to each of the Expts III and IV, in addition to that given in the table. The material was sonicated for 3 min prior to fractionation on isopycnic gradients ranging from 0.1–0.6 M sucrose.

ed to be potentiated proportional to the enrichment in phospholipids. However, when the specific enzyme activity was plotted against the ratio of lipid-*P*/protein (Fig. 4A) a sigmoidal curve relationship was obtained, indicative of cooperative

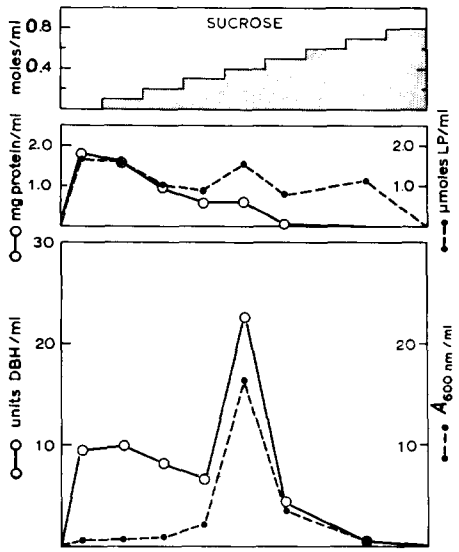


Fig. 3. Sedimentation patterns of phospholipid–chromogranin particles. The suspension of matrix protein and membrane lipids was sonicated in the presence of 60 mM KCl, 5 mM sodium succinate, 10 mM ATP, 0.1 mM adrenaline in 0.002% NaN_3, pH 6.0 prior to fractionation through the discontinuous sucrose gradient ranging from 0.1–0.8 M sucrose. The fractions of the developed gradient were assayed for total protein and lipid-*P* (LP), for dopamine-β-hydroxylase (DBH) activity and light-scattering material ($A_{600\ nm}$).

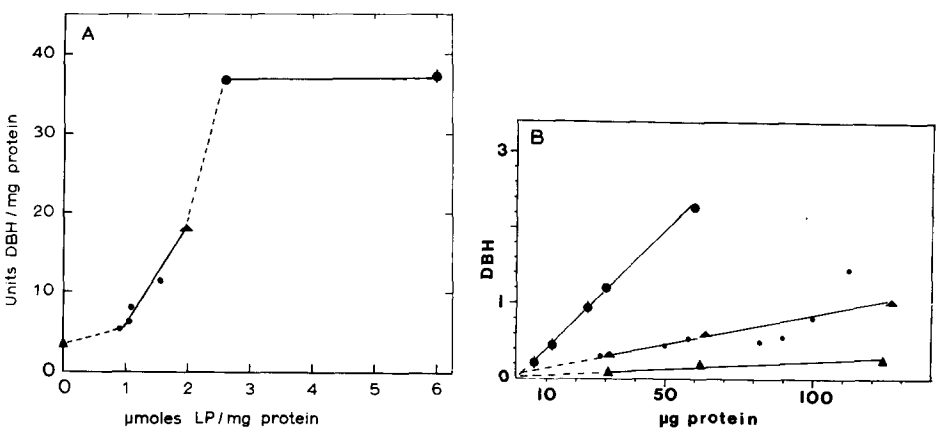


Fig. 4. The specific dopamine-β-hydroxylase (DBH) activities related to lipid-*P* and protein in the fractions obtained by isopycnic gradient centrifugation. A. The specific dopamine-β-hydroxylase activity has been plotted against the ratio of lipid-*P*/protein in each fraction (*cf.* Table III). B. The dopamine-β-hydroxylase activity of each fraction was determined at 3 different protein concentrations. The fractions were: ▲, the soluble chromogranins prior to addition of lipid-*P*; ▲, the sonicated suspension prior to gradient fractionation; •, Fractions 1–4 (0–0.3 M sucrose); ●, Fraction 5 (0.4 M sucrose layer); ●, Fraction 6 (0.5 M sucrose layer).

TABLE III

PARTICLE FORMATION IN SONICATED SUSPENSIONS OF PHOSPHOLIPIDS AND CHROMOGRANINS

	M sucrose in layer	*% of total protein*	*% of total phospholipid*	*μmoles phospho-lipid/mg protein*	*% of total units dopamine-β-hydro-xylase*	*Units dopamine-β-hydro-xylase/mg protein*
Whole suspension		100	100	1.99	100	18.0*
Gradient fractions						
1	0	27.7**	13.1	0.93	12.4	5.3
2	0.1	25.5	12.6	1.04	13.0	6.1
3	0.2	20.0	10.6	1.08	13.0	8.0
4	0.3	8.9	7.0	1.54	13.0	11.4
5	0.4	11.6	15.3	2.60	37.0***	36.7
6	0.5	2.7	8.4	6.0	8.7	37.0

* The mean specific dopamine-β-hydroxylase activity of the material after gradient fractionation was 11.9 units/mg. The data represent mean values of three parallel gradients (Ref. legend Fig. 3).

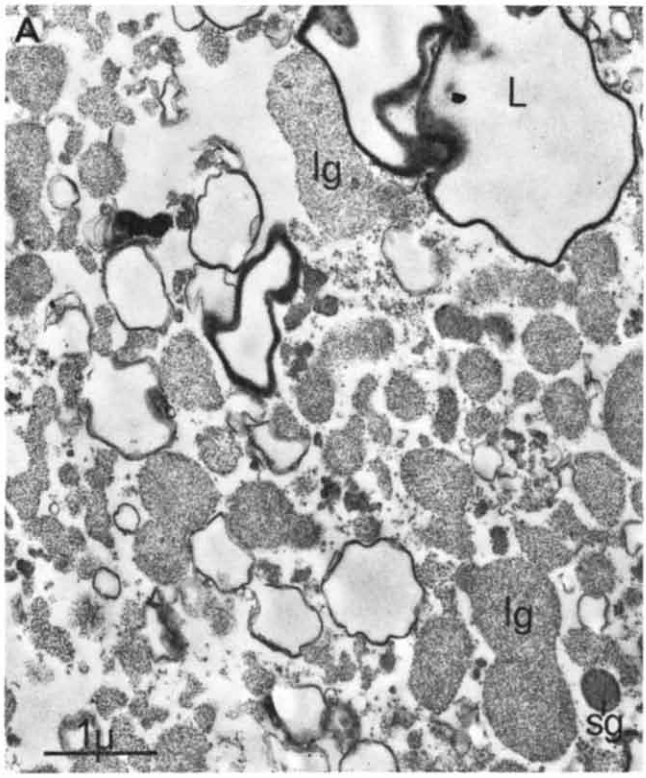
** The amount of chromogranin A detected by immunological titration of the Fractions 1–5 was proportional the amount of total protein in the fractions.

*** This fraction contained ATP but no ATP hydrolysis could be observed during 5-min periods of incubation at 37 °C in the presence or absence of 5 mM $MgCl_2$, even after repeated cycles of freezing and thawing.

changes in the enzyme structure induced by the presence of the phospholipids. When the enzyme activity was related to protein concentration (Fig. 4B) inhibitory effects of protein dilution could not be observed for the gradient fractions, and the potentiation of the enzyme activity could thus only be related to increase in ratio of lipid-*P*/protein. The particles in the 0.6–0.8 M sucrose layers were an exception, as despite their extremely high ratio of lipid-*P*/protein, their specific dopamine-β-hydroxylase activity was of the same order as that observed for the phospholipid-low fraction in the 0.2 M sucrose layer.

The ultrastructure of the particles in the 0.4 M sucrose layer (Fig. 5A) revealed the presence of two major types; one vesicular and one granular. The former type resembled the structure of liposomes[9], consisting of multiple, concentric bilayers of phospholipids. The latter type of particles exhibited a moderate electron density due to their granulated substructure which was strikingly similar to that of the matrix phase of the intact chromaffin granules (Figs 5B and 6C). The diameters of the matrices varied from 0.1 to more than 1 μm. The granular particles were mainly oval or spherical and indications of particle fusions were numerous. Within the particles the

Fig. 5. Electron micrographs of phospholipid–chromogranin particles ($\rho \approx 0.4$ M sucrose). The fraction contained 2.6 μmoles lipid-*P*/mg protein and the dopamine-β-hydroxylase specific activity was 37 units/mg. A. The heterogenous population of particles contained liposomes (L) and granules of large (lg) and small (sg) diameters. B. Enlargement of a small granule (sg in A). C. Intact chromaffin granules in 1.6 M sucrose (*cf.* Helle *et al.*, ref. 10).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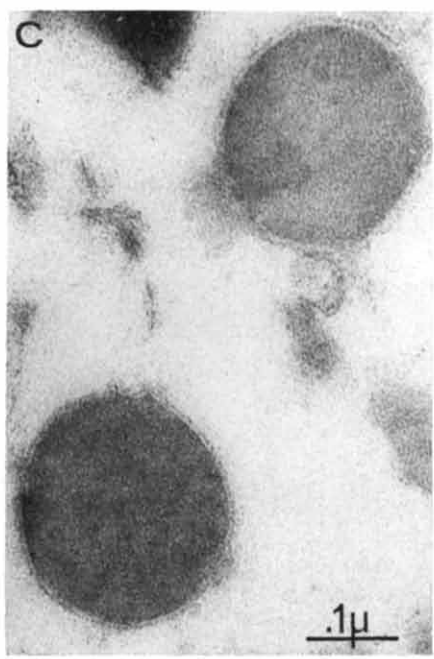
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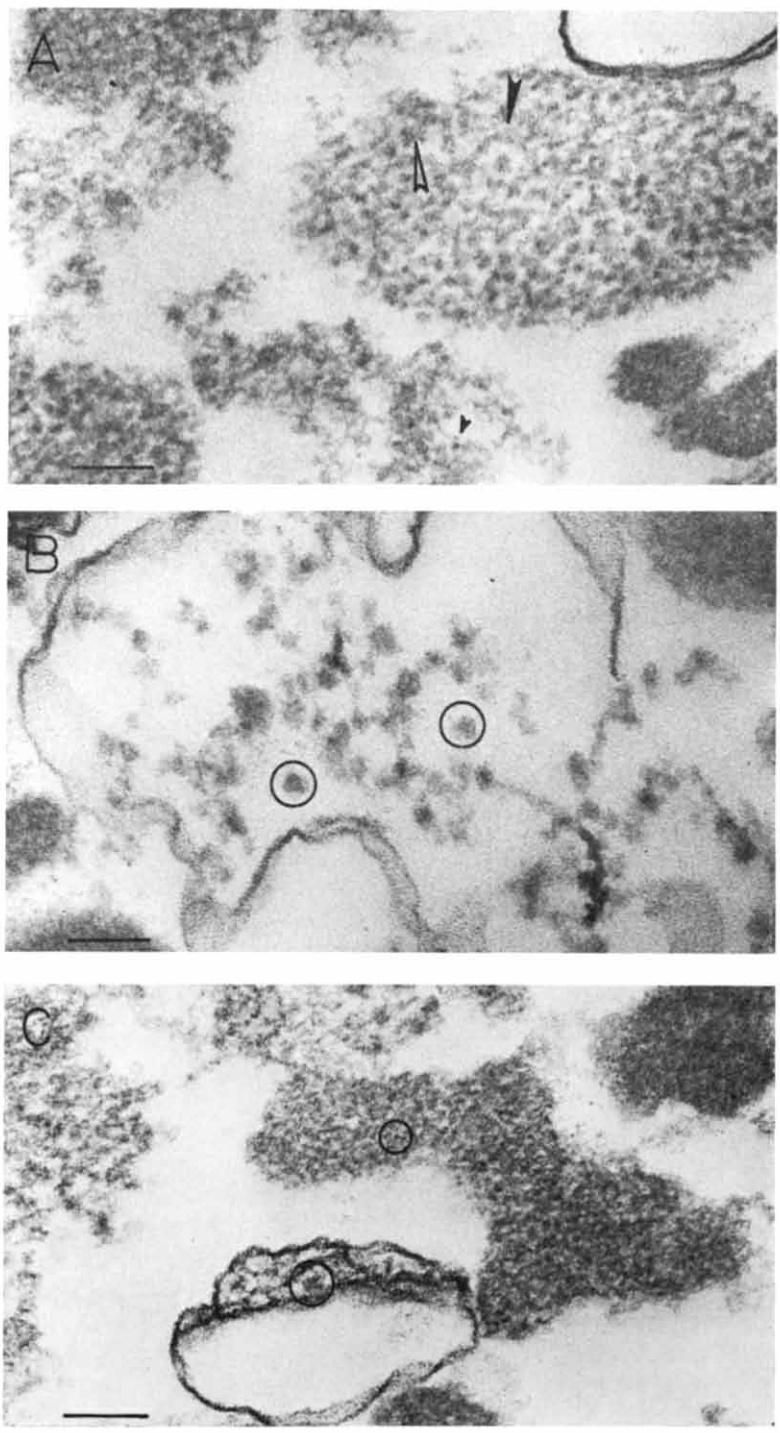
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smallest units (Fig. 6A) had diameters of the order of 30–40 Å. In some of the granular aggregates the ultrastructure revealed a distinct pattern of closed rings of moderately electron-dense material around a globular core (Fig. 6). The diameters of these cores were 200–300 Å and such units could be seen also within the liposomal vesicles (Fig. 6B), often in open rings or in ribbon-like patterns. In some instances globular units could be detected in the spacings between the bilayers (Fig. 6C). Thus, these electron micrographs were suggestive of protein cores of diameters 200–300 Å, possibly composed of subunits (30–40 Å diameters) and apparently surrounded by ring-like enclosures of protein material.

DISCUSSION

The present study has provided the first evidence for particle formation from the soluble chromogranins in the presence of phospholipids. The artificial particles which could be isolated from the 0.4–0.6 M fractions of the sucrose density gradients, moreover, exhibited an ultrastructure which closely resembled that of the intact adrenomedullary storage granules, particularly the matrix phase from which the soluble chromogranins had been derived. In view of the fact that the formation of these artificial matrices corresponded to the potentiation of the dopamine-β-hydroxylase activity, the results make it evident that the aggregated state of the soluble chromogranins is relevant to the integrity of the highly active form of dopamine-β-hydroxylase.

The source of soluble chromogranins used in the present experiments was the supernatant fraction of extensively dialysed chromaffin granules[11], *i.e.* the matrix protein fraction. Soluble chromogranins prepared in this manner (*cf.* ref. 11) were devoid of phospholipids and exhibited a specific enzyme activity of 3.4 units/mg protein (*i.e.* a specific activity comparable to that of the phospholipid-rich membrane fractions[15]) due to the high ratio of Cu/protein (6 nmoles/mg) in these preparations of soluble chromogranins[11]. Control experiments (unpublished) confirmed that the preparations of the latter were devoid of particles which could be sedimented into sucrose of buoyant density above that of 0.2 M.

The phospholipid fraction used for the present experiments was obtained as a protein-free, chloroform extract of the phospholipid-rich, multivesicular liposomal structures derived from isolated membranes subjected to freezing and thawing[11]. In view of the fact that the main purpose of the present study was to investigate the effects of granule phospholipids on the aggregation of the soluble chromogranins,

Fig. 6. Electron micrographs of substructural details in phospholipid–soluble chromogranin particles. A. A ring-like pattern of electron-dense material (diameter 1000 Å) can be clearly seen at upper, dark arrow. The core unit ($\approx$250 Å) in this pattern can be seen to contain an electron-dense subunit (30–40 Å diameter) centrally. Similar subunits can also be detected elsewhere in the granular aggregates, *e.g.* at the lower, dark arrow. The diameter of the core unit (at open arrow) exhibits a less condensed organization of subunits (diameter of core 300 Å). B. Core units of 300 Å diameters (encircled) have also been seen in less regular patterns within the phospholipid bilayers or between these and the granular aggregates. Ribbon-like strands of units are also apparants in this micrograph. C. Between the bilayers of some liposomal vesicles small electron-dense subunits can be observed in the 250 Å core (encircled).

the unfractionated chloroform phase was employed as a source of phospholipids. Similar preparations of chromaffin granule lipids have been shown to contain mainly cholesterol and phospholipids such as lecithin, lysolecithin, phosphatidylethanolamine and phosphatidylserine[16].

The idea of lipoprotein interactions between the soluble chromogranins and phospholipids is by no means new. Phospholipids have previously been reported to be a constituent of the solubilized matrix phase, *i.e.* in association with the water-soluble chromogranins of the lysed granules, indicating that the soluble chromogranins may occur as high-density lipoproteins[17,18,19]. Further support for the presence of phospholipids as true constituents of the matrix phase has been presented in the accompanying paper[11]. Aggregation of the soluble chromogranins has also been observed in earlier studies[3] and was found to occur when adrenaline and ATP–Mg^{2+} were added to ethanol-precipitated preparations of the soluble chromogranins which contained light scattering material, probably due to the presence of phospholipids (Protein VII-I)[20]. Divalent metals such as Mg^{2+} and Ca^{2+} have, however, been omitted from the present preparations in order to avoid interfering aggregations due to metal–protein, metal–phospholipid and metal–ATP–catecholamine interactions[4].

The method employed for particle formation between phospholipids and the aqueous chromogranins was that of Calissano and Bangham[8], designed primarily for the formation of unilayered, liposomal vesicles enveloping an aqueous phase containing the solutes. By using the chromogranins as solutes in addition to salt and buffer ions, it was hoped in the present study to obtain the chromogranins enveloped by the phospholipid bilayers. Sonication was applied as the force needed to break the strong concentric bilayers of the large liposomal structures which form when the aqueous phase enters the anhydrous, liquid phospholipid crystals[9]. The first portion of aqueous phase which enters the crystal has been reported of form tubes of diameters up to 30 Å; tubes which are lined by the polar head groups of the phospholipids and organized as a hexagonal mesophase of phospholipids and water. Upon further entry of the aqueous phase the hexagonal pattern changes into a lamellar, smectic mesophase which consists of concentric bilayers of phospholipids. Thus the aqueous phase becomes sandwiched between the phospholipid bilayers, and exchange between two aqueous phases would be restricted to that of diffusion across the interlaying bilayers[9].

The ultrastructual study of the phospholipid particles which were formed in the present experiments (Fig. 5 and Fig. 6) revealed two types of organization patterns between the phospholipids and the aqueous chromogranins, namely those of liposomal vesicles (L) and large (lg) and small (sg) granules (Fig. 5A). Contrary to what had been anticipated, the liposomal vesicles were on the whole "empty", *i.e.* apparently devoid of granular material, while the granular aggregates were seemingly without an outer membrane-like bilayer of phospholipids. Nevertheless, the granular aggregates were oval or spherical in form, as for lipid droplets, and were on the whole strikingly similar to those of the matrix phase of the intact chromaffin granule (Fig. 5B and 5C).

The fine structure of the artificial particles provided indications of certain regularity in the organization pattern in the granular aggregates (Figs 6A, 6B and 6C), seemingly forming lattices of globular units, often encircling core units, 200–200 Å in diameter. In the variety of granular aggregates, differing in their electron-dense appearance, the distances between the electron-dense, globular units occurred as the variable parameter, while the diameters of the core units appeared more constant. In

view of the lack of electron density exhibited by the hydrocarbon chain moiety of the phospholipids by the glutaraldehyde-osmium fixation, it is not unreasonable to assume that the electron-light intervals mainly represent the hydrocarbon chains of the phospholipids. By similar reasoning it seems most probable to assume that the electron-dense material in the present micrographs is representative of the aqueous chromogranins *plus* the polar head groups of the phospholipids. A hexagonal pattern of phase organization between the phospholipids and the soluble chromogranins would be compatible with that to be expected from phase interactions between phospholipids and aqueous solutes, provided the aqueous phase accounted for less than 30% of the aqueous phospholipid crystals[9]. The ring-like structures which could be seen in some of the granular aggregates might point to hexagonal-like arrays and thus suggesting that the granular aggregates might account for particles with a high ratio of phospholipid/aqueous phase.

If on the other hand, the granular aggregates were devoid of phospholipids, the enzymic activity of the soluble chromogranins should be unaffected by the presence of phospholipid vesicles in this fraction. However, the specific dopamine-β-hydroxylase activity used as a "marker" for the soluble chromogranins, was 4-fold potentiated by the interaction with phospholipids (Table I, AI). The sonication procedure further enhanced the specific enzyme activity parallel to an increase in the amount of chromogranins sedimented into the 0.4 M sucrose layer; findings which support the assumption that the phospholipid–chromogranin interactions were the reason for the potentiation of the specific enzyme activity of the particles. Furthermore, the specific enzyme activity of the particles in the 0.4 M sucrose layer (Table I, AIII) was identical to that of the purified enzyme obtained from the Triton X-100-solubilized membrane phase and from water-soluble chromogranins in earlier work, *i.e.* chromogranin A_M and A_I respectively[19]. The former contained an average of 1.5 μmoles lipid-*P*/mg protein while the latter was free of lipid and dissociated upon dilution into the monomer of chromogranin A (A_{III}). Taken together, these data strongly support the earlier assumptions[19] that phospholipid–chromogranin interactions lead to a stabilization of higher molecular weight complexes of potentiated enzymic activity, with phospholipids as a "glue" or molecular "cement"[21].

The data (Table III and Fig. 3) revealed a direct proportionality between the amount of protein in the fraction and the dopamine-β-hydroxylase activity (Fig. 4B); findings which made it unlikely that protein dilution effects[22] were the reasons for the different levels of enzyme activity in the phospholipid-rich fractions compared to that of the lipid-free chromogranins. The apparently sigmoidal curve relationship between the specific dopamine-β-hydroxylase activity and the lipid-*P*/protein ratio of the particle fraction was on the other hand suggestive of conformational changes in the enzyme protein due to the presence of phospholipids. Such effects of phospholipids have been described for a number of membrane-associated enzymes and have been assigned to catalytic functions of the phospholipids[23] in addition to their action as a molecular "dement"[21].

The elevated levels of dopamine-β-hydroxylase activity in the particle fractions were up to 10 times higher than the levels of the enzyme activity in the membrane and matrix phases derived from the lysed chromaffin granules[11,15]. However, in these fractions a small amount of catecholamines remained firmly bound, even after prolonged dialysis[24,25]. The present study has shown that the presence of $3 \cdot 10^{-6}$ M

noradrenaline exhibited a pronounced inhibitory effect on the enzyme activity (Table II, Fig. 1B and Fig. 2), while the presence of a low concentration of adrenaline (10^{-4}M) in the presence of 10 mM ATP appeared without inhibitory effect (Fig. 3 and Table III). It is thus quite likely that the noradrenaline present in the catecholamine fraction bound to the membrane phase protein[24,25] could be sufficient for the inhibition of the enzyme to the level observed for the artificial particles in the presence of noradrenaline (Table II and Fig. 2). By such an inhibitory effect the product of the enzymic hydroxylation might excert a negative feed-back, regulatory action on the enzyme activity which might be of relevance to a "secondary" function of the enzyme protein as the storage matrix of the product.

The artificial particles of the 0.4 M sucrose layer took up ATP and noradrenaline during further sonication of the preformed particles. However, the two low molecular weight constituents did not seem to accumulate in a quite parallel manner. Thus the ATP/protein ratio was higher at a lipid-*P*/protein ratio of 2.6 μmoles/mg (Fraction 5, Table III) than in the particles which contained 18 μmoles lipid-*P*/mg protein (Fraction 7, Table III). Noradrenaline, on the other hand, was higher in the latter fraction than in the former (*cf.* Fig. 1B). As far as these findings can be taken, they provide additional support for the postulation[19] that phospholipids and ATP may compete for the same binding sites on the chromogranins and thus lead to inverse relationships between phospholipids and ATP/protein. Noradrenaline, on the other hand, appears to have a basic affinity for phosphates, either phospholipids or ATP, and thus may accumulate at high levels where the sum of phosphates is high, *e.g.* in the 0.6 M sucrose layers in the present experiments, and in the 0.4 M sucrose layer containing liposomal vesicles derived from the lysis-resistant fraction of intact chromaffin granules[26,11].

Due to scarcity of material only the ultrastructure of the major fraction of particles, *i.e.* those of buoyant density equivalent to that of 0.4 M sucrose, could be examined (Figs 5 and 6). This material contained 4 μmoles ATP and 2.6 μmoles lipid-*P*/mg protein and exhibited the highest specific dopamine-β-hydroxylase activity observed (37 units/mg, Table III) in the presence of chromogranin A in ratios equivalent to that observed for the less dense particle fractions (Fractions 1–4, Table III). Based on the present data it seems probable that these properties must be confined to the electron-dense material in the granular aggregates rather than to the bilayers of the liposomal vesicles. As discussed above, the granulation seems to contain lattices, possibly of hexagonal organization, of protein units around a core. In this connection it is of interest to recall that 4–7 moles of Cu have been reported for the purified dopamine-β-hydroxylase (mol. wt 290000)[27]. This leaves us with a minimum mol. wt of 41000/mole Cu, suggestive of a subunit molecular weight of the order of chromogranin A_{III} (32000–40000)[19,28,29]. A diameter of 30–40 Å (the smallest subunit detected in the electron micrographs) would be compatible with a $\times$40000 mol. wt subunit, which might be surrounded by a layer of polar head groups of the phospholipids, as in the hexagonal mesophase of the aqueous phase in the liquid crystals of phospholipids[9]. It is, therefore, not unlikely that the cores seen in the granular particles represent the unit structure of the dopamine-β-hydroxylase activity, *i.e.* with diameters approximately 300 Å due to phospholipids between the subunits. The apparently ring-like organization of electron-dense material around the 300 Å cores suggests that this might be an organization pattern which might be comparable to that observed

for the matrix phase of the intact chromaffin granule[30]. For aqueous phospholipid crystals of more than 30% water, the hexagonal mesophase, however, changes into the lamellar, smectic mesophase; a pattern which is illustrated by the vesicles of phospholipid bilayers, *e.g.* (L) in Fig. 5. For the intact chromaffin granule, the bilayered membrane could be clearly seen at the site of influx of the aqueous phase[10,24]. This furthermore suggests that not only the artificial particles but also the intact chromaffin granule may merely represent two different phases of phospholipids and aqueous solutes; with the lamellar organization pattern as a diffusion barrier between the cytosol and the hexagonal mesophase of the intragranular matrix phase.

Although in many ways strikingly similar to the intact chromaffin granule the presently described particles of phospholipids and chromogranins are lacking in two major constituents of the intact granules, namely ATPase and cytochrome b_{561} (refs 31, 32). These constituents persisted in the membrane fragments depleted of phospholipids, Cu, dopamine-β-hydroxylase and chromogranin A by freezing and thawing of the lysis-resistant fraction[11] and may thus be assumed to be true constituents of the hydrophobic phases of the phospholipid bilayers. In future studies it would be of interest to investigate the mode of incorporation of these constituents into the artificial particles in order to obtain a complete model of the functional amine storage organelle.

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