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Membranes of Adrenal Chromaffin Granules

Solubilization and Partial Characterization of the Mg^{++}-Dependent Adenosine Triphosphatase

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SUMMARY

Membrane proteins were solubilized upon treatment of membranes obtained from adrenal medullary chromaffin granules with detergents (0.1–0.5% sodium dodecyl sulfate, 0.1% sodium deoxycholate, 0.02% Triton X-100, and 0.2–2.0% Lubrol PX). All solubilizing agents except Lubrol inactivated the chromaffin granule ATPase. Lubrol not only solubilized the membrane ATPase, but also increased the specific activity of the preparation 2.5 times.

Lubrol-solubilized membrane proteins were separated by gel filtration and by ion-exchange chromatography. The elution pattern of the granule ATPase changed with the Lubrol to protein ratio used during solubilization. With low ratios (4:1), the ATPase was eluted with the void volume from a Sephadex G-200 column, and immediately after the void volume from a Sepharose 6B column (K_{av} = 0.037), whereas when a 15:1 ratio was used the ATPase had a larger partition coefficient (K_{av} = 0.383).

Chromatography on DEAE-Sephadex A-25 showed that a fraction containing the ATPase activity was eluted at the ionic strength of 0.2, and that in the presence of Mg^{++} transphosphorylation from ATP to granule protein was observed only in this fraction. The specific activity of the ATPase and the transphosphorylation from ATP in this fraction were 8–15 times higher than when tested in intact granule membranes.

INTRODUCTION

Catecholamines are stored in the adrenal medulla together with soluble proteins and adenine nucleotide within specific storage particles, the chromaffin granules (1, 2). When the gland is stimulated the chromaffin granule discharges its soluble contents to the cell exterior by exocytosis, and the granule membrane remains within the cell as an empty vesicle (3–6). The series of events involved in the secretory process has been named "stimulus-secretion coupling" (7) because of their similarity to "excitation-contracting coupling" in muscle. Like muscle contraction, the secretory process is Ca^{++}-dependent (8), and because secretion is blocked by inhibitors of oxidative phosphorylation and glycolysis (9, 10), it seems likely that the production of ATP is necessary for maintaining secretion (11).

Although the molecular events involved in the secretory process have not yet been completely elucidated, much information has been obtained by studying the effects of ATP on isolated chromaffin granules. This nucleotide, in the presence of Mg^{++}, is hydrolyzed by the granule membrane ATPase, and the P_i liberated is transphosphorylated to the granule membrane (12). Concomitant with

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this process are both the release of granule contents (catecholamines and other soluble components) (13, 14) and structural modification of the granules as revealed by light scattering changes (15–17). The effects of ATP on chromaffin granules can be blocked at early (ATP hydrolysis, transphosphorylation) or subsequent (conformational changes) steps (18).

Although it is not yet known whether this effect of ATP on chromaffin granules occurs during stimulation of the gland *in vivo*, it is possible that the chromaffin granule ATPase plays an important role in stimulus-secretion coupling, either during the interaction between the chromaffin granule membrane and the plasma membrane or during the discharge of the soluble granule contents to the cell exterior. It was therefore decided to study the membrane-bound ATPase of the chromaffin granules, in order to obtain a better understanding of the molecular events involved in the secretory process.

This paper describes the solubilization, isolation, and partial characterization of the Mg^{++}-dependent ATPase of chromaffin granules. A preliminary report of some of these data has been presented (19).

METHODS

Isolation of adrenal chromaffin granules. Bovine adrenal glands obtained from a slaughterhouse were kept on ice, and the medullae were separated from the cortices. Each medulla was homogenized in 4 volumes of ice-cold 0.3 M sucrose (pH 7.0). A low-speed sediment was removed by centrifugation at 800 × *g* for 10 min, and the supernatant fraction was recentrifuged at 20,000 × *g* for 20 min. The pellet thus obtained (crude granule fraction) was resuspended in 2.0 ml of 0.3 M sucrose, layered on 7.5 ml of 1.6 M sucrose, and centrifuged at 100,000 × *g* for 60 min. The final pellet contained chromaffin granules (20) and was a relatively pure preparation as indicated by the enzyme determinations used as mitochondrial (succinate dehydrogenase and monoamine oxidase), lysosomal (β-glucuronidase), and microsomal (glucose 6-phosphatase) markers. The enzymatic activities in the crude granule fraction were: monoamine oxidase, 0.440 μg of *p*-hydroxyphenylacetaldehyde produced per milligram of protein per hour; succinate dehydrogenase, 133 μg of neotetrazolium reduced per milligram of protein per hour; and β-glucuronidase, 108 μg of phenolphthalein liberated per milligram of protein per hour. In contrast, the enzymatic activities in the chromaffin granule fraction were: monoamine oxidase, 0.018; succinate dehydrogenase, 2.9; and β-glucuronidase, 3.05 μg/mg of protein per hour. The microsome fraction (sediment obtained after centrifuging the 20,000 × *g* supernatant fraction at 100,000 × *g* for 60 min) of the adrenal medulla had a glucose 6-phosphatase activity of 65.4 μg of P_i per milligram of protein per hour, whereas the granule fraction had a specific activity of 1.25 μg/mg/hr.

Preparation of chromaffin granule membranes. Purified granules were prepared every day from 60–80 g of medullae and were kept frozen for 2–3 days. These granule fractions, obtained from 180–240 g of medullae, were combined and resuspended in cold (4°c) 0.005 M Tris-HCl buffer (pH 7.0) for 30 min and centrifuged at 100,000 × *g* for 60 min. This resuspension and centrifugation process was repeated three times. Chromaffin membranes thus prepared had an ATPase activity of 0.9–1.02 μmoles of P_i per milligram of protein per hour.

Chemical determinations. Catecholamines were assayed by the trihydroxyindole fluorometric method (21), and protein was determined by the method of Lowry *et al.* (22).

ATPase activity. Samples of granule membranes were incubated in centrifuge tubes for various periods of time at 30° in a total volume of 2.0 ml. The standard incubation medium contained KCl, 160 mM; NaCl, 5 mM; Tris-HCl Buffer (pH 6.5), 10 mM; and $MgCl_2$, 1.0 mM; this was varied in some experiments as described below. [γ-^{32}P]ATP (specific activity, 1.8 mCi/μmole), synthesized in our laboratory according to a modification by Post and Sen (23) of the method of Glynn and Chappell (24), was added to the medium to give a concentration of 1.0 mM. Incubation was stopped by adding ice-cold trichloracetic acid (10% final concentration) containing 0.2 mM K_2HPO_4 and 0.2 mM ATP. The tubes were cooled in an ice-water bath for a few minutes and centrifuged in the cold (4°) at 20,000 × *g* for 10 min.

Aliquots (1.0 ml) of the supernatant fluid thus obtained were treated with 100 mg of Norit A charcoal to remove [γ-^{32}P]ATP, and the mixture was centrifuged at 20,000 × *g* for 10 min. Radioactivity was determined by adding an aliquot of the supernatant fractions to scintillation vials containing 0.5 ml of "NCS" (Nuclear-Chicago) and 15.0 ml of the mixture previously described (25, 26). The ^{32}P radioactivity was measured with counting efficiencies between 85 and 92%; the results were corrected to 100% efficiency and expressed in micromoles of P_i per milligram of protein, calculated on the basis of the specific activity of the added [γ-^{32}P]ATP. Aliquots of the fractions obtained during elution of the columns were assayed in the same manner. Controls with the eluent fluids used in the different chromatographic procedures were run as well as samples incubated in the absence of $MgCl_2$ and the presence of 2.0 mM EDTA. In all experiments zero-time samples were prepared by adding the [γ-^{32}P]-ATP after treating the incubation medium containing the protein aliquots with trichloracetic acid (10% final concentration). The transphosphorylation from [γ-^{32}P]ATP to granule protein was measured as described previously (12).

β-Glucuronidase activity was measured by the method described by Gianetto and de Duve (27). Succinate dehydrogenase and monoamine oxidase activities were measured by the methods of Pennington (28) and Wurtman and Axelrod (29), respectively, but following the modifications previously described (30). Glucose 6-phosphatase was determined by the method of de Duve *et al.* (31).

Sephadex chromatography. Sephadex G-200 (particle size, 40–120 μ) was swollen for 24–48 hr at room temperature in a medium containing 160 mM kCl, 5 mM NaCl, 10 mM Tris-HCl buffer (pH 6.5), and 0.2% (w/v) Lubrol PX. Sodium azide (0.02%) was added to the medium to inhibit bacterial growth. A glass column (Pharmacia) was packed, and the Sephadex bed (2.5 × 37 cm) was equilibrated and eluted by upward perfusion with the same medium used for swelling, at a flow rate of 12 ml/hr. Samples containing 8–12 mg of granule membrane protein in 3 ml of the medium were applied to the column.

Sepharose chromatography. A glass column (Pharmacia) was slowly filled with a slurry of Sepharose 6B (particle size, 40–210 μ). The Sepharose bed (2.6 × 90 cm) was equilibrated and eluted by upward perfusion with the same medium used for Sephadex G-200, at a flow rate of 13.0 ml/hr. Samples containing 10–15 mg of Lubrol-solubilized membrane proteins in 8.0 ml of medium were applied to the column. To calibrate the column, a mixture of known proteins (30 mg of total protein in 8.0 ml of medium) was applied to the column. The proteins used were thyroglobulin (mol wt 670,000), apoferritin (mol wt 460,000), and pyruvate kinase (mol wt 237,000). The void volume of the column, determined with blue dextran 2000, was 156 ml, and represented 33% of the bed volume (V_t) of the column. The V_t value calculated on the basis of the dimensions of the bed volume was 477 ml, and that obtained from the elution volume of catecholamines was 480 ml. The results obtained with gel filtration chromatography are expressed in terms of a column partition coefficient, K_{av} (32).

Ion-exchange chromatography. Nine grams of DEAE-Sephadex A-25 (particle size, 40–120 μ) were swollen at room temperature for 24 hr in 0.005 M Tris-HCl buffer (pH 6.5). A glass column (Fischer and Porter Company) was packed, and the bed (1.5 × 28 cm) was equilibrated by perfusion through the column with 200–300 ml of starting medium (0.005 M Tris-HCl buffer, pH 6.5, and 0.2% Lubrol). After application of the samples (15–25 mg of Lubrol-solubilized granule membrane protein in 15–25 ml of starting buffer), 40–50 ml of starting buffer were run through the column. This was followed by elution with either a linear (NaCl, I = 0 to I = 0.8, in starting buffer) or a stepwise ionic strength gradient (NaCl, I = 0.0, 0.2, 0.4, 0.6, and 0.8 in starting buffer).

All columns were perfused in the cold (4°) at a constant flow rate with an LKB peristaltic pump (Varioperpex), and elution was always monitored at 280 nm by means of an LKB Uvicord II apparatus. The fractions (4 ml) collected were assayed for protein content, ATPase activity, and catecholamines.

Polyacrylamide gel electrophoresis. Polyacrylamide disc electrophoresis was per-

formed as described by Davis (33). Samples (50–150 μg of protein) in 0.1% Lubrol were applied to the gels. Electrophoresis was carried out for 70–90 min with a current of 3 mamp/tube. Gels were stained for 1 hr in 7% (v/v) acetic acid containing 0.2% (w/v) Buffalo black. Destaining was performed electrophoretically in 7% acetic acid.

Chemicals. Triton X-100, sodium deoxycholate, Tris buffer, pyruvate kinase, ATP, phenolphthalein-glucuronic acid (sodium salt), and glucose 6-phosphate were obtained from Sigma Chemical Company; Sephadex G-200, Sepharose 6B, DEAE-Sephadex A-25, and blue dextran 2000, from Pharmacia; sodium dodecyl sulfate, Norit A, and EDTA, from Fisher Scientific Company; thyroglobulin and neotetrazolium, from Nutritional Biochemicals Corporations; Lubrol PX, from CIL Chemicals, Montreal; apoferritin, from Schwarz BioResearch, Inc.; [^{14}C]1-tyramine hydrochloride, from Nuclear-Chicago Corporation; $^{32}P_i$ (carrier-free), from New England Nuclear Corporation; and Buffalo black NBR, from Allied Chemical and Dye Corporation.

RESULTS

Effect of detergents on solubilization and activity of Mg^{++}-dependent ATPase of chromaffin granule membranes. Chromaffin granule membranes prepared as indicated in METHODS were treated with the detergents Triton X-100, sodium deoxycholate, sodium dodecyl sulfate, and Lubrol PX at the concentrations and for the times indicated in Table 1. The granule membrane suspensions were then centrifuged at 100,000 × *g* for 60 min, and the supernatant solutions thus obtained were assayed for protein and Mg^{++}-dependent ATPase activity. Table 1 shows the effectiveness of the different detergents on solubilizing granule membrane proteins; all of them except Lubrol PX inactivated the ATPase activity of these preparations. Lubrol not only solubilized the membrane ATPase, but also increased the specific activity of the preparation for all concentrations of Lubrol tested (Table 1). The following experiment showed that this increase in the specific activity was due to selective solubilization of the ATPase rather than to activation of the enzyme. Granule membranes were treated with 2% Lubrol; protein, total and specific ATPase activities were determined before and after treatment of the membranes with Lubrol and in the supernatant fluid obtained after centrifugation (100,000 × *g* for 60 min) of the Lubrol-treated membrane preparations. Table 2 shows that there was a small increase in the total ATPase activity after treatment of the membranes with Lubrol. However, the specific activity of the ATPase (1.11 ± 0.31 μmoles of P_i per milligram of protein per hour) was not significantly increased by the Lubrol treatment. After centrifugation of the Lubrol-treated preparations, 92% and 44% of the total ATPase activity and protein, respectively, were recovered in the supernatant fraction (Lubrol-extracted protein). No significant ATPase activity was detected in the sedi-

TABLE 1

Solubilization of chromaffin granule membrane proteins by different agents

Chromaffin granule membranes were prepared as indicated in METHODS and were suspended (1–2 mg of protein per milliliter) in ice-cold solutions containing the different detergents at the concentrations indicated below. After treatment with a solubilizing agent (sodium dodecyl sulfate, Triton X-100, and deoxycholate, 10 min; Lubrol, 30 min), the preparations were centrifuged at 100,000 × *g* for 60 min, and the supernatant fractions were assayed for protein and Mg^{++}-dependent ATPase activity as indicated in METHODS.

Detergent	Concentration	Solubilized protein	Solubilized ATPase[a]
	%	*% total membrane protein*	*% specific activity*
Sodium dodecyl sulfate	0.1	13 (4)[b]	3.5
	0.3	54 (5)	3.0
	0.5	76 (5)	2.7
Triton X-100	0.02	74 (3)	1.6
Sodium deoxycholate	0.1	76 (3)	10.3
Lubrol PX	0.2	36 (5)	250
	0.4	41 (4)	230
	0.8	43 (3)	242
	1.0	42 (4)	253
	2.0	57 (4)	215

[a] The ATPase specific activity was 0.92 μmole of P_i per milligram of protein per hour.

[b] Number of observations is given in parentheses.

TABLE 2

Partial purification of chromaffin granule membrane ATPase

Chromaffin granule membranes were prepared as indicated in METHODS and suspended in ice-cold solutions containing 2% Lubrol. Protein, total and specific ATPase activities were determined before and after Lubrol treatment of the membranes, in the Lubrol-extracted membrane proteins, and in a fraction (peak II) obtained by DEAE-Sephadex A-25 chromatography. The Lubrol-extracted membrane protein fraction is the supernatant fluid obtained by centrifuging the Lubrol-treated granule membranes at 100,000 × *g* for 60 min. Results are averages of four different experiments, except for those obtained from peak II, which are averages of two separate determinations.

Purification step	Protein	Mg^{++}-ATPase	
		Total Activity	Specific activity
	mg	*μmoles P_i/hr*	*μmoles P_i/hr/mg*
Granule membranes	55.4	54.79	0.99 ± 0.08[a]
Lubrol-treated granule membranes	55.4	61.27	1.11 ± 0.31
Lubrol-extracted membrane proteins	24.4	56.61	2.32 ± 0.41
Peak II from DEAE-Sephadex A-25	3.6	54.20	15.05

[a] Mean ± standard error.

ment. However, as expected, there was an increase in the ATPase specific activity in the Lubrol-extracted protein (Table 2).

Therefore, Lubrol was used as a solubilizing agent in all experiments described below.

Molecular sieve chromatography of solubilized chromaffin granule membrane components and determination of "apparent" molecular weight of Mg^{++}-dependent ATPase. The supernatant fractions (100,000 × *g*, 60 min) from Lubrol-treated membrane preparations were subjected to molecular sieve chromatography on Sephadex G-200 as indicated in METHODS. Elution was carried out with a solution containing 160 mM KCl, 10 mM Tris-HCl buffer (pH 6.5), and 0.2% Lubrol. This pH was chosen because it is the optimal pH for granule ATPase activity (12). Under these conditions, the ATPase was eluted from the column with the void volume, and had a K_{av} value of 0.009. This result indicates that the ATPase has an "apparent" molecular weight equal to or greater than the exclusion limit for Sephadex G-200. Therefore, gel filtration chromatography was performed next on a Sepharose 6B column. The ratio between Lubrol and membrane protein used was 4:1 (w/w). The column was eluted as described above, and a fraction containing 60% of the total protein applied to the column, as well as all the ATPase activity, was eluted immediately after the void volume (Fig. 1). The K_{av} value obtained in this experiment was 0.037 (Table 3). Since it had been reported that higher Lubrol to protein ratios decrease the aggregation of membrane-bound protein molecules (34), granule membrane proteins were solubilized in a solution containing a Lubrol to protein ratio of 15:1. Upon chromatography on the same Sepharose 6B column, the ATPase activity was eluted as a single peak (Fig. 2), and had a partition coefficient (K_{av}) of 0.383. This was compared with the K_{av} value of substances of known molecular weight (Table 3). The "apparent" molecular weight of the ATPase under these experimental conditions was between 490,000 and 500,000 (Fig. 2).

The protein recovery from the column was 95–98%, and the total ATPase activity was between 90 and 95% of that applied to the column. The protein in the ATPase peak accounted for 41% of the total protein applied to the column. Polyacrylamide disc electrophoresis of these proteins showed a pattern of six bands, which corresponded to the slowly moving bands of the total membrane proteins that were extracted by Lubrol (Fig. 3). The specific activity of the ATPase was 4–5 times higher than that in the chromaffin granule membranes.

Ion-exchange chromatography of chromaffin granule membrane proteins solubilized by Lubrol. Chromaffin granule membrane proteins solubilized in Lubrol (Lubrol to protein ra-

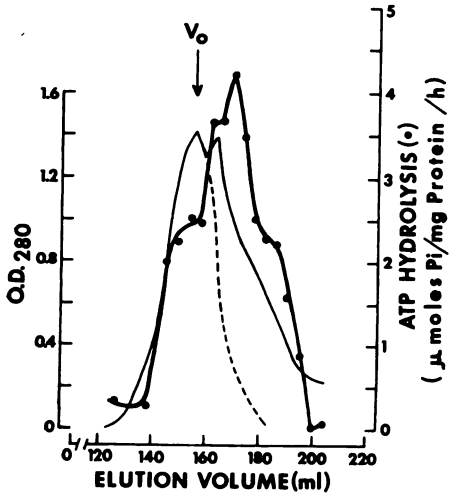

FIG. 1. *Molecular sieve chromatography of Lubrol-solubilized proteins of chromaffin granule membranes*

Chromaffin granule membrane proteins were solubilized with Lubrol (Lubrol to protein ratio, 4:1) and fractionated on a Sepharose 6B column. A sample (6 mg of membrane protein in 8 ml of elution medium) was applied to the column, and eluted by upward perfusion as indicated in METHODS. The effluents from the column were monitored at 280 nm (solid line), and 4-ml samples were collected and assayed for protein and ATPase activity. The void volume is indicated by the broken line, and the ATPase activity by the solid circles.

TABLE 3

Column partition coefficients (K_{av}) of Lubrol-solubilized chromaffin granule ATPase compared with those of substances of known molecular weight

The K_{av} values were calculated from the elution volume (V_e) of each substance as indicated in METHODS.

Substance	K_{av}
Blue dextran 2000	0.0
ATPase (Lubrol:protein, 4:1)[a]	0.037
Thyroglobulin	0.210
ATPase (Lubrol:protein, 15:1)[a]	0.383
Apoferritin	0.433
Pyruvate kinase	0.855

[a] These values represent the ratios (by weight) between Lubrol and chromaffin granule membrane protein used during solubilization of the membranes.

tio, 15:1) were eluted from DEAE-Sephadex A-25 as indicated in METHODS. Samples were applied in the starting buffer (0.005 M Tris-HCl, pH 6.5, and 0.2% Lubrol), and 40 ml of this buffer were perfused through the column. This was followed by stepwise elution with NaCl solutions of increasing ionic strength (I = 0 to I = 0.8) (Fig. 4). Five protein peaks (Fig. 4) were eluted from the column, and their protein content and ATPase activity were determined. Peak II (NaCl, I = 0.2) contained all the ATPase activity eluted from the column (Fig. 4). The protein content in peak II represented 14–16% of the total protein applied to the column, and the specific activity of ATPase in this fraction was 8–15 times higher than that of the granule membranes (Fig. 4 and Table 2). Polyacrylamide disc electrophoresis of the protein recovered in peak II showed two bands, which corresponded to the first two slowly moving bands of the membrane proteins that were extracted by Lubrol (Fig. 5). Similar results were obtained when a gradient of linear ionic strength was used.

There was no difference in the specific activity of the ATPase when the enzyme activity recovered in peak II after one-step chromatography on DEAE-Sephadex A-25 was compared with that obtained in peak II after two-step chromatography on Sepharose 6B followed by DEAE-Sephadex A-25.

Transphosphorylation from [γ-^{32}P]ATP to chromaffin granule membrane proteins. We have previously demonstrated that when ATP is hydrolyzed by the granule ATPase, part of the P_i from ATP is transphosphorylated to the chromaffin granule membranes (12). The five peaks obtained by ion-exchange chromatography on DEAE-Sephadex A-25 were also tested for protein phosphorylation. When fractions from peak II (the peak that contained all the ATPase activity; Fig. 4) were tested in the absence of Mg^{++} (presence of 2.0 mM EDTA), there was no transphosphorylation from ATP to the protein. On the other hand, the same fractions tested in the presence of Mg^{++} (0.5 mM) gave values of P_i transphosphorylation from ATP of 676 ± 14 pmoles of P_i per milligram of protein per minute. This figure

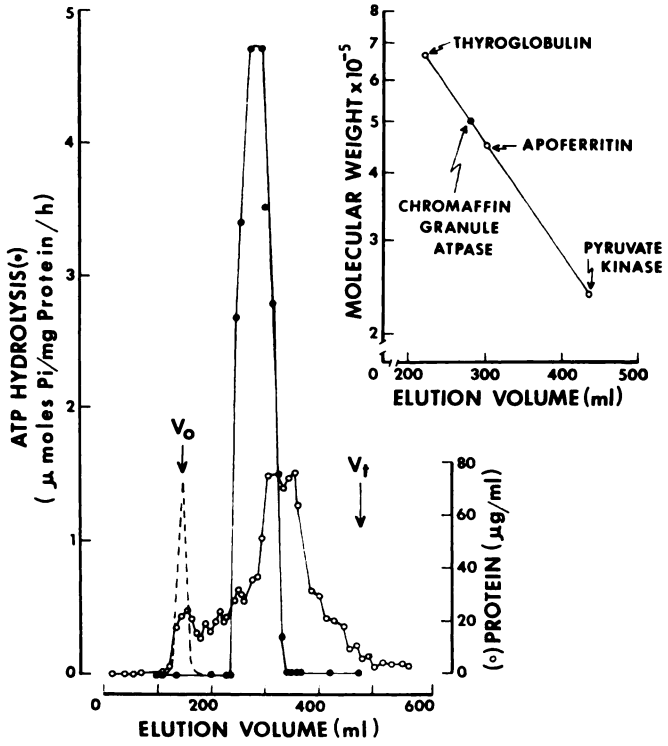

FIG. 2. *Molecular sieve chromatography of Lubrol-solubilized proteins of chromaffin granule membranes*
Proteins of the granule membranes were solubilized by Lubrol (Lubrol to protein ratio, 15:1). A sample (10 mg of membrane protein in 8 ml of medium) was applied and eluted from a Sepharose 6B column in the same manner as indicated in Fig. 1. Fractions (4 ml) were collected and assayed for protein (○) and ATPase activity (●) as indicated in METHODS. The void volume (V_0) is indicated by the broken line. The inset shows the "apparent" molecular weight of the granule ATPase calculated from the value obtained when its elution volume (V_e) was compared with those of substances of known molecular weight.

is 11.3 times higher than that obtained when intact chromaffin granule membranes were tested (60 ± 4 pmoles/mg of protein per minute).

DISCUSSION

We have previously demonstrated that ATP causes structural changes leading to release of catecholamines, endogenous ATP, and soluble proteins from chromaffin granules (13–15), and that part of the P_i liberated from ATP by the granule ATPase is incorporated into chromaffin granule membranes (12).

The present results show that granule membrane proteins are solubilized when granule membranes are treated with detergents. Winkler *et al.* (35) have also recently shown that sodium dodecyl sulfate, Triton WR-1339, and deoxycholate effectively solubilize the chromaffin granule membrane proteins. However, these workers did not measure ATPase or other enzyme activities in the detergent-treated preparations (35).

The present experiments show that of all the detergents tested only Lubrol solubilized a protein fraction which contained ATPase activity. This solubilized fraction contained 40–50% of the membrane protein and all of the ATPase activity, which means that the specific activity of the enzyme preparation was increased 2–2.5 times.

Attempts to purify the granule ATPase that was solubilized in low Lubrol to protein mixtures were only partly successful; ATPase activity was eluted with or close to the void volume from Sephadex G-200 or Sepharose 6B. However, when the Lubrol to protein

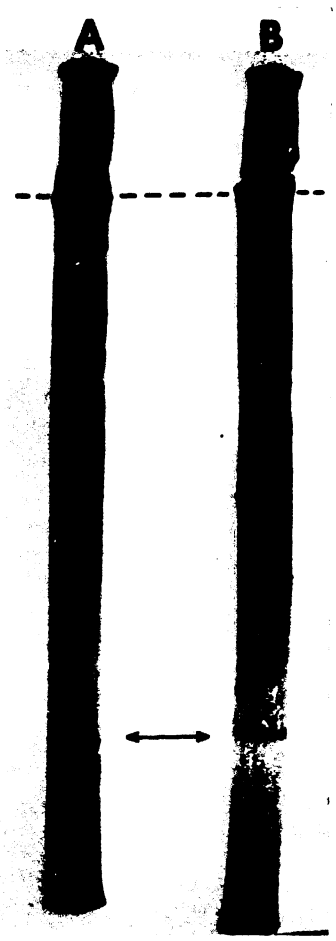

FIG. 3. *Polyacrylamide gel electrophoresis of chromaffin granule membrane proteins before and after Sepharose 6B chromatography*

Chromaffin granule membranes were treated with Lubrol and centrifuged as indicated in the legend to Table 1. The electrophoretic pattern of the proteins present in the fraction with the greatest ATPase activity obtained after gel filtration on Sepharose 6B is indicated in panel *A*. This gel shows six bands with mobilities similar to the six slowly moving bands of the total Lubrol-extracted granule membrane proteins (*B*). Electrophoresis was performed as indicated in METHODS. The direction of migration was from top to bottom, which was the anode. The broken line indicates the interphase between the stacking and running gels. The arrow indicates the position of the tracking rings.

ratios were increased to 10–15: 1, the ATPase showed a different elution pattern and had a K_{av} of 0.383. This difference in the elution patterns suggests that high Lubrol to protein ratios may decrease the aggregation of different membrane proteins, or that the ATPase is formed of subunits, which can be dissociated when high Lubrol to protein ratios are used.

The elution peak from Sepharose 6B with the highest ATPase activity did not coincide with a protein peak. This indicates that the ATPase activity was eluted together with other membrane proteins from the Sepharose 6B column, and this was confirmed by polyacrylamide disc electrophoresis. In addition, 41 % of the protein applied to the column was recovered in the fraction containing the ATPase. This figure represents more than double the amount of protein recovered in the ATPase peak, when the chromatography was performed on DEAE-Sephadex A-25.

On Sepharose 6B the elution volume and K_{av} obtained for the ATPase suggest that the enzyme has an "apparent" molecular weight of 500,000, when compared with the V_e and K_{av} values of globular proteins of known molecular weight. However, variations in the elution positions can be due to molecular asymmetry and to the degree of hydration of the molecules (36). The hydrodynamic radius of a protein also changes with the ionic strength of the medium used during elution (36). Therefore, the elution pattern of substances during gel filtration is related more to their Stokes radius than to their molecular weight (36). Furthermore, polyacrylamide disc electrophoresis showed that the proteins present in the ATPase fraction penetrated 7.0 % acrylamide gels, and this indicates that these proteins have a molecular weight smaller than 500,000. Therefore, other methods for estimation of the molecular weight of the granule ATPase must be used before any conclusion about its molecular weight can be reached.

In the present experiments all chromatographic procedures were done in the presence of Lubrol. Although it is obvious that Lubrol interacts strongly with the ATPase, it is not known whether Lubrol binds to the enzyme extensively, or whether binding changes with the Lubrol to protein ratio used. However, proteins of known molecular weights gave a straight line when their distribution coefficients were plotted against their molecular weights, and this suggests either that Lubrol

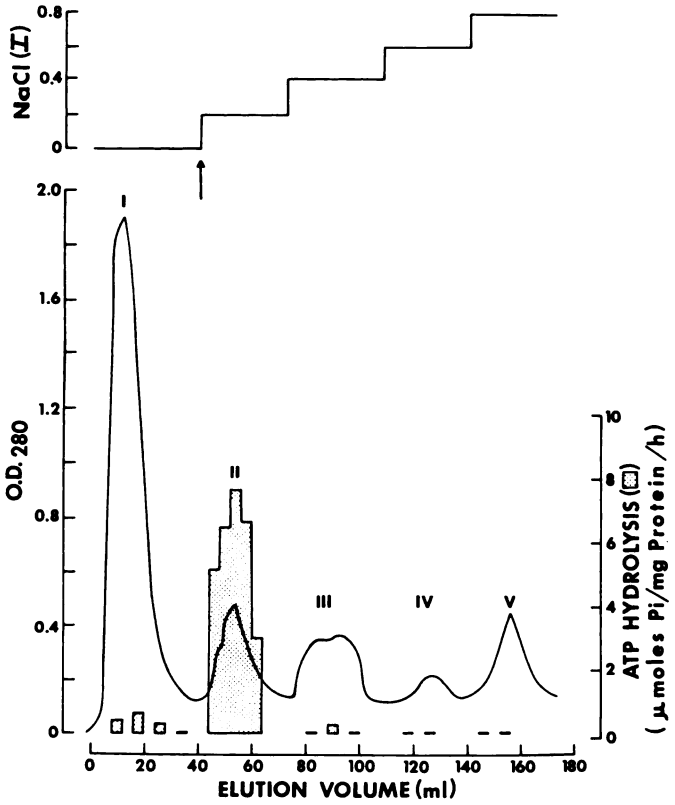

FIG. 4. *Ion-exchange chromatography of Lubrol-solubilized proteins of chromaffin granule membranes*
Chromaffin granule proteins were solubilized with Lubrol (Lubrol to protein ratio, 15:1). A sample containing 18 mg of protein in 15 ml of starting buffer (0.005 M Tris-HCl buffer, pH 6.5, and 0.2% Lubrol) was applied to a DEAE-Sephadex A-25 column. Forty milliliters of starting buffer were run through the column. At the point indicated by the arrow, a stepwise ionic strength gradient of NaCl (*I* from 0 to 0.8) in starting buffer was begun. Fractions (4 ml) were collected and were assayed for protein content and ATPase activity as indicated in METHODS.

did not bind to these proteins to a large exent or that Lubrol binds uniformly to quite different proteins. The latter possibility is known to be true for the binding of sodium dodecyl sulfate to a range of proteins (37).

When Lubrol-solubilized membrane proteins were separated by ion-exchange chromatography on DEAE-Sephadex A-25, the ATPase activity was recovered in the protein peak (peak II) eluted with NaCl at an ionic strength of 0.2. About 14–16% of the total protein applied to the column was recovered in the ATPase peak, and since Lubrol solubilized about 50% of the membrane protein (Table 1) and no appreciable ATPase activity was detected in the sediment (Lubrol-insoluble fraction), it seems that ATPase represents no more than 7–8% of the total proteins of the granule membrane.

The protein present in peak II (ATPase fraction) was phosphorylated by ATP, and this is in agreement with our previous demonstration of the transphosphorylation from ATP to chromaffin granule membranes (12). These experiments have shown that Mg^{++} is required for the transphosphorylation from ATP to chromaffin granule membranes (12) as well as for ATPase activity (12) and the ATP-evoked release of catecholamines (13–15). Transphosphorylation from ATP to the protein present in peak II occurred only in the presence of Mg^{++}. The amount of P_i transphosphorylated per milligram of protein was 11.3 times higher than that observed when chromaffin granule membranes were tested. This figure agrees with the 8–15-fold increase in the specific activity of the ATPase found in peak II over that in chromaffin

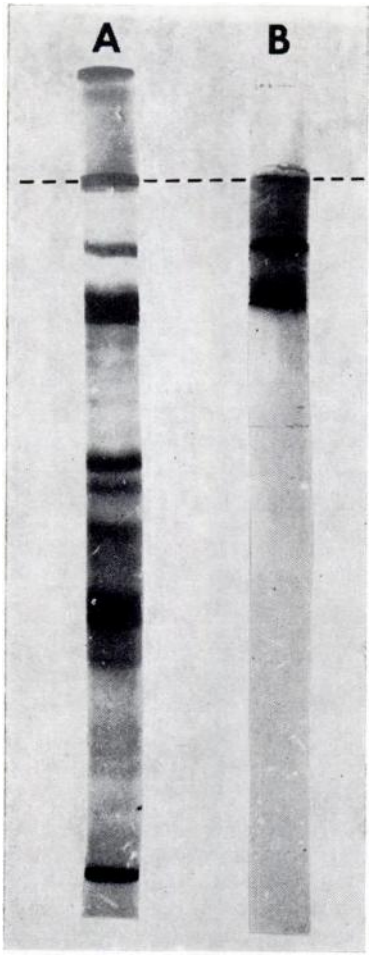

FIG. 5. *Polyacrylamide gel electrophoresis of chromaffin granule membrane proteins before (A) and after (B) DEAE-Sephadex A-25 chromatography*

Chromaffin granule membranes were treated with Lubrol and centrifuged as indicated in the legend to Table 1. Electrophoresis was performed as indicated in METHODS. B represents the electrophoretic pattern of the protein recovered in peak II after DEAE Sephadex A-25 chromatatography. The direction of migration was from top to bottom, which was the anode. The broken line indicates the interphase between the stacking and running gels.

granule membranes. These values indicate the degree of enzyme purification achieved and show that the ATPase represents no more than 7–12% of the membrane proteins. This value agrees with the 7–8% value calculated on the basis of the recovery of protein in peak II.

Finally, the fact that the chromaffin granule ATPase can be solubilized and can be chromatographed on columns without loss of its activity provides hope that a considerable degree of purification can eventually be achieved. Further work on the characterization and on the physicochemical properties of the enzyme is under way in our laboratory. Chromaffin granule membranes have other constituents than ATPase; they contain chromogranin A (38, 39), dopamine β-hydroxylase (40), and cytochrome b_{559} (41, 42). It is possible that the other elution peaks (I, III, IV, and V) obtained during ion-exchange chromatography contain these proteins.

The results reported in this paper are, to our knowledge, the first demonstration of the solubilization and partial characterization of the ATPase present in the membranes of granules that store or release hormones.

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REFERENCES

1. H. Blaschko and A. D. Welch, *Arch. Exp. Pathol. Pharmakol. (Naunyn-Schmiedebergs)* **219**, 17 (1953).
2. N. Å. Hillarp, S. Legerstedt and B. Nilson, *Acta Physiol. Scand.* **29**, 251 (1953).
3. J. M. Trifaró, A. M. Poisner and W. W. Douglas, *Biochem. Pharmacol.* **16**, 2095 (1967).
4. A. M. Poisner, J. M. Trifaró and W. W. Douglas, *Biochem. Pharmacol.* **16**, 2101 (1967).
5. O. Diner, *C. R. Hebd. Seances Acad. Sci. Paris* **265D**, 616 (1967).
6. S. Malamed, A. M. Poisner, J. M. Trifaró and W. W. Douglas, *Biochem. Pharmacol.* **17**, 241 (1968).
7. W. W. Douglas, *Brit. J. Pharmacol. Chemother.* **34**, 451 (1968).
8. W. W. Douglas and R. P. Rubin, *J. Physiol. (London)* **159**, 288 (1961).
9. N. Kirshner and W. J. Smith, *Science* **154**, 422 (1966).
10. R. P. Rubin, *J. Physiol. (London)* **202**, 197 (1969).
11. J. M. Trifaró, *Endocrinol. Exp.* **4**, 225 (1970).

12. J. M. Trifaró and J. Dworkind, *Mol. Pharmacol.* **7**, 52 (1971).
13. A. M. Poisner and J. M. Trifaró, *Mol. Pharmacol.* **3**, 561 (1967).
14. A. M. Poisner and J. M. Trifaró, *Mol. Pharmacol.* **5**, 294 (1969).
15. J. M. Trifaró and A. M. Poisner, *Mol. Pharmacol.* **3**, 572 (1967).
16. M. Oka, T. Ohuchi, H. Yoshida and R. Imaizumi, *Jap. J. Pharmacol.* **17**, 199 (1967).
17. A. M. Poisner and J. M. Trifaró, *Mol. Pharmacol.* **4**, 196 (1968).
18. J. Dworkind and J. M. Trifaró, *Experientia.* **27**, 1277 (1971).
19. J. M. Trifaró and M. Warner, *Pharmacologist* **13**, 228 (1971).
20. A. D. Smith and H. Winkler, *Biochem. J.* **103**, 480 (1967).
21. A. H. Anton and D. F. Sayre, *J. Pharmacol. Exp. Ther.* **138**, 360 (1962).
22. O. H. Lowry, N. J. Rosebrough, A. L. Farr and R. J. Randall, *J. Biol. Chem.* **193**, 265 (1951).
23. R. L. Post and A. K. Sen, *Methods Enzymol.* **10**, 762 (1967).
24. I. M. Glynn and J. B. Chappell, *Biochem. J.* **90**, 147 (1964).
25. J. M. Trifaró, *Mol. Pharmacol.* **5**, 382 (1969).
26. J. M. Trifaró *Mol. Pharmacol.* **5**, 424 (1969).
27. R. Gianetto and C. de Duve, *Biochem. J.* **59**, 433 (1955).
28. R. J. Pennington, *Biochem. J.* **80**, 649 (1961).
29. R. J. Wurtman and J. Axelrod, *Biochem. Pharmacol.* **12**, 1439 (1963).
30. J. M. Trifaró and J. Dworkind, *Anal. Biochem.* **34**, 403 (1970).
31. C. de Duve, B. C. Pressman, R. Gianetto, R. Wattiaux and F. Appelmans, *Biochem. J.* **60**, 604 (1955).
32. L. Fisher, *in* "An Introduction to Gel Chromatography" (T. S. Work and E. Work, eds.), p. 168. North Holland Publishing Co., Amsterdam, 1969.
33. B. J. Davis, *Ann. N. Y. Acad. Sci.* **121**, 404 (1964).
34. S. Uesugi, N. C. Dulak, J. F. Dixon, T. D. Hexum, J. L. Dahl, J. F. Perdue and L. E. Hokin, *J. Biol. Chem.* **246**, 531 (1971).
35. H. Winkler, H. Hörtnagl, H. Hörtnagl and A. D. Smith, *Biochem. J.* **118**, 303 (1970).
36. G. K. Ackers, *Biochemistry* **3**, 723 (1964).
37. J. A. Reynolds and C. Tanford, *Proc. Nat. Acad. Sci. U. S. A.* **66**, 1002 (1970).
38. K. B. Helle, *in* "New Aspects of Storage and Release Mechanisms of Catecholamines" (H. J. Schümann and G. Kroneberg, eds.), p. 45. Springer, Berlin, 1970.
39. G. Serck-Hanssen, *Biochem. Pharmacol.* **20**, 361 (1971).
40. N. Kirshner, *Pharmacol. Rev.* **11**, 350 (1959).
41. Y. Ichikawa and T. Yamano, *Biochem. Biophys. Res. Commun.* **20**, 263 (1965).
42. P. Banks, *Biochem. J.* **95**, 490 (1965).