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BIOCHEMICAL STUDIES OF THE CHROMAFFIN GRANULE

I. DISTRIBUTION OF CHROMOGRANIN A AND DOPAMINE-β-HYDROXYLASE ACTIVITY IN THE MEMBRANE AND WATER-SOLUBLE GRANULE FRACTIONS

KAREN B. HELLE

Institute of Physiology, University of Bergen, Bergen (Norway)

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SUMMARY

1. Chromogranin A was detected in a water-insoluble form by immunological assay of the washed granule membrane fraction after solubilization in Triton X-100. This fraction of bovine chromaffin granules accounted for 17 and 19% of the total granule pool of chromogranin A and protein, respectively. The water-insoluble chromogranin A had a lower electrophoretic mobility than that of the purified, water-soluble form.

2. Dopamine-β-hydroxylase in the membrane fraction represented 43% of the total granule activity of this enzyme and the specific activity of the water-insoluble form of the enzyme was 6-fold higher than that observed for the water-soluble activity. The low specific activity appeared to be associated with most of the water-soluble proteins, the chromogranins, suggesting that the latter contain subunits of the membrane complex of dopamine-β-hydroxylase.

3. Catecholamines and ATP were found to be bound to the proteins of the membrane fraction in small amounts with a molar ratio of 2:1. EDTA in the lysis medium was found to inhibit the granule ATPase leading to a ratio of ATP/extracted protein of 0.6 μmole/mg; a value similar to that obtained for intact granules. The addition of ATP was moreover found to have a significant solubilizing effect on the water-insoluble chromogranin A and dopamine-β-hydroxylase activity of the membrane fraction.

INTRODUCTION

The chromaffin granule of the bovine adrenal medulla contains in addition to catecholamines and adenosine triphosphate (ATP) several constituents of protein nature that are specific to the catecholamine-storing organelle, namely the chromogranins[1] and the enzyme dopamine-β-hydroxylase[2]. The former have been considered to be proteins specific to the water-soluble fraction while dopamine-β-hydroxylase at first was detected as an enzyme confined to the granule membrane.

More recently chromogranin A[3,4] and dopamine-β-hydroxylase[5,6] have been

reported to be present in both granule phases. However, the relative amounts of chromogranin A that can be recovered in the water-insoluble membrane fraction are a subject of controversy[7]. It appears to depend on the procedure employed in the solubilization of the granule proteins as well as on the immunological technique used for the assay of the solubilized chromogranin A.

The chromogranins evidently occur as lipoprotein complexes also in their water-soluble forms[8,9]. It may therefore be assumed that a dissociation between the lipids and the protein moieties of the chromogranins occurs when these lipoproteins are subjected to unfavourable ionic environments. Thus in distinguishing between the protein components of the inner matrix and the surrounding membrane phases it is essential to take into account the conditions employed in the solubilization of the granule proteins and enzyme activities.

The aim of the present study has been to compare the percentage distribution of chromogranin A concomitantly with that of dopamine-β-hydroxylase activity in the water-soluble and membrane fractions of highly purified chromaffin granules as a function of the ionic strength and composition. The amounts of chromogranin A and dopamine-β-hydroxylase activity have also been related to that of catecholamines, ATP and total protein. The results obtained reinforces those previously reported in that chromogranin A as well as the dopamine-β-hydroxylase activity are quantitatively important constituents of the membrane fraction. Furthermore the present results suggest that chromogranin A represents a subunit of the enzyme dopamine-β-hydroxylase, as studied more extensively in the accompanying paper[10].

The two proteins have previously been shown to be secreted from the stimulated gland by an exocytotic mechanism of release[1,11,12]. The physiological implication of the dual distribution of chromogranin A and dopamine-β-hydroxylase is, however, not clear but a possible explanation for this phenomenon may be sought in the hypothesis[13] that proteins to be secreted from a cell would also be expected to be a part of the membrane of their specific storage organelle.

MATERIALS AND METHODS

Bovine adrenal glands were obtained at Bergen Municipal Slaughterhouse with the kind assistance of Mr. Bjånesö. The glands were cut out 20–30 min after the death of the animal and kept on ice until dissection could be carried out in the laboratory, usually within 2 h.

Anti-chromogranin A sera were obtained from rabbits injected with bovine water-soluble chromogranin A[4,14].

Chromaffin granules were obtained as a pellet in 1.6 M sucrose as described elsewhere[15].

Lysis of granules was achieved by a reduction in osmolarity of the granule suspension. In order to obtain a high protein concentration of the first water-soluble extract (SN1) the granule pellet was diluted 2-fold with buffer and dialysed in the cold against 500 vol. of buffer with 2 changes for periods of 24 h each. The supernatant SN1 of the dialysed material was obtained after centrifugation of the dialysed granule protein at $55.2 \cdot 10^5$ $g_{av} \cdot$min in the MSE Superspeed 40 ultracentrifuge. The pellet was washed twice with 10 vol. of buffer and centrifuged as before. The composition of the buffers are given in the tables. The supernatants thus obtained (SN2 and SN3) were kept separate for analyses. The washed pellet of water-insoluble protein

was suspended in 1 vol. of solvent and aliquots solubilized in 1 % Triton X-100 in 170 mM NaCl for assays (S4).

Electron microscopic examination of the water-insoluble granule fraction: Fraction S4, obtained as a pellet after centrifugation at $55 \cdot 10^6$ $g_{av} \cdot$min, was fixed in 2.5 % glutaraldehyde in 0.03 M sodium phosphate (pH 6.3) with post-fixation in OsO_4 essentially as previously described[15].

Catecholamines were determined as the sum of noradrenaline *plus* adrenaline according to the method of BERTLER *et al.*[16] using a Farrand Spectrofluorimeter exiting at wavelength 390 nm and analyzing at wavelength 540 nm.

ATP was assayed by a modified firefly method as described by HOLMSEN *et al.*[17].

Protein was assayed in the dialysed protein fractions by the method of LOWRY *et al.*[18].

Chromogranin A was assayed immunologically using the double diffusion technique on agarose plates[4, 14].

Dopamine-β-hydroxylase was assayed according to the method of GIBB *et al.*[19] with tyramine as the substrate. The unit of enzyme activity is taken as $1 \cdot 10^{-5}$ moles of product formed after 10 min of incubation in the assay medium.

Cu, Fe, Ca and Mg were assayed in the dialysed water-soluble protein fractions and in detergent-treated suspensions of the membrane fraction by the standard procedures of Perkin–Elmer, using their Model 303 atomic absorption spectrophotometer for the determinations.

Disc electrophoresis was carried out as described by DAVIS[20] using a stacking gel of Sephadex G-200 and a running gel of 7.5 % polyacrylamide in Tris–HCl (pH 8.9) with Tris–glycine (pH 9.5) in the electrode compartments. A current of 3 mA was applied to each gel and the running time was 45–60 min. The gels were stained for at least 2 h in 10 % (v/v) acetic acid containing 45 % (v/v) methanol and 1 % (v/v) Amido black. Destaining was carried out electrophoretically in 10 % acetic acid.

For the immunological assay of chromogranin A and the detection of dopamine-β-hydroxylase activity associated with the electrophoretically separated protein components the polyacrylamide gels were cut into sections immediately after termination of the electrophoresis run.

For the detection of chromogranin A the gels were cut into 2-mm-thick discs, and each disc was transferred to a well in the agarose plate used for immunodiffusion experiments. The discs were moistened with saline and thus assayed for immunologically active chromogranin A by immunodiffusion as previously described[4, 14].

For the estimation of dopamine-β-hydroxylase activity electrophoresis conditions similar to that described by GIBB *et al.*[19] were also employed; electrophoresis was carried out with (a) 3 mA/gel for 1 h at room temperature and (b) 5 mA/gel for 3 h in the cold. The gels were cut into discs of 4-mm thickness and each disc was extracted with 0.5 ml 0.2 M potassium phosphate (pH 6.0) for 24 h. Dopamine-β-hydroxylase activity was assayed in the extracts as described above[19].

RESULTS

In vitro lysis and solubilization of granule constituents

Protein

Highly purified chromaffin granules were prepared as described[14] and a series of batches were compared for solubility of granule constituents. An average of 66 %

of the total protein could be rendered water-soluble at pH 6.0 (Table I). No clear effect of the chemical composition, ionic strength of pH was apparent with respect to efficiency of lysis since for parallel experiments the values were within the limits of the range of deviation obtained for the whole series.

At best the water-soluble protein could account for 85 % of the total granule

TABLE I

GRANULE CONSTITUENTS IN THE WATER-INSOLUBLE PROTEIN FRACTION S_4

Lysis of the highly purified chromaffin granules was carried out at pH 6.0 as described in MATERIALS AND METHODS. The recovery of total protein and chromogranin A has been expressed as the arithmetic mean of the values obtained for each batch of granules subjected to lysis in the given buffer. Each batch usually contained 100–150 mg total protein. A mean value of 1.48 ± 1.04 μmoles catecholamine/mg protein was obtained for these batches (n = 19) while the value for S_4 was 3.6 ± 1.6 nmoles catecholamine/mg protein (n = 19).

Expt. No.	*Buffer composition*	n	*Granule constituents in the water-insoluble protein fraction S4 (% of total granule content)*			
			Protein		*Chromogranin A*	
			Range	*Mean*	*Range*	*Mean*
I	Water	2	27.9–41.5	34.7	50.0–69.0	59.5
II	0.02 % EDTA in 1 mM sodium succinate	1		35.2		28.6
III	5 mM sodium succinate	3	30.0–39.0	35.0	32.5–52.0	45.5
IV	10 mM sodium succinate	1		43.3		20.5
V	25 mM sodium succinate	1		43.0		16.8
VI	50 mM sodium succinate	2	23.6–39.1	31.4	53.3–63.9	58.6
VII	5 mM sodium succinate + 40 mM $CaCl_2$	1		39.1		30.0
VIII	50 mM sodium succinate + 50 mM $CaCl_2$	2	24.0–30.0	27.0	18.4–37.0	27.7
IX	100 mM sodium phosphate	2	33.0–41.0	37.0	30.0–34.8	32.4
X	100 mM potassium phosphate	1		15.0		32.5
		16	15.0–43.3	34.1	16.8–69.0	35.2

TABLE II

GRANULE CONSTITUENTS IN THE WATER-INSOLUBLE PROTEIN FRACTION S_4

Lysis was carried out in 5 mM sodium succinate pH 6.0 and the experiments represent 6 aliquots of one batch of purified chromaffin granules (same as in Table III).

Expt. No.	*Granule constituents in the water-insoluble protein fraction S4 (% of total granule content)*		
	Protein	*Chromogranin A*	*Dopamine-β-hydroxylase*
I	15.4	16.3	35.8
II	19.7	34.0	41.5
III	22.1	12.1	33.2
IV	22.4	11.5	59.4
V	19.6	6.4	49.7
IV	14.8	20.0	40.2
Mean	19.0	16.7	43.3
± S.D.	3.3	9.4	9.2
(n)	(6)	(6)	(6)

protein while the lowest value detected was 57 %. With one particular batch of highly purified chromaffin granules the water-insoluble granule protein averaged 19 % and the standard deviation (Table II) indicates that the methodological variations cannot fully account for the large deviations observed when different batches were compared (Table I). These experiments thus suggest that there may be inherent differences in the solubility properties of the different batches of chromaffin granules that may be related rather to differences in the excited state of the animals prior to slaughter than to methodological variations in the *in vitro* lysis procedure.

The membrane fraction

The morphological apparence of the water-insoluble protein fraction S4 indicates, as shown in Fig. 1, that this fraction contains mainly membranous structures with some amorphous material inbetween. About 80 % of the bound phosphates and 90–95 % of the total content of Fe were confined to this fraction while Ca and Mg were solubilized in a manner parallel to that of the total granule protein.

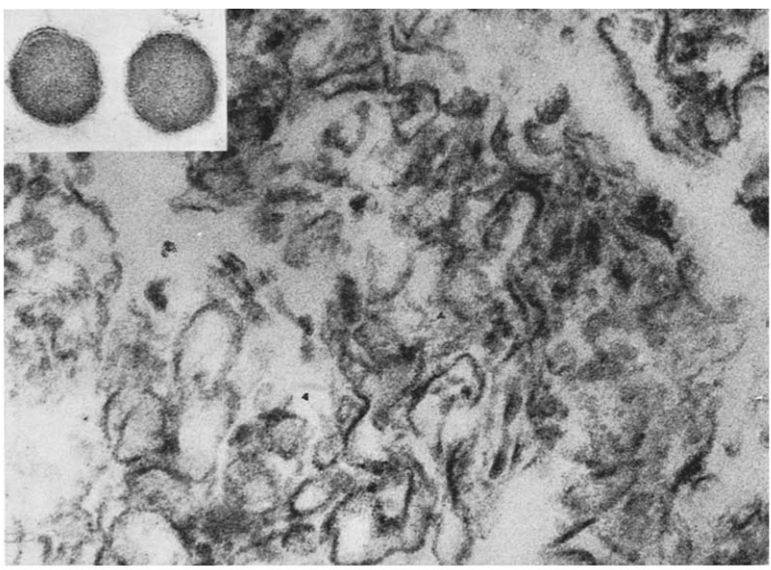

Fig. 1. Electron micrograph of the water-insoluble granule fraction S4. Lysis of the intact granules (insert) was carried out in 5 mM sodium succinate (pH 6.0) (Expt. I, Table II). Magnification: 60 000 ×.

Dopamine-β-hydroxylase

Enzyme activity was found to be present in both the water-soluble and insoluble granule fractions. The results presented in Table II show that in experiments in which the water-insoluble granule fraction accounted for 19 % of the total protein, the dopamine-β-hydroxylase activity in this fraction represented 43 % of the total granule activity. Thus, in these experiments slightly more dopamine-β-hydroxylase activity was water-soluble than insoluble. However, the specific activity of the enzyme in the former fraction (SN1) was much lower than that of the water-insoluble fraction (S4) in presence of Triton X-100, with average values of 0.3 and 2.1 units dopamine-β-hydroxylase/mg protein in SN1 and S4, respectively (Table III).

TABLE III

GRANULE CONSTITUENTS IN THE WATER-INSOLUBLE PROTEIN FRACTION S_4

The experiments were carried out as described in Table II. In the water-soluble protein fraction SN1 of these experiments average ratios of 0.27 ± 0.11 chromogranin A/mg protein and 0.31 ± 0.12 unit dopamine-β-hydroxylase/mg protein were obtained. The ratio of dopamine-β-hydroxylase/chromogranin A in SN1 averaged 1.34 ± 0.69 units/mg.

Expt. No.	*Granule constituents in the water-insoluble protein fraction S4*					
	nmoles catecholamine / mg protein	*nmoles ATP / mg protein*	*Catecholamine / ATP*	*mg chromogranin A / mg protein*	*Units dopamine-β-hydroxylase / mg protein*	*Units dopamine-β-hydroxylase / mg chromogranin A*
I	5.9	7.6	0.78	0.15	1.36	9.1
II	5.7	2.34	2.45	0.35	2.03	5.8
III	6.8	2.60	2.60	0.15	1.87	12.4
IV	4.0	2.00	2.00	0.20	2.49	12.4
V	4.7	1.55	3.00	0.23	2.68	11.7
VI	4.8	2.07	2.30	0.15	2.10	13.2
Mean	5.3	3.02	2.19	0.21	2.09	10.8
± S.D.	1.0	2.27	0.77	0.08	0.47	2.8
(*n*)	(6)	(6)	(6)	(6)	(6)	(6)

Copper has been shown to be a constituent of the purified dopamine-β-hydroxylase[21] when isolated from Triton extracts of whole granules, and in the present experiments granule-bound copper was solubilized in a manner parallel to the solubilization of dopamine-β-hydroxylase activity. SN1 contained about 2 nmoles Cu^{2+}/mg protein while a value of 1.6 nmoles Cu^{2+}/mg protein was obtained for S4. A further addition of Cu^{2+} to the assay medium did not increase the dopamine-β-hydroxylase activity of SN1.

The present findings thus indicate that the dopamine-β-hydroxylase activity of the water-soluble fractions may represent less active subunits of the highly active enzyme complex in the water-insoluble granule fraction.

Chromogranin A

Immunologically active chromogranin A detected in the water-insoluble protein accounted for an average of 35 % of the total granule pool of this protein in a series of granule batches having an average of 34 % water-insoluble protein (Table I). In other experiments (Table II) aliquots of a single batch of granules were used and again the distribution of chromogranin A paralleled that of the total protein in solubility properties, with average values of 16.7 and 19.0 % in the water-insoluble granule fraction for chromogranin A and total protein respectively.

Catecholamines and ATP

A certain core of catecholamines and ATP could always be detected in the water-insoluble granule fractions and the molar ratio of these constituents in fraction S4 was 2.2:1 for catecholamines:ATP (Table III).

When EDTA was present in the buffer used for lysis of the granules, more ATP than usual remained in all the granule protein fractions after the extensive dialysis. Table IV shows that the 2 washings, SN2 and SN3, contained more ATP/mg protein than did SN1 or S4. Moreover the ratios of ATP–protein in fractions SN2 and SN3 were high (0.6 μmole/mg protein) and of the same order as that found in the intact granule (0.4 μmole/mg protein). The presence of EDTA also appears to have increased the percentage of immunologically active chromogranin A in the solubilized protein (SN2) as well as the specific activity of dopamine-β-hydroxylase (SN2 and SN3) compared to that solubilized in absence of EDTA (Table IV).

Immunelectrophoresis of chromogranin A

Anti-chromogranin A sera were used for the experiments and in keeping with earlier observations[4,14] only one precipitin arc could be obtained with concentrated protein of the water-soluble and insoluble granule fractions. The latter fraction was solubilized in Triton X-100 and the immunoprecipitin arc obtained with the detergent-solubilized fraction S4 was identical to that obtained with the water-soluble fractions and with purified chromogranin A[14].

Protein constituents of fractions SN1 and S4 were separated by disc electrophoresis (see MATERIALS AND METHODS) and the individual bands so obtained were examined by immunodiffusion against an antiserum to chromogranin A (see MATERIALS AND METHODS). Immunologically active chromogranin A could be detected in the first and the second bands counting from the cathode side of the electropherogram of SN1 (Fig. 2a), indicating that both components contain the same antigen. The immunological activity of the detergent-solubilized protein of S4 was found to correspond to that of a conspicuously stained component in the electropherogram with

TABLE IV

GRANULE CONSTITUENTS IN THE WATER-INSOLUBLE PROTEIN FRACTION S_4

The batch of chromaffin granules had been dialysed against large volumes of 0.02% EDTA in 1 mM sodium succinate, pH 6.0 prior to the first centrifugation (see MATERIALS AND METHODS). By this procedure 99.8% of the total catecholamine of the preparation had been removed. ATP removed by dialysis was not determined.

Fraction	*Granule constituents (% of total)*				*Granule constituents/mg protein in fraction*			*Ratios of granule constituents*	
	ATP	*Protein*	*Chromogranin A*	*Dopamine-β-hydroxylase*	*ATP (nmoles)*	*Chromogranin A (mg)*	*Dopamine-β-hydroxylase (units)*	*ATP/chromogranin A (nmoles/mg)*	*Dopamine-β-hydroxylase/chromogranin A (units/mg)*
SN_1	32	58	50	30	66.6	0.36	4.9	184	12.5
SN_2	48	6.5	21.5	4.8	660	0.40	7.1	1650	17.8
SN_3	13	0.8	0	23.2	540	0	176.7	0	0
S_4	7.5	35.2	28.6	42.0	9.3	0.34	11.5	27.5	33.8

a mobility similar to that of the first, slow component of SN1 (A_I). By comparison of the immunodiffusion patterns a complete identity of the protein material in SN1 and S4 was obtained, indicating that these two fractions contain immunologically identical protein in keeping with earlier observations[3,4,14].

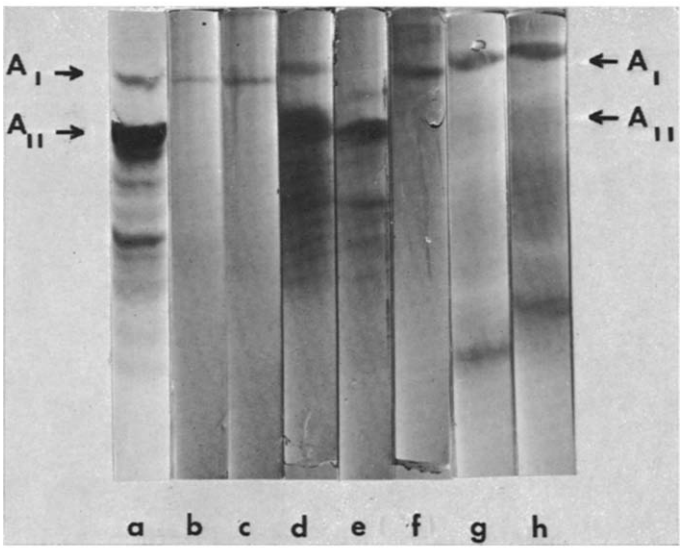

Fig. 2. Polyacrylamide disc electrophoresis of membrane and water-soluble protein of bovine chromaffin granules. Water-soluble granule fraction SN1 was obtained from batches of chromaffin granules lysed in sodium succinate (pH 6.0) at different ionic strengths. Electrophoresis was carried out with 3 mA/gel for 1 h on samples containing 0.06 mg protein. The proportion of water-soluble protein in each experiment is given in brackets together with the corresponding number referring to the experiment in the tables. The ionic strengths of the samples were: a, 0.012 (80 %; II, Table II); b, 0.012 (61 %; III, Table I); c, 0.024 (57 %; IV, Table I); d, 0.120 (61 %; VI, Table I); e, 0.220 (70 %, VIII, Table I). Membrane protein was solubilized in: f, 1 % Triton X-100 in 170 mM NaCl; g, 100 mM sodium phosphate (pH 6.0) and h, 100 mM ATP (pH 6.0). The latter experiments correspond to those presented in Table V.

These findings confirm and extend the previous results in that chromogranin A can be separated electrophoretically into two molecular forms that contain the same antigen. The two components carrying identical immunological properties have been numbered according to their position in the electropherograms and will be referred to as chromogranin A_I and chromogranin A_{II}.

A complex pattern of electrophoretically distinct components can be observed for fraction SN1 in experiments containing 70–80 % of the total granule protein in a water-soluble form. In Fig. 2a such a pattern is given and when compared with the patterns obtained for SN1 of experiments having about 60 % water-soluble protein, one notices a marked difference in the electropherograms (Figs. 2 (b–e)). In the latter case only one conspicuously stained component could be detected when lysis had been carried out at low ionic strength (Figs. 2b, 2c). The mobility of this component paralleled that of chromogranin A_I; not that of purified chromogranin A (A_{II}). At high ionic strength chromogranin A_I was present as a quantitatively small component on these electropherograms and chromogranin A_{II} appeared as the predominant species of the water-soluble proteins (Figs. 2d, 2e).

In the electropherograms obtained with detergent-solubilized protein of S4 (Fig. 2f) a stained component of mobility similar to that of chromogranin A_1 was the main component moving into the gel.

Electrophoretic mobility of dopamine-β-hydroxylase activity

The patterns of dopamine-β-hydroxylase activity in the electropherograms of water-soluble and insoluble protein fractions are given in Fig. 3. From this figure it may be seen that when electrophoresis was carried out under conditions identical to that described in Fig. 2, the enzyme activity was distributed throughout the whole length of the gel.

The main dopamine-β-hydroxylase activity of the water-insoluble fraction when suspended in the potassium phosphate buffer (Fig. 3a), moved with the buffer boundary and the mobility of this activity corresponded to that of the densely stained component of fast mobility seen in Fig. 2g.

When the water-insoluble protein was solubilized in Triton X-100 a different distribution pattern for the dopamine-β-hydroxylase activity was obtained (Fig. 3b). This figure should be compared with Fig. 2f and it may be seen that the conspicuously

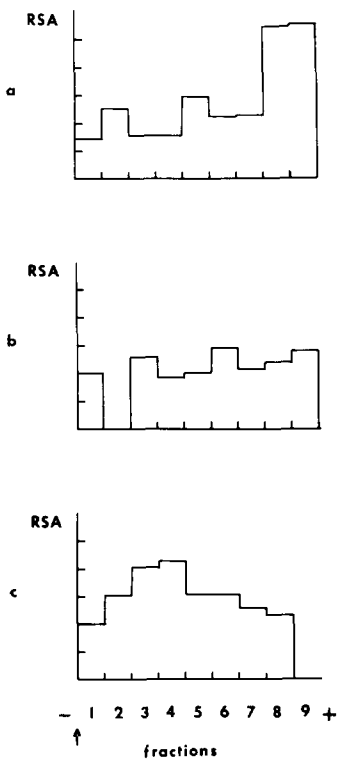

Fig. 3. Distribution of dopamine-β-hydroxylase in sections of polyacrylamide gels after disc electrophoresis of membrane and water-soluble protein. Electrophoresis was carried out at 3 mA/gel for 1 h and the gels were cut in sections of 0.4 cm thickness. Dopamine-β-hydroxylase was assayed in the extracted protein as described in MATERIALS AND METHODS and is given as relative specific activity (RSA). The samples were: a, membrane protein in 200 mM potassium phosphate (pH 6.0); and b, membrane protein in 1 % Triton X-100 in 170 mM NaCl; and c, water-soluble protein (SN1; 80 % water-soluble of total granule protein) in 5 mM sodium succinate (pH 6.0).

TABLE V

SOLUBILIZED GRANULE CONSTITUENTS

Aliquots of the pellet S_4, containing the water-insoluble protein of granules lysed in 0.02 % EDTA in 1 mM sodium succinate, pH 5.0 (24.5 % of the total protein), were suspended in the solvents given. The suspensions were centrifuged at $55 \cdot 10^5$ $g_{av} \cdot$ min and the supernatants SN_4 were removed. The pellets were resuspended in the respective solvents and centrifuged as before. The supernatants thus obtained are referred to as SN_5.

Solubilizing agent	*Solubilized granule constituents*								
	S4 protein (mg)	*Protein (mg)*		*mg chromogranin A / mg protein*		*Dopamine-β-hydroxylase units / mg protein*		*Dopamine-β-hydroxylase units / mg chromogranin A*	
		SN4	*SN5*	*SN4*	*SN5*	*SN4*	*SN5*	*SN4*	*SN5*
100 mM ATP (pH 6.0)	16.2	8.4	0.8	0.68	0.75	0.148	0.171	0.22	0.23
100 mM sodium phosphate (pH 6.0)	15.2	6.4	1.2	0.76	0.38	0.090	0.118	0.12	0.31
1 % Triton X-100 in 170 mM NaCl	23.5	11.6	0.94	0.31	0.14	0.048	0.140	0.15	1.00

stained component of mobility similar to chromogranin A_I carried relatively little of the totally detected enzyme activity. The main dopamine-β-hydroxylase-activity had a faster mobility than both chromogranin A_I and A_{II}.

The peak of the dopamine-β-hydroxylase activity detected in the water-soluble fraction SN1 (Fig. 3c) corresponded in mobility to that of the main water-soluble protein in the purified form, chromogranin A_{II}, although material with faster as well as slower electrophoretic mobility also carried enzyme activity.

When dopamine-β-hydroxylase activity was assayed in sections of gels subjected to conditions comparable to that used in the study of purified dopamine-β-hydroxylase[19], the main enzyme activity of the water-insoluble protein was detected near the negative pole when potassium phosphate was used as the suspending medium. With Triton X-100, the main activity had a faster mobility.

Effect of ATP and P_i on the solubility of granule protein

In a few experiments water-insoluble granule protein was suspended in 100 mM ATP or P_i at pH 6.0 and the effect of these substances on the solubility of membrane protein was compared with that of Triton X-100 in 170 mM NaCl. The results are presented in Table V where it may be seen that both chromogranin A and dopamine-β-hydroxylase activity were brought into solution by these agents. The amount of solubilized protein that could be accounted for as chromogranin A was much higher with ATP and P_i than with detergent and the specific activity of dopamine-β-hydroxylase had increased in the presence of ATP (see also Table IV) but the ratio of dopamine-β-hydroxylase/chromogranin A of the solubilized protein was 10-fold less than that found in the water-soluble granule fraction (Table III, SN1).

The electropherograms of the solubilized protein (Fig. 2) show that components similar to chromogranin A_I could be seen with ATP, P_i and Triton X-100 and in addition a conspicuous component close to the anode was present when ATP and P_i were the solubilizing agents.

As far as any conclusions can be drawn from these experiments they suggest that ATP and to a lesser degree P_i have a solubilizing effect on components in the water-insoluble granule fraction which differs from that of the detergent in that relatively more chromogranin A and dopamine-β-hydroxylase activity than total protein was brought into solutions by the former than by treatment with detergent.

DISCUSSION

The present results indicate that the water-solubility of highly purified chromaffin granules differs for a series of batches in a manner which points to inherent differences in the state of the source material rather than to large methodological variations in the *in vitro* lysis procedure. Thus the values obtained with a series of granule preparations range from 57–85 % with an average of 66 % water-soluble protein, in keeping with earlier observations by EADE[22] and others[9,23]. In a number of the granule preparations about 80 % of the total granule protein could be rendered water-soluble, thus in agreement with reports by HILLARP[24] and by WINKLER *et al.*[8]. The latter authors assume that with 22 % of the total protein remaining in the water-insoluble protein this fraction can be considered representative of the granule membrane protein, an assumption confirmed by the present electron microscopic study

of the water-insoluble protein fraction S4. For this reason observations on the distribution of chromogranin A and dopamine-β-hydroxylase activity in the membrane fraction which have been carried out on granule preparations containing about 80 % of their protein in a water-soluble form have been presented.

In agreement with observations by LADURON AND BELPAIRE[5] and since confirmed by DUCH *et al.*[6] dopamine-β-hydroxylase activity appears evenly distributed over the water-soluble and membrane fractions. However, the specific activity of this enzyme in the water-soluble granule fractions is 6-fold less than that found for the detergent-solubilized membrane protein, but added Cu^{2+} had no effect on the water-soluble enzyme activity. Thus the low enzyme activity in the water-soluble fractions may not be attributed to the presence of endogeneous inhibitors[6] in the extract but more likely to the presence of less active subunits of the membrane-bound complex of dopamine-β-hydroxylase.

Chromogranin A, first assumed to be exclusively water-soluble, has since by immunological methods been detected as a quantitatively important constituent of the granule membrane fraction[3,4]. The present findings support the earlier observations that chromogranin A parallels the total protein in solubility properties; *i.e.* chromogranin A accounts for as much of the water-soluble as of the granule membrane protein.

The detection of chromogranin A in the membrane fraction depends on the solubilization of the protein with detergents. Thus an apparent lack of chromogranin A in the granule membrane protein reported by others[7] may be understood when one takes into consideration that they employed another immunological assay in absence of detergents.

Two components carrying immunologically active chromogranin A have been separated by disc electrophoresis, chromogranin A_I predominant in the membrane fraction and chromogranin A_{II} mainly water-soluble. Chromogranin A_I could under certain experimental conditions also be detected in the water-soluble protein as the main component of SN1 in granule preparations with a relatively low percentage of water-soluble protein. Purified chromogranin A from these fractions[10] had, however, the electrophoretic mobility characteristic of chromogranin A purified by other workers[25] indicating that a dissociation of chromogranin A_I into chromogranin A_{II} may occur also as a result of protein dilution.

The detection of significant amounts of ATP associated with the granule protein solubilized in the presence of EDTA is of particular interest. The granule protein reportedly contains a (Ca^{2+}–Mg^{2+})activated ATPase[26] and the inhibitory effect of EDTA on this enzyme activity is therefore not unexpected. Yet it is surprising to find as much as 0.6 μmole ATP/mg protein in the washings of the granule membrane protein when the ratios of these parameters in SN1 and S4 of this experiment were 0.06 and 0.01 μmole/mg, respectively. The effect of ATP and P_i on the preferential solubilization of membrane-bound chromogranin A and dopamine-β-hydroxylase activity lends further support to the assumption that ATP in the intact granule is involved in keeping these macromolecular constituents in a water-soluble state.

The effects of Triton X-100 and of P_i on the electrophoretic mobility of the solubilized dopamine-β-hydroxylase activity moreover suggest that a dissociation of the membrane-bound enzyme complex has taken place in the presence of these agents, in keeping with observations by FLATMARK *et al.*[27]. One of the polypeptide

moities of the membrane-bound complex of dopamine-β-hydroxylase may be chromogranin A while the other, faster moving components of the membrane-bound enzyme apparently differ from chromogranin A in antigenicity as well as in specific enzyme activity.

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