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DISTRIBUTION OF CHROMOGRANIN IN THE CHROMAFFIN VESICLE OF THE BOVINE ADRENAL MEDULLA

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Abstract—The distribution of chromogranin has been studied in purified chromaffin granules and in particulate fractions of isolated bovine adrenals perfused *in vitro* with Krebs–Henseleit bicarbonate buffer. When purified chromaffin granules were lysed and recentrifuged on a sucrose density gradient ranging from 1·4 to 2·5 M, chromogranin was recovered in two fractions corresponding to emptied granule membranes at the top of the gradient and intact granules at the bottom of the gradient. When compared with control glands, the ratio of chromogranin to catecholamines in the large granule fraction and in the fraction containing free membranes obtained from stimulated glands was increased markedly. The amount of chromogranin in the large granule fraction was the same before and after stimulation of the perfused gland, supporting earlier findings of chromogranin as a major constituent of the granule membrane.

The present findings are in agreement with the idea that the emptied granule vesicle is retained within the cell after secretion of the soluble constituents by means of an exocytotic mechanism.

THE CATECHOLAMINES, synthesized and stored in the chromaffin cells of the adrenal medulla, are secreted upon stimulation. Based on studies of the chromaffin cell in the electronmicroscope, release by exocytosis was first suggested by De Robertis and Vaz Ferreira[1] and biochemical support for this suggestion has been obtained in recent years. As no increase in efflux of phospholipids and cholesterol can be observed during secretion of catecholamines the discharge of the intact chromaffin granule across the cell membrane seems unlikely.[2] In isolated perfused bovine adrenals stimulated *in vitro* ATP and protein are released concomitantly with the catecholamines.[3,4] The ratio of catecholamines to protein in the effluent is of the same order of magnitude as found in the soluble phase of the chromaffin granule.[5] This indicates that the granule content is released directly to the exterior of the cell. Chemical and electronmicroscopical evidence obtained indicates that the empty granule membranes are not incorporated in the cell membrane but are retained within the cell after secretion.[6,7]

Chromogranin, the major water-soluble protein of the chromaffin granule, has recently been found to be an important constituent of the granule membrane. In membranes obtained from isolated chromaffin granules lysed by osmotic shock or sonication, chromogranin accounted for more than 50 per cent of total protein.[8] The present work was undertaken in order to study the distribution of chromagranin in subfractions of granules which have released their content of catecholamines under more physiological conditions.

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Isolated bovine adrenals, perfused *in vitro*, were stimulated with acetylcholine. Cell fractions were isolated and analysed for their content of chromogranin. The greater part of chromogranin was shown to be retained within the cell after catecholamine secretion in accordance with earlier findings indicating that the major part of chromogranin is bound to the granule membrane.

A preliminary report of these results has been presented elsewhere.[9]

METHODS

Bovine adrenals were obtained at the Bergen Municipal Slaughterhouse. The glands were removed from the animals about 30 min after death and carried to the laboratory on ice.

Perfusion of adrenals, starting within 1–2 hr after death, was carried out in the retrograd fashion as described by Banks.[10] The perfusion medium, Krebs–Henseleit original Ringer bicarbonate buffer (118 mM NaCl, 4·73 mM KCl, 2·54 mM $CaCl_2$, 1·18 mM KH_2PO_4, 1·18 mM $MgSO_4$. $7H_2O$, 25 mM $NaHCO_3$ + 11·5 mM glucose) was gassed with 95% O_2 + 5% CO_2 and maintained at 37°. The glands were suspended in air at 20°. The perfusion rate was adjusted to about 18 ml/min and the total perfusion period was 3 hr and 15 min. The glands were stimulated by injecting acetylcholine chloride (Sigma) in the tube inserted in the vein just prior to entering the gland. Four ml of a solution of acetylcholine chloride in the perfusion buffer, final concentration of $3{\cdot}8 \times 10^{-6}$ M, was injected after 15 min of perfusion over a period of 4 min. Equivalent amount of acetylcholine chloride was injected every 15 min 12 times to give a total dose of 1 m-mole acetylcholine chloride. Both glands of the same animal were perfused in all experiments, one serving as control. After perfusion the glands were cooled in ice and the medulla was dissected free of cortex.

Cell fractionation. The weighed medulla was homogenized in 0·3 M sucrose of 0° in a volume five times the weight of tissue, and all centrifugations were performed at 4°. Nuclei and cell debris were removed by centrifugation for 15 min at 800 *g* in a Sorvall centrifuge, model RC2. The 800 *g* supernatant was centrifuged at 20,000 *g* for 20 min in the Spinco centrifuge, model L. The sediment obtained will be referred to as the large granule fraction and contained mitochondria and lysosomes in addition to chromaffin granules.[11] The large granule fraction was suspended in 0·3 M sucrose and the suspension was further fractionated on a sucrose density gradient, ranging from 1·4 to 2·5 M sucrose, as described by Banks[11] and used for the resolution of subcellular particles in the 20,000 *g* sediment. After centrifugation for 1 hr at 36,000 rev/min in the Spinco centrifuge, rotor SW 39, fractions as indicated in the figures were aspirated and analysed for the content of protein, chromogranin, and catecholamines. Fractions were washed once by diluting with 10 ml of 170 mM NaCl of 0°, and recentrifuged for 1 hr at 105,000 *g*. The pellets obtained were suspended in 1 per cent sodium deoxycholate in 170 mM NaCl.

Chromaffin granules, used for lysis experiments, were obtained from a suspension of the large granule fraction in 0·3 M sucrose by centrifugation through 1·6 M sucrose as described by Smith and Winkler.[12]

The fractionation procedure is summarized in Fig. 1.

Fractionation of chromaffin granules. Chromaffin granules were suspended in 170 mM NaCl and sonified for 2 min at 0°, using a Branson Sonic Power Sonifier. The suspension was adjusted to a sucrose concentration of 0·3 M. This method was adopted

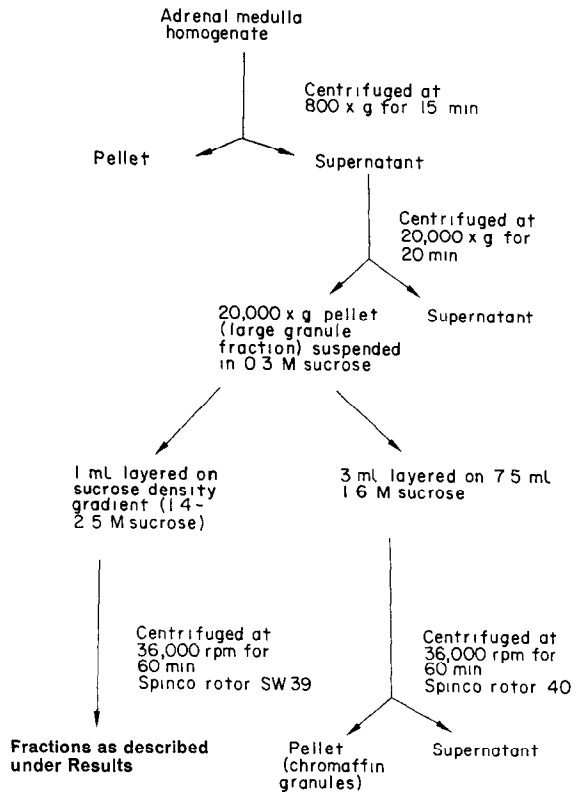

FIG. 1. Scheme of fractionation procedure. For details see Methods.

as the presence of sucrose during sonication in some cases led to the loss of immunologically active chromogranin. The suspension of lysed granules was fractionated on a sucrose density gradient as described above.

Chemical analysis. Protein was estimated by the method of Lowry *et al.*[13] using bovine serum albumin as standard. As the catecholamines interfere with the method, the amines have to be removed from all samples. Therefore protein in the perfusates was determined in samples concentrated by ultrafiltration or in samples dialysed against the perfusion medium. The protein in cell fractions was precipitated by 5% w/v trichloroacetic acid (TCA), washed in 5% TCA and fat extracting solvents and dissolved in 0·1 N NaOH. Chromogranin was quantitatively measured by immunological titration on agarose plates using a rabbit antiserum prepared against purified bovine chromogranin.[14] Catecholamines were determined spectrofluorometrically by the method of Bertler *et al.*,[15] using an Eppendorf spectrophotometer with a Fluorometer attachment. L-adrenaline was used as standard. The catecholamines in cell fractions were extracted with 5% TCA. Pooled perfusates were used for catecholamine determinations without any further purification.

Expression of results. The amount of catecholamines in the large granule fraction is expressed as micromoles per millilitre of original homogenate and the amount of protein and chromogranin in the large granule fraction is expressed as milligrams per millilitre of original homogenate. The results are corrected for lysis and efficiency of

homogenization, based on the assumption that the catecholamines are mainly located in the chromaffin granules. The degree of lysis was calculated from the amount of catecholamines found in the 20,000 *g* supernatant. About 30 per cent of total catecholamines were recovered in this supernatant from both stimulated and control glands. The efficiency of homogenization, varying between 85–100 per cent for both types of glands, was calculated from the difference in content of catecholamines in total homogenate and the 800 *g* supernatant. The corrected values for the protein content in the large granule fraction are based on a ratio of catecholamines to protein in chromaffin granules equal to 4 μmoles/mg, the mean value of ratios obtained for eight different preparations of chromaffin granules.

As the amount of tissue per ml homogenization buffer was the same in all experiments the results can be compared directly without referring to the weight of tissue used in each experiment.

RESULTS

Fractionation of lysed granule suspension on sucrose density gradient

In a previous report we described experiments in which the membranes of lysed granules were separated from the soluble phase by centrifugation at high speed.[8] Chromaffin granules isolated by centrifugation through 1·6 M sucrose underwent some lysis when suspended in 170 mM NaCl and nearly complete lysis was obtained by sonication.

In the present experiments suspensions of granules lysed by these two methods were fractioned on a sucrose density gradient as described under Methods. Lysis of granules by osmotic shock gave rise to particular material of lighter density than that of intact granules (Fig. 2). Sonication displaced almost completely particulate material from denser to lighter layers on the gradient. Figure 3 shows the distribution of protein and catecholamines on the gradient as obtained in one experiment and the histograms clearly demonstrate that after lysis by sonication most of the material was recovered in the lighter layers of the gradient.

For granules lysed by sonication fraction 1 contained solubilized protein and catecholamines in a ratio of 6·7 μmoles catecholamines per mg protein (Table 1). The amount of chromogranin in this fraction accounted for 19 per cent of the protein. When granules were lysed by osmotic shock the amount of chromogranin represented 16 per cent of the solubilized protein in fraction 1.

Fraction 2, overlapping the volume layered on the gradient, contained some solubilized protein and catecholamines in addition to particulate material of fluffy appearance and reddish in colour. After washing in 170 mM NaCl about 50 per cent of the protein and 97 per cent of the catecholamines in this fraction from both gradients remained in the supernatant after centrifugation. Chromogranin accounted for approximately 20 per cent of the sedimented protein obtained from granules lysed by either sonication or osmotic shock. These results suggest that the particulate material in fraction 2 of the gradients contained protein originating from the granules and insoluble in salt solution. This material, insoluble in 170 mM NaCl and sedimented by centrifugation for 60 min at 105,000 *g*, will be referred to as granule membranes.

Furthermore these results show that the insoluble protein obtained from granules lysed by the two different methods of lysis move to the same level of the gradient, in

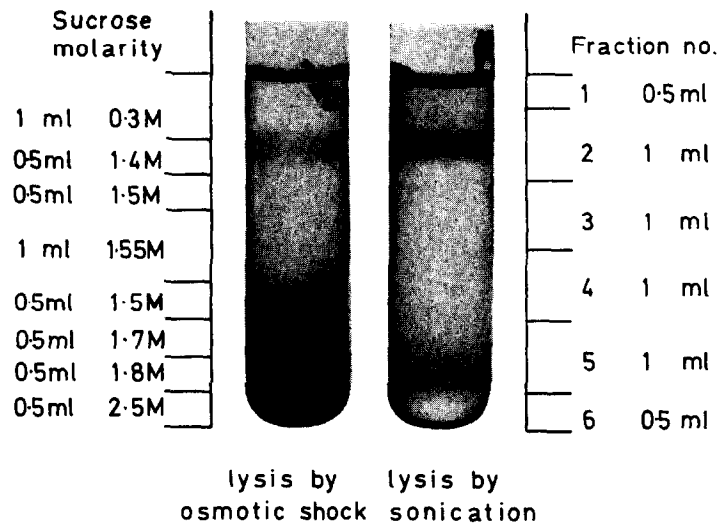

FIG. 2. Distribution of particulate material from lysed chromaffin granules on a sucrose density gradient. One ml of a suspension of lysed chromaffin granules in 0·3 M sucrose was layered on a sucrose density gradient made up as shown on the left scale. After centrifugation for 60 min in the SW 39 of the Spinco model L the gradient was divided in six fractions as indicated on the right scale.

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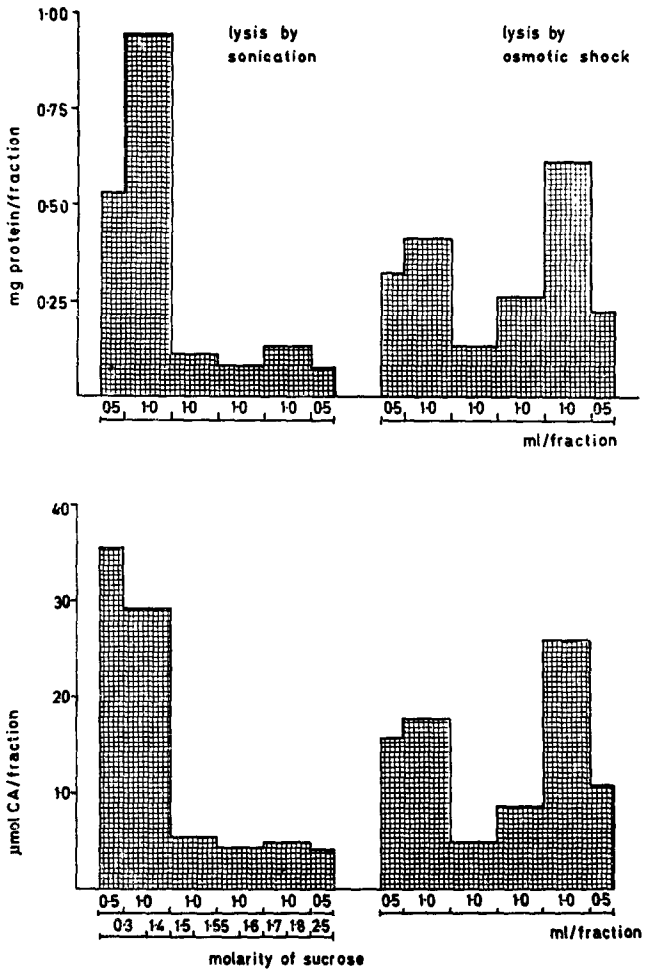

FIG. 3. Distribution of protein and catecholamines of lysed chromaffin granules on a sucrose density gradient. The gradient was made up and centrifugation performed as described in Fig. 2. Catecholamines (CA) and protein were analyzed as mentioned under Methods. Recovery of material layered on the gradients:

Lysis by sonication:	protein	93 per cent
	CA	101 per cent
Lysis by osmotic shock:	protein	94 per cent
	CA	91 per cent.

which also phospholipids and cholesterol originating from granules lysed by osmotic shock are found.[6]

Fraction 5 of both gradients contained intact granules as indicated by the catecholamine–protein ratio of approximately 4 μmoles/mg protein (Table 1).

Chemical composition of fractions obtained from isolated bovine adrenals perfused in vitro.

(a) *The perfusate.* Protein and catecholamines were released from isolated bovine adrenals perfused *in vitro* with Krebs–Henseleit bicarbonate buffer, indicating a

TABLE 1. CATECHOLAMINE–PROTEIN RATIOS OF SUBFRACTIONS OF LYSED CHROMAFFIN GRANULES

Fraction no.	μmoles CA*/mg protein	
	Lysis by sonication	Lysis by osmotic shock
1	6·71	4·90
2	3·11	4·37
— after washing	0·09	0·34
5	3·72	4·20
suspension of lysed granules	4·04	4·39

One ml of a suspension of chromaffin granules lysed by sonication or osmotic shock was layered on a sucrose density gradient made up as indicated in Fig. 2. After centrifugation for 60 min at 36,000 rev/min in the Spinco, rotor SW 39, the gradients were divided in six fractions as shown in Fig. 2. Fraction 2 was washed as described under Methods.

* Catecholamines.

spontaneous secretion. However, the amount of total protein, chromogranin, and catecholamines in the perfusate during periods of stimulation and rest showed that an increased output of the components took place in response to acetylcholine chloride. The amount of catecholamines secreted by stimulated glands was about two to four times the amount released from control glands under the same experimental conditions. Calculated as mg adrenaline per gland the total amount of catecholamines secreted from stimulated glands varied between 3·5 and 8·3 mg per gland (mean value 6·3 mg) in five experiments. Corrected for catecholamines released from the control glands the amount of catecholamines secreted in response to acetylcholine chloride varied between 2·05 and 6·45 mg per gland.

(b) *The large granule fraction.* According to Poisner *et al.*[6] the empty granule vesicles sediment with the large granule fraction. The large granule fraction obtained from the 800 *g* supernatant by centrifugation at 20,000 *g* for 20 min was analysed for the content of protein, catecholamines, and chromogranin. The results of three experiments are given in Table 2. A small or no difference in protein content in the large granule fraction from stimulated and control glands was observed. Poisner *et al.* have reported a loss of about 30 per cent of total protein in the large granule fraction from glands that had secreted about the same amount of catecholamines as measured in the present experiments. According to our calculations (to be published) the loss of protein due to acetylcholine-induced secretion would at most be about 10 per cent of total protein in the large granule fraction. This amount of protein is in the order of 0·1–0·2 mg protein per ml (Table 2) in our experiments, and may be lost during the isolation of the protein.

As indicated by the catecholamine content, the large granule fraction from control glands contained twice the amount of chromaffin granules found in the large granule fraction from stimulated glands. The amount of chromogranin, however, was shown to be about the same for stimulated and control glands in each of the experiments. The ratios of catecholamines to chromogranin thus indicate that on stimulation a higher loss of catecholamines than that of chromogranin took place.

(c) *Sucrose gradient subfractions of the large granule fraction.* Membranes from isolated, lysed granules can, as shown, be separated from intact granules on a sucrose

TABLE 2. THE CONTENT OF PROTEIN, CATECHOLAMINES AND CHROMOGRANIN IN THE LARGE GRANULE FRACTION FROM ISOLATED BOVINE ADRENALS PERFUSED *in vitro* WITH KREBS–HENSELEIT ORIGINAL RINGER BICARBONATE BUFFER

	Expt. No.	protein (mg/ml)	CA* (μmoles/ml)	μmoles CA/ mg protein	chrom.† (mg/ml)	chrom. calcul.‡ (mg/ml)	μmoles CA/ mg chrom.
I	Stim.§	1·77	0·76	0·43	0·28	0·13	2·72
	Contr.	1·72	1·84	1·07	0·28	0·32	6·56
II	Stim.	1·91	0·70	0·37	0·47	0·12	1·49
	Contr.	1·92	1·56	0·81	0·49	0·27	3·18
III	Stim.	1·28‖	0·19	0·15	0·29	0·04	0·66
	Contr.	1·83	1·09	0·60	0·49	0·19	2·01

The isolated bovine adrenals were perfused in a retrograd fashion over a period of total 195 min. The stimulated glands received acetylcholine chloride every 15 min, in total 1 m-mole acetylcholine chloride dissolved in the perfusion buffer. The large granule fractions were obtained from the 800 *g* supernatant by centrifugation at 20,000 *g* for 20 min in the Spinco L. All values, corrected for lysis and efficiency of homogenization (see Methods), are expressed as the amount present per ml homogenate.

* CA = catecholamines,

† chrom. = chromogranin.

‡ mg chromogranin expected to be found, assuming it being present only in intact granules and accounting for 70 per cent of total granule protein.[8] Calculated on basis of CA found in fraction and a CA–protein ratio for intact granules = 4 μmoles CA/mg protein.

§ Stim. = stimulated gland, Contr. = control gland.

‖ Some material lost during isolation of the large granule fraction.

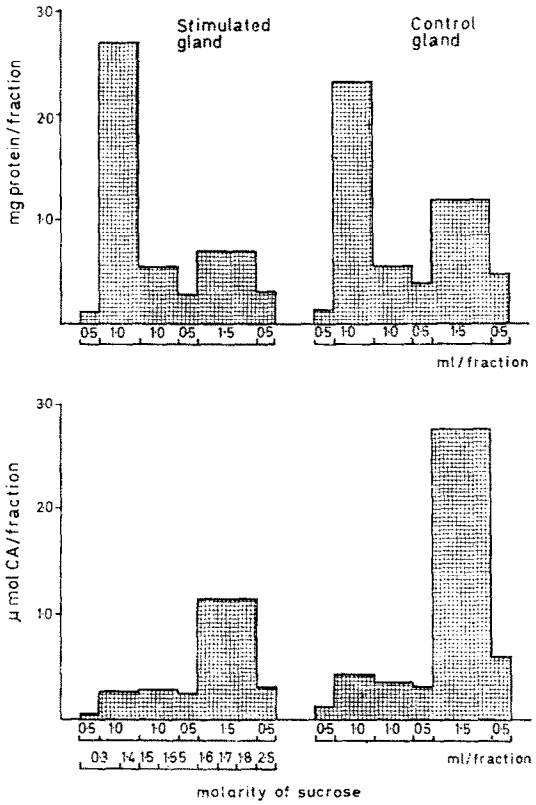

FIG. 4. Distribution of protein and catecholamines of the large granule fraction of stimulated and control glands on a sucrose density gradient. The density gradient was made up as indicated in Fig. 2. One ml of large granule suspensions, containing 4·73 mg of protein, was layered on the gradients. After centrifugation for 60 min in the SW 39 rotor of the Spinco model L, six fractions were obtained by aspiration. The fractions, from top to bottom, were numbered 1–6 and the fraction volumes are indicated below the histograms. Recovery of material layered on the gradients:

Stimulated gland:	protein	97 per cent
	CA	97 per cent
Control gland:	protein	105 per cent
	CA	110 per cent.

density gradient. The same density gradient was used for further resolution of the large granule fractions. Figure 4 gives the result obtained in one experiment and shows the distribution of protein and catecholamines on the gradient. In this experiment the large granule fraction from stimulated gland contained 6 per cent less protein than the large granule fraction from the control gland. The large granule fractions suspended in 0·3 M sucrose were adjusted to the same concentration based on protein content and 1·0 ml of the suspensions were layered on the gradient. This adjustment of concentrations was done in order to observe the effect of stimulation on the redistribution of protein and catecholamines on the gradient.

Reduction of chromaffin granules from the large granule fraction of stimulated gland was shown by the decrease in amount of protein and amines from the layers of 1·6–1·8 M sucrose. The decrease in protein and amines was 40 and 60 per cent respectively of that found for the control gland giving an approximate 50 per cent reduction

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TABLE 3. THE CONTENT OF PROTEIN, CATECHOLAMINES AND CHROMOGRANIN IN FRACTION 2 FROM SUCROSE DENSITY GRADIENTS BEFORE AND AFTER WASHING IN 170 mM NaCl

	Unwashed fractions				Washed fractions			
Expt. No.	Protein (mg)	CA* (μmoles)	chrom.† (mg)	μmoles CA/ mg chrom.	protein (mg)	CA (μmoles)	chrom. (mg)	μmoles CA/ mg chrom.
I Stim.	2·69	0·25	0·79	0·32	2·36	0·02	0·41	0·05
Contr.	2·31	0·42	0·60	0·70	1·92	0·02	0·39	0·05
II Stim.	2·77	0·32	0·67	0·47	1·91	—	0·31	—
Contr.	2·90	0·58	0·64	0·91	2·03	—	0·30	—

The fractions were obtained from the large granule fraction as described under Methods. The fractions in experiment I were taken from the gradient shown in Fig. 4.

* Catecholamines.

† Chromogranin.

in granule content. A ratio of catecholamines to protein lower than the usual ratio for chromaffin granules was found for the granules recovered in the lower layers of the gradient. The ratios for the chromaffin granules in the two experiments referred to in Table 3 were for stimulated glands 1·49 and 1·64 μmoles catecholamines per mg protein and for control glands 2·65 and 2·33 μmoles catecholamines per mg protein. As can be seen the low ratio was most pronounced for the granules from stimulated glands.

Mitochondria at the interphase between 0·3 M and 1·4 M sucrose were slightly contaminated by intact granules as indicated by the catecholamine profile. Compared with the control gland an increase of 16 per cent in protein in this layer, fraction 2, was observed on the gradient of stimulated gland.

Chromogranin was shown to be present in fraction 2 as well as in the chromaffin granules in the lower layers of the two gradients. No chromogranin was detected in fraction 1.

The amount of protein, chromogranin, and catecholamines in fraction 2 from the gradients of stimulated and control glands were determined before and after washing in 170 mM NaCl. The results obtained in two experiments are given in Table 3. Experiment I is the same as shown in Fig. 4. Chromogranin was found to represent between 20 and 30 per cent of total protein in all fractions. The ratios of catecholamines to chromogranin were lower than the ratios found for the total large granule fractions. This is to be expected for suspensions containing more empty granule vesicles relatively to intact granules.

After washing of insoluble material in fraction 2 in 170 mM NaCl 5–8 per cent of catecholamines and 70–88 per cent of the protein were recovered in the pellets sedimented by high speed centrifugation. The residual amines may be due to traces of intact granules as well as to free amines present in the saline trapped within the pellets. Thus complete or nearly complete lysis of the granules was obtained by the washing procedure. However, 50–60 per cent of the chromogranin was recovered in the pellets.

The ratios of catecholamines to chromogranin in the washed pellets were less than $\frac{1}{100}$ of that found for intact granules (approximately 5 μmoles catecholamine per mg chromogranin) and no difference in the ratios was found for stimulated and control glands. These results indicate that the washed pellets of fraction 2 contained the vesicles of emptied granules, devoid of intact granules.

DISCUSSION

Isolated bovine adrenals stimulated *in vitro* with acetylcholine secrete catecholamines by an exocytotic mechanism. Evidence has been presented by Poisner *et al.*[6] and Malamed *et al.*[7] that the empty granule vesicles remain within the cell after secretion of the soluble constituents. In the present work chromogranin, a constituent of the granule soluble fraction as well as the insoluble fraction,[8] has been shown to be of use as a marker of the empty granule vesicle after secretion.

The presence of chromogranin in the granule insoluble fraction was used to determine the density of the granule membranes on the sucrose density gradient applied for fractionation of preparations of large granule fractions. The presence of chromogranin and the absence of catecholamines in the particulate material in fraction 2 (Figs. 2 and 3) indicated that this material represented the granule membranes of the lysed chromaffin granules.

In cell fractions obtained from isolated perfused adrenals the amount of catecholamines and chromogranin was used as a measure for the content of chromaffin granules and granule membranes. The crude fractions of large granules were shown to contain chromogranin, see Table 2, in accordance with the presence of chromaffin granules. After stimulation the large granule fraction contained catecholamines and chromogranin in a ratio of $\frac{1}{2}$–$\frac{1}{3}$ of that found for the control experiments, suggesting that as a result of stimulation catecholamines left the gland in a higher proportion than chromogranin. Since no chromogranin was detected in fraction 1 (Fig. 4), containing solubilized proteins, the retained chromogranin must remain within the cell in an insoluble form, i.e. as membrane-bound protein. No reduction in chromogranin was observed in the large granule fraction from stimulated glands. According to Table 2, the acetylcholine-induced secretion of amines amounts to about 1 μmole catecholamines per ml of homogenate. This amount of catecholamines represents about 0·25 mg chromaffin granules expressed as protein. Assuming that chromogranin accounts for 40 per cent of soluble granular protein,[8] the amount of chromogranin discharged from 0·25 mg of granules upon stimulation would be in the order of 0·05 mg. This amount of chromogranin may not be detected by the immunological method applied.

On further resolution of the large granule fractions from stimulated and control glands, chromogranin was recovered in two fractions, corresponding to that of intact granules and granule membranes. The catecholamine–chromogranin ratios of the fractions containing the granule membranes showed that the presence of chromogranin in the fractions was not caused by contamination of chromaffin granules.

It can be argued, however, that the chromogranin found in the membrane fraction originates from the soluble phase of granules lysed during homogenization and that it has adsorbed to the granule membranes. The following evidence is against this view:

(1) As shown in Table 3, about the same amount of chromogranin was found in the fractions containing the granule vesicles from the two types of glands. If the presence of chromogranin was due to unspecific adsorption of the protein, one would expect, however, that the more chromogranin solubilized by homogenization the more would adsorb to the granule membranes. In accordance with the relatively lower proportion of granules in the stimulated glands (see above), the number of granules lysed during homogenization of stimulated glands was about half of that found for the controls. Thus the amount of chromogranin solubilized by lysis was less for the stimulated glands than the controls.

(2) More chromogranin was found in the fractions containing the granule vesicles than can be accounted for by chromogranin solubilized by lysis. Based on the content of protein, the chromaffin granules represented about 30 per cent of the large granule fraction of control gland layered on the sucrose density gradient, see Fig. 4. This equals about 1·2 mg protein. Corrected for lysis due to homogenization the amount of granule protein originally present in the large granule fraction is calculated to 1·45 mg protein. 30 per cent lysed granules expressed as protein thus equals 0·44 mg protein. About 70 per cent of total granule protein is solubilized on lysis and chromogranin accounts for 40 per cent of solubilized protein. From this follows that chromogranin set free due to lysis amounts to about 0·12 mg. This is far less than the amount of chromogranin found in fraction 2, containing the free membranes, of both stimulated and control gland, see Table 3.

It therefore seems justified to assume that the chromogranin associated with the membranes of light density is actually part of the membrane and not only attached to it in some artificial way. The present findings thus confirm and extend our previous results showing that chromogranin is an important constituent of the membrane fraction of granules lysed by osmotic shock or sonication.[8]

The present experiments were conducted under conditions similar to those described by Poisner *et al.*,[6] and the amount of catecholamines secreted was of the same order of magnitude as in their experiments. The membrane-bound chromogranin shows the same pattern of distribution in cell fractions as the phospholipids assumed by these authors to represent the empty granule vesicles. Our results thus provide additional evidence for the granule vesicles being retained within the cell after secretion. A recent report by Viveros *et al.*[16] shows that on insulin-induced secretion from rabbit adrenals the content of dopamine-β-hydroxylase, known to exist in both a soluble and a membrane-bound form,[17,18] is reduced. The reduction is due to loss of the soluble form of the enzyme. Chromogranin and dopamine-β-hydroxylase therefore seem to have similar properties in regard to their distribution in the soluble and membraneous phase of the chromaffin granule as well as in behaviour during secretion.

The fate of the empty granule vesicles within the cell after secretion is not known. If they are emptied completely, reutilization seems unlikely as they in addition to catecholamines have to be refilled with protein. The present findings as well as those of Poisner *et al.*[6] and Viveros *et al.*[19] indicate that secretion leads to accumulation of granule vesicles of the same density as granule vesicles obtained from isolated granules by lysis. This supports the view that the granules release their total content of soluble material. This would require a complete resynthesis of granules after secretion, analogous to synthesis of other secretory granules as the zymogen particles of the pancreas.[20]

As mentioned above the chromaffin granules of perfused glands recovered from the sucrose density gradient had an unusual low ratio of catecholamines to protein. This indicates that these granules or some of these granules contained less catecholamines per milligram protein than usually found for chromaffin granules. A similar reduction in catecholamine–protein ratio for granules from stimulated glands can be calculated from the results obtained by Poisner *et al.*[6] In the present experiment both stimulated and control glands released catecholamines during perfusion. As the ratio of catecholamines to protein for granules obtained from the stimulated glands was even lower than the ratio for control glands, there seems to be some connection between secretion and the low ratios observed. The low catecholamine–protein ratios can be explained in two ways.

The granules or part of the granules consist of:

(1) Premature granules not being fully filled with catecholamines. Evidence for the existence of such granules has been obtained by Hillarp.[21] The accumulation of premature granules is consistent with mature granules being preferentially emptied during secretion.

(2) Granules which have released only part of their soluble content. This is consistent with a gradual release of the protein component of the soluble phase of the granules on repeated stimulation as proposed by Stjärne for the granules of adrenergic nerve-endings.[22]

Further investigation is therefore needed to settle the question whether the chro-

maffin granules of the adrenal medulla release their total soluble content or only part of it during secretion.

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