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ISOLATION OF AND PRODUCTION OF ANTIBODY TO COW CHROMAFFIN GRANULE MEMBRANE*

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Abstract—Cow adrenal medulla chromaffin granules were obtained relatively free of mitochondrial, lysosomal or mircosomal contamination by differential and sucrose density gradient centrifugation. The granule membrane was prepared by lysis and subsequent centrifugation of the granules and was purified further by sucrose density gradient centrifugation. The membrane fraction contained cytochrome b-559, adenosine triphosphatase and 50 per cent of the chromaffin granule dopamine-β-hydroxylase activity. Injection of the membrane preparation into rabbits resulted in the production of an antibody which was used for micro-immunodiffusion and for complement fixation. Membrane antigen was detected in cow adrenal medulla microsomes and in homogenates of sheep, pig, dog and rabbit adrenal medullae.

BIOCHEMICAL and morphological evidence suggests that catecholamines are released from chromaffin tissue by the process of exocytosis.[1] The cellular events associated with exocytosis include: (a) stimulation of the cell by an appropriate agonist, such as acetylcholine, which leads to, (b) an increase in intracellular free calcium; (c) association of the catecholamine-containing chromaffin granule with the cell membrane, followed by, (d) efflux of the soluble constituents of the chromaffin granule into the extracellular space. The fate of the empty chromaffin granule remaining in the cell is not yet known. However, it has been shown that after secretion of catecholamines, chromaffin granule membrane markers remain within the tissue[2–5] and are found by density gradient centrifugation to have a density less than that of intact chromaffin granules.[2,3] Furthermore, electron microscopy has shown the presence of empty vesicles, similar in size to normal chromaffin granules, in adrenal medullae that have been stimulated to secrete their catecholamines.[6–8]

Understanding of the cellular events that occur during exocytosis depends upon knowledge of the properties of the membranes involved, namely, the chromaffin granule membrane and the chromaffin cell membrane. Additionally, the ability to follow the fate of the chromaffin granule after it has lost its stored materials depends upon the ability to identify and measure specific components of the membrane. The work reported here was undertaken in order to obtain a pure preparation of chromaffin granule membrane and to produce an antibody to it. Winkler *et al.*[9,10] have reported enzyme and lipid analyses and gel electrophoresis studies of a membrane preparation obtained from cow adrenal medulla chromaffin granules, and Asamer *et al.*[11] have reported the production of an antiserum to a specific protein isolated from cow adrenal medulla chromaffin granule membrane.

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MATERIALS AND METHODS

Fractionation of homogenates. Adrenal glands were removed from cows within 15 min after death and stored in ice until they were used. The cortex was cut away and the medulla was placed in several volumes of chilled 0·3 M sucrose. The following operations were carried out at temperatures near 3°. The tissue was minced with a knife blade, then homogenized in 3 vol. of 0·3 M sucrose with three strokes using a Potter–Elvehjem glass homogenization vessel and Teflon pestle. The homogenate was centrifuged at 550 *g* for 15 min and the resulting supernatant (cytoplasmic extract) was centrifuged at 12,500 *g* for 20 min to yield the sediment (large-granule fraction) and the postlarge-granule fraction supernatant. (All *g* forces are given for the bottom of the tubes.) The large-granule fraction obtained by centrifugation at 12,500 *g* was resuspended in 1 vol. (w/v) of 0·3 M sucrose and centrifuged again at 12,500 *g* for 20 min. The resulting supernatant was added to the postlarge-granule fraction supernatant. In some experiments the combined supernatants were centrifuged at 120,000 *g* for 60 min to yield a microsomal pellet and a high-speed supernatant.

The large-granule fraction was resuspended in 2 vol. (w/v) of 0·3 M sucrose and further fractionated to obtain chromaffin granules by centrifugation at 100,000 *g* for 120 min over one-step gradients consisting of either 7 ml of 1·6 M sucrose[12] or 7 ml of 1·8 M sucrose.[13] Chromaffin granules were washed with sucrose of the same molarity as that used to make the gradient and lysed in Tris–sodium succinate buffer according to the procedure described by Smith and Winkler.[12] Discontinuous sucrose gradient centrifugation of lysed chromaffin granules was carried out either in the Spinco swinging bucket type 25·3 rotor at 100,000 *g* for 120 min or over 7 ml of 1·0 M sucrose at 80,000 *g* for 90 min. The chromaffin granule soluble proteins (chromogranins) were separated from the granule membrane by centrifugation of the lysate at 100,000 *g* for 120 min; the chromogranins remained in the supernatant. Dialysis or hypotonic lysis of tissue fractions was carried out with 5×10^{-3} M Tris–sodium succinate buffer (pH 5·9) or with 5×10^{-2} M Tris buffer (pH 7·2).

Analytical methods. Catecholamines were assayed by the colorimetric method of von Euler and Hamberg[14] using citrate–phosphate buffer at pH 6·0, and are expressed as micromoles of epinephrine. Protein was precipitated by trichloroacetic acid (final concentration, 5%; w/v) and measured by the microbiuret method according to Goa.[15] Protein assays were standardized with bovine serum albumin.

Succinic dehydrogenase activity of fractions dialyzed against 5×10^{-2} M Tris buffer was measured at 23° by the method of Arrigoni and Singer[16] and fumarase activity was measured at 23° according to Racker.[17] Arylsulfatase C, a marker for endoplasmic reticulum, was assayed at pH 8·0 and at 23° by the procedure of Milsom *et al.*,[18] glucose 6-phosphatase was measured at 37° by the procedure of de Duve *et al.*,[19] and β-glucuronidase was measured by the procedure of Gianetto and de Duve.[20] Mg^{2+}-stimulated adenosine triphosphatase (ATPase) activity was measured according to the procedure described by Banks,[21] using Tris–HCl buffer at pH 7·4 containing 5 mm $MgCl_2$ and at 37°. Dopamine-β-hydroxylase activity was measured at 37° by the procedure of Viveros *et al.*,[4] except that the assay mixture contained $5{\cdot}7 \times 10^{-4}$ M tyramine; dopamine-β-hydroxylase activity was expressed as nanomoles of octopamine formed during 1 min. Lactate deyhdrogenase activity was measured at 23° by the method of Wroblewski and LaDue.[22]

For analysis of cytochrome b-559 content, absorption spectra (370–480 nm) of

chromaffin granule membrane preparations were obtained with a Beckman DU spectrophotometer using cells with a 1-cm path length. Samples were dissolved in 1×10^{-1} M glycylglycine buffer, pH 7·4, containing 2% (w/v) sodium deoxycholate. Spectra were obtained from samples in 2 ml that had been oxidized by the addition of three drops of 0·25% (w/v) aqueous potassium ferricyanide and from samples in 2 ml that had been reduced by the addition of 1 mg sodium dithionite.[21]

Lipids of chromaffin granule membrane preparations were extracted by the method of Folch *et al.*[23] Total cholesterol was measured by the method of Zlatkis *et al.*,[24] and total lipid phosphorus was measured by the method of Fiske and Subbarow,[25] after digestion according to the method of Bosmann *et al.*[26]

Electron microscopy. The chromaffin granule membrane fraction was fixed for electron microscopy in glutaraldehyde and osmium tetroxide in 1×10^{-1} M cacodylate buffer, pH 7·4, according to the procedure of Hirsch and Fedorko.[27]

Immunochemical assays. Rabbit antiserum was prepared against the chromaffin granule membrane fraction according to the following procedure. Rabbits were injected with chromaffin granule membrane fraction mixed with an equal volume of Freund's complete adjuvant (final concentrations, 1 mg protein/ml) in the popliteal lymph nodes of the hind legs; the volume injected was 0·2 ml. Intramuscular injections were given 7 days later with 1 mg of membrane protein in a volume of 0·5 ml mixed with 0·5 ml of Freund's complete adjuvant. Blood was collected from the marginal ear vein between 3 and 4 weeks after the final injection. Thereafter, intramuscular injections of the same volume of protein (1 mg)–adjuvant mixture were given every 4–6 weeks and the rabbits were bled 2–3 weeks after each injection. The atiserum was stored at 4° in the presence of 0·01% merthiolate and 25% undiluted complement; it was incubated for 30 min at 56° immediately before use for complement fixation in order to destroy complement. The microcomplement fixation method used has been described in detail previously.[28] In the present study, complement was used at a dilution which provided five 50 per cent lysis units. The micro-immunodiffusion technique used was similar to that described by Crowle[29] using 0·5% agar in 0·9% saline diffusion media. The agar immunodiffusion plates were allowed to develop for 3–5 days at 4° in sealed petri dishes. At the end of this time, the agar plates were washed with isotonic saline for 24–36 hr, stained for 4 hr with thiazine red R (0·1 g in 100 ml of 1% acetic acid), and washed with 70% ethanol containing 1% acetic acid. The plates were photographed by passing light through the plate onto Kodak Kodabromide contrast F.5 photographic film.

RESULTS

Preparation of chromaffin granules. Centrifugation of medulla homogenates at 550 *g* for 15 min yielded 74·9 $\pm$ 6·9 per cent ($n = 8$) of the tissue catecholamines in the supernatant; the catecholamine content of the medulla was 51·3 $\pm$ 1·9 μmoles/g of tissue ($n = 10$). Table 1 shows the occurrence of certain cytoplasmic extract constituents in the large-granule fraction after centrifugation at 12,500 *g* for 20 min. A comparison of chromaffin granules prepared from the large-granule fraction by centrifugation over single-step gradients of either 1·6 or 1·8 M sucrose is also given (Table 1). Neither preparation was contaminated with cytoplasm or with mitochondria; the 1·8 M sucrose preparation contained less lysosomal and microsomal

TABLE 1. DISTRIBUTION OF COW ADRENAL MEDULLA CONSTITUENTS*

	Distribution (%)†					
	CA	Protein	LDH	Fum	β-Gluc	G-6-Pase
$\frac{\text{LGF}}{\text{CE}} \times 100$	40 ± 3 (15)	25 ± 4 (15)	0 (2)	74 ± 7 (6)	41 ± 7 (10)	8 ± 2 (7)
$\frac{\text{1·6 M sucrose}}{\text{LGF}} \times 100$	71 ± 10 (7)	40 ± 4 (7)	0 (3)	0 (5)	5 ± 1 (3)	28 ± 10 (7)
$\frac{\text{1·8 M sucrose}}{\text{LGF}} \times 100$	46 ± 6 (7)	30 ± 5 (7)	0 (1)	0 (3)	4 ± 1 (6)	4 ± 2 (7)

* Centrifugation procedures are described in the Methods section. CE represents the cytoplasmic extract; LGF represents the large-granule fraction; 1·6 M sucrose and 1·8 M sucrose represent the chromaffin granule fractions obtained by centrifugation of the LGF over 1·6 and 1·8 M sucrose respectively. CA represents catecholamines, LDH represents lactate dehydrogenase activity, Fum represents fumarase activity, β-Gluc represents β-glucuronidase activity and G-6-Pase represents glucose 6-phosphate activity. The amount of each constituent in 1 ml of the cytoplasmic extract (representing 0·25 g of original wet wt of tissue) was 10·3 ± 1·3 μmoles for catecholamines, 16·8 ± 2·2 mg for protein, 1·3 μmoles/min for LDH, 6·2 ± 1·9 μmoles/min for fumarase, 1·0 ± 0·1 μmol/hr for β-glucuronidase and 1·2 ± 0·2 μmoles/hr for glucose 6-phosphatase. Recovery of cytoplasmic extract catecholamines, protein and enzyme activities in the LGF and in the LGF supernatant ranged between 85% for protein and 100% for β-glucuronidase. The limit of sensitivity for the detection of activity by the procedure used would have allowed the detection of LDH at 0·05% the activity of that of the cytoplasmic extract; for fumarase, the limit of sensitivity would be 0·02 per cent of the activity in the cytoplasmic extract.

† The figures represent the per cent ± standard error; the values were calculated as indicated in the column on the left. The numbers in parentheses are the number of experiments.

contamination than did the 1·6 M sucrose preparation. Centrifugation over 1·8 M sucrose was routinely used for subsequent preparation of chromaffin granules.

Preparation of chromaffin granule membrane. Exposure of chromaffin granules prepared in 1·8 M sucrose to hypotonic buffer caused the release of protein and catecholamines. Two hypotonic washes were sufficient to remove essentially all the soluble protein from the granules; after four washes, the insoluble sediment contained no detectable catecholamines and 16·6 ± 2·7 per cent of the protein of the intact chromaffin granules. The amount of dopamine-β-hydroxylase activity removed by the washes was 53·1 ± 15·1 per cent, and that remaining in the sediment was 54·4 ± 19·7 per cent of the intact granule (n = 6). Dopamine-β-hydroxylase activity in the wash and in the sediment was not stimulated by Cu^{2+}, although Cu^{2+} (5×10^{-4} M) did stimulate dopamine-β-hydroxylase activity in the cytoplasmic extract. In contrast, Cu^{2+} inhibited enzyme activity in the chromaffin granule fractions; 5×10^{-4} $Cu(NO_3)_2$ inhibited soluble dopamine-β-hydroxylase 67 per cent and inhibited in the insoluble dopamine-β-hydroxylase 88 per cent.

To further purify lysed chromaffin granules, the washed preparation was subjected to sucrose density gradient centrifugation (Fig. 1). The major portion of the dopamine-β-hydroxylase activity was in a band equivalent in location to 0·8 M sucrose and just above the 1·0 M sucrose layer. The 0·8 M sucrose fraction was free of succinic dehydrogenase, glucose 6-phosphatase and arylsulfatase C activity. The limits of sensitivity of the assays employed would allow the detection of enzyme activity at a level of 0·2 per cent of the specific activity of medulla large-granule fraction succinic

dehydrogenase and 2 and 4 per cent, respectively, of the specific activity of medulla microsomal glucose 6-phosphatase and arylsulfatase C. Subsequent preparation of chromaffin granule membrane was carried out by centrifugation of lysed chromaffin granules over a one-step gradient consisting of 1·0 M sucrose. The membrane fraction layered over the 1 M sucrose, as indicated by the localization of dopamine-β-hydroxylase activity.

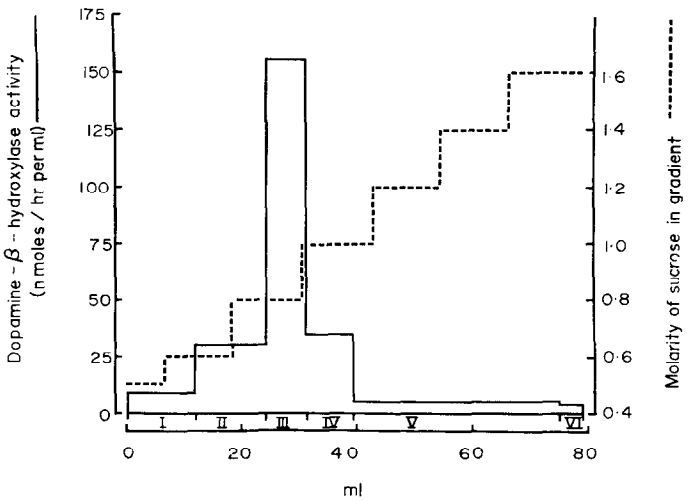

FIG. 1 Centrifugation of the washed membrane fraction from lysed cow adrenal medulla chromaffin granules over sucrose density gradients. Fractions obtained from five gradient tubes were combined the volume of the fractions is shown on the abscissa and the Roman numerals indicate the fraction number. The fractions were obtained with a Spinco tube cutter and are numbered from top to bottom. Dopamine-β-hydroxylase activity and the molarity of the sucrose at various positions of gradient are shown on the ordinates.

An electron micrograph of the chromaffin granule membrane fraction prepared by the 1·0 M sucrose one-step gradient method is shown in Fig. 2. The preparation consists primarily of membrane, usually in the form of an "empty bag". Vesicles that appear to contain granulated material were also occasionally present, possibly representing unlysed chromffin granules. The diameter of the empty vesicles was 150–550 nm, with an average of approximately 250 nm.

Constituents of the chromaffin granule membrane prepation. The membrane fraction contained 2·06 ± 0·06 (n = 5) μmoles of lipid P and 1·63 ± 0·28 (n = 6) μmoles cholesterol per mg of protein. Cytochrome b-559 was also present; in 1% Triton X-100 the λ_{max} of the oxidized form was 415 nm and the λ_{max} of the reduced form was 426 nm. The absorbance of the oxidized form was 0·652 and of the reduced form was 0·902 per mg of protein. ATPase activity was associated with the chromaffin granule membrane; at pH 7·4 in the presence of 5 mM Mg^{2+}, the activity was 2·81 μmoles Pi formed/hr/mg of protein. Dopamine-β-hydroxylase activity of the membrane was 212 ± 39 (n = 5) nmoles/hr/mg of protein; there was no difference between this value and that obtained in the presence of 10^{-3} M *p*-chloromercuribenzoate.

Production and detection of antiserum to chromaffin granule membrane. Analysis by micro-immunodiffusion of serum from rabbits immunized with chromaffin granule

membrane showed the presence of antibody to the membrane fraction (Fig. 3). It is evident from the immunodiffusion pattern that there is more than one antigenic component present in the membrane preparation. Addition of Triton X-100 to the antigen (final concentration, 1 per cent) increased the rate at which the slower migrating membrane antigen diffused. Centrifugation of a sonicated membrane preparation showed that the antigenic component is not readily solubilized. There was cross-reaction between chromaffin granule membrane antiserum and the chromogranins, and the immunodiffusion pattern indicates that the chromogranin antigens are similar to those of the membrane.

Complement fixation was also used to measure the reaction between chromaffin granule membrane antisera and antigens. Table 2 shows the protein concentration of the dilution of various tissue preparations that gives a 50 per cent lysis endpoint with chromaffin granule membrane antiserum. A high protein concentration at the endpoint reflects a low concentration of antigen in the sample. Complement fixation also indicated a cross-reaction between chromaffin granule membrane antiserum and chromogranins; there was also a cross-reaction with cow adrenal medulla microsomes, although not with microsomes from cow liver or atria. Antiserum of the cow membrane antigen cross-reacts with adrenal protein of sheep, pig, dog and rabbit; there was less of a reaction with cat and rat.

TABLE 2. REACTION BETWEEN COW CHROMAFFIN GRANULE MEMBRANE ANTISERUM AND VARIOUS TISSUE PREPARATIONS*

Tissue preparations	Protein concentration at 50% lysis endpoint (μg/ml)
Chromaffin granule membrane (17)	4·4 ± 0·6
Chromogranins (13)	220 ± 65
Cow adrenal LGF	145
Cow adrenal medulla microsomes (4)	63·4 (range, 22–126)
Cow liver microsomes	2,498
Cow atria microsomes	Not detected, > 7,300
Sheep adrenal medulla homog. (3)	73·2, 57·5, 45·7
Pig adrenal medulla homog.	68
Rabbit adrenal gland homog. (2)	103, 120
Dog adrenal gland homog. (2)	68, 52
Cat adrenal medulla homog.	865
Rat adrenal medulla homog.	1,000
Rat liver homog.	Not detected, > 11,900
Rat brain homog.	Not detected, > 7,000

* Complement fixation (see Methods for description of procedure) was used to measure the reaction between rabbit antiserum to cow adrenal medulla chromaffin granule membrane and various tissue preparations. The values given are the concentration of protein at the dilution giving a 50 per cent lysis of red blood cells (50 per cent endpoint). In some cases the mean ± standard error is given with the number of experiments shown in parentheses. In other cases, when n is small, the individual values or a mean value and the range are given.

DISCUSSION

The preparation of chromaffin granules described in this report is similar to the method used by Burack *et al.*[13] for preparation of fowl adrenal medulla chromaffin granules. However, since slight contamination by lysosomal and microsomal constituents was evident, further purification of membrane fraction obtained by hypotonic

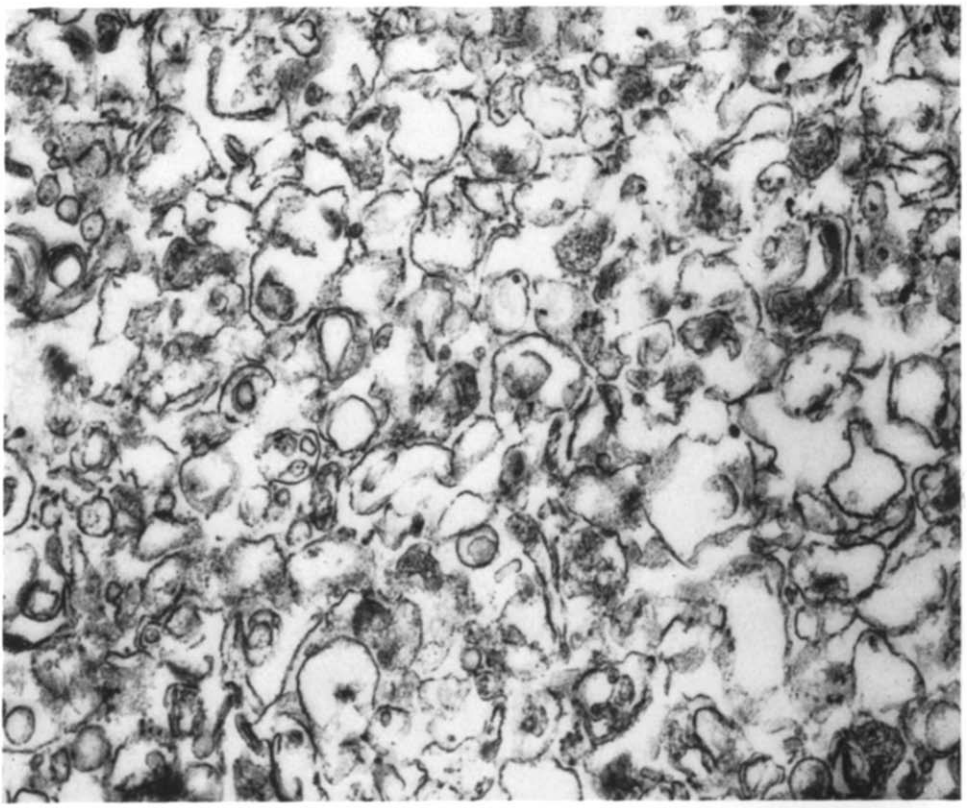

FIG. 2. Electron micrograph of the cow adrenal medulla chromaffin granule membrane preparation The magnification was at 55,000.

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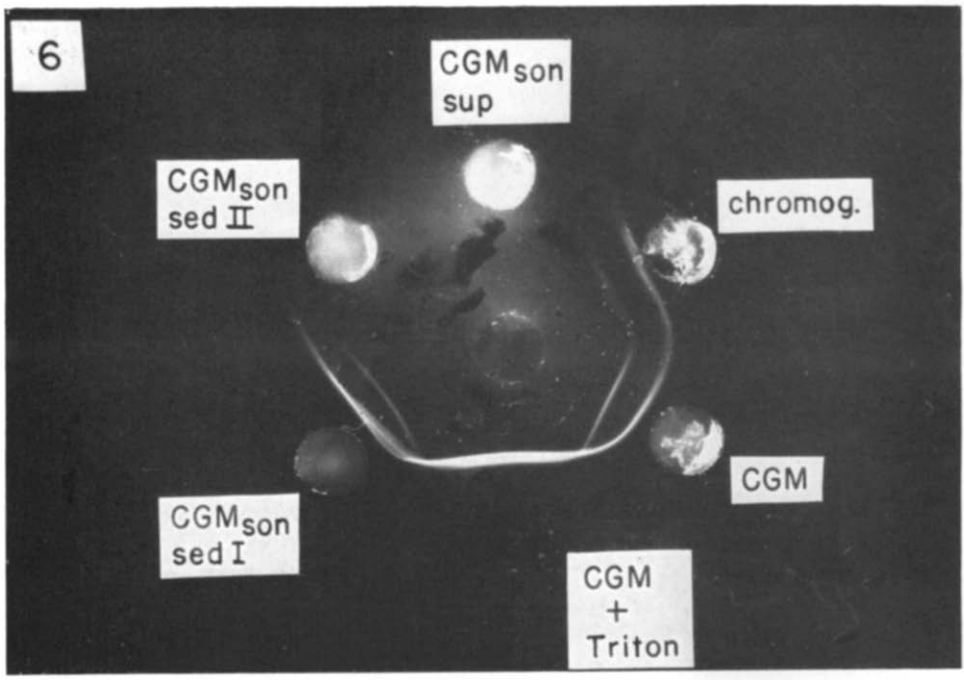

FIG. 3. Micro-immunodiffusion pattern for cow adrenal medulla chromaffin granule membrane antiserum and chromaffin granule constituents. Immunodiffusion was carried out on 0·5% agar on microscope slides (see Methods). The center well contained rabbit antiserum to the membrane fraction; the "chromog." well contained cow adrenal medulla chromogranins (0·72 mg/ml); the "CGM" well contained chromaffin granule membrane (0·56 mg protein/ml); the "CGM + Triton" well contained chromaffin granule membrane (0·56 mg/ml) in 1% Triton X-100; the "CGM_{son}, sed I" well contained the sediment obtained by centrifugation at 7000 *g* for 15 min of chromaffin granule membrane that had been sonicated for six 20-sec periods (the sediment was resuspended in a volume of 9·0% NaCl equal in volume to the volume of the membrane fraction); the "CGM_{son}, sed II" well contained the sediment obtained by centrifugation at 60,000 *g* for 30 min of the supernatant obtained by centrifugation of the sonicate at 7000 *g* (the sediment was resuspended in a volume of 0·9% NaCl equal in volume to the volume of the membrane fraction); "CGM_{son}, sup" well contained the supernatant obtained by centrifugation at 60,000 *g*.

lysis of the granules was carried out by another gradient centrifugation step. Centrifugation of the lysed granule sediment over 1·0 M sucrose provided a highly purified preparation of chromaffin granule membranes.

The identity of the chromaffin granule membrane was verified by the presence of dopamine-β-hydroxylase,[9,30] cytochrome b-559[9,31] and ATPase.[9,21,32] The content of lipid phosphorous and cholesterol was very similar to the values found byWinkler *et al.*[9] for their cow adrenal medulla chromaffin granule membrane fraction (Winkler *et al.* reported 2·4 and 1·66 μmoles/mg of protein, respectively). These findings, in conjunction with the absence of other cell organelle markers, indicate that the preparation obtained was a relatively pure preparation of chromaffin granule membrane. This finding is supported by the results of an electron microscopic examination which showed that the predominant constituent of the preparation was membrane material. The membrane fragments and empty vesicles were similar in appearance to the empty chromaffin granules isolated by Malamed *et al.*[8] in cat adrenal medullae that had been depleted of catecholamines by stimulation with acetylcholine.

The membrane was found to be antigenic in rabbits and yielded an antiserum that could be used to detect membrane by immunodiffusion and by complement fixation. The latter method is quite sensitive, since membrane can be detected in a sample containing as little as 4·4 μg/ml of membrane protein. The antigenic site appears to be bound tightly to the membrane, since it was not removed by sonication. The slight degree of cross-reaction between the membrane antiserum and chromogranins, indicated by immunodiffusion and complement fixation, may reflect antibody produced to constituents that are found in both fractions of the chromaffin granule, such as dopamine-β-hydroxylase. Chromogranin A has also been reported to occur to a varying extent to the membrane portion of the chromaffin granule.[33–35] Cross-reaction between the antiserum and cow adrenal medulla microsomes is in agreement with the hypothesis that the granules are synthesized in the Golgi apparatus (see reference 35), since the microsomal preparation contains antigen common to both. The specificity of the cross-reaction with adrenal medulla microsomal antigens is substantiated by the finding that there was no cross-reaction with cow liver or atria microsomes. In addition, there was little or no reaction with rat liver or brain homogenates. Cross-reactions with preparations of adrenal gland homogenates of sheep, pig, rabbit and dog indicate the presence of common antigens. Cross-reaction between the antiserum to cow chromaffin granule membrane and adrenal gland from cat and rat was approximately $\frac{1}{20}$ as intensive as with the other species tested. Asamer *et al.*[11] recently reported immuno-histochemical evidence that antiserum to a specific protein (component B) of cow adrenal medulla chromaffin granules cross-reacted with adrenal medulla of horse, pig, cat and rat, as well as with bovine splenic nerves. If the antiserum used in the present study cross-reacts with norepinephrine-containing vesicles membrane, the antigen must have been present in the cow atria microsome preparation in a concentration below the sensitivity of the methods used, since there was no evidence of a reaction in the complement fixation assay.

The results reported here show that cow adrenal medulla chromaffin granule membrane produces an immune response in rabbits; the antibody produced reacts with components of chromaffin granules and with adrenal medulla microsomes. This method provides an additional approach to investigating mechanisms of catecholamine storage and release in the adrenal medulla.

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