Mechanism of Secretion from the Adrenal Medulla

I. A Microquantitative Immunologic Assay for Bovine Adrenal Catecholamine Storage Vesicle Protein and Its Application to Studies of the Secretory Process

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SUMMARY

Rabbit antisera have been prepared against a protein contained within the catecholamine storage vesicles of cow adrenal glands. Although the vesicle protein appeared homogeneous by physical and chemical criteria, immunologic analysis showed the presence of small amounts of highly immunogenic contaminants representing less than 1% of the total protein. The major contaminant was shown to be dopamine-β-oxidase, and antibodies to this contaminant could be removed from the antisera by absorption with a dopamine-β-oxidase-containing fraction obtained from a DEAE-cellulose fractionation of a crude catecholamine storage vesicle lysate. The resultant purified antivesicle protein sera were used to measure the release of the vesicle protein during stimulation of isolated perfused adrenal glands. It was found that catecholamines and vesicle protein appeared in the perfusates in ratios very similar to that present in the whole gland.

INTRODUCTION

Biochemical evidence has recently been presented to support the hypothesis that catecholamines are secreted directly to the exterior of the adrenal medullary cells. Douglas and co-workers (1–3) have shown that, after stimulation of the adrenal gland, catecholamines and adenine nucleotides appear in perfusion fluids in ratios very similar to that in intact catecholamine storage vesicles. Banks and Helle (4) were able to detect in perfusates of stimulated glands the presence of a specific protein found within the storage vesicles. These workers employed Ouchterlony immunodiffusion to detect the storage vesicle protein. Kirshner *et al.* (5), also using immunological methods, reported quantitative data on the release of the vesicle protein and catecholamines in isolated perfused bovine adrenal glands. It was found that conditions which stimulated the release of catecholamines caused a parallel release of the vesicle protein. The ratios of catecholamines to specific vesicle protein in perfusates and in intact storage vesicles were very similar. This report describes the preparation and immunologic properties of rabbit antisera to purified bovine storage vesicle protein. It also describes the use of rabbit antisera to measure, qualitatively and quantitatively, the presence of this protein in perfusates of bovine adrenal glands under resting conditions and during stimulation. Subsequent reports[3] will give a more detailed analysis of the release of catecholamines, total protein, and vesicle

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protein in a variety of experimental conditions.

MATERIALS AND METHODS

Purified bovine adrenal medulla catecholamine storage vesicle protein (purified vesicle protein). The preparation and properties of the purified vesicle protein have been previously described (6). The purification procedure briefly consisted of (a) dissection of the medullas from fresh bovine adrenals, (b) homogenization of the medullas in 0.25 M sucrose, (c) isolation of the catecholamine storage vesicles by differential centrifugation, (d) lysis of the storage vesicles with distilled water, (e) removal of the low molecular weight materials by Sephadex G-25 chromatography, and (f) fractionation of the soluble vesicle protein by DEAE-cellulose and Sephadex G-200 chromatography. The purified vesicle protein was homogeneous by ultracentrifugal and disc electrophoretic analysis, and the amino acid composition and fingerprints of tryptic digest were constant from preparation to preparation. However, the protein contained small amounts of dopamine-β-oxidase activity as measured by published procedures (7).

Antisera. Rabbit antisera were prepared against the purified vesicle protein and the crude vesicle lysate. Three courses of 3 or 10 mg protein in complete Freund's adjuvant (Difco) were given at monthly intervals to animals of various strains. The first course was given half in the hind foot pads and half subcutaneously at multiple sites. The second and third courses were given subcutaneously. Bleedings were made on days 12 and 14 after course 1 and on days 6, 8, and 10 after each of the other courses. Sera were stored either frozen or at 4° in a solution containing 0.001% merthiolate. These sera are referred to in the text as Ra x course 1, Ra x course 2, etc., identifying the rabbit and the immunization schedule for each of the antisera.

Catecholamines. Catecholamines in the various adrenal medulla fractions and in perfusates of the gland were determined by the trihydroxyindole method (8). Trichloroacetic acid extracts (5%) of the various adrenal fractions were prepared and diluted for fluorometric assay. The perfusates were merely diluted 1:50 with distilled water prior to assay. Purification of the catecholamines by absorption on alumina was omitted.

Protein measurement. The absorbance at 280 mμ of a 1 mg/ml solution of a salt-free and carefully dried sample of purified vesicle protein was 0.71 (6). This extinction coefficient was used for determining the concentration of solutions of purified protein. The protein concentrations of crude fractions and of total protein in perfusion experiments were determined by the method of Moore and Stein (9) or by the method of Lowry *et al.* (10).

Immunodiffusion and immunoelectrophoresis. Ouchterlony agar double diffusion and immunoelectrophoresis were performed on microscope slides according to standard procedures using the LKB model 6800 A immunoelectrophoresis and immunodiffusion apparatus.

Complement fixation analysis. Quantitative measurements of the vesicle protein concentration in purified protein preparations, lysates, perfusates, and fractions of the adrenal medulla were performed by a modification of the micro complement fixation technique of Wasserman and Levine (11). Guinea pig complement (C′), hemolysin, and sheep red cells were purchased from Baltimore Biological Laboratories. Absorbed antisera against purified vesicle protein, prepared as described below, were used in all C′ fixation studies. The amount of vesicle protein in an unknown solution was determined as follows: A serial 2-fold dilution of the unknown was prepared along with an identical serial 2-fold dilution of purified vesicle protein of known concentration. Duplicate micro C′ fixation analyses were done on the samples and the dilutions of unknown and control giving peak C′ fixation were determined. The dilutions which gave peak C′ fixation in the duplicate determinations were usually identical. Any determination in which the dilutions which gave peak fixation varied by more than one tube or where the points on the C′ fixation curve did not agree with

each other was repeated. The concentration of vesicle protein in the unknown was calculated from the ratio of the dilutions of the unknown and control solutions giving peak fixation. Because the control solution almost always gave peak C′ fixation at 0.15 μg/ml under our conditions, this usually meant that the amount of vesicle protein in the unknown was equal to the reciprocal of the dilution giving peak fixation multiplied by 0.15 μg/ml.

Sucrose gradient. Purified storage vesicles were obtained by centrifugation of the heavy granule fraction through a continuous hyperbolic sucrose density gradient[4] varying in concentration from 1.0 to 2.25 M. The tubes were centrifuged for 3 hr at 24,000 rpm in a Spinco SW25 rotor. Fractions of 40 drops were collected and lysed by adding 10 ml of distilled water, freezing and thawing. The insoluble residue was removed by centrifugation and the concentration of total protein, catecholamines, and vesicle protein in the supernatant was measured.

Perfusion of adrenal glands. Adrenal glands were obtained from cows 20–30 minutes after slaughter. Immediately upon removal, the glands were cannulated through the adrenal vein, flushed with 20 ml of ice cold Locke's solution (154 mM NaCl, 5.6 mM KCl, 2.2 mM $CaCl_2$ 2.15 mM Na_2HPO_4, 0.85 mM NaH_2PO_4, 10 mM glucose) and transported in ice to the laboratory (45 min to 1 hr). The glands were perfused at 30° with Locke's solution at a flow rate of 4 ml/min for 45 min before samples were collected. Stimulation was evoked by changing to Locke's solution containing 10^{-5} g/ml of acetylcholine for 2 min. Samples were collected for 2-min intervals and assayed for catecholamines and vesicle protein. At the end of the perfusion experiment, the medulla was dissected from the cortex, blotted dry, and weighed. The medulla was then homogenized in 0.25 M sucrose using a conical Potter-Elvehjem glass homogenizer and centrifuged at 800 *g* for 8 min to remove heavy debris. The supernatant was centrifuged for 20 min at 25,000 *g*. The pellet was resuspended in distilled water, and aliquots of the whole homogenate, low speed supernatant, high speed supernatant, and high speed sediment were assayed for catecholamines, total protein, and specific vesicle protein.

[4] D. Duch, A. Moore, and N. Kirshner, unpublished observations.

RESULTS

Immunochemical Heterogeneity of the Purified Vesicle Protein

Purified vesicle protein preparations appeared homogeneous by the usual protein physical-chemical analytical methods. The protein sedimented as a single component in the ultracentrifuge and showed essentially a single electrophoretic component by

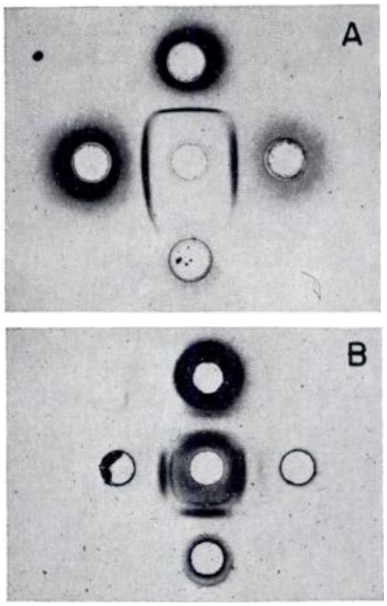

FIG. 1. *Ouchterlony immunodiffusion studies*

(A) Reaction of purified vesicle protein with four sera prepared against vesicle protein. Center well: purified vesicle protein 15 mg/ml. Upper well: Ra 12 course 2; right well, Ra 1 course 1; bottom well: Ra 22 course 2 (nonresponder); left well: Ra 14 course 2.

(B) Reaction of crude vesicle lysates and concentrated perfusates with antiserum Ra 14 course 2. Center well: Ra 14 course 2. Top and bottom wells: 2 vesicle lysate preparations 15 mg protein/ml. Side wells: concentrated perfusates ~5 mg protein/ml.

disc and by cellulose acetate electrophoresis. The amino acid composition and fingerprints of tryptic digests of several different preparations were essentially constant (6).

Of six rabbits injected with purified vesicle protein, two responded by producing antibody after one course. Five responded after the second course. These antisera gave a single precipitation line with the purified vesicle protein by Ouchterlony analysis (Fig. 1A). When crude vesicle lysates were examined (Fig. 1B), one or more other lines (always including a faint line close to the antibody well) were usually noted.

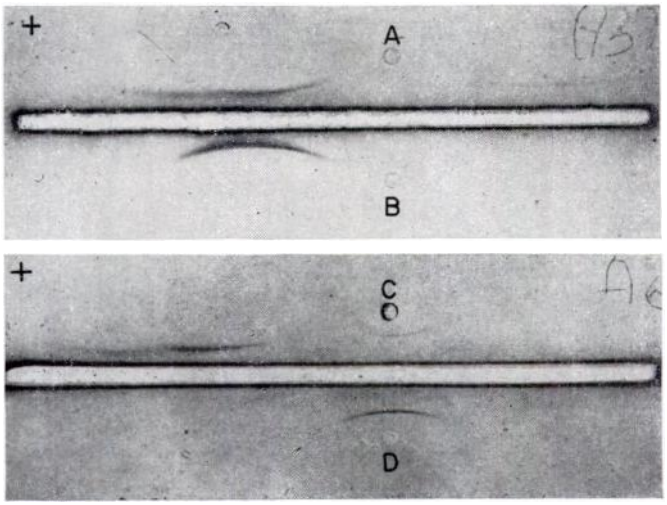

FIG. 2. *Immunoelectrophoresis studies*

Well A: concentrated perfusate obtained during stimulation period. Top trough: Ra 14 course 2. Well B: purified vesicle protein 5 mg/ml. Well C: crude vesicle lysate 15 mg/ml. Bottom trough: Ra 12 course 2. Well D: fraction containing dopamine-β-oxidase 3 mg protein/ml.

Immunoelectrophoresis demonstrated the heterogeneity of purified vesicle protein more graphically. When purified vesicle protein was subjected to immunoelectrophoretic analysis using antiserum prepared against the purified protein, only a single precipitation arc could be seen (Fig. 2B). Crude vesicle lysate showed three precipitation arcs (Fig. 2C). With every antiserum tested, 2–4 arcs could be demonstrated with the vesicle lysate although the purified protein showed a single arc. The purified protein must therefore have contained minute amounts of impurities which were good immunogens. Antibodies were made against these impurities and showed precipitation bands with the crude vesicle lysate, but the purified protein contained such small amounts of these impurities that no precipitation arcs could be seen. The Ouchterlony technique is generally capable of detecting 50–100 μg of antibody per milliliter. At equivalence the amount of antigen in an antigen-antibody precipitate is 5–30% of the antibody for most protein antigens. The Ouchterlony technique should therefore have been able to detect as little as 30 μg of antigen per milliliter. The concentration of purified vesicle protein in the antigen well was 15 mg/ml. The absence of any second precipitating arcs would indicate that none of the immunogenic impurities were present in amounts greater than 0.3% of the total protein.

The various antisera did not give precipitation lines with extracts of adrenal glands of the dog, cat, rat, and rabbit. Aqueous extracts of cow sympathetic nerve, spleen, heart, and adrenal cortex gave a common faint precipitation line on Ouchterlony plates with certain antisera. This line was shown not to be related to the vesicle protein by immunoelectrophoresis. None of the aqueous extracts contained the vesicle

protein in detectable amounts. The protein contents of these preparations were 70–160 mg/ml. Helle (12) has recently reported that her preparation of bovine vesicle protein antisera gave precipitation lines with aqueous extracts of adrenal medulla granules from pig and horse which were not identifiable with the precipitation line obtained with the purified bovine vesicle protein; a similar extract of sheep adrenal medulla gave a precipitation line which was identifiable with that obtained from purified bovine vesicle protein.

Preparation of Purified Antisera

All the antisera prepared against the purified protein contained appreciable amounts of antibodies directed against an impurity which did not migrate from the starting point in immunoelectrophoresis. With one antiserum (Ra 1 course 1) these were the only components. With a second antiserum (Ra 12 course 2) a very small amount of a third antibody was seen, the corresponding antigen migrating toward the anode slightly ahead of the purified vesicle protein (Fig. 2C). In order to use an antiserum for quantitative measurement of the vesicle protein, it was necessary to remove the antibodies directed against the impurities. Because the vesicle protein and nonmigrating impurity were very different electrophoretically, it was decided to look for the impurity in a DEAE-cellulose fractionation of the crude vesicle lysate. Using Ouchterlony analysis and immunoelectrophoresis, this impurity was located in a protein peak just preceding the vesicle protein (Fig. 3). This peak contained a high dopamine-β-oxidase activity, none of the vesicle protein, and a single immunoelectrophoretic component corresponding to the impurity (Fig. 2D). That the antibody directed against this impurity was anti-dopamine-β-oxidase was shown by the fact that (a) the enzyme activity was completely precipitable by the antiserum (Table 1), (b) the washed precipitate upon resuspension had dopamine-β-oxidase activity, and (c) the absorbed antiserum no longer showed a precipitation arc with the impurity-containing DEAE-cellulose fraction. Purified antisera could be made against either the vesicle protein or the dopamine-β-oxidase by appropriate absorption procedures. The procedure for the preparation of antivesicle protein sera was as follows;

TABLE 1

Absorption of dopamine-β-oxidase with rabbit antiserum

Fractions containing dopamine-β-oxidase were mixed in optimal proportion with Ra 12 course 2 antiserum, incubated at 37° for 30 min, and overnight at 4°. The resultant precipitate was centrifuged, washed 3 times with cold saline, and resuspended in saline. Normal rabbit serum, equal to the amounts of antiserum used to precipitate the enzyme, were added to the resuspended enzyme and the untreated enzyme prior to assay.

Sample	Per cent of enzyme activity
Untreated enzyme	100
Enzyme–antibody precipitate, washed and resuspended	75
Supernatant of enzyme–antiserum reaction	4

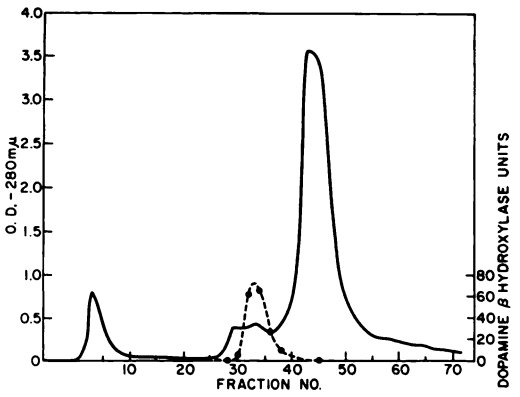

FIG. 3. *Purification of vesicle protein on DEAE-cellulose*

The DEAE-cellulose was equilibrated with 0.005 M phosphate pH 7.4 prior to adding the protein. The protein was eluted with a linear gradient of 0–0.8 *N* NaCl in 0.01 M phosphate pH 7.4. Solid line, optical density; dashed line, dopamine-β-oxidase. A unit of enzyme activity is here defined as micrograms of noradrenaline (norepinephrine) formed per 15 min per 0.1 ml of enzyme solution. The fractions containing the peak of enzyme activity were used to prepare the purified antisera.

A standard flocculation test was performed on a fraction containing dopamine-β-oxidase obtained from the DEAE-cellulose column shown in Fig. 3, and the optimal proportions of antigen and antiserum determined. Optimal proportions of the dopamine-β-oxidase fraction and antiserum were mixed, then incubated at 37° for 30 min and 1–2 days at 4°; the precipitate centrifuged down at 12,000 *g* for 30 minutes. The supernatant contained the vesicle protein antibodies. By a similar procedure an anti-dopamine-β-oxidase could be produced by absorption with purified vesicle protein. C′ fixation studies (Fig. 4) show the results of these absorption procedures. With unabsorbed antiserum, two C′ fixation peaks were seen when a purified vesicle protein preparation was tested (Fig. 4). Absorption of antiserum with dopamine-β-oxidase removed that antibody directed against the component present in very small proportion. Absorption with purified vesicle protein removed that antibody directed against the major antigenic component. The dilutions of antigen required to cause peak fixation of the antidopamine-β-oxidase and antivesicle protein were 1:9 and 1:6561, respectively, indicating that the latter protein was present in much greater relative amount. From the dopamine-β-oxidase activity present in the purified vesicle protein and the specific activity of the purified enzyme reported by Friedman and Kaufman (13), it was calculated that the dopamine-β-oxidase represented only 0.25% of the total protein.

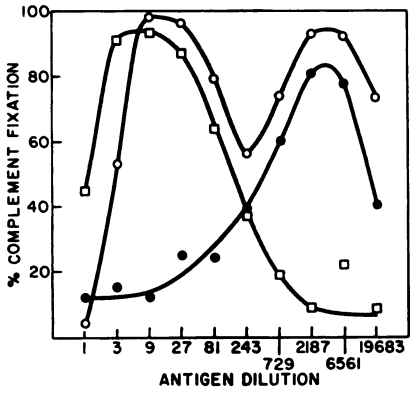

FIG. 4. *Complement fixation curves of purified vesicle protein versus absorbed and nonabsorbed rabbit antiserum prepared against purified vesicle protein*

Unabsorbed antiserum Ra 12 course 2 ○——○. Ra course 2 absorbed with purified vesicle protein, □——□. Ra 12 course 2 absorbed with dopamine-β-oxidase fraction, ●——●.

Localization of the Vesicle Protein within Catecholamine Storage Vesicles

Catecholamine storage vesicles were purified by centrifugation through a sucrose density gradient, and the total protein, the vesicle protein, and the catecholamine contents of the various fractions were measured. The distribution of total protein, catecholamines, and vesicle protein in the density gradient is shown in Fig. 5. The

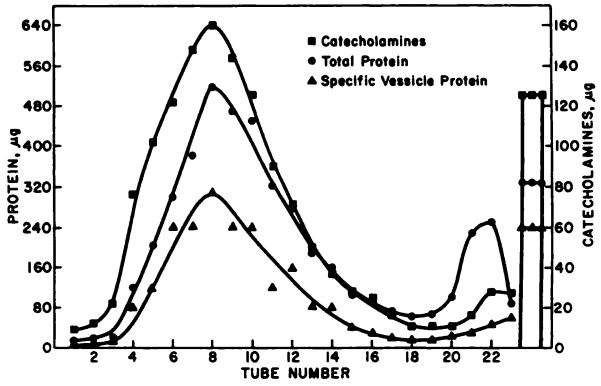

FIG. 5. *Distribution of catecholamines, total soluble protein, and specific antigenic vesicle protein in a sucrose gradient*

See methods and text for details.

distribution of specific antigenic protein closely paralleled the distribution of catecholamines. The ratio of catecholamines to antigenic protein in fractions 4 through 12 was 0.64; the ratio of antigenic protein to total protein in these fractions was 0.59. The ratios of catecholamines to antigenic proteins and antigenic protein to total protein in the unfractionated heavy granules (indicated by the bar on the right of Fig. 5) were 0.53 and 0.74, respectively. The ratios observed in the peak fractions 4–12 and in the unfractionated material were within experimental error. Fractions 4–14 contained 75% of the total protein, 86% of the antigenic protein, and 83% of the catecholamines. Table 2 shows the ratios of catecholamine:antigenic protein, catecholamines:total protein, and antigenic protein:total protein. The ratio catecholamine:antigenic protein was essentially constant over the entire gradient while the other two ratios showed an almost continual decline as a function of fraction number. The ratio of antigenic protein to total protein at the catecholamine peak was approximately 0.7, whereas at the minimum (fraction 18) the ratio was only 0.13.

TABLE 2

Ratios of catecholamines, vesicle protein, and total protein in sucrose gradient

Ratios were calculated from the amounts of each material in micrograms. Catecholamines were calculated as the free base. CA, catecholamines; VP, vesicle protein; TP, total protein.

Fraction no.	CA:VP	CA:TP	VP:TP
1	1.50	0.69	0.46
2	1.00	0.66	0.67
3	1.10	1.22	1.11
4	0.95	0.63	0.67
5	0.85	0.50	0.59
6	0.51	0.41	0.81
7	0.61	0.59	0.63
8	0.52	0.31	0.63
9	0.60	0.31	0.51
10	0.52	0.28	0.54
11	0.75	0.28	0.38
12	0.44	0.26	0.58
13	0.62	0.27	0.43
14	0.46	0.23	0.50
15	0.70	0.27	0.38
16	0.80	0.26	0.32
17	0.80	0.21	0.27
18	0.69	0.17	0.25
19	0.69	0.16	0.24
20	0.46	0.11	0.23
21	0.56	0.07	0.13
22	0.60	0.12	0.19
23	0.47	0.33	0.72
24[a]	0.53	0.39	0.74

[a] Unfractionated material.

Identification of Vesicle Protein in Perfusates

Perfusates from a 10-min stimulation period were pooled and concentrated approximately 10-fold. The concentrated perfusate, containing 3 mg/ml protein, was subjected to immunoelectrophoresis using Ra 12 course 2 antiserum. Figure 2A shows the presence of vesicle protein in the concentrated perfusate. Also present was the impurity with the faster mobility mentioned previously. Dopamine-β-oxidase was not detectable in the perfusate either by immunoelectrophoresis or by enzymic assay.

Perfusion Studies

A study of the release of catecholamines and vesicle protein during acetylcholine stimulation was performed as described in the Materials and Methods section. After an initial 2-min stimulation period, four additional 2-min samples were collected during perfusion with Locke's solution. In the second part of the experiment, the gland was continuously perfused with Locke's solution containing 10^{-5} g of acetylcholine per milliliter and samples were collected at 10-minute intervals. Figure 6 shows the analysis of catecholamines and antigenic protein for this perfusion. In the two 2-min intervals preceding stimulation, the average output of catecholamines and specific vesicle protein was 23 and 6 μg/min, respectively, giving a catecholamine to protein ratio of 3.8. In the five 2-minute intervals during and following the administration of acetylcholine, the average catecholamine and vesicle protein secretion rate increased to 84 and 35 μg/min, respectively. The net increases were 61 and 29 μg/min,

respectively, for catecholamines and protein, giving a catecholamine to protein ratio of 2.13. During the second stimulation period of 30 min, the average net increases above the resting levels were 55 μg/min of catecholamines and 32 μg/min of protein, giving a net catecholamine to protein ratio of 1.67. The catecholamine to vesicle protein ratios in the various fraction of homogenates of the perfused gland were: low speed supernatant, 1.25; high speed supernatant, 1.38; high speed sediment, 0.89. In

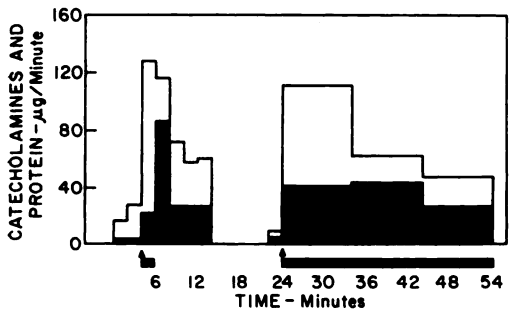

FIG. 6. *Secretion of catecholamines and vesicle protein*

Stimulation of secretion was evoked by adding acetylcholine to the perfusing medium. The clear bars represent catecholamines, the solid bars vesicle protein. The solid bars below the histogram indicate the time acetylcholine was present in the perfusing medium. The vesicle protein was determined by complement fixation.

the nonperfused contralateral gland which was removed from the animal at the same time as the perfused gland and kept in ice throughout the experimental period, the catecholamine to protein ratios were: whole homogenate, 1.21; low speed sediment, 1.36; low speed supernatant, 1.81; high speed supernatant, 1.53. The catecholamine to protein ratios observed in the perfusates and in the various fractions of the homogenates agree within the limits of experimental error.

Complement fixation analyses of vesicle protein released during the resting period and during and after stimulation with acetylcholine are shown in Fig. 7 together with a control purified vesicle protein solution. A parallel series of curves was obtained, the position of which was related to the concentration of vesicle protein in each solution. The C′ fixation curves of the purified protein and the perfusion samples were not always completely superimposable. This was because (a) some perfusion samples were slightly anticomplementary while the purified protein was not and (b) the small amount of antibody to the unidentified impurity reacted with the corresponding impurity in the perfusion

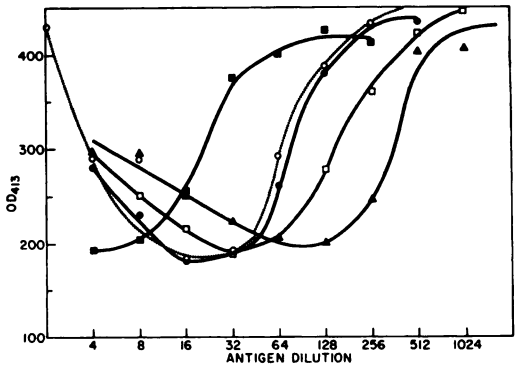

FIG. 7. *C′ fixation studies of purified vesicle protein and selected fractions from adrenal perfusates versus purified Ra 12 course 2 antiserum*

The perfusates are those represented in the histogram on the left of Fig. 6. Purified vesicle protein, 4 μg/ml, O----O; fraction 1, prestimulation period, ■——■; fraction 4, peak release, ▲-----▲; fraction 7, □——□; fraction 10, post-stimulation, ●——●.

samples while the amount of this impurity in the purified protein was so small as to not affect the C′ fixation curve of the purified material. The differences in the C′ fixation curves, when they occurred, were not large and were always confined to the very large antigen excess region of the curve. Estimations of antigen concentration were made either at the peak C′ fixation or the antibody excess region of the C′ fixation curve where neither of these complications was a factor. The method of C′ fixation could detect as little as 0.05 μg/ml of vesicle protein reproducibly. Being a dilution method, it is subject to an error of 0.5–2 times the reported values.

DISCUSSION

By ultracentrifugation, electrophoresis, Ouchterlony immunodiffusion, and immuno-

electrophoresis, purified vesicle protein appeared to be a homogeneous preparation. Closer analysis revealed the presence of minute amounts of highly immunogenic contaminants. One of these contaminants, dopamine-β-oxidase, was particularly immunogenic compared to the vesicle protein. The identity of the other impurities has not been established. However, the concentrations of antibodies made against these were usually very low in first and second course bleedings. Antisera could be chosen in which the amounts of these antibodies were either not detectable or so low as to cause no problems in their use as analytical reagents. A relatively "mono specific" antiserum could be easily produced by appropriate absorption procedures. Previous reports (14) have utilized antisera to purified vesicle protein preparations produced by a different procedure. In these reports Ouchterlony patterns showed a major precipitation line and a slowly developing second line which the authors ascribed to a polymerized form of the vesicle protein (13). Our antisera were produced by an entirely different immunization schedule than those used by these authors. It is possible that the slowly developing line was, in reality, a second antigen-antibody system.

The availability of purified antisera has made it possible to obtain further evidence for the hypothesis that catecholamines are released from the storage vesicles directly to the exterior of the cell membrane. The finding that catecholamines and a specific protein contained within the storage vesicle appear in the perfusates of stimulated glands in relative amounts very similar to those present in the gland provides strong support for the above hypotheses.

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