# Some Chemical and Physical Properties of the Soluble Protein Fraction of Bovine Adrenal Chromaffin Granules

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SUMMARY

1. The soluble protein fraction, 70% of the total protein of the chromaffin granules of the bovine adrenal medulla, has been prepared by ethanol precipitation. The protein fraction was freed of nucleotides by gel filtration, and its sedimentation properties and amino acid composition have been studied.

2. The sedimentation pattern showed a single peak with a slight asymmetry which is interpreted as indicative of a molecular heterogeneous system. A sedimentation constant of $s^0_{20,w} = 2.0$ and an average apparent molecular weight of 25,000 was calculated for this peak.

3. The soluble protein fraction was very rich in glutamyl (or glutaminyl) residues (25.4% by weight), and its proline content was high. No cysteine was found. The negatively charged groups were found to outnumber the positively charged groups by 7 residues per protein molecule.

4. This peak was further fractionated into two subfractions which had identical electrophoretic mobility patterns but differed in their sedimentation properties.

5. It is suggested that the soluble protein interacts with low molecular weight ions, leading to physically distinguishable molecular forms of the same protein unit.

## INTRODUCTION

It is known that the hormones of the adrenal medulla are stored in specific organelles, the chromaffin granules. These granules have been studied by both cytological and biochemical methods (1). The hormones in these granules are present together with ATP and other adenine nucleotides (2), but the mechanism by which they are retained is not known.

The chromaffin granules have been separated from other intracellular particles by ultracentrifugation in a sucrose density gradient (3, 4). It was established that the granules are rich in protein. Much of the granule protein is extractable in hypotonic media and is recovered in the supernatant on further centrifugation, whereas some insoluble protein is sedimented. We owe a more detailed study of the proteins from chromaffin granules to Hillarp (5, 6), who found that over 70% of the protein is water soluble and is precipitated at pH 4.0 in 50% ethanol in the cold.

The most characteristic property of the chromaffin granules is their ability to store catecholamines and ATP. Little is known about the characteristics of the soluble protein. The present work was undertaken in order to obtain more information on the chemical and physical properties of the soluble protein fraction.

Purified fractions of the protein have been studied by centrifugation in the analytical ultracentrifuge and by electrophoresis. It will be shown that the extractable protein fraction is not monodisperse, and evidence for the presence of two different molecular forms of the soluble protein has been obtained.

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Some of the results obtained in this work, which was carried out in 1962–1963, have already been briefly reported (7).

## METHODS

### *Centrifugation of Bovine Adrenal Medullary Homogenates*

The method used was essentially that described by Blaschko *et al.* (4), but with some minor modifications. The tissue was homogenized in 0.3 M sucrose, and the final concentration was chosen to give 5 ml of homogenate (fraction I, Fig. 1) from 1 g of tissue. Coarse debris and nuclei were removed by centrifugation at 600 *g* for 15 min. The supernatant was centrifuged at 10,300 *g* for 15 min in the Spinco preparative centrifuge, giving a pellet of "large granules" (fraction II, Fig. 1). The pellet was resuspended in 0.3 M sucrose before resolution on the sucrose density gradient. In order to reduce the loss of chromaffin granules in the nuclear sediment, the first centrifugation was less strong than that used by Blaschko *et al.* The second centrifugation was shorter than that used earlier, to reduce the microsomal contamination of the "large granules" fraction.

The sucrose density gradient used in the further resolution of the "large granules" fraction was set up as described by Banks (8):

| Sucrose | ml |
|---|---|
| 1.40 M | 0.5 |
| 1.50 M | 0.5 |
| 1.55 M | 1.0 |
| 1.60 M | 0.5 |
| 1.70 M | 0.5 |
| 1.80 M | 0.5 |
| 2.50 M | 0.25 |

The gradient tubes were always prepared about 21 hr prior to adding 1.0–1.5 ml of the resuspended "large granules." Centrifugation was carried out at 103,000 *g* for 60 min in the Spinco SW 39L, and the slightly pink and opaque zone seen at the level of the 1.7–1.8 M sucrose layers was collected from each tube by means of a cutter designed by Dr. E. J. Schuster. The chromaffin granules (fraction III, Fig. 1) thus collected were used for further studies.

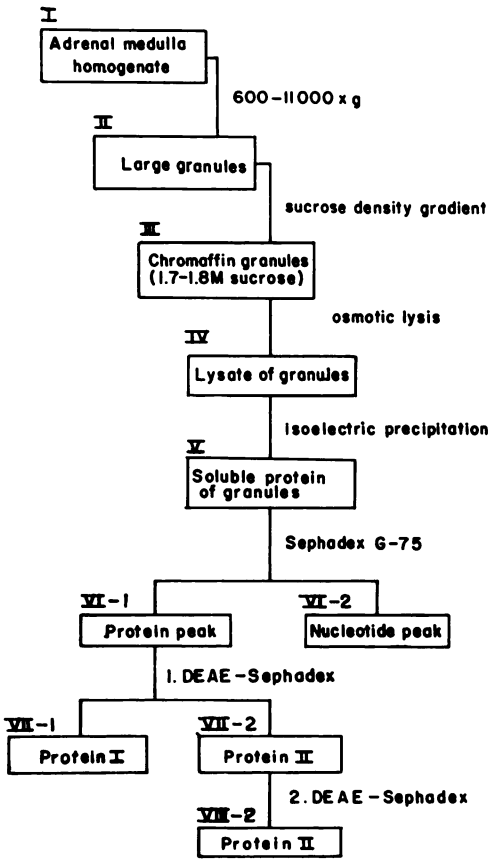


FIG. 1. *Flow sheet of fractionation procedures*

The stages of purification of the soluble protein are shown in a schematic form. Each fraction has been assigned a number to which reference is made throughout.

### *Lysis of Granules and Ethanol Precipitation of Soluble Protein*

The chromaffin granule suspension obtained from the sucrose density gradient was mixed with 2 volumes of ice-cold distilled water and centrifuged immediately at 70,000 *g* for 30 min. The sediment was washed with 0.03 M sucrose of approximately the same volume as that of the distilled water. The sample was stirred, then the centrifugation was repeated and the supernatant added to that collected in the first run. The granular lysate thus obtained

(fraction IV, Fig. 1) was dialyzed against four changes of large volumes of 0.03 M sucrose.

Ethanol precipitation was carried out by slowly adding 1 volume of ethanol, precooled to −15°, to the lysate, the temperature being kept below +5°. The lysed material usually had a pH of 5.5–5.6; on addition of ethanol, the pH rose to 6.5. The pH was adjusted to 4.0 by dropwise addition to 1.0 M acetic acid, and the mixture was allowed to stand for 15 min at −20° before centrifugation at 20,000 *g* for 15 min. The precipitate was drained and dissolved in a small volume of 0.1 M sodium phosphate buffer of pH 7.0 to make the protein concentration 0.5–1.0%. Traces of ethanol were removed by dialysis against four changes of buffer. The resulting, somewhat opaque, solution was centrifuged for 30 min at 70,000 *g*; the clear supernatant thus obtained will hereinafter be called the soluble protein (fraction V, Fig. 1).

*Chemical Estimations*

*Protein.* In samples that also contained nucleotides the protein was determined by the micro-Kjeldahl method; the digestion mixture of Campbell and Hanna (9) and the Markham still (10) were used. In nucleotide-free samples, protein was determined spectrophotometrically (11).

*Amino acids.* Acid hydrolyzates of protein samples were analyzed on an E.E.L. automatic amino acid analyzer (12).

*Catecholamines* were determined spectrophotofluorometrically, the Farrand spectrophotofluorometer (13) being used.

*Adenine nucleotides* were determined by ultraviolet spectrophotometry, the relative absorbance being measured at 260 and 275 mμ. ATP was assayed by the firefly-luminescence method (14); ADP was determined by the myokinase (15) combined with the firefly method.

*Gel Fractionation*

*Sephadex G-75* was prepared as described by Porath (16), and the gel was equilibrated in buffers of 0.026 M ($I$ = 0.05) or 0.1 M ($I$ = 0.2) sodium phosphate at pH 7.0. The gels were packed in columns of bed volume of approximately 120 ml (2 × 40 cm).

*DEAE-Sephadex A-50* (100–200 mesh) was prepared according to the method of Porath and Lindner (17) and equilibrated in 0.1 M $NaH_2PO_4$ (pH 4.5). The columns (1 × 20 cm) were eluted by a gradient of $NaH_2PO_4$, with 200 ml 0.1 M solution in the mixing flask and 1.0 M solution in the reservoir.

All column experiments were carried out in the cold.

*Electrophoresis*

Horizontal starch gels (18) were prepared in a buffer of pH 8.8 (19) (0.076 M Tris–0.005 M citric acid) or at pH 7.0 in a phosphate buffer (20) (0.0114 M $Na_2HPO_4$–0.0064 M $NaH_2PO_4$–0.16 M NaCl; $I$ = 0.1). Samples of 0.05 ml were applied, containing 0.3–1.0% protein, and electrophoresis was performed using a voltage of 5.25 volts/cm for 6 hr at room temperature. Proteins were detected by staining the starch gels with nigrosin in methanol–water–acetic acid (50:50:10) (18).

*Ultracentrifugation Analysis*

The Spinco Model E analytical ultracentrifuge was used for the analysis of the various protein fractions (21). All runs were made at +20°, and observations were recorded by schlieren optics.

Sedimentation coefficients were determined from photographic exposures taken at 16-min intervals when the samples were centrifuged at 59,780 rpm in single-sector cells. Lengths of runs were usually 144 min. The sedimentation coefficients were corrected to water at +20°.

The degree of homogeneity of the samples was found by plotting the apparent diffusion, $D_{app}$, calculated from the exposures obtained during the sedimentation-velocity runs, against time. An estimation of the degree of homogeneity as well as an apparent molecular weight were obtained from centrifugation in a double-sectored cell at low speed, 31,410 rpm, acording to the method of "approach to equilibrium" sedimentation described by Trautman and Crampton (22).

The diffusion coefficient was determined using the synthetic boundary cell at low speed, 10,589 rpm, corrected to water at +20°.

The partial specific volume, $\bar{V}$, was determined from density measurements and from the amino acid composition (21).

A minimum molecular weight was calculated from the amino acid composition (23).

## RESULTS

In all experiments the chromaffin granules (fraction III) were first lysed. All catecholamines and most of the ATP were removed by extensive dialysis of the lysate (fraction IV). Ethanol precipitation of lysates prior to dialysis showed that the precipitation point of the protein in presence of the amines and ATP was raised to pH 5.3 as compared to pH 4.0 in the dialyzed samples.

In experiments where the soluble protein (fraction V) was dissolved in buffers of low ionic strength ($I = 0.05$), the solubility of the protein was rather low. Increasing the ionic strength to $I = 0.2$ greatly enhanced the solubility, giving clear solutions of protein which kept for some time. For this reason a phosphate concentration of 0.1 M ($I = 0.2$) was used for most experiments.

### *Fractionation of Soluble Protein on Sephadex G-75*

Gel fractionation of the soluble protein (fraction V) was performed on columns of Sephadex G-75 at ionic strengths $I = 0.05$ and $I = 0.2$ (see Methods). The results of two such fractionations are given in Fig. 2. It can be seen that the core of nucleotides (fraction VI-2), firmly attached to the extensively dialyzed soluble protein, was by this procedure well separated from the protein component (fraction VI-1) at both ionic strengths employed.

At low ionic strength of the column buffer system ($I = 0.05$) the protein fraction appeared as an irregular peak on the chromatogram, consisting of 5 minor peaks. Chromatograms developed in buffer of higher ionic strength ($I = 0.2$) showed only two peaks in the protein fraction. All attempts to resolve the protein fraction in-

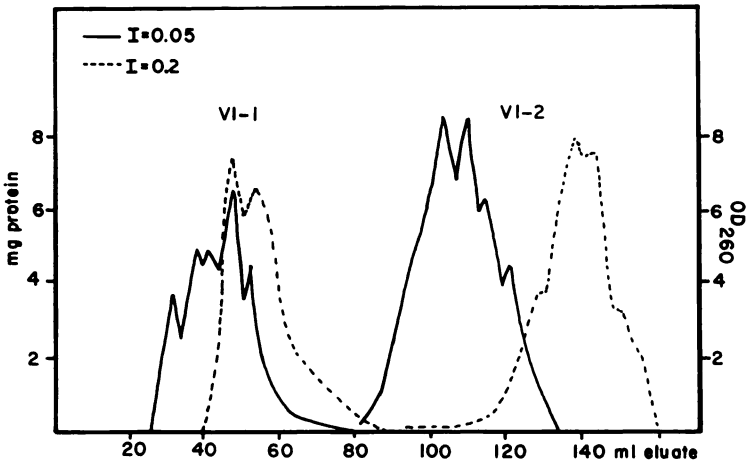


FIG. 2. *Gel fractionation of soluble protein on Sephadex G-75 at low and high ionic strength*

$I = 0.05$: Fraction V, experiment A (199 mg protein) was fractionated on Sephadex G-75 at pH 7.0 (see Methods), and 94 mg protein was recovered as protein VI-1. $I = 0.2$: Fraction V, experiment D (171 mg protein) was fractionated on a similar column as for experiment A. In this experiment 115 mg protein was recovered as protein VI-1.

In both experiments, fractions VI-2 contained nucleotide material only. The void volumes of the columns were about 45 ml. The eluates were collected in aliquots of 1.3 ml; the protein in peaks VI-1 was followed spectrophotometrically, and the nucleotides in peaks VI-2 by optical density at 260 mμ.

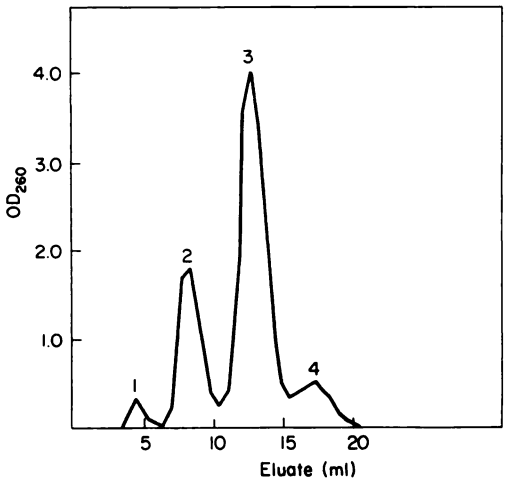


FIG. 3. *Separation of nucleotides removed from the soluble protein (fraction V)*

This figure shows the chromatogram obtained by applying the nucleotide fraction VI-2 obtained in experiment A to a 15-ml column of DEAE-Sephadex A-50 and eluting the nucleotides with a gradient of $NaH_2PO_4$, using 250 ml 0.1 M sodium phosphate buffer pH 7.0 in the mixing flask and 1.0 M $NaH_2PO_4$ in the reservoir. The eluate was collected in aliquots of 5 ml and the nucleotide content was followed by optical density at 260 mμ.

to well separated subfractions on Sephadex G-75 were unsuccessful.

The nucleotide fraction (VI-2) obtained by gel fractionation was resolved into 4 subfractions on a short column of DEAE-Sephadex (see Methods). The results obtained in experiment A (Table 1) are shown in Fig. 3. Peak 1 was identified as AMP by its absorption spectrum and the negative reaction with the firefly-luminescence reagents both in presence and absence of myokinase. Peak 2 was identified as ADP and peak 3 as ATP according to Methods. Peak 4 was assumed to be GTP from both its absorption spectrum and its position on the chromatogram.

The amount of nucleotide material adhering to the soluble protein fraction (V) in experiment A represented 1.47 OD units at 260 mμ per milligram of nucleotide-free protein (fraction VI-1). Assuming the nucleotides were correctly identified, they were distributed as shown in the accompanying tabulation.

| | | Nucleotide | |
|---|---|---|---|
| Peak | $OD_{275}/OD_{260}$ | Micromoles | Micromoles per 25000 μg of protein |
| 1. AMP | 0.32 | 0.32 | 0.09 |
| 2. ADP | 0.37 | 2.43 | 0.85 |
| 3. ATP | 0.35 | 6.15 | 1.85 |
| 4. GTP(?) | 0.64 | 0.10 | 0.03 |

The tabulated results show that of the number of adenine nucleotide molecules separated from the extensively dialyzed soluble protein (V) by gel filtration, as many as two-thirds could be isolated in the form of ATP.

*a. Chemical properties of protein fraction VI-1.* The protein fraction VI-1 was analyzed for contamination with adenine nucleotides. No ATP could be detected by means of the firefly-luminescence system, and the ratio of absorbancy at 280 and 260 mμ of 1.7 for protein fractions obtained by gel filtration at $I = 0.05$ indicated also the absence of other nucleotides. In view of the observation by Philippu and Schümann (24) that RNA is released from the chromaffin granules, contamination of the protein fraction by RNA was investigated by means of the orcinol reaction (25) in one experiment (experiment B, Table 1) for which a ratio of absorbancies at 280 and 260 mμ of 1.0 had been obtained. The spectrum seen with the protein fraction by this method was not that characteristic of reference samples of ATP. In addition to the ribose maximum at 660 mμ, strong absorbancies in the region of 460–580 mμ were also observed, indicating contamination of this protein fraction also by carbohydrates other than ribose. Assuming that all absorption at 660 mμ was due to RNA, the protein fraction was found to be contaminated by RNA to the extent of not more than 0.6% by weight.

A sample of protein fraction VI-1 obtained by gel fractionation at $I = 0.05$ (experiment A, Table 1) was prepared for amino acid analysis. The results of the analysis are shown in Table 2, and some interesting features may be pointed out.

TABLE 1
*Recoveries of protein in various fractions of chromaffin granules*

| | | Experiments | | | | |
|---|---|---|---|---|---|---|
| | Fraction | A | B | C[a] | D | |
| Wet weight of adrenal medullae (mg) | I | 69,150 | 92,570 | 48,940 | 75,750 | |
| | | Protein in fractions (mg) | | | | Per cent recovery in D |
| Chromaffin granules | III | — | — | — | 410 | 100 |
| Lysate of granules | IV | — | — | — | 282 | 69.1 |
| Soluble protein | V | 199 | 171 | — | 176 | 43.6 |
| Protein from Sephadex G-75 | VI-1 | 94 | 115 | 77 | 116 | 28.3 |
| Subfraction I | VII-1 | — | 0 | 21 | 6 | 1.5 |
| Subfraction II | VII-2 | — | 72 | 27 | 60 | 14.6 |
| Subfraction II | VIII-2 | — | 18 | — | — | — |

[a] In this experiment the soluble protein was prepared by lysing the "large granules" (omitting the sucrose gradient step).

First, there was an abundance of glutamic acid in the hydrolyzate, representing 25.4% of the total weight. Secondly, the proline content was unusually high (8.2%). No cysteine was found in this protein preparation.

*b. Physical properties of protein fraction VI-1.* In a preliminary centrifugation of the

TABLE 2
*Amino acid analysis and calculated minimum molecular weight of soluble protein (fraction VI-1)*

| Amino acid | Per cent of weight | Moles/$10^4$g protein | Minimum mol. wt. | Assumed number of residues | Mol. wt., calculated |
|---|---|---|---|---|---|
| Lysine | 8.3 | 6.54 | 1529 | 17 | 25.993 |
| Histidine | 2.4 | 1.737 | 5757 | 5 | 28.785 |
| $NH_3$ | 1.7 | 10.088 | 990 | 26 | 25.740 |
| Arginine | 9.8 | 6.3076 | 1585 | 16 | 25.360 |
| Cystine | 0 | 0 | — | — | — |
| Aspartic | 8.4 | 7.2709 | 1370 | 19 | 26.030 |
| Threonine | 2.5 | 2.535 | 3944 | 7 | 27.608 |
| Serine | 5.8 | 6.713 | 1489 | 18 | 26.802 |
| Glutamic | 25.4 | 19.748 | 506 | 52 | 26.328 |
| Proline | 8.2 | 8.019 | 1247 | 21 | 26.187 |
| Glycine | 4.3 | 7.573 | 1320 | 20 | 26.410 |
| Alanine | 5.4 | 7.6098 | 1314 | 20 | 26.280 |
| 1/2-Cystine | 0 | 0 | — | — | — |
| Valine | 3.4 | 3.437 | 2900 | 9 | 26.100 |
| Methionine | 1.6 | 1.232 | 8110 | 3 | 24.330 |
| Isoleucine | 1.3 | 1.133 | 8826 | 3 | 26.478 |
| Leucine | 7.2 | 6.331 | 1570 | 17 | 26.690 |
| Tyrosine | 2.1 | 1.314 | 7610 | 3 | 22.830 |
| Phenylalanine | 2.3 | 1.528 | 6544 | 4 | 26.176 |
| | 100.1 | 89.03 | | 234 | Mean: 26.125 |

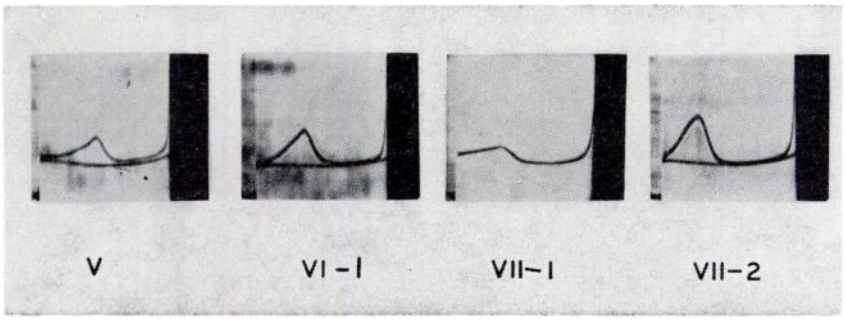


FIG. 4. *Sedimentation of protein fractions*

Samples of protein fractions were centrifuged at 59,780 rpm; the patterns obtained after 144 min centrifugation are given in the figure.

| Fraction | Protein content (%) | $s_{20,w}$ |
|---|---|---|
| V | 0.66 | 2.4 |
| VI-1 | 0.74 | 1.69 |
| VII-1 | 0.58 | 2.6 |
| VII-2 | 0.99 | 1.37 |

soluble protein (fraction V) one single, asymmetric, peak was observed during the 144-min run. An apparent sedimentation coefficient of $s_{20,w} = 2.40$ could be calculated for this peak at 0.66% protein in 0.05 M phosphate buffer at pH 7.0 (Fig. 4).

After removal of the nucleotides present in this fraction, the sedimentation coefficient for protein fraction VI-1 (experiment A) was again determined by ultracentrifugation, and a value of $s_{20,w} = 1.69$ at a protein concentration of 0.74% was found. The sedimentation pattern of this peak is shown in Fig. 4. An apparent diffusion coefficient was determined for this preparation in an independent centrifugation at 10,589 rpm, giving a value of $D_{20,w} = 5.06$ (Fig. 5). The apparent partial specific volume was determined for this sample to be 0.653 ml/g in the buffer used for this experiment ($I = 0.05$).

From these data an approximate average

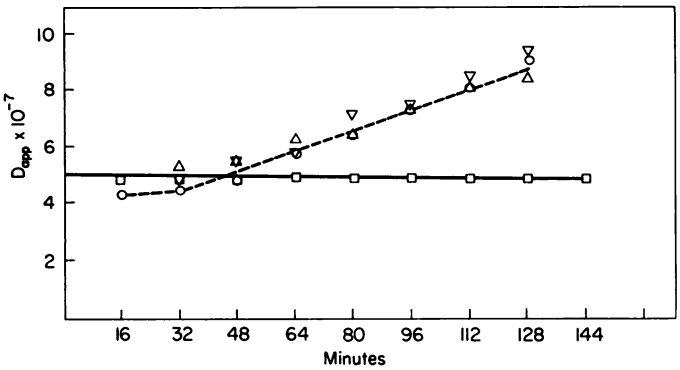


FIG. 5. *Diffusion coefficients of protein fractions*

This figure shows the diffusion coefficient obtained for protein fraction VI-1, using the synthetic boundary cell at 10,589 rpm (□——□) at 0.57% protein concentration. The apparent diffusion coefficients obtained at 59,780 rpm for protein VI-1 at 0.57% (△---△) and 0.74% (▽---▽) protein concentration and protein VII-2 at 0.99% protein (○——○) are also given in this figure.

molecular weight of 24,000 was calculated. When the same sample was used for an independent determination of the molecular weight by "approach to equilibrium" centrifugation, a value of the apparent molecular weight of 26,800 was obtained from the slope of the regression line calculated for the observed points given in Fig. 6. The value of $\bar{V}_{app} = 0.653$ ml/g was used for this calculation.

The two independent sedimentation methods used to determine the apparent molecular weight of this sample of protein, fraction VI-1, also give two independent evaluations of the homogeneity of the sample. In the sedimentation-velocity centrifugations polydispersity would make itself evident by an increase in $D_{app}$ with time. Aggregating protein systems would also cause such an increase in $D_{app}$ with time. In the "approach to equilibrium" centrifugation polydispersity would cause the regression line to deviate from a straight line. It can be seen that protein VI-1 appears homogeneous by the latter method (Fig. 6), while it does exhibit polydispersity as given by the former (Fig. 5).

The value of the apparent partial specific volume ($\bar{V}_{app} = 0.653$ ml/g) arrived at by density measurements in the phosphate buffer of $I = 0.05$ at pH 7.0 was notably lower than the range of values given for most proteins (0.70–0.75 ml/g). A determination of $\bar{V}_{app}$ for this protein fraction in the buffer of higher ionic strength ($I = 0.2$) gave an even lower value ($\bar{V}_{app} = 0.623$ ml/g), while a calculation of this parameter from the amino acid composition gave a value of 0.7057 ml/g. A high content of dicarboxylic acids in the soluble protein would be expected to contribute to a low value of $\bar{V}$, as the specific volumes of glutamic and aspartic acids are 0.67 and 0.60 ml/g, respectively (21). The decrease in the value of $\bar{V}_{app}$ with increase in ionic strength of the solvent indicates that the shape and volume of the soluble protein are quite sensitive to changes in ionic strength of the surrounding medium. An increase in the ionic strength of the medium seems to cause the protein to become more densely packed.

The sedimentation coefficients obtained with protein fraction VI-1 showed a strong dependence upon protein concentration. A sedimentation constant of $s^0_{20,w} = 2.03$ was calculated for this protein fraction.

The patterns of nigrosin staining bands obtained in starch gel electrophoresis experiments with protein fraction VI-1

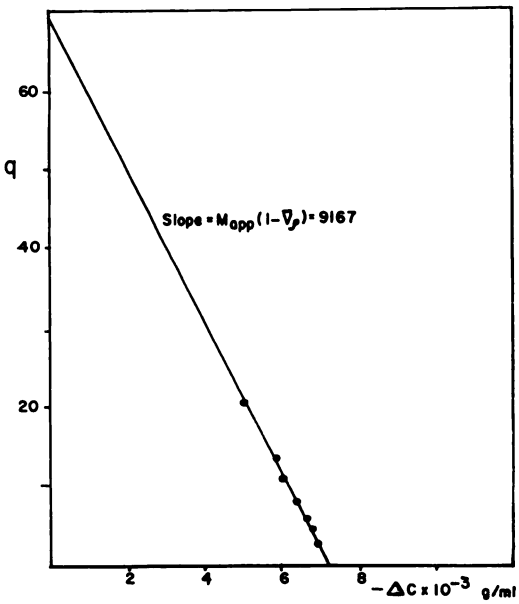


FIG. 6. *Molecular weight determination of protein VI-1.*

Approach to equilibrium centrifugation was performed at 31,410 rpm of a sample containing 0.74% protein in phosphate buffer pH 7.0, $I = 0.05$. The theoretical basis for the Trautman plot given in this figure is:

$$M_{app}(1 - \bar{V}\rho) = \frac{RT(\delta c/\delta r)r_a}{\omega^2 r_a c_a} \equiv \text{slope of regression line}$$

*Abscissa:* $q = \frac{RT(\delta c/\delta r)}{\omega^2 r_a} r_a$;

*ordinate:* $-\Delta C = (c_a - c_o)$

showed two strong bands using a phosphate buffer of pH 7.0 (see Methods). A better resolution was obtained using the Poulik buffer system of pH 8.8. In these experiments one main and a number of less predominant bands were routinely observed (see Fig. 7). The main band seemed to contain two overlapping fractions. Whether

the fine resolution seen in the electrophoresis experiments in the Poulik buffer system at pH 8.8 is indicative of a corresponding number of unrelated protein species or of various complexes of protein–protein or protein–salt nature, remains open. It may be mentioned that in previous experiments (7) the presence of low molecular weight components such as ATP plus $Mg^{++}$ or ATP plus epinephrine together

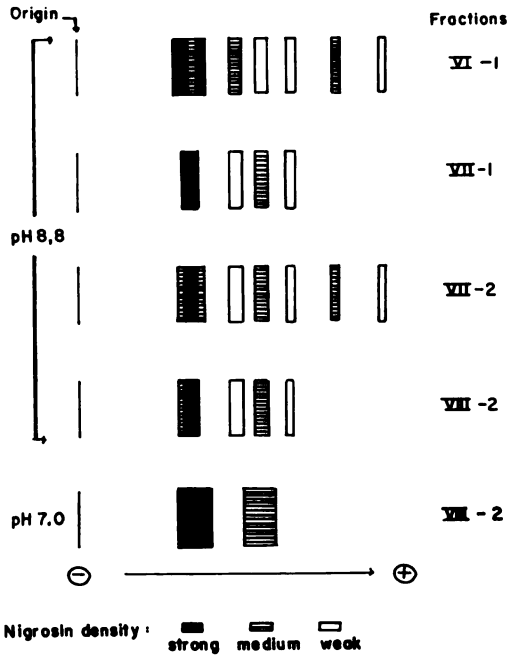


FIG. 7. *Horizontal starch gel electrophoresis*

Low voltage electrophoresis was carried out on horizontal starch gels at pH 8.8 and 7.0 (see Methods. The schematic drawings are based on measurements made on the photographic copies of the stained gels.

with this protein preparation altered significantly the electrophoretic mobility pattern at both values of pH investigated. In presence of these additions only one broad, fast moving line was seen at pH 7.0, and at pH 8.8 the conspicuous, slow and overlapping lines had almost disappeared while the faster moving lines had increased in color density. The number of bands detectable by nigrosin staining on the starch gels may therefore vary with the nature of the low molecular weight ions present in the solvent.

*Subfractionation of Protein VI-1 on DEAE-Sephadex*

The results of experiments carried out on preparations of protein fraction VI-1 indicated the presence of at least two, possibly more, subfractions. In order to isolate subfractions, protein fraction VI-1 was chromatographed on columns of DEAE-Sephadex (see Methods).

The protein was held very strongly by columns equilibrated in 0.1 M phosphate buffer at pH 7.0 and was released as a single peak after the salt concentration had been raised to 0.5 M by $NaH_2PO_4$ or NaCl (experiment B, Table 1). Separation into two subfractions was achieved when the protein was chromatographed on columns equilibrated at pH 6.0 in 0.1 M phosphate buffer; one-third of the recovered protein was not absorbed by the column and appeared in the first eluate (fraction VII-1), whereas two-thirds of the protein emerged from the column when the salt concentration of the eluate was raised to 0.5 M (fraction VII-2). Equally good separations were achieved when columns equilibrated in 0.1 M $NaH_2PO_4$ (pH 4.5) were used, indicating that protein VII-1 was near its isoelectric point at pH 6.0. It is also evident that protein VII-2 was strongly ionized at pH 4.5 as the salt concentration necessary to release this protein from the column at this pH was 0.5 M.

The procedure already described under Methods was therefore adopted for the following experiments.

In the experiment shown in Fig. 8 (experiment D), only 57% of the protein applied was recovered from the column (5% in fraction VII-1 and 52% in fraction VII-2). Full recoveries for this experiment are given in Table 1.

When the soluble protein fraction V was prepared by lysis of the "large granules" (fraction II), omitting the sucrose gradient, and fractionated under otherwise similar conditions as in the experiment shown in Fig. 8, the distribution of protein material in the two subfractions was more uniform; as much as 36% of the protein applied was recovered as protein VII-1 and 37% as protein VII-2 (experiment C, Table 1).

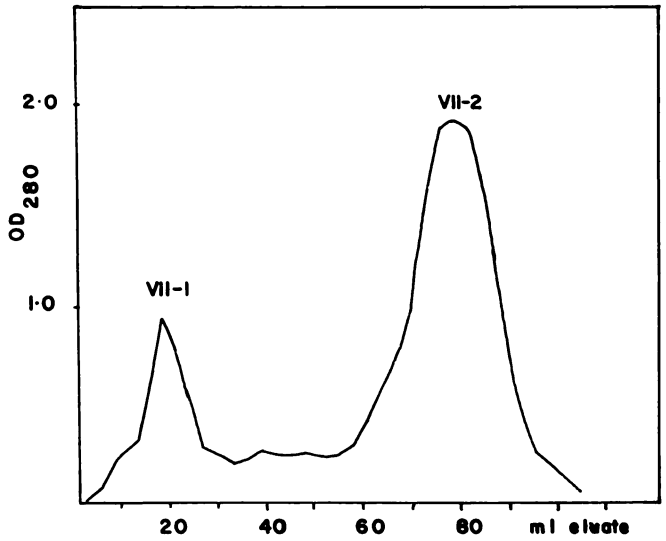


FIG. 8. *Separation of subfractions from protein VI-1 by chromatography of DEAE-Sephadex.*

Fraction VI-1, experiment D (115 mg protein) was applied to a column of DEAE-Sephadex (see Methods) and eluted by a gradient of $NaH_2PO_4$. Two subfractions were recovered, protein VII-1 (6 mg protein) and protein VII-2 (60 mg protein). Ratios of absorbancies at 280 and 260 mμ were 1.2 for fraction VII-1 and 1.6 for fraction VII-2.

Eluate was collected in aliquots of 3 ml, and the protein content was followed spectrophoretically.

Most of the experiments on protein VII-1 to be described below were carried out on this material, the unresolved "large granules" being used as a source, and not material prepared from chromaffin granules isolated on the sucrose gradient.

Electrophoresis experiments showed that an enrichment of the conspicuous, slow moving band was accomplished by the fractionation procedures described. This band was seen in the electrophoresis patterns of both subfractions VII-1 and VII-2. Two of the weak, fast moving bands still present in protein VII-2 could not be detected in protein VII-1 (see Fig. 7).

*Physical Properties of Subfractions*

The sedimentation properties of proteins VII-1 and VII-2 were studied. Comparing the sedimentation patterns obtained after 144 min of centrifugation at 59,780 rpm (Fig. 4), it is apparent that there is a marked difference in the shape of the sedimentation curves of the two subfractions. The sedimentation coefficients, $s_{20,w}$, calculated were, respectively, 2.6 for protein VII-1 (at 0.58% protein concentration) and 1.37 for protein VII-2 (at 0.99% protein concentration).

The peak seen with protein VII-1 was still asymmetric; its sedimentation coefficient was similar to that of 2.4 (at 0.66% protein concentration) found for the soluble protein (fraction V, Fig. 4) which was still contaminated with nucleotides.

Protein VII-2 exhibited the same increase in $D_{app}$ with time (Fig. 5), as was previously found for the unresolved protein VI-1, although the sedimentation pattern obtained for protein VII-2 appeared more symmetrical than that of protein VI-1 (Fig. 4).

*Rechromatography of Protein VII-2 on DEAE-Sephadex*

A sample of protein VII-2 was chromatographed on a second column of DEAE-Sephadex, using a column bed of $2 \times 40$ cm and a gel equilibrated at pH 7.0 in 0.1 M phosphate buffer. The column was eluted with a gradient of $NaH_2PO_4$; 0.1 M buffer was used in the mixing flask, and 1.0 M $NaH_2PO_4$ in the reservoir. The protein was released from the column as one

single peak, protein VIII-2 (Fig. 1), after the pH had been lowered to 4.9. The electrophoresis of protein VIII-2 is shown in Fig. 7. At pH 8.8 the pattern obtained was very similar to that seen with protein VII-1; at pH 7.0 the slow moving band was predominant.

Thus, while no significant differences between the two subfractions, protein VII-1 and protein VIII-2, were detected by electrophoresis, these two subfractions differed in their chromatographic behavior as well as in their sedimentation properties (protein VII-1 as compared to protein VII-2).

## DISCUSSION

The distinctive feature of the chromaffin granules is their very high content of catecholamines and ATP. If one assumes that in the retention of these low molecular weight substances a protein is involved, one would expect such a protein to be a major constituent of the storage granule. For this reason interest is directed to the soluble protein fraction of the granules. The yield of about 70% of the total granular protein as water soluble is in good agreement with earlier figures already discussed.

The present observations have shown that the soluble protein associated with the chromaffin granules can be resolved into two subfractions. Since these experiments were completed (7), there have been two reports on the soluble protein (26, 27). Although the procedures of isolation of the soluble protein differ from the method applied here, these two reports are in agreement with the finding of two subfractions.

In the present work it has been shown that the two subfractions isolated from the soluble protein precipitated by ethanol at pH 4.0 appeared indistinguishable with respect to their electrophoretic mobility patterns but differed significantly in their sedimentation properties. The existence of two physically different forms of the same protein unit might account for these observations.

In view of their similarity in electrophoretic mobility, the great difference in affinity for the DEAE-Sephadex columns exhibited by the two subfractions is not readily understood. It is suggested that charged low molecular weight ions present in the protein solution or in the buffer systems may be engaged in "neutralizing" some of the many charged residues in this protein, or in forming labile intermolecular linkages between charged groups of adjacent protein molecules. Ions of neutral and buffer salts are known to have specific and nonspecific effects on the sedimentation of proteins (28). A number of anions bind to certain proteins and thereby change their isoelectric points, resulting in different values of the sedimentation coefficients, and alkali ions may be bound to proteins at pH above their isoelectric points with a resulting increase in the sedimentation coefficients.

Interactions like the above would explain the higher isoelectric point observed for protein VII-1 on the DEAE-Sephadex columns and also account for the higher sedimentation coefficient determined for this protein fraction in comparison with the more acid protein VII-2. A specific interaction of adenine nucleotides with the soluble protein would explain the higher sedimentation coefficient observed for protein V, containing 2.8 $\mu$moles adenine nucleotides per 25,000 $\mu$g protein, in comparison with the sedimentation coefficient observed for the nucleotide-free protein VI-1. A similarly increased sedimentation coefficient was found when epinephrine, ATP plus $Mg^{++}$ was added to the nucleotide-free protein VI-1 in concentrations equal to those found in the granular lysate. In this experiment the sedimentation coefficient was raised from $s_{20,w} = 1.8$ to 2.4, and upon removal of the additions by extensive dialysis the sedimentation coefficient was again 1.8 (7). Malic dehydrogenase, has been reported to exhibit a marked decrease in sedimentation coefficient with decrease of salt in the protein solution, due to a less compact structure of the enzymically inactive protein in absence of salt (29). The lower value of $\bar{V}_{app}$ found here for protein VI-1 in phosphate buffer of $I = 0.2$ than in phosphate buffer of $I = 0.05$, suggests that a decrease in salt in the soluble protein similarly results in a less compact structure of the protein.

The sedimentation coefficients determined for the unresolved protein VI-1 as well as for the two subfractions, decreased with increasing protein concentrations, though less so for protein VII-1 than for proteins VI-1 and VII-2. Such a decrease in sedimentation coefficients with concentration is normally found for homogeneous proteins in presence of neutral and buffer salts and is known to depend on the net charge of the protein and of the ionic strength (28).

The increase in $D_{app}$ with time at high speed centrifugations found with protein VI-1 as well as with protein VII-2 is known to be characteristic of heterogeneous protein solutions (21), but it is not known whether this effect can be explained by a heterogeneity due to interactions between a protein and low molecular weight ions.

The results of chromatography on DEAE-Sephadex suggest that the major part of the unresolved protein VI-1 (at ionic strengths from 0.05 to 0.2) is present as the more acid subfraction. Therefore the average molecular weight calculated for protein VI-1 (25,000) is representative of the more acid subfraction, protein VII-2. Due to scarcity of material of protein VII-1, a molecular weight has not yet been obtained for this protein fraction.

The determination of the amino acid composition of the unresolved protein VI-1 has brought to light a number of interesting features. For instance, little if any cysteine could be detected among the amino acids recovered. Smith *et al.* (26) have since stated that no free sulfhydryl groups have been detected in the preparation of their heavier subfraction, although this fraction did yield a small amount of cysteine upon acid hydrolysis.

Another outstanding feature of the soluble protein is its high content of dicarboxylic acids, particularly glutamic acid. Glutamyl residues represented 25 of the 31% by weight of dicarboxylic acid residues recovered in the acid hydrolyzate of protein VI-1. A comparably high content of glutamic acid has only been reported for the highly asymmetric tropomyosin of rabbit (30), which contains 31% of this acid. The high content of dicarboxylic acids in the soluble protein may be related to its great sensitivity to changes in ionic strength, resulting in a more dense packing of the protein with increase in ionic strength of the solvent. The proline content is also rather high, like that reported for $\alpha$-casein of bovine origin (31).

According to Smith *et al.* (26) the amino acid fingerprint pattern revealed little difference for the two subfractions. This is in keeping with the present observation on the similarity in electrophoretic mobility observed for the two subfractions. It seems therefore permissible to calculate a minimum molecular weight (23) for the soluble protein based on the amino acid analysis of the unresolved protein VI-1, containing most of its protein as protein VII-2. The value of $M_{min} = 26{,}100$ obtained is in good agreement with the values of 24,000 and 26,800 arrived at from the two independent ultracentrifugation methods.

An isoelectric point of 4.0 has been obtained for the ethanol-precipitated soluble protein. The acidic nature of this protein is explained by the high number of negatively charged groups present, outnumbering the positively charged groups by 7 residues per protein molecule of molecular weight 26,100.

While the present investigations suggest an average molecular weight of 25,000 for the more acid subfraction exhibiting the lowest sedimentation coefficients ($s^0_{20,w} = 2.03$) molecular weights of 40,000 (26) and 30,000 (27) have been reported for the less acid subfraction isolated by methods omitting the ethanol precipitation step used in the present investigations. It is likely that the ethanol precipitation destroys some of the tertiary structures in the soluble protein and therefore gives higher yields of the most acid subfraction than the methods reported above (26, 27).

No enzymic activity has been described for the soluble protein fraction of the chromaffin granules. It is therefore tempting to assume that this protein is in some way related to the storage of catecholamines. In the preliminary report (7) observations were reported in which epinephrine, ATP plus $Mg^{++}$ produced reversible changes in the physical properties of the nucleotide-free protein VI-1. The nucleotides still

firmly attached to the soluble protein V after extensive dialysis, could only be removed by gel filtration on Sephadex-G-75, and two-thirds of the nucleotide molecules removed from the protein by this procedure was recovered in the form of ATP. This indicates that ATP is strongly held by the soluble protein, and that its presence alters the physical properties of the protein. The possibility therefore might be suggested that the soluble protein in form of the ATP-protein complex is engaged in some way, not yet understood, in holding epinephrine in the storage position. This suggestion will be the subject of further investigations.

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