A Specific Soluble Protein from the Catecholamine Storage Vesicles of Bovine Adrenal Medulla

I. Purification and Chemical Characterization

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SUMMARY

The soluble proteins obtained by osmotic lysis of the catecholamine storage vesicles have been purified by chromatography on DEAE-cellulose and Sephadex G-200. The major protein fraction (S_1) appears to be highly purified: it sedimented as a single homogeneous fraction in the ultracentrifuge; it gave, with few exceptions, a single protein spot upon electrophoresis at different pH values, and its amino acid composition and fingerprints of tryptic digests were constant from preparation to preparation. Immunological and enzymic analysis of this protein indicated that it contained less than 1% of highly immunogenic contaminants, one of which was dopamine-β-oxidase.

In addition to the major protein fraction, two other protein peaks were obtained from the Sephadex G-200 column. One of these (S_2) appeared homogeneous in the ultracentrifuge and had an $s_{25,w}$ of 1.4 at a protein concentration of 3 mg/ml. This fraction was less pure than S_1.

Studies of the amino acid composition, fingerprints of tryptic digests and the molecular weight of S_1 indicate that the protein may consist of two identical subunits having a molecular weight of 40,000. The data are consistent with a unit structure of (a) a single peptide chain with one intrachain disulfide bond, or (b) two or more different chains with one intra- or one interchain disulfide bond.

Studies of the binding capacity of S_1 showed that it did not, by itself, or in the presence of Mg^{++} and ATP, bind sufficient amounts of catecholamines to be able to account for the stability of a significant fraction of the catecholamines within the storage vesicles.

INTRODUCTION

Most of the adrenaline and noradrenaline of adrenal glands can be isolated in membrane-limited vesicles, the catecholamine storage vesicles, by differential centrifugation of medulla homogenates (1, 2). The vesicles are osmotically sensitive, and the catecholamines and other vesicle contents can be released by hypotonic lysis (2, 3). After lysis, approximately 80% of the total protein of the vesicles can be recovered in the supernatant fraction following removal of vesicle debris by centrifugation for 20 min at 25,000 *g* (2). Findings that the crude protein was quite acidic prompted theories that the protein was involved in the catecholamine storage process by forming a nondiffusible complex with catecholamines, ATP and Mg^{++} (4). The present paper is concerned with the purification of the sol-

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uble protein components obtained by hypotonic lysis of the storage vesicles and with the chemical characterization of the major component and its ability to bind catecholamines. Preliminary reports on the purification and properties of these proteins have appeared from this and other laboratories (5–8). The purification procedure recently described by Helle (9), and the properties of the protein thus obtained, differ significantly from those reported here.

METHODS

Protein concentration was measured spectrophotometrically at 280 mμ. Sedimentation velocities were determined in a Model E Spinco at 25°C and expressed as $s_{25,w}$ values. Starch gel electrophoresis was performed according to Smithies (10). Tryptic fingerprints were prepared by the method of Ingram (11) as modified by Kimmel *et al.* (12). The tryptic digestion was performed on a 1% protein solution using trypsin (Worthington Biochemical Corporation lot 591) in an amount equal to 1% of the substrate protein. The pH of the incubation was maintained between 8 and 9 during digestion by addition of 0.1 *N* NaOH. A time series indicated that 2 hr was sufficient to complete the digestion. Electrophoresis at 1200 volts for 1 hr was enough to move the fastest peptides almost to the edge of the paper. After chromatographic development in pyridine:butanol: acetic acid:water 10:15:3:12 for 13 hr in the second dimension, the spots were made visible by spraying with ninhydrin and allowing to stand at room temperature. Amino acid analyses were obtained by the method of Spackman *et al.* (13). Cysteine was measured as cysteic acid according to Moore (14). Tryptophan in the protein was measured photometrically by the method of Spies and Chambers (15). Tryptophan-tyrosine ratios were obtained spectrometrically in 0.1 N NaOH according to Bencze and Schmid (16). Dopamine-β-oxidase was assayed by determining fluorometrically the amount of noradrenaline produced in 15-minute incubations at 37° under O_2 in a medium containing 2 μmoles dopamine, 5 μmoles ascorbic acid, 10 μmoles fumarate, 10 μmoles ATP, 100 μmoles phosphate buffer, pH 5.5, and 0.1 ml enzyme in a total volume of 1 ml.

RESULTS

Isolation of Protein

Catecholamine storage vesicles from bovine adrenal medullas were prepared according to Hillarp (17). Typically, one hundred glands were used and yielded about 250 g of medulla. The vesicles were lysed by suspension in 5 volumes of ice cold distilled water which liberated catecholamines and large quantities of soluble protein. Insoluble material was removed by centrifugation at 25,000 *g* for 20 min. The protein was lyophilized, resuspended in water, and separated from catecholamines and other small molecules by passage through a 5 × 40 cm column of Sephadex G-25 which was previously equilibrated in 0.005 M phosphate at pH 7.4. The protein was lyophilized, resuspended in 0.001 M phosphate, pH 7.4, and dialyzed several hours against 0.001 M phosphate pH 7.4. Sedimentation velocity centrifugation of this protein solution revealed heterogeneity; but most of the protein sedimented within a narrow (1 s to 3 s) velocity range. The protein was then chromatographed on DEAE-cellulose equilibrated with 0.01 M phosphate, pH 7.4. A 0 to 0.8 N NaCl linear gradient (1200 ml) in 0.01 M phosphate buffer pH 7.4 was used to elute the protein. Usually about 1 g of protein in 20–30 ml was applied to a 2.5 × 50-cm column. The resulting elution pattern is shown in Fig. 1. The first peak, which did not bind to the DEAE-cellulose, was largely hemoglobin. Two or three minor peaks were usually obtained. One of these, as indicated in Fig. 1, was found to contain dopamine-β-oxidase of surprisingly high specific activity (in one run about 2 μmoles noradrenaline produced 15 min/mg protein). The largest peak comprised about 60–70% of the eluted protein. This peak contained the protein of interest in this paper. A third small peak was sometimes eluted after the large peak. The large protein peak was usually then prepared for application to Sephadex G-200. More

recently, however, the protein was rechromatographed on DEAE-cellulose (just as above except that dialysis was substituted for Sephadex G-25 filtration) in order to remove more of the minor components which trail into the region of the major peak. Figure 1 shows the pattern of the second DEAE column. The large peak was pooled, dialyzed, against 0.01 M phosphate buffer pH 6.8, lyophilized and applied in as

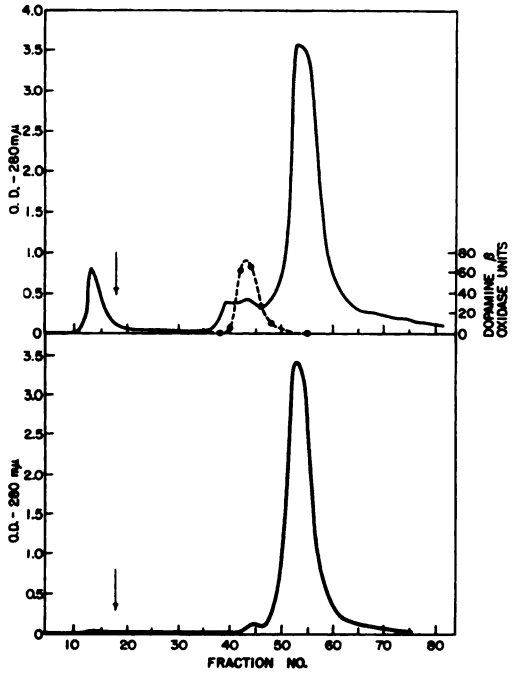

FIG. 1. *Chromatography of soluble vesicle protein on DEAE-cellulose*

Top: Crude protein fraction obtained from Sephadex-G-25 column. *Bottom:* Rechromatography of major DEAE-cellulose fraction. Broken lines indicate location of dopamine-β-oxidase activity. Arrows indicate beginning of gradient elution.

small a volume as possible (10–20 ml) to a 5 × 95 cm Sephadex G-200 column previously equilibrated with 0.4 N NaCl in 0.01 M phosphate buffer, pH 6.8. A fine nylon mesh applicator was used to obtain a sharp leading boundary and to prevent inversion of the dense protein solution into the gel. Column flow rate was about 10–20 ml per hour. The gel filtration pattern obtained is shown in Fig. 2. The small peak that emerged in the exclusion water was, mostly, a yellow colored insoluble material. The first major peak (S_1) comprised about 50% of the eluted material with peak II (S_2) and peak III (S_3) containing most of

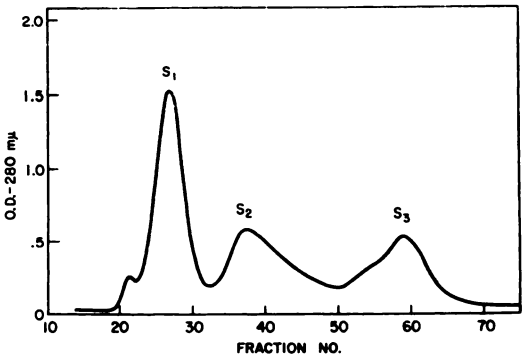

FIG. 2. *Chromatography of major DEAE-cellulose fraction on Sephadex G-200*

the rest. For subsequent analysis only the middle fractions of peak I were used and usually represented 15–30% of the soluble protein obtained from the granules. All fractionation procedures were carried out at 5°.

Criteria of Purity

The S_1 protein was found to exhibit a high degree of homogeneity as measured by several criteria. Rechromatography on Sephadex G-200 yielded only one peak. Sedimentation velocity centrifugation showed one peak with no detectable impurities Fig. 3). The $s_{25,w}$ at pH 6.8 extrapolated to zero protein concentration was 3.8.[3] The sedimentation velocity was highly dependent upon the protein concentration and the salt concentration of the solvent and will be described more fully in a subsequent report. The S_2 protein also appeared homogeneous in the ultracentrifuge and had an $s_{25,w}$ of 1.4 at a protein concentration of 3 mg/ml.[3]

Electrophoresis of the S_1 protein on starch gel at pH 7.4, on cellulose acetate at pH 6.0 and on polyacrylamide gel at pH 8.9 usually gave only one band of protein, but sometimes trace amounts of an impurity were indicated by a very faint slower-

[3] A. G. Kirshner, unpublished observation.

moving band. Amino acid analyses of several different preparations (Table 1) gave identical compositions within experimental error and fingerprints of tryptic digests (Fig. 4) were as reproducible as those of other pure proteins. However, immunological analysis of the protein[4] showed the presence of two or three very minor contaminants. By microcomplement fixation,

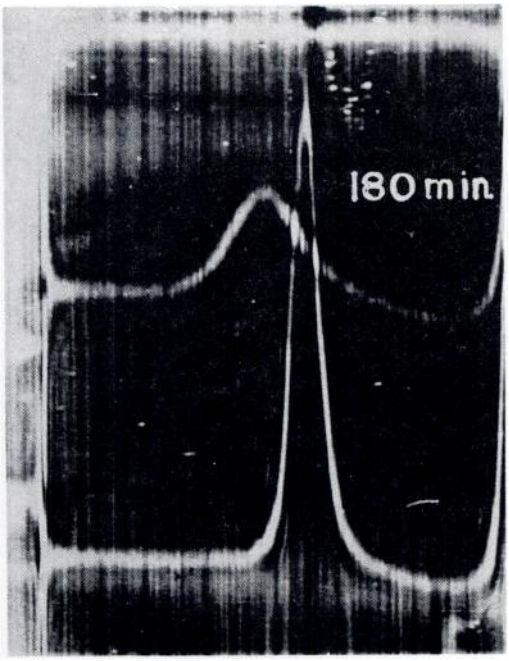

FIG. 3. *Sedimentation patterns of S_1 (bottom) and S_2 (top) in 0.05 M phosphate pH 6.8*

Protein concentrations were 7 and 3 mg/ml, respectively, for S_1 and S_2. Rotor speed was 59,780 rpm at 25°. Direction of sedimentation is from left to right.

these contaminants were estimated to represent less than 1% of the total protein. Immunological and enzymic analyses have identified dopamine-β-oxidase as the major contaminant.[4] From the activity of the enzyme in our preparation and from the specific activity of the enzyme reported by Friedman and Kaufman (18), it was calculated that dopamine-β-oxidase represented from 0.3 to 0.5% of the total protein.

[4] H. J. Sage, N. Kirshner, and W. J. Smith, in preparation.

Molecular Weight

A previous estimate (8) of 39,000 for the molecular weight of the S_1 protein was based on preliminary experiments using sedimentation equilibrium and amino acid analysis. Further studies showed that the protein was not stable under the conditions normally employed in the short-column Yphantis method (19) for sedimentation equilibrium. After about 18 hours the curvature of the interference pattern began to decrease, and after 30–36 hr it had almost completely disappeared. Therefore, the molecular weight was redetermined by osmometry using a high speed membrane osmometer (Mechrolab model 503, F and M Division of Hewlitt-Packard, Avondale, Pennsylvania). In either 0.3 M NaCl or 0.1 M $MgCl_2$ the molecular weight was found to be 80,000. If the weight determined by sedimentation equilibrium is approximately correct, then the data obtained by osmometry indicate that under the conditions employed for the sedimentation equilibrium studies dissociation into subunits occurred or that association of subunits occurred under the conditions used for osmometry. Further studies of the molecular weight are in progress and will be reported subsequently. The fingerprints of tryptic digests indicate a unit of repeating subunit structure having a molecular weight of 40,000. For this reason the amino acid composition was calculated on a molecular (or subunit) weight of 40,000.

Amino Acid Composition

Table 1 gives the amounts of each amino acid found in 12, 24, and 48-hr hydrolyzates of two different preparations of S_1. Either the maximum amount of each amino acid released in the time series or the average amount was chosen to most accurately represent the amounts actually present in the protein. The number of residues was then calculated by assuming the molecular weight of 40,000 for the protein. On the basis of the molecular weight, tryptophan was photometrically estimated at 5 residues. In addition, two residues of cysteine were estimated on the amino acid analyzer

TABLE 1

Amino acid analysis of S_1 protein

Experiments 1 and 2 were performed on different preparations of S_1 protein and on different modifications of the amino acid analyzer which required different quantities of protein for analysis. The protein was hydrolyzed in constant boiling HCl at 110° for the times indicated in sealed glass ampules.

Amino acid residue	Expt. 1 μMoles of amino acid				Expt. 2 μMoles of amino acid				Expt. 1 Residues per 40,000 g	Expt. 2 Residues per 40,000 g	Average of Expt. 1 and Expt. 2 Residues per 40,000 g
	12 hr	24 hr	48 hr	Value chosen	12 hr	24 hr	48 hr	Value chosen			
Lys	0.452	0.536	0.545	0.545	0.0822	0.0829	0.0685	0.0829	33	30	32
His	0.075	0.076	0.104	0.104	0.0153	0.0147	0.0160	0.0160	6	6	6
Arg	0.191	0.275	0.293	0.293	0.0444	0.0491	0.0483	0.0487	18	18	18
Asp	0.489	0.468	0.478	0.489	0.0792	0.0809	0.0806	0.0807	30	30	30
Thr	0.164	0.167	0.158	0.167	0.0264	0.0272	0.0268	0.0272	10	10	10
Ser	0.485	0.422	0.402	0.485	0.0723	0.0746	0.0698	0.0746	29	27	28
Glu	1.351	1.353	1.39	1.39	0.2350	0.2320	0.2390	0.2350	84	86	85
Pro	0.452	0.536	0.533	0.536	0.0946	0.0939	0.0912	0.0943	32	34	33
Gly	0.418	0.392	0.389	0.418	0.0670	0.0679	0.0679	0.0679	25	25	25
Ala	0.505	0.480	0.478	0.505	0.0806	0.0822	0.0812	0.0819	30	30	30
Val	0.200	0.219	0.229	0.229	0.0348	0.0312	0.0368	0.0368	14	14	14
Met	0.070	0.100	0.094	0.100	0.0195	0.0186	0.0236	0.0205	6	7	7
Ile	0.062	0.076	0.079	0.079	0.0127	0.0100	0.0136	0.0136	5	5	5
Leu	0.378	0.353	0.365	0.378	0.0635	0.0630	0.0648	0.0648	23	24	23
Tyr	0.045	0.047	0.056	0.056	0.0092	0.0079	0.0081	0.0083	3	3	3
Phe	0.078	0.073	0.069	0.078	0.0121	0.0140	0.0117	0.0140	5	5	5
Cys/2									2	2	2
Try									5	5	5
									360	361	361

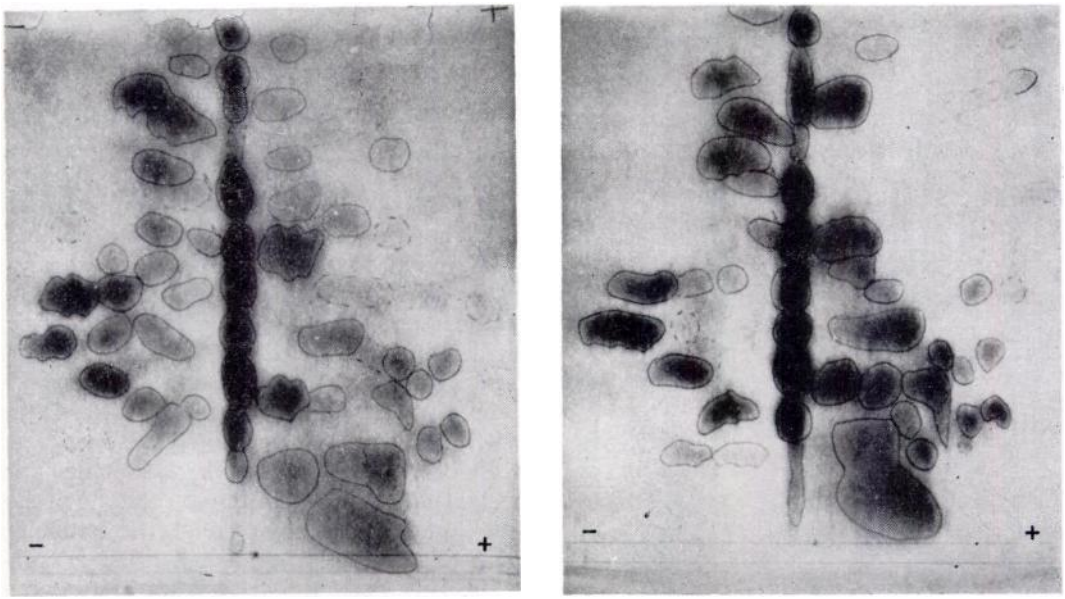

FIG. 4. *Fingerprints of tryptic digests of S_1 (right) and S_2 (left)*

TABLE 2

Comparison of amino acid analyses of S_1, S_2, and IV-1 preparation of Helle (9)

The values for S_1 protein are those from Table 1. The values for S_2 protein are from one 24-hr hydrolyzate. The values of Helle (9) for her preparation IV-1 are converted from residues per 25,000 g to residues per 40,000 g.

Amino acid	Residues/40,000 g			Weight per cent		
	S_1	S_2	Helle, IV-1	S_1	S_2	Helle, IV-1
Lys	32	28	27	10.2	8.9	8.4
His	6	6	8	2.0	2.0	2.7
Arg	18	18	26	7.0	7.0	9.9
Asp	30	29	30	8.5	8.2	8.3
Thr	10	7	11	2.5	1.8	2.7
Ser	28	25	29	6.1	5.4	6.1
Glu	85	105	83	27.3	33.7	26.0
Pro	33	39	34	8.0	9.4	8.0
Gly	25	32	32	3.5	4.5	4.4
Ala	30	39	32	5.3	6.9	5.5
Val	14	10	14	3.5	2.5	3.4
Met	7	4	5	2.3	1.2	1.6
Ile	5	1	5	1.4	0.3	1.4
Leu	23	19	27	6.5	5.4	7.4
Tyr	3	3	5	1.2	1.2	2.0
Phe	5	4	6	1.8	1.5	2.2
Cys/2[a]	2	—	0	0.6	—	0
Try[b]	5	—	—	2.3	—	—
	361	369	374	100.0	99.9	100.0

[a] Determined as cysteic acid.

[b] Determined colorimetrically on separate sample.

as cysteic acid. The acidic amino acids were present in much larger amounts than the basic amino acids. Aspartic and glutamic acid represented 113 of the 361 residues; arginine and lysine 47. Proline was also present in unusually large amounts representing 9% of the total number of residues.

The amino acid composition of S_2 was determined only after one 24-hr hydrolysis. Since data are lacking on the molecular weight of this protein, its relative amino acid composition was calculated on the basis of molecular weight of 40,000 to show its similarity to S_1. The data reported by Helle (9) were also converted to a molecular weight basis of 40,000 and are shown in Table 2. Since extensive studies on S_2 were not carried out, its chemical purity is somewhat questionable. However, its relative amino acid composition is similar to that of S_1. This similarity and the $s_{25,w}$ values of peaks S_1 and S_2 suggested the possibility that S_2 may consist of subunits of S_1.

Fingerprints of Tryptic Digests

Fingerprints of S_1 and S_2 were prepared to obtain further information on the structure and relationship of these two proteins. From the number of lysine and arginine residues in the S_1 protein one would expect a maximum of 48 ninhydrin spots for a single peptide chain having a molecular weight of 40,000. Fingerprints of S_1 gave 46 ninhydrin spots (Fig. 4), indicating that it consisted of: (a), a single polypeptide chain having a molecular weight of 40,000; or (b), two or more peptide chains having a combined molecular weight of 40,000; or (c), identical subunits of either a or b. Studies now in progress should resolve these alternatives.

Fingerprints of S_2 yielded about 51 spots, and the pattern was largely similar to that of S_1. However, a number of spots on S_2 fingerprints were not present on S_1 fingerprints and the same was true for the reverse comparison. These differences would indicate that S_2 did not consist of subunits of S_1, but the large number of common spots and the similar amino acid composition indicate many similar amino acid sequences in the two proteins.

Studies of Sulfhydryl Groups

The sulfhydryl groups of S_1 were not titratable with *p*-hydroxymercuribenzoate (PMB) as measured spectrophotometrically (20) under a variety of conditions. In salt concentrations (0.01 M and 0.4 M NaCl) which caused marked differences (7) in the $s_{25,w}$ value of the protein no titratable —SH groups could be detected. When butanol was added to the titration medium at a concentration of 0.25 M in an effort to penetrate and expand any hydrophobic pockets in the protein which might contain —SH groups, no reaction with PMB could be detected. Finally the protein was digested with enzymic amounts of trypsin before

titrating with PMB. Still no free —SH groups could be detected. The cysteine residues in the protein are therefore presumed to be present in disulfide linkage.

Molar Absorbance of the Protein

Dry weights of S_1 were determined so that optical density measurements at 280 mμ could be related to the protein concentration. Duplicate samples of S_1 solution in tared glass weighing bottles were dried at 107° for 6 hr and then weighed and replaced in the oven until constant weight was attained. It was found that a 1 mg/ml solution exhibited an optical density of 0.708 at 280 mμ. A molecule of 40,000 molecular weight would then have a molar extinction of 28,320.

Spectrophotometric Estimation of Tyrosine Tryptophan Ratio

The tyrosine:tryptophan ratio of S_1 was estimated spectrophotometrically (16) at pH 13 in several preparations. When the protein was diluted in NaOH and immediately assayed, the ratio was found to be relatively constant at 0.4. However, it was found that at least one of the tyrosines in the protein ionized slowly at pH 13 and that maximum absorbance was attained 12–24 hours after titration to pH 13. The tyrosine:tryptophan ratio thus obtained was 0.57, which is in good agreement with the ratio of 0.6 calculated from the tyrosine content determined by the amino acid analyzer and from the tryptophan content determined by colorimetric assay (15).

From the 5 tryptophans, 3 tyrosines, and 2 cysteines (assuming the 2 cysteines are present as cystine) per protein molecule or subunit of 40,000 molecular weight, the extinction of the protein at 280 mμ in neutral solution was calculated by adding 5550 O.D. units/tryptophan, 1340 O.D. units/tyrosine, and 150 O.D. units/cystine (21). The value of 31,920 obtained by this method agrees well with the value of 28,320 obtained experimentally.

Catecholamine Binding Studies

The ability of the purified S_1 protein to bind catecholamines was of interest since it has been proposed that the storage mechanism involves a nondiffusible catecholamine–protein–ATP–Mg^{++} complex (4). The capacity of the S_1 protein to bind catecholamines was investigated by equilibrium dialysis as described in Table 3.

From experiment 1 it can be seen that the S_1 protein, under our experimental conditions, can establish a concentration gradient with adrenaline about 2.5 times that found with bovine serum albumin and on a molar basis can bind 14–16 times as much as bovine serum albumin. At lower concentrations of adrenaline the concentration gradient was steeper but much less of the amine was bound (1b).

When ATP and Mg^{++} were present in the same concentrations as adrenaline (1d, 3b) the binding of adrenaline was slightly decreased. However, a large excess of Mg^{++} (2c) or NaCl (2d) markedly decreased the binding. The concentration of phosphate buffer also affected the amount of adrenaline bound. In 0.5 mM phosphate 50–60% more adrenaline was bound than in 1.0 mM phosphate. When the concentration of adrenaline added to the external solutions was increased to 100 mM, no concentration gradient was detectable. Under the conditions of these experiments the binding capacity appeared to be saturated at an external adrenaline concentration of 1 mM (3a,c). The binding was readily reversible. Protein solutions equilibrated with ^{14}C-adrenaline released nearly all the label when dialyzed against a 0.5 mM phosphate buffer.

The data from experiment 4 (Table 3) indicate that the binding is relatively nonspecific. D-Adrenaline, L-adrenaline, and tyramine were bound equally well; dopamine was bound slightly better; and noradrenaline best. If one compares the binding of noradrenaline and adrenaline, of noradrenaline and dopamine, and of dopamine and tyramine, it appears that the primary amino group of noradrenaline contribute more to the binding than the substituted amino group of adrenaline and that the β-hydroxyl group and the catechol group are also involved. Because of the limited amount of S_1 protein available, ex-

TABLE 3

Binding of amines to S_1 protein

The ^{14}C-adrenaline, ^{14}C-noradrenaline, ^{14}C-dopamine and ^{14}C-tyramine used in these studies were obtained from New England Nuclear Corporation. The ^{14}C-adrenaline and ^{14}C-noradrenaline were racemic mixtures and were diluted with unlabeled D-noradrenaline or D-adrenaline except in 4b where L-adrenaline was used. The optical configuration refers to the configuration of the added unlabeled isomer. The general procedure for the equilibrium dialysis studies was as follows: 0.2 ml of S_1 protein solution containing 60 mg of protein/ml buffered at pH 6.4 with either 1 mM phosphate (Expt. 1) or 0.5 mM phosphate (Expt. 2, 3, 4) was placed inside a dialysis sack (Visking Size 8DC) and dialyzed for 24 hr at 5° against the corresponding phosphate buffer containing the compounds at the concentrations listed in the table. In experiments 2, 3, and 4, ATP, NaCl, and Mg^{++}, where indicated, were also added at the beginning of the experiment to the inside of the dialysis sack. In all cases the amines were placed only in the outside solutions. At the end of the experiment aliquots of the solutions from inside and outside the sack were assayed for radioactivity. The concentrations were calculated from the cpm of the material inside the sack and the specific activity of the compounds in the dialyzate.

In experiment 3 nitrogen was bubbled through the external solution throughout the period of dialysis; in the other experiments the solutions were exposed to air. The concentration gradient in the table is the ratio cpm/ml inside solution: cpm/ml dialyzate. The moles of amine bound to protein were calculated by subtracting the concentration of amines in the external solution from the concentration in the dialysis sack. At the end of the experiment the volume of solution inside the dialysis sack had increased to give a protein concentration of 40 mg/ml.

Expt. No.	Additions	Conc. gradient	Moles amine / moles protein
1	a Adr., 1 mM	2.4	1.4
	b Adr., 0.1 mM	3.6	0.3
	c Adr., 1 mM	2.6	1.6
	d Adr., ATP, Mg, 1 mM each	2.5	1.4
	e Adr., 1 mM, bovine serum albumin instead of S_1 protein	1.1	0.1
2			
	a Adr., 1 mM	3.1	2.1
	b Adr., ATP, 1 mM each	2.9	1.9
	c Adr., 1 mM, Mg^{++} 20 mM	1.3	0.3
	d Adr., 1 mM, NaCl 100 mM	1.5	0.5
3	a Adr., 1 mM	3.3	2.3
	b Adr., ATP,M Mg, 1 mM each	2.9	1.9
	c Adr., 10 m	1.2	2.0
	d Adr., 100 mM	0.9	
	e Adr., ATP ,Mg, 100 mM each	0.8	
4	a D-Adr., 1mM	3.5	2.5
	b L-Adr., 1mM	3.7	2.7
	c D-Nor., 1 mM	5.6	4.6
	d Dopamine, 1 mM	4.2	3.2
	e Tyramine, 1 mM	3.2	2.2

tensive studies to delineate the various factors which contribute to binding were not conducted.

DISCUSSION

The procedure described here for the purification of the major soluble protein component of the catecholamine storage vesicles gave a protein preparation that appeared homogeneous by the criteria of sedimentation velocity centrifugation, by electrophoresis at several different pH values and in different supporting media, and by constancy of amino acid composition and fingerprints of tryptic digests. However, immunological analysis[4] has shown that the protein contained 2 or 3 minor contaminants which account for less than 1% of the total protein.

The molecular weight of the S_1 protein

appears to be either 40,000 or 80,000. Assuming a molecular weight of 40,000, then the amino acid composition, the fingerprints of tryptic digests, and the nonreactivity of the protein with PMB is consistent with (a), a molecule composed of a single peptide chain with one intrachain disulfide bond, or (b), a molecule composed of two or more dissimilar chains with either one intra- or one interchain disulfide bridge. If the molecular weight were 80,000, then the data would indicate that the molecule was made up of two identical subunits of either a or b.

The purification procedure described here differs from that of Helle (9) in several ways and provides a highly purified preparation of the major soluble protein of the storage vesicles. In our procedure separation of the storage vesicles from other particulate matter by density gradient centrifugation and isoelectric precipitation of the protein in alcohol is avoided. Chromatography on Sephadex G-200 of the large protein fraction obtained from the DEAE-cellulose column resolves these proteins into the purified major component and at least two other smaller fractions.

The apparent molecular weight of 25,000 reported by Helle (9) is not consistent with the fingerprints of tryptic digests reported here. On a molecular weight basis of 25,000 the molecule contains a total of 33 arginine plus lysine residues. Fingerprints of tryptic digests of such a molecule would provide a maximum of $33 + n$ ninhydrin spots (where n = the number of peptide chains). The fingerprints we obtained contained 46 ninhydrin spots, which is consistent with a molecular or subunit weight of 40,000. The molecular weight obtained by Helle (9) may have been due to dissociation of the molecule into subunits as a result of the isoelectric precipitation in alcohol. Early attempts in our laboratory to fractionate the crude protein in alcohol always gave a preparation which had a low $s_{25,w}$ (1.4). Studies on the subunit structure of the major component are in progress.

If the data presented by Helle (9) on the amino acid composition of the protein are converted to a molecular weight basis of 40,000, they agree remarkably well with our data on the composition of the S_1 protein (Table 2). The only significant differences are that Helle (9) finds 8, 7, and 4 more residues, respectively, of arginine, glycine, and leucine and 5 fewer residues of lysine. Smaller differences of two amino acid residues were also present among several other amino acids. The similarity in the amino acid composition between the two protein preparations and the difference in the molecular weights obtained by Helle (9) and that reported here are consistent with the view that the former author's preparation consisted largely of denatured fragments of the S_1 protein.

Although it has been proposed that the catecholamines of the adrenal medulla are held within the storage vesicles by the formation of a complex with other intravesicular components (4), there is little direct evidence to support this concept. At various times it was proposed that the complex consisted of catecholamines and ATP; catecholamines, ATP, and Mg; catecholamines, ATP, Mg^{++}, and protein (4, 22–24), and catecholamines, ATP, Mg^{++}, protein, RNA, and Ca^{++} (25). In early experiments Carlsson and Hillarp (26) could find no evidence for an interaction among the various components of the vesicles. Subsequently Weiner and Jardetsky (27) using nuclear magnetic resonance did show an interaction between catecholamines and ATP. Colburn and Maas (28) also obtained evidence that ATP, Mg^{++}, and catecholamines formed complexes in solution, but these were readily reversible and could not account for the stability of the storage mechanism.

The evidence for the existence of a storage mechanism is compelling (3). The concentration of catecholamines and ATP within the vesicles is about 0.5 M and 0.125 M, respectively, yet the vesicles are stable in isotonic media, indicating that catecholamines and ATP are osmotically inactive. At temperatures of 1° to 5° the vesicles can maintain their contents for several days, indicating that no energy source is required for maintaining the very high concentration difference. Also at 1° to 5° the mem-

brane is readily permeable to adrenaline, but added adrenaline does not exchange with endogenous adrenaline, nor does it become bound (26, 29). At higher temperatures, 30° for example, the vesicles can take up catecholamines and store them in a manner indistinguishable from their endogenous contents by a process which is stimulated by Mg^{++} plus ATP and inhibited by reserpine and by sulfhydryl reagents (30, 31). However, when the vesicles are placed in a hypotonic medium, the catecholamines are immediately released. Catecholamines are stored in the vesicles in a nondiffusible manner but the nature of the storage mechanism is entirely obscure.

The studies on the binding of catecholamines by the major soluble protein component (S_1) of the vesicles indicate that this protein, by itself, or in the presence of ATP and Mg^{++} cannot bind sufficient amounts of catecholamines to account for a significant fraction of the catecholamines stored within the vesicles. Immunological assays of the S_1 protein[4] showed that the ratio of catecholamines:S_1 protein in the storage vesicles is approximately 1 on a weight basis. Based on a molecular weight of 40,000 for the S_1 protein, the molal ratio of catecholamines:S_1 in the storage vesicles is 200. Thus, the binding we observed would account for only 1–2% of the stored catecholamines. If the storage mechanism consisted of a complex of adrenaline and one or more of the intravesicular components, the experimental observations would require that this complex, once formed, should not readily dissociate or exchange with added catecholamines at 0–5°. From the studies reported here and by others (4, 25, 27, 28) it would seem that the mechanism of storage cannot be accounted for solely by complex formation of catecholamines with other components of the vesicles, but must also involve, in some unknown manner, the integrity of the vesicle membrane.

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