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Purification and Properties of an Acidic Protein from Chromaffin Granules of Bovine Adrenal Medulla

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1. A soluble protein has been purified from an aqueous extract of bovine adrenal chromaffin granules by chromatography on Sephadex G-200. This protein comprises 25% of the total protein of the granules and gave a single band on gel electrophoresis. 2. The protein is unusually rich in acidic amino acids, notably glutamic acid (26·0%, w/w); it is also relatively rich in proline (8·6%, w/w) but poor in cystine (0·35%, w/w). 3. A molecular weight of 77000 was obtained from sedimentation and diffusion measurements on the protein, and approach-to-equilibrium measurements gave apparent molecular weights of the same order. 4. A molecular weight 7 times that given above was estimated from the results of chromatography on a column of Sephadex G-200 that had been calibrated with globular proteins. However, good agreement between the ultracentrifuge and Sephadex experiments was obtained on the assumption that Sephadex chromatography depends on the effective hydrodynamic radii of proteins and not on their molecular weights. 5. The hydrodynamic properties of the protein differed from those of a typical globular protein. Thus the protein had a high intrinsic viscosity, a high frictional ratio and a large effective hydrodynamic volume. 6. The hydrodynamic properties of the protein, but not its molecular weight, were dependent on the ionic strength of the solvent. Increasing the ionic strength caused an increase in the sedimentation and diffusion coefficients, but a decrease in the intrinsic viscosity and in the frictional ratio of the protein. 7. Optical-rotatory-dispersion measurements indicated that only a small part of the polypeptide chain was in an α-helical conformation. 8. These results are compatible with the protein's having a conformation approaching that of a random-coil polypeptide, the volume occupied by the molecule being determined by electrostatic repulsion between the excess of negative charges.

Chromaffin granules contain, in addition to the catecholamines, high concentrations of ATP and water-soluble proteins (Blaschko, Born, d'Iorio & Eade, 1956). Hillarp (1958*b*) found that 77% of the protein from bovine chromaffin granules was recovered in a high-speed supernatant after lysing the granules in hypo-osmotic solution. Chromaffin granules from horse and pig adrenals also contain a high proportion of soluble protein (Winkler, Ziegler & Strieder, 1966). It has been suggested (Hillarp, 1958*a*; Blaschko, 1960) that the soluble proteins, together with ATP, might be involved in the binding of the catecholamines within the chromaffin granules. The first studies on the soluble proteins were reported by Blaschko & Helle (1963), who achieved a partial purification of one of the proteins. More recently it has been found that the main component of the soluble proteins is secreted from the perfused bovine adrenal gland when the gland is stimulated to release catecholamines (Banks & Helle, 1965).

The present paper describes the purification and some physical and chemical properties of the major component of the soluble proteins from chromaffin granules of the bovine adrenal medulla. Preliminary communications describing some of these results have already been published (Smith & Winkler, 1965; Blaschko, Smith & Winkler, 1966).

METHODS

Tris–sodium succinate buffer. For much of the work described in this paper a buffer was required that had the following properties: a pH below 6·5 (to avoid rapid oxidation of catecholamines), no absorption of ultraviolet light and containing no phosphate. A satisfactory buffer was found by titration of an equimolar mixture of tris and succinic acid with NaOH. The buffer had pH 5·9 and I approx. 0·3; it was composed of tris (0·1 M), succinic acid (0·1 M) and NaOH (0·09 N). For some experiments the buffer was diluted 20-fold to give I approx. 0·015.

Preparation of a soluble lysate of chromaffin granules. Chromaffin granules were isolated from an homogenate of bovine adrenal medullae by the method described in the

preceding paper (Smith & Winkler, 1967). Each pink pellet of chromaffin granules was suspended in 1 ml. of ice-cold tris–sodium succinate buffer, pH 5·9 and I 0·015, to lyse the granules. In the centrifugal data given below the value of g was calculated by using the radius from the centre of rotation to the bottom of the tube, and the centrifugal force is given as g-min. The suspensions of lysed granules were pooled, left for 15 min. and then centrifuged at $22 \times 10^5 g$-min. (A40 head, Spinco ultracentrifuge, 2°). The supernatant was decanted and kept ice-cold; the sediment was resuspended in about twice its volume of the tris–sodium succinate buffer and centrifuged at $22 \times 10^5 g$-min. This procedure was repeated once more, and the combined supernatants were finally centrifuged at $66 \times 10^5 g$-min. to remove traces of insoluble material: the clear supernatant is called the soluble lysate. The soluble lysate was either used immediately or stored at −20°.

Gel electrophoresis. Vertical starch-gel electrophoresis was carried out with an apparatus of the kind described by Smithies (1959), except that platinum electrodes were employed. The discontinuous buffer system of Poulik (1957) was used. Polyacrylamide-gel electrophoresis was performed by the simplified procedure of Clarke (1964).

Sephadex chromatography. Dry Sephadex (bead type) was sieved and that fraction passing through 120 mesh (U.S. standard sieve) and remaining on 200 mesh was used for chromatography. Sephadex types G-25, G-75 and G-100 were suspended in tris–sodium succinate buffer, pH 5·9, and allowed to swell for 3 days at 4°. Sephadex G-200 was swollen for 2–3 months before use; $CHCl_3$ (0·05 ml./l.) was added to the suspension as a preservative and was also present during chromatography. For experiments with buffers of I greater than 0·3, KCl was added to the tris–sodium succinate buffer.

Glass columns of two sizes were used: for analytical studies the columns were 150 cm. long × 1·8 cm. diam.; for preparative separations the columns were 150 cm. long × 4·8 cm. diam. The dead space below the Sephadex was kept as small as possible by using glass wool and a layer of acid-washed sand (0·5 cm.) at the base of the column. To avoid packing down of the Sephadex in the column, the hydrostatic pressure was kept below 100 cm. H_2O during both the pouring of the columns and their subsequent use. The upper surface of columns of Sephadex G-100 and G-200 was stabilized by a 1 cm. layer of Sephadex G-25. The volume of the sample applied to the columns was very small in relation to the capacity of the column: the maximum volumes employed were 4 ml. for the analytical columns and 12 ml. for the preparative columns. All chromatography experiments were carried out at 4°.

Fractions of volume 3 ml. were collected from the analytical columns and fractions of volume 6–8 ml. from the preparative columns. The fractions were analysed by their ultraviolet absorption and by means of the Folin reaction as modified by Lowry, Rosebrough, Farr & Randall (1951).

The results of Sephadex chromatography are expressed in terms of the column distribution coefficient, K_d, which has been defined by Gelotte (1960) in terms of the elution volume (V_e) of a substance, the void volume (V_0) of the column and V_i, the volume of water imbibed by the gel, thus:

$$V_e = V_0 + K_d V_i$$

The value of V_i was not determined directly, but was calculated from the elution volume of KCl.

Concentration of the fractions from the columns was achieved by ultrafiltration with an apparatus similar to that described by Sober, Gutter, Wyckoff & Peterson (1956). The ultrafiltration was carried out at 4°, with 8/32 Visking dialysis tubing that had been washed with double-distilled water.

Analytical ultracentrifugation. These experiments were carried out with a Spinco model E ultracentrifuge at a rotor temperature of 20°, and the changes in concentration were recorded by schlieren optics. All the measurements were made on protein solutions that had been concentrated by ultrafiltration and then dialysed against buffer solution. Tris–sodium succinate buffers, pH 5·9 and I approx. 0·015 and 0·3, were used.

Sedimentation coefficients were determined with standard cells containing Kel-F centre-pieces. The rotor speed was 59780 rev./min., and photographs were taken every 16 min. for a total of 144 min. The x value corresponding to the maximum of the schlieren peak was used in the calculations. The decrease in protein concentration at the boundary during the run was allowed for by calculating the sedimentation coefficient corresponding to the initial protein concentration, according to the procedure of Alberty (1954). For this purpose, the zero time for each run was determined by the method of Elias (1959). The sedimentation coefficients were corrected by the standard method (Svedberg & Pederson, 1940) to the value in water at 20°.

Diffusion measurements were made with the double-sector synthetic-boundary cell (valve type). The protein solutions were dialysed against an excess of buffer solution for 24 hr. at 4°, and the solvent outside the dialysis sac was used as the upper phase. The rotor speed was 10589 rev./min., and photographs were taken every 16 min. for 144 min. Measurements of height and area of the schlieren peak were made from × 10 enlargements of the negatives. The apparent diffusion coefficient at zero time was determined by a linear regression of at least six values of $D_{app.}$, and was corrected to water at 20° by the standard procedure.

Molecular-weight measurements were made by the approach-to-equilibrium method as described by Trautman & Crampton (1959), by using only the solution-to-air meniscus. For each experiment, 15–20 measurements of the protein concentration and concentration gradient were made and the best straight line was calculated by the method of least squares. After each experiment, the protein concentration was determined with a synthetic-boundary cell from the area under the schlieren peak, assuming a specific refractive increment of 186×10^{-5}.

Viscosity measurements. These were carried out with a rotating-cylinder viscometer (kindly lent by Dr I. O. Walker) of the kind described by Zimm & Crothers (1962). At least three measurements were made at each protein concentration. The temperature was 25° and the solvents were tris–sodium succinate buffers, pH 5·9 and I 0·015 and 0·3.

Optical-rotatory-dispersion measurements. A Bendix–Ericsson automatic recording spectropolarimeter (Polarmatic 62) was used to measure optical rotations at 25°. The slit widths were fixed at 1·0 mm. (entrance slit) and 0·8 mm. (exit slit). Rectangular cells with a path length of 4 cm. were used, and the protein concentration was in the range 0·3–0·5 mg./ml.

Amino acid analysis. Solutions of the purified protein were dialysed exhaustively against tris–sodium succinate

buffer, pH 5·9 and *I* 0·015, and a small volume (0·2–0·3 ml.), containing 4–6 mg. of protein, was placed in a hydrolysis tube. Then 3·0 ml. of constant-boiling HCl was added and the hydrolysis was carried out as described by Crestfield, Moore & Stein (1963) for 17 hr. at 110°. Chromatography and quantitative determination of the amino acids in the hydrolysate were performed in an automatic amino acid analyser (Evans Electroselenium Ltd., Halstead, Essex) similar in design and operation to that described by Moore, Spackman & Stein (1958) and Spackman, Stein & Moore (1958).

Materials. Blue Dextran 2000 and Sephadex cross-linked dextran gels were purchased from Pharmacia (Uppsala, Sweden). Tris was obtained under the name Trizma from Sigma Chemical Co. (St Louis, Mo., U.S.A.), and the same firm also supplied bovine serum albumin (type V), thyroglobulin, cytochrome *c*, bovine γ-globulin and urease (type C-2). All other substances were of analytical grade and were purchased from British Drug Houses Ltd. (Poole, Dorset).

RESULTS

Chromatography of the soluble proteins on Sephadex

A high-speed supernatant, called the soluble lysate, was prepared from chromaffin granules that had been lysed by osmotic 'shock', as described in the Methods section. The soluble proteins could be separated from the catecholamines and ATP, also present in the soluble lysate, by chromatography on columns of Sephadex G-75, G-100 or G-200. No significant fractionation of the proteins was obtained with Sephadex G-75 or G-100: the bulk of the protein was eluted in one peak at the void volume of the column. Some fractionation of the proteins was obtained by chromatography of the soluble lysate on Sephadex G-200, but the degree of fractionation depended markedly on the ionic strength of the buffer used as eluent. About 70% of the protein was eluted at the void volume of the column when the ionic strength of the buffer was between 0·15 and 0·95. However, when the ionic strength was decreased to 0·015, several peaks of ultraviolet-absorbing material were detected in the eluate. The result of a typical experiment with a column of Sephadex G-200 is shown in Fig. 1. The adrenaline and ATP were eluted, with other low-molecular-weight material, between 1·5 l. and 1·75 l. and gave rise to a large ultraviolet-absorbing peak with a distribution coefficient (K_d) of 1. Three other major ultraviolet-absorbing peaks were obtained, as well as several smaller peaks: the peaks have been numbered 1–7 in order of elution from the column.

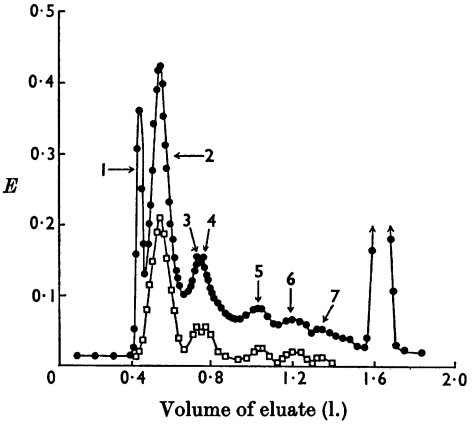

Fig. 1. Molecular-exclusion chromatography on Sephadex G-200 of soluble lysate. The column (150 cm. × 4·8 cm.) was equilibrated with tris–sodium succinate buffer, pH 5·9 and *I* 0·015, and, after 10 ml. of soluble lysate had been applied, it was eluted with the same buffer at a flow rate of 10 ml./hr. ●, E_{280}; □, E_{720} after treating 0·2 ml. of each fraction with Folin reagent.

Fractions from each peak were examined by vertical starch-gel electrophoresis and a diagram representing the results is given in Fig. 2. The results of this experiment confirmed that fractionation of the proteins had been achieved. Seven bands that were stained by Nigrosine were obtained on electrophoresis of the soluble lysate. The material from peak 2 gave a single densely staining, but rather diffuse, band that corresponded to the major component of the soluble lysate. Very similar results were obtained when fractions from the Sephadex column were examined by polyacrylamide-gel electrophoresis. In both methods the mobility of the proteins in the gel on electrophoresis was inversely related to their order of elution from the Sephadex column.

Calibration of the Sephadex column. The elution volumes of six compounds of known molecular

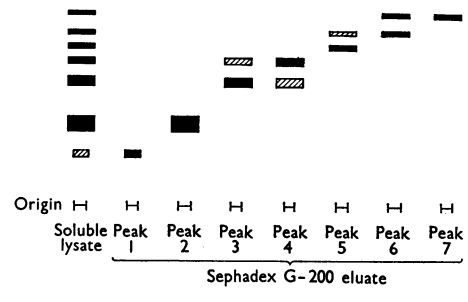

Fig. 2. Vertical starch-gel electrophoresis of soluble lysate and of fractions from the Sephadex G-200 eluate. The discontinuous buffer system (pH 8·3) of Poulik (1957) was used. Bands stained by Nigrosine (0·05%) are shown thus: ■, densely stained; ■, lightly stained. The material migrated towards the anode.

weight were determined in order to calibrate the Sephadex G-200 column according to the procedures given by Ackers (1964) and Andrews (1965). The column distribution coefficients were calculated from the results and are given in Table 1 along with the K_d values of the first two ultraviolet-absorbing peaks arising out of chromatography of the soluble lysate. The material of peak 1 was eluted ahead of Blue Dextran, which is widely used to determine the void volume of Sephadex columns (see the Discussion section). The protein giving rise to peak 2 was eluted after thyroglobulin (mol.wt. 670000) but before urease (mol.wt. 490000). The use of these observations to estimate the molecular weight of the protein in peak 2 is critically examined in the Discussion section.

Nature of the material from peak 1

The fractions from the column of Sephadex G-200 were analysed for protein with Folin's reagent by the method of Lowry *et al.* (1951). The results (Fig. 1) agreed very well with those obtained by measuring the ultraviolet absorption of the fractions except for peak 1: the material in this peak did not react with Folin's reagent. Further analysis of this material showed that it did not have an ultraviolet spectrum characteristic of either protein or nucleic acid, and that it contained organic phosphate. The organic phosphate could be extracted into chloroform by the method of Folch, Lees & Sloane-Stanley (1957) and this extract gave a positive hydroxamic acid test for carboxylic esters. Thin-layer chromatography of the chloroform extract established that it contained the following phospholipids: ethanolamine- and serine-containing phospholipids, lecithin, lysolecithin and sphingomyelin. Quantitative analysis by the method of Skipski, Peterson & Barclay (1964) revealed a phospholipid composition very similar to that of whole chromaffin granules (Blaschko, Firemark, Smith & Winkler, 1966); these particles are characterized by a relatively high content of lysolecithin.

Table 1. *Column distribution coefficients* (K_d) *of components of the soluble lysate compared with those of substances of known molecular weight*

The K_d values were calculated from the elution volume of each compound from a column (150cm.×4·8cm.) of Sephadex G-200 equilibrated with tris–sodium succinate buffer, pH 5·9 and I 0·015.

Substance	K_d
Peak 1 (soluble lysate)	0·0
Blue Dextran 2000	0·025
Thyroglobulin	0·032
Peak 2 (soluble lysate)	0·077
Urease	0·158
γ-Globulin (bovine)	0·207
Serum albumin (bovine)	0·403
Cytochrome *c*	0·641

Properties of the main component of the soluble proteins

The protein giving rise to peak 2 of the Sephadex G-200 eluate produced a single band in gel electrophoresis and was the major component of the soluble proteins. An estimate of the relative amount of the protein recovered in peak 2 was obtained from the area under the peak: it comprised $38 \pm 2\%$ ($n = 4$) of the total 280mμ-absorbing material, excluding peak 1. Some of the physical and chemical properties of this protein were determined and are described below. For these experiments the protein was obtained by pooling the fractions that formed the upper third of peak 2 in the eluate from the Sephadex G-200 column. The pooled fractions were concentrated by ultrafiltration as described under methods. Freeze-drying was not used as a means of concentrating the protein solutions, since this caused a change in the electrophoretic mobility of the protein.

Sedimentation-velocity measurements. The protein

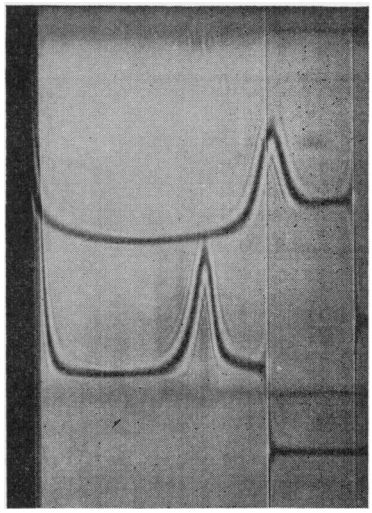

Fig. 3. Ultracentrifugation of the purified protein (peak 2 of the Sephadex G-200 eluate). Schlieren photographs taken after 106min. at 59780rev./min.: sedimentation is from right to left. The upper photograph is of protein (4·8mg./ml.) in tris–sodium succinate buffer, pH 5·9 and I 0·3; the lower photograph is of protein (4·6mg./ml.) in the same buffer but of I 0·015. The temperature was 20° and the phase-plate angle was 60°.

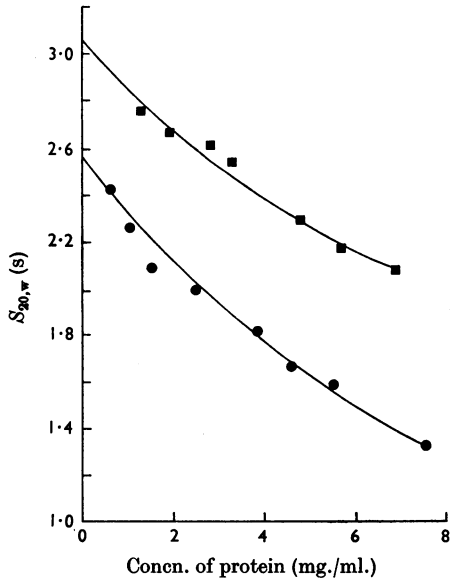

Fig. 4. Effect of protein concentration and ionic strength on the sedimentation coefficient of the purified protein. The conditions were: 59780rev./min.; temperature, 20°; ●, tris–sodium succinate buffer, pH5·9 and I 0·015; ■, tris–sodium succinate buffer, pH5·9 and I 0·3. The lines have been extrapolated to the values of $S^0_{20,w}$ calculated from the reciprocal plot (see Fig. 5).

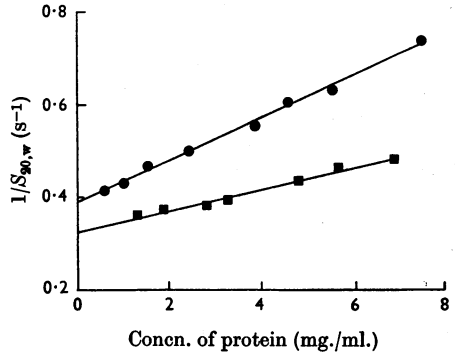

Fig. 5. Effect of protein concentration and ionic strength on the reciprocal of the sedimentation coefficient of the purified protein. The solvents were: ●, tris–sodium succinate buffer, pH5·9 and I 0·015; ■, tris–sodium succinate buffer, pH5·9 and I 0·3. The lines were calculated by the method of least squares and gave values for $S^0_{20,w}$ of 2·57s at I 0·015 and 3·05s at I 0·3.

gave a single boundary when examined in the analytical ultracentrifuge by the schlieren optical system. A typical schlieren photograph is shown in Fig. 3. The boundary showed a tendency to hypersharpening, which was especially marked at protein concentrations above 5mg./ml. The sedimentation coefficient of the protein, determined at several protein concentrations in the range 0·6–8mg./ml., decreased very markedly with increasing protein concentration. The results of these measurements on samples of protein that had been dialysed against buffers of either high ionic strength (0·3) or low ionic strength (0·015) are given in Fig. 4. At both ionic strengths the sedimentation coefficient decreased in a non-linear manner with increasing protein concentration. However, when the reciprocals of the sedimentation coefficients were plotted against protein concentration (Fig. 5), a straight line was obtained. The equation of the straight line can be written as:

$$1/S_{20,w} = 1/S^0_{20,w} + K_s c/S^0_{20,w}$$

where K_s is a constant and c is the protein concentration. The value of the sedimentation coefficient at zero protein concentration ($S^0_{20,w}$) can then be estimated. For the protein in buffer of low ionic strength, $S^0_{20,w}$ was 2·57s and K_s was 116ml./g.; for the protein in buffer of high ionic strength, $S^0_{20,w}$ was 3·05s and K_s was 69ml./g.

Diffusion coefficient and molecular weight. The apparent diffusion coefficient ($D_{app.}$) of the protein in buffer of I 0·015 increased markedly with increasing protein concentration: $D_{app.}$ was 3·2 Fick units at 1mg./ml. and 5·0 Fick units at 6mg./ml. Five experimental values of $D_{app.}$ were extrapolated linearly to give a $D^0_{20,w}$ of 2·87 Fick units. At high ionic strength (0·3) the concentration-dependence of $D_{app.}$ was less marked and the value of $D^0_{20,w}$ was 3·47 Fick units.

The molecular weight of the protein was calculated from $S^0_{20,w}$ and $D^0_{20,w}$ by means of the Svedberg equation and the partial specific volume obtained from the amino acid composition (see below). There was no significant difference between the molecular weights of the protein in buffers of high and low ionic strength: at I 0·015 the value was 76610 and at I 0·3 the value was 76880. The estimate of the molecular weight given in a preliminary communication (Smith & Winkler, 1965) was incorrect since it was calculated from values of $S_{20,w}$ and $D_{20,w}$ at a finite protein concentration.

Molecular weight by the approach-to-equilibrium method. Measurements of the protein concentration and the concentration gradient at the water–air meniscus were made with the schlieren optical system of the analytical ultracentrifuge. Straight lines were obtained when the results were plotted according to the method of Trautman & Crampton (1959). At I 0·015 and a protein concentration of 2·05mg./ml. the apparent molecular weight was 70800. Apparent molecular weights of the protein

in buffer of I 0·3 were 73400 and 72600 at 2·4mg./ml. and 5·6mg./ml. respectively. The data are not sufficient to calculate the true molecular weight, but the values of $M_{app.}$ are of the same order as the molecular weights calculated from the sedimentation and diffusion measurements.

Viscosity measurements. The relative viscosities of solutions of the protein were determined over the concentration range 2–10mg./ml. The rotating-cylinder viscometer used had a shear rate of 1·5sec.$^{-1}$. The reduced viscosities ($\eta_{sp.}/c$) at I 0·015 and I 0·3 are plotted against protein concentration in Fig. 6. Linear extrapolation to zero protein concentration gave values of 42·4ml./g. (I 0·015) and 18·9ml./g. (I 0·3) for the intrinsic viscosity, $[\eta]$, of the protein. Thus, although the molecular weight of the protein remained the same, the intrinsic viscosity decreased markedly with an increase in the ionic strength of the solvent.

Optical rotatory dispersion. The optical rotation of protein solutions was measured at several wavelengths in the range 250–500mμ. The ionic strength of the buffer had only a small effect: the rotation was slightly more negative in the buffer of low ionic strength. Straight lines were obtained when the results were plotted according to the simple Drude equation (for definition of the symbols see Urnes & Doty, 1961):

$$[m']_\lambda = a_c\lambda_c^2/(\lambda^2-\lambda_c^2)$$

The dispersion constant λ_c was 222mμ at I 0·015 and 226mμ at I 0·3: these values are similar to those obtained for denatured proteins and random-coil polypeptides (Yang & Doty, 1957; Schellman & Schellman, 1961). The percentage of residues in a right-handed α-helix was estimated to be about 9% from the data given for other proteins by Yang & Doty (1957).

The results of the optical-rotation measurements in the range 300–500mμ have been plotted in Fig. 7 according to the Moffit (1956) equation:

$$[m']_\lambda = a_0\lambda_0^2/(\lambda^2-\lambda_0^2)+b_0\lambda_0^4/(\lambda^2-\lambda_0^2)^2$$

A value of 212mμ was used for λ_0, as recommended by Yang (1965). At each ionic strength the experimental points lay on a straight line, and the values of the Moffit constants a_0 and b_0 were calculated from the intercepts and slopes respectively. At the low ionic strength $a_0 = -655$ and $b_0 = -90$, and at the high ionic strength $a_0 = -598$ and $b_0 = -85$. Both the low negative values of b_0 and the high negative values of a_0 are characteristic of denatured proteins and of polypeptides in the random-coil form (Urnes & Doty, 1961). The values of b_0 at high and low ionic strength do not differ significantly and they indicate that approx. 14% of the amino acid residues are in the form of an α-helix.

Amino acid analysis. The amino acid compositions of the acid hydrolysates of three different samples of protein were determined and the results are given in Table 2. The recovery of nitrogen

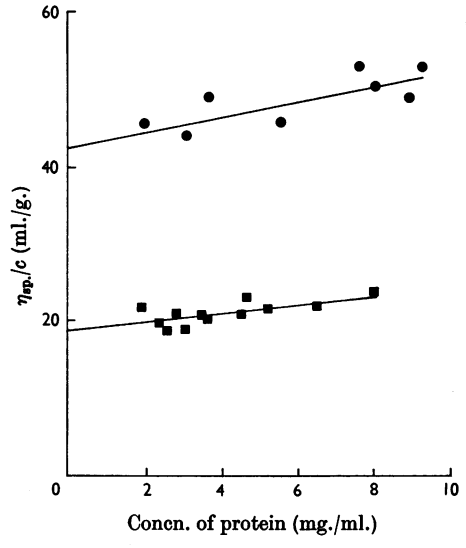

Fig. 6. Effect of protein concentration and ionic strength on the reduced viscosity of the protein. Relative viscosities were determined with a rotating-cylinder viscometer. The solvents were: ●, tris–sodium succinate, pH5·9 and I 0·015; ■, tris–sodium succinate, pH5·9 and I 0·3. The lines were calculated by the method of least squares and gave intrinsic viscosities, $[\eta]$, of 42·4ml./g. at I 0·015 and of 18·9ml./g. at I 0·3.

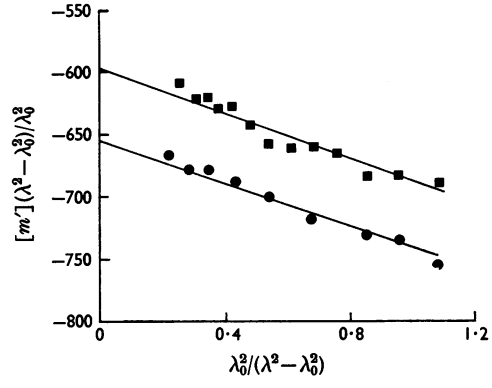

Fig. 7. Moffit plots of the optical-rotatory-dispersion data for the purified protein. The solvents were: ●, tris–sodium succinate, pH5·9 and I 0·015; ■, tris–sodium succinate, pH5·9 and I 0·3. The lines were calculated by the method of least squares.

Table 2. *Amino acid composition of the purified protein*

Three different preparations of the protein (peak 2 of the Sephadex G-200 eluate) were hydrolysed for 17hr. with HCl as described in the Methods section. The values given for half-cystine, serine and threonine were not corrected for any destruction of these amino acids during hydrolysis, nor was that for leucine corrected for incomplete hydrolysis. A molecular weight of 77390 for the protein was used in the calculations.

Amino acid	Amino acid content (g./100g.) (mean ± S.D.)	Amino acid content (moles/mole)
Glu	26·01 ± 0·45	156
NH_3	1·44 ± 0·07	70
Pro	8·56 ± 0·13	68
Lys	9·43 ± 0·23	57
Asp	8·35 ± 0·16	56
Ser	6·20 ± 0·11	55
Ala	5·03 ± 0·17	55
Gly	3·86 ± 0·31	52
Leu	7·31 ± 0·37	50
Arg	8·49 ± 0·48	42
Val	3·25 ± 0·05	25
Thr	2·45 ± 0·11	19
His	2·35 ± 0·27	13
Met	2·24 ± 0·04	13
Phe	2·08 ± 0·16	11
Tyr	1·67 ± 0·19	8
Ile	1·08 ± 0·07	7
CyS	0·347 ± 0·16	3

(excluding that from tryptophan) from the columns of the amino acid analyser was 92% of that applied. Minimum molecular weights for the protein were calculated from the amounts of three of the least abundant amino acids (tyrosine, phenylalanine, methionine). A mean molecular weight of 77390 was then obtained on the assumption that there were 8 residues of tyrosine, 11 of phenylalanine and 13 of methionine.

The protein is very rich in the highly polar amino acids glutamate, aspartate, lysine and arginine; it is also rich in proline. When the ratio of polar to non-polar amino acid residues is calculated by the procedure given by Hatch (1965) a ratio of 2·21 is obtained: this ratio is exceeded only by tropomyosin (2·99).

The amino acid composition of the protein was used to calculate its partial specific volume from the data for each amino acid given by Edsall (1953). A value of 0·717ml./g. was obtained; this has been used in the calculations of the molecular weight from the ultracentrifuge experiments.

The acid hydrolysate used for amino acid analysis contained glucosamine and galactosamine, which were eluted from the long column of the amino acid analyser after the last amino acid had emerged. The amount of amino sugars present in the protein was estimated by using the amino acid analyser after acid hydrolysis under mild conditions (4N-hydrochloric acid for 6hr. at 110°): it contained 0·5% by weight of glucosamine and 1·1% by weight of galactosamine, i.e. approx. 2 residues of glucosamine and 4 residues of galactosamine/mol. An orcinol reaction (Winzler, 1955) was carried out on a sample of the protein that had been extensively dialysed, and this indicated the presence of about 2% by weight of neutral sugars. The protein may therefore be a glycoprotein, but the possibility of contamination with a trace of mucopolysaccharide cannot be excluded.

DISCUSSION

The experiments described above show that it is possible to isolate the main component of the soluble proteins in a single chromatographic step. The purified protein comprises 38% of the soluble protein or, in other words, 25% of the total protein of the granules. The results obtained with gel electrophoresis indicate that the protein was unchanged in the isolation procedure, since a band identical in appearance and mobility with that given by the isolated protein was present in electrophoretograms of the soluble lysate.

Sephadex chromatography. The chromatography of the soluble lysate on Sephadex G-200 showed some interesting features. First, fractionation of the material was only achieved when the column was eluted with a buffer of relatively low ionic strength: the main component of the proteins was eluted together with the phospholipids when the ionic strength was above 0·015. Secondly, the finding that phospholipids were eluted at the void volume of the column means that these low-molecular-weight compounds must have been present as micelles of high molecular weight. This also raises the question of the definition of the void volume of a column of Sephadex G-200. The void volume is usually considered to be identical with the elution volume of Blue Dextran (see Andrews, 1965), which has a molecular weight of 2×10^6. However, in the present work it was found that the phospholipid micelles were eluted before Blue Dextran. The molecular weights of phospholipid sols and micelles are about 10×10^6 (Saunders, Perrin & Gammack, 1962). Andrews (1965) has suggested that the lower molecular-weight limit for complete exclusion from Sephadex G-200 may be as high as 3×10^6. It is therefore likely that the elution volume of the phospholipids is closer to the void volume (if not identical with it) than is the elution volume of Blue Dextran.

A third point of interest emerges from the results

of Sephadex chromatography: the main protein component (peak 2) is eluted close to the void volume of the column. When the molecular weight of this protein was estimated from the plot of log(mol.wt.) versus elution volume of proteins of known molecular weight, a value of about 5×10^5 was obtained. This is in contrast with the much lower molecular weight of 77 000 calculated from the results of analytical ultracentrifugation. It is shown below that the apparent discrepancy is a consequence of the unusual hydrodynamic properties of this protein.

Physical and chemical properties of the purified protein. Analytical ultracentrifugation established that the molecular weight of the protein is close to 77 000 and that there was no change in molecular weight on going from a solvent of I 0·3 to one of I 0·015. However, if the hydrodynamic properties of the protein are compared with those of globular proteins of similar molecular weight a number of differences are found: the sedimentation and diffusion coefficients of the protein are lower; the intrinsic viscosity is much higher; the sedimentation coefficient is markedly dependent on protein concentration; and, finally, each of these hydrodynamic properties varies with the ionic strength of the solvent. The most probable explanation of these observations is that the protein does not have a compact globular conformation, but that it has a conformation approaching that of a random-coil polymer: the evidence that leads to this conclusion is discussed below.

Both the frictional ratio (f/f_0) and the viscosity increment ($[\eta]/\bar{v}$) of the protein are very high, and they also depend on the ionic strength of the solvent: f/f_0 is 2·69 at I 0·015 and 2·04 at I 0·3; the viscosity increment is 69 at the low ionic strength and 26 at the high ionic strength. These values for the frictional ratio and the viscosity increment indicate that the protein molecule in solution is not a compact sphere: it may be spherical but of expanded form, or it may be a highly asymmetric molecule, or it may have a structure intermediate between these alternatives. It is possible to distinguish between the contributions of expanded form and of asymmetry to the frictional properties of a macromolecule from a knowledge of the ratio $K_s/[\eta]$ (Ogston, 1953; Creeth & Knight, 1965). K_s is the coefficient of concentration-dependence of the reciprocal sedimentation coefficient, and the values for the purified protein have been given in the Results section. Creeth & Knight (1965) found that for asymmetric protein molecules the ratio $K_s/[\eta]$ was less than 1·5, whereas the ratio was equal to or greater than 1·5 for spherical molecules. In the present work the ratios were 2·73 at I 0·015 and 3·63 at I 0·3; this implies that the protein molecule is approximately spherical. The viscosity increment of the protein is much higher than the value for a compact sphere (2·5) and so the molecule must be a highly expanded sphere.

The marked change in the hydrodynamic parameters, notably the viscosity, when the ionic strength of the solvent is altered shows that the expanded form of the molecule is not rigid, but is like a flexible polyelectrolyte. The dependence of the viscosity of a macromolecule on the ionic strength of the solvent is typical of polypeptides in the random-coil form (Iizuka & Yang, 1965), other polyelectrolytes such as hyaluronic acid (Preston, Davies & Ogston, 1965) and denatured proteins (for reviews see Tanford, 1958, 1961). The increase in intrinsic viscosity and the decrease in both S_{20} and D_{20} that accompanied a fall in the ionic strength of the solvent reflect the importance of electrostatic interactions in determining the overall shape of the molecule: at low ionic strength the repulsion between like charges is increased, causing the molecule to expand. If it is assumed that the molecule is spherical, then the effective hydrodynamic volume at I 0·3 is 7·5ml./g. and this is increased to 17ml./g. at I 0·015. The large effective hydrodynamic volume of the protein probably accounts for the concentration-dependence of its sedimentation coefficient and also for its lack of retardation on Sephadex chromatography.

The concentration-dependence of the sedimentation coefficient of the protein is very marked at both high and low ionic strengths. The slopes of the $1/S$ versus c plots are among the steepest that have been reported for a naturally occurring protein, and are similar to those given by the highly asymmetric protein collagen (Nishihara & Doty, 1958) or random-coil proteins such as gelatin (Gouinlock, Flory & Scheraga, 1955) and denatured myosin (Kielley & Harrington, 1960).

Two kinds of phenomena can contribute to the concentration-dependence of the sedimentation coefficient of a protein: hydrodynamic factors and electrical-charge effects. Electrical-charge effects in sedimentation have been described by Pedersen (1958) for serum albumin and by Sitaramaiah, Robertson & Goring (1962) for CM-cellulose. These authors showed that the primary charge effect on the sedimentation of serum albumin and CM-cellulose was almost entirely overcome by increasing the ionic strength of the solvent to 0·05–0·1. By analogy, it is likely that the concentration-dependence of S for the chromaffin-granule protein at I 0·3 was due to hydrodynamic factors, whereas at low ionic strength (I 0·015) the concentration-dependence may have been influenced in addition by charge effects. The hydrodynamic factors causing S to vary with c have been discussed by Schachman (1959) and by Gilbert (1959, 1960). The type of variation of S with c described by Gilbert (1959, 1960) for aggre-

gating systems has not been found in the present work. One of the principal factors causing S to decrease with increasing concentration is the backward flow of solvent (Cheng & Schachman, 1955; Schachman, 1959), which is greatly enhanced when the macromolecule has a large effective hydrodynamic volume. The concentration-dependence of S found in the present work is therefore most readily explained by the large hydrodynamic volume of the protein.

The chromaffin-granule protein was eluted from a column of Sephadex G-200 between thyroglobulin (mol.wt. 670000) and urease (mol.wt. 490000) implying that, under these conditions, it had a molecular weight of about 500000. This is nearly 7 times the value of 77000 found by ultracentrifugal analysis of the protein dissolved in the same buffer (I 0·015) that had been used for Sephadex chromatography. The reason for this apparent discrepancy is that the proteins used to calibrate the Sephadex G-200 column were all globular proteins of small effective hydrodynamic volume (about 2–3ml./g.), whereas the effective hydrodynamic volume of the chromaffin-granule protein was about 17ml./g. at I 0·015. Ackers (1964) and Laurent & Killander (1964) have pointed out that it is the effective hydrodynamic radius of a protein, and not its molecular weight, that determines its elution volume from Sephadex. The procedure given by Ackers (1964) allows the calculation of the effective hydrodynamic radius, and hence the diffusion coefficient, of a protein from its column distribution coefficient (K_d). By this method, an effective hydrodynamic radius of 77Å (which gives a D_{20} value of 2·78 Fick units) was calculated from the elution volume of the peak 2 protein: this agrees very well with the radius of 75Å that is obtained from the $D^0_{20,w}$ value of 2·87 Fick units found with the analytical ultracentrifuge. These results therefore confirm the analysis of molecular-exclusion chromatography given by Ackers, and emphasize that the estimation of the molecular weight of a protein from the results of Sephadex chromatography alone should be done with caution.

A characteristic feature of our protein is its unusual amino acid composition: it is very rich in polar amino acids, in particular glutamic acid (26%, w/w) and in proline (8·6%, w/w), but it contains very little cyst(e)ine (0·35%, w/w). Only two other animal proteins contain more glutamic acid: tropomyosin contains 29% (see Tristram & Smith, 1963) and an acidic protein from ox brain contains 30% (Moore, 1965). The protein will carry a large excess of negative charge in solution at neutral pH: allowing for the amide groups, there is an excess of 43 acidic amino acid residues/mol. of protein.

The high proline content of the protein is noteworthy in relation to the secondary structure of proteins. Low & Edsall (1956) showed by model building that proline cannot be accommodated in an α-helix except at the N-terminal end, and Szent-Györgyi & Cohen (1957) predicted that proteins containing about 8% of proline would behave as random-coil polypeptides. X-ray-crystallographic studies (see Perutz, 1962) have shown that in myoglobin and haemoglobin the proline residues all lie in corners or in non-helical regions of the chain. The fact that, in the chromaffin-granule protein, one residue in 11 is proline therefore accounts for the results of the optical-rotatory-dispersion measurements, which indicated a low content of α-helix. Since the protein contains few, if any, disulphide bridges the low content of α-helix is consistent with a random-coil conformation. This being so, the excess of acidic amino acid residues explains why the hydrodynamic properties of the molecule were dependent on the ionic strength of the solvent.

The amino acid composition of the protein agrees closely with that reported by Helle (1966*a*) for a protein prepared from bovine chromaffin granules by ethanol precipitation at pH 4, although Helle (1966*a*) could not detect any cystine. A similar amino acid composition was also reported by Kirshner, Holloway, Smith & Kirshner (1966*a*). However, the molecular weight of the protein studied by Helle (1966*a*) was 25000 and that of the protein studies by Kirshner *et al.* (1966*a*) was about 40000. Both these values for the molecular weight are lower than that found in the present work. It is possible that the methods of purification used by Helle (1966*a*) and by Kirshner *et al.* (1966a) caused the protein to dissociate into lower-molecular-weight sub-units. The protein studied by Kirshner *et al.* (1966*a*) was reported to change its molecular weight according to the composition of the solvent.

The present work has shown that the main component of the soluble proteins from bovine chromaffin granules has some interesting physicochemical properties, but it is not at present possible to infer from these properties anything about the function of the protein. However, it is noteworthy that the same, or a very similar, protein has been identified in the soluble lysate of chromaffin granules from pig and horse adrenals by starch-gel electrophoresis (Winkler *et al.* 1966), and that the proteins from pig, horse and sheep granules gave a precipitation line with antiserum to the bovine protein (Helle, 1966*b*). Banks & Helle (1965) have found that the protein is recovered in the perfusate when the isolated bovine adrenal gland is stimulated to release catecholamines, and this observation has been confirmed by Kirshner, Sage, Smith & Kirshner (1966*b*). Preliminary experiments (R. S. Comline, M. Silver & A. D. Smith, unpublished work) have

shown that the protein is secreted into the blood from the adrenal gland of the calf when the splanchnic nerve is stimulated.

The question as to the physiological significance of the soluble proteins of chromaffin granules remains open. The earlier work of Blaschko & Helle (1963) was begun to find whether the protein was involved in the binding of adrenaline, but the work quoted above makes it possible that the soluble protein represents a secretion product of the chromaffin cell that has hitherto escaped detection.

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