Transcortin. A Corticosteroid-Binding Protein of Plasma. IX. Isolation and Characterization*

W. Roy Slaunwhite, Jr., Sara Schneider, Franklyn C. Wissler, and Avery A. Sandberg

ABSTRACT: Plasma from male subjects with cancer of the prostate, who were receiving diethylstilbestrol treatment, was fractionated successively on columns of diethylaminoethylcellulose, Sephadex G-25, hydroxylapatite, and Sephadex G-75. Transcortin was detected by virtue of its ability to bind [^{14}C]cortisol. The final product (12 mg from 300 ml of plasma) was purified as judged by polyacrylamide gel electrophoresis and ultracentrifugation ($s_{20,w}$ 4.1). Its molecular weight was 58,500 (calcd). An amino acid analysis is given.

The initial demonstrations of the existence of transcortin, a corticosteroid-binding protein in human plasma (Sandberg *et al.*, 1957; Bush, 1957; Daughaday, 1958a), were rapidly followed by the partial resolution and identification of this component of plasma protein, using the technique of continuous-flow electrophoresis (Daughaday, 1958b; Slaunwhite and Sandberg, 1959). The preparation of highly purified transcortin by Seal and Doe (1962a,b) enabled these authors to characterize the protein more completely than had previously been possible.

The continuing study of transcortin in any significant detail is dependent upon the availability of highly purified material for analysis. In order to obtain sufficient amounts of highly purified material for study, a modification of the methods of fractionation suggested by Seal and Doe (1962a,b) was developed. Utilizing sequential ion-exchange, molecular sieve, and adsorption chromatography of plasma from estrogen-treated subjects, it was possible to obtain a highly purified protein preparation whose molecular weight could be determined with a high degree of accuracy.

Materials and Methods

Materials. Human plasma was obtained from male subjects with cancer of the prostate who had received 15 mg of diethylstilbestrol/day for 5 days preceding the withdrawal of blood. This regimen has been shown to raise the plasma transcortin level significantly (Sandberg *et al.*, 1960).

Radioactive [4-^{14}C]cortisol was obtained from New England Nuclear Corp. and contained 70 μc/mg of cortisol. *N,N*-Diethylaminoethylcellulose was obtained from Eastman Chemical Co., Rochester, N.Y.; Sephadex G-25 and G-75 from Pharmacia, Uppsala, Sweden; and hydroxylapatite, under the trade name of Hypatite-C, from Clarkson Chemical Co., Williamsport, Pa.

Preparation of Columns. DEAE-cellulose was prepared for use by washing the fresh adsorbent with 1 N NaOH until no discoloration of a fresh alkali wash could be observed. This procedure was followed by thorough washing of the material on a Büchner funnel until the filtrate was neutral. The adsorbent was then suspended in the initial eluting buffer (see Experimental Procedures) and adjusted to a pH of 5.0. The columns were packed in the cold (4°) with prechilled absorbent suspension. The particles of DEAE-cellulose were allowed to settle under gravity-flow conditions as described by Flodin and Kupke (1956). The packed column was then washed with 2–3 l. of buffer, care being taken to maintain a hydrostatic head of 125 ml above the cellulose bed for a 40 × 4 cm (i.d.) column. The dextran gels Sephadex G-25 and G-75 were prepared and packed in columns according to the methods of Flodin (1961) and Porath and Flodin (1959).

Hydroxylapatite columns were always freshly packed in the cold and thoroughly equilibrated with the cold eluting buffer. Prior to the packing of a column, the Hypatite-C was carefully equilibrated at 4° with the eluting buffer. Upon standing, spontaneous changes in the adsorbent occurred which were recognizable by concurrent changes in the pH and in the ionic strength of the effluent of the columns. These changes in the nature of the adsorbent eventually caused irreversible adsorption of protein. Hypatite-C obtained from the Clarkson Chemical Co. was rated by means of the batchwise absorption of human serum albumin. When rated at 80–90 mg adsorbed/ml of suspended adsorbent, the hydroxylapatite was found to be satisfactory in the procedure described. Hypatite-C possessing a higher rating is not suitable for use.

Assay of Column Eluent. Fractions eluted from the DEAE-C, Sephadex, and Hypatite-C columns were assayed in the following manner. The radioactivity in 1 ml of every fraction was measured in a dual-channel liquid scintillation spectrometer to determine the content of [4-^{14}C]cortisol. A solution containing 6

* From the Roswell Park Memorial Institute, Buffalo, New York. *Received January 3, 1966; revised August 5, 1966.* This work was supported in part by a grant (A-1240) from the U. S. Public Health Service.

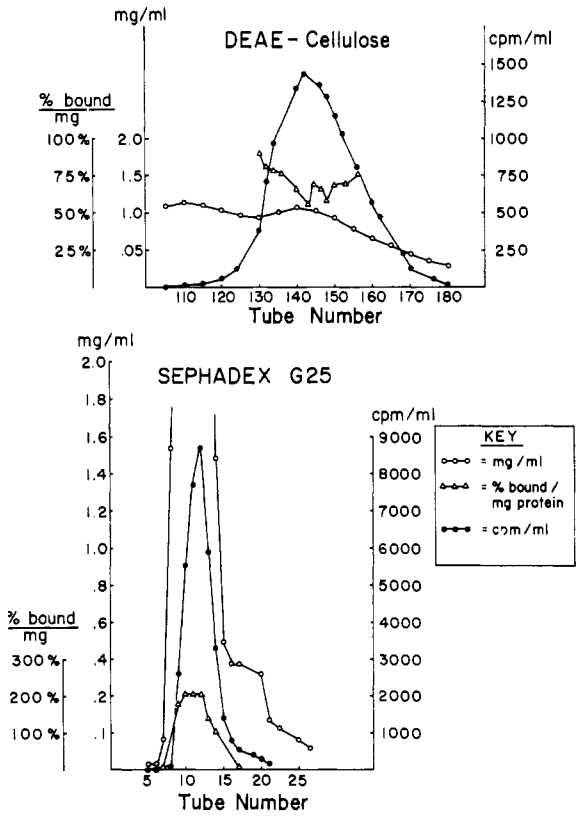

FIGURE 1: Column chromatography of plasma from subjects treated with diethylstilbestrol. Experimental details are contained in the text. In the case of the DEAE-cellulose column, only the portion relevant to transcortin is shown.

g % PPO,[1] 0.05 g % POPOP, and 5 g % naphthalene in a 5:1 (v/v) mixture of dioxane and 2-ethoxyethanol was used (Bruno and Christian, 1961). The ultraviolet absorbance at 280 and 260 mμ was measured on a Beckman DU spectrophotometer and the protein concentration determined by assuming a value of $E^{1\%}_{1cm}$ of 14.8 at 280 mμ. The binding of [^{14}C]cortisol was performed by equilibrium dialysis (Sandberg *et al.*, 1957) on serial dilutions of each fraction in the radioactivity peak. This was accomplished by diluting each aliquot to 3 ml with 0.155 M saline at pH 7.0 and dialyzing against 14 ml of saline containing 0.005 μc (0.07 μg) of [4-^{14}C]cortisol. Each solution (1 ml, inside and outside the casing) was assayed for radioactivity. That dilution which exhibited binding between 40 and 60% was divided by the protein concentration of that dilution to give the per cent binding per milligram of protein present. Aliquots of approximately 150 μg from each fraction were examined in disc electrophoresis (Ornstein and Davis, 1962) using 7.5% polyacrylamide gel obtained from the Canalco Corp.[2]

[1] Abbreviations used: PPO, 2,5-diphenyloxazole; POPOP, 1,4-bis[2-(5-phenyloxazolyl)]benzene.

[2] Canal Industrial Corp., Bethesda, 14, Md.

Electrophoresis was performed in the standard Tris–glycine buffer system in which proteins in the gel migrate according to their mobilities at pH 8.6. The gel columns were preserved and compared with those obtained after additional purification. The electrolyte conductance of eluates of all columns except those of the DEAE-cellulose were measured on a standard conductance bridge.

Physical Methods. The molecular weight was determined by the high-speed sedimentation equilibrium method of Yphantis (1964). Amino acid analysis was performed on a Technicon amino acid analyzer using a single column with a buffer gradient according to the method described by Piez and Morris (1960).

Experimental Procedures

Anion-Exchange Chromatography on DEAE-Cellulose. Plasma (300 ml) collected from diethylstilbestrol-treated patients was dialyzed overnight at 4° against ten volumes of distilled water. The plasma was adjusted to a pH of 5.0 and centrifuged to remove precipitated material. The electrolytic conductance of the plasma was measured to give assurance that the ionic strength of the plasma was lower than that of the initial eluting buffer which was 0.025 M in NaCl and 0.015 M in NaH_2PO_4 at pH 5.0. After the plasma had percolated into the bed at a maximum flow rate of 2 ml/min, 1 μc of [4-^{14}C]cortisol (14 μg) in the same buffer was added directly to the top of the column and allowed to settle. In this manner, there was sufficient time for the equilibration of [4-^{14}C]cortisol with the transcortin at the top of the column. Elution was begun with the initial buffer, maintaining a hydrostatic head of 125 ml, and was continued until the optical density of the effluent at 280 mμ was between 0.2 and 0.6. A gradient in ionic strength at a constant pH of 5.0 was then begun using 1200 ml of the initial buffer in a mixing chamber consisting of a 2-l. aspirator bottle equipped with a magnetic stirrer, and dropping into it from a stoppered separatory funnel a buffer consisting of 1:9 (v/v) 0.2 M NaH_2PO_4–0.09 M NaCl at pH 5.0. Fractions of 30-ml volume (with 1 drop of 0.001 M ascorbate added as a preservative) were collected at a flow rate of less than 1 ml/min until the characteristic blue caeruloplasmin band had passed out of the column. The flow rate was regulated by the rate of flow of buffer from the mixing chamber to the stoppered top of the column. The protein concentration, content of radioactivity, and binding ability of the effluent were determined as described. Those fractions demonstrating maximum elution of radioactivity and maximum binding per milligram of protein present were pooled and lyophilized (Figure 1).

Sephadex G-25 Column. The lyophilized eluate pools selected on the basis of the close coincidence of the [^{14}C]cortisol effluent with maximum binding prowess were dissolved in 0.001 M NaH_2PO_4 at pH 6.8. The protein solution (5–10 mg/ml) was adjusted to pH 6.8 and applied to a Sephadex G-25, medium grade, column of appropriate size (sample volume equaling

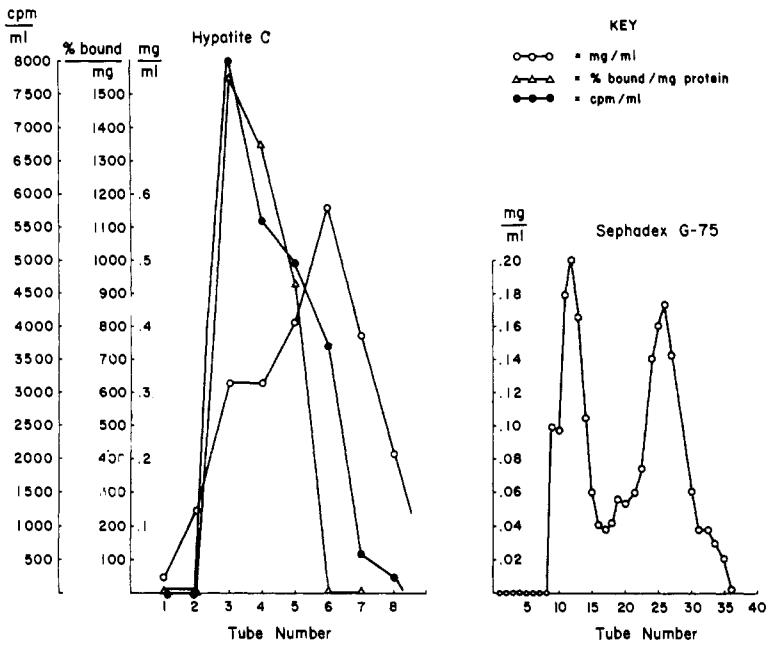

FIGURE 2: Same as Figure 1. Radioactivity and binding measurements were made on pooled material from the Sephadex G-75 column. The frontal peak contained all the radioactivity and exhibited all the binding ability.

20% of total bed volume). Most often the total volume of sample was approximately 100 ml and the column size 40 × 4 cm i.d. To assure the presence of sufficient [^{14}C]cortisol, an additional 1 μc of [^{14}C]cortisol was added in a manner similar to the application on the DEAE-cellulose column.

The protein solution was allowed to settle at a controlled rate of 2–3 ml/min. Elution was performed with a 0.001 M NaH_2PO_4 buffer at a constant pH of 6.8 utilizing the same apparatus as was used for the DEAE-cellulose column. The rate of elution was regulated so that one 15-ml fraction was collected approximately every 30 min. The optical density at 280 mμ and the radioactivity of each fraction were measured as previously described. The progress of the elution was monitored by the use of conductance measurements on each fraction as the column was running. All fractions containing protein were examined in serial dilution for binding of [^{14}C]cortisol as described. Aliquots of each fraction were run on disc electrophoresis. The appropriate fractions were pooled and lyophilized (Figure 1).

Hydroxylapatite Adsorption Chromatography. The nearly salt-free protein resulting from the treatment with Sephadex G-25 was dissolved in 50–60 ml of 0.001 M NaH_2PO_4 buffer at pH 6.8 and applied in a concentration of 5 mg/ml to a 40 × 2 cm column of the commercially prepared hydroxylapatite known as Hypatite-C. Fractions were collected at the rate of 10 ml every 20 min. This combination of flow rate with column dimensions provided the best resolution of our material from its impurities with a minimum of any tailing effect. The fractions containing the maximum amount of radioactivity exhibited the maximum amount of binding per milligram of protein present and were preserved (Figure 2). If disc electrophoresis of 150–200 μg of this material revealed the presence of more than two bands, the lyophilized material was recycled through a similar hydroxylapatite column using a similar technique.

Sephadex G-75. A persistent contaminant of the eluate from hydroxylapatite column was detected by the presence of a minor second band in disc electrophoresis and by the presence of a small amount of a slow sedimenting material in trial sedimentation velocity runs in the ultracentrifuge. The highly purified material, dissolved in 0.001 M NaH_2PO_4 at pH 6.8, was applied to a Sephadex G-75, fine grade, column in a concentration of 1 mg/ml. The dimensions of the column were chosen so that the sample volume was never in excess of 10% of the bed volume. Elution was accomplished at the maximum flow rate attainable without the application of pressure using 0.001 M NaH_2PO_4, pH 6.8 buffer (Figure 2). To obtain the maximum amount of recovery of transcortin from the plasma available, the fractions adjacent to those of maximum binding values and maximum concentration of radioactivity in each column were also purified further.

Unlike Seal and Doe (1962a,b) we have used ordinary distilled water. Zn^{2+}, Cu^{2+}, Fe^{2+}, and Fe^{3+} at 10^{-4}–10^{-6} M concentrations have no effect on the binding of [^{14}C]cortisol, at least after the first step of purification.

Physical Characterization. In one sector of a 12-mm Epon aluminum-filled, double-sector centerpiece was placed a small amount of Dow Corning 555 silicone fluid plus 0.05 ml of buffer (0.033 M sodium phosphate

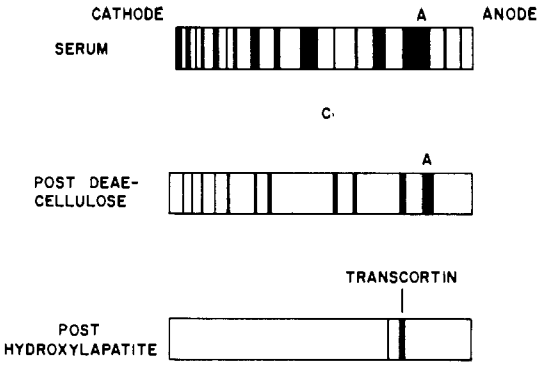

FIGURE 3: Polyacrylamide gel electrophoresis at various stages of purification. Details are given in the text. After passage through Sephadex G-75, 200 μg of product revealed a single band (not shown).

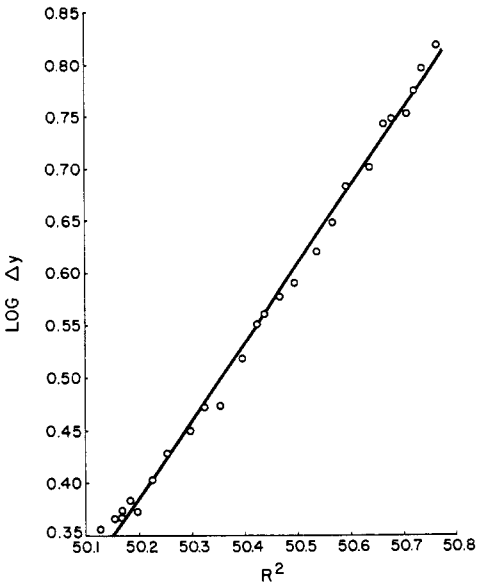

FIGURE 4: Equilibrium sedimentation analysis by the method of Yphantis (1964). From the slope of the line determined by plotting the logarithm of the fringe displacement *vs.* the square of the radial distance from the center of rotation, a value of 3.45 was calculated for the parameter σ. The linearity of the experimental points is an indication of the homogeneity of the transcortin.

at pH 7.2) and 0.05 ml of a 0.133 g% solution of transcortin in the same buffer. Buffer was added to the other sector so as to slightly overlap the solution column. Centrifugation was continued for 23 hr at 27° at a speed of 21,740 rpm in a Spinco Model E ultracentrifuge equipped with a Rayleigh interference optical system.

At the conclusion of the run, the fringes in the neighborhood of the meniscus were parallel with those in the air space indicating zero-protein concentration at the meniscus, a necessary condition for the success of this method. The photographic plates were measured with a Bausch and Lomb two-way optical comparator. The average of five fringe displacements were taken for each radial distance.

The sedimentation constant was determined from a velocity run of the same solution on the same instrument. The initial temperature was 10°; the temperature at the end of the run was 16°.

Results

Recoveries and Yield of Transcortin. The recoveries of protein and radioactivity from the Sephadex and hydroxylapatite columns were essentially quantitative. If spontaneous changes had occurred in the Hypatite-C, however, there was a protein and radioactivity loss of 40–60%. Approximately 12 mg of highly purified transcortin was obtained from 300 ml of plasma with a known loss of 2–3 mg occurring through use of the assay procedure. Throughout the procedure a persistent increase in the binding ability of the column eluates was noted (Table I).

Prior to the removal of the final contaminant, which was detectable only when highly purified material was carefully scrutinized on disc electrophoresis and in the ultracentrifuge, approximately 22 mg of protein remained from the original plasma. Examination of the effluent from the Sephadex G-75 columns revealed two protein peaks representing nearly 100% recovery of protein. The frontal peak, possessing a maximum absorption band at 280 mμ, was eluted in 0.001 M NaH_2PO_4, pH 6.8, and exhibited binding ability when equilibrium dialysis was performed with [4-^{14}C]-cortisol. The second peak did not display maximum absorption at 280 mμ nor binding with [^{14}C]cortisol.

TABLE I: Increase in % C^{14}-Cortisol Bound/mg of Protein.

Eluate	% Bound/mg of Protein (%)[a]	No. of Bands in Disc Electrophoresis
Cellulose column	68	6–7
Sephadex G-25	203	6–7
Hypatite-C	1550	2
Sephadex G-75	*b*	1

[a] Equilibrium dialysis of eluates was performed to a constant end point as described in the text. The calculation of milligrams of protein per milliliter of eluate was based upon an extinction coefficient of $E^{1\%}_{1cm}$ 14.8 at 280 mμ. [b] The value determined was unexpectedly high, perhaps due to the inaccuracy in measurements at the high dilutions used.

TABLE II: Preliminary Estimation of Amino Acid Composition of Transcortin.[a]

Residue	g of Amino Acid/100 g of Protein[a]			Moles of Amino Acid[d] 50,250 g of Protein	Moles of Amino Acid × Residue Wt
	Slaunwhite (A)	Seal (B)[d]	A/B		
Aspartic	10.81	7.73	1.40	48.9	5628
Threonine[b]	6.05	4.69	1.29	30.1	3043
Serine[b]	6.42	4.87	1.32	37.0	3222
Glutamic	12.36	11.60	1.07	48.1	6205
Proline	5.12	6.40	0.80	26.5	2574
Glycine	3.61	3.64	0.99	31.8	1815
Alanine	4.94	4.76	1.04	34.9	2481
Valine[c]	7.15	4.34	1.65	36.2	3589
Methionine	2.42	1.04	2.33	9.3	1220
Isoleucine[c]	4.57	2.84	1.61	20.3	2297
Leucine	12.94	11.25	1.14	57.0	6450
Tyrosine[b]	3.87	2.52	1.53	11.9	1942
Phenylalanine	6.86	5.36	1.28	23.4	3444
Lysine	4.05	3.39	1.20	15.9	2038
Histidine	3.14	3.25	0.97	11.5	1577
Arginine	5.05	6.87	0.74	16.2	2530
				Total	50055

[a] A 20-hr hydrolysis in 6 N HCl at 110°. [b] Uncorrected for destruction during hydrolysis. [c] Uncorrected for incomplete hydrolysis after 20 hr. [d] Weight of protein was based on a total molecular weight of 58,500, with the published carbohydrate content of 14.1% (Seal and Doe, 1962a). Amino acid analysis taken from the same paper. [e] Independent analyses for cysteine and for tryptophan were not performed.

No additional material could be obtained from the fringe fractions of each of the columns described previously. The DEAE-cellulose fringe fractions when examined on Sephadex G-25 did not elute with the [^{14}C]cortisol and displayed no binding activity. The protein in Sephadex G-25 fringe fractions, resulting from the desalting of the transcortin-containing fractions, did not elute with the 0.001 M buffer when analyzed on Hypatite-C, but could be eluted by stepwise elution with higher concentrations of buffer. The eluted material displayed no binding ability and five to six bands on disc electrophoresis.

Evidences of Homogeneity of Purified Transcortin. Disc electrophoresis (Figure 3) provided evidence of increasing purity with each procedure. The material that accompanies transcortin after hydroxylapatite chromatography was first detected during a velocity run on the ultracentrifuge. Although this impurity, according to ultraviolet absorbance measurements at 280 mμ, constitutes nearly one-half of the material (Figure 2, Sephadex G-75), it apparently stains very poorly, for it is barely discernible after disc electrophoresis (Figure 3, post-hydroxylapatite). Disc electrophoresis of 200 μg of the final material revealed a single band of protein in this highly sensitive technique. The sedimentation velocity run on the ultracentrifuge revealed one component of $s_{20,w}$ = 4.1 S. The microsedimentation equilibrium method used for the determination of molecular weight also revealed a homogeneous preparation (see below).

Identity of Purified Protein and Transcortin. In every step of the purification procedure coincidence of the radioactive peak of [^{14}C]cortisol with the maximum binding fractions was the requisite for additional purification to be performed on protein eluates. The final preparation, when run on disc electrophoresis, migrated as an α_1-globulin.

Physical Properties. From the equilibrium sedimentation analysis, a plot of the log of the fringe displacement, ΔY, *vs.* the square of the radial distance from the center of rotation was made (Yphantis, 1964) (Figure 4). The linearity of this plot indicates molecular weight homogeneity of the sample. A value of 3.45 was calculated for the "effective reduced molecular weight parameter", σ, from the slope of this plot.

An "apparent molecular weight" can be calculated from the relation $\sigma RT/\omega^2(1 - \bar{v}\rho)$, where R is the gas constant, T is the temperature, ω is the angular velocity, ρ is the density of the solution, and $\bar{v}$ is the partial specific volume of the solute. The value of $\bar{v}$ was taken to be 0.718 ml/g (Seal and Doe, 1962a). From these values an "apparent molecular weight" of 58,500 was obtained for this sample of transcortin.

Calculations of Amino Acid Composition. The nitrogen content of the protein was not determined. The weight per cent of each amino acid anhydro residue

was calculated on the best available dry weight[3] of protein as recommended by other authors (Mahowald *et al.*, 1962; Spero *et al.*, 1965). The sum of these values was then divided into 100 to obtain a correction factor for the analysis values. The weight per cent of each amino acid was then multiplied by this factor and each amino acid expressed as g of anhydro amino acid/100 g of protein. The approximate amino acid composition derived by this procedure is shown in Table II. Independent analyses for cysteine and tryptophan, which are destroyed during hydrolysis of the protein, were not performed.

Discussion

The procedure described in this communication differs from that of Seal and Doe (1962a,b) in only a few respects. Whereas they have used DEAE-cellulose for a rough, preliminary fractionation of plasma proteins followed by repeated chromatography on hydroxylapatite, we have employed a fine gradient elution from DEAE-cellulose which has eliminated most of the unwanted protein in the first step. Therefore, only one or two fractionations on hydroxylapatite are required instead of five (Seal and Doe, 1962b).

The dextran gel, Sephadex G-25, was used primarily to desalt the protein. Under the proper conditions, however, columns of this material were found to function as an additional anion-exchange column. A twofold purification was accomplished in conjunction with the desalting operation. This potential use of the Sephadex gel was noted by Porath (1960). This gel has also been utilized by other authors in the study of transcortincortisol binding (DeMoor *et al.*, 1963; Seal and Doe, 1963; Doe *et al.*, 1964).

Although our isolated material differs in sedimentation constant and molecular weight from that reported originally by Seal and Doe (1962b) under nearly identical conditions, it is in reasonably close agreement with more recent values found by Seal (4.0 and 52,600, respectively; private communication). Thus, we might tentatively conclude that the two proteins are identical. Yet there are serious discrepancies in the amino acid analysis (Table II). While there is excellent agreement on the values for glutamic acid, glycine, alanine, and histidine, and good agreement for proline, leucine, and lysine, the values for valine, methionine, isoleucine, and tyrosine differ by more than 50%. Seal (private communication) has found that the carbohydrate present in the protein may be responsible for destruction of several amino acids during hydrolysis. Values for five of the amino acids are increased if sialic acid and fucose are removed prior to the amino acid analysis.

[3] Carbohydrate content (Seal and Doe, 1962b) taken into account; analysis revealed 0.34% ash.

References

Bruno, G. A., and Christian, J. E. (1961), *Anal. Chem. 33*, 1216.

Bush, I. E. (1957), *in* Ciba Foundation Colloquia on Endocrinology, Vol. 11, Wolstenholme, G. E., Ed., Boston, Mass., Little, Brown, p 263.

Daughaday, W. H. (1958a) *J. Clin. Invest. 37*, 511.

Daughaday, W. H. (1958b), *J. Clin. Invest. 37*, 5

DeMoor, P., Heirwegh, K., Heremans, J. F., Declerck-Raskin, M. (1963), *J. Clin. Invest. 41*, 816.

Doe, R. P., Fernadez, R., and Seal, U. S. (1964), *J. Clin. Endocrinol. Metab. 24*, 1029.

Flodin, P. (1961), *J. Chromatog. 5*, 103.

Flodin, P., and Kupke, D. W. (1956), *Biochim. Biophys. Acta 21*, 368.

Mahowald, T. A., Noltman, E. A., and Kuby, S. A. (1962), *J. Biol. Chem. 237*, 1138.

Ornstein, L., and Davis, B. J. (1962), Disc Electrophoresis, Rochester, N. Y., Distillation Products Industries.

Piez, K. A., and Morris, L. (1960), *Anal. Biochem. 1*, 187.

Porath, J. (1960), *Biochim. Biophys. Acta 39*, 193.

Porath, J., and Flodin, P. (1959), *Nature 183*, 1657.

Sandberg, A. A., Slaunwhite, W. R., Jr., and Antoniades, H. N. (1957), *Recent Progr. Hormone Res. 13*, 209.

Sandberg, A. A., Slaunwhite, W. R., Jr., and Carter, A. C. (1960), *J. Clin. Invest. 39*, 1914.

Seal, U. S., and Doe, R. P. (1962a), *Cancer Chemotherapy Rept. 16*, 329.

Seal, U. S., and Doe, R. P. (1962b), *J. Biol. Chem. 237*, 3136.

Seal, U. S., and Doe, R. P. (1963), *Endocrinology 73*, 371.

Slaunwhite, W. R., Jr., and Sandberg, A. A. (1959), *J. Clin. Invest. 38*, 384.

Spero, L., Stefanye, D., Brecher, P. I., Jacoby, H. M., Dalidowicz, J. E., and Schantz, E. J. (1965), *Biochemistry 4*, 1024.

Yphantis, D. A. (1964), *Biochemistry 3*, 297.