Isolation and Properties of Cortisol Metabolite Binding Proteins of Rat Liver Cytosol*

K. S. Morey and Gerald Litwack†

ABSTRACT: Two proteins which bind the two most negatively charged metabolites of radioactive cortisol formed in 45 min were isolated from rat liver cytosol. Protein-bound radioactivity was separated initially from unbound radioactivity by column chromatography on Bio-Gel P-100. It then was fractionated into two protein–radioactivity complexes upon chromatography on DEAE-Sephadex A-50. Each binding protein was purified to homogeneity. The large binder was purified by chromatography on Cellex-phosphate followed by Sephadex G-75. The small binder was purified by chromatography and rechromatography on Sephadex G-75. The large binder had an estimated molecular weight of 37,000 calculated from amino acid analysis and from sedimentation velocity and diffusion data, although a value of 50,000 ± 6,000 was obtained by chromatography on Sephadex G-75. The sedimentation coefficient was 3.47 S, a value apparently independent of protein concentration and the isoelectric point was at pH 8.9. It was homogeneous in the analytical ultracentrifuge, by moving-boundary electrophoresis, by column chromatography, and by pH electrofocusing. The small binder had a molecular weight of 6950 calculated from amino acid analysis and 4000–5000 by chromatography on Sephadex G-75. It had a low tyrosine content and an absorbance maximum at 260 mμ suggesting the presence of nucleotides. The isoelectric point was at pH 5.4. It was homogeneous by column chromatography and by pH electrofocusing. The cortisol metabolite binders were specific in binding. The large binder had only the most negatively charged metabolite of cortisol associated with it, while the small binder was bound to the metabolite of intermediate negative charge. The third metabolite of cortisol, with the least anionic charge did not appear to be bound to proteins.

In order to understand the mode of action of a hormone, its distribution and uptake by various tissues of the body and the biochemical transformations of the molecule *in vivo* have to be investigated. Bradlow and coworkers (1954) demonstrated that 5 min after an intravenous injection of tritiated cortisone to mice most of the radioactivity was taken up by the liver with only a trace left in the blood. Bellamy *et al.* (1962) studied the tissue distribution of radioactivity from [4-^{14}C]cortisol 30 min after an intravenous injection into male rats. Only the liver, of all the organs tested, contained more radioactivity per unit weight of tissue than the blood. Thus, the liver plays a central role in the metabolism of corticosteroids. Litwack *et al.* (1963) studied the uptake and subcellular distribution of radioactivity from [4-^{14}C]cortisol in rat liver and observed that the radioactivity was concentrated rapidly in the cytosol and microsomes. Accumulation was maximal by 45 min following an intraperitoneal injection of the hormone and it declined rapidly thereafter. The subcellular distribution of radioactivity from [4-^{14}C]cortisol has been confirmed by Morris and Barnes (1967) who observed that a significant amount of radioactivity from intravenous injections of [4-^{14}C]cortisol was distributed in the supernatant, microsomes, and to a lesser extent in the mitochondria of rat liver as early as 30 sec after the injection.

Recently it was observed that radioactive cortisol was converted rapidly into three anionic metabolites *in vivo* and a significant portion of the radioactive metabolites was bound to proteins soluble in the liver cytosol (Litwack *et al.*, 1965; Fiala and Litwack, 1966) In this communication, we report the purification and properties of two proteins soluble in the rat liver cytosol that bind the two most negatively charged metabolites of radioactive cortisol. The larger of the two proteins was observed to bind the most negatively charged metabolites whereas the metabolite of intermediate negative charge was bound to the smaller protein. The metabolite with the least negative charge did not appear to be bound to proteins. At the moment, specific physiological functions cannot be assigned to these binding proteins or to the anionic metabolites of cortisol. The binders may be involved in regulation of protein synthesis, in intracellular transport of metabolites for excretion or in an as yet undefined role. A preliminary report of this work has been made (Morey and Litwack, 1968).

Experimental Section

Chemicals and Isotopes. [1,2-^{3}H]Cortisol (specific activity, 55 Ci/mmole) and [4-^{14}C]cortisol (specific activity, 53.8 mCi/mmole), obtained from New England Nuclear Corp., were checked for purity by paper chromatography or by high-voltage paper electrophoresis prior to use. Cortisol and cortisol acetate were purchased from Nutritional Biochemicals Corporation. There were no differences in experimental results when either cortisol alcohol or cortisol acetate was used as a carrier. Bovine γ-globulin and beef heart cytochrome *c* were obtained from Sigma Chemical Company.

* From the Fels Research Institute and Department of Biochemistry, Temple University School of Medicine, Philadelphia, Pennsylvania 19140. *Received June 9, 1969.* Supported by Research Grant AM-08350 from the National Institute of Arthritis and Metabolic Diseases, U. S. Public Health Service.

† Research Career Development Awardee 3K3-AM-16,568, National Institute of Arthritis and Metabolic Diseases, U. S. Public Health Service.

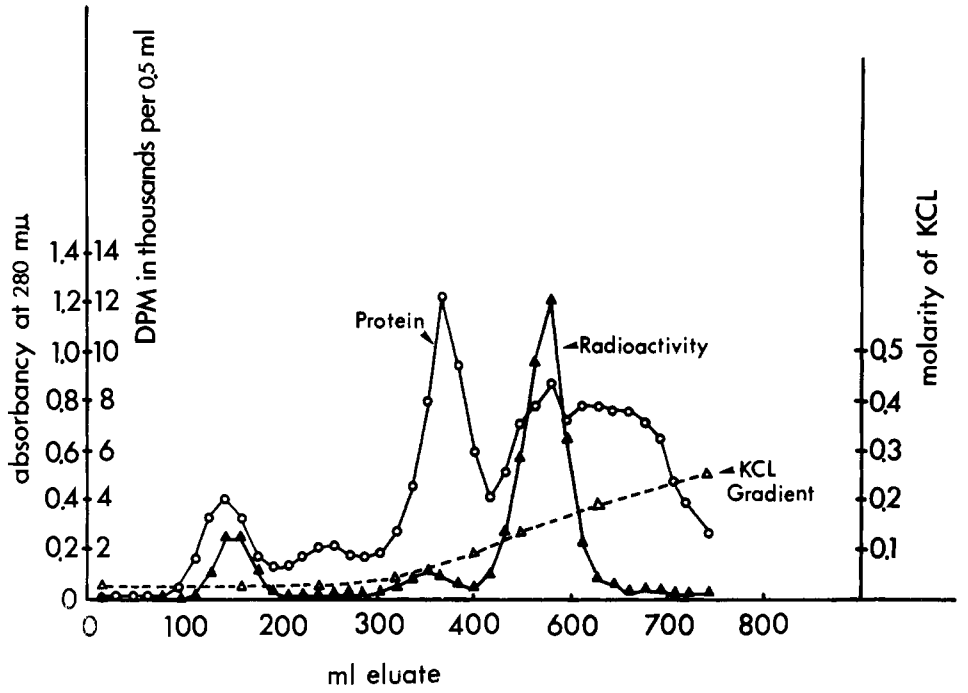

FIGURE 1: Fractionation of protein-bound radioactivity on DEAE-Sephadex A-50. A 2.8 × 70 cm column was used. A linear KCl gradient from 0 to 0.5 M KCl was used for the elution (see Methods); 2.9 × 10^7 dpm was applied, 61% of which was recovered in the bound form. The values of micrograms of steroid in bound form per milligram protein for the peak tube of the large binder is 0.15 and for the small binder (acidic) is 0.39.

Pronase was purchased from K & K Laboratories. Bio-Gels P-2, P-10, P-100, and Cellex-phosphate were purchased from Calbiochem. DEAE-Sephadex A-50, Sephadex G-10, and G-75 were purchased from Pharmacia Fine Chemicals. Ribonuclease and deoxyribonuclease were purchased from Worthington Biochemical Corporation, and human hemoglobin from Pentex Laboratories. Reagent grade chemicals were used throughout.

Animals and Injection. Male adrenalectomized rats ranging in weight from 150 to 200 g were purchased from Charles River Breeding Laboratories and maintained on 1% NaCl and Wayne Laboratory Blox *ad libitum*. They were used for experiment within 7–10 days after adrenalectomy. Radioactive cortisol was dried in a stream of filtered nitrogen and was mixed with unlabeled hormone previously suspended in saline (pH 7.0) prior to the intraperitoneal injection. Usually 100 μCi of [1,2-^{3}H]cortisol or 15 μCi of [4-^{14}C]cortisol were injected per animal mixed with 60 mg of carrier/kg body weight.

Preparation of Liver Cytosol. All fractionation and purification procedures were performed at 2–4° unless otherwise indicated. Animals were decapitated 45 min after injection. Livers were perfused with cold physiological saline and homogenized with an equal volume of 0.25 M sucrose–0.05 M Tris-HCl (pH 7.5). The homogenate was centrifuged in the Sorvall RC-2 centrifuge at 30,000*g* for 20 min. The resulting supernatant was filtered through cheesecloth and centrifuged at 100,000*g* for 60 min in a Spinco Model L-2 ultracentrifuge. The clear supernatant was used as the cytosol preparation.

Preparation of Bound Radioactive Material and Chromatographic Fractionation. The cytosol was fractionated on a 5.0 × 50 cm column of Bio-Gel P-100 using water as the eluent. By this means protein-bound metabolites were separated from unbound metabolites. Protein-bound radioactivity was pooled and concentrated by ultrafiltration. The resulting concentrate was centrifuged at 30,000*g* for 30 min and the supernatant was chromatographed on a 2.8 × 70 cm column of DEAE-Sephadex A-50. A linear gradient from 0 to 0.5 M KCl was established by mixing equal volumes of 0.05 M Tris-HCl (pH 7.5) and 0.05 M Tris-HCl containing 1 M KCl (pH 7.5).

Individual radioactive protein peaks from DEAE-Sephadex A-50 were concentrated by ultrafiltration using UM-1 and UM-2 Amicon membranes. These membranes retained the macromolecules under positive pressure of nitrogen. The leading protein peak from DEAE-Sephadex was purified further on a 2.8 × 35 cm column of Cellex-phosphate. Linear gradient elution with KCl was carried out as described above. The protein-bound radioactivity was pooled, concentrated by ultrafiltration, and chromatographed on a 2.8 × 70 cm column of Sephadex G-75 eluted with 0.05 M Tris-HCl (pH 7.5). The trailing radioactive protein peak from DEAE-Sephadex was purified by chromatography and rechromatography on 2.8 × 47 and 2.8 × 70 cm columns of Sephadex G-75, respectively. Elution was carried out with 0.05 M Tris-HCl (pH 7.5) and this buffer was used for all chromatographic elutions unless specified differently. Desalting of the purified trailing protein peak from DEAE-Sephadex was performed by chromatography on a 2.8 × 47 cm column of Bio-Gel P-2 with water as the eluting agent.

Physical and Chemical Properties of Binders. Molecular weight determinations of purified binders[1] were made

[1] The protein–radioactivity complex eluting first upon DEAE-Sephadex A-50 chromatography is referred to as *large binder*, whereas

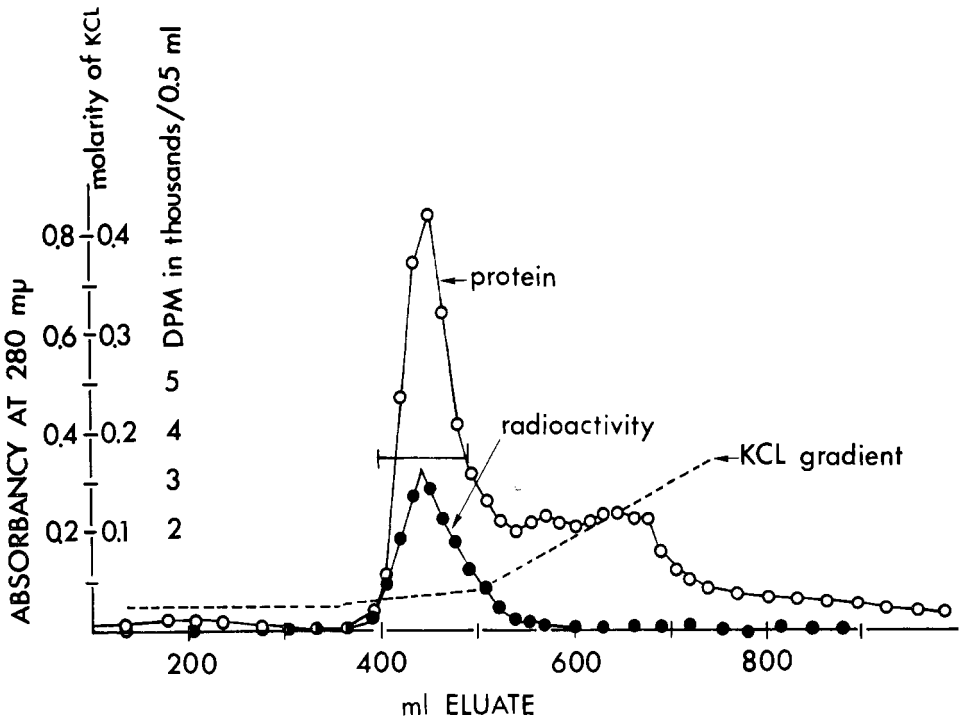

FIGURE 2: Purification of the large binder on Cellex-phosphate. A 2.8 × 35 cm column was used. A linear KCl gradient from 0 to 0.5 M kc was used; 1.7×10^6 dpm was applied, 87% of which was recovered in the bound form. The value of micrograms of steroid in bound form per milligram of protein for the peak tube of the large binder is 0.33.

with a 2.8 × 77 cm column of Sephadex G-75 using bovine γ-globulin, human hemoglobulin, and beef heart cytochrome *c* as marker proteins. Sedimentation velocity experiments were run in the Spinco Model E analytical ultracentrifuge with the AN-D rotor at 56,000 rpm and 20°. Concentration dependence of the sedimentation coefficient of the large binder was studied in 0.05 M Tris-HCl (pH 7.5) at 60,000 rpm and 20° using protein concentrations ranging from 1.5 to 3.8 mg per ml. Moving-boundary electrophoresis of the large binder using the 2-ml cell of the Model 038 Perkin-Elmer Tiselius apparatus was performed at 1° in 0.1 M acetate buffer (pH 4.0). Isoelectric focusing using a pH gradient of 3–10 was performed on the purified large and small binders in 110-ml LKB electrofocusing columns at 300 V, 0.5 mA for 60–65 hr at 4°. The bound radioactivity was treated with hydrolytic enzymes, such as trypsin, pronase, ribonuclease, and deoxyribonuclease overnight at 4° with continuous stirring. Following enzymatic treatment the extent of binding remaining was determined by ultrafiltration. For amino acid analysis, the purified binders were hydrolyzed in 6 N HCl at 110° for 18, 24, and 48 hr in evaculated sealed tubes. The hydrolysates were used for automated amino acid analyses in a Beckman-Spinco analyzer. Radioactivity associated with the large and small binders was extracted with cold 1-butanol as described previously (Fiala and Litwack, 1966). High-voltage electrophoresis was performed at 4° on the extract using Whatman No. 3MM paper and the Savant flat-plate type apparatus; 18–20 V/cm for 3–4 hr was applied in all runs. A caffeine marker was used to indicate the electro-osmosis-compensated origin and a picric acid marker was used to indicate visually the extent of the run. Electrophoresis was carried out in 0.05 M sodium tetraborate (pH 9.2). Ultrafiltration was performed in the PR-2 International centrifuge at 1200 rpm for 2.5–3 hr at 2–4° to determine per cent binding by the technique described previously (Fiala and Litwack, 1966). Protein concentrations were determined by the biuret method (Gornall *et al.*, 1949) and

the late eluting protein–radioactivity complex is referred to as the *small binder*.

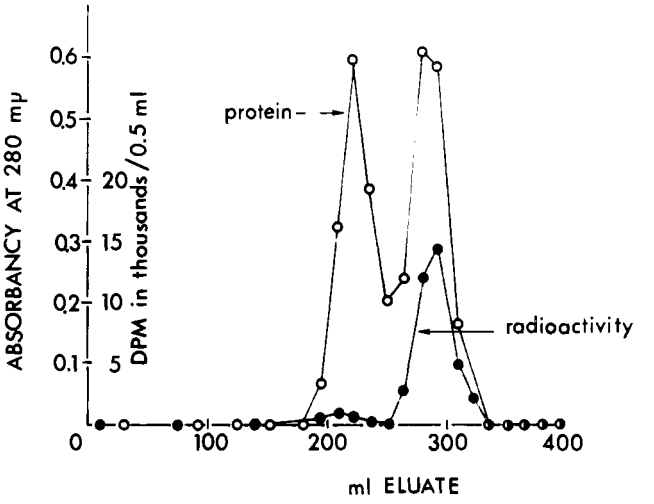

FIGURE 3: Purification of the large binder on Sephadex G-75. A 2.8 × 70 cm column was used; 7.7×10^5 dpm was applied. Recovery as bound radioactivity was 100%. The value of micrograms of steroid in bound form per milligram of protein for the peak tube of the large binder is 0.43.

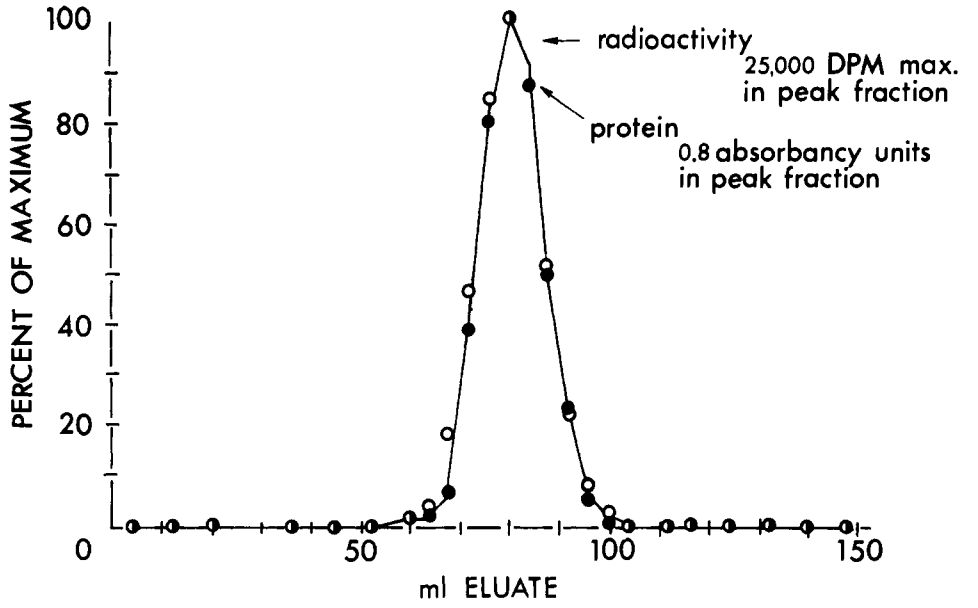

FIGURE 4: Chromatogram showing the homogeneity of the large binder on Sephadex G-75. A 2 × 48 cm column was used; 6.8×10^5 dpm was applied of which 100% was recovered as the bound form.

by the method of Lowry *et al.* (1951) or qualitatively by measuring the ultraviolet absorbance at 280 mμ. Protein–anionic metabolite complex was analyzed as protein. Molarity of fractions from salt gradient elutions was calculated from resistance measured in a Perkin-Elmer conductivity cell with a Leeds and Northrup Model 1563539 electrolytic resistance indicator.

Determination of Radioactivity. Radioactivity determinations were carried out in the dioxane–napththalene–Cab-O-Sil scintillation system described previously (Weinstein *et al.*, 1967). Radioactivity was measured in the Nuclear-Chicago Model 723 scintillation spectrometer. Efficiency was determined by the channels ratio method and was usually 15–20% for tritium and 55–70% for ^{14}C. Radioactivity from electrophoresis strips was determined in a toluene-based scintillation system after sectioning the paper strips into squares (2 × 2 cm).

TABLE 1: Enzymatic Treatment of Bound Radioactivity Eluted from Bio-Gel P-100 Columns.

Enzymatic Treatment[a]	% Binding by Ultrafiltration	% Change
Experiment I		
Control (no enzyme)	66	
RNase[b]	61	(−8)
DNase[c]	66	(0)
Trypsin[d]	46	(−30)
Experiment II		
Control	43	
+200 μg of trypsin	17	(−60)
+400 μg of trypsin	0	(−100)

[a] 60 mg of complex + 100 μg of enzyme; 4° for 18 hr with stirring. [b] 20 μg of RNA made acid soluble per μg of enzyme per min; 38°. [c] 21 μg of DNA made acid soluble per μg of enzyme per min; 38°. [d] 67 mμg of bovine serum albumin made acid soluble per μg of enzyme per min; 38°.

Results

Fractionation of the cytosol derived from perfused liver on Bio-Gel P-100 was similar to that reported by Litwack *et al.* (1965). The protein-bound radioactivity eluted before the unbound radioactivity. Radioactivity from the protein-bound fractions could be rendered dialyzable by ultrafiltration after proteolysis with trypsin or pronase or by extraction with butanol. Ribonuclease and deoxyribonuclease were without effect (Table I). Therefore the binding agents for cortisol metabolites appeared to be proteins.

The protein-bound radioactivity eluted from Bio-Gel P-100 was pooled, concentrated by ultrafiltration, and fractionated on a column of DEAE-Sephadex A-50 (Figure 1). Most of the radioactivity was associated with the leading and trailing protein peaks. The two radioactive protein peaks were purified individually as indicated in the Experimental Section.

Large Binder. After concentration by ultrafiltration the leading protein peak from DEAE-Sephadex was subjected to column chromatography on Cellex-phosphate resulting in significant purification (Figure 2). The radioactivity was recovered in the first eluting protein peak. This protein-bound radioactivity was pooled, concentrated by ultrafiltration, and chromatographed on Sephadex G-75 (Figure 3). Two protein peaks were nearly completely resolved and the radioactivity was associated almost entirely with the late-eluting peak. The protein-bound radioactivity eluted from Sephadex G-75 was pooled, concentrated by ultrafiltration or by freeze drying, and rechromatographed on Sephadex G-75. At this point it was homogeneous by column chromatography (Figure 4). It also behaved as a single component in sedi-

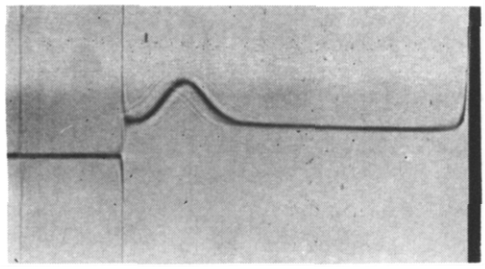

FIGURE 5: Analytical ultracentrifuge pattern of large cortisol metabolite binding protein. Photograph was taken 24 min after reaching speed of 56,000 rpm in the AN-D rotor at 20° with a bar angle of 80°. The protein concentration was 4 mg/ml. Solvent was 0.05 M Tris-HCl (pH 7.5). Sedimentation is from left to right. A single symmetrical peak was visible throughout the run of 104-min duration. Two other runs on different preparations confirm these results.

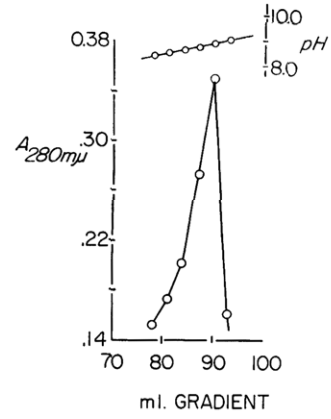

FIGURE 7: Isoelectric focusing of large binder in a pH gradient. Electrofocusing was carried out for 65 hr at 4°, using 300 V and 0.5 mA. Protein used was 7.9 mg.

mentation velocity experiments in the analytical ultracentrifuge (Figure 5). Its sedimentation coefficient at 56,000 rpm and 20° was 3.47 S. This value appeared to be independent of its protein concentration at 60,000 rpm, 20° from 1.5 to 3.8 mg per ml. By least-squares analysis the line extrapolated to zero protein concentration gave a sedimentation coefficient of 3.47 S. Moving-boundary electrophoresis in 0.1 M acetate (pH 4.0) gave a single peak suggesting lack of contaminating proteins with significantly different electrophoretic mobilities (Figure 6). The purified large binder had an isoelectric point at pH 8.9 as determined by isoelectric focusing (Figure 7).

Figure 8 shows the calibration curve used for the determination of the molecular weight of the large binder along with marker proteins on Sephadex G-75. It gave a value of 50,000 ± 6,000 in five experiments. This value remained unchanged even when the binder was incubated overnight in 8 M urea at 4° and then was chromatographed along with marker proteins at room temperature on Sephadex G-75 equilibrated in 8 M urea. The position of the γ-globulin marker on the calibration curve is fortuitous in that it is excluded by this molecular sieve. Amino acid composition of the large binder (Table II) indicated the presence of almost equal numbers of both acidic and basic amino acid residues. It had a high content of hydroxylated amino acids. Such was the case with the leucine-binding protein from *Escherichia coli* (Penrose *et al.*, 1968). Since the large binder behaved as a basic protein upon ion-exchange chromatography, electrophoresis, and pH isoelectric focusing, many of the glutamic and aspartic acid residues must be present in this protein as glutamine and asparagine. Tryptophan content determined by the method of Barman and Koshland (1967) indicated the presence of two residues for a molecular weight of about 37,000. Only trace amounts of cysteine were present. Calculation of the molecular weight from the estimated number of amino acid residues after correcting for the water of hydrolysis gave a value of 37,250.

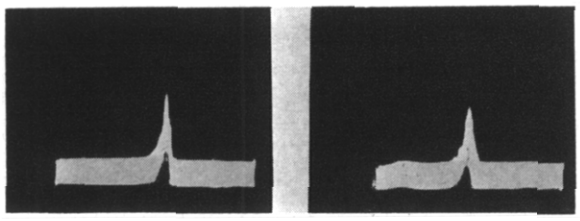

FIGURE 6: Moving-boundary electrophoresis patterns of the purified large binder. Electrophoresis was done in a 2-ml rectangular Tiselius cell at 1°; 200 V, 4.4 mA in 0.1 M acetate buffer (pH 4.0). Protein concentration was 2.7 mg/ml. Photographs were taken 14 min (ascending pattern on left) and 14 min (descending pattern on right) after start of current.

TABLE II: Amino Acid Composition of Large Cortisol Metabolite Binder.

Amino Acid	Av No. of Residues[a]	Mol Wt × No. of Residues
Aspartic acid	28	3,724
Threonine	12	1,428
Serine	12	1,260
Proline	16	1,840
Glutamic acid	36	5,292
Glycine	20	1,500
Alanine	24	2,136
Valine	20	2,340
Methionine	12	1,788
Isoleucine	20	2,620
Leucine	44	5,764
Tyrosine	12	2,172
Phenylalanine	16	2,640
Lysine	28	4,088
Histidine	4	620
Arginine	20	3,480
Tryptophan[b]	2	408
	Total = 326	Total = 43,100
Corrected for 325 peptide bonds (water of hydrolysis)		−5,850
Corrected molecular weight		37,250

[a] Average number of residues calculated from four experiments. Protein was hydrolyzed in 6 N HCl for 18, 24, or 48 hr at 110° after evacuation of sample. [b] Determined by the method of Barman and Koshland (1967).

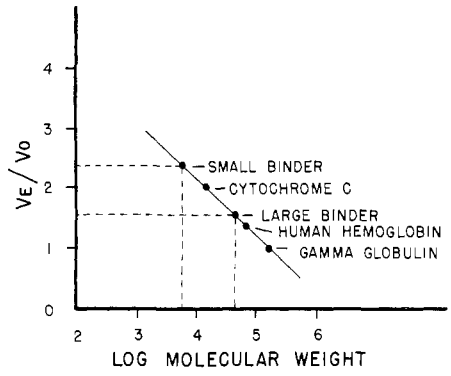

FIGURE 8: Calibration curve showing the molecular weight of the large and the small binder as estimated on Sephadex G-75. A 2.8 × 77 cm column was used. Position of γ-globulin is fortuitous since it is excluded on this molecular sieve.

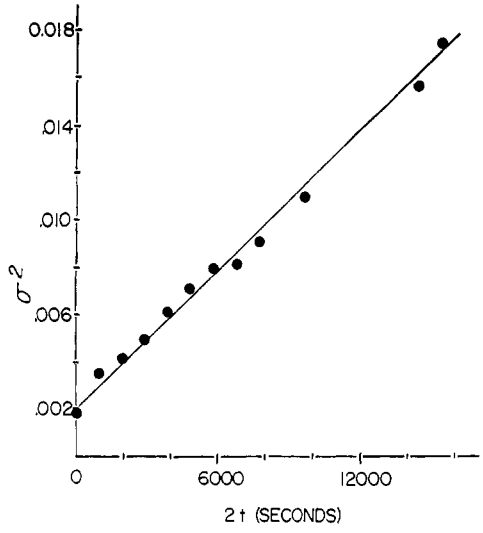

FIGURE 9: Diffusion coefficient of large binder calculated from the slope of the plot of σ^2 *vs.* $2t$. Centrifugation was at 20,000 rpm, 20°.

It compared well with the value of 36,598, obtained by using the sedimentation and diffusion coefficients from the Spinco Model E analytical ultracentrifuge. A diffusion coefficient of 10.1 × 10^{-7} cm^2/sec was calculated from the slope of σ^2 *vs.* $2t$ plot (Figure 9).

The radioactivity associated with the large binder was released by extraction with cold 1-butanol. High-voltage electrophoresis of the radioactive material so obtained (Figure 10) revealed that the most anionic of the three negatively charged cortisol metabolites was bound to this protein. The ultraviolet spectrum of the large binder is shown in Figure 11A.

Small Binder. The trailing peak of protein-bound radioactivity from chromatography on DEAE-Sephadex (Figure 1) was purified further by chromatography on Sephadex G-75 (Figure 12). This resulted in a very large purification. The binder was included and a protein of relatively large molecular weight was eluted before it. The protein-bound radioactivity was concentrated by freeze drying and an aliquot was used for molecular weight estimation on Sephadex

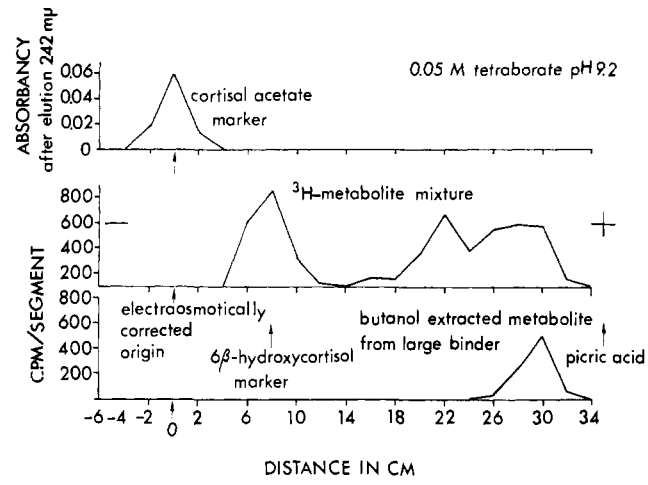

FIGURE 10: Paper electrophoresis of radioactive material released from the large binder after extraction with cold 1-butanol, and of a mixture of unbound metabolites of [^{3}H]cortisol. See Methods for procedure.

TABLE III: Amino Acid Composition of Small Cortisol Metabolite Binder.[a]

Amino Acid	Av No. of Residues[b]	Mol Wt × No. Residues
Aspartic acid	4	532
Threonine	4	476
Serine	2	210
Proline	3	345
Glutamic acid	6	882
Glycine	6	450
Alanine	5	445
Valine	6	702
Methionine	1	149
Isoleucine	5	655
Leucine	8	1,048
Tyrosine	1	181
Phenylalanine	2	330
Lysine	7	1,022
Histidine	2	310
Arginine	2	348
	Total = 64	Total = 8,085
Correction for 63 peptide bonds (water of hydrolysis)		−1,134
Corrected molecular weight		6,951

[a] Protein hydrolyzed for 24 or 48 hr in 6 N HCl at 110° after evacuation of sample. [b] Average of two determinations. Tryptophan content not determined. See text for explanation.

G-75 producing a value in the range of 4000–5000 (Figure 8). The small binder was desalted chromatographically on Bio-Gel P-2 which demonstrated homogeneity (Figure 13). Isoelectric focusing of the small binder indicated the homogeneous peak had an isoelectric point at pH 5.4 (Figure 14). The amino acid composition of the small binder (Table III)

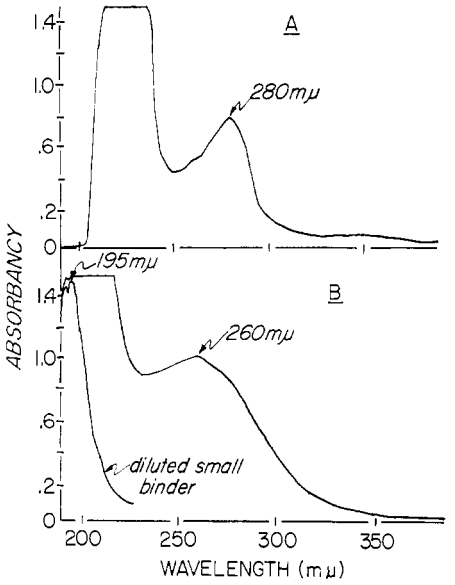

FIGURE 11: Ultraviolet spectra. (A) Of purified large binder in 0.05 M Tris-HCl (pH 7.5). (B) Of purified small binder in deionized water.

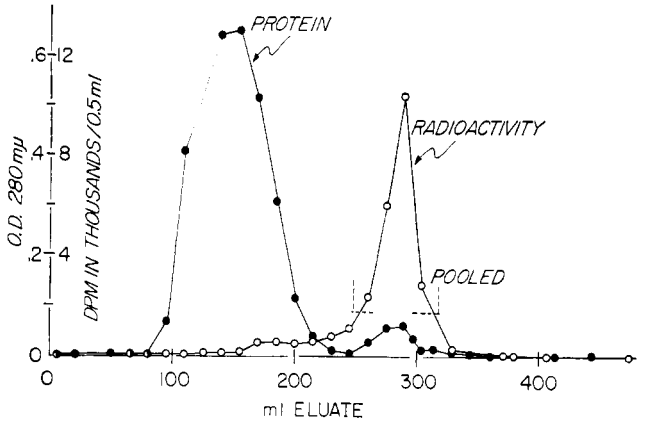

FIGURE 12: Purification of the small binder on Sephadex G-75. A 2.8 × 47 cm column was used; 5.0×10^6 dpm was applied of which 81% was recovered in the bound form. The value of micrograms of steroid in bound form per milligram of protein for the peak tube of the small binder is 6.8.

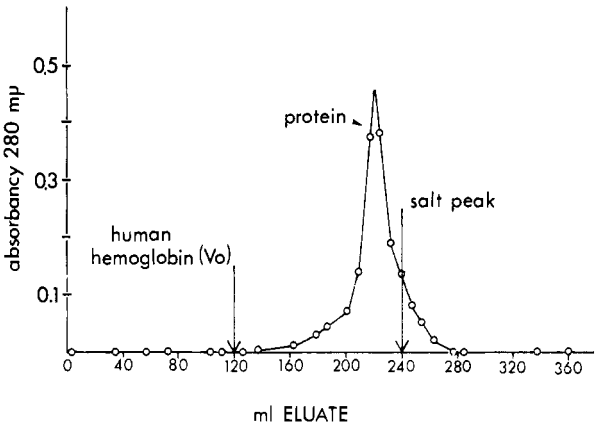

FIGURE 13: Chromatography of small binder on Bio-Gel P-2 showing homogeneity. A 2.8 × 47 cm column was used. Eluent was deionized water.

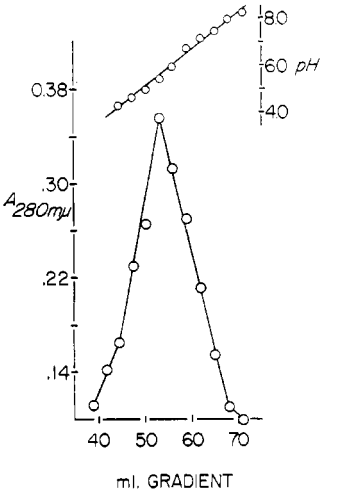

FIGURE 14: Isoelectric focusing of small binder in a pH gradient. Electrofocusing was carried out for 65 hr at 4°, using 300 V and 0.5 mA. Protein used was 11 mg.

indicated a very low tyrosine content which was reflected in the lack of an absorbance maximum of 280 mμ. Attempts to determine the tryptophan content of the small binder using Barman and Koshland's procedure (1967) were unsuccessful since the protein is too small to be precipitated out by trichloroacetic acid. Only trace amounts of cysteine were present. The molecular weight, determined after correcting for the water of hydrolysis, was 6950 which was slightly higher than the molecular weight of 4000–5000 obtained by gel filtration.

The radioactivity associated with the small binder was released by extraction with 1-butanol. High-voltage electrophoresis (Figure 15) characterized the radioactive material as the cortisol metabolite of intermediate negative charge. In the ultraviolet spectrum of the small binder (Figure 11B), the absorbance maximum at 260 mμ indicated the presence of nucleotides. The absence of an absorbance maximum at 280 mμ verified the low content of tyrosine in this protein.

Studies with proteolytic enzymes revealed the proteinaceous nature of the large cortisol metabolite binder. Since both RNase and DNase were without effect on binding after appropriate incubation and the absorption spectrum contained no 260-mμ maximum, the role of polynucleotides can be ruled out in case of the large binder. The amino acid analysis showed that the small binder was also a protein, but it had an absorbance maximum at 260 mμ. This suggested a content of one or more nucleotides, a conclusion supported further by a positive orcinol reaction, a negative diphenylamine reaction, and the ability to label the small but not the large binder *in vivo* by injection of [^{14}C]adenosine[2] Of interest is the report by Hariharan and Barnes (1968) which indicated covalent-bond formation between radioactivity from corticosterone and a nucleotide shortly

[2] K. S. Morey and G. Litwack, unpublished data.

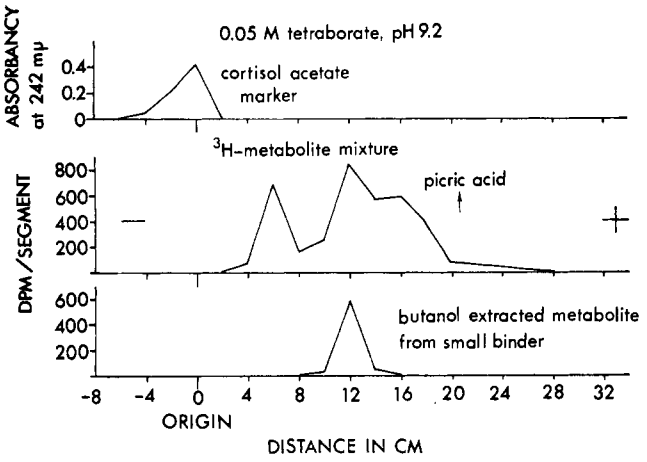

FIGURE 15: Paper electrophoresis of radioactive material released from the small binder after extraction with cold 1-butanol, and of a mixture of unbound metabolites of [^{3}H]cortisol. See Methods for procedure.

after intravenous injection in the rat. It is possible that a complex of this type may later associate with the polypeptide portion of the binder. The amino acid analysis of the large and small binders showed the presence of only trace amounts of cysteine. This suggested that sulfhydryl groups may not be involved in the cortisol metabolite binding to these proteins.

On the basis of protein concentration recovered in successive fractionation steps, the large binder was purified about 100-fold over the protein-bound peak obtained after chromatography on Bio-Gel P-100 and the small binder was purified about 115-fold from the same starting point. The values of micrograms of steroid per milligram of protein given in the legends of figures depicting various fractionation steps are misleading because the radioactive steroid is also dissociated away during purification. Thus, in the case of the large binder, there is about a 45-fold loss of the radioactivity, and in the case of the small binder, about a 10-fold loss of radioactivity, from the Bio-Gel P-100 step.

Discussion

Binding of hormones to intracellular protein receptors may be an early event in the actions of hormones. This concept has been well supported in the case of estrogens by Noteboom and Gorski (1965) and by Jensen *et al.* (1968). Aldosterone–protein complexes have been isolated from the nuclear and cytosol fractions of rat kidneys by Herman *et al.* (1968). There are notable differences between the intracellular binding of estrogens and aldosterone and that of cortisol in liver. Parent estrogens and aldosterone were bound directly to intracellular proteins in the target tissue. Previous studies on cortisol binding (Fiala and Litwack, 1966), however, have shown that only 3% of unchanged cortisol was bound to proteins in the liver cytosol *in vitro* in spite of the rather extensive binding observed *in vivo*. This suggested that a metabolically altered form of cortisol was bound *in vivo* in 45 min. No more than 6% of the total radioactivity in the liver cytosol could be accounted for by metabolically unaltered cortisol. Another significant difference from binding of estrogens and aldosterone is that cortisol was accumulated to a very small extent in the nucleus of the hepatocyte as observed by Litwack and Baserga (1967) using the technique of autoradiography.

Values for the molecular weight of about 37,000 for the large binder, obtained by calculation from the amino acid composition, and that calculated from sedimentation velocity–diffusion agree very well. The higher molecular weight of 50,000 ± 6,000 obtained from gel filtration was not reduced by prolonged incubation in 8 M urea. Therefore aggregation based on hydrogen-bond formation on Sephadex G-75 is probably ruled out. Interestingly, the molecular weight of the leucine binding protein of *E. coli* also appeared to be much larger when estimated by gel filtration compared with that calculated from ultracentrifuge data (Penrose *et al.*, 1968).

The molecular weight for the small binder was 6950 when calculated from the amino acid analysis data, whereas a value of 4000–5000 was obtained on a calibrated Sephadex G-75 column. The former is probably more correct. Some proteins show anamolous behavior on Sephadex (Janson, 1967) as well as on polyacrylamide gels either due to absorption or slight electrostatic interaction. Combination of both these effects would result in retardation of certain proteins on these gels. Uziel and Cohn (1965) observed that ribonucleosides and deoxyribonucleosides were slightly adsorbed on Bio-Gel P-2 columns thereby being retarded during their elution. Schwartz *et al.* (1965) also noted similar retardation of nucleic acid bases on Bio-Gel P-2. The ring structure of nucleic acid bases probably contributed toward their retardation on these columns. Since nucleotides may be present in the small binder, it might be retarded during chromatography on both Sephadex and polyacrylamide gels, causing the unexpected inclusion of the protein on Bio-Gel P-2 columns. The binding between the protein and the cortisol metabolites may be noncovalent as suggested previously (Fiala and Litwack, 1968). Upon repeated column chromatography the steroid could be stripped from the small binder. The binding appeared specific since electrophoresis of the radioactive material extracted from the binders revealed that only the most anionic metabolite of radioactive cortisol was bound to the large binder, whereas the metabolite of intermediate negative charge was associated with the small binder.

It was already established that the proteins binding the cortisol metabolites in liver cytosol were different from the proteins that bound unmetabolized cortisol in the serum (Fiala and Litwack, 1968). At present no definite function can be assigned to either of the two binders but specific binding may indicate specific function. Studies on subcellular distribution of cortisol (Litwack *et al.*, 1963), autoradiographic experiments (Litwack and Baserga, 1967), and the early effects of cortisol on the liver parenchymal cell by electron microscopy (Rancourt and Litwack, 1968) suggested that the early action of the hormone occurred in the cytoplasmic compartment. However, these studies did not rule out the possibility that following the early events in the cytoplasm subsequent changes might occur in the cell nucleus. Since the events occurring in the cytoplasmic compartment were important, there exists the possibility that one or both binders is a repressor of translation capable of the removal from the site of inhibition by formation of a complex with a cortisol metabolite. Another possible function for the binders is that of intracellular transport. As transporting

agents in secretion the binders would be expected to complex the metabolites and enter the bile canaliculi of liver in the bound form, or release the metabolite into the blood stream. A third possibility is that the binders are enzymes or parts of enzymes involved in cortisol metabolism. Appropriate experiments to test these possibilities will be carried out.

Currently, studies are being carried out in our laboratory in order to characterize the metabolites of cortisol. Results from double-label experiments using a [^{35}S]H_2SO_4 injection followed by [1,2-^{3}H]cortisol injection suggested that at least one anionic metabolite is a sulfate.[3] The least anionic metabolite of cortisol (which did not bind to protein), having an electrophoretic mobility in tetraborate buffer similar to authentic 6β-hydroxycortisol, did not appear to be sulfated. It is possible that these metabolites may be the physiologically active forms of cortisol in certain metabolic reactions.

Acknowledgment

The authors are grateful to Dr. M. Prince Brigham for allowing us the use of the Beckman-Spinco amino acid analyzer and to Mr. Earl Pearson for running the analyses. The excellent technical assistance of Miss Helene Pavlov is appreciated.

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