Characterization and Partial Purification of the Specific Glucocorticoid-Binding Component from Mouse Fibroblasts*

John F. Hackney† and William B. Pratt‡

ABSTRACT: The glucocorticoid-binding component (receptor) isolated from the soluble fraction of mouse fibroblasts behaves as a large lipoprotein complex. The binding complex has a molecular weight of 620,000 as determined by filtration on Sephadex G-200 and a sedimentation coefficient in a sucrose density gradient of 5.5 S. It is weakly acidic with an isoelectric point of 6.4. The size of the receptor may be reduced by digestion with phospholipase A or C. The complex of receptor and bound radioactive triamcinolone acetonide has been purified 2100-fold.

The rate of replication of mouse fibroblasts is inhibited by low concentrations of glucocorticoids (Pratt and Aronow, 1966; Ruhmann and Berliner, 1967). It has been demonstrated that the inhibition of growth *in vitro* of strain L929 cells is preceded by a decrease in the rate of incorporation of nucleic acid precursors into macromolecular cell components (Pratt and Aronow, 1966; Kemper *et al.*, 1969). These effects on nucleic acid synthesis are in turn preceded by a decrease in the rate of uptake of hexoses into the cell (Gray *et al.*, 1971).

In a recent study Hackney *et al.* (1970) identified in L cells a binding component for triamcinolone acetonide[1] which was distributed intracellularly largely in the 105,000*g* supernatant. The glucocorticoid-binding component possessed certain qualities characteristic of a "receptor" for the growth inhibition response. The binding component became saturated between 10^{-8} and 5×10^{-8} M triamcinolone acetonide, a concentration range where maximum growth inhibition is achieved. The structure–activity relationship of steroids active in growth inhibition is paralleled exactly by the ability of these steroids to compete for the association of [^{3}H]triamcinolone acetonide with the binding component. A subline of L929 cells which was resistant to the growth inhibitory effects of glucocorticoids had a markedly diminished ability to bind [^{3}H]triamcinolone acetonide to the high-affinity binding component in comparison with sensitive cells. The binding of triamcinolone acetonide with its binding component was demonstrated to be noncovalent, and chemically unaltered triamcinolone acetonide could be released from the bound complex by brief digestion with a proteolytic enzyme.

In the present study, we have investigated the sedimentation and gel filtration characteristics of the binding component as it exists in the soluble fraction of the cell. In addition a partial purification of the binding material has been achieved.

Materials and Methods

Materials. [1,2,4-^{3}H]Triamcinolone acetonide (20 Ci/mmole) was purchased from Schwarz BioResearch Inc., Orangeburg, N. Y. Alcohol dehydrogenase from horse liver, catalase from bovine liver, and phospholipase C from *Clostridium welchii* were obtained from Sigma Chemical Co., St. Louis, Mo. β-Galactosidase from *Escherichia coli* was obtained from Worthington Biochemical Corp., Freehold, N. J. A boiled snake venom preparation (protease free) with a high activity of phospholipase A was a gift from Dr. Lawrence Eng, Department of Pathology, Stanford University. Aldolase, ovalbumin, and chymotrypsinogen were purchased as a calibration kit from Pharmacia Fine Chemicals Inc., Piscataway, N. J. Myoglobin from whale skeletal muscle was obtained from Calbiochem, Los Angeles, Calif.

Cell Culture. Spinner cultures of L929 cells were maintained in basal medium (Eagle, 1955) modified such that the amino acids were concentrated five times, L-glutamine three times, vitamins two times, glucose five times, NaH_2PO_4 ten times, and $CaCl_2$ and $NaHCO_3$ were omitted. This medium was supplemented with 150,000 units/l. of penicillin, 100 mg/l. of streptomycin sulfate, 10% bovine serum, and 0.1% methylcellulose (15 cP). Cultures were maintained at 37° with constant stirring in an atmosphere of humidified air. Glucocorticoid-resistant L cells were prepared as described previously (Hackney *et al.*, 1970). They were cloned by isolating a single cell on a sparsely seeded monolayer culture and allowing it to replicate for several generations in 50% reconstituted medium until the resulting clone was large enough to allow serial passage in monolayer culture in prescription bottles with the normal medium.

Incubation of Cells with Steroid and Cell Fractionation. Cells were harvested from suspension culture by centrifugation at 600*g* for 10 min and washed twice by resuspension in four to six volumes of Earle's saline and centrifugation at 600*g*. The washed cells were then suspended in Earle's saline supplemented with 0.1% glucose at a density of 2 ml of packed cells/40 ml of saline. The cell suspension was then added to an erlenmeyer flask containing [^{3}H]triamcinolone acetonide in ethanol and an atmosphere of 5% CO_2 in air. The final concentration of [^{3}H]triamcinolone acetonide was 10^{-8} M and the final concentration of ethanol was never more than 0.25%. The cells were then incubated at 37° with mild shaking for 30 min.

After incubation with the labeled steroid, cells were centri-

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† Predoctoral Fellow: Public Health Service Training Grant GM 322 from the Division of General Medical Sciences. Present address: Department of Pharmacology, University of Wisconsin Medical School, Madison, Wis.

‡ To whom to address correspondence.

[1] Triamcinolone acetonide, 9α-fluoro-11β,16α,17α,21-tetrahydroxypregna-1,4-diene-3,20-dione 16,17-acetonide.

fuged at 600*g* for 3 min at 4°. All subsequent procedures, including chromatography, density gradient centrifugation, and isoelectric focusing, were carried out at 0–4°. The cell pellet was washed twice by resuspension in 40 ml of Earle's saline and low-speed centrifugation. The washed cells were suspended in four volumes of hypotonic solution of 0.01 M Tris buffer at pH 7.5 and 0.1 mM EDTA for 5 min, and homogenized with 15 strokes of a tight-fitting pestle in a Dounce-type glass homogenizer. After homogenization, exactly one-tenth volume of hypertonic buffer (1.43 M NaCl, 0.11 M KCl, 0.033 M $MgCl_2$, and 0.11 M Tris, pH 7.5) was added to bring the broken cell suspension to isotonicity. The broken cell suspension was centrifuged at 600*g* for 10 min and the supernatant was centrifuged at 105,000*g* for 1 hr.

Gel Filtration. Solutes in the 105,000*g* supernatant were separated according to molecular size on columns of Sephadex G-200 or 6% Agarose (Bio-Gel A-5m, Bio-Rad Laboratories, Richmond, Calif.). In both cases, 2.0 ml of 105,000*g* supernatant prepared from cells incubated with 10^{-8} M [^{3}H]-triamcinolone acetonide was chromatographed on a 2.5 × 36 cm column with an elution buffer of 0.01 M Tris–0.04 M KCl (pH 7.8). In some of the experiments, a crude separation of bound from free steroid was carried out by passing the 105,000*g* material through a similar column of Sephadex G-25. The fractions comprising the macromolecular peak were pooled and submitted to subsequent separation procedures.

Chromatography on DEAE-cellulose. DEAE-cellulose was washed with 0.5 N HCl for 30 min, rinsed to pH 5 with distilled water, washed with 0.5 N NaOH for 30 min, and again rinsed to pH 8. It was then equilibrated with the 0.01 M Tris–0.04 M KCl (pH 7.2). The preparation of bound [^{3}H]triamcinolone acetonide was then adsorbed onto a 1.5 × 22 cm column of DEAE-cellulose, followed by 100 ml of the starting buffer, and eluted with a 0.04–0.3 M KCl gradient–0.01 M Tris (pH 7.2). Samples were assayed for chloride content, radioactivity, and optical density at 280 mμ.

Isoelectric Focusing. The macromolecular peak (6 ml) obtained from Sephadex G-25 filtration of the 105,000*g* supernatant was diluted to 55 ml with distilled water and 0.63 ml of 40% (w/v) ampholine preparation, pH 5–8, was added. A total of 1.9 ml of 40% ampholines (pH 5–8 was added to 55 ml of a 47% solution of sucrose in distilled water and the light and dense solutions were added to 110-ml isoelectric focusing column as a linear gradient. The column was run for 24 hr at 700 V at 4° with the anode at the bottom and the cathode at the top. Concentrated solutions of acid and base were added at the anode and cathode ends of the gradient, respectively. At the end of the procedure, 2.0-ml samples were collected and analyzed for pH, radioactivity, and optical density at 280 mμ. Fractions 8–18 contained a visible precipitate and they were centrifuged before carrying out the above assays on the supernatant.

Sucrose Gradient Centrifugation. The Sephadex G-25 macromolecular elution or the appropriate ammonium sulfate fractionated sample (0.1 ml) was placed on a 4.6-ml 5–20% sucrose gradient. The sample contained 1.0 mg of marker alcohol dehydrogenase. Gradients were centrifuged for 21 hr in the SW50 rotor of a Spinco Model L centrifuge at 39,000 rpm. Fractions were collected by puncturing the bottoms of the centrifuge tubes. Assays for radioactivity and enzyme activity were carried out on each fraction. Sedimentation coefficients were calculated according to the method of Martin and Ames (1961).

Enzyme Assays. Alcohol dehydrogenase (alcohol:NAD-oxidoreductase, EC 1.1.1.1) was assayed by a modification of the method of Vallee and Hoch (1955). One unit of enzyme activity is defined here as that amount of enzyme which reduced 3.2 mmoles of NAD/ml per min. Catalase (hydrogen peroxide:hydrogen peroxide oxidoreductase, EC 1.11.1.6) activity was assayed by a modification of the technique of Beers and Sizer (1952). β-Galactosidase (β-D-galactoside galactohydrolase, EC 3.2.1.23) activity was measured according to the method of Craven *et al.* (1965). The myoglobin marker was localized by its optical density at 407 mμ. The other proteins utilized as molecular weight standards were localized by their absorption at 280 mμ.

Assay for Radioactivity and Chemical Assays. The aliquot to be counted was added to 10 ml of scintillation solution prepared according to the method of Bray (1960), and assayed in a Packard Tri-Carb liquid scintillation spectrometer, Model 3310.

Protein determinations were carried out according to the method of Oyama and Eagle (1956) using crystalline bovine albumin (Armour Pharmaceutical Co.) as a standard. Micrograms of protein nitrogen were converted into micrograms of protein by multiplying by 6.25. Chloride was assayed with an Aminco Cotlove chloride titrator.

Results

Characterization of the Glucocorticoid Binding Component

Gel Filtration of the Bound Triamcinolone Acetonide. Radioactive triamcinolone acetonide was added to a suspension of L cells in a balanced salt solution supplemented with glucose and incubated as described in Materials and Methods. Triamcinolone acetonide completely equilibrates between L cells and the suspending medium within 5 min. A 105,000*g* supernatant fraction was prepared, and an aliquot was filtered through a column of Sephadex G-200. It can be seen (Figure 1A) that the bound steroid is eluted between the excluded material indicated by the sharp solid-line peak at fraction 22, and the arrow indicating the location of *E. coli* β-galactosidase, mol wt 540,000 (Craven *et al.*, 1965). As the bound material was eluted from the Sephadex G-200 in such proximity to the exclusion limits of the gel, a similar experiment was carried out with a column of 6% Agarose which has a dextran exclusion size of 5×10^6. The bound radioactivity as recovered from the 105,000*g* supernatant is again eluted from the column just before the β-galactosidase marker enzyme (Figure 2A). There is also a small amount of the radioactivity which is eluted in the void volume of the column. Identical experiments with the cloned steroid-resistant subline of L-929 cells also yield a binding component for triamcinolone acetonide which has the same elution volume as that from sensitive cells on chromatography both on Sephadex (G-200 Figure 1A) and on 6% Agarose (not shown). There is, however, only 10–15% as much radioactive triamcinolone acetonide associated with the binding component from resistant cells at 10^{-8} M concentration of steroid, as with sensitive cells.

If the 105,000*g* fraction containing bound radioactive triamcinolone acetonide is filtered through Sephadex G-25 and the fractions comprising the macromolecular peak are combined and precipitated with 25% ammonium sulfate, about 60% of the radioactivity is precipitated (Table I). When this 25% ammonium sulfate precipitated material is redissolved and filtered through a column of Sephadex G-200 (Figure 1B) or 6% Agarose (Figure 2B), a large fraction of the radioactivity is eluted between the β-galactosidase (mol wt 540,000) and catalase (mol wt 244,000, Samejima *et al.*, 1962; mol wt 250,-

TABLE I: Ammonium Sulfate Precipitation of the Macromolecular Fraction from Sephadex G-25.[a]

Fraction	Protein Content (mg of Protein)	% of Total	Radioactivity (cpm × 10^{-3})	% of Total	Sp Act. (cpm/μg of Protein)	-Fold Purifcn
Pooled G-25 macromolecular fractions	194	100	1049	100	5.4	1
Ammonium sulfate concentration (% of saturation)						
0–10	0.7	0.3	4	0.3	5.4	1
10–15	1.0	0.5	23	2.2	22.7	4.2
15–20	5.6	3	164	16	29.4	5.4
20–25	15.9	8	395	38	25.3	4.6
25–30	20.4	10	179	17	8.8	1.6
30–35	25.8	13	79	7.5	3.0	0.6
35–50	58.8	30	30	2.9	0.5	0.1
50–100	43.2	22	18	1.7	0.4	0.1
100% supernatant			1	0.1		

[a] The fractions comprising the macromolecular peak after Sephadex G-25 filtration of a 105,000*g* supernatant prepared from L cells incubated with 10^{-8} M radioactive triamcinolone acetonide were combined and precipitated with increasing concentrations of ammonium sulfate. 87% of the original protein and 86% of the original radioactivity was accounted for in the procedure.

000, Kiseler *et al.*, 1967) marker enzymes. In addition, there is a peak of radioactivity which coincides with the excluded material on both columns. The amount of radioactivity which elutes with excluded material varies from 10 to 50% of the total radioactivity precipitated by ammonium sulfate.

An estimation of the size of the binding component in the soluble phase of the cell and that found after precipitation with ammonium sulfate was made by comparing the elution volume of the binding components on Sephadex G-200 to those of a number of globular proteins of known molecular weight. These results are present in Figure 3. By this method the glucocorticoid-binding component as it exists in the 105,000*g* fraction behaves as would a globular protein of molecular weight of approximately 620,000. The binding component recovered after ammonium sulfate treatment corresponds to a molecular weight of approximately 330,000.

Sedimentation of the Glucocorticoid-Binding Component in a Sucrose Gradient. If the 105,000*g* cell supernatant fraction is filtered through Sephadex G-25, the binding component remains in the large (mol wt 620,000) form. An aliquot of Sephadex G-25 filtered material and an aliquot of the redissolved ammonium sulfate precipitate containing bound radioactive triamcinolone acetonide were centrifuged in 5–20% sucrose gradients with alcohol dehydrogenase, 5.11 S (Ellfolk, 1960) as a marker. The results which are presented in Figure 4 demonstrate that the binding component before precipitation with ammonium sulfate has a sedimentation value of 5.5 S and after

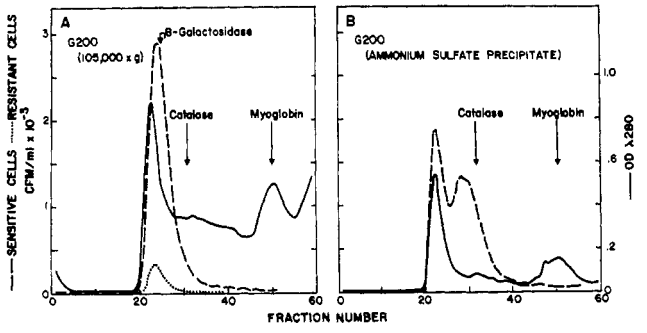

FIGURE 1: Gel filtration of triamcinolone acetonide binding component on Sephadex G-200. (A) The 105,000*g* supernatant fraction (2.0 ml; containing 12.5 mg of protein) prepared from L cells incubated with 10^{-8} M [^{3}H]triamcinolone acetonide (4.3 Ci/mmole) was filtered through a Sephadex G-200 column. The radioactivity, the activity of the designated marker enzymes, and the absorbance at 280 mμ were measured in the appropriate fractions as described under Materials and Methods. (B) A 105,000*g* supernatant fraction prepared as described above was precipitated with ammonium sulfate. The protein which precipitated out of solution between 10 and 25% ammonium sulfate saturation was dissolved in the standard Tris buffer, and 2.0 ml containing 4.7 mg of protein was filtered through Sephadex G-200. In both cases the locations of marker proteins (1 mg of catalase, 2 mg of β-galactosidase, 3 mg of myoglobin per sample) are indicated by arrows. The solid line in each section of the figure represents the absorbance at 280 mμ. The dashed line represents the radioactivity from sensitive cells incubated with [^{3}H]triamcinolone acetonide and the dotted line represents the radioactivity from an identical incubation with glucocorticoid-resistant cells. The unbound steroid is eluted in fractions 60–70 and has not been included in the figure.

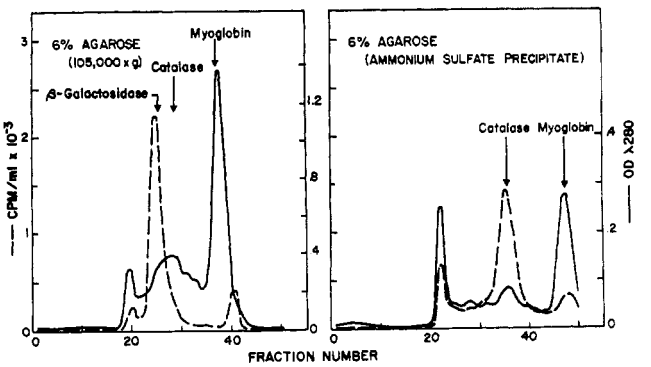

FIGURE 2: Gel filtration of triamcinolone acetonide binding component on a column of 6% Agarose. Experimental details are as described in the legend to Figure 1.

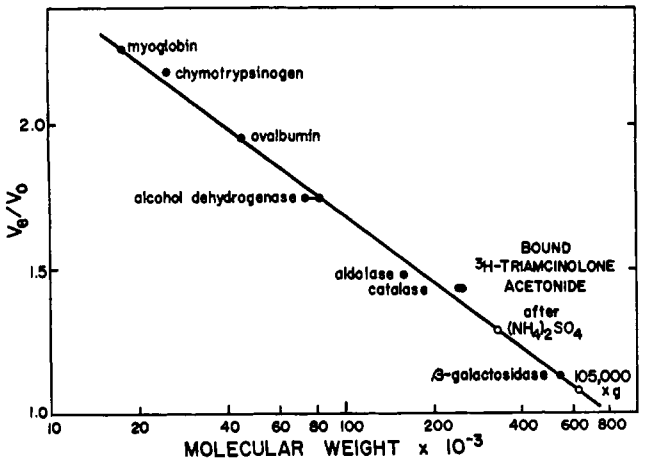

FIGURE 3: Apparent molecular weight of the soluble binding component and the binding component after ammonium sulfate precipitation as determined by filtration on Sephadex G-200. The figure presents a plot of the ratio of the elution volume to the void volume against the log of the molecular weight for a number of protein standards on a Sephadex G-200 column (2.5 × 36 cm) at pH 7.8. The determination of the location of the various protein standards is described under Materials and Methods. The closed circles represent the location of the protein standards and the open circles the binding component.

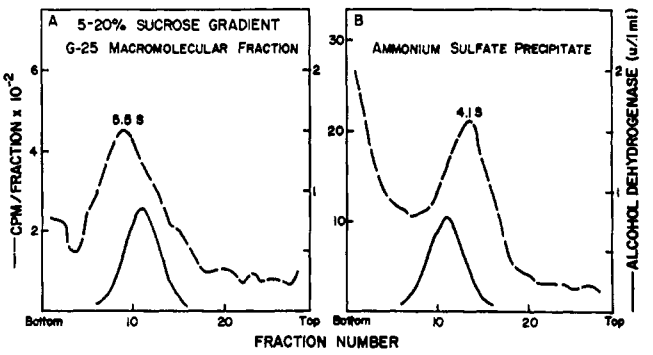

FIGURE 4: Centrifugation of the binding component on a 5–20% sucrose gradient. The 105,000*g* supernatant prepared from fibroblasts incubated with [^{3}H]triamcinolone acetonide was filtered through a column of Sephadex G-25 using the standard Tris buffer. The fractions comprising the macromolecular peak were combined and a portion was precipitated at a saturation of 25% with respect to ammonium sulfate. The Sephadex G-25 macromolecular fraction (0.1 ml; 865 μg of protein) and the redissolved ammonium sulfate precipitate (0.1 ml; 768 μg of protein) were centrifuged with 1 mg of alcohol dehydrogenase as a marker on a 5–20% sucrose gradient as described in the Materials and Methods. The solid line represents the sedimentation profile for alcohol dehydrogenase activity (5.1 S) and the dashed line that for radioactivity.

precipitation, a sedimentation value of 4.1 S. The same sedimentation values were obtained on other gradients employing catalase, 11.3 S (Svedberg and Pedersen, 1940) as the standard.

Determination of the Isoelectric Point of the Binding Component. The weakly acidic nature of the binding component is seen in its behavior on isoelectric focusing (Figure 5). By this procedure, the soluble bound radioactivity concentrates in a major peak at pH 6.4. The minor peak seen in the early fractions is due to counts bound to precipitated material in the high-density sucrose. Although this procedure yields a good estimation of the isoelectric point of the binding component it is not useful for purification as there is no enrichment of the specific activity of binding due to significant precipitation of the bound radioactivity.

Effect of Phospholipase Digestion on the Size of the Glucocorticoid-Binding Component. Replicate aliquots of a Sephadex G-25 macromolecular fraction containing bound [^{3}H]triamcinolone acetonide were incubated for various intervals of time with a preparation of phospholipase C from *Cl. welchii* or with a preparation of phospholipase A from *Crotalus adamanteus* venom. The results are presented in Figure 6. Digestion with the phospholipase C results in the gradual appearance of a binding peak which elutes on Sephadex G-200 slightly before myoglobin (mol wt 17,800). As demonstrated in the second profile of Figure 7, a 10-min digestion with the phospholipase A preparation results in a shift of the major portion of the bound steroid to material which also elutes just prior to the myoglobin marker. This shift is not accompanied by dissociation of the steroid to the free form during the early time intervals. After 2-hr incubation, however, a significant portion of the radioactivity is recovered in the free form. During both treatments there is some shift in the optical density profile from large- to smaller-sized material. The sharp optical density peak under the indicator line for myoglobin is due to the absorbance of that marker protein.

Both the phospholipase C and phospholipase A enzyme preparations were incubated with purified β-galactosidase for 1 hr under the conditions described for the experiment carried out in Figure 6. At the end of the incubation, both the activity of the β-galactosidase and its molecular size as determined by filtration on Sephadex G-200 were unaltered. In addition, the phospholipase A preparation was further examined for protease contamination by incubating it for one hour under the experimental conditions with purified bovine serum albumin and purified ovalbumin (crystallized two times). The incubations were terminated by precipitation in cold 4% trichloroacetic acid and electrophoresed in a 7.5% sodium dodecyl sulfate acrylamide gel as described by Palmiter *et al.* (1971). There was no evidence that there was

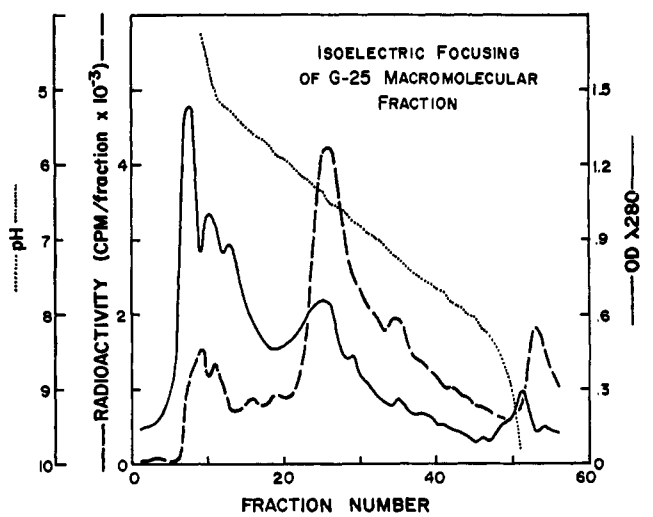

FIGURE 5: Isoelectric focusing of the soluble binding component from L cells. Sephadex G-25 macromolecular material (6 ml) prepared from 105,000*g* supernatant of L cells incubated with [^{3}H]-triamcinolone acetonide (20 Ci/mmole) containing 53 mg of protein were placed in the light chamber of a sucrose density gradient maker and the column was formed as described under Materials and Methods. At the completion of the run, 3-ml fractions were collected, and values for pH (. . . .) radioactivity (---) and absorbance at 280 mμ (—) were determined.

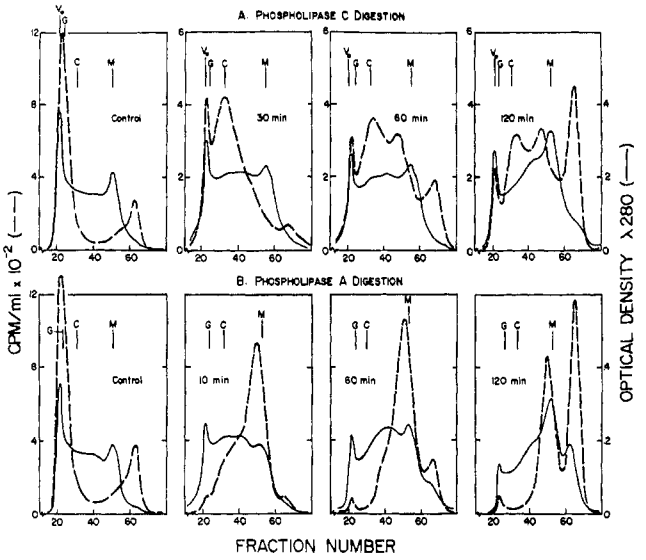

FIGURE 6: Digestion of the Sephadex G-25 macromolecular fraction containing bound [^{3}H]triamcinolone acetonide with phospholipase C and phospholipase A preparations; 0.5 ml of 0.1 M Tris (pH 7.3)–0.2 M $CaCl_2$ containing 2 mg of phospholipase C or A preparation was added to replicate 1.5-ml (24 ml of protein) samples of the Sephadex G-25 macromolecular material prepared in the usual manner. The resulting solutions were incubated for various times with shaking at 23°. At the end of each incubation the samples were cooled in ice, centrifuged at 10,000*g* for 10 min, and the supernatant was transferred onto a column of Sephadex G-200 after the addition of marker proteins as described in the Legend to Figure 1. The solid line in each set of figures represents the optical density at 280 mμ and the dashed lines represent the radioactivity. The following abbreviations identify the location of marker peaks: void volume (V_0), β-galactosidase (G), catalase (C), and myoglobin (M). Part A, digestion with phospholipase C. The control in this case was a 60-min incubation in the same buffer without phospholipase C. Part B, digestion with phospholipase A. The control was a 120-min incubation without phospholipase A.

any digestion of the purified proteins to smaller molecular weight species.

Partial Purification of the Binding Component

Ammonium Sulfate Precipitation of the Glucocorticoid-Binding Component. Table I presents the results of ammonium sulfate fractionation of the triamcinolone acetonide binding component in the macromolecular fraction from Sephadex G-25. There is an enrichment in specific activity of approximately 5-fold in the 15–20% precipitation. The largest portion of the radioactivity, however, is recovered in the fraction precipitated between 20 and 25%.

Chromatography of the Binding Component on DEAE-cellulose. Macromolecular material obtained from Sephadex G-25 chromatography was absorbed to a column of DEAE-cellulose and eluted with a 0.04–0.3 M KCl gradient. The binding component is eluted between 0.10 and 0.15 M KCl (Figure 7.) Such a procedure results in five-to sixfold increase in the radioactivity to protein ratio over the Sephadex G-25 macromolecular fraction.

Purification of the Glucocorticoid-Binding Component. A number of different purification schemes were devised utilizing the various procedures of gel filtration, ammonium sulfate precipitation, and ion-exchange chromatography. Table II presents the purification which can be achieved by that scheme which results in the greatest purification. However, under such conditions only 0.5–1% of the original binding component is recovered.

TABLE II: Purification of the Triamcinolone Acetonide Binding Component.[a]

	Protein (mg)	Bound Radioactivity (cpm × 10^{-3})	Uncor Sp Act. (cpm/mg of Protein × 10^{-3})	-Fold Purifcn
Cell homogenate	2388	3950[b]	1.65	1
105,000*g* supernatant	750	3160	4.21	2.6
6% Agarose column	79.2	2280	28.8	17
0–25% Ammonium sulfate precipitate	2.86	425	149	90
Sephadex G-25 column	2.20	235	107	65
DEAE column	0.007	26	3459	2096

[a] Washed, packed L cells (50 ml) were incubated for 30 min with 10^{-8} M [^{3}H]triamcinolone acetonide (20 Ci/mmole) at 37° and the cells were washed and collected in the usual manner. A 105,000*g* supernatant was prepared and passed through a 5 × 75 cm column of 6% Agarose, and the elution of the binding component was monitored by measuring the radioactivity in each sample. The samples included within the peak of bound radioactivity were combined and precipitated at 25% of saturation with ammonium sulfate. The ammonium sulfate precipitated material was redissolved in buffer and filtered on a 1 × 25 cm column of Sephadex G-25. The fractions comprising the macromolecular peak from the G-25 column were combined absorbed on a 1 × 9 cm column of DEAE and eluted with a gradient of KCl as described in the legend to Figure 7. The fractions containing the bound radioactivity from DEAE chromatography were combined and concentrated by vacuum dialysis for protein assay. [b] The [^{3}H]triamcinolone acetonide in L cells which is bound in a specific manner is distributed 80% in the 105,000*g* supernatant fraction and 20% in the 600*g* sediment fraction (Hackney *et al.*, 1970). The total specifically bound radioactivity in the cell homogenate was calculated from that found in the 105,000*g* supernatant corrected for that lost in the low-speed centrifugation prior to preparation of the 105,000*g* supernatant fraction.

The purification of the binding component is made difficult by two recurring problems. Firstly, the steroid–macromolecular complex is continually dissociating. If this loss of steroid is ignored, the apparent purification achieved is 2100-fold. One possible way to circumvent this difficulty would be to assay the amount of binding component recovered at each step by rebinding with radioactive triamcinolone acetonide and assaying the amount of binding over small Sephadex G-25 columns, or by equilibrium dialysis. However, we have not been able to accomplish such a rebinding of the partially purified material. For this reason the purification and all of the experiments presented in this work were carried out with radioactive triamcinolone acetonide initially bound to the binding component in the intact cell.

The second problem is that ammonium sulfate precipitation of the partially purified binding component from the Agarose column results in an aggregation of the binding component, either with itself or with other material in the fraction, to yield a complex which is eluted in the void volume on 6% Agarose. The form of the partially purified binding component resulting from the above purification sequence is an aggregate. Unfortunately other conditions which result in concentration (such as vacuum dialysis) or exposure to high salt concentration (such as 1.0 M KCl) also yield aggregation of the binding component.

The greatest purification that we have been able to obtain for the large form of the binding component (app mol wt 620,000) is 15- to 20-fold when compared to the cell homogenate. This is obtained by filtering the 105,000*g* supernatant on 6% Agarose. A 20- to 30-fold purification (uncorrected for dissociation) of the smaller unit of app mol wt 330,000 may be obtained by precipitating the 105,000*g* supernatant with ammonium sulfate and filtering the redissolved precipitate on a column of 6% Agarose.

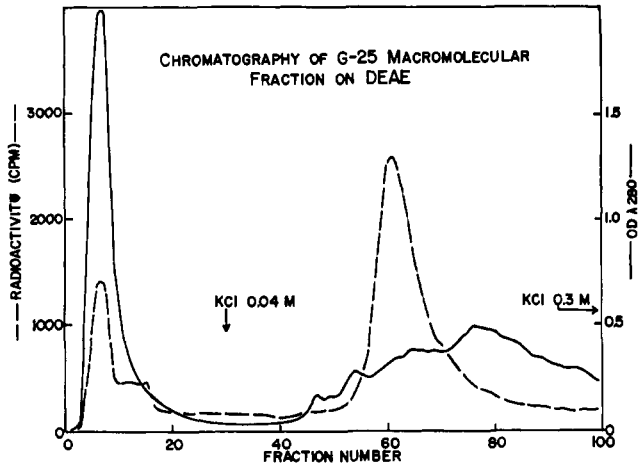

FIGURE 7: Chromatography of the triamcinolone acetonide binding component on a DEAE-cellulose column. A 105,000*g* supernatant was prepared from L cells incubated with 10^{-8} M [^{3}H]triamcinolone acetonide (4.3 Ci/mmole). After passage of the supernatant through a Sephadex G-25 column, 20 ml of the macromolecular peak containing 163 mg of protein was adsorbed on 22 × 1.5 cm DEAE column equilibrated with 0.01 M Tris–0.04 M KCl (pH 7.2). The column was then washed with this buffer until 30 fractions were collected. At this point, a linear KCl gradient was begun ranging from 0.04 to 0.3 M KCl. The dashed line represents radioactivity and the solid line optical density at 280 mμ for each fraction.

Discussion

Approximately 95% of the bound triamcinolone acetonide found in the 105,000*g* supernatant fraction of the cell is bound in a manner which is specific for the action of the glucocorticoids in inhibiting the growth of mouse fibroblasts (Hackney *et al.*, 1970). From Figures 1 and 2, it is clear that this binding component elutes as a molecule of very large size. Indeed, it behaves on gel filtration as would a globular protein of mol wt 620,000. This apparent large size is not reflected in the behavior of the binding component on centrifugation in a sucrose gradient. The sedimentation value of 5.5 S for the binding component in the 105,000*g* supernatant fraction corresponds to the sedimentation properties expected of a globular protein with a molecular weight of about 85,000. Such a deviation from the behavior expected for a spherical molecule composed entirely of amino acids could be due to three things. (A) The binding component may be reduced to a smaller molecular size when it is centrifuged in sucrose. This is, however, not the case as the binding component can be recovered from the sucrose gradient and filtered through a Sephadex G-200 column as the large unit which elutes with an apparent molecular weight of 620,000. (B) The binding component may be fibrous in nature. (C) The binding component may contain lipid or other nonprotein material which would decrease its density.

In order to test this last possibility, the 105,000*g* supernatant fraction was digested with phospholipase in order to separate possible lipid and protein components. It is known (Hackney *et al.*, 1970) that at least part of the binding component is protein, as the steroid is readily released from bound form by mild digestion with Pronase. The results of the experiment presented in Figure 6 demonstrate that digestion with both phospholipase C and a snake venom phospholipase A preparation will effect a marked reduction in the size of the binding component as determined by filtration on Sephadex G-200. It is likely that the phospholipase enzyme is the principal agent responsible for this change as both enzyme preparations were able to change the molecular size of the binding component to the small unit, and there was no detectable proteolytic activity in either phospholipase preparation. We have demonstrated that even after two hours of digestion with the phospholipase A preparation there is no further reduction in the size of the binding component. If one were to postulate that the shift in molecular size of the binding complex is the result of digestion with a proteolytic enzyme contaminating the phospholipase preparation, then the proteolytic enzyme would have to be capable of only limited rather than extensive attack on protein, and the binding complex would have to be more sensitive to such attack than the bulk protein represented by the optical density at 280 mμ. In addition such a postulated protease contaminant would have to have an unusual heat stability as the phospholipase A preparation is completely active in reducing the size of the binding complex even after it has been boiled for three minutes. Such extreme heat stability is a characteristic of this enzyme. We conclude that the shift in molecular weight is probably due to the removal of lipid from the binding component. We cannot say definitively, at the present time that the resulting binding unit with an approximate molecular weight of 25,000–45,000 is solely protein in nature or that this smaller unit and lipid are the only components of the large 620,000 molecular weight binding complex. It is not likely that digestion with phospholipase results in the production of new substances with which the steroid can associate and to which it transfers. A 105,000*g* supernatant fraction prepared from L cells which were not exposed to steroid was incubated with the phospholipase preparations and at the completion of the digestion, radioactive triamcinolone acetonide was added to the samples and they were filtered through Sephadex G-200. All of the steroid emerged as the free form.

From the observation that the binding complex in the 105,000*g* supernatant fraction has a very low sedimentation constant in comparison with its molecular size on gel filtration and from the observation that the molecular size is markedly reduced by digestion with phospholipase, we postulate that the glucocorticoid-binding component exists in the form of a lipoprotein in the cell. Although it is clear that the majority of the specifically bound triamcinolone acetonide is recovered in the soluble fraction of the cell (Hackney *et al.*,

1970), it is by no means clear that this is the normal location of the binding component in the intact cell. All of the studies which have been carried out on the glucocorticoid-binding component in fibroblasts have involved the same method of hypotonic rupture and subsequent fractionation. It is certainly possible that the methods employed could result in a redistribution of the binding component, from particulate material to the soluble fraction.

Extensive purification of the binding component is compromised by the problems of aggregation, loss of the radioactive marker at each step and low yield. The conventional methods of purification presented here probably will not suffice in arriving at the ultimate goal of purification of a significant amount of the protein portion of the binding component to homogeneity. The experiments with phospholipase A digestion presented in Figure 6 suggest that the use of this enzyme may become a useful tool in the eventual purification of the protein portion of the binding component.

The continuous loss of radioactivity to the free form is a problem which can possibly be solved by affinity-site labeling or the development of a good subcellular binding assay. As triamcinolone acetonide is the most active glucocorticoid which can be obtained commercially in the radioactive form, we are already employing the most potent radioactive probe available for the identification of the receptor. The ED_{50} for triamcinolone acetonide as an inhibitor of growth in mouse fibroblasts is about 3×10^{-9} M (Gray *et al.*, 1971).

It is interesting to compare the properties of the glucocorticoid-binding component with the estrogen-binding component from uterus—another system in which an extensive purification of the binding component, 2500-fold, has been achieved (DeSombre *et al.*, 1969). In the uterine cells, estradiol concentrates in the nuclear fraction (Noteboom and Gorski, 1965; Stumpf and Roth, 1966), whereas in the fibroblast the bulk of the glucocorticoid is recovered in the soluble fraction (Hackney *et al.*, 1970). The molecular weight of the 8S and 4S estrogen-binding components are 200,000 and 75,000, respectively, as determined by gel filtration on Sephadex G-200 (DeSombre *et al.*, 1969). These sedimentation values are consonant with the behavior expected of globular proteins of those molecular weights. The glucocorticoid-binding component from mouse fibroblasts has a molecular weight of 620,000 by gel chromatography and a very low sedimentation constant of 5.5. There is no indication that the estrogen-binding component might be a lipoprotein. Both the glucocorticoid- and estrogen-binding components are weakly acidic, precipitate at low ammonium sulfate concentrations and tend to form aggregates upon precipitation with ammonium sulfate. The aggregation seen with the estrogen-binding component is decreased by pretreatment with 1 M KCl; however, the same conditions lead to complete aggregation of the L-cell glucocorticoid receptor.

There appear to be relatively few estrogen-binding components per cell. Noteboom and Gorski (1965) estimate that there may be 1700–2500 binding sites/cell in the rat uterus. Knowing the total amount of triamcinolone acetonide bound per milligram of cell protein (Table II) and the amount of protein per L cell, we can calculate that there are approximately 30,000 specific binding sites for glucocorticoid per fibroblast.

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