

Synthesis and Secretion of Corticosteroid-Binding Globulin by Rat Liver

A SOURCE OF HETEROGENEITY OF HEPATIC CORTICOSTEROID-BINDERS

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ABSTRACT Classical glucocorticoid receptors (type II) have a high affinity for synthetic and natural glucocorticoids. We have previously demonstrated an additional binding site in kidney cytosol (type III) which has a high affinity for corticosterone but a low affinity for dexamethasone. In many ways, this binder resembles plasma corticosteroid-binding globulin (CBG). The first goal of this study was to determine the organ distribution of the type III binding sites. Cytosol was prepared from isolated cells to avoid plasma contamination. Of the tissues examined, type III sites were found only in liver and kidney; sites were absent from thymocytes, IM-9 lymphocytes, adipocytes, and bone cells. The second goal of this study was to ascertain whether CBG is synthesized in liver and kidney. Liver and kidney slices were incubated *in vitro* and the concentration of type III sites was seen to rise in hepatic cytosol and incubating medium but not kidney. To verify the impression that liver was synthesizing and secreting CBG, the following experiments were performed: (a) To demonstrate that type III sites were CBG, steroid-binding profiles and migration on polyacrylamide gel electrophoresis were shown to be identical for hepatic type III sites and serum. (b) To indicate that the rise in type III sites was dependent on protein synthesis, it was shown that cycloheximide blocked the appearance of new type III sites. (c) To establish that the type III sites were being secreted, *in situ* liver perfusion experiments showed time-de-

pendent release of new sites into the perfusate. In conclusion, liver synthesizes and secretes type III sites, a finding previously suspected but never proved. The presence of type III sites in kidney remains to be explained.

INTRODUCTION

Heterogeneity of glucocorticoid-binding proteins has been described in a variety of target tissues (1-4). We have previously demonstrated three distinct corticoid binders in rat kidney: type I, characterized by high affinity for aldosterone and low affinity for corticosterone, was felt to be the mineralocorticoid receptor (5); type II, characterized by high affinity for dexamethasone and corticosterone, was felt to represent the glucocorticoid receptor (6); and type III, characterized by high affinity for corticosterone but low affinity for dexamethasone (7), was not possible to classify according to function. The type III binding site was recognized to be indistinguishable in binding properties from plasma corticosteroid-binding globulin (CBG).¹ In addition to classical glucocorticoid receptors (type II) several laboratories have described "CBG-like" binders in a variety of other tissues including liver (2, 3), brain and pituitary (8-9), muscle (10), lung (11), and uterus (12). We believe the CBG-like binders in other tissues and the type III binder in kidney may be the same but will use the designation type III binding in this paper to specify our experimental conditions. Because the binding properties of these cellular binders are virtually identical to CBG (7), we have been concerned about the difficulty in discriminating between a true intracellular molecule and extracellular fluid contamination of the tissues. At least for the kidney, we

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¹Abbreviations used in this paper: CBG, corticosteroid-binding globulin; KRB, Krebs-Ringer Bicarbonate; MEM, minimal essential medium.

were able to demonstrate that the type III sites were intracellular with autoradiographic techniques (13).

The first aim of this study was to ascertain the organ distribution of these type III (CBG-like) binding sites (i.e., sites exhibiting a high affinity for corticosterone and low affinity for dexamethasone). We believed that knowing whether the binder was present in all tissues or restricted to only selected targets would have implications in a consideration of potential functions of this molecule. Once it was determined that, of the tissues examined, the binder was limited to liver and kidney, we turned our attention to the possibility that these organs might be sites of biosynthesis and secretion of plasma CBG, thus explaining the intracellular presence of type III binders. The liver has usually been considered the site of CBG synthesis but this fact has not been adequately documented. As will be detailed in this paper, the present data indicate that the liver does indeed synthesize and secrete type III binders which presumably represent CBG. This finding provides a basis for the intracellular localization of type III sites in liver. The presence of type III binding sites in the kidney remains to be explained.

METHODS

[1,2-³H]Corticosterone (48 Ci/mmol), [1,3,4-³H]dexamethasone (23 Ci/mmol), and [U-¹⁴C]amino acid mixture (\approx 260 mCi/mmol) were purchased from New England Nuclear (Boston, Mass.). Unlabeled steroids (A grade) were obtained from Calbiochem-Behring Corp. (San Diego, Calif.). Conventional reagents were of analytical grade and were purchased from Sigma Chemical Co. (St. Louis, Mo.) unless otherwise specified. Sprague-Dawley, 160–200 g, male rats (Simonsen Laboratories, Gilroy, Calif.) were adrenalectomized before use and maintained on 0.9% saline drinking water and Purina chow pellets (Ralston Purina Co., St. Louis, Mo.) ad libitum. Rats were sacrificed by decapitation.

Preparation of isolated cells. All experiments employed adrenalectomized rats. Livers and kidneys were removed after the viscera were extensively perfused via the vena cava with 50 ml of iced Ca⁺⁺-free Krebs-Ringer bicarbonate (KRB) buffer (pH 7.4) to wash out blood and then with 20 ml of Ca⁺⁺-free KRB containing 1 mg/ml of crude collagenase (Worthington Biochemical Corp., Freehold, N. J.: CLS II). Each kidney was decapsulated, halved, and the papilla excised and discarded. The tissue was rinsed, blotted, and sliced (275 μ m thickness) with a McIlwain tissue chopper (5–7). Slices were placed in a KRB solution with 1 mg/ml crude collagenase, 0.2% glucose, and 0.5% bovine serum albumin (Grand Island Biological Co., Grand Island, N. Y.) and incubated for 60 min at 37°C in a vigorously shaking water bath in a 95:5%–O₂:CO₂ atmosphere. Cells were filtered through a 250 μ m nylon mesh, centrifuged at 100 g, and resuspended three times with cold KRB. This yielded isolated hepatocytes and renal tubules with excellent viability assessed by trypan blue exclusion.

Adipocytes were obtained from epididymal fat pads which were diced into a Ca⁺⁺-free KRB solution containing 3 mg/ml crude collagenase, 3 mM glucose, and 4% BSA in a Nalgene flask (Nalge Co., Nalgene Labware Div., Rochester, N. Y.) (14). The tissue was incubated as above, washed twice with iced

KRB containing 1% bovine serum albumin and once with KRB, and collected by flotation at 200 g.

The thymus was removed, cleaned of connective tissue, and placed in cold 0.9% saline. Pieces of thymus were rinsed through a steel screen (100 μ m pore size) with iced incubating solution. Thymocytes were centrifuged at 250 g and washed once in 0.84% NH₄Cl to eliminate erythrocytes and twice with incubating solution.

The primary culture of bone cells from fetal rat calvaria has been described (15). Heat-inactivated calf serum was substituted in the procedure to avoid contamination with CBG. Monolayers were incubated with 1 mg/ml crude collagenase to remove extracellular collagen. The bone cells were centrifuged at 200 g and brought up twice in incubating solution.

Cultured IM-9 human lymphocytes (National Institutes of Health, Bethesda, Md.) were grown in a minimal essential medium (MEM) with Earle's salts (F-17, Grand Island Biological Co.) that included 10% heat-inactivated calf serum. Cells were collected from the suspension at 250 g and washed twice with incubating solution.

Preparation of tissue slices. Livers and/or kidneys were perfused and sliced as described for isolated cell preparation, except the perfusion medium contained only iced 0.9% saline. Slices were carefully separated and rinsed several times with a cold KRB solution. For each sample, 1 g of slices was added to 4 ml of sterile F-17 MEM (pH 7.4) with 25 mM Hepes in a Nalgene flask. All flasks were incubated with continuous 95:5%–O₂:CO₂ at 37°C in a shaking water bath. At the end of the incubation, the flasks were placed on ice and the contents were homogenized with 20 strokes of a motor-driven Teflon (Du Pont Co., Wilmington, Del.) glass homogenizer. The homogenates were centrifuged at 50,000 g for 40 min. The resulting high-speed supernate was stored at –20°C for subsequent assay.

Binding assays. Type III binding was carried out by incubation in the presence of 52 nM [³H]corticosterone plus 10-fold excess of aldosterone and dexamethasone to prevent isotope binding to type I (mineralocorticoid receptor) and type II (glucocorticoid receptor) (5–7). Type II binding was carried out by incubation in the presence of 26 nM [³H]dexamethasone which binds only minimally to type III sites (6, 7). In both systems, nonspecific binding (<20% of the total) was defined as that binding resistant to 100-fold unlabeled steroid and was subtracted from total binding to give specific binding. Type II binding was performed immediately on fresh tissue, whereas type III binding could be performed on frozen material without loss of binding activity. Type II incubations were carried out at 0°C for 150 min and type III at 37°C for 30 min. Pilot studies verified that the conditions employed measured peak steady-state levels of binding in both assays. In both systems, unbound steroid was removed by charcoal adsorption. Norite A 5% and dextran T70 0.5% were prepared in a buffer of 10 mM Tris-HCl and 1.5 mM EDTA (pH 7.4). A one-tenth volume of the dextran-coated charcoal solution was added to cytosol or serum, vortexed, and allowed to sit for 20 min at 0°C. Charcoal was removed by centrifugation at 2,500 g for 10 min and the supernate radioassayed. The conditions of the assay gave similar results when samples were measured by Sephadex G-50 chromatography (Pharmacia Fine Chemicals, Div. of Pharmacia Inc., Piscataway, N. J.) (7). Protein was measured with Coomassie Brilliant Blue G-250 dye (Bio-Rad Laboratories, Richmond, Calif.) after the method of Bradford (16). Data are usually expressed per milligram of cytosol protein or where medium plus cytosol is used, per milligram of supernatant protein.

When the starting material was isolated cells, the washed cells were suspended in hypotonic buffer (10 mM Tris-HCl, 1.5 mM EDTA, 10 mM monothioglycerol (pH 7.4), and lysed

by sonication. A high-speed supernate (50,000 *g* for 40 min) was prepared from the lysate and used for binding studies. High-speed supernatant fractions from tissue slice experiments were thawed and diluted with hypotonic buffer to assay plasma CBG and(or) type III binding by similar procedures.

In whole cell binding studies, aliquots of cell suspensions were first counted electronically with a Coulter counter (Coulter Electronics Inc., Hialeah, Fla.). Cells were then incubated in F-17 MEM with appropriate concentrations of steroids for 30 min at 37°C in a 95:5%–O₂:CO₂ atmosphere while shaking. 1-ml aliquots were then removed, placed in 1.5 ml micro test tubes, and centrifuged at 800 *g* for 1 min. After two washes with 1 ml of cold incubating solution, the cells were poured onto Whatman GF/A filter paper (Whatman, Inc., Clifton, N. J.) in a filtration manifold under vacuum. Filters were rinsed with 10 ml of incubating solution which eliminated unbound steroids, and the dried filter paper was radioassayed. "Nonspecific" binding varied between 15 and 20% of total.

Cycloheximide experiments. The effect of cycloheximide on the incorporation of amino acids into proteins and the synthesis of CBG binding sites was measured in parallel experiments. After preincubations with various concentrations of cycloheximide for 90 min, amino acid incorporation with a [¹⁴C]amino acid mixture was determined over the subsequent 120 min. TCA (10% final concentration) was added to high-speed supernates of homogenates containing [¹⁴C]amino acids. TCA precipitable protein was collected at 2,500 *g* for 10 min and washed twice with TCA (5% final concentration). The precipitate was dissolved by incubation in 0.5 ml of NCS tissue solubilizer (Amersham Corp., Arlington Heights, Ill.) for 24 h at 50°C and radioassayed. Parallel flasks of slices were treated similarly with cycloheximide and the type III binding site content of slices plus medium was measured.

Polyacrylamide gel electrophoresis experiments. Liver slices were incubated and aliquots of cytosol, incubation medium, and serum were labeled for type III sites as described above. In parallel experiments, unlabeled corticosterone was used and the medium pulsed for the final 60 min of a 4-h incubation with 0.5 μ Ci of a [¹⁴C]amino acid mixture. Samples were then run on a slab gel electrophoresis apparatus (Pharmacia Fine Chemicals) at 2°C for 4 h at 6 mA/sample. The gel was 7.5% polyacrylamide in a buffer of 0.05 M Tris-HCl (pH 8.5). The gels were stained for protein or sliced into 2.5-mm fractions and the radioactivity determined.

RESULTS

Tissue distribution of type III binding: cytoplasmic assay. The distribution of type III binding sites in various tissues was examined. The selection of organs was based, in part, on our ability to prepare isolated cells and, thus avoid extracellular fluid contamination. Binding studies were performed on thymocytes, adipocytes, IM-9 lymphocytes, bone cells, hepatocytes, and renal tubules. As indicated in Fig. 1, with the exception of hepatocytes and renal tubules, none of these tissues exhibited cytoplasmic type III binders. Type II binding, was measured as a necessary condition for type III binding; the presence of the labile type II binding site implied that the relatively stable type III binder had not been destroyed during cell preparation or in the binding procedures. The number of cytoplasmic type

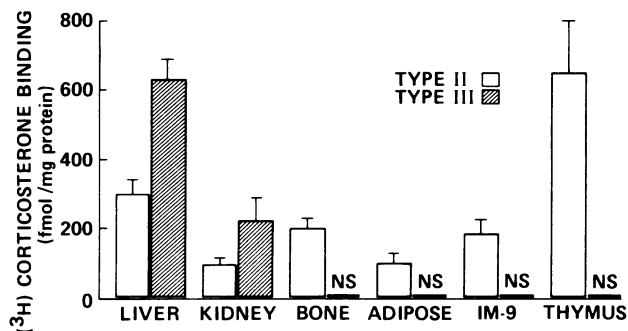


FIGURE 1 Glucocorticoid binding sites in cytosol prepared from isolated cells. Isolated cells were prepared as described in Methods. Cells were sonicated in hypotonic buffer and cytosol obtained. Binding studies were then performed with fresh cytosol; type II binding with 26 nM [³H]dexamethasone and type III binding with 52 nM [³H]corticosterone in the presence of 10-fold unlabeled dexamethasone and aldosterone. Bound and free steroid were separated by charcoal. Nonspecific binding was \approx 23% of total binding and was subtracted in each case. Results are expressed per milligram cytosol protein. NS indicates that the difference between total binding and nonspecific binding was not significant. Values shown are mean \pm SE. *n* = 6 for liver and kidney and *n* = 4 for the other organs.

III sites in renal tubules (212 fmol/mg cytosol protein) and in hepatocytes (638 fmol/mg cytosol protein) was two to three times that of the type II value in the same tissue prepared with the same experimental procedure. In our hands, the concentration of type II receptors is always lower after isolated cell preparation than when cytosol is directly prepared from intact tissues.

Tissue distribution of type III binding: whole cell assay. It has been suggested that the subcellular location of CBG-like glucocorticoid binding may be on the plasma membrane of pituitary cells (17), or liver cells (18), or within the nucleus of hepatocytes (19). Because our experiments with high-speed supernates assayed mainly cytoplasmic corticosterone binders, we also used a whole-cell binding technique with isolated cells to include all subcellular components as potential sites of high affinity binding. As shown in Fig. 2, the results of whole cell binding procedures, like the cytoplasmic binding assays, showed type III binding only in kidney of the cell types examined. Because of variable and methodologically inconsistent results, we do not have confidence in similar experiments done with hepatocytes that gave exceptionally high values and adipocytes which, because they floated, were difficult to quantitatively transfer to the filters (data not shown).

Because the type III binder was not present in all tissues, we next turned our attention to the possibility that intracellular CBG-like material in liver and kidney might be the result of synthesis of plasma CBG by those organs.

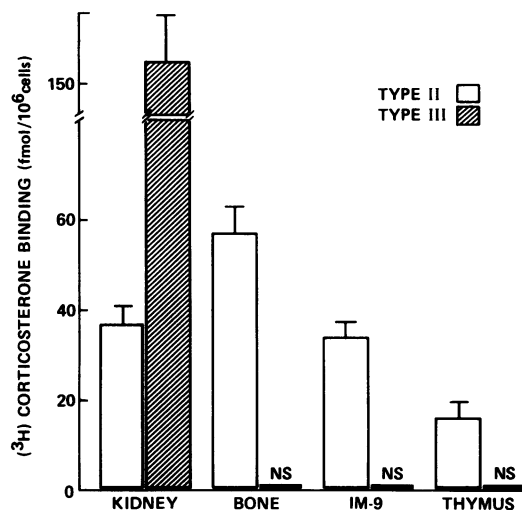


FIGURE 2 Glucocorticoid binding sites in whole cells. Isolated cells were prepared as described in Methods. Binding was performed by the whole cell technique; type II binding with 26 nM [³H]dexamethasone and type III binding with 52 nM [³H]corticosterone in the presence of 10-fold unlabeled aldosterone and dexamethasone. Bound and free steroids were separated by washing cells on a filter. Nonspecific binding was $\approx 20\%$ of total binding and was subtracted in each case. NS indicates that the difference between total and nonspecific binding was not significant. Values shown are mean \pm SE. $n = 4$.

Measurement of type III binding site production by liver and kidney slices. Liver and kidney slices were incubated for 240 min and the total number of type III binding sites was determined in tissue plus medium at hourly intervals. As shown in Fig. 3, the number of type III binding sites per milligram of supernatant

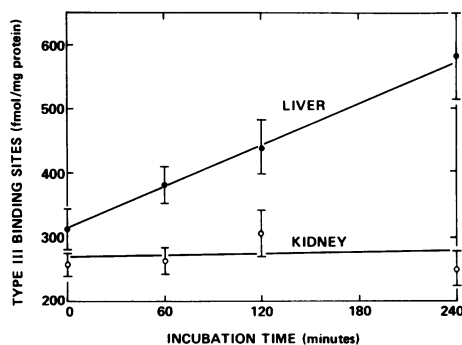


FIGURE 3 The effect of incubation time on the total number of type III binding sites in liver and kidney slices. Slices of liver and kidney were incubated at 37°C in a series of flasks. At the indicated time points, the tissue and medium in a given flask were homogenized and the high-speed supernate prepared and frozen. The concentration of total specific type III binding sites in cytosol plus medium was determined by the charcoal method and is expressed per milligram of supernatant protein. Each value is the mean \pm SE. $n = 4$ experiments for both liver and kidney.

protein was unchanged over time in kidney slices but linearly increased in liver slices from 309 ± 32 fmol/mg protein at 0 min to 579 ± 86 fmol/mg protein after 240 min of incubation at 37°C. The almost doubling of the total number of type III sites per flask appeared to indicate that liver was a site of type III production.

Is the type III binder CBG? Anti-rat CBG antibody was not available to us for this study. In the absence of specific antibody, we employed the steroidal-binding properties as a means of characterizing the protein. The ability of a variety of steroids to compete for type III hepatic [³H]corticosterone binding sites was determined and the results compared to values for plasma CBG binding sites. The hepatic samples were taken from the bathing medium after 240-min liver slice incubations which contained $\approx 66\%$ newly synthesized binding sites. The results are shown in Fig. 4. The order of affinities for [³H]corticosterone binding sites in both hepatic and plasma components was corticosterone > cortisol = progesterone > aldosterone > dexamethasone > estradiol. Estradiol potentiated the binding of [³H]corticosterone by apparently occupying low affinity sites. The close correspondence between plasma CBG and liver slice high-speed supernatant binding is evidence that the newly produced binding sites in the liver are indistinguishable from plasma CBG binding sites.

To further substantiate that the proteins themselves are identical, polyacrylamide gel electrophoresis experiments were performed. The data indicate that plasma CBG and the hepatic protein from both cytosol and medium migrate in an identical fashion (Fig. 5). The nature of the slower migrating [³H]corticosterone binder in the medium is not clear. Taken with the binding data, these results strongly suggest type III binding sites represent CBG molecules.

Is the type III binder newly synthesized protein? Liver slices were treated with increasing concentrations of cycloheximide and [¹⁴C]amino acid incorporation into TCA precipitable material assessed. As shown in Fig. 6B, a concentration of 20 μ g/ml of cycloheximide blocked $\approx 90\%$ of protein synthesis. As shown in Fig. 6A, this concentration of cycloheximide blocked 94% of the increase in type III binding as compared to untreated control slices. Lesser concentrations of cycloheximide caused comparably less inhibition of protein synthesis and comparably less blockage of type III production. The data suggest that the production of type III sites is dependent on protein synthesis.

Polyacrylamide electrophoresis data confirm that one of several protein peaks from medium and cytosol possessing newly incorporated [¹⁴C]amino acids comigrates with plasma CBG binding sites labeled with [³H]corticosterone (Fig. 7).

Is the type III binder secreted by liver in situ? In isolated *in situ* liver perfusion experiments, type III

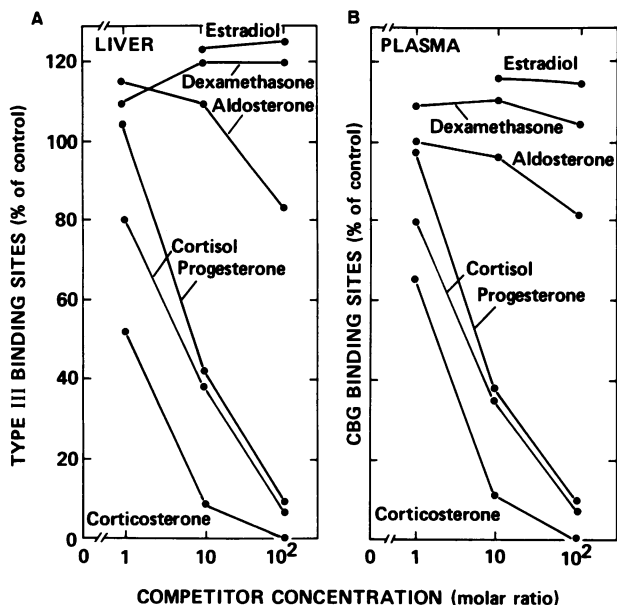


FIGURE 4 A comparison of the steroid binding profile of type III sites in liver cytosol and plasma. (A) Liver cytosol after 240 min slice incubation. (B) Plasma obtained from adrenalectomized rats. Cytosol or plasma was incubated with 2.6 nM [³H]corticosterone plus increasing concentrations of the indicated unlabeled steroid for 30 min at 37°C. Nonspecific binding was subtracted from all values. Total binding in the absence of competitor was taken as 100% and was 2,180 fmol/mg plasma protein and 127 fmol/mg cytosol protein.

binding could be found in increasing amounts in the perfusate (Table I). Livers from intact rats were perfused *in situ* with a KRB buffer solution containing 38% erythrocytes and 3% bovine serum albumin according to the method of Mortimore (20). The quantity of type III binding sites in the perfusate increased in a roughly linear fashion between 30 and 120 min. The variability in the initial rate of secretion (0–30 min period) reflects the fact that the liver of rat 2 was better washed out before the perfusion was initiated. The high initial levels are, therefore, felt to represent washout of residual plasma CBG plus the secretion rate of the first 30 min.

DISCUSSION

This work began as an attempt to elucidate the nature of the type III or CBG-like binding site in kidney. The organ distribution data (Figs. 1 and 2) indicate that type III binding is not universally distributed along with type II or classical glucocorticoid receptors but rather that its presence is restricted to certain organs. Of those organs tested, only the liver and kidney possessed the type III binding site. However, in our hands, all tissues appeared to possess the binder until extensive efforts at washing and cell isolation were undertaken

to remove extracellular fluid. It is difficult to reconcile our findings with those of Amaral et al. (19) and Werthamer and colleagues (21) who found, by immunologic methods, a CBG-like protein in lymphocytes and liver nuclei.

The realization that the type III binder was limited to certain organs caused us to reconsider our previous position that the binder was related to hormone action (7, 13) and to examine other possibilities. Because the liver and kidney both synthesize many products and because the liver has long been suspected of being the site of CBG synthesis, we examined the hypothesis that type III intracellular binders might represent CBG synthesis. This does not appear to be the case for kidney (Fig. 3).

The following data indicate that the liver synthesizes and secretes CBG and that the type III binders represent intracellular CBG. First, the type III binders appear indistinguishable from CBG. A comparison of the type III steroid binding site and plasma CBG gives similar results (Fig. 4). Scatchard analysis has shown

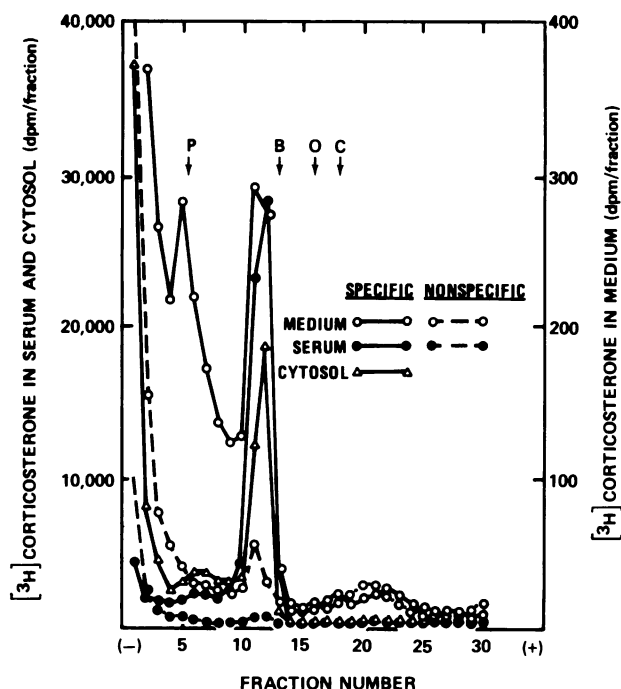


FIGURE 5 Polyacrylamide gel electrophoresis of [³H]corticosterone binders. Liver slices were incubated for 4 h at 37°C. At the conclusion of the incubation, aliquots of liver cytosol and medium as well as serum were incubated with 26 nM [³H]corticosterone \pm 250-fold unlabeled corticosterone for 30 min at 37°C. These samples were then subjected to slab gel electrophoresis on 7.5% polyacrylamide in a buffer containing 0.05 M Tris-HCl (pH 8.5) for 4 h at 2°C at 6 mA/sample. The standards and molecular weights are: P, phosphorylase b (94,000); B, bovine serum albumin (67,000); O, ovalbumin (43,000); and C, carbonic anhydrase (30,000).

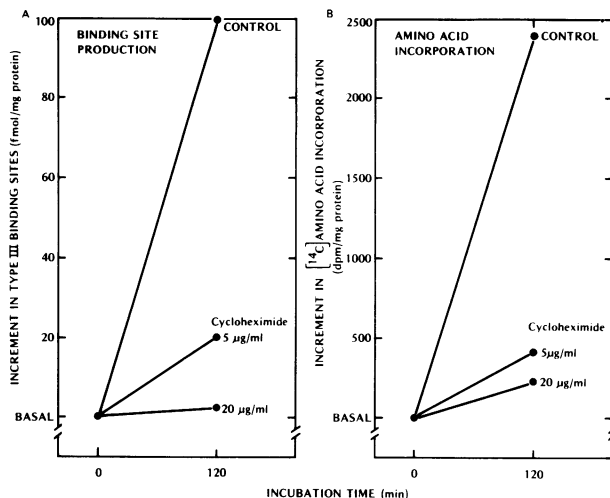


FIGURE 6 The effect of cycloheximide on type III binding site production and amino acid incorporation in liver slices. Slices were preincubated in cycloheximide for 90 min. (A) Increment in type III binding site production. The binding sites represent tissue plus medium. The basal level was 214 fmol/mg of supernatant protein. (B) Inhibition of amino acid incorporation into TCA precipitable protein. After a 90-min preincubation in medium plus or minus cycloheximide, [^{14}C]amino acids were added for the next 120 min.

that both proteins possess the same affinity for [^3H]-corticosterone (data not shown). Furthermore, electrophoresis on polyacrylamide gel shows identical mobility of plasma, cytosol, and medium [^3H]corticosterone binders (Fig. 5). Previous studies showed multiple other properties in common (7).

Second, the liver appears to be synthesizing the binder. Support for this argument is found in studies showing a rise in type III sites over time (Fig. 3). [^{14}C]-Amino acids are incorporated into a protein that comigrates with serum labeled with [^3H]corticosterone (Fig. 7). Inhibition of the rise in type III binding by concentrations of cycloheximide that inhibit $\approx 90\%$ of amino acid incorporation is further evidence that the rise in binding site content is dependent on protein synthesis.

Third, the liver secretes type III binders *in vitro* and *in situ*. *In vitro*, the liver slices exhibited a time-dependent release of type III sites into the medium bathing the slices. This process was also inhibited by cycloheximide. Our initial studies using an *in situ* liver perfusion system, indicated a roughly linear secretion of type III binding sites into the perfusing medium (Table I).

Although the data indicate that the liver is synthesizing and secreting CBG, we cannot rule out the possibility that intracellular CBG plays an additional physiological role in liver. Because the kidney does not

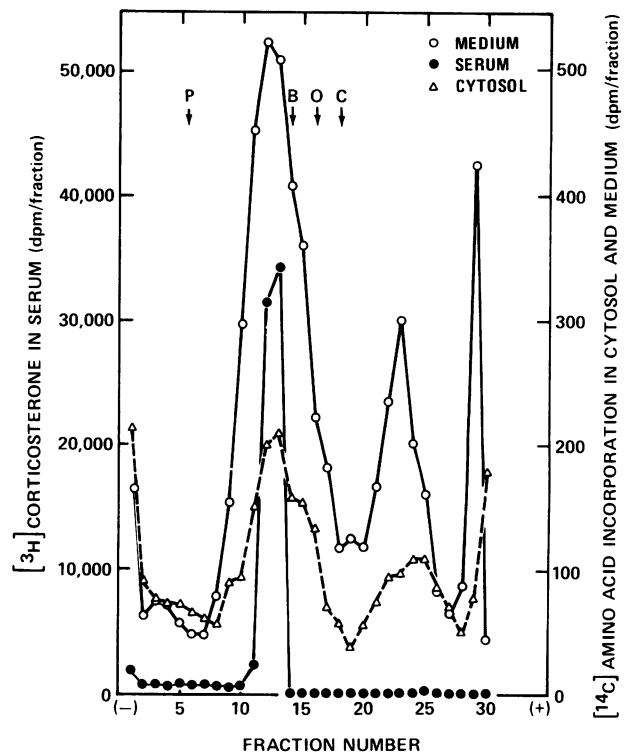


FIGURE 7 Polyacrylamide gel electrophoresis after [^{14}C]-amino acid incorporation into hepatic cytosol. Liver slices were incubated for 4 h at 37°C in a medium that included [^{14}C]amino acid mixture. At the conclusion of the incubation, aliquots of liver cytosol and medium as well as serum incubated with [^3H]corticosterone were subjected to slab gel electrophoresis as described in the legend in Fig. 5.

TABLE I
Time-Course of CBG Secretion by *In Situ* Perfused Liver

Time	[^3H]Corticosterone binding sites			
	Rat 1		Rat 2	
	Cumulative secretion	Incremental secretion	Cumulative secretion	Incremental secretion
	pmol			
min				
0-30	112	112	54	54
0-60	153	41	89	35
0-90	184	31	135	46
0-120	236	52	179	44

Intact rats underwent *in situ* liver perfusion with serum-free perfusate. Aliquots of perfusate were sampled at the indicated time points for [^3H]corticosterone binding sites by the charcoal method. The first 30-min period represents some liver washout of plasma CBG in addition to the new secretion. The data is expressed as total picomoles secreted into the perfusion medium per unit time.

appear to synthesize CBG, a role for the type III binder in that tissue remains to be determined.

The studies documenting CBG-like binders in other organs (8–12) have, to a variable degree, attempted to exclude extracellular fluid as a source of the binding protein. However, as has become apparent with binding systems for other steroid hormones, the exclusion of plasma contamination is a difficult problem. Alpha-fetoprotein, an important estrogen plasma binding protein in perinatal rats, must be carefully distinguished from the true intracellular estrogen receptor (22). Additionally, it is now believed that the widespread occurrence of 25-hydroxycholecalciferol binding protein in tissues is the result of plasma contamination (23). In this later case, the issue is more complex, as it appears that a tissue and a plasma component interact to yield the binding protein.

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