Heterogeneity and Properties of Hepatic Dexamethasone-Binding Proteins

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Evidence from experiments *in vivo* and *in vitro* is presented for the presence of three species of dexamethasone-binding proteins in rat liver, which are identified by chromatography on Sepharose 6B or by isoelectric focusing. Although two of these species (DI and DII) possess properties characteristic of a true receptor, the third binding protein (i.e. DIII), which migrates most slowly on Sepharose 6B, but has stability properties similar to protein DII, exhibits a 3-fold lower affinity for dexamethasone and the activated complex neither binds to DNA–cellulose nor translocates to the nucleus. Only the predominant liver receptor (DI), which is eluted first from Sepharose 6B, is present in Novikoff-hepatoma cytosol, suggesting that the major and minor species are not interconverted through simple dissociation during their isolation. The binding activities of all three species in the liver cytosol increase approx. 2-fold *in vivo* after adrenalectomy and show a transient 2-fold fall *in vivo* after the administration of cortisol. These changes *in vivo* in protein DIII shows a marked lag compared with those in proteins DI and DII, which change in parallel. It is therefore proposed that rat liver cytosol contains two dexamethasone receptors and a dexamethasone-binding protein that may be derived from these receptors.

Evidence obtained by fractionating rat liver cytosol on DEAE-Sephadex (Litwack & Rosenfield, 1975) or Sepharose 6B (Liu & Webb, 1977a) columns indicates that rat liver contains at least two dexamethasone-binding proteins. The major component, DI, eluted first from Sepharose 6B, appears to be similar in physical properties to the glucocorticoid-receptor protein, binder II, identified on DEAE-Sephadex. The minor component (DII) similarly appears to have the same molecular weight as binder Ib. Both components DI and DII undergo nuclear translocation (Liu & Webb, 1977a). Furthermore, the two receptors appear to have very similar affinities for dexamethasone, since the corresponding Scatchard plot was linear (Liu & Webb, 1977a). Two dexamethasone receptors were identified when adult-liver cytosol was chromatographed on DEAE-cellulose columns (Agarwal, 1976). In contrast with adult rat liver, only one receptor (DI) was identified in embryonic liver and hepatomas (Liu & Webb, 1977a). In addition, the unactivated form of the receptors (which are more negatively charged than the activated form) could be seen as a separate species on appropriate ion-exchange columns if strict isolation and chromatographic conditions were observed (Sakaue & Thompson, 1977; Parchman & Litwack, 1977). In all previous studies only the activated form of the glucocorticoid receptor complex was apparently detected, owing to the use of Tris buffer (an activator) and extended periods of chromatography (cf. Parchman & Litwack, 1977). Furthermore, the activated and unactivated forms are not distinguish-

able by the Sepharose 6B chromatographic procedure used in our laboratory; thus the two dexamethasone receptors identified earlier in this medium (Liu & Webb, 1977a) do not represent the activated and unactivated forms.

In subsequent experiments we were able to identify consistently, by chromatography on a Sepharose 6B column, a third dexamethasone-binding protein. Although this component is easily observed when the receptors are first saturated with [³H]dexamethasone *in vivo*, our previous failure to observe this component (Liu & Webb, 1977a) is tentatively attributed to its lower affinity for dexamethasone. While these studies were in progress, Schmid *et al.* (1976) reported the separation on DEAE-cellulose columns of three dexamethasone-binding proteins from rat liver cytosol. The present paper presents the results of a comparative study on the three binders, with emphasis on the evidence for and properties of the third component.

Materials and Methods

Animals

Sprague–Dawley rats (Laboratory Supplies, Indianapolis, IN, U.S.A.) weighing 190–210 g were maintained on Purina chow with water *ad libitum*; bilaterally adrenalectomized rats were maintained with 0.9% NaCl in their drinking water. The Novikoff hepatoma was carried as the ascites form in the intraperitoneal cavity of female Sprague–Dawley rats (cf. Liu & Webb, 1977b; Liu *et al.*, 1975).

Chemicals

[³H]Dexamethasone (20–26 Ci/mmol) was purchased from Amersham/Searle Corp., Chicago, IL, U.S.A., and unlabelled dexamethasone phosphate was a gift of Dr. C. A. Stone, Merck, Sharp and Dohme, Westpoint, PA, U.S.A. Sepharose 6B was purchased from Pharmacia Fine Chemicals, Uppsala, Sweden. Calf thymus DNA was purchased from Calbiochem, La Jolla, CA, U.S.A., and the Whatman CF-11 cellulose powder used in the preparation of the DNA-cellulose columns was obtained from H. Reeve Angel and Co., Clifton, NJ, U.S.A. Acrylamide, *NN'*-methylenebisacrylamide and Bio-Lyte 6/8 and 3/10 used for the isoelectric-focusing experiments were obtained from Bio-Rad, Richmond, CA, U.S.A.; riboflavin (Sigma Chemical Co., St. Louis, MO, U.S.A.) was used to initiate the polymerization of the gel.

Preparation of the steroid-receptor complex

The cytosol and nuclear fractions were prepared as previously described (Liu & Webb, 1977b), except that 0.25 M-sucrose was included in the homogenization buffer. The cytosol fraction was incubated with labelled dexamethasone (20000 c.p.m./pmol; 60 nM) at 4°C for 2h, or for the indicated time period, then treated with dextran-coated charcoal to remove unbound steroid (Beato & Feigelson, 1972). In one protocol, the cytosol was stored at 4°C without exogenous steroid for 24h before charging with dexamethasone, in order to determine the relative stabilities of the three binders. Other variations of the basic assay conditions are noted in the Results section. Protein was determined with the biuret reagent (Gornall *et al.*, 1948).

Sepharose 6B chromatography

As previously described (Liu & Webb, 1977b), a portion of the cytosol containing 64mg of cytosol protein was loaded on a Sepharose 6B column (55 cm × 0.64 cm²) pre-equilibrated with the eluting buffer, i.e. 10 mM-Tris/HCl/1.0 mM-EDTA/0.5 mM-dithiothreitol, pH 7.2. Where indicated, the [³H]dexamethasone-charged binders were activated by incubation at 20°C for 30 min before Sepharose 6B chromatography. In other experiments the [³H]dexamethasone-charged cytosol was preincubated with an excess of nuclei (5.3 × 10⁷/ml) at 20°C for 30 min in order to estimate the maximum nuclear uptake of the three dexamethasone-binding-protein complexes. In all experiments 0.2 ml samples of each column fraction were radioassayed in Scintisol (Isolab Inc., Akron, OH, U.S.A.).

Receptor binding to DNA-cellulose

DNA-cellulose was prepared by the procedure of Alberts & Herrick (1974). The relative affinity of the

glucocorticoid-binding proteins for the DNA-cellulose was determined (Kalimi *et al.*, 1973) after separation of the three species on a Sepharose 6B column. A 0.2 ml and a 0.4 ml sample of the pooled fractions from each peak were mixed with 100 μl of packed DNA-cellulose or cellulose (control), then incubated at 0°C for 45 min with gentle shaking. After incubation, the mixture was diluted with 2.0 ml of 10.0 mM-Tris/1.0 mM-EDTA, pH 7.5, then centrifuged at 1500g for 5 min; the cellulose was washed twice with the dilution buffer before radioassay. Binding activities are expressed as the percentage of the total added radioactivity that was specifically bound to the DNA-cellulose.

Isoelectric focusing

Polyacrylamide gels, prepared from 7.5 % acrylamide, 0.19 % *NN'*-methylenebisacrylamide, 5 % (v/v) glycerol, 2 % Bio-Lyte and 0.28 mg % of riboflavin, were polymerized in 10 cm × 0.5 cm (internal diam.) tubes. The upper (cathode) and lower (anode) electrolyte chambers of the electrophoresis apparatus contained 0.1 M-NaOH and 0.05 M-H₂SO₄ respectively. A sample (40 μl) of [³H]dexamethasone-charged cytosol, desalted on a Sephadex G-25 column (0.64 cm² × 12 cm) pre-equilibrated with 25 % (w/v) sucrose, was loaded on the top of the gel and overlaid with 100 μl each of unbuffered 20% and 10% sucrose. After electrofocusing for 17 h at 200 V at 4°C, the 8.5 cm gel was cut into 2.2 mm slices for radioassay. Every second (alternate) gel slice was extracted for 60 min with 1.5 ml of water for determination of the pH profile.

Results

Sepharose-6B profiles of [³H]dexamethasone-binding proteins prelabelled in vitro

In our earlier studies (Liu & Webb, 1977a), where only DI and DII components were identified, the liver was homogenized in buffer (50 mM-Tris/HCl/1.0 mM-MgCl₂/2.0 mM-CaCl₂/1.0 mM-dithiothreitol, pH 7.8) and the receptors were saturated with [³H]dexamethasone by incubation at 4°C for 90 min. Subsequent studies used the same buffer containing 0.25 M-sucrose as the isolation medium and the incubation time was increased to 120 min; under the latter conditions three peaks were observed (cf. Liu *et al.*, 1978). It has now been determined that the incubation time and not the absence or presence of 0.25 M-sucrose is the critical factor. Fig. 1 shows the chromatographic profile of the dexamethasone-binding proteins from the liver of adrenalectomized rats. Thus three dexamethasone-binding components were observed, designated DI, DII and DIII in order of elution, when the cytosol was preincubated with [³H]dexamethasone for 120 min before charcoal treatment

Fig. 1. *Sepharose-6B* profiles of the dexamethasone binders in the cytosol prepared from the liver of 47 h-adrenalectomized rats. A 0.2 ml portion of each 0.5 ml fraction was radioassayed and the receptor concentration was calculated from the known specific radioactivity of the $[^3\text{H}]$ dexamethasone. (a) 120 min preincubation with $[^3\text{H}]$ dexamethasone, cytosol prepared in media containing 0.25 M-sucrose; (b) 90 min preincubation with $[^3\text{H}]$ dexamethasone. In this and all subsequent profiles, the small peak to the right labelled DEX represents free (unbound) $[^3\text{H}]$ dexamethasone.

and application to the column (Fig. 1a). In contrast, when 90 min incubation was used, component DIII was visible only as slight shoulder on peak II (Fig. 1b). A 90 min incubation period was sufficient to saturate components DI and DII. These differences may be related to differences in the K_d of these binding proteins (see below). To assess the physiological significance of binding proteins DI, DII and DIII, a 120 min incubation period was used in all subsequent experiments. However, 0.25 M-sucrose is now routinely included in the isolation medium.

Sepharose-6B profile of $[^3\text{H}]$ dexamethasone-binding proteins prelabelled *in vivo*

To rule out the possibility that the multiple dexamethasone-binding proteins are formed during the 2 h incubation of the cytosol *in vitro*, this step was eliminated by prelabelling *in vivo*. In this experiment an adrenalectomized rat was injected via the portal vein with 200 μCi of $[^3\text{H}]$ dexamethasone (24 Ci/mmol) and 3 min later the liver was perfused, excised and immediately homogenized. The *Sepharose-6B* chromatographic profile of the dexamethasone-binding proteins labelled *in vivo* is shown in Fig. 2. At the dexamethasone concentration used (i.e. 8.33 nmol/rat) the pattern was very similar to the pattern *in vitro* (Fig. 1a), where 0.1 nmol/ml was determined to be saturating. Thus, after charging

Fig. 2. *Sepharose-6B* profile of dexamethasone binders in the liver of an intact rat prelabelled *in vivo* with $[^3\text{H}]$ -dexamethasone

The rat was injected via the portal vein with 200 μCi of $[^3\text{H}]$ dexamethasone (8.33 nmol) 3 min before removal of the liver. Other conditions were as for Fig. 1.

in vivo, three distinct peaks of dexamethasone binders were observed, component DI being predominant. This pattern was reproducible when 8.33 nmol

(200 μ Ci) of [3 H]dexamethasone was injected into a 230g rat, but peak DIII in particular was decreased in size and appeared as a shoulder on chromatographic peak DII when the rat received half this dose.

Differential stabilities of the dexamethasone-binding proteins at 4°C

Cytosols from both intact and adrenalectomized rats were stored at 4°C for 24h before charging *in vitro* with [3 H]dexamethasone and Sepharose 6B chromatography. As indicated by the data in Table 1, species DI is stable, whereas species DII lost approx. 50 % of its binding activity, regardless of whether the liver cytosol came from intact or adrenalectomized rats. Species DIII was also relatively labile, but appeared to be stabilized by endogenous steroids; it was approx. 72 and 40% of the control values in cytosol derived respectively from intact and adrenalectomized rats.

Nuclear uptake and DNA binding of the three dexamethasone-binding-protein complexes

The differential nuclear uptakes of the three dexamethasone-binding-protein complexes were analysed by comparing the Sepharose 6B profiles of the cytosol after (a) charging the cytosol with [3 H]-dexamethasone at 4°C for 120min, (b) charging the cytosol as in (a), then activating by heating at 20°C for 30min, and (c) charging the cytosol as in (a), then incubating with an excess of nuclei at 20°C for 30min. Further details are given in the Materials and

Methods section. Binding activities, expressed as percentages of total protein-bound radioactivity of the control sample, are summarized in Table 2. Thus approx. 45.5% of component DI and 55.5% of component DII translocated to the liver nuclei under the conditions specified above. In contrast, the binding activity of component DIII showed a slight increase during incubation with the nuclei. It is concluded that the dexamethasone complex of component DIII does not translocate to the nucleus.

Since binding to DNA is also a characteristic of glucocorticoid receptors, this parameter was also assessed. The [3 H]dexamethasone-binding proteins were fractionated on a Sepharose 6B column, then the active fractions of each peak were pooled and assayed for binding to DNA-cellulose (see the Materials and Methods section): 56.1% of component DI and 32.3% of component DII bound to DNA-cellulose. However, there was no perceptible DNA binding of component DIII. The degree of nuclear uptake and DNA binding obtained for components DI and DII are equal to or exceed the values reported by others for dexamethasone receptors (Eisen & Glinsmann, 1978; Parchman *et al.*, 1977; Sakaue & Thompson, 1977).

Isoelectric focusing of the [3 H]dexamethasone-binding proteins

Cytosol, pre-charged with [3 H]dexamethasone at 4°C and desalted with Sephadex G-25, was analysed by isoelectric focusing on a polyacrylamide gel; the pH gradient varied over the range 4.5–8.0. The profile

Table 1. *Stability in vitro of the dexamethasone-binding proteins at 4°C*

Values in parentheses are from a second experiment in which the cytosol was incubated for 24h at 4°C in the presence of 60nM-[3 H]dexamethasone. Control cytosol was incubated with [3 H]dexamethasone for 2h immediately after preparation, and the experimental cytol was stored at 4°C for 24h before charging with [3 H]dexamethasone (see the text for further details).

Hormonal status of donor rat	Binding activity (% of fresh control)		
	DI	DII	DIII
Intact	117 (116)	48 (48)	72 (71)
Adrenalectomized	102	49	40

Table 2. *Nuclear uptake of [3 H]dexamethasone-binding proteins*

Control cytosol charged with [3 H]dexamethasone at 4°C (1) was heat-activated at 20°C in the absence (2) or presence (3) of nuclei. See the text for details. Total protein-bound radioactivity in the control was 484 600 c.p.m. The proteins were prelabelled *in vivo*.

Binding proteins	Cytosol-bound dexamethasone (% of total)		
	(1) Control cytosol	(2) Heat-activated cytosol	(3) Cytosol incubated with nuclei
DI	74.0	63.2	34.4
DII	9.2	10.5	4.7
DIII	16.8	20.8	24.4

(Fig. 3) shows four dexamethasone-binding components. The presence of at least four components is predicted, since electrophoresis, but not the Sepharose 6B column, would distinguish between the activated and unactivated forms of at least the predominant receptor (cf. Parchman & Litwack, 1977). In fact, the form with the lowest pI increases significantly after heat activation, i.e. after incubation of the charged, desalted cytosol at 25°C for 20 min. Two of the components appeared to decrease after heat activation.

Affinity of the binding proteins for dexamethasone

The K_d (dissociation constant) of the dexamethasone-binder complexes was estimated from the kinetic data by the method of Scatchard (1949). Since the binding proteins were relatively unstable in the absence of steroid, portions of the cytosol were first charged with one of four concentrations of [3H]-

dexamethasone, then the four samples were separated simultaneously on separate Sepharose 6B columns. Binding activities were estimated from comparisons of the bound radioactivity in the corresponding peaks from each column. As shown in Table 3, the K_d values of components DI and DII were comparable and similar to that of unfractionated liver cytosol, i.e. $K_d = 13$, whereas that for component DIII was 3-4 times as high.

Response in vivo of binders to adrenalectomy and glucocorticoid administration

The results of an earlier study (Beato *et al.*, 1974) showed that the total dexamethasone-binding activities in rat liver cytosol increased sharply after adrenalectomy, reaching a maximum 2 days after operation. It was therefore decided to investigate the specific changes in components DI, DII and DIII after adrenalectomy. As shown in Fig. 4, component DI

Fig. 3. Isoelectric-focusing profile of liver cytosol from an adrenalectomized rat precharged with [3H]dexamethasone at 4°C before (○) and after (Δ) a further 20 min pre-incubation at 25°C. The pH gradient formed during the 17h run is also shown (●). The anode is to the left.

Fig. 4. Time-course changes in the concentration of the three dexamethasone binders in the livers of rats at the indicated times after adrenalectomy. The cytosols were prepared in media containing 0.25M-sucrose. The data shown represent the peak concentrations of components DI (●), DII (Δ) and DIII (○).

Table 3. *Affinity of binding proteins for dexamethasone*

The parameters were estimated from Scatchard (1949) plots of the binding data for each binder at four different dexamethasone concentrations, which varied from 10 to 60 nM. For details see the text.

Binder	Concn. (n) (pmol/mg of protein)	Dissociation constant, K_d (nM)
DI	0.45	13
DII	0.05	17
DIII	0.15	46
Unfractionated cytosol	0.63	13

increased sharply, then reached a plateau at twice the activity in the intact rat by approx. 40h after adrenalectomy. The binding activity of component DII also doubled within 20h after operation, but then slowly declined toward the initial value. A slight but transient increase was also observed in the binding activity of component DIII. It is noteworthy that the changes in components DI and DII more or less parallel one another, whereas the changes in component DIII, which more closely reflect those in component DII, showed a marked lag.

In an earlier study (Wira & Munck, 1974) it was shown that the total cytoplasmic concentration of glucocorticoid-receptor complex in the intact cell decreased after glucocorticoid administration, and that this decrease coincided with the translocation of the complex to the nucleus. Fig. 5 shows the differential effect on hepatic components DI, DII and DIII of pretreating the rat with cortisol. This was selected because it is rapidly cleared from the animal, permitting the observation of any reversible changes in their concentration *in vivo*. Within 1h after the administration of the steroid to intact rats the [3 H]-dexamethasone-binding activity of component DI in the liver cytosol fraction decreased by 70%, whereas that of components DII and DIII decreased by 50%. The dexamethasone-binding activities of all three components in the cytosol returned to within 80% of control values by 24h after cortisol administration. Although suggestive of nuclear uptake of all three dexamethasone-binder complexes *in vivo*, the data must be interpreted in the light of the studies *in vitro*. Thus component DIII probably does not undergo nuclear translocation *in vivo*, but rather the changes observed reflect the dependence of the concentration of component DIII on the cytoplasmic concentration

of one or both of components DI and DII, which undergo nuclear translocation. Again the lag between the concentration changes in components DIII and DI or DII are consistent with this possibility.

Dexamethasone receptors in hepatoma cells

As shown in Fig. 6, only one dexamethasone receptor (DI) is present in the cytosol prepared from Novikoff-hepatoma cells in medium containing 0.25M-sucrose. These results not only confirm earlier indications (Liu & Webb, 1977a) that the hepatomas contain fewer receptors than do normal liver cells, but also show there is no simple equilibrium between the major component (DI) and the other two receptors identified in liver.

Discussion

Although several proteins have been identified in rat liver which bind cortisol and its metabolites (Litwack *et al.*, 1973; Agarwal, 1976), interest has centred on those components that bind the biologically active synthetic steroid, dexamethasone, since this fraction must contain the receptors that modify genetic activity (Rousseau *et al.*, 1973). As indicated in the introduction, several laboratories, including ours, earlier identified two dexamethasone-binding proteins in rat liver cytosol by using different chromatographic media. The results of the present study now suggest that there are three (one major and two minor) dexamethasone-binding proteins of different concentration, stability and possibly function in rat liver. Only two of these appear to be receptors, undergoing nuclear translocation *in vitro* and having the capability for binding DNA-cellulose. There is the possibility that the third (minor) component (DIII) may be derived from one of the other two, since (i) changes in concentration of this component *in vivo* after adrenalectomy or administration of

Fig. 5. Time-course changes in the concentration of the three dexamethasone binders in the livers of intact rats after the administration of 40mg of cortisol hemisuccinate. The cytosols were prepared in media containing 0.25M-sucrose. The data shown represent the peak concentrations of components DI (●), DII (△) and DIII (○).

Fig. 6. Sepharose-6B profile of the dexamethasone binders in cytosol prepared from Novikoff-hepatoma cells. The binders were saturated with [3 H]dexamethasone *in vitro*. Other conditions were as for Fig. 1.

dexamethasone lag behind the predictable changes in components DI and DII (which parallel one another), (ii) components DI and DII have similar stabilities and (iii) apparently components DII and DIII are both absent from Novikoff-hepatoma cells, which exhibit normal concentrations of component DI. The latter observation suggests that species DIII may be derived from species DII, but not DI. However, none of these observations prove a product-precursor relationship.

The significance of multiple forms of the dexamethasone receptors is at present unclear. However, the clear demonstration of their existence after pre-saturation with [^3H]dexamethasone *in vivo* indicates that they are not an artifact of the incubation procedures *in vitro* normally used, although the administration of [^3H]dexamethasone dosages much below 35 nmol/kg is not sufficient to saturate component DIII *in vivo*. Any attempts to demonstrate the inter-conversion of the binding proteins *in vitro* must take into account the relatively greater lability of components DII and DIII compared with component DI. Furthermore, mutant cells, including neoplastic cells, may provide us with some clues about their function in target cells. For example, the growth sensitivity of target cells to glucocorticoids appears to reside with component DI, since it is the only form present in the glucocorticoid-sensitive Novikoff-hepatoma cells (cf. Liu *et al.*, 1975). Component DI is the only form present in embryonic liver (Liu & Webb, 1977a).

The failure to observe component DIII in an earlier study (Liu & Webb, 1977a) using Sepharose 6B chromatography of the [^3H]dexamethasone-charged cytosol is attributed to its lower affinity for dexamethasone. However, even under the earlier assay conditions, component DIII was sometimes observed as a small shoulder on the DII peak. It is assumed that the extended time required for the saturation of this component *in vitro* is related to its comparatively lower affinity for dexamethasone. Component DIII may be a degradative product of component DII, and the corresponding glucocorticoid complex probably does not affect genetic activity as do the two receptors. However, its presence could result in an error of approx. 20% in the estimation of the dexamethasone-receptor activity in the unfractionated cytosol.

Finally, recent studies (Carlstedt-Duke *et al.*, 1977; Wrangé & Gustafsson, 1978) indicate that the main dexamethasone-receptor complex in rat liver cytosol is convertible by proteolysis (e.g. trypsin) into two discrete fragments with Stokes radii of approximately one-half and one third that of the former. Interestingly, the smaller complex does not bind to nuclei or to DNA, whereas the intermediate-sized fragment resembles the nuclear form of the dexamethasone-receptor complex. We have also found (S.-L. Liu &

T. E. Webb, unpublished work) a decrease in component DI with an equivalent increase in component DII and a slight increase in component DIII on treating cytosol with 3 μg of trypsin/mg of cytosol protein. Such data provide only circumstantial evidence that component DII may be derived from DI, and of course component DIII from DII. If these proteolytic conversions *in vitro* have a normal counterpart *in vivo*, then the two minor components identified in the present study may correspond to these proteolytic fragments. Although such a conversion does not occur in our untreated cytosol preparations, nor does it appear to occur in the Novikoff-hepatoma cells, it may very well occur in intact normal hepatic cells.

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