Interaction of Glucocorticoids with Rat Liver Nuclei. II. Studies on the Nature of the Cytosol Transfer Factor and the Nuclear Acceptor Site†

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ABSTRACT: The ability of rat liver cytosol to enhance the uptake of [^{3}H]dexamethasone into liver nuclei isolated from adrenalectomized rats is shown to be due to the presence of a specific glucocorticoid receptor in the hepatic cytosol. Neither of the transcortin-like proteins of liver cytosol enhanced the nuclear uptake of glucocorticoids *in vitro*. *In vitro* incubation of cytosol with nuclei and dexamethasone depletes the cytosol of glucocorticoid receptor concurrently with the generation of "new" steroid binding sites within the cell nucleus. In the absence of dexamethasone neither the concentration of receptor in the cytosol nor the number of nuclear glucocorticoid binding sites changed upon *in vitro* incubation. Nuclear fractionation studies showed that the nucleolus is not a preferential site of glucocorticoid binding; both in the absence and presence of cytosol receptor most of the nuclear bound dexamethasone is located in the extranucleolar chromatin. Treatment of nuclei with 0.4 M NaCl or deoxyribonuclease I, after incubation with [^{3}H]dexamethasone bound to the cytosol receptor, solubilizes a complex of radioactive steroid and protein which sediments in the 4S region of a sucrose gradient and tends to aggregate in the absence of salt. In the presence of dexamethasone, but not in its absence, the cytosol receptor binds to protein-free DNA purified from rat liver or *Escherichia coli* and adsorbed to cellulose. These findings support a model in which the intracellular location of the liver glucocorticoid receptor is determined by the concentration of circulating corticosteroids and by the nuclear acceptor capacity. In the absence of glucocorticoids, *e.g.*, after adrenalectomy, the receptor is found in the cytosol fraction. In the presence of the steroid a temperature-dependent modification of the receptor–glucocorticoid complex takes place, leading to its migration into the cell nucleus. The intracellular translocation of the steroid into the nucleus is, at least partly, a consequence of the high affinity of the transformed receptor–dexamethasone complex for DNA-containing structures.

In the previous papers the interaction of glucocorticoid hormones with liver nuclei from intact and adrenalectomized rats has been studied (Beato *et al.*, 1973; Kalimi *et al.*, 1973). It was shown that liver nuclei from intact rats are able to incorporate glucocorticoids *in vitro*, and that this nuclear capacity is progressively lost following bilateral adrenalectomy. Data were presented demonstrating the existence in liver cytosol of a protein factor able to enhance the glucocorticoid binding capacity of liver nuclei from adrenalectomized rats. This cytosol factor was found in lower concentrations in kidney and spleen and interacted preferentially with liver nuclei than with nuclei of kidney or spleen (Kalimi *et al.*, 1973).

In this paper we present investigations upon the identity of the protein factor in liver cytosol which binds and is responsible for the transfer of glucocorticoids into the nucleus. These studies show that this function is performed by a specific cytosol glucocorticoid receptor and not by the transcortin-like proteins of liver cytosol (Beato and Feigelson, 1972). Furthermore, the intranuclear localization of the transported dexamethasone has been studied and some of the properties of the nuclear dexamethasone–protein complex have been compared with those of the cytosol glucocorticoid receptor.

Materials and Methods

The radiochemicals and other materials used for these experiments have been described previously (Beato *et al.*, 1973).

The preparation of liver cytosol and the binding assay of [^{3}H]dexamethasone to the cytosol proteins were as previously reported (Beato *et al.*, 1969a; Beato and Feigelson, 1972). Liver nuclei were prepared either with nonionic detergents or with hypertonic sucrose as described (Beato *et al.*, 1969b). The conditions for the nuclear steroid binding assay in the presence and absence of cytosol have been specified in the previous papers (Beato *et al.*, 1973; Kalimi *et al.*, 1973).

Fractionation of the Nuclei. Nuclei purified through hypertonic sucrose were resuspended in 50 mM Tris-HCl (pH 7.55) containing 250 mM sucrose, 3 mM $MgCl_2$, 25 mM KCl, 1 mM Na_2EDTA, and 1 mM mercaptoethanol (homogenization buffer) and incubated with [^{3}H]dexamethasone as indicated in the legend to Table II. The nuclei were then reisolated by centrifugation through 5% Dextran-500 in homogenization buffer, and nucleoli were prepared by sonication and centrifugation through 0.88 M sucrose as originally described by Muramatsu *et al.* (1970) and adapted by Yu and Feigelson (1971). The extranucleolar chromatin in the supernatant was centrifuged through 1 M sucrose at 240,000*g* for 90 min. Both the nucleolar and the extranucleolar chromatin pellets were resuspended in homogenization buffer and used for optical density and radioactive measurements (Beato *et al.*, 1970b).

Receptor Binding to DNA-Cellulose. Rat liver DNA was prepared from isolated chromatin by a modification of the procedure of Marmur (1961) including treatment with pancreatic ribonuclease and Pronase (Beato *et al.*, 1970b). The

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TABLE I: Effect of Liver Cytosol Fractions on the Uptake of Glucocorticoids by Rat Liver Nuclei.[a]

Fraction Added	No. of Expts	Radioactivity Incorpd (dpm/ml of Protein) [^{3}H]-Cortisol	[^{3}H]Dexamethasone
None	5	389 ± 41	210 ± 29
Cytosol	5	525 ± 76	605 ± 93
Cytosol heated[b]	2	352 ± 48	225 ± 51
A protein fraction[c]	4	371 ± 63	230 ± 71
B protein fraction[c]	3	295 ± 35	198 ± 22
Serum transcortin[c]	2	300 ± 16	215 ± 15
Receptor protein fraction[c]	3	482 ± 52	509 ± 64

[a] Purified liver nuclei were incubated at 20° for 20 min with [^{3}H]cortisol or [^{3}H]dexamethasone (final concentration 2×10^{-8} M), preincubated either with buffer or with the indicated protein fractions. Blanks were taken with a 1000-fold excess of the corresponding nonradioactive steroid. After reisolation of the nuclei, the amount of specifically bound radioactivity and the protein content were measured (Beato *et al.*, 1973). [b] The cytosol was heated at 40° for 10 min and centrifuged at 10,000*g* for 10 min before incubation with the steroid. [c] The A and B proteins and rat serum transcortin were separated from each other by DEAE-cellulose column chromatography and Sephadex G-150 gel filtration (Beato *et al.*, 1972). The receptor protein was separated from the A and B proteins by ammonium sulfate fractionation and Sephadex G-150 gel filtration (Beato and Feigelson, 1972). In each case, the amount of active binding proteins added to the nuclei corresponded to their respective concentrations in liver cytosol.

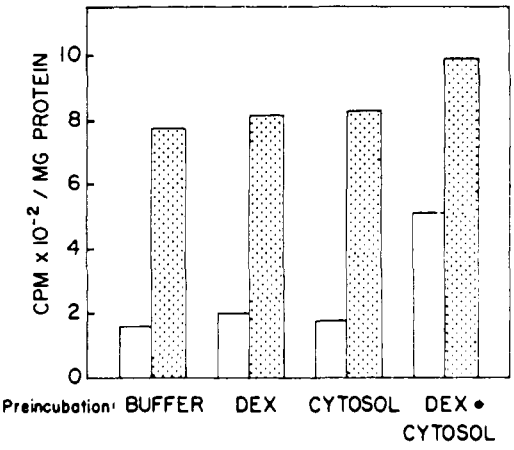

FIGURE 1: Effect of preincubation of liver nuclei with dexamethasone or liver cytosol receptor or both together on the subsequent incorporation of [^{3}H]dexamethasone in the absence and presence of liver cytosol receptor. Liver nuclei and cytosol were prepared from 5-day adrenalectomized rats. The purified nuclei were incubated at 20° for 20 min with either buffer, dexamethasone, cytosol, or cytosol preincubated at 20° for 20 min with dexamethasone. The final concentration of dexamethasone in the nuclear incubation was 5×10^{-8} M. At the end of the incubation, the nuclei were reisolated through 5% Dextran-500 (Beato *et al.*, 1973) and resuspended in homogenization buffer. The reisolated nuclei were used for a "transfer experiment" (Kalimi *et al.*, 1973) using [^{3}H]dexamethasone in buffer (open bars) or preincubated at 20° for 20 min with liver cytosol (dotted bars). The amount of specifically bound [^{3}H]dexamethasone and the protein content were determined in the reisolated nuclei. The values are the average of two experiments.

purified DNA was coupled to cellulose (Sigmacell type 38 from Sigma Co.) by the procedure of Alberts *et al.* (1968). The binding of [^{3}H]dexamethasone alone or preincubated with cytosol to the DNA-cellulose suspension was evaluated after incubation at 20° for 40 min. The DNA-cellulose was then washed twice at 0° with 50 vol of 10 mM Tris-HCl (pH 7.6) containing 1 mM mercaptoethanol. The pellet obtained after the second centrifugation was counted in 10 ml of Bray's scintillation solution (Bray, 1960). Controls were taken with plain cellulose as well as with a 1000-fold excess of nonradioactive dexamethasone.

Results

Experiments directed to elucidate the identity of the cytosol factor responsible for the enhancement of nuclear glucocorticoid uptake *in vitro* are summarized in Table I. The fact that the effect of cytosol on nuclear steroid uptake is much more evident when [^{3}H]dexamethasone is used instead of [^{3}H]-cortisol suggests that the transcortin-like proteins of liver cytosol are not responsible for the transfer of the steroid into the nucleus, since they have negligible affinities for the 9α-fluorinated glucocorticoids (Beato and Feigelson, 1972). Furthermore, preincubating the cytosol at 40° for 10 min abolishes its enhancement of nuclear steroid uptake (Table I). Since it is known that the transcortin-like proteins of liver cytosol are stable at this temperature, while the glucocorticoid receptor is inactivated by this treatment (Koblinsky *et al.*, 1972), the assumption is further substantiated that the glucocorticoid receptor is indeed the responsible cytoplasmic factor required for enhanced nuclear steroid uptake.

To directly test this hypothesis, the ability to enhance nuclear steroid uptake was evaluated using liver cytosol fractions, each containing only one of the three glucocorticoid binding proteins. The A protein is without effect and the B protein or serum transcortin actually inhibits cortisol incorporation by isolated liver nuclei (Table I). None of these preparations had a significant effect on nuclear uptake of [^{3}H]dexamethasone, whereas the fraction containing the glucocorticoid receptor markedly enhances the uptake of [^{3}H]dexamethasone and [^{3}H]cortisol into the liver nuclei.

Considering that the cytosol receptor is responsible for the transfer of the steroid into the nucleus, we then explored whether the receptor molecule actually enters the nucleus and the possible role of the hormonal steroid on this process. Experiments were conducted in which the nuclei were preincubated at 20° with either steroid alone or cytosol alone or both together. The nuclei were then reisolated and their content of dexamethasone binding sites evaluated in the presence and absence of liver cytosol (Figure 1). Preincubation with dexamethasone or cytosol alone did not affect the subsequent incorporation of [^{3}H]dexamethasone by isolated nuclei, but preincubation with cytosol and dexamethasone together considerably elevates the capacity of these nuclei to later bind [^{3}H]dexamethasone in the absence of cytosol receptor. These results indicate that nuclei preincubated with receptor–dexamethasone complex have acquired the capability of autonomous steroid uptake which is characteristic of liver nuclei from intact rats (Beato *et al.*, 1973), thus suggesting the transport of cytoplasmic receptor–steroid complex into the nucleus.

We then determined whether the acquisition of the nuclear ability to incorporate [^{3}H]dexamethasone, effected by pre-

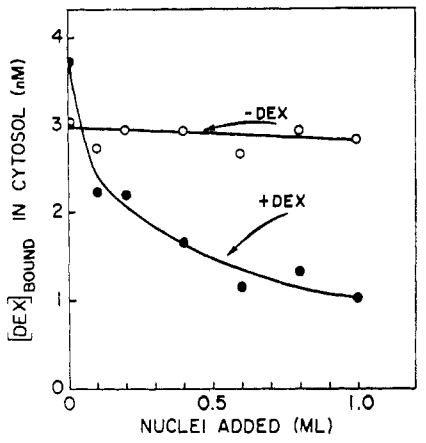

FIGURE 2: Depletion of liver cytosol receptor after incubation with liver nuclei in the presence of dexamethasone. Liver nuclei and cytosol were prepared from 4-day adrenalectomized rats. The cytosol was incubated at 20° for 20 min with either buffer or [^{3}H]dexamethasone (5×10^{-8} M). To 1-ml aliquots of cytosol, increasing amounts of a liver nuclear suspension were then added in a final volume of 1 ml of homogenization buffer, and incubation continued at 20° for 40 min. After centrifugation of the nuclei at 3000*g* for 10 min, the supernatant was used for the determination of the concentration of specific dexamethasone binding sites. Five concentrations of [^{3}H]-dexamethasone ranging from 2.5×10^{-8} to 2×10^{-7} M were used alone or with the addition of a 1000-fold excess of nonradioactive dexamethasone. Incubation was for 24 hr at 4° and the amount of specifically bound [^{3}H]dexamethasone was determined using the charcoal technique (Beato and Feigelson, 1972). For the calculation of the concentration of dexamethasone binding sites, *n*, in the cytosol the Scatchard plot was used (Scatchard, 1949).

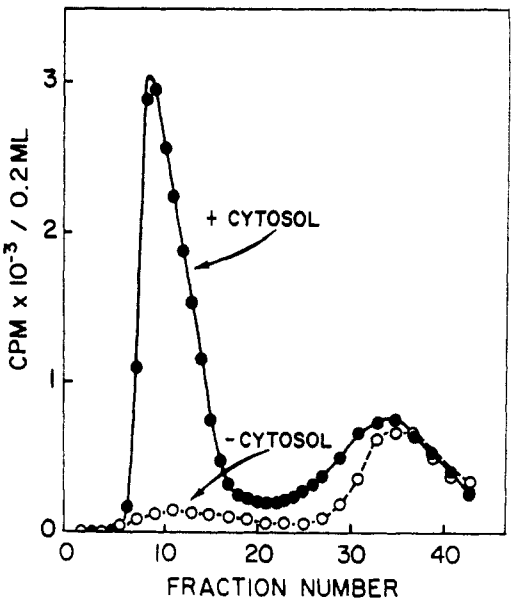

FIGURE 3: Column chromatography on Sephadex G-25 of NaCl extracts from liver nuclei incubated with [^{3}H]dexamethasone in the absence and in the presence of liver cytosol. Liver nuclei and cytosol were prepared from 5-day adrenalectomized rats. One milliliter of nuclear suspension was incubated at 20° for 40 min with 2×10^{-8} M [^{3}H]dexamethasone alone or after preincubation with 1 ml of liver cytosol (20°, 20 min). The nuclear pellet was reisolated through 5% Dextran-500 and resuspended in 0.5 ml of 10 mM Tris-HCl buffer (pH 7.5) containing 1 mM mercaptoethanol and 0.3 M NaCl. After gentle stirring at 0° for 30 min, the mixture was centrifuged at 15,000*g* for 15 min and the supernatant was applied to a column of Sephadex G-25 (0.8 × 20 cm) equilibrated with 10 mM Tris-HCl buffer (pH 7.5) containing 1 mM mercaptoethanol. Fractions (0.3 ml) were collected and 0.2 ml was used for radioactivity determination.

incubation of the nuclei with receptor–dexamethasone complex, is accompanied by the disappearance of these receptor molecules from the cytosol.

A constant volume of cytosol was incubated at 20° for 40 min with an increasing number of nuclei in the presence or absence of a fixed level of [^{3}H]dexamethasone. After removal of the nuclei by centrifugation, the concentration of dexamethasone binding sites remaining in each supernatant was determined by Scatchard analysis of equilibrium binding data obtained at 0° (Beato and Feigelson, 1972). As shown in Figure 2, incubation with nuclei in the presence of [^{3}H]-dexamethasone results in a decrease in the concentration of receptor in the cytosol, which is dependent on the amount of nuclei added. In the absence of the steroid, there is no significant loss of cytoplasmic dexamethasone receptor after incubation with liver nuclei. Stabilization of the receptor by dexamethasone accounts for the lower receptor level observed in the absence of steroid. These findings are compatible with the migration of the receptor–dexamethasone complex from the cytoplasm into the cell nucleus.

TABLE II: Intranuclear Distribution of [^{3}H]Dexamethasone.

Fraction	[^{3}H]Dexamethasone Incorpd (cpm/10^8 Nuclei)		Cytosol Buffer
	Buffer	+Cytosol	
Nuclei	2410	6950	2.9
Nucleoli	160	289	1.8
Extranucleolar chromatin			
(a) Crude supernatant	2505	6650	2.8
(b) Centrifuged through sucrose	630	2090	3.0

[a] Liver nuclei were prepared from 4-day adrenalectomized rats, using hypertonic sucrose (Beato *et al.*, 1969b). After incubation, at 20° for 40 min with 2×10^{-8} M [^{3}H]dexamethasone, alone or preincubated with cytosol, the nuclei were reisolated through Dextran-500 and resuspended in homogenization buffer. Sonication and separation of nuclear and extranucleolar fractions were as described under Materials and Methods. The values represent the average of two experiments.

Intranuclear Distribution of [^{3}H]Dexamethasone. In order to study the intranuclear localization of dexamethasone, sonication of the nuclei followed by separation of the nucleolar and extranucleolar fractions was performed after incubation of the nuclei with [^{3}H]dexamethasone in the absence or in the presence of liver cytosol receptor (Table II). Only 6% of the radioactivity incorporated into the nuclei in the absence of cytosol was found to be bound to the isolated nucleolar fraction, the remainder existing in the nucleoplasmic supernatant. In the presence of the cytosol receptor, a threefold increase in nuclear steroid uptake was observed. More radioactivity was incorporated into the nucleoli but this accounted for only 4% of the total nuclear radioactivity, thus showing that the nucleolus is neither a preferential site for binding of the steroid nor an efficient acceptor site for the receptor–dexamethasone complex. After centrifugation of the extranucleolar supernatant fraction through 1 M sucrose, the resulting chromatin pellet contains considerably more bound radioactivity than the nucleolar fraction. Furthermore, the cytosol-

TABLE III: Solubilization by DNase I of Nuclear-Bound Radioactivity from Liver Nuclei Incubated with [^{3}H]Dexamethasone in the Absence or Presence of Liver Cytosol.[a]

	[^{3}H]Dexamethasone (cpm/10^8 Nuclei)	
	Buffer	Cytosol
Total nuclei	1960	6040
DNase I extract		
Total	890	3070
Macromolecular bound	96	1640

[a] Liver nuclei from 4-day adrenalectomized rats were incubated at 20° for 40 min with 2×10^{-8} M [^{3}H]dexamethasone alone or preincubated with liver cytosol (20°, 20 min). The nuclei were reisolated through Dextran-500 and resuspended in homogenization buffer. Aliquots were taken from radioactivity measurements and enzyme treatment. Pancreatic deoxyribonuclease I (Worthington, Inc.) was added to a final concentration of 100 μg/ml and the nuclei incubated at 5° for 40 min. After centrifugation at 40,000g for 20 min, the supernatant was used for measurement of radioactivity and OD_{260}. Aliquots of the supernatants were passed through Sephadex G-25 columns and the amount of radioactivity in the excluded volume was determined.

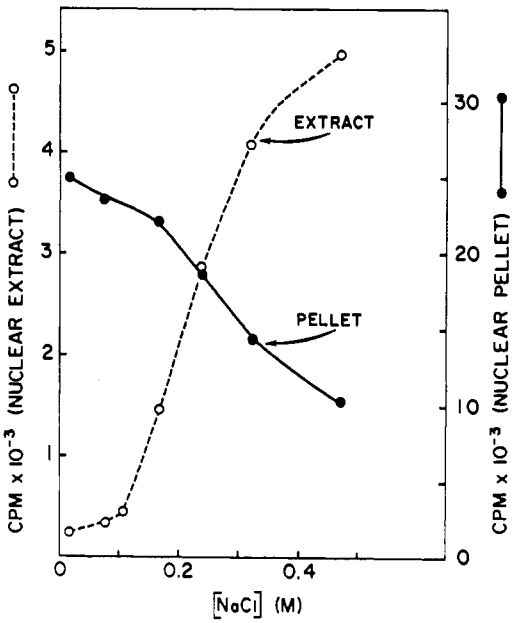

FIGURE 4: Extraction with NaCl of protein-bound radioactivity from liver nuclei incubated with [^{3}H]dexamethasone and liver cytosol. Liver nuclei and cytosol were prepared from 5-day adrenalectomized rats. Aliquots (0.5 ml) of the nuclear suspension were incubated at 20° for 40 min with 2×10^{-8} M [^{3}H]dexamethasone preincubated at 20° for 20 min with 0.5 ml of liver cytosol. The nuclei were reisolated through 5% Dextran-500 and resuspended in 10 mM Tris-HCl (pH 7.5) containing 1 mM mercaptoethanol and NaCl to the indicated concentrations. After stirring for 30 min at 0°, the mixture was centrifuged at 15,000g for 15 min. The pellet was washed in 5 ml of homogenization buffer and counted in 20 ml of Bray's solution after digestion with 1 ml of Nuclear-Chicago solubilizer. No variation in the OD_{260} of the nuclear pellet was observed up to NaCl concentrations of 0.4 M. The supernatant was treated with 0.1 vol of dextran-coated charcoal at 0° for 10 min, and the protein-bound radioactivity was estimated in the supernatant after centrifugation at 3000g for 10 min (Beato and Feigelson, 1972). The values represent the average of two experiments performed in duplicate.

mediated enhancement of the dexamethasone uptake is more pronounced in the extranucleolar chromatin. The fact that a cytosol enhancement is also observed in the nucleolar binding of dexamethasone may reflect unavoidable contamination of this fraction with extranucleolar chromatin.

Nature of the Nuclear Dexamethasone–Macromolecular Complex. Extraction with 0.3 M NaCl of nuclei preincubated with [^{3}H]dexamethasone leads to the solubilization of about half the radioactivity incorporated into the nuclei. Filtration of the NaCl nuclear extract through a column of Sephadex G-25 results in characteristic elution profiles depending on whether or not the cytosol receptor has been present during the incubation of the nuclei with the steroid (Figure 3). The extract from nuclei incubated with [^{3}H]dexamethasone alone shows neglectable amounts of radioactivity in the macromolecular peak, whereas the extract from nuclei incubated with the steroid in the presence of cytosol receptor contains largely macromolecular-bound radioactivity. Treatment of the nuclei with pancreatic deoxyribonuclease I (DNase I) after similar preincubation with the steroid also leads to the solubilization of about half the nuclear bound radioactivity, which behaves upon Sephadex G-25 chromatography similarly to the NaCl extractable nuclear radioactivity (Table III). This procedure indicates that preincubation with cytosol receptor protein greatly augments the nuclear level of macromolecular bound [^{3}H[dexamethasone.

The amount of macromolecular-bound [^{3}H]dexamethasone in the nuclear extracts can also be determined by treating aliquots with dextran-coated charcoal and measuring the nonadsorbed (*i.e.*, protein-bound) radioactivity (Beato and Feigelson, 1972). Using this procedure we studied the salt extractability of the macromolecular-bound dexamethasone incorporated into the nuclei in the presence of the cytosol receptor (Figure 4). At salt concentrations below the physiological level very little macromolecular-bound radioactivity can be extracted from the nuclei, but between 0.15 and 0.30 M NaCl the amount of protein-bound dexamethasone extracted from the nuclei increases linearly. Above 0.4 M NaCl, the chromatin itself begins to go into solution, thus interfering with the use of the charcoal assay to estimate the amount of macromolecular-bound radioactivity in the nuclear extract.

When a 0.4 M NaCl nuclear extract obtained from nuclei incubated with [^{3}H]dexamethasone in the presence of cytosol receptor is passed through a column of Sephadex G-25 and the resultant protein-bound [^{3}H]dexamethasone is applied to a 5–20% sucrose gradient, a radioactive peak in the $4S_{20,w}$ region is observed (Figure 5). The position of this peak is not affected by the presence in the gradient buffer of 0.3 M NaCl. Moreover, in the low salt gradients a considerable amount of radioactivity is found in the pellet and in the midregion of the gradient. This may be due either to the existence of a heterogeneous population of nuclear binding proteins or, more probably, to aggregation of a single steroid–protein complex (Beato and Feigelson, 1972). Treatment of nuclei, preincubated with [^{3}H]dexamethasone and cytosol, with DNase I also released a [^{3}H]dexamethasone–protein complex sedimenting at 4 S in sucrose gradients. If nuclei are incubated with the radioactive steroid in the absence of the cytosol receptor and then extracted with either 0.4 M NaCl or DNase I treatment, very little protein-bound radioactivity is solubilized, and after sucrose gradient centrifugation, no radioactive peak in the 4S region is detected.

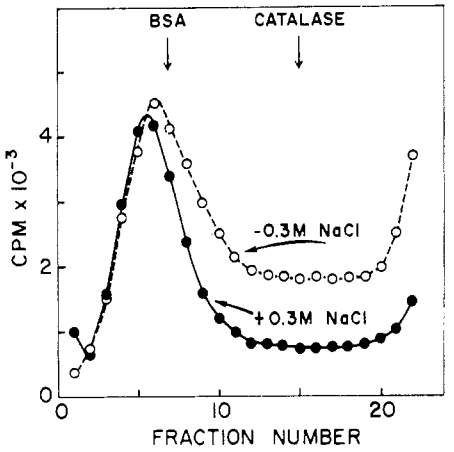

FIGURE 5: Sucrose gradient centrifugation of the NaCl extract of liver nuclei incubated with [^{3}H]dexamethasone and liver cytosol. Liver nuclei prepared from 4-day adrenalectomized rats were incubated at 20° for 40 min with 2×10^{-8} M [^{3}H]dexamethasone preincubated with homologous cytosol (20° for 20 min). The reisolated nuclei were extracted at 0° for 30 min with 0.4 M NaCl dissolved in 10 mM Tris-HCl buffer (pH 7.5) containing 1 mM mercaptoethanol and centrifuged at 15,000*g* for 15 min. Aliquots (0.5 ml) of the supernatant were applied to 5–20% linear sucrose gradients preformed in 10 mM Tris buffer (pH 7.5) containing 1 mM mercaptoethanol, 1×10^{-10} M [^{3}H]dexamethasone (about 400 cpm/0.2 ml), and, where indicated, 0.3 M NaCl. After centrifugation at 40,000 rpm for 20 hr in the Spinco SW-41 rotor, 0.5-ml fractions were collected and counted in 10 ml of Bray's solution (Bray, 1960). Bovine serum albumin and beef liver catalase were used as markers.

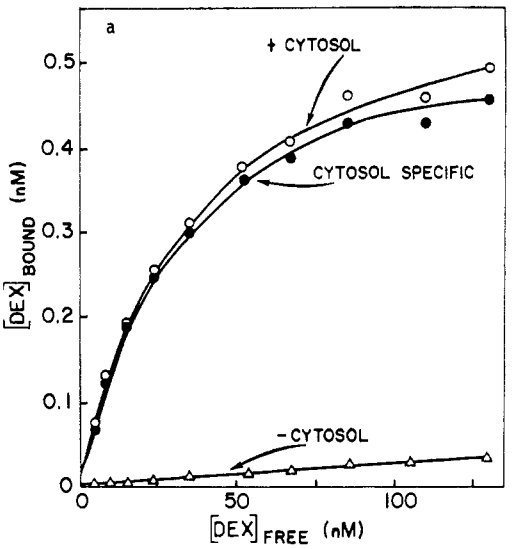

FIGURE 6: Binding of [^{3}H]dexamethasone to liver nuclei in the absence and presence of liver cytosol; protein-bound radioactivity in NaCl nuclear extract; linear plot of the concentration of free dexamethasone against the concentration of protein-bound dexamethasone. Liver nuclei from 5-day adrenalectomized rats were incubated at 20° for 60 min with ten different concentrations of [^{3}H]dexamethasone alone or preincubated at 20° for 20 min with liver cytosol. Parallel assays were performed to which a 1000-fold excess of nonradioactive dexamethasone was added before the cytosol, to determine nonspecific steroid binding. The nuclei were reisolated through 5% dextran and extracted with 0.4 M NaCl in Tris buffer as indicated in the legends to Figures 3 and 5. The nuclear extracts were then treated with charcoal-coated dextran to determine the amount of protein-bound radioactivity (Beato and Feigelson, 1972). The values obtained in the presence of nonradioactive steroid (less than 2% of the total counts per minute in this case) were subtracted.

In order to further understand the nature of the binding of [^{3}H]dexamethasone to the NaCl-extractable nuclear proteins we performed a Scatchard analysis of the binding data (Figure 6). Liver nuclei from adrenalectomized rats were incubated at 20° for 60 min with different concentrations of [^{3}H]dexamethasone in the absence or presence of cytosol receptor. The nuclei were then reisolated by centrifugation through 5% Dextran-500 and extracted with 0.4 M NaCl. The protein-bound radioactivity in the extract was determined using the charcoal technique (Beato and Feigelson, 1972). Figure 6a shows that there is very little protein-bound dexamethasone in the extract from nuclei incubated with the steroid in the absence of cytosol receptor. In the extract from nuclei incubated in the presence of cytosol receptor, a saturable binding of [^{3}H]dexamethasone to proteins is observed. Appropriate calculations indicate the existence of about 3000 steroid binding sites per nucleus (range 1500 to 3800 in four determinations) with an affinity for dexamethasone of $2\text{–}4 \times 10^7$ M^{-1} at 20°.

The pellet obtained after extraction of the nuclei with 0.4 M NaCl contains about half of the nuclear-bound [^{3}H]dexamethasone. The saturation of the binding sites in the nuclear pellet can be studied by incubating the nuclei with different concentrations of the steroid, and measuring the amount of steroid retained in the pellet after 0.4 M NaCl extraction (Figure 7). It can be seen that there is considerable binding in the absence of cytosol receptor, but that the affinity for the steroid of the cytosol-dependent binding is not significantly different from that observed in the NaCl nuclear extract. The number of these cytosol dependent binding sites in the nuclear pellet varied between 7,000 and 12,000 per nucleus.

Nature of the Nuclear Acceptor Site. The experiments with DNase I digestion already suggested that the nuclear DNA played a role in binding the receptor–dexamethasone complex. In order to test the possibility of a direct interaction between the cytosol glucocorticoid receptor and DNA, we studied the binding of [^{3}H]dexamethasone alone or in the presence of liver cytosol receptor to purified native rat liver DNA coupled to cellulose (Alberts *et al.*, 1968). As a control for the specificity of the binding, native DNA purified from *Escherichia coli* was used in parallel assays. In the absence of the cytosol receptor, very little steroid binding to either rat liver or *E. coli* DNA was observed. In the presence of cytosol receptor, both types of DNA were able to retain a considerable amount of the radioactivity (Table IV). These experiments were performed with native double-stranded DNA but receptor dependent binding was also observed when the DNA was heat denatured prior to its coupling to cellulose (Beato, M., Kalimi, M., and Feigelson, P., unpublished).

We then explored whether the cytosol receptor simply facilitates the interaction of the steroid with the DNA or whether the protein itself is participating in a binding complex involving the DNA and the steroid. To test that possibility, we studied the depletion of glucorticoid receptor from the cytosol as a function of the incubation with DNA-cellulose (Table V). As in the experiments with isolated nuclei, a marked decrease in the concentration of dexamethasone binding protein was observed after incubation of the cytosol with DNA-cellulose, thus indicating that the receptor is either inactivated in the process of transferring the steroid into the DNA or more probably is an integral component generating a steroid–receptor–DNA complex. It is also obvious from Table V that this depletion only occurs when dexamethasone is present during the incubation of the cytosol with the DNA-cellulose, demonstrating that the cytosol receptor does not bind to DNA in the absence of the steroid.

TABLE IV: Binding of [^{3}H]Dexamethasone Alone or Preincubated with Liver Cytosol to DNA-Cellulose.[a]

[^{3}H]Dexamethasone Preincubated with	[^{3}H]Dexamethasone Bound (cpm)		
	Cellulose Alone	Rat Liver DNA-Cellulose	*E. coli* DNA-cellulose
Buffer	850	1010	982
Cytosol	1400	9650	7075
Heated cytosol	1280	1680	1892

[a] Liver cytosol prepared from 5-day adrenalectomized rats was incubated with [^{3}H]dexamethasone at 20° for 20 min. Purified DNA prepared from rat liver or *E. coli* was coupled to cellulose (Sigmacell type 38, Sigma) as indicated under Materials and Methods. Fifty microliters of packed cellulose, containing 25 μg of DNA, in 10 mM Tris-HCl (pH 7.6)–1 mM mercaptoethanol, was added to 2×10^{-8} M [^{3}H]dexamethasone preincubated at 20° for 20 min with either homogenization buffer or liver cytosol. As a control, cytosol was heated at 40° for 10 min before use. Incubation with DNA-cellulose or cellulose alone was performed in round-bottomed tubes at 20° for 40 min with gentle shaking. At the end of the incubation, 1 ml of cold Tris–mercaptoethanol buffer was added and the cellulose centrifuged at 3000*g* for 5 min. After washing twice with 1 ml of cold Tris–mercaptoethanol buffer, the samples were counted in 10 ml of Bray's solution (Bray, 1960). The values obtained in assays containing a 1000-fold excess of nonradioactive dexamethasone were subtracted from all samples.

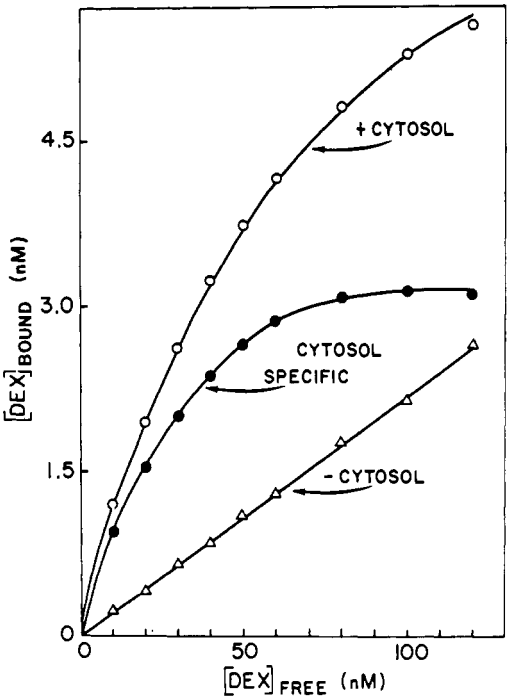

FIGURE 7: Binding of [^{3}H]dexamethasone to liver nuclei in the absence and presence of liver cytosol; NaCl nuclear pellet. Liver nuclei were prepared and incubated with different concentrations of [^{3}H]dexamethasone as described in the legend to Figure 6. The pellets obtained after NaCl extraction were washed 5 min in 5 ml of 10 mM Tris-HCl buffer (pH 7.5) containing 1 mM mercaptoethanol and counted in 20 ml of Bray's solution after digestion with 1 ml of Nuclear-Chicago solubilizer, as shown in the legend to Figure 6.

Discussion

We have reported the presence in rat liver cytosol of a protein factor responsible for the intracellular translocation of glucocorticoid hormones from the cytoplasm into the cell nucleus (Beato *et al.*, 1973; Kalimi *et al.*, 1973; Beato *et al.*, 1970a). In the present study, we have endeavored to further characterize this cytosol protein and its intracellular interactions. Liver cytosol contains three proteins with high affinity for glucocorticoids; two of them, the A and B proteins, have transcortin-like steroid binding properties, whereas the third one, the G protein, binds synthetic as well as natural glucocorticoids with high affinity and specificity (Beato and Feigelson, 1972; Koblinsky *et al.*, 1972; Beato *et al.*, 1972b). Since the degree of *in vivo* saturation of the G protein after intraperitoneal injection of cortisol correlates closely with the extent of hormonal induction of the enzymes tryptophan oxygenase and tyrosine aminotransferase, we inferred that the G protein may indeed be the physiologically significant glucocorticoid receptor of rat liver cytosol (Beato *et al.*, 1972a). We then inquired whether any of the three glucocorticoid-binding proteins from rat liver cytosol could be identified as being responsible for the intracellular transfer of the adrenal corticosteroids into the cell nucleus. The fact that this intranuclear transfer could be reproduced in an *in vitro* system using the 9α-fluorinated synthetic glucocorticoid, dexamethasone, renders a direct involvement of the transcortin-like proteins improbable, as these proteins do not detectably bind dexamethasone (Beato and Feigelson, 1972; Koblinsky *et al.*, 1972). Support for the participation of the glucocorticoid receptor in the cytosol-mediated enhancement of steroid uptake by nuclei *in vitro* came from experiments in which cytosol was preheated at 40° before the transfer experiment. This treatment, which is known to inactivate the cytosol receptor but not the transcortin-like proteins (Koblinsky *et al.*, 1972), completely abolished the cytosol transfer function.

More direct evidence for involvement of the glucocorticoid receptor in the nuclear uptake of steroid required, however, the separation of the three glucocorticoid binding proteins of liver cytosol. The A, B, and G proteins were separated by ammonium sulfate fractionation and subsequent ionic exchange chromatography or Sephadex gel filtration (Beato *et al.*, 1972a; Beato and Feigelson, 1972). Experiments with the individual cytosol fractions showed that only the fraction containing the glucocorticoid receptor (G protein) was able to stimulate the nuclear uptake of [^{3}H]dexamethasone *in vitro* (Table I). These findings indicate that the glucocorticoid receptor of liver cytosol may be the factor responsible for the enhanced nuclear uptake of glucocorticoids, and clearly eliminate direct involvement of the transcortin-like proteins in this function. Experiments in which the cytosol receptor was added to the nuclei alone or in combination with A or B proteins showed that these other proteins do not detectably influence the glucocorticoid transfer function of the receptor, thus arguing against indirect participation of the transcortin-like proteins in the cytoplasmic–nuclear translocation of the hormone.

The observation that in the presence of dexamethasone the concentration of receptor in the cytosol decreases after incubation with the nuclei suggested that the receptor either actually moves into the cell nucleus or is inactivated during the transfer process. In the absence of steroid, no change in the cytosol concentration of receptor was observed after incubation with the nuclei, indicating that glucocorticoids are re-

TABLE V: Depletion of Glucocorticoid Receptor from Liver Cytosol after Incubation with DNA-Cellulose.[a]

Cytosol Incubated with	Dexamethasone	Concn of Dexamethasone Binding Sites in Cytosol (nM)
Cellulose	−	11.2
	+	13.5
Rat liver DNA-cellulose	−	10.8
	+	5.3
E. coli DNA-cellulose	−	10.6
	+	6.7

[a] Liver cytosol was prepared from 5-day adrenalectomized rats and preincubated at 20° for 20 min with either buffer or 2×10^{-8} M [^{3}H]dexamethasone. Five hundred microliters of packed cellulose alone or with rat liver or *E. coli* DNA (250 μg) coupled were added to 2 ml of cytosol and incubation was continued at 20° for 40 min. The cellulose was then removed by centrifugation and the supernatant used for the determination of dexamethasone binding sites. Five concentrations of [^{3}H]dexamethasone ranging from 2×10^{-8} to 2×10^{-7} M were used alone or in the presence of a 1000-fold excess of nonradioactive dexamethasone. Incubation was at 4° for 24 hr and the binding data were plotted according to Scatchard to obtain "*n*" (Beato and Feigelson, 1972; Scatchard, 1949).

quired for the nuclear-dependent depletion of cytosol receptor. Preincubation of the nuclei with either dexamethasone alone or cytosol receptor alone did not alter the subsequent nuclear uptake of dexamethasone. After preincubation of nuclei with the unlabeled dexamethasone–receptor complex, their ability to subsequently incorporate free [^{3}H]dexamethasone was markedly increased. The nuclear ability to incorporate free [^{3}H]dexamethasone, we presume, reflects the nuclear concentration of glucocorticoid–receptor complex wherein the receptor-bound steroid exchanges with the free [^{3}H]dexamethasone. These findings indicate that the intranuclear migration of the cytosol receptor requires the presence of glucocorticoids.

Further support for this mechanism is derived from the analysis of the material extracted from the nuclei containing receptor–dexamethasone using 0.3–0.4 M NaCl or after DNase I treatment. Upon centrifugation in linear sucrose gradients, the macromolecular-bound [^{3}H]dexamethasone extracted from these nuclei sedimented in the 4S region in a way indistinguishable from the cytosol receptor at high ionic strength (Beato and Feigelson, 1972).

The equilibrium association constants of dexamethasone binding to the proteins extracted from the nuclei with NaCl and to those remaining in the nuclear pellet were essentially identical and were one-fifth that observed for binding of the steroid to the cytosol receptor (Kalimi *et al.*, 1973; Beato and Feigelson, 1972). As these measurements were made under different ionic conditions, the data do not enable one to evaluate whether the cytoplasmic receptor underwent modification in the nucleus.

The question remains as to what are the chemical and physical processes underlying the cytoplasmic–nuclear translocation of the steroid–receptor complex. The striking affinity of the receptor dexamethasone complex for purified DNA may ultimately provide the basis for a better understanding of the forces responsible for intranuclear migration of the steroid. In the absence of glucocorticoids the receptor does not have a tendency to bind to either isolated nuclei or pure DNA, but after combining with the steroid and upon incubation at 20°, the receptor–steroid complex develops a marked affinity for both nuclei and DNA. A similar interaction between the glucorcorticoid receptor and DNA-containing structures has been described in hepatoma cells in culture (Baxter *et al.*, 1972), as well as for other steroid hormone receptors (King *et al.*, 1971; Tymoczko and Liao, 1971; Musliner and Chader, 1972; Yamamoto and Alberts, 1972; Clemens and Kleinsmith, 1972; O'Malley *et al.*, 1972). This affinity for DNA-containing structures may suffice to explain the tight binding of the steroid–receptor to nuclei; dependence on its interaction with the steroid would also account for the variations in intracellular distribution of the glucocorticoid receptor observed following adrenalectomy or cortisol injection (Beato *et al.*, 1973).

The fact that in adrenalectomized animals, the receptor is not randomly distributed inside the cell, but concentrates in the nucleus, suggests that either the unbound receptor cannot diffuse freely across the nuclear membrane, or the intranuclearly located fraction of the receptor is washed out of the nuclei during cell fractionation due to its lack of affinity for DNA in the absence of the steroid. Even at concentrations of dexamethasone resulting in full saturation of the receptor, only a fraction of the cytosol receptor molecules can be bound to the nucleus, both *in vivo* and *in vitro* (Beato *et al.*, 1973; Kalimi *et al.*, 1973). At saturation about one molecule of receptor dexamethasone complex is bound per 10^5 nucleotide pairs in the nuclear DNA. Thus, either a very marked sequence specificity is required for the interaction of the receptor with the DNA or most of the potential DNA binding sites are masked by chromosomal proteins (King and Gordon, 1972).

Although an active participation of the chromosomal protein in binding of the receptor–steroid complex to the nucleus cannot be ruled out, the intracellular distribution of the receptor as well as the *in vitro* nuclear binding experiments can be sufficiently explained in terms of the affinity of the receptor–steroid complex for a set of DNA binding sites showing limited availability.

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Reaction of Bovine and Ovine Pituitary Growth Hormones with Tetranitromethane†

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ABSTRACT: Bovine and ovine pituitary growth hormones, two structurally similar proteins, were allowed to react with tetranitromethane at 0°. Data obtained from studies of the relative rates of trypsin digestion, elution volumes from exclusion chromatography, circular dichroism spectra, and biological activities indicate close structural similarity of the two modified hormones, both in relationship to their parent compounds, and to each other. Both proteins were shown to have two completely modified, two partially modified, and two unmodified tyrosines at identical sites along the chain. It was also demonstrated that all six tyrosine residues in the bovine hormone are to some extent accessible to the nitrating agent at 25°. The nitrated bovine hormone was also reduced to its amino derivative which, on limited investigation, appears to have similar properties to the native and nitrated proteins.

Comparison of the reported sequences of bovine (Fernández *et al.*, 1972) and ovine (Li *et al.*, 1972) pituitary growth hormones shows a very high degree of homology between these two proteins. In addition, studies of their respective states of aggregation (Dellacha *et al.*, 1968; Bewley and Li, 1972), circular dichroism (Edelhoch and Lippoldt, 1970; Bewley and Li, 1972), and immunological and biological responses (Papkoff and Li, 1958; Moudgal and Li, 1961; Hayashida and Li, 1959) are indicative of a similar molecular architecture. Sedimentation and exclusion chromatographic studies (Dellacha *et al.*, 1968) have shown that BGH[1] exists in slightly basic solutions as a dimer, whereas dissociation to a monomeric form occurs under acidic conditions in low ionic strength media. Osmotic pressure and exclusion chromatography measurements have confirmed this finding for BGH and extended it to SGH (Bewley and Li, 1972). A helix content of 40–50% was found under several conditions (Edelhoch and Lippoldt, 1970; Bewley and Li, 1972). Additional studies performed on BGH have indicated that a molecular transition accompanied by increased unfolding occurs on acidification from pH 5 to 2 (Burger *et al.*, 1966). The effect of urea on the conformation of BGH was studied and several new molecular forms were inferred, based on data from fluorescence, polarization of fluorescence, and ultraviolet difference spectroscopy (Edelhoch and Burger, 1966). In the latter study, optical rotary dispersion indicated that the helical regions remain largely stable, even under conditions leading to considerable disruption of tertiary structure. This may account for the reported stability of hormonal activity under conditions of strong alkali, acid, and heat (Li and Papkoff, 1953; Ellis *et al.*, 1956).

The relationship between the chemical structure of a protein and its biological activity has undergone increased chemical probing in recent years with the advent of milder and more specific reagents for the modification of side-chain residues (Glazer, 1970; Riordan and Sokolovsky, 1971). One of the most successfully employed procedures has been the nitration of tyrosine residues with tetranitromethane (Sokolovsky *et al.*, 1966). Details of this selective chemical reaction on BGH and SGH and the subsequent functional and structural studies of the modified proteins are the subject of this report. Nitrotyrosyl-BGH, prepared at 0°, was also reduced to the corresponding aminotyrosyl derivative and this product was investigated by circular dichroism and bioassay.

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[1] Abbreviations used are: BGH, bovine pituitary growth hormone; SGH, ovine pituitary growth hormone; nitrotyrosyl-BGH, the product formed on reaction of BGH with tetranitromethane; nitrotyrosyl-SGH, the product formed on reaction of SGH with tetranitromethane; aminotyrosyl-BGH, the product formed on reduction of nitrotyrosyl-BGH with sodium hydrosulfite to its amino derivative; $C(NO_2)_4$, tetranitromethane; CD, circular dichroism.