Glucocorticoid Receptors in Mouse Pituitary Tumor Cells. II. Nuclear Binding†

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ABSTRACT: The binding of tritiated triamcinolone acetonide to receptors in the cytosol and nuclei of AtT-20 mouse pituitary tumor cells grown in tissue culture has been examined using whole cells or cell fractions to characterize the receptors and determine their interrelationship. When intact cells were incubated with triamcinolone acetonide at 37°, the hormone was found predominantly in the nucleus after initial binding in the cytoplasm. The nuclear receptor complex appeared to be bound to chromatin and was readily solubilized in 0.4 M KCl. The appearance of the nuclear receptor complex was associated with a loss of cytosol-binding capacity. Isolated nuclei bound the steroid hormone following incubation at 20° with cytosol containing bound triamcinolone acetonide. This nuclear uptake process appeared to be hormone specific and temperature dependent, and required the presence of the cytosol hormone–receptor complex. The nuclear complex had sedimentation coefficients of 5.0 S in 0.01 M KCl and 3.8 S in 0.4 M KCl, values comparable to those displayed by the cytosol receptor. Like the cytosol complex, it was destroyed by Pronase, *N*-ethylmaleimide, and heat treatment, but resistant to ribonuclease and deoxyribonuclease. These results suggested a temperature-dependent transfer of hormone–receptor complex from cytosol to nucleus in these pituitary cells.

According to current concepts, a major component in the mechanism of steroid hormone action involves an interaction with the genome resulting in an alteration of transcription or RNA synthesis within the nucleus. Steroid hormones are bound to chromatin proteins and/or DNA after initial binding to cytoplasmic receptors in target tissues (Jensen and De Sombre, 1972).

In the case of glucocorticoid hormones, cytoplasmic and nuclear receptors have been described in several systems, and binding of glucocorticoids or glucocorticoid–receptor complexes to chromatin (Sluyser, 1966; Dastague *et al.*, 1971) and DNA (Baxter *et al.*, 1972) in the nuclei has been reported. These hormones have stimulatory and inhibitory effects on macromolecular synthesis and metabolism in organs in which they have anabolic (liver) and catabolic (lymphoid tissue, skin, bone, muscle, adipose tissue) actions, respectively. Thus, in organs such as the liver in which they induce enzymes involved in gluconeogenesis, they increase RNA synthesis (Feigelson *et al.*, 1962), RNA polymerase activity (Barnabei *et al.*, 1966), and chromatin template activity (Dahmus and Bonner, 1965). In glucocorticoid-inhibited systems, in contrast, an inhibition of RNA polymerase activity (Nakagawa and White, 1966), synthesis of protein and nucleic acids (Makman *et al.*, 1966), cell growth (Gabourel and Aronow, 1962), and uptake of glucose and precursors of nucleic acid and protein into cells (Makman *et al.*, 1966; Peck *et al.*, 1969; Hallahan *et al.*, 1973) has been detected. Some of these effects may depend on new RNA and protein synthesis (Hallahan *et al.*, 1973). These studies indicating alteration of biochemical events within the nucleus are compatible with an action of glucocorticoid hormones on the genome.

We have been concerned with the inhibitory action of glucocorticoid hormones on the production of ACTH[1] by the pituitary, and have reported studies using a mouse pituitary tumor cell line grown in tissue culture, in which ACTH production is selectively suppressed by glucocorticoids without a significant effect on cell growth (Watanabe *et al.*, 1973a). Specific glucocorticoid receptors were found in the cytoplasm of these cells and shown to be proteins which bound natural and synthetic glucocorticoids with high affinity to a small number of saturable binding sites (Watanabe *et al.*, 1973b).

Since the inhibitory action of glucocorticoid hormones on ACTH production is likely to be exerted, at least in part, within the nucleus and to involve the glucocorticoid receptor, it was important to determine whether glucocorticoid hormones were taken up and retained in the nucleus of pituitary cells. In the present study, we describe results confirming the presence of glucocorticoid receptors in the nuclear fraction of AtT-20 mouse pituitary tumor cells and characterizing their relationship with cytosol binding sites and their interaction with chromatin.

Materials and Methods

Chemicals. [1,2,4-^{3}H]Triamcinolone acetonide[2] (10.7 Ci/mmol) and Ultra Pure sucrose were obtained from Schwarz/Mann, nonradioactive steroids and Norit A from Sigma, dextran 80 from Pharmacia, Triton X-100 from Beckman, Spectrafluor from Amersham/Searle, and AtT-20 cells from American Type Culture Collection.

Steroids. The methods for preparation of steroid solutions

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[1] Abbreviations used are: ACTH, adrenocorticotropic hormone; [^{3}H]triamcinolone acetonide, [1,2,4-^{3}H]triamcinolone acetonide.

[2] Trivial names used are: triamcinolone acetonide, 9α-fluoro-11β,-16α,17α,21-tetrahydroxypregna-1,4-diene-3,20-dione 16α,17α-acetonide; dexamethasone, 9α-fluoro-11β,17α,21-trihydroxy-16α-methylpregna-1,4-diene-3,20-dione.

and counting of radioactivity have been previously described (Watanabe *et al.*, 1973b).

Cell Incubation Studies. AtT-20 cells were grown in F10 medium as previously described (Watanabe *et al.*, 1973a). The cells were incubated at 37° with 1×10^{-8} M [^{3}H]triamcinolone acetonide, harvested, washed by centrifugation in phosphate-buffered saline, and homogenized lightly (Polytron PT-10, Brinkmann, rheostat setting 4) with three 5-sec bursts in buffer A (0.02 M *N*-tris(hydroxymethyl)methylglycine–2 mM $CaCl_2$–1 mM $MgCl_2$, pH 7.5). The homogenate was centrifuged at 800*g* for 10 min and the supernatant was centrifuged at 120,000*g* for 1 hr to obtain the cytoplasmic soluble fraction (cytosol). The 800*g* pellet was washed twice with buffer A containing 0.25 M sucrose and extracted first with buffer B (0.4 M KCl–0.01 M Tris-HCl–1 mM EDTA–0.1 M thioglycerol, pH 7.6) to obtain salt-extractable radioactivity and then with ethanol to determine total nuclear [^{3}H]triamcinolone acetonide.

Binding of [^{3}H]Triamcinolone Acetonide to Isolated Nuclei. Cells were homogenized in buffer C (0.05 M Tris-HCl–0.025 M KCl–0.002 M $MgCl_2$, pH 7.5) containing 0.5 M sucrose with a Teflon glass homogenizer and centrifuged at 5000*g* for 10 min. The pellet was resuspended with several strokes of the homogenizer in 1.75 M sucrose in buffer C and centrifuged at 25,000*g* for 10 min. The pellet was then resuspended in buffer C containing 0.5 M sucrose. Aliquots containing about 10^8 purified nuclei were incubated at 20°, usually for 1 hr, with an equal volume of cytosol (0.5–1 ml, prepared as outlined in previous paragraph) which had been preincubated with 2×10^{-8} M [^{3}H]triamcinolone acetonide at 0° to obtain hormone–receptor complex (Watanabe *et al.*, 1973b). Tubes containing cytosol preincubated with a 500-fold excess of nonradioactive dexamethasone in addition to [^{3}H]triamcinolone acetonide were used to determine nonspecific binding. Following incubation, the nuclei were recovered by centrifugation at 800*g* for 10 min, washed twice with buffer C containing 0.25 M sucrose, and then extracted with buffer B and ethanol to obtain a measure of bound and total [^{3}H]triamcinolone acetonide.

Extraction of Nuclei. To extract bound [^{3}H]triamcinolone acetonide from nuclei, the nuclei were suspended in buffer B, allowed to stand for 10 min at 0°, and centrifuged at 800*g* for 10 min. Separation of bound and free [^{3}H]triamcinolone acetonide in the nuclear extracts (supernatant) was achieved by adsorption of free steroid to dextran-coated charcoal, as described below. To determine total [^{3}H]triamcinolone acetonide in the nuclei, the nuclei were suspended in ethanol and centrifuged at 800*g* for 10 min, and the supernatant was counted for radioactivity.

Chromatin Isolation. Chromatin was isolated from purified nuclei and analyzed as described by Spelsberg (Spelsberg *et al.*, 1971). After centrifugation in 1.75 M sucrose (as described above), nuclei were resuspended in buffer C containing 0.2% Triton X-100, and centrifuged 5 min at 10,000*g*. Chromatin was then isolated and purified by successive homogenization and centrifugation in 0.05 M NaCl–0.02 M EDTA (pH 6.3) (three times) and 2 mM Tris-HCl–0.1 mM EDTA (pH 7.5) (twice).

Protein and DNA analyses of chromatin and nuclear pellets were performed by the methods of Lowry (Lowry *et al.*, 1951) and Burton (Burton, 1956), respectively. Histones were extracted from chromatin with 0.4 N H_2SO_4. Analysis of purified chromatin indicated ratios of protein:DNA of 1.72, histone:DNA of 1.23, and non-histone:DNA of 0.58.

Sucrose Density Gradient Centrifugation. Samples (0.3 ml) of cytosol or nuclear 0.4 M KCl extract were layered on 4.6-ml linear gradients of 5 to 20% sucrose in buffer D (0.01 M Tris-HCl–1 mM EDTA–0.01 M thioglycerol, pH 7.6) containing 0.01 or 0.4 M KCl, and centrifuged at 2° for 16 hr at 200,000*g*. Fifteen-drop fractions were collected and counted for radioactivity.

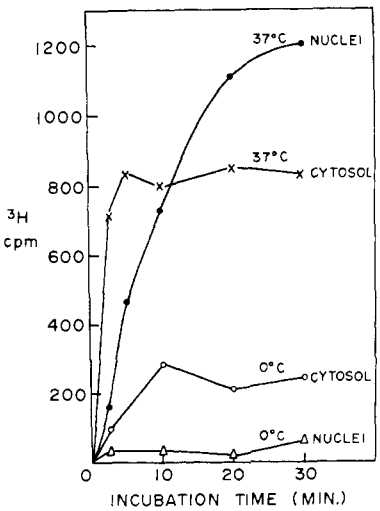

FIGURE 1: Uptake of triamcinolone acetonide into cytosol and nuclei at 37 and 0°. Cells were incubated at 37 or 0° with 1×10^{-8} M [^{3}H]triamcinolone acetonide in duplicate tubes with or without a 250-fold excess of nonradioactive dexamethasone to determine specific uptake of triamcinolone acetonide. Radioactivity in cytosol and ethanol extracts of nuclei was measured at various times after steroid addition.

Charcoal Binding Assays. To 0.4-ml aliquots of cytosol or nuclear 0.4 M KCl extract, 0.5 ml of a charcoal suspension containing 0.5% Norit A and 0.05% dextran 80 in buffer D was added. After 10 min of incubation at 0°, the samples were centrifuged at 600*g* for 10 min and the radioactivity in the supernatants was determined.

Results

Uptake into Cytosol and Nuclei. Uptake of [^{3}H]triamcinolone acetonide into nuclei was first examined by incubating cells at 37 and 0° with the steroid and comparing the kinetics and temperature requirements for uptake into cytosol and nuclei. An excess of nonradioactive dexamethasone was added to duplicate samples to compete for the limited specific binding sites. Specific uptake of triamcinolone acetonide was determined by subtracting the radioactivity in samples containing dexamethasone from the values obtained in duplicate samples without the competitor steroid. At 37° there was immediate uptake into the cytosol fraction followed by appearance of ^{3}H in the nuclear fraction (Figure 1). Cytosol uptake was maximal at about 5 min in this experiment whereas nuclear uptake continued to increase to 30 min and exceeded cytosol uptake. At 0°, there was limited uptake into the cytosol and essentially none into the nuclear fraction. These results suggested a transfer of hormone or hormone–receptor complex from cytosol to nuclei at 37° but not 0°.

Demonstration of Binding to Nuclear Receptor. That the [^{3}H]triamcinolone acetonide taken up into the nucleus was bound to a distinct macromolecular component was demonstrated by sucrose density gradient analysis. After incubation of cells with [^{3}H]triamcinolone acetonide, the nuclei were isolated and extracted with 0.4 M KCl, and aliquots of this nuclear extract were analyzed on sucrose density gradients. In cells

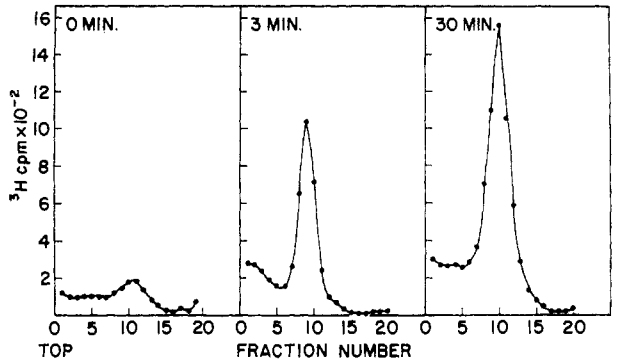

FIGURE 2: Sucrose density analysis of nuclear complexes. Cells were incubated for 3 and 30 min in the presence of 1.8×10^{-8} M [^{3}H]-triamcinolone acetonide. A control sample was left at 0° (0 min). Nuclei were extracted with 0.4 M KCl. Aliquots of the nuclear extracts were layered on sucrose gradients containing 0.4 M KCl, and centrifuged at 200,000g for 16 hr at 2°.

incubated at 0°, there was virtually no detectable binding in nuclei. A nuclear receptor–hormone peak was detectable, however, in cells incubated at 37° and was greater at 30 than at 3 min of incubation (Figure 2).

Depletion of Cytosol Receptor Capacity. Studies in other systems have illustrated a depletion of total receptor capacity in the cytosol as the hormone–receptor complex moves from the cytoplasm to the nucleus (Shyamala and Gorski, 1969; O'Malley *et al.*, 1971; Jensen *et al.*, 1968). To determine if this phenomenon occurred in AtT-20 cells, kinetic studies of receptor capacity were performed. Cells were incubated at 37° with [^{3}H]triamcinolone acetonide for varying times (0, 3, and 45 min). Aliquots of cytosol and nuclear extract prepared from these cells were further exposed for several hours to [^{3}H]-triamcinolone acetonide at 0° to ensure complete saturation of the total receptor sites. The samples were then analyzed by

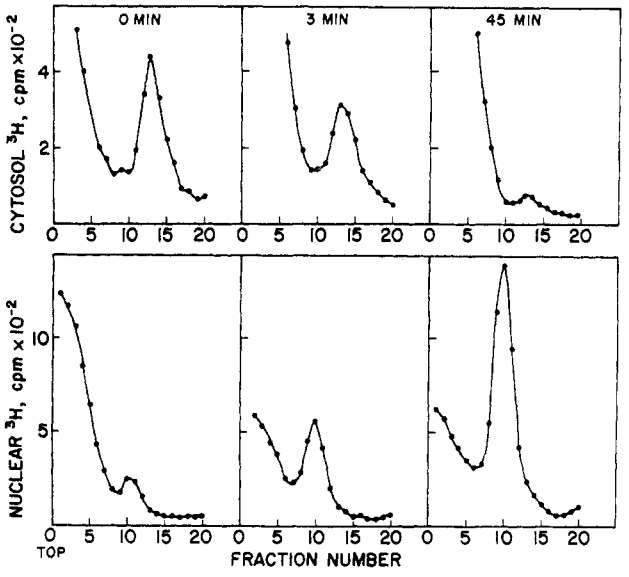

FIGURE 3: Nuclear and cytosol receptor capacities. Cells were incubated at 37° with 1×10^{-8} M [^{3}H]triamcinolone acetonide for 0, 3, and 45 min. Cytosol and nuclear (0.4 M KCl) extracts were prepared and incubated at 0° with 2×10^{-8} M [^{3}H]triamcinolone acetonide for 8 hr. Unbound steroid was removed by treatment with dextran-coated charcoal, and aliquots were layered on sucrose density gradients containing 0.01 or 0.4 M KCl, respectively.

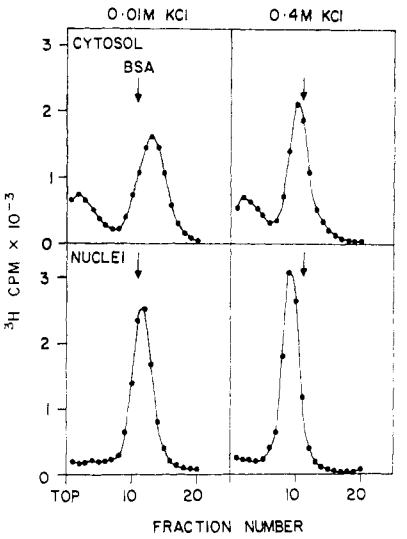

FIGURE 4: Effect of salt concentration on sedimentation of nuclear and cytosol complexes. Cells were incubated at 37° with 2×10^{-8} M [^{3}H]triamcinolone acetonide for 30 min, and aliquots of cytosol and 0.4 M KCl nuclear extract were layered on sucrose density gradients containing 0.01 or 0.4 M KCl. Bovine serum albumin was used as a marker (4.6 S, arrow).

sucrose density centrifugation to obtain a measure of total receptor capacity.

Cytosol of cells previously unexposed to triamcinolone acetonide bound [^{3}H]triamcinolone acetonide at 0°, but the nuclei of these cells contained essentially no binding activity for triamcinolone acetonide (Figure 3). In cells incubated with triamcinolone acetonide at 37°, the binding capacity of the cytosol rapidly declined, while the nuclear binding capacity increased with time of incubation. The simultaneous disappearance of cytosol receptor capacity and appearance of nuclear capacity was compatible with a migration of the hormone–receptor complex from the cytosol into the nucleus. Total receptor capacity (cytoplasmic plus nuclear) appeared to increase with time, but this was difficult to accurately quantitate in view of the lability of the cytoplasmic receptor in the absence of bound steroid (Watanabe *et al.*, 1973b).

Sedimentation Behavior of Nuclear Triamcinolone Acetonide–Receptor Complex. Physicochemical similarity of nuclear and cytosol complexes would be compatible with a migration of cytosol complexes to nuclear binding sites. The sedimentation characteristics of nuclear and cytosol hormone–receptor complexes formed in cells incubated at 37° with [^{3}H]triamcinolone acetonide were examined in high- and low-salt density gradients.

The cytosol complex had a sedimentation coefficient of about 5.4 S in 0.01 M KCl and 4.1 S in 0.4 M KCl (Figure 4), like the cytosol complex formed at 0° after incubation of isolated cytosol with [^{3}H]triamcinolone acetonide (Watanabe *et al.*, 1973b). The sedimentation coefficients of the nuclear complex in low and high salt were calculated to be 5.0 and 3.8 S, respectively. Thus, both nuclear and cytosol complexes appeared to be similarly affected by high-salt treatment, and the complexes were almost identical in sedimentation behavior.

Chemical Nature of Nuclear Receptor. The cytosol receptor for triamcinolone acetonide in these cells has been shown to be sensitive to heat, *N*-ethylmaleimide, and Pronase treatment (Watanabe *et al.*, 1973b). When nuclei or nuclear extract of cells labeled at 37° with [^{3}H]triamcinolone acetonide were incubated with Pronase or *N*-ethylmaleimide or heated to 60°

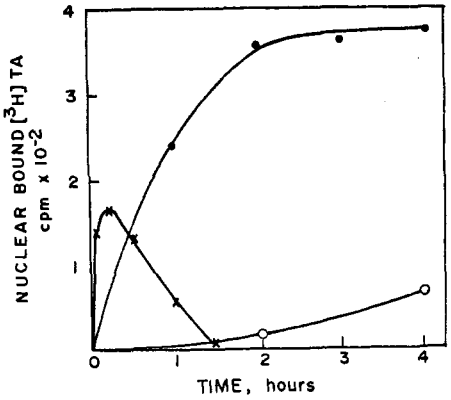

FIGURE 5: Effect of temperature on binding of triamcinolone acetonide to isolated nuclei. Isolated nuclei were incubated with [^{3}H]-triamcinolone acetonide–cytosol receptor preparation at 0° (○), 20° (●), or 37° (×) for various times. The samples were then chilled and the nuclei were recovered and extracted with 0.4 M KCl. Charcoal binding assays were performed on the nuclear extracts to measure bound ^{3}H.

prior to sucrose density gradient centrifugation, there was a loss of the peak of bound hormone seen in the control sample or in the sample treated with RNase or DNase (not shown). Thus, nuclear binding also appeared to be dependent on a protein moiety with functional sulfhydryl groups.

Binding of [^{3}H]Triamcinolone Acetonide to Isolated Nuclei. Though the intact cell experiments suggested transfer of triamcinolone acetonide–receptor complex from cytosol to nuclei, this was more directly demonstrated by reconstitution experiments in which cytosol, preincubated with [^{3}H]triamcinolone acetonide to obtain hormone–receptor complexes, was incubated with isolated nuclei and the amount of [^{3}H]-triamcinolone acetonide bound in the nuclei was determined. Nonspecific binding was measured by incubating nuclei with cytosol preincubated with [^{3}H]triamcinolone acetonide and a 500-fold excess of nonradioactive dexamethasone and subtracted from the total nuclear binding to determine specifically bound [^{3}H]triamcinolone acetonide.

Isolated nuclei incubated with [^{3}H]triamcinolone acetonide did not bind the steroid, but when cytosol preparation was added, triamcinolone acetonide was taken up and bound triamcinolone acetonide was recovered from the nuclei. The effect of temperature on the rate of uptake in nuclei was determined by incubating nuclei and [^{3}H]triamcinolone acetonide cytosol preparation at 0, 20, and 37° (Figure 5). At 0°, there was negligible bound triamcinolone acetonide in the nuclei after 4 hr of incubation. At 20°, the amount of bound [^{3}H]-triamcinolone acetonide in the nuclei increased with time to a maximum at 2 hr of incubation. At 37°, there was a more rapid initial rate of appearance of bound nuclear triamcinolone acetonide with a maximum at 15 min, followed by a rapid decline, probably due to heat denaturation or enzymatic degradation of the receptor. The cell-free nuclear uptake process was therefore time and temperature dependent.

The sedimentation coefficients of the nuclear complex extracted from isolated nuclei incubated at 20° with cytosol receptor complexes were 4.6 S in 0.01 M KCl and 3.8 S in 0.4 M KCl, values very similar to those of the nuclear complex generated in intact cells (Figure 6). The reconstitution conditions therefore appeared to be successful in producing a nuclear hormone–receptor complex analogous to that formed in whole cell incubations.

Hormone Specificity of Nuclear Binding. Reconstitution experiments were used to examine the hormone specificity of

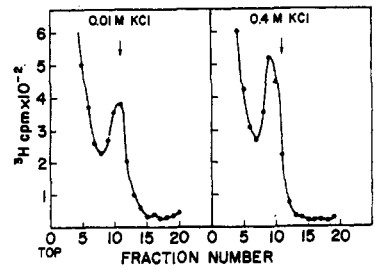

FIGURE 6: Sucrose density gradient analysis of [^{3}H]triamcinolone acetonide bound by isolated nuclei. Nuclei were incubated with [^{3}H]triamcinolone acetonide–cytosol receptor preparation at 20° as described in Methods and extracted with 0.4 M KCl. Aliquots (0.3 ml) of the extracts were layered on sucrose density gradients containing 0.01 or 0.4 M KCl. Bovine serum albumin (4.6 S, arrow) was used as a marker.

the nuclear uptake process. Cells were incubated at 37° with excess nonradioactive triamcinolone acetonide, dexamethasone, and testosterone to saturate the nuclear binding sites. These nuclei were then isolated and incubated with [^{3}H]-triamcinolone acetonide–cytosol receptor complex at 20°. Triamcinolone acetonide and dexamethasone reduced the amount of triamcinolone acetonide–receptor complex recovered in the nucleus, while testosterone had no inhibitory effect (Figure 7). Thus, the process of uptake of bound [^{3}H]triamcinolone acetonide into the nuclei was inhibited by glucocorticoids but not by non-glucocorticoid hormones such as testosterone.

Extraction of Bound Triamcinolone Acetonide from Chromatin. Studies in several systems have demonstrated that steroid–receptor complexes are bound to chromatin and/or DNA. When chromatin was prepared from cells incubated with [^{3}H]-triamcinolone acetonide, a triamcinolone acetonide–receptor complex could be extracted from the chromatin with 0.4 M KCl. The sedimentation coefficients of this complex on sucrose density gradient analysis appeared to be slightly smaller than

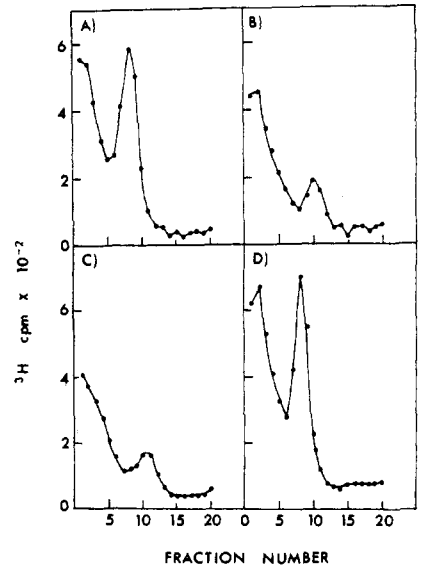

FIGURE 7: Hormone specificity of nuclear binding. Nuclei were isolated from cells incubated with no steroid (control, A) or with 2.5 × 10^{-7} M nonradioactive triamcinolone acetonide (B), dexamethasone (C), or testosterone (D) at 37° for 1 hr. These nuclei were then incubated with [^{3}H]triamcinolone acetonide–receptor preparation as described in Methods and extracted with 0.4 M KCl. Aliquots of nuclear extract were then applied to sucrose density gradients containing 0.4 M KCl.

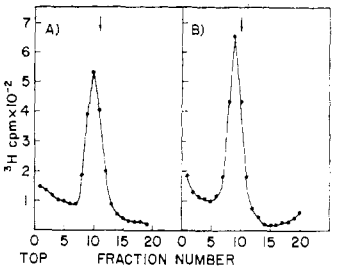

FIGURE 8: Sucrose density gradient centrifugation of triamcinolone acetonide–receptor complex extracted from chromatin. Chromatin was isolated from cells incubated with [^{3}H]triamcinolone acetonide at 37° for 45 min, purified, and extracted with 0.4 M KCl. Aliquots of the extract were layered on sucrose gradients containing 0.01M KCl (A) or 0.4 M KCl (B). The arrow indicates the position of migration of bovine serum albumin.

those of the cytosol or nuclear complexes previously described, being 4.2 S in 0.01 M KCl and 3.7 S in 0.4 M KCl (Figure 8).

Analysis of Bound Radioactivity. To determine the nature of the steroid bound after incubation of cells at 37° with [^{3}H]triamcinolone acetonide, the steroid was extracted with ethyl acetate from the medium, cytosol, and nuclear and chromatin extracts, and analyzed by thin-layer chromatography on silica gel F22 (Brinkmann Instruments) using the system ethyl acetate–chloroform 50:1. The recovery of triamcinolone acetonide was virtually 100% in each case and the radioactivity cochromatographed with the triamcinolone acetonide standard, indicating essentially no metabolism or degradation of triamcinolone acetonide during the incubation.

Discussion

These studies have demonstrated that triamcinolone acetonide is rapidly taken up into nuclei of AtT-20 pituitary cells and exists within the nuclei bound to a receptor protein. The term "nuclear receptor" has been used in this report to refer to this binding moiety located within the nucleus which has apparently arisen by migration from the cytoplasm. The receptor has been partially characterized and shown to resemble the cytosol receptor for this steroid in its requirement for sulfhydryl groups and protein integrity, and its resistance to RNase and DNase treatment. The sedimentation constants of the nuclear hormone complex were only slightly smaller than those of the cytosol complex in high and low salt. Whether there is a true difference between the sedimentation constants of the cytosol and nuclear complexes is uncertain since the apparent sedimentation may be affected by experimental conditions such as protein concentration, ionic environment, etc. Though the nuclear and cytosol complexes appeared very similar, nuclear binding activity was not due to cytoplasmic contamination or adsorption of steroid to the outer nuclear membrane. Nuclei treated with Triton X-100 to remove the outer nuclear membrane contained as much bound steroid as did untreated nuclei (unpublished observation).

The characteristics of nuclear uptake and binding of [^{3}H]-triamcinolone acetonide were examined in whole cells and isolated nuclei, and the relationship of nuclear and cytosol complexes was studied. Analyses of the effects of time and temperature on nuclear uptake and binding indicated that nuclear uptake followed cytosol uptake and occurred only at elevated temperatures. Higher levels of triamcinolone acetonide were taken up and bound in the nuclei than in the cytosol, though initially receptor activity was present only in the cytosol. The uptake and binding of [^{3}H]triamcinolone acetonide in the nuclei appeared to be a hormone-specific process. Within the nuclei, the steroid was localized to a large extent in chromatin.

It is generally believed that the nuclear hormone–receptor complex arises by a transfer of the cytosol complex to the nucleus rather than by binding of the hormone to a different or separate nuclear receptor. Evidence cited in support of this idea includes an obligatory binding of hormone with cytosol receptor prior to detection of nuclear binding, apparent physicochemical similarity of cytosol and nuclear receptors and depletion of cytosol receptor as nuclear binding increases in some systems (Shyamala and Gorski, 1969; O'Malley *et al.*, 1971; Jensen *et al.*, 1968). The sequential appearance of [^{3}H]-triamcinolone acetonide in cytosol and nuclei of AtT-20 cells, lack of binding of hormone to isolated nuclei in the absence of cytosol complex, decrease in cytosol binding capacity accompanying increase in nuclear receptor activity, and the similarity of nuclear and cytosol receptors in sedimentation behavior were all compatible with migration of the cytosol complexes into the nuclei.

It is likely that the receptors which we have identified play an essential role in the suppression of ACTH synthesis in pituitary cells by glucocorticoid hormones, and that a major part of their action is exerted within the nucleus where transcriptional events are probably affected as in other systems. An interesting possibility is that the glucocorticoid receptor, free or complexed with the steroid hormone, may act as a form of repressor for the gene which codes for ACTH synthesis. Further studies will be required to test this and other possible modes of action of glucocorticoid hormones and their receptors in this system.

References

Barnabei, O., Romano, B., Bitonto, G., and Tomasi, V. (1966), *Arch. Biochem. Biophys. 113*, 478.

Baxter, J. D., Rousseau, G. G., Benson, M. C., Garcea, R. L., Ito, J., and Tomkins, G. M. (1972), *Proc. Nat. Acad. Sci. U. S. 69*, 1892.

Burton, K. (1956), *Biochem. J. 62*, 315.

Dahmus, M. E., and Bonner, J. (1965), *Proc. Nat. Acad. Sci. U. S. 54*, 1370.

Dastague, B., Defer, N., and Kruh, J. (1971), *FEBS (Fed. Eur. Biochem. Soc.) Lett. 16*, 121.

Feigelson, M., Gross, P. R., and Feigelson, P. (1962), *Biochim. Biophys. Acta 55*, 495.

Gabourel, J. D., and Aronow, L. (1962), *J. Pharmacol. Exp. Ther. 136*, 213.

Hallahan, C., Young, D. A., and Munck, A. (1973), *J. Biol. Chem. 248*, 2922.

Jensen, E. V., and De Sombre, E. R. (1972), *Annu. Rev. Biochem. 41*, 203.

Jensen, E. V., Suzuki, T., Kawashima, T., Stumpf, W. E., Jungblut, P. W., and De Sombre, E. R. (1968), *Proc. Nat. Acad. Sci. U. S. 68*, 632.

Lowry, O. H., Rosebrough, N. J., Farr, A. L., and Randall, R. J. (1951), *J. Biol. Chem. 193*, 265.

Makman, H., Dvorkin, B., and White, A. (1966), *J. Biol. Chem. 243*, 1485.

Nakagawa, S., and White, A. (1966), *Proc. Nat. Acad. Sci. U. S. 55*, 900.

O'Malley, B. W., Toft, D. O., and Sherman, M. R. (1971), *J. Biol. Chem. 246*, 1117.

Peck, W. A., Messenger, K., Brandt, J., and Carpenter, J. (1969), *J. Biol. Chem. 244*, 4174.

Shyamala, G., and Gorski, J. (1969), *J. Biol. Chem. 244*, 1097.
Sluyser, M. (1966), *J. Mol. Biol. 19*, 591.
Spelsberg, T. C., Steggles, A. W., and O'Malley, B. W. (1971), *J. Biol. Chem. 246*, 4188.
Watanabe, H., Nicholson, W., and Orth, D. N. (1973a) *Endocrinology 93*, 411.
Watanabe, H., Orth, D. N., and Toft, D. O. (1973b), *J. Biol. Chem. 248*, 7625.

Inhibition of *in Vitro* Amino Acid Incorporation by Sodium Selenite†

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ABSTRACT: Nanomole quantities of sodium selenite completely inhibited amino acid incorporation in a system consisting of free polyribosomes and a 150,000*g* supernatant prepared from rat liver, fortified with cofactors. The 150,000*g* supernatant contained a factor required for maximum expression of the inhibitory effect. This factor could be replaced by thiols, *e.g.*, glutathione. Incorporation of leucine from L-[U-^{14}C]-leucyl-tRNA into protein by free polyribosomes and partly purified transferases was not at all affected by Na_2SeO_3, but 100% inhibition was obtained by Na_2SeO_3 in the additional presence of glutathione. A reaction product between Na_2SeO_3 and glutathione, which could be the selenotrisulfide derivative of glutathione (selenodiglutathione, GSSeSG), was found to be a very potent inhibitor of amino acid incorporation, abolishing the amino acid incorporation process beyond aminoacyl-tRNA formation.

Various hepatocarcinogens cause peroxidation of the unsaturated fatty acids of liver endoplasmic reticulum membranes (Rao and Recknagel, 1968; Jose and Slater, 1970). Lipoperoxidation could be involved in the acute toxic effects of the carcinogens on liver, and accompany liver carcinogenesis as a symptom of the metabolic production of free radicals from these carcinogens (Recknagel, 1967).

Seleno compounds have been shown to act as antioxidants and free-radical scavengers (Tappel, 1965). During experiments in which antioxidants were applied to counteract liver microsomal lipoperoxidation, we found that sodium selenite exerted a pronounced inhibition of amino acid incorporation by membrane-bound polyribosomes from rat liver *in vitro*. In order to exclude membrane effects, the inhibitory activity of selenite was studied on free polyribosomes, and the results are reported in the present paper. We were motivated to investigate this aspect of the biological action of selenite in view of the known toxicity of this and other seleno compounds (Rosenfeld and Beath, 1964; Harr and Muth, 1972), and the tumor growth inhibitory properties of some seleno compounds (Harr and Muth, 1972; Shapiro, 1972).

Materials and Methods

Chemicals. DL-[1-^{14}C]Leucine was obtained from the Radiochemical Centre, and L-[U-^{14}C]leucyl-tRNA from New England Nuclear Corp. Glutathione was purchased from Sigma, oxidized glutathione, phosphoenolpyruvate, and pyruvate kinase from Boehringer, sodium selenite from Merck, and Dowex 50W-X4 from Bio-Rad.

Preparation of Free Polyribosomes. Free polyribosomes were isolated according to the method of Bloemendal *et al.* (1967).

Preparation of the 150,000g Supernatant. Rat liver was homogenized in 2 ml of ice-cold buffer A per g (wet weight) of tissue. Buffer A consisted of 50 mM Tris-HCl (pH 7.6), 25 mM KCl, 10 mM $MgCl_2$, and 0.35 M sucrose. The homogenate was centrifuged at 15,000*g* for 10 min and the 15,000*g* supernatant was centrifuged for 1 hr at 150,000*g*. As indicated and described in the text, the 150,000*g* supernatant was submitted to gel filtration over Sephadex G-25.

Preparation of Aminoacyl Transferases. The method of Gasior and Moldave (1965) has been followed partially. The pH of the 150,000*g* supernatant was lowered to 5.2 by adding 1 N acetic acid. The resulting suspension was kept in an ice bath for 15 min and then centrifuged at 10,000*g* for 10 min. The remaining supernatant was passed through a column of Sephadex G-25 (1.7 × 30 cm), equilibrated and eluted with buffer A. The first peak, containing the transferases, was used in the amino acid incorporation experiments.

Preparation of Aminoacyl-tRNA. The method of Bloemendal *et al.* (1962) has been followed partially. Equal volumes of 105,000*g* rat liver supernatant and a phenol–cresol mixture as described by Kirby (1965) were mixed and shaken for 10 min at 4°. Following centrifugation for 20 min at 35,000*g* the two layers were separated and the phenol layer was extracted once with an equal volume of a 10^{-4} M EDTA (pH 4.5) solution. The water layers were combined and a twofold volume of 96% ethanol supplemented with 2% ammonium acetate was added. The suspension was kept at −20° for at least 2 hr and centrifuged. The RNA precipitate was dissolved in water and centrifuged and the RNA solution was precipitated once with ethanol. After dissolving the precipitate in water, the solution was applied to a column of Sephadex G-25. The first peak eluted contained aminoacyl-tRNA. The final solution was concentrated by lyophilization.

Incubation of [^{14}C]Leucine with Polyribosomes. The incubation mixture contained 0.5 μmol of ATP, 0.25 μmol of GTP, 5.0 μmol of phosphoenolpyruvate, 25 μg of pyruvate kinase, 0.175 μCi of DL-[1-^{14}C]leucine (sp act. 7 Ci/mol), 5

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