Evidence for Glucocorticoid Transport into AtT-20/D-1 Cells†

Robert Walker Harrison, III,*,‡ Sandra Fairfield, and David Nelson Orth§

ABSTRACT: Glucocorticoid uptake by AtT-20/D-1 mouse pituitary adenocarcinoma cells grown in tissue culture was examined. The binding of triamcinolone acetonide, a potent synthetic glucocorticoid, by intact cells and by cell cytosol was studied at both 4 and 25°. Specific binding of [^{3}H]triamcinolone acetonide by intact cells was markedly different from cell-free cytosol binding at 4°. Intact cells bound a relatively small amount of labeled steroid within 2 min, after which no further binding was observed. In contrast, the receptor in a cell-free cytosol preparation was capable of binding steroid progressively at 4°, indicating that the limited binding by intact cells was not a consequence of receptor characteristics. At 25°, uptake by intact cells and cytosol was nearly identical and appeared to be limited only by the binding kinetics of the cytosol receptor. Estradiol-17β, a nonglucocorticoid steroid, was not bound by the AtT-20/D-1 cell at 4°. Triamcinolone was not bound significantly at 4 or 25° by an adrenal carcinoma cell that does not appear to be a glucocorticoid target cell. An Arrhenius plot of cell steroid uptake vs. the reciprocal of absolute temperature revealed an abrupt change in slope at 16°, which is compatible with the temperature-dependent mechanism involved in glucocorticoid uptake being associated with lipid constituents of the cell membrane. These data suggest that glucocorticoid uptake by this target cell involves a mechanism of specific, temperature-dependent transport through the cell membrane.

Although it has generally been assumed that steroid hormones enter their target cells by passive diffusion (O'Malley, 1971), it has recently been suggested that steroid uptake by intact tissue is temperature dependent (Williams and Gorski, 1971). Since binding of the steroid by the cytosol[1] receptor under cell-free conditions is not prevented at 4° (Williams and Gorski, 1971), the nature of this temperature-dependent function in intact tissue binding has been unclear. We postulated that transport of the steroid into its target cell might be the early, temperature-dependent step. This hypothesis was tested using the functional, cloned, mouse pituitary tumor cell line, AtT-20/D-1 (Buonassisi et al., 1962). These cells secrete ACTH (Orth et al., 1973), the synthesis of which is inhibited by glucocorticoids (Watanabe et al., 1973a). These cells have been shown to contain glucocorticoid receptors (Watanabe et al., 1973b, 1974) and thus function in tissue culture as glucocorticoid-sensitive target cells.

The experiments reported here suggest that glucocorticoids are transported into the AtT-20 cell by a temperature-sensitive mechanism involving the cell membrane.

Materials and Methods

1,2,4-[^{3}H]Triamcinolone acetonide[2] (4 Ci/mmol) and uniformly labeled [^{3}H]valine (16 Ci/mmol) were purchased from Schwarz/Mann, [^{3}H]estradiol-17β from New England Nuclear, toluene from Baker, Spectrafluor-PPO-POPOP from Amersham-Searle, and Dextran 80 from Pharmacia. All other chemicals and unlabeled steroids were obtained from Sigma, unless otherwise noted.

Cell Culture. AtT-20/D-1 cells (American Type Culture Collection CCL-89) were grown in 9-l. spinner flasks containing Eagle's Minimal Essential Medium (Dulbecco's modification) (North American Biological), supplemented with 15% horse serum and 2.5% fetal calf serum (Grand Island Biological), under a 10% CO_2 atmosphere. Cells were allowed to settle undisturbed for 30 min and were harvested by gentle aspiration with a large bore pipette. Y-1 murine adrenocortical carcinoma cells (American Type Culture Collection CCL-79) were grown in roller bottle culture in Ham's F-10 Nutrient Medium (Grand Island Biological), supplemented with 15% horse serum and 2.5% fetal calf serum, under a 5% CO_2 atmosphere. Cells were harvested by gentle scraping with a rubber policeman.

Cell Incubations. Cells were diluted with fresh medium to a final concentration of 5×10^6/ml. Incubations were begun with the addition of 1.15×10^{-8} *M* [^{3}H]TA dissolved in ethanol. A 500-fold excess (5×10^{-6} *M*) of unlabeled dexamethasone, also in ethanol, was added to duplicate samples for determination of the displaceable binding, which represented more than 80% of total binding in most experiments. Incubations were terminated by pipetting 1-ml aliquots of the incubation mixtures into tubes containing 4 ml of ice-cold Tris-saline buffer (10 m*M* Tris–154 m*M* NaCl (pH 7.5)), which were immediately centrifuged for 5 min at 4° at 800*g*. The cell pellets were washed three times by resuspending them with vigorous vortexing in 2 ml of ice-cold Tris-saline and centrifuging them as before. The radioactivity retained in the pellets was then determined.

† From the Department of Medicine and Cancer Research Center, Vanderbilt University School of Medicine, Nashville, Tennessee 37232. *Received October 25, 1974.* Supported in part by National Institutes of Health National Cancer Institute Research Grant 5-ROI-CA 11685 and American Cancer Society Institutional Research Grant IN-25.

‡ Josiah Macy, Jr., Faculty Fellow.

§ Investigator of the Howard Hughes Medical Institute.

[1] Abbreviations used are: ACTH, adrenocorticotrophic hormone; [^{3}H]TA, 1,2,4-[^{3}H]triamcinolone acetonide; cytosol, cytoplasmic soluble fraction; PPO, 2,5-diphenyloxazole; POPOP, 1,4-bis[2-(5-phenyloxazolyl)]benzene.

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

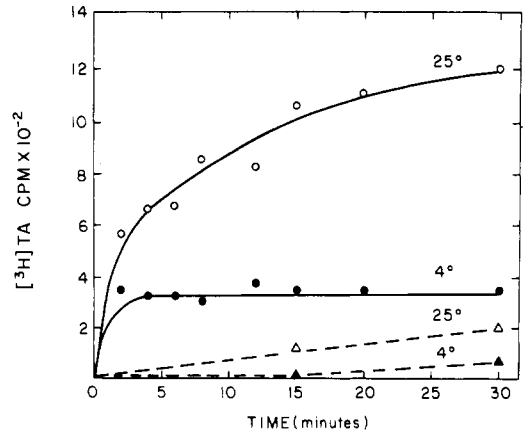

FIGURE 1: Uptake of [^{3}H]triamcinolone by intact cells. AtT-20/D-1 cells, shown by the circles, or Y-1 cells, shown by triangles, were incubated for various times at either 4 or 25°, as described under Materials and Methods, with [^{3}H]TA. Only "specific binding", defined as the difference in bound counts between duplicate tubes, one of which contained a 500-fold excess of unlabeled dexamethasone throughout the experiment, is shown in this and all subsequent figures.

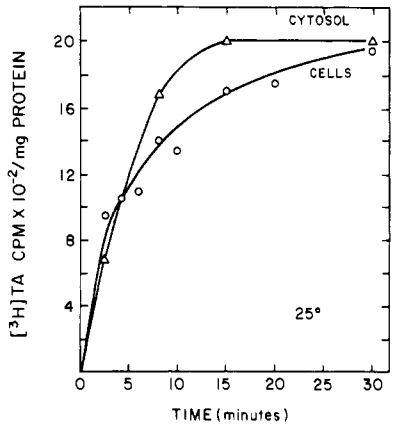

FIGURE 2: Binding of [^{3}H]triamcinolone by cytosol and intact cells at 25°. Intact AtT-20/D-1 cells or cytosol preparations were incubated at 25° with 11.5 × 10^{-9} *M* [^{3}H]TA for various time intervals. Specific binding of labeled steroid per mg of protein is plotted.

Cytosol Preparation and Incubations. Cells used for preparation of cytosol were harvested on ice and centrifuged immediately at 4° for 4 min at 600*g*. All subsequent steps were also carried out at 4°. The medium was aspirated, and the cell pellet was washed once with a small volume of Tris-saline. The washed cell pellet was homogenized in four volumes of 50 m*M* Tris-1 m*M* EDTA-10 m*M* thioglycerol (pH 7.5) buffer, using a Brinkman PT-10 Polytron, and cytosol was prepared as previously described (Harrison and Toft, 1973). Aliquots of cytosol were incubated with [^{3}H]TA (1.15 × 10^{-8} *M*). The amount of [^{3}H]TA that was protein bound was determined by a charcoal assay.

Charcoal Assay. This procedure was carried out at 4° and was based on ability of charcoal, in suspension, to adsorb steroid not bound to protein (Korenman, 1970). A 0.5-ml aliquot of charcoal suspension (0.5% Norit A, 10 m*M* Tris-HCl, and 1 m*M* EDTA (pH 7.5)) was added to 0.3-ml samples of previously labeled cytosol. Following a 10-min incubation, the mixture was centrifuged at 800*g* for 10 min and the radioactivity of the supernate, which contained the protein-bound steroid, was determined.

Radioactivity Determination. Radioactivity was determined by extracting the cell pellets with multiple aliquots of a scintillation fluid (total volume 9 ml) consisting of toluene and Spectrafluor-PPO-POPOP, 2366:151 (v/v). This simple procedure was found to be as effective as solubilization with NCS (Amersham-Searle). Samples thus extracted were counted in a Beckman Model LS-233 liquid scintillation spectrometer with an average efficiency for tritium of 55%. [^{3}H]TA bound by the receptor in cytosol experiments was determined by counting the supernate from charcoal assays in 10 ml of scintillation fluid.

Miscellaneous. Quantitative protein measurements were performed according to the method of Lowry et al. (1951), using bovine serum albumin as a standard. Cell pellets were solubilized in 0.1 *N* NaOH at 100° for 10 min in order to perform protein determinations; cytosol protein was assayed directly.

Results

Validation of Assay Procedures. In the experiments to be described, cells were washed following incubation at 4 or 25° by vigorous resuspension in Tris-saline buffer in order to remove adsorbed radioactivity. The wash procedure resulted in negligible loss of cells, since trichloroacetic acid precipitable, [^{3}H]valine-prelabeled cell protein after four washes was still 99% that of control. Cell viability was determined by the ability of trypsinized cells to exclude 0.025% nigrosin during a 10-min incubation at 25°. Cell viability after four washes was still 84% of control. The uptake of labeled TA by cells washed in this manner was directly proportional to cell number at both 4 and 25°, up to a cell concentration four times greater than that used in these experiments.

Effect of Temperature on Cell Uptake. When intact cells were incubated at 4° in media containing labeled triamcinolone (Figure 1), binding was maximal within 2 min, and there was no further uptake of steroid for as long as 30 min. When a similar incubation was performed at 25°, uptake was initially somewhat more rapid than that seen at 4° and by 30 min was approximately three times greater than seen at 4°. The binding shown in this and all subsequent figures represents "specific" binding, or that amount which was displaced by the addition of a 500-fold excess of unlabeled dexamethasone. This figure also shows the results of similar incubations with a presumed nontarget cell, the mouse adrenocortical carcinoma clonal cell line, Y-1. When Y-1 cells were incubated at 25°, a minimal amount of uptake was seen, the possible significance of which was not further investigated. Incubation at 4° resulted in no significant uptake. Incubation of AtT-20 cells with labeled estradiol-17β resulted in no detectable uptake at either 4 or 25°. Incubation of [^{3}H]TA with the sera contained in the tissue culture medium resulted in no detectable binding to serum proteins.

Comparison of Glucocorticoid Binding by Intact Cells and Cytosol Preparations. It was possible that the striking temperature dependence observed for intact cell uptake in Figure 1 might have been the result of decreased binding activity of the cytosol receptor at 4°. Therefore, intact cell uptake and cytosol binding were compared at both 4 and 25°. As shown in Figure 2, uptake by intact cells and cytosol at 25° was almost identical, suggesting that access of steroid to the cytosol receptor is unhindered at this temperature and that it is the kinetics of the steroid–receptor binding which limits cell uptake. In similar experiments performed at 4°, cell-free binding occurred in a progressive fashion over the 30-min period of study (Figure 3). This was in marked contrast to the results with intact cells incu-

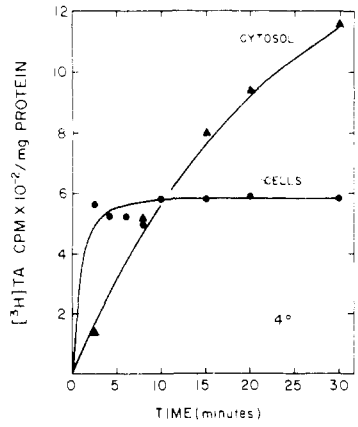

FIGURE 3: Binding of [^{3}H]triamcinolone by cytosol and intact cells at 4°. Intact AtT-20/D-1 cells or cytosol preparations were incubated at 4° with 11.5 × 10^{-9} *M* [^{3}H]TA for various time intervals. Specific binding of [^{3}H]TA per mg of protein is plotted.

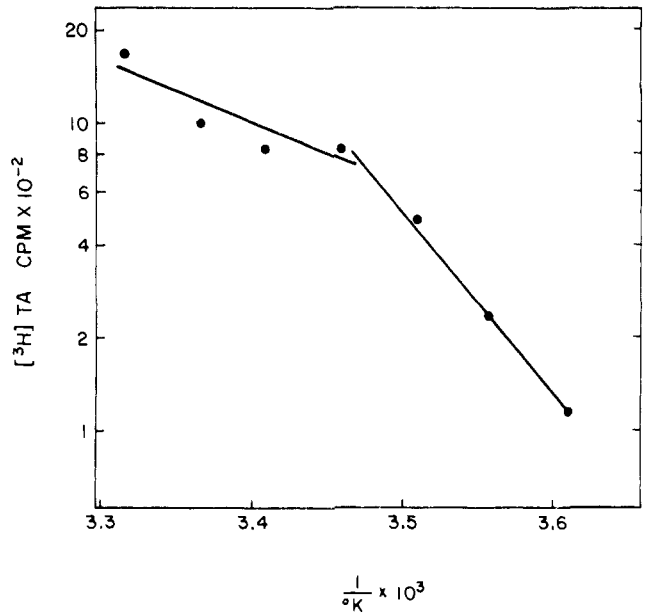

FIGURE 4: Arrhenius plot of intact cell uptake vs. temperature. Cells were incubated with [^{3}H]TA at 4° intervals from 4 to 28° for 30 min. Specific binding is plotted vs. the reciprocal of the incubation temperature in degrees Kelvin. The lower curve (4-16°) is expressed by the equation $y = 26 - 56x$ ($r = -0.98$), and the upper curve (16-28°) by the equation $y = 14.2 - 0.22x$ ($r = -0.80$). The slopes of the two curves are significantly different ($p < 0.006$).

bated at 4°, in which initial uptake was more rapid, but was complete by 2 min.

Arrhenius Plot of Steroid Uptake vs. Temperature. Uptake of [^{3}H]triamcinolone acetonide was measured at temperature intervals from 4 to 28°. The relationship of specific [^{3}H]TA binding to the reciprocal of degrees Kelvin (Figure 4) demonstrates that the energy of activation (West, 1963) of the binding reaction was abruptly lowered when the reaction temperature was raised above approximately 16°.

Discussion

The AtT-20/D-1 clonal cell line has retained certain differentiated pituitary cell functions, despite the fact that it is a tumor cell and has been carried in continuous culture for 18 years. The advantages of studying steroid-cell interactions in this homogeneous clonal glucocorticoid target cell line, rather than in the heterogeneous populations of cells which constitute whole tissues, are obvious.

A temperature-dependent phenomenon has been observed to be involved in the uptake of steroids by rat uterus (Williams and Gorski, 1971), chick oviduct (R. W. Harrison, unpublished observations), and, in the present study, the AtT-20/D-1 pituitary cell, but has not been observed in HTC cells (Rousseau et al., 1973). There is no ready explanation for the apparent absence of this phenomenon in HTC cells, but one possibility is that the differentiated function of steroid membrane transport has been lost during the process of establishing the HTC cell as a continuous tissue culture line.

It has generally been assumed that steroids diffuse passively into cells and that, in their target cells, they are concentrated by binding to a specific cytosol receptor protein (O'Malley, 1971). Thus, our suggestion that steroids may be transported across the cell membrane by a specific, temperature-dependent process deserves careful scrutiny. This report describes the temperature-dependent binding of glucocorticoids to specific sites on or in the AtT-20/D-1 cell. Further characterization of the 4° binding component and comparisons with the cytosol receptor will be presented in a subsequent paper.[3]

The first question is whether or not the labeled glucocorticoid is bound to an intrinsic cell component or to an adsorbed serum protein, such as corticosteroid-binding globulin. Although it is known that triamcinolone acetonide is not bound significantly by corticosteroid-binding globulin (Florini and Buyske, 1961), and although the cells were washed extensively, the ability of fetal calf serum and horse serum to bind [^{3}H]TA was specifically tested, and none was detected.

Another possible explanation for these findings might be found in the relative solubilities of steroids in the lipid constituents of the AtT-20/D-1 cell membrane at 4 and at 25°. Since the lipid constituents of the membrane are thought to be generally similar from one cell to another (Law and Snyder, 1972), we examined the uptake of labeled TA in another murine cell which we anticipated might not be a glucocorticoid target cell, namely, a cell that synthesizes glucocorticoids. The mouse adrenocortical carcinoma cell, Y-1, exhibited a minimal capacity for taking up labeled TA at 25° and bound virtually no steroid at 4°, indicating that the phenomenon we have observed is not characteristic of all cells.

The glucocorticoid binding reported here is specific, in the sense that only those [^{3}H]TA counts displaced by an excess of unlabeled dexamethasone have been considered. Furthermore, the AtT-20/D-1 cells failed to bind significant quantities of labeled estradiol, a nonglucocorticoid steroid hormone. The saturability and high binding affinity of the specific glucocorticoid binding sites of the AtT-20/D-1 cell will be discussed elsewhere.[3]

It might be argued that the differences in the uptake of [^{3}H]TA by intact cells at 4 and 25° simply reflected effects of temperature on the binding characteristics of the cytosol receptor that has been described in the AtT-20/D-1 cell (Watanabe et al., 1973b). However, when cytosol preparations were incubated with labeled TA at both temperatures and compared with intact cell uptake, the cytosol receptor bound steroid progressively for 30 min at both 25 and 4°.

[3] Submitted.

Thus, an intrinsic characteristic of the cytosol receptor would not appear to explain the temperature dependence of steroid uptake by intact cells.

Finally, there is the question of the subcellular localization of the 4° binding component. The unusual biphasic nature of the temperature dependence of membrane-associated biochemical events was first observed in regard to the effects of membrane lipid composition on galactoside transport in *Escherichia coli* (Schairer and Overath, 1969; Wilson and Fox, 1971). It appeared that the transition temperature in the Arrhenius plot was related to fluidization of lipid components in the bacterial cell membrane (Wilson and Fox, 1971). This phenomenon has since been shown to occur in animal cell membranes as well (Inesi et al., 1973), and studies on (Na^+K^+)ATPase have confirmed the lipid dependency of this phenomenon (Grisham and Barnett, 1973; Kimelberg and Papahadjopoulos, 1974). The biphasic temperature dependence of the phenomenon observed in the AtT-20/D-1 cell suggests that the 4° steroid binding mechanism is associated with a membrane component of the cell. The observation that access to the cytosol receptor and total cell uptake are limited when binding to the cell is limited by lowering the temperature to 4° suggests that binding at 4° is to a component of the cell surface membrane. Further evidence in support of this conclusion is presented in another paper (Harrison et al., 1974).

Although the possibility that steroids are transported into their target cells has been considered by other investigators (Milgrom et al., 1973), the demonstration of temperature-dependent glucocorticoid uptake in this homogeneous target cell model system provides the clearest evidence to date in support of such a mechanism. The generality of the phenomenon of steroid transport is as yet uncertain, but the possible existence of a discriminant step prior to cytosol receptor–steroid interaction has considerable biological significance and may represent an important conceptual link in our evolving understanding of the mechanism of steroid hormone action.

Acknowledgments

We thank Drs. Grant W. Liddle and Robert L. Post for their many useful suggestions during this study, George Frater who supplied the AtT-20/D-1 cells, and Ms. Nancy Schultz who assisted in the preparation of this manuscript.

References

Buonassisi, V., Sato, G., and Cohen, A. I. (1962), *Proc. Natl. Acad. Sci. U.S.A. 48,* 1184–1190.

Florini, J. R., and Buyske, D. A. (1961), *J. Biol. Chem. 236,* 247–251.

Grisham, C. M., and Barnett, R. W. (1973), *Biochemistry 12,* 2635–2637.

Harrison, R. W., Fairfield, S., and Orth, D. N. (1974), *Biochem. Biophys. Res. Commun. 61,* 1262–1267.

Harrison, R. W., and Toft, D. O. (1973), *Biochem. Biophys. Res. Commun. 55,* 857–863.

Inesi, G., Millman, M., and Eletr, S. (1973), *J. Mol. Biol. 81,* 483–504.

Kimelberg, H. K., and Papahadjopoulos, D. (1974), *J. Biol. Chem. 249,* 1071–1080.

Korenman, S. G. (1970), *Endocrinology 87,* 1119–1123.

Law, J. H., and Snyder, W. R. (1972), *in* Membrane Molecular Biology, Fox, C. F., and Keith, A., Ed., Stamford, Conn., Sinauer Associates, pp 3–26.

Lowry, O. H., Rosebrough, N. J., Farr, A. L., and Randall, R. J. (1951), *J. Biol. Chem. 193,* 265–275.

Milgrom, E., Atger, M., and Baulieu, E. E. (1973), *Biochim. Biophys. Acta 320,* 267–283.

O'Malley, B. W. (1971), *Metabolism 20,* 981–988.

Orth, D. N., Nicholson, W. E., Mitchell, W. M., Island, D. P., Shapiro, M., and Byyny, R. L. (1973), *Endocrinology 92,* 385–393.

Rousseau, G. G., Baxter, J. D., Higgins, S. J., and Tomkins, G. M. (1973), *J. Mol. Biol. 79,* 539–554.

Schairer, H. U., and Overath, P. O. (1969), *J. Mol. Biol. 44,* 209–214.

Watanabe, H., Nicholson, W. E., and Orth, D. N. (1973a), *Endocrinology 93,* 411–416.

Watanabe, H., Orth, D. N., and Toft, D. O. (1973b), *J. Biol. Chem. 248,* 7625–7630.

Watanabe, H., Orth, D. N., and Toft, D. O. (1974), *Biochemistry 13,* 332–337.

West, E. S. (1963), *in* Textbook of Biophysical Chemistry, West, E. S. Ed., New York, N.Y., MacMillan, pp 311–312.

Williams, D., and Gorski, J. (1971), *Biochem. Biophys. Res. Commun. 45,* 258–264.

Wilson, G., and Fox, C. F. (1971), *J. Mol. Biol. 55,* 49–60.