THE OUTWARD TRANSPORT OF CORTISOL
BY MAMMALIAN CELLS IN VITRO

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ABSTRACT

It has been determined that cortisol and a few other steroids are transported outward from certain mammalian cells growing in vitro. The extrusion process is temperature dependent, glucose dependent, saturable, and operates for only a few selected steroids. Many, but not all, steroids are able to block the extrusion process but are not themselves transported. The outward transport process for steroids has been found in mouse fibroblasts, mouse lymphoma cells, and functional mouse adrenal gland tumor cells growing in vitro. The transport process is not present in two varieties of cells cultured from human sources—HeLa or diploid fibroblasts, WI-38.

The rate of replication of mouse fibroblasts L-929 growing in vitro is inhibited by low concentrations of glucocorticoids in the growth medium (1, 2). In the course of experiments designed to locate intracellular binding components for cortisol in these cells, we found that the amount of cortisol present inside the cell, which was normally quite low, could be increased by lowering the temperature, by increasing the external cortisol concentration, or by depriving the cells of glucose under certain conditions. A recent report from this laboratory (3) postulated that cortisol was actively extruded from fibroblasts. In this communication, we present additional data in support of the hypothesis that there is an energy-dependent outward transport (4, 5) of cortisol from mouse fibroblasts and from mouse adrenal gland cells in tissue culture.

Previous studies on uptake of materials by mammalian cells in culture have established the occurrence of transport of certain sugars (6), amino acids (7), nucleosides (8), and the folic acid analogue methotrexate (9). Only uptake of amino acids and sugars has been studied in strain L-929 cells (10, 11). Although exit components have been described for the aforementioned substances (8, 9, 12) and characterized as temperature dependent, saturable, and stereospecific, they are, as yet, poorly delineated. There is, to our knowledge, no previous report of carrier-mediated transport of a steroid molecule by mammalian cells.

EXPERIMENTAL PROCEDURE

Materials

Cortisol-1,2-^{3}H, 32 c/mmole; coricosterone-1,2-^{3}H, 39 c/mmole; cortisone-1,2-^{3}H, 35 c/mmole; and testosterone-1,2-^{3}H, 44 c/mmole were obtained from Amersham/Searle Corp. Des Plaines, Ill.[1] Choles-

[1] The trivial names for steroids used are: dehydroepiandrosterone, 3β-hydroxyandrost-5-ene-17-one; Reichstein's S, 17α,21-dihydroxypregn-4-ene-3,20-dione; dexamethasone, 9α-fluoro-16α-methyl-11β,17α,21-trihydroxypregna-1,4-diene-3,20-dione; prednisolone, 11β,17α,21-trihydroxy-pregna-1,4-diene-3,20-dione; triamcinolone acetonide, 9α-fluoro-11β,16α,17α,21-tetrahydroxy-pregna-1,4-diene-3,20-dione-16,17-acetonide; fluocinolone acetonide, 6α,9α-difluoro-11β,16α,17α,21-tetrahydroxypregna-1,4-diene-3,20-dione-16,17-acetonide. N-ethylmaleimide is abbreviated NEM.

terol-1,2-³H, 53 c/mmole; deoxycorticosterone-1,2-³H, 41 c/mmole; dehydroepiandrosterone-7-³H, 10 c/mmole; estradiol-17β-6,7-³H, 48 c/mmole; Reichstein's S-1,2-³H, 5.2 c/mmole; progesterone-1,2-³H, 55 c/mmole; sucrose-¹⁴C(U), 4.9 mc/mmole; and tritiated water were bought from New England Nuclear Corp. Boston, Mass. Dexamethasone-1,2,4-³H, 4 c/mmole; prednisolone-1,2,4-³H, 1 c/mmole; and triamcinolone acetonide-1,2,4-³H, 4.3 c/mmole were obtained from Schwarz Bio Research Inc., Orangeburg, N. Y. Fluocinolone acetonide-³H, 53 mc/mmole, was a gift of Dr. Ralph Dorfman of the Syntex Laboratories, Inc., Palo Alto, Calif. Nonradioactive-labeled steroids were obtained from commercial sources. The cyclic 3',5'-monophosphate of adenosine was bought from Boehringer Mannheim Corp., New York.

Cell Culture

Mouse fibroblasts, L-strain, and HeLa cells were grown in Joklik medium (Schwarz Bio Research) supplemented with 10% bovine serum as described previously (1). Mouse lymphoma cells, ML-388, (originally obtained from Dr. Robert A. Roosa) were grown on Eagle's minimal medium (Grand Island Biological Co., Grand Island, N.Y.) supplemented with serine (10^{-3} M), pyruvate (10^{-4} M), and 6% calf serum. Human diploid fibroblasts, WI-38, were obtained from Dr. Leonard Hayflick at the 26th passage and were maintained as previously described (13). Mouse adrenal tumor cells, Y-1290S3, were a gift of Dr. Bernard Schimmer and Dr. Gordon Sato of Brandeis University and were grown in Nutrient Mixture F-10 (Grand Island Biological Co.) supplemented with 15% horse serum and 2.5% fetal calf serum (14). All cells were grown as monolayer cultures in Roux bottles containing 70 ml of medium in an atmosphere of 5% carbon dioxide in air.

Incubation of Cells with Steroid

Cells were harvested by scraping with a rubber policeman in growth medium, collected by centrifugation at 600 g, and resuspended either in fresh culture medium or in Earle's salt solution (pH 7.2) at a cell density approximating 3 × 10⁶ cells/ml. Sucrose-¹⁴C (New England Nuclear) 4.86 mc/mmole was added to the cell suspension at a final concentration of approximately 10^{-5} M. Inulin-¹⁴C was used in place of sucrose-¹⁴C in a few experiments. Replicate aliquots of the cell suspension were distributed into stoppered flasks with an atmosphere of 5% CO_2 in air. Cortisol-³H (or another steroid) was added to some cultures to give a final concentration of 1.0–3.3 × 10^{-8} M, while replicate cultures received the same amount of nonradioactive cortisol and water-³H, 1.1 μc/ml. Cells were incubated with mild shaking either in an ice bath or at 37°. At appropriate

intervals, aliquots were removed, and the cell suspension was centrifuged at 500 g for 5 min at the same temperature at which they were incubated. The supernatant solution was carefully removed with a fine-tipped pipette and set aside. The unwashed cell pellet (usually about 0.1–0.2 ml) was suspended directly in 3 ml of distilled water, and the resulting suspension was sonicated for 5 sec with a Bronwill Biosonik III (Bronwill Scientific, Rochester, N. Y.) at a setting of 30 so that there was complete cell disruption. Duplicate 1 ml samples were added to 10 ml of scintillation solution prepared according to the method of Bray (15). Protein assays were performed on 0.5 ml samples of the sonicated cell suspension after the method of Oyama and Eagle (16). 0.05 ml aliquots of the incubation medium (i.e., the supernatant obtained after centrifugation at 500 g) were added to 10 ml of scintillator fluid, as were separate samples of the radioactive labeled cortisol, sucrose, and water.

Radioactive Assay

All radioactive assays were carried out in a Packard Tri-Carb liquid scintillation spectrometer, Model 3310, (Packard Instrument Co., Downers Grove, Ill.), with the channels set so that one channel counted ¹⁴C exclusively. The lower energy channel, the one used for assaying tritium, also detected carbon[14], and the ratio of ¹⁴C counts appearing in the two channels was determined in each experiment with the sucrose-¹⁴C sample only. These determinations permitted a correction of the apparent tritium count in the lower energy channel so that, after correction for the small amount of extracellular fluid trapped in the cell pellet[2], the absolute amount of cortisol-³H associated with the cell sample was known. Similar corrections were applied to the water-³H counts, thereby allowing a calculation of the intracellular water space. The actual cortisol-³H counts associated with the cells were divided by the cortisol-³H count/ml of medium. This calculation yields an apparent volume of distribution for the cell-associated cortisol-³H. The results are expressed as apparent volumes of distribution/μg of cellular protein nitrogen.

Paper Chromatography

Descending paper chromatography was performed as described by Neher (17) as modified from Zaf-

[2] The sucrose-¹⁴C count in the cell pellet was multiplied by the ratio of cortisol tritium counts over the ¹⁴carbon sucrose counts in the incubation medium to determine the amount of extracellular cortisol-³H present in the resuspended cell pellet. This cortisol-³H contributed by medium was subtracted from the total tritium counts to yield the amount of cortisol-³H associated with the cells themselves.

faroni (18). The following formamide-saturated solvent systems were employed with formamide-impregnated Whatman No. 1 (H. Reeve Angel and Co., Clifton, N.J.) chromatography paper: (a) chloroform, (b) benzene, (c) butyl acetate:water:ethyl acetate (15:5:15), and (d) n-butanol:n-butyl acetate:water (15:85:5). The chromatograms were allowed to run 30 cm, dried, and then cut into small squares. Each square piece was placed in a vial with 5 ml of scintillator solution and assayed for radioactivity in the scintillation spectrometer.

RESULTS

The apparent volume of distribution of tritium-labeled cortisol as a function of cortisol concentration is presented in Fig. 1. Nonradioactive cortisol
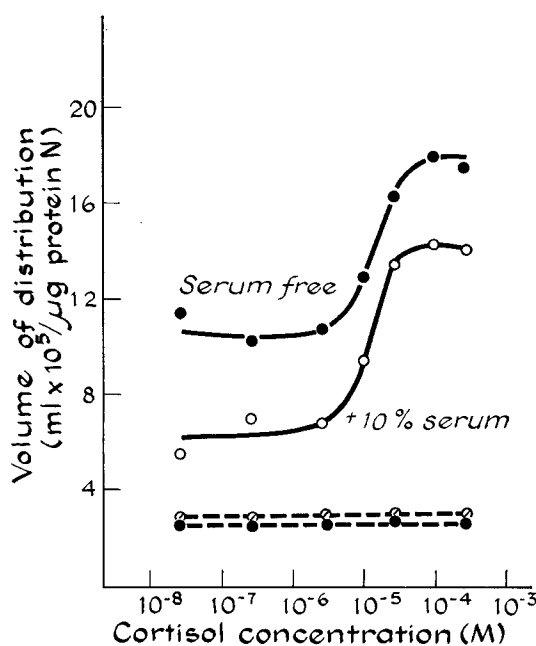

FIGURE 1 The effect of increasing concentrations of cortisol on the apparent volume of distribution of cortisol and water in L cells. Replicate suspensions of L cells in Earle's salt solution with or without supplementation with 10% bovine serum were incubated for 1 hr at 37°C with sucrose-^{14}C, 3.3 × 10^{-8} M cortisol-^{3}H or an equal μc amount of ^{3}H$_2$O, and increasing concentrations of nonradioactive cortisol. At the end of the incubation, the apparent volume of distribution of cortisol or water was determined for duplicate samples from each culture as described under Experimental Procedure. Volume of distribution of cortisol-^{3}H (●——●, serum-free medium; O——O, medium supplemented with 10% bovine serum); volume of distribution of ^{3}H$_2$O (●----●, serum-free; ⊘----,⊘ plus serum).

was added in various concentrations to replicate suspensions of mouse fibroblasts in Earle's saline either with or without 10% bovine serum. Each suspension contained the same amount of radioactivity from tritiated cortisol or tritiated water. As the concentration of cortisol in the medium rises, the apparent volume of distribution of tritium-labeled cortisol remains constant until 10^{-5} M and there sharply increases to values that are again constant at concentrations higher than 5 × 10^{-5} M. This increase is not the result of cell swelling, because the volume of distribution of tritiated water in the cell pellet remains constant. Similarly, cell rupture resulting in exposure of previously inaccessible binding sites could not have occurred. The apparent volume of distribution of cortisol at low concentration in cells suspended in the salt solution supplemented with 10% serum is somewhat higher than the water volume and lower than the apparent volume of distribution of cortisol in cells suspended in the salt solution without serum. An apparent volume of distribution higher than the water volume indicates binding phenomena of some sort, either within the cell or on the cell surface, or concentration of the steroid by the cells.

A determination of the true distribution ratio (steroid concentration in cell water/steroid concentration in extracellular water) would require a determination of the free intracellular cortisol concentration, and we were unsuccessful in attempts to measure this parameter. Simple division of the apparent volume of distribution of cortisol by the cellular water space gives an apparent distribution ratio, usually a number around 2–4 at low cortisol concentrations (cf. Figs. 1, 3), but of course this would include the amount present intracellularly in a bound form.

The addition of N-ethylmaleimide (NEM) to cells suspended in serum-containing medium re sults in a linear increase in the apparent volume of distribution of cortisol (Fig. 2) when the steroid is present at low concentration, 3 × 10^{-8} M. If after 30 min the cells are centrifuged and resuspended in medium without NEM, there is no decrease in the apparent volume of distribution of cortisol such as occurs very rapidly (see insert, Fig. 2) when control cells suspended in 10^{-4} M cortisol are resuspended in medium containing a low concentration (3 × 10^{-8} M) of radioactive cortisol.

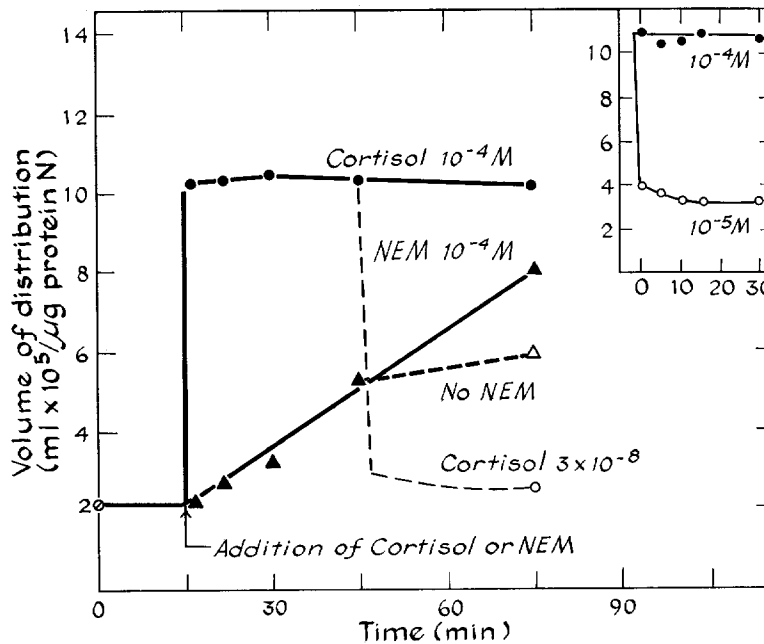As reported in a previous study (3), the apparent volume of distribution of cortisol in L cells

FIGURE 2   The effect of N-ethylmaleimide and a high concentration of cortisol on the apparent volume of distribution of cortisol in L cells. A suspension of L cells was prepared in Joklik medium supplemented with 10% bovine serum, and inulin-^{14}C and cortisol-^{3}H (3.3×10^{-8} M) were added. After 45 min of incubation at 37°, duplicate samples were obtained from the culture for the determination of the apparent volume of distribution of cortisol ($\varnothing$) as described under Experimental Procedure. At the time indicated by the arrow, the culture was divided into two equal parts, and cortisol (10^{-4} M) or N-ethylmaleimide (10^{-4} M) was added to the two cultures. The apparent volume of distribution of cortisol was determined on duplicate samples at various times thereafter. After an additional 30 min of incubation, part of each culture was centrifuged, and the pelleted cells were resuspended in incubation medium containing the same concentration of cortisol-^{3}H and inulin-^{14}C as the original incubation medium (used for the zero time determination). The volume of distribution of cortisol in these cultures was determined 30 min later ($\triangle$, N-ethylmaleimide culture; $\bigcirc$, high concentration of cortisol). A separate experiment (see insert) shows the time course of the change in the apparent volume of distribution of cortisol when L cells are removed from a high concentration of cortisol (10^{-4} M) and are placed in a lower one (10^{-5} M).

is temperature dependent. When L cells equilibrated with a low concentration of radioactive labeled cortisol at 20° are incubated in an ice bath, the apparent volume of distribution of cortisol rises (Fig. 3). The amount of cortisol associated with cells kept at 20° remains constant. If cells that have been incubated in an ice bath are placed at 20°, the apparent volume of distribution of cortisol is markedly reduced. Apparently all of the cortisol allowed into the cell on incubation in the cold is extruded upon raising the incubating temperature. The water volume of the cells is unaltered by the changes in temperature.

Since the data of Fig. 3 could result from the presence of cellular binding sites with negative temperature coefficients (19, 20), the binding of

cortisol to broken cells was measured by equilibrium dialysis at 0° and 37°. The results (Table I) show that the amount of cortisol bound at both temperatures is constant over the time range studied and that more, not less, cortisol is bound to broken cells at 37° than at 0°. And finally, there is more cortisol bound to broken cell components at 37° than is associated with intact cells of a control incubation at 37°, a finding that contrasts with the results obtained at 0°.

The change in the apparent volume of distribution of cortisol at different temperatures is examined as a function of cortisol concentration in Fig. 4. At all cortisol concentrations except the highest, 7.8×10^{-4} M, the least amount of cortisol associated with the cell occurs at 20–25°. The
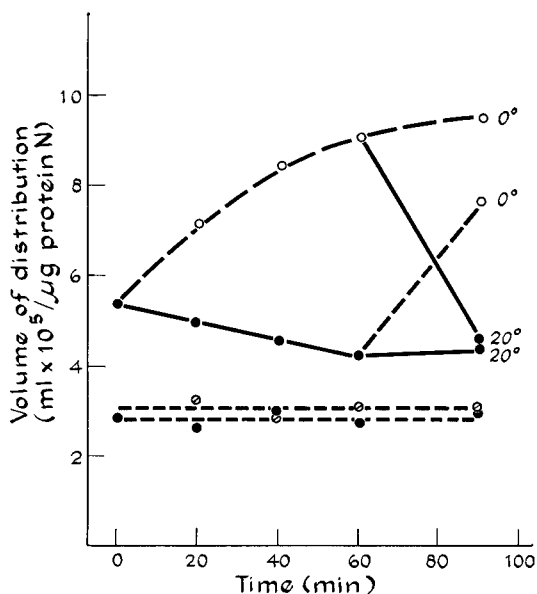
FIGURE 3 The effect of temperature on the apparent volume of distribution of cortisol-³H and ³H₂O in L cells. Sucrose-¹⁴C and cortisol-³H (3.3 × 10⁻⁸ M) or an equal μc amount of ³H₂O were added to a suspension of L cells in Earle's saline as described in Experimental Procedure. The cultures were sampled in duplicate and divided. One-half was incubated at 20°C, and the other at 0°C. After 60 min of incubation, one half of the cortisol culture at 0°C was placed at 20°, and one half of the culture incubating at 20°C was placed in an ice bath. The apparent volume of distribution of cortisol and water was determined on duplicate samples at the intervals indicated in the figure. Volume of distribution of cortisol-³H (●——●, 20°C incubation; O-----O, 0°C incubation); volume of distribution of ³H₂O (●-----●, 20°C incubation; ⊘-----⊘, 0°C incubation).

amount of cortisol associated with the cell at this optimum temperature is progressively higher at cortisol concentrations of 1 × 10⁻⁵ M, 2 × 10⁻⁵ M, and 7.8 × 10⁻⁴ M, as would be predicted from the concentration-distribution curves at 37° (Fig. 1). At low cortisol concentration (Fig. 4), the apparent volume of distribution of tritium at 0° is not the maximum attained at the two high concentrations, possibly indicating that some cortisol is kept out of the cell even at the lower temperatures. Note that at the highest cortisol concentration, 7.8 × 10⁻⁴ M, there is little difference in the amount of steroid associated with the cells at any temperature tested.

Incubation of L cells under 95% N₂ and 5% CO₂, in the absence of glucose, or in the presence

of 1 mM concentrations of sodium azide, sodium cyanide, dinitrophenol, sodium arsenate, or sodium fluoride, does not result in any change in the apparent volume of distribution of cortisol at low concentrations. Glucose dependence could be shown (Fig. 5) in an experiment in which cells were preincubated in the absence of glucose for 24 hr and then suspended in Earle's salt solution containing deoxyglucose (1 mM). After addition of radioactive labeled cortisol (3.3 × 10⁻⁸ M) and sucrose, the cell suspension was divided into four parts. The replicate suspensions received either unlabeled cortisol at high concentration (3.3 × 10⁻⁴ M), 1 mM sodium cyanide, or 1 mM dinitrophenol. The fourth replicate suspension served as a control without any additions. After incubation for ½ hr, each suspension was divided into three identical portions and incubated for a further 30 min with glucose (10⁻² M), with glucose (10⁻² M) and deoxyglucose (5 × 10⁻² M), or with no added sugar. The apparent volume of distribution of cortisol was then determined. The data presented in Fig. 5 demonstrated that the amount of cortisol associated with the cell rises in the presence of cortisol, cyanide, or dinitrophenol, and that the increase seen after cyanide or dinitrophenol can be reversed by the addition of glucose alone. This glucose effect was blocked by deoxyglucose, but deoxyglucose alone (control cultures) was not capable of producing an increase in the volume of distribution of cortisol. The addition of glucose to cells that contained higher levels of steroid radioactivity as a result of addition of a large amount of unlabeled cortisol was without effect; this result would have been expected from the concentration-distribution curve shown in Fig. 1. Preincubation in the absence of glucose is necessary to demonstrate the effect of cyanide or dinitrophenol on the amount of cortisol associated with cells.

High concentrations of several steroids can affect the volume of distribution of cortisol at 3.3 × 10⁻⁸ M (Fig. 6). Active steroids include dexamethasone, prednisolone, fluocinolone acetonide, deoxycorticosterone, corticosterone, cortisone, Reichstein's S, progesterone, dehydroepiandrosterone, and triamcinolone acetonide. Cholesterol, testosterone, and estradiol are inactive. Fluocinolone and triamcinolone, both potent growth-inhibitory steroids, are also seemingly inactive. These negative observations obtained with steroids that are growth inhibitory are subject to some uncertainty in their interpretation. It is possible that

TABLE I

Effect of Temperature on the Amount of Cortisol Associated with Whole and Broken Cells

A suspension of L cells in Earle's salt solution was divided into two parts. One part was divided equally among six cultures. Three of the resulting suspensions were incubated at 37°C and three at 0°C. After 1 hr of incubation with 5×10^{-6} M cortisol, the apparent volume of distribution of cortisol was determined for duplicate samples from each culture as described under Experimental Procedure. These results are given in the table as "whole cells."

The second part was sonicated for 30 sec with a Bronwill Biosonik III at a setting of 50 and divided in 14–2.0 ml parts that were each placed in a separate dialysis bag. Both the broken cell suspensions and control dialysis bags with salt solution only were dialyzed for up to 22 hr against 10 times the volume of Earle's saline with 5×10^{-6} M tritium-labeled cortisol. At the end of the dialysis, the radioactivity inside the dialysis bags and of the dialyzate was measured, and the amount associated with nondialyzable material was determined. The results are given as the volume of the dialyzate containing that amount of cortisol associated with the broken cell material and expressed as the volume divided by the protein content of the dialysis bag (which did not change during dialysis). The numbers with standard errors are the means of three separate determinations; the others are means of two determinations.

Cell preparation	Time of dialysis	Amount of cortisol associated with whole cells or with broken cell material		p value
		37°C	0°C	
	hr	*ml* $\times$ *10⁵/µg protein N*		
Whole cells	—	11.0 ± 0.2	15.3 ± 0.5	<0.01
Broken cells	6	19.7	12.1	
	11	18.0	11.8	
	22	18.1 ± 0.4	12.3 ± 0.3	<0.01

these compounds compete for cortisol binding sites within the cell. Thus, even if they interfered with the outward transport of cortisol, no predictable changes in the volume of distribution of cortisol could have been observed.

The problem was therefore approached in another way. The volumes of distribution of a number of radioactively-labeled steroids were determined in L cells suspended in Earle's salt solution without serum (Table II). Replicate cell suspensions were incubated for 1 hr in the presence of a variety of radioactive steroids at 1×10^{-8} M with and without dehydroepiandrosterone at 3×10^{-5} M. This C-19 steroid is not a growth-inhibitory agent (and therefore presumably does not interact with the glucocorticoid-specific receptors in the cell), but it does interfere with cortisol transport (Fig. 6). As shown in Table II, high concentrations of dehydroepiandrosterone increase the amount of labeled cortisol and dexamethasone associated with the cell. There are also small increases in the amounts of prednisolone and fluocinolone acetonide in the cell fraction. But the volumes of distribution of a large variety of close structural analogues of cortisol, as well as the

few structurally dissimilar steroids that were tested, were not changed.

The extrusion of cortisol and dexamethasone from the cell was measured in the following manner. L cells were preincubated for 20 hr in the absence of glucose and then suspended in 20–30 vol of glucose-free Earle's salt solution which contained NaCN and radioactive steroid. After a 20-min incubation, the cells were divided, centrifuged, resuspended in 2–3 vol of the decanted medium supplemented with either glucose or deoxyglucose, and incubated for 30 min. The amount of steroid extruded from the cell then can be determined from the difference between the counts associated with the cells in the presence and in the absence of glucose as is demonstrated in Fig. 5 b. The amount of steroid extruded from the cells should be accounted for by the amount of steroid recovered in the supernatant medium. The results of several such experiments are presented in Table III. Here it can be seen that the addition of glucose to glucose-depleted L cells resulted in the extrusion of both cortisol and dexamethasone into the medium. But no effect was observed with cortisone, a steroid with which the volume of
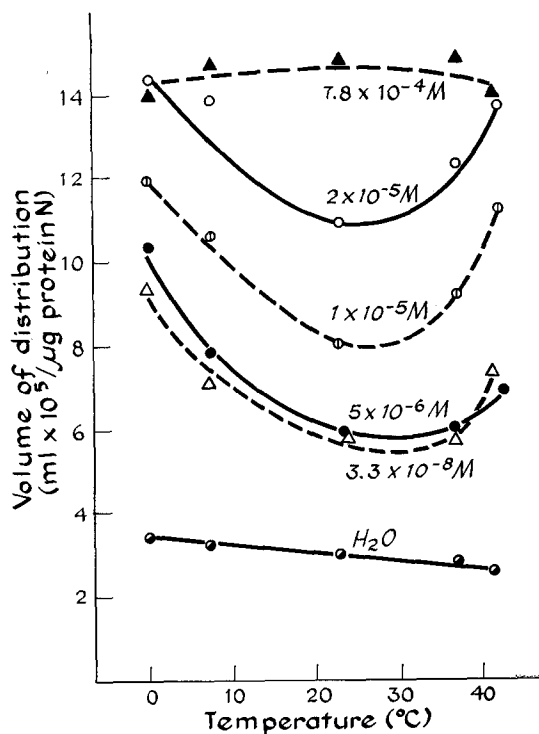
FIGURE 4 The effect of temperature on the apparent volume of distribution of cortisol in L cells incubated with varying concentrations of cortisol. Replicate suspensions of L cells in Earle's salt solution were incubated at the temperatures indicated in the figure for 1 hr with sucrose-^{14}C and cortisol-^{3}H (specific activity diluted to give final cortisol concentrations as shown in the figure) or an equal μc amount of ^{3}H$_2$O. At the end of the incubation, the apparent volume of distribution of cortisol and water was determined for duplicate samples as described under Experimental Procedure. Centrifugations of cell suspensions were done at the same temperatures at which the cells were incubated.

distribution is unchanged by dehydroepiandrosterone (Table II). There was no difference in the water volume of energy-depleted and glucose-supplemented cells.

The effect of addition of high concentrations of nonradioactive cortisol on the apparent volume of distribution of radioactively labeled cortisol in a variety of cell types is presented in Fig. 7. The apparent volume of distribution of cortisol is elevated in cells of three origins: mouse fibroblasts, L-929; mouse lymphoma cells, ML-388; and mouse adrenal cells, Y-1290S3. There is no change in the volume of distribution of radioactive

labeled cortisol in HeLa cells or in human diploid fibroblasts, WI-38. Both L cells and ML-388 cells are sensitive to the growth inhibitory effects of glucocorticoids, but HeLa cell and human diploid fibroblast growth is not. The mouse adrenal cells used in these experiments were demonstrated in our laboratory to increase their production of Δ^4-3-ketosteroids in response to the presence of cyclic 3',5'-AMP or pregnenolone as described by Buonassisi et al. (21). L cells do not secrete Δ^4-3-ketosteroids into the culture medium under these conditions. This result indicates that the Y-1290S3 cells growing in vitro retain certain properties of functioning adrenal cells.

DISCUSSION

The experiments presented in this paper demonstrate that at low concentrations cortisol is excluded from mouse fibroblasts, lymphoma cells, and adrenal tumor cells growing in cell culture. The results presented here support the hypothesis that the exclusion mechanism is saturable, temperature dependent, energy dependent, limited to a few steroids, competed for by a large number of steroids, and operative in a variety of, but not all, cultured cells.

The exclusion mechanism is saturable at a cortisol concentration of about 5×10^{-5} M (Fig. 1). The time courses of both the saturation effects and its reversal at low cortisol concentrations (Fig. 2) are so rapid that they cannot be measured with our present methods, which require approximately 30 sec to separate the cells from their incubation medium. Further demonstrations of the kinetics of the exclusion process depend upon the development of an extremely rapid method of separating cells from their suspending medium.

The exclusion mechanism is overcome by incubation at 0°C (Fig. 3), and there appears to be a temperature optimum for the exclusion process at about 25°C (Fig. 4). The observation that broken cells bind less cortisol at 0°C than at 37°C (Table I) argues strongly against the existence of intracellular binding components for cortisol with negative temperature coefficients (19, 20) as an explanation for the data of Fig. 3. The increase in the volume of distribution of cortisol at low concentrations seen on incubation at 0°C may actually be the summation of two separate effects of low temperature. There is an inhibition of the extrusion process, possibly even
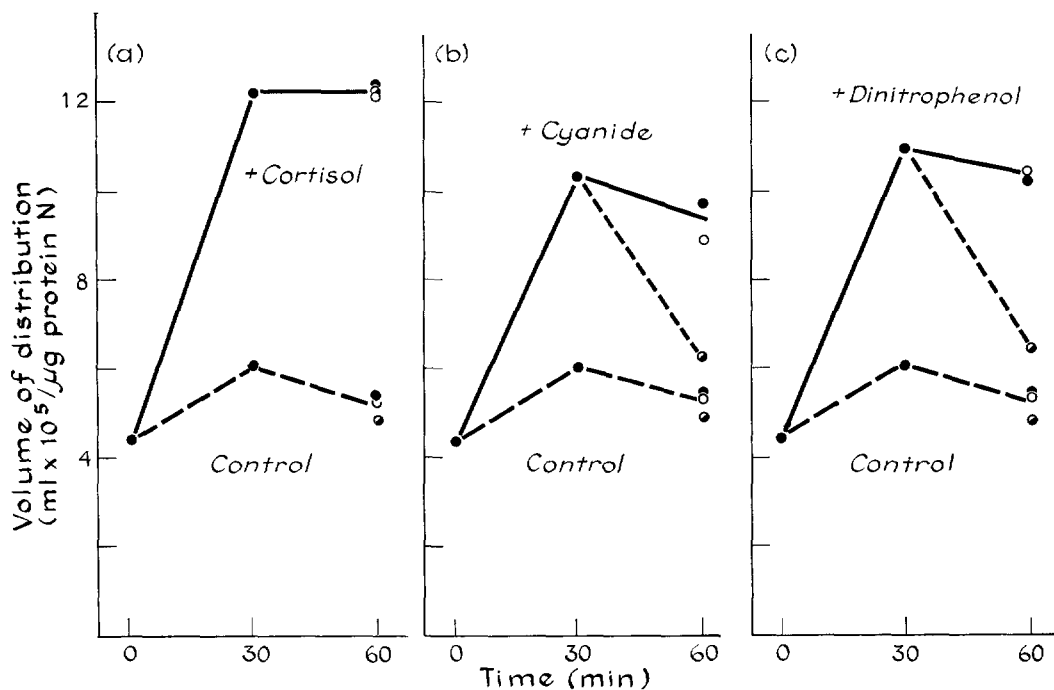
FIGURE 5 Glucose dependence of the apparent volume of distribution of cortisol in L cells. L cells were grown in monolayer for 24 hr in glucose-free Foley's medium supplemented with 10% bovine serum. After harvesting, the cells were suspended in glucose-free Earle's salt solution supplemented with 10% bovine serum, 2-deoxyglucose (1 mM), sucrose-^{14}C, and cortisol-^{3}H (3.3 × 10^{-8} M). After thorough mixing, duplicate samples were removed and the apparent volume of distribution of cortisol was determined. The culture was divided into four parts, and each of the resulting cell suspensions received either vehicle (control), nonradioactive cortisol (3.3 × 10^{-4} M), dinitrophenol (1 mM), or sodium cyanide (1 mM). After 30 min of incubation at 37°, duplicate samples were removed from each culture for determination of the volume of distribution of cortisol. The four primary cultures were then divided into three parts, each of which received either vehicle (●); 10 mM glucose (◒); or glucose, 10 mM, and 2-deoxyglucose, 50 mM, (○). After an additional 30 min of incubation, the apparent volume of distribution of cortisol was determined on duplicate samples from each culture.

complete, which would increase the apparent volume of distribution. And there is a decrease in the amount of steroid bound to intracellular components, which would tend to decrease the observed apparent volume of distribution of cortisol.

N-ethylmaleimide, a sulfhydryl binding reagent, irreversibly inhibits the extrusion mechanism (Fig. 2). This could result from an interaction of NEM with a postulated transport molecule as has been described in the lactose transport system of E. coli. (22). We have, however, no evidence that NEM interacts with any protein component of the cortisol transport system. The increased cortisol volume of distribution produced by NEM is unlikely to have resulted from nonspecific

damage to the cellular integrity, because NEM blocks cortisol transport at concentrations that do not affect the cellular water space.

Although glucose is probably the principal energy source of L cells (23), incubation of cells in glucose-free medium for up to 5 hr does not inhibit the exclusion mechanism for cortisol. To demonstrate glucose dependence, the cells must be preincubated under glucose-free conditions. After these cells have been suspended in glucose-free medium with sodium cyanide or dinitrophenol, the extrusion of steroid can be demonstrated to take place on the addition of glucose. Sodium cyanide or dinitrophenol does not appreciably affect the extrusion of cortisol if the cells are incubated in the presence of glucose or if
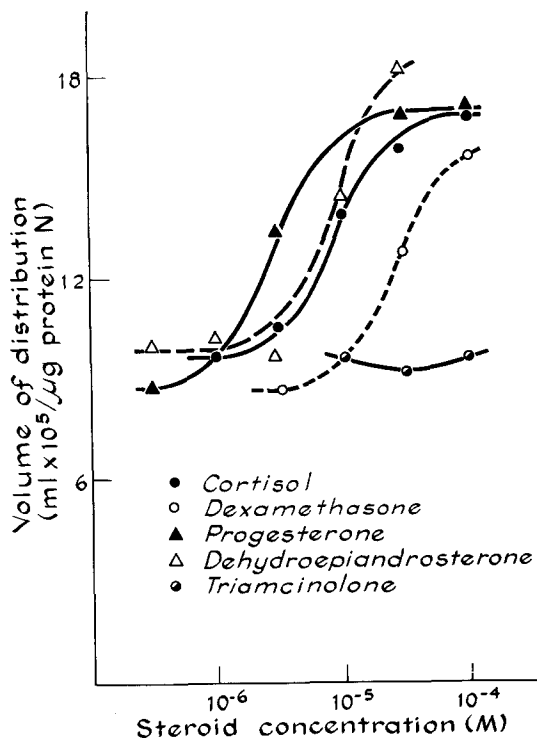
FIGURE 6 The effect of various steroids on the apparent volume of distribution of cortisol in L cells. Replicate suspensions of L cells in Earle's salt solution were incubated for 1 hr at 37°C with sucrose-^{14}C, cortisol-^{3}H (3.3 × 10^{-8} M), and increasing concentrations of a nonradioactive steroid as indicated in the figure. At the termination of the incubation, the volume of distribution of cortisol was determined for duplicate samples from each culture as described under Experimental Procedure. Each value represents the average of two experiments.
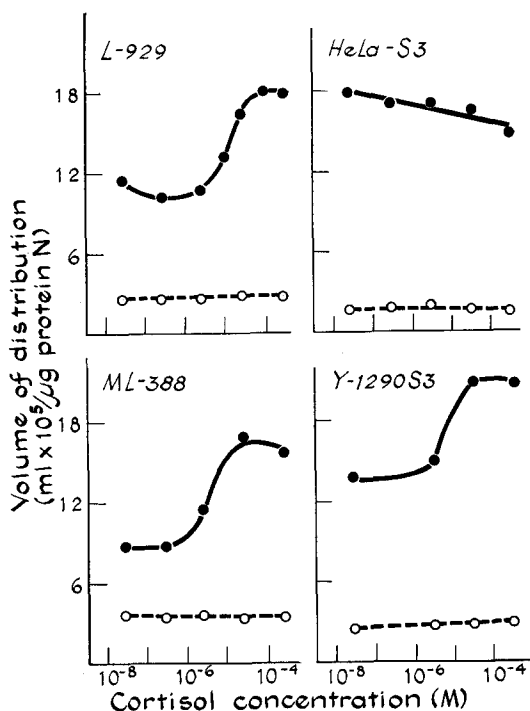
FIGURE 7 The apparent volume of distribution of cortisol as a function of the cortisol concentration of the incubation medium in several types of cultured mammalian cells. Replicate suspensions of cultured mammalian cells, as identified in the figure, were incubated for 1 hr at 37°C in Earle's salt solution with sucrose-^{14}C, cortisol-^{3}H (3.3 × 10^{-8} M) or an equal μc amount of ^{3}H$_2$O, and increasing concentrations of nonradioactive cortisol. At the end of the incubation, the apparent volume of distribution of cortisol and water was determined for duplicate samples of each culture as described under Experimental Procedure. ●——●, volume of distribution of cortisol-^{3}H; O-----O, volume of distribution of ^{3}H$_2$O.

there is no period of preincubation under glucose-free conditions. Sodium cyanide and dinitrophenol do obstruct respiration in whole L cells at concentrations considerably lower than those used in our experiments (24, 25). To provide a perspective for the interpretation of these results, it should be noted that incubation in a glucose-free salt solution did not inhibit the uptake of α-aminoisobutyric acid or galactose by L cells (10, 11), of glycine by mouse ascites-tumor cells (26), or of methotrexate by L1210 leukemia cells (9). Moreover, agents like sodium cyanide, dinitrophenol, or sodium azide when present in the concentrations used in these experiments had little or no effect on these same transport systems, as well as other

transport systems in cultured cells (6, 27). And in all of these systems the substrate was shown to be concentrated within the cell by a temperature-dependent process. For mammalian cells in culture, it has proven difficult to unequivocally demonstrate energy dependence for transport processes, although active transport processes undoubtedly exist. We believe that the temperature dependence and the effects of glucose deprivation described above support the hypothesis that the extrusion process is energy dependent.

By preincubating cells under glucose-free conditions and then suspending them in glucose-

TABLE II

The Volumes of Distribution of Various Steroids in L Cells, and the Effect of Dehydroepiandrosterone on their Distribution

Replicate suspensions of L cells in Earle's salt solution were incubated at 37°C for 1 hr with sucrose-^{14}C, a radioactive-labeled steroid (1×10^{-8} M) and either vehicle or nonradioactive dehydroepiandrosterone at 3×10^{-5} M. At the end of the incubation, the apparent volume of distribution of each steroid was determined for duplicate samples as described under Experimental Procedure. The figures in the table represent the means and standard errors of three separate experiments.

Steroid	Control	+ Dehydroepi-androsterone (3×10^{-5} M)	Ratio of dehydroepi-androsterone treated to control	p value
1×10^{-8} M	*ml $\times$ 10^5/μg protein N*			
H$_2$O	2.4 ± 0.2	2.5 ± 0.2	1.02	N.S.
Cortisol	13.0 ± 1.4	22.1 ± 0.7	1.66	0.01
Dexamethasone	37.5 ± 1.8	56.7 ± 0.4	1.51	0.01
Prednisolone	30.3 ± 2.8	36.7 ± 1.7	1.21	0.05
Fluocinolone acetonide (10^{-6} M)	79.1 ± 1.2	88.4 ± 1.4	1.12	0.01
Deoxycorticosterone	134.2 ± 9.2	131.4 ± 8.5	0.98	N.S.
Corticosterone	47.8 ± 1.8	49.1 ± 1.0	1.03	N.S.
Cortisone	17.0 ± 0.7	17.5 ± 0.9	1.03	N.S.
Reichstein's S	60.9 ± 1.2	62.7 ± 2.8	1.03	N.S.
Progesterone	504.7 ± 6.5	452.2 ± 3.7	0.90	0.05
Dehydroepiandrosterone	168.5 ± 3.6	159.2 ± 1.8	0.94	0.05
Triamcinolone acetonide	93.5 ± 3.7	93.3 ± 4.0	1.00	N.S.
Cholesterol	1339 ± 200	1415 ± 121	1.06	N.S.
Testosterone	149.1 ± 1.5	141.6 ± 1.2	0.95	N.S.
Estradiol	312.5 ± 4.4	304.4 ± 0.9	0.97	N.S.

free medium with sodium cyanide, the transport process can be inhibited, and the cells "loaded" with the tritium label. The addition of glucose will result in extrusion of tritium into the incubation medium and, by using a sufficiently concentrated cell suspension, the increase in the tritium content of the incubation medium can be measured. All of the tritium in the medium under such conditions chromatographs with cortisol in four different solvent systems by paper chromatography. And the tritium label associated with the cells after 1 hr incubation at a low cortisol concentration behaves like cortisol upon extraction from the cells with an organic solvent and paper chromatography in two solvent systems. It is still possible that the exclusion process may be mediated by a temporary conjugation of the steroid with glucuronide or sulfate; however, there is at present no evidence for such a mechanism. In any case, the net effect must be the extrusion of cortisol by L cells.

Since the compounds that show an increase in their own volume of distribution in the presence of dehydroepiandrosterone (Table II) were also able to increase the volume of distribution of cortisol, these compounds (cortisol, dexamethasone, prednisolone, and possibly fluocinolone acetonide) are apparently suitable substrates for the transport process and can be extruded from the cell. A large number of other steroids, including dehydroepiandrosterone (Table II), evidently interfere with the extrusion mechanism, allowing cortisol concentrations in the cell to rise, but are not themselves extruded from the cell. It is therefore difficult at the present time to specify the stereospecific requirements of the transport system.

The increased amount of steroid associated with cells upon incubation at 0°, as demonstrated in Figs. 3 and 4, has also been seen after incubation of thymocytes with cortisol-^{3}H or corticosterone-^{3}H in vitro (28, 29), but no radioactive-labeled cortisol was found associated with leukocytes incubated at 0° (30). In marked contrast with the results of Fig. 1, the uptake of cortisol in vitro by

TABLE III

Glucose-Dependent Secretion of Steroids from L Cells

L cells were grown in monolayers for 24 hr in glucose-free Foley's medium supplemented with 10% bovine serum. After harvesting, the cells were suspended in 20–30 vol of glucose-free Earle's salt solution with NaCN (1 mM), sucrose-^{14}C, and radioactive steroid (1 × 10^{-8} M) or water. After incubation for 20 min, the cells were centrifuged at 600 g and resuspended in 2–3 vol of the incubation medium, glucose-free Earle's salt solution with NaCN (1 mM), sucrose-^{14}C, and radioactive steroid or water. Then unlabeled glucose (1 g/L) or deoxyglucose (1 g/L) was added to identical replicate suspensions. After 20 additional min of incubation at 37°C, the amount of steroid associated with the cells and the amount present in the suspending medium were determined by the usual methods. The difference between the samples which received glucose and those which received deoxyglucose was calculated. The numbers in the table represent the means and standard error of the mean for the number of determinations noted in the right hand column.

Steroid	Amount lost from cells	Amount of excess steroid recovered in the medium	No. of experiments
equivalent ml of medium/100 mg cellular protein N			
H$_2$O	0.2 ± 0.2	−0.2 ± 0.1	3
μμg of steroid/10 mg cellular protein N			
Cortisol	2.6 ± 0.1	2.6 ± 0.2	9
Dexamethasone	3.0 ± 0.7	4.7 ± 1.1	3
Cortisone	0.2 ± 0.4	−0.2 ± 0.3	3

thymocytes (10^{-6} − 2 × 10^{-4} M, 5 × 10^{-7} − 3 × 10^{-5} M), (28, 31), leukocytes (10^{-6} − 3 × 10^{-5} M), (29), and isolated liver cells (10^{-6} − 10^{-5} M), (32), was proportional to the external steroid con-

centration over the concentration ranges listed in parentheses.

The observation that the exclusion process is saturable, temperature dependent, glucose dependent, and limited to a few close structural analogues strongly supports the hypothesis that certain steroids are actively transported out of cultured fibroblasts, lymphoma cells, and adrenal cells. To our knowledge this is the first such transport process demonstrated with steroid hormones. It has been demonstrated that the rat intestine is capable of transferring estradiol, testosterone, and androstenedione from the mucosal to the serosal side by an energy-dependent mechanism involving conjugation of the steroids as glucuronides (33, 34). The conditions of the experiments, however, indicate that the phenomenon observed was an energy-dependent conjugation with resultant trapping of the poorly lipid-soluble glucuronide and not the active transport of steroid.

It is of great interest that the exclusion mechanism is found to operate in functioning cells of adrenal origin. This transport process may constitute a mechanism whereby cells of the adrenal cortex normally secrete glucocorticoids. This hypothesis as it stands is probably too simplistic, because the principal glucocorticoid secreted from mouse adrenal cortex is corticosterone (35, 36), a steroid for which we can find no evidence of transport.

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