# Effect of Glucocorticoids on Hexose Uptake by Mouse Fibroblasts *in Vitro**

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ABSTRACT: Triamcinolone acetonide ($10^{-7}$ M) has been found to inhibit the uptake of radioactive galactose by L 929 cells suspended in a salt solution. The uptakes of glucose and deoxyglucose, hexoses which utilize the same uptake process as galactose, are also inhibited. Glucocorticoids also inhibit amino acid transport although the effect is delayed relative to the effect on hexose uptake. The structure–activity relationships of the steroids active in growth inhibition is paralleled by their ability to inhibit hexose uptake. Cells which are resistant to glucocorticoids demonstrate no inhibition of hexose uptake when exposed to triamcinolone acetonide. Fructose will support L cell growth provided its external concentration is high, but it is not taken into the cell by the same process as galactose or glucose. Fructose-supported growth is shown to be as sensitive to inhibition by triamcinolone acetonide as growth on glucose. These data indicate that steroid-mediated inhibition of hexose uptake is probably secondary to some other effect of the steroid, possibly an alteration in the phosphorylation or energy-yielding metabolic fate of carbohydrates in these cells.

The rate of replication of mouse fibroblasts, strain L 929, growing *in vitro* is inhibited by low doses of glucocorticoids (Pratt and Aronow, 1966; Ruhmann and Berliner, 1967). Previous investigations concerning the mechanism of growth inhibition brought about by steroids in these cells have centered on inhibitions of the rate of incorporation of radioactive-labeled precursors into macromolecular cell components (Pratt and Aronow, 1966; Seifert and Hilz, 1966) and on inhibitions in the rate of nucleic acid synthesis measured in subcellular systems (Kemper *et al.*, 1969). Recently, a macromolecule which specifically binds glucocorticoids has been identified in these cells (Hackney *et al.*, 1970).

In thymus cells Munck (1968) has demonstrated that there is an inhibition of glucose uptake which precedes the steroid-mediated inhibitions of nucleic acid metabolism. Similar steroid-mediated inhibitions of glucose uptake have been demonstrated in adipose tissue (Fain, 1964; Munck, 1962), lymphosarcoma cells (Rosen *et al.*, 1970), and skin (Overell *et al.*, 1960). It has proved difficult however to demonstrate whether the effect of the steroid is on the uptake mechanism or whether uptake is depressed as a secondary phenomenon resulting perhaps from decreased hexose phosphorylation or further energy-yielding metabolism. The present study is concerned with the steroid-mediated inhibition of hexose uptake mouse fibroblasts. The data presented in this work support the hypothesis that in these cells, the inhibition of hexose uptake is secondary to other effects of the steroid in the cell.

## Materials and Methods

### *Materials*

D-Galactose-1-$^{14}C$ (48.1 mCi/mmole, 2-deoxy-D-glucose-1-$^{14}C$ (2.29 mCi/mmole), D-fructose-*U*-$^{14}C$ (121 mCi/mmole), and $\alpha$-aminoisobutyric-1-$^{14}C$ acid (8.7 mCi/mmole) were obtained from New England Nuclear Corp., Boston, Mass. D-Glucose-*U*-$^{14}C$ (240 mCi/mmole) and inulin-*t* (150 mCi/mmole) were purchased from Schwarz BioResearch Inc., Orangeburg, N. Y. Dexamethasone was a gift of Dr. Ralph Dorfman of Syntex Corp. Triamcinolone acetonide was donated by Lederle Laboratories and 11-$\alpha$-cortisol[1] by the Squibb Institute for Medical Research. 11-$\beta$-Cortisol was purchased from Mann Research Laboratories. Nonradioactive-labeled sugars were purchased from commercial sources. $\beta$-D-Fructose obtained from Sigma Chemical Co., St. Louis, Mo., was demonstrated to be free of detectable contamination with glucose by paper chromatography in methanol–isopropyl alcohol–ammonia–water (45:30:15:10, v/v).

*Cell Cultures.* Monolayer cultures of mouse fibroblasts (L cells) were maintained on Joklik medium obtained from Grand Island Biological Co., Berkeley, Calif., supplemented with 10% bovine serum in an atmosphere of 5% $CO_2$ and 95% air. Spinner cultures of L cells were maintained in basal medium (Eagle, 1955) modified such that the amino acids were concentrated five times, L-glutamine three times, vitamins two times, glucose five times, $NaH_2PO_4$ ten times, and $CaCl_2$ and $NaCHO_3$ were omitted. This medium was supplemented with 150,000 units/l. of penicillin, 100 mg/l. of streptomycin sulfate, 10% bovine serum, and 0.1% methylcellulose (15 cP). Cultures were maintained at 37° with constant stirring in an atmosphere of humidified air. The pH of this culture medium is 7.2. Resistant cultures were made by growing stock cultures in $5 \times 10^{-6}$ M cortisol for 2 months and in $10^{-5}$ M cortisol for 19 months. At that time the growth rate in the presence and absence of $10^{-5}$ M cortisol or triamcinolone acetonide (a glucocorticoid with 100 times the growth inhibitory potency of cortisol) was the same.

*Dose–Response Assay for Steroid Inhibition of Growth and Hexose-Supported Growth.* Replicate cultures of fibroblasts were grown in 6-oz prescription bottles inoculated with $1 \times$

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[1] The trivial names for steroids used are: triamcinolone acetonide, 9$\alpha$-fluoro-11$\beta$,16$\alpha$,17$\alpha$,21-tetrahydroxypregna-1,4-diene-3,20-dione 16,17-acetonide; dexamethasone, 9$\alpha$-fluoro-16$\alpha$-methyl-11$\beta$,17$\alpha$,21-trihydroxypregna-1,4-diene-3,20-dione; 11$\alpha$-cortisol, 11$\alpha$,17$\alpha$,21-trihydroxypregn-4-ene-3,20-dione; 11$\beta$-cortisol, 11$\beta$,17$\alpha$,21-trihydroxypregn-4-ene-3,20-dione.

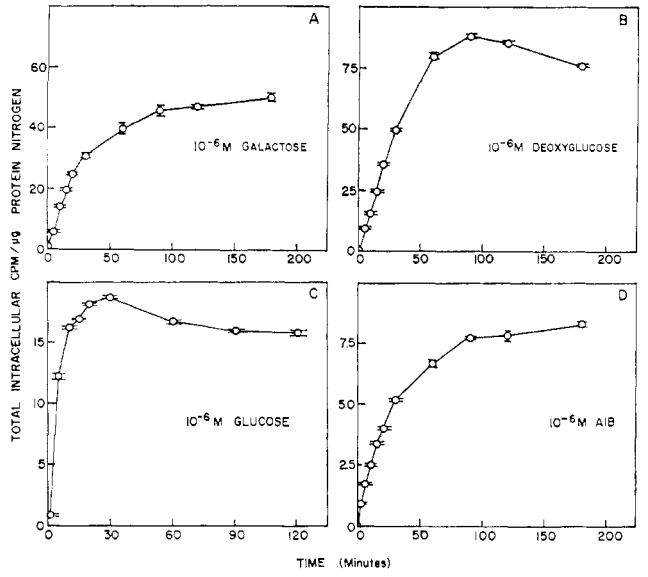


FIGURE 1: Time course of uptake of radioactive galactose, 2-deoxyglucose, glucose, and AIB by mouse fibroblasts. Two milliliters of washed, packed fibroblasts was added to 38 ml of Earle's saline (pH 7.2) and incubated at 37° with $10^{-6}$ M D-galactose-1-$^{14}C$ (48.1 mCi/mmole), 2-deoxy-D-glucose-1-$^{14}C$ (2.29 mCi/mmole), D-glucose-*U*-$^{14}C$ (6.0 mCi/mmole), α-aminoisobutyric-1-$^{14}C$ acid (8.7 mCi/mmole), or inulin-*t* (150 mCi/mmole). After various intervals of incubation, 3.0-ml aliquots were removed and assayed for radioactivity as described under Materials and Methods. The amount of radioactivity associated with the cells was determined after appropriate correction for the contribution of extracellular radioactivity. The values in the figure represent the mean and standard error of three separate experiments expressed as counts per minute of radioactive substrate associated with the cells per microgram of cell protein N.

$10^5$ cells in 15.0 ml of Joklik medium supplemented with 10% bovine serum. The cultures were allowed to grow for 2 days and the medium was discarded, the cultures were washed with 15 ml of Joklik medium without glucose (also obtained from Grand Island Biological Co.), and 15 ml of glucose-free medium supplemented with 10% dialyzed calf serum (Grand Island Biological Co.) was added. The cultures were incubated in the absence of any externally supplied hexose for 5 hr at 37°. At the end of the incubation, the medium was again discarded and 15 ml of hexose-free Joklik medium supplemented with 10% dialyzed calf serum was added to each culture. Various concentrations of glucose or fructose were then added to each culture and incubation was allowed to proceed for 4 days, at the end of which time the number of cells per culture were assayed as described previously (Pratt and Aronow, 1966). Similarly the effects of various concentrations of steroids on the growth of L cells over a 4-day period were determined as described previously.

*Uptake of Radioactive Hexoses by L Cells.* Cells were harvested from suspension culture by centrifugation at 600*g* for 10 min and washed twice by resuspension in four to six volumes of Earle's saline and centrifugation at 600*g*. The washed cells were then suspended in Earle's saline (approximately 1.2 ml of packed cells in 20 ml of saline). Replicate 20-ml samples of cell suspension were distributed to 50-ml erlenmeyer flasks containing either steroid in ethanol or ethanol alone and an atmosphere of 5% $CO_2$ in air. The final concentration of ethanol was never more than 0.25% (a concentration of ethanol which has no effect on glucose uptake or cell growth). In the majority of experiments, $10^{-7}$ M triamcinolone acetonide was used and the ethanol concentration in this case was 0.0025%. The cell suspensions were incubated at 37° with mild shaking for the appropriate time interval. All incubations were done in duplicate.

After the required interval of incubation with the steroid, radioactive-labeled substrate dissolved in Earle's saline was added to each vial to give a final concentration of $10^{-6}$ M. The initial rate of uptake of substrate was measured by removing 3-ml aliquots at appropriate intervals and centrifuging at 1500*g* for 30 sec. The supernatant was rapidly removed from the cell pellet with a pasteur pipet. The unwashed cell pellets were suspended directly in 3 ml of distilled water and the resulting suspension was sonicated for 5 sec with a Bronwill Biosonik III at a setting of 30 such that there was complete cell disruption. Duplicate 1-ml samples of cell sonicate were added to 10 ml of scintillation solution prepared according to the method of Bray (1960). Protein assays were performed on 0.5-ml samples of the sonicated cell suspensions after the method of Oyama and Eagle (1956). Duplicate 1-ml samples of the incubation medium (*i.e.*, the supernatant obtained after centrifugation at 1500*g*) were also added to 10 ml of scintillator fluid and assayed for radioactivity. Initial rates of uptake were calculated from the slope of each uptake curve which was computed by the method of least squares.

*Radioactive Assay.* All radioactive assays were carried out in a Packard Tri-Carb liquid scintillation spectrometer, Model 3310, with the channels set so that one channel counted $^{14}C$ exclusively. In order to correct the $^{14}C$ substrate counts associated with the cell pellet for any contribution made by contaminating extracellular fluid, inulin-*t* was used as a marker for extracellular fluid as described by Gross *et al.* (1968). The amount of extracellular contamination remained relatively constant from experiment to experiment, averaging between 25 and 30% of the volume of the cell pellet.

## Results

*Uptake of Hexoses and α-Aminoisobutyric Acid by L Cells.* Radioactive-labeled hexose or AIB was added to replicate suspensions of mouse fibroblasts in Earle's saline and the amount of radioactivity associated with the cells was determined at various time intervals after correction for extracellular contamination as described in the Experimental Procedure. It can be seen (Figure 1) that the uptake of galactose, 2-deoxyglucose, and AIB remains linear for approximately 30 min. In L cells galactose is converted into galactose 1-phosphate and uridine diphosphogalactose but these cells lack the enzyme uridine diphosphogalactose-4-epimerase (Maio and Rickenberg, 1961) and therefore galactose cannot be utilized as an energy source (Eagle *et al.*, 1958). The uptake of galactose and 2-deoxyglucose, another nonenergy yielding hexose (Barban and Schulze, 1961), can be considered an effective measure of hexose uptake and phosphorylation. The time course of glucose uptake (Figure 1C) is considerably more rapid than that of the nonutilizable hexoses. AIB is an amino acid which is actively transported into L cells by a process shared with the naturally occurring L-amino acids, but it is not utilized by the cells (Kuchler and Marlowe-Kuchler, 1965). All subsequent hexose and AIB experiments presented in this work were carried out by measuring initial rates of uptake determined from the early linear portion of the uptake curve.

The uptake of radioactive-labeled galactose ($10^{-6}$ M) is inhibited by high concentrations of nonradioactive glucose (Figure 2A). Similarly, nonradioactive galactose inhibits the uptake of glucose-$^{14}C$ present in low concentrations (Figure

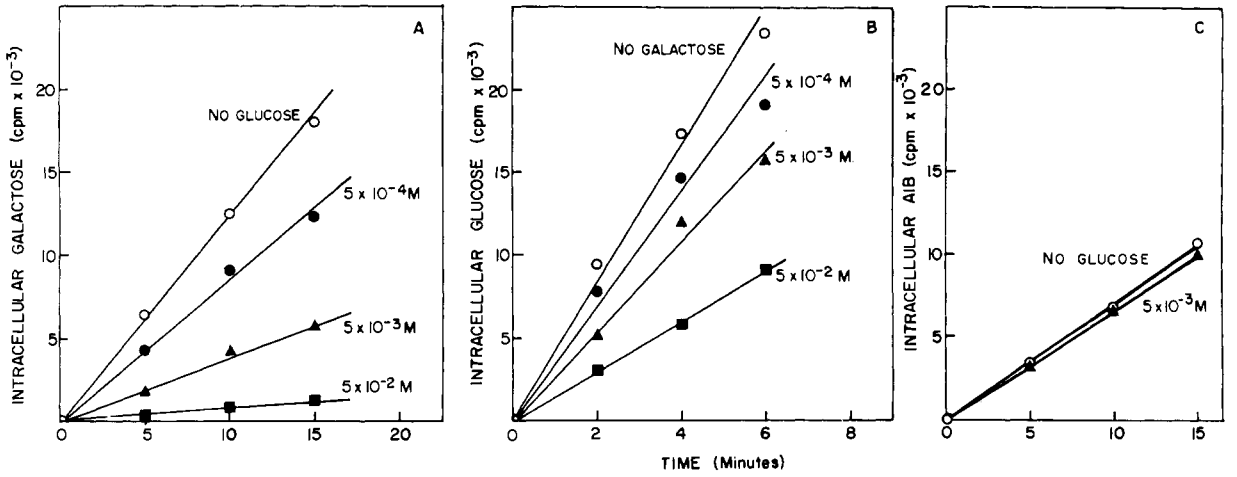


FIGURE 2: Competition of hexoses for the uptake of glucose, galactose, and AIB. Replicate 20-ml suspensions of L cells in Earle's saline concontaining 1.2 ml of packed cells/suspension were incubated in duplicate with $10^{-6}$ M radioactive-labeled galactose (part A), glucose (part B), or AIB (part C) and varying concentrations of nonradioactive glucose or galactose as demonstrated in the figure. The cell suspensions were incubated at 37°; 3.0-ml aliquots were removed at the indicated intervals. The amount of radioactivity associated with the cell was determined after appropriate correction for extracellular contamination determined as described in Materials and Methods. Each value in the figure represents the average of single determinations from duplicate incubations. (A) The uptake of galactose-1-$^{14}C$ in the presence of varying concentrations of nonradioactive glucose; (B) the uptake of glucose-*U*-$^{14}C$ in the presence of varying concentrations of nonradioactive galactose; (C) the uptake of $\alpha$-aminoisobutyric-1-$^{14}C$ acid in the absence of glucose and in the presence of $5 \times 10^{-3}$ M glucose.

2B). As presented in part C of Figure 2, AIB uptake is uninfluenced by the presence of glucose at $5 \times 10^{-3}$ M.

In L cells the measurement of galactose uptake is therefore an effective measure of hexose uptake for two reasons: (1) as this sugar is not utilized by the cells, the uptake process is distinguished from cellular carbohydrate metabolism; and (2) the uptake mechanism appears to be shared with some other hexoses, such as glucose, which are used as the prime energy source by the cells.

*Effect of Triamcinolone Acetonide on the Uptake of Hexoses and AIB by L Cells.* The effect of $10^{-7}$ M triamcinolone acetonide on the uptake of galactose-$^{14}C$ was measured at various time intervals and the results are presented in Figure 3. The rate of galactose uptake by control cell suspensions remained reasonably constant even after 6-hr incubation at 37°. Suspensions of fibroblasts which were incubated in the presence of triamcinolone acetonide, however, demonstrated progressive inhibitions in the rate of uptake of radioactive-labeled galactose.

The effect of triamcinolone acetonide ($10^{-7}$ M) on the uptake of deoxyglucose, glucose, and AIB was assayed after 3-hr incubation in the presence of the steroid. These results are presented in Table I and compared to the inhibition of galactose uptake. The uptakes of all three of the hexoses as well as that of AIB were significantly inhibited by the glucocorticoid. The inhibition of AIB uptake has a somewhat delayed time course and is not measurable until 3 hr after addition of steroid to the cells.

*Effect of Glucose on the Inhibition of Galactose-$^{14}C$ Uptake by Triamcinolone Acetonide.* The effect of $5 \times 10^{-3}$ M glucose on the time course of the steroid-mediated inhibition of galactose-$^{14}C$ uptake is examined in Figure 4. The data presented in this figure were obtained in the following manner. Control and steroid-treated incubations were carried out either in the absence of hexose or in the presence of $5 \times 10^{-3}$ M nonradioactive glucose. In the presence of glucose there is approximately a 60% inhibition of galactose-$^{14}C$ uptake (Figure 2A). The values in the figure represent the difference between the rates of initial galactose uptake in control and steroid-treated cultures determined as presented in Figure 3. The data in Figure 4 demonstrate that even in the absence of glucose, steroid treatment results in a time-dependent inhibition of the rate of galactose uptake. In the presence of glucose the inhibition of galactose-$^{14}C$ uptake by triamcinolone acetonide is much greater at the earlier time intervals and a maximum inhibition of 60% is attained within 2 hr.

The enhancement of the steroid effect by glucose is examined further in Table II. In this experiment galactose-$^{14}C$ uptake by cells which were preincubated for 90 min in the presence of glucose was inhibited 42%. Galactose uptake by control cells which were preincubated in the absence of glucose was inhibited only 25%. When cells were preincubated in

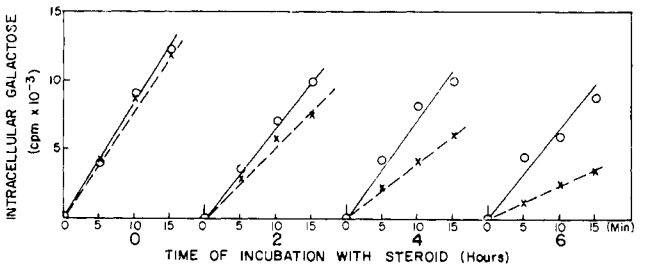


FIGURE 3: The effect of triamcinolone acetonide on galactose uptake. Replicate 20-ml suspensions of fibroblasts were prepared as described in the legend to Figure 2. Triamcinolone acetonide ($10^{-7}$ M) was added to 8 cell suspensions and the appropriate amount of ethanol vehicle (0.0025%) was added to eight control cell suspensions. The cells suspended in Earle's saline were then incubated at 37°. At the indicated times $10^{-6}$ M glactose-1-$^{14}C$ was added to duplicate control and duplicate steroid-containing cultures and 15-min uptake rates were determined. The intracellular galatose radioactivity was determined as described in the legend to Figure 1. Each value in the figure represents the average of single determinations from duplicate cultures. The large numbers on the abscissa refer to the number of hours of incubation in the presence of triamcinolone acetonide and the smaller numbers represent the time course of the galacotse uptake. (○—○) Control cultures; (×--×) $10^{-7}$ M triamcinolone acetonide.

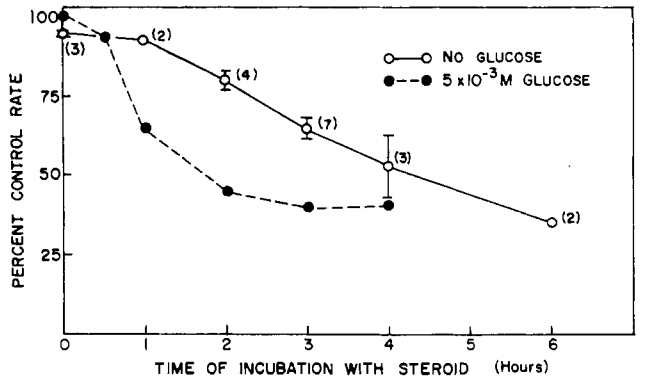


FIGURE 4: Time course of the steroid inhibition of radioactive galactose uptake in L cells incubated in the presence and absence of 5 mM glucose. Suspensions (20 ml) of L cells in Earle's saline were incubated for various times in the presence or absence of $10^{-7}$ M triamcinolone acetonide as described in the legend to Figure 3. After incubating for the interval indicated, $10^{-6}$ M galactose-1-$^{14}C$ was added to duplicate steroid-treated and control cultures and 15-min rates of uptake were determined. The values in the figure represent the rate of uptake of galactose in steroid-treated cultures expressed as a per cent of the uptake in control incubations—all uptake rates were measured as counts per minute associated with the cells per $\mu$g of protein N per 5 min. The numbers in parentheses represent the number of experiments carried out at each time interval. (O-O) Inhibition of galactose uptake by triamcinolone acetonide in cultures incubated in the absence of nonradioactive hexose; (●- -●) inhibition of galactose uptake in cultures to which $5 \times 10^{-3}$ M nonradioactive glucose was added at the same time as the steroid (each value here represents the average of two experiments, each carried out in duplicate).

the absence of glucose but galactose-$^{14}C$ uptake was studied in the presence of glucose, the steroid inhibition was only 27%. These results indicate that the enhancing effect of glucose on steroid inhibition of galactose uptake is a time dependent phenomenon and not merely the enhancement of the steroid effect by the presence of large amounts of extracellular glucose or an artifact introduced by the competition of glucose for galactose uptake.

TABLE I: Effect of a 3-hr Incubation with $10^{-7}$ M Triamcinolone Acetonide on the Uptake of Sugars and $\alpha$-Aminoisobutyric Acid by L Cels.[a]

| Radioactive-Labeled Compd | Rate of Uptake[b] (cpm/$\mu$g of Protein N per 5 min) | | Inhibition[c] (%) |
|---|---|---|---|
| | Control | +Steroid | |
| Galactose (5) | 10.1 ± 0.9 | 7.2 ± 1.1 | 29 |
| Deoxyglucose (2) | 10.8 | 8.2 | 24 |
| Glucose (5) | 10.05 ± 1.31 | 6.70 ± 0.86 | 34 |
| AIB (3) | 0.897 ± 0.103 | 0.520 ± 0.005 | 42 |

[a] Replicate 20-ml suspensions of L cells were prepared as described in the Legend of Figure 3. After 3-hr incubation, $10^{-6}$ M radioactive substrate was added to duplicate control and duplicate steroid-containing cultures and the uptake rates were determined as described under Materials and Methods. [b] The figures in the table represent the mean and standard error of the number of experiments listed in parentheses. Where standard errors are not presented, the figure represents the mean of two separate experiments each with duplicate determinations. [c] All steroid-treated cultures are significantly different from control at $p < 0.01$ (paired comparisons for incubations in each experiment).

TABLE II: Effect of Glucose on Steroid-Mediated Inhibition of Galactose Uptake.[a]

| Glucose Concn | | Rate of Uptake of Galactose[b] (cpm/$\mu$g of Protein N per 5 min) | | Inhibition[c] (%) |
|---|---|---|---|---|
| Preincubn Medium | Assay Medium | Control | +Steroid | |
| None | None | 10.82 ± 0.43 | 8.17 ± 0.48 | 25 |
| 5 mM | 5 mM | 4.69 ± 0.35 | 2.72 ± 0.15 | 42 |
| None | 5 mM | 4.71 ± 0.63 | 3.45 ± 0.55 | 27 |

[a] Replicate 20-ml suspensions of L cells in Earle's saline, with or without 5 mM glucose present, containing 1.2 ml of packed cells/suspension were incubated in the presence or absence of $10^{-7}$ M triamcinolone acetonide. After the 90-min preincubation, $10^{-6}$ M galactose-1-$^{14}C$ was added to all suspensions. At the same time, 5 mM unlabeled glucose was added to two sets of the incubated vials and the initial rate of uptake of galactose-$^{14}C$ was measured. [b] The figures in the table represent the mean and standard errors of three experiments done in duplicate. [c] All steroid-treated cultures are significantly different from controls at $p < 0.01$ (paired comparisons for incubations in each experiment).

*Correlation of Structure and Activity.* In order to determine whether the steroid-mediated inhibition of hexose uptake is related to the inhibitory effects of glucocorticoids on the growth of L cells, the potency of various steroids as inhibitors of galactose uptake was compared to their potency as inhibitors of cell growth. The growth-inhibiting effects of four different steroids are presented in Figure 5A in the form of dose response curves. 11$\beta$-Cortisol is approximately 0.1 as active as dexamethasone and 100th as active as triamcinolone acetonide. The 11$\alpha$ isomer of cortisol was inactive as an inhibitor of cell growth even at a concentration of $10^{-5}$ M. The ability of various concentrations of each of these steroids to inhibit the uptake of galactose-$^{14}C$ at 3 hr is presented in Figure 5B. From a comparison of the data presented in parts A and B of Figure 5, it is evident that the ability of a steroid to inhibit the uptake of hexose is paralleled by the potency of the steroid as a growth inhibitor. 11$\alpha$-Cortisol was also inactive as an inhibitor of galactose uptake.

*Effect of Triamcinolone Acetonide on Hexose Uptake by L Cells Which Are Resistant to Growth Inhibition by Glucocorticoids.* A steroid-resistant subline of L 929 cells was developed using procedures similar to those described by Aronow and Gabourel (1963), for mouse lymphoma cells *in vitro*. In these resistant cells the rate of growth is not inhibited by triamcinolone acetonide at $10^{-5}$ M, a concentration almost three orders of magnitude higher than the dose yielding maximal growth inhibition in sensitive cells. As presented in Table III, 3-hr incubation of sensitive cells with $10^{-7}$ M triamcinolone acetonide resulted in about 42% inhibition of hexose uptake whereas the cells which are resistant to the growth inhibitory effects of glucocorticoids responded to triamcinolone acetonide with only a 6% inhibition in hexose uptake.

*Effect of Triamcinolone Acetonide on the Replication of L Cells Growing in a Medium with Fructose as the Energy Supply.*

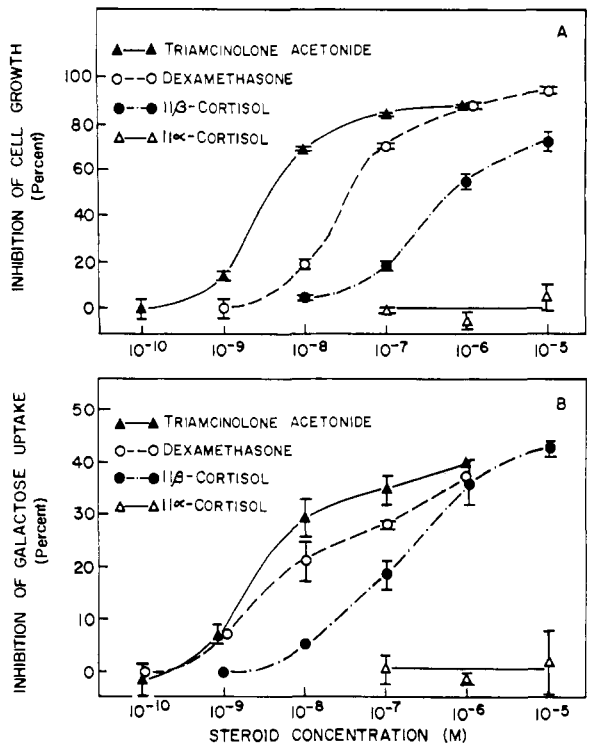


FIGURE 5: The relationship between the growth inhibitory potency of three active glucocorticoids and 11α-cortisol and the ability of these same steroids to affect galactose uptake. Part A, dose–response experiments for growth inhibition were carried out on cells growing in monolayer culture as described under Materials and Methods. Each value represents the mean and standard error of three replicate cultures expressed as the per cent of inhibition of growth as compared to control cultures which received vehicle alone. In part B replicate suspensions of fibroblasts in Earle's saline were incubated with various concentrations of steroids for 3 hr at 37° and 15-min rates of galactose-1-$^{14}C$ uptake were carried out as described in the legend to Figure 3. The uptake rates were calculated as counts per minute associated with the cells per μg of protein N per 5 min and are expressed as per cent of inhibition of uptake as compared to control cultures which received vehicle alone. The figures represent the mean and standard error of three experiments, or, where standard errors are not presented, the average of two separate experiments each with duplicate determinations.

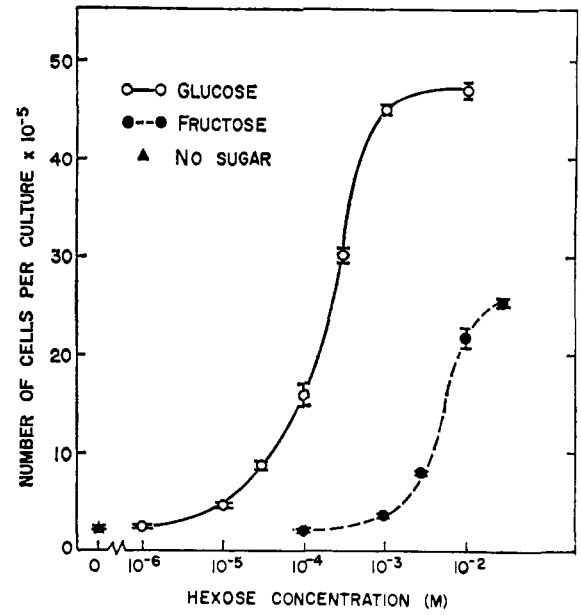


FIGURE 6: The growth of L cells in the presence of various concentrations of glucose and fructose. Replicate cultures of fibroblasts growing in monolayer were washed and grown for 4 days in triplicate in the presence of various concentrations of glucose or fructose as described in Materials and Methods. At the end of 4 days, the cells were counted and the results, expressed as the number of cells per culture, represent the mean and standard error of three separate cultures. The cell count at the time of hexose addition was 2.9 ± 0.21 × $10^5$ cells/culture.

L cells are able to grow in a medium in which fructose is substituted for glucose (Figure 6). Fructose is an effective source of energy for L cells although it is effective only at two orders of magnitude higher concentration than glucose. The effect of triamcinolone acetonide in inhibiting cell replication is the same regardless which of the two sugars is supplied (Table IV).

In view of the fact that fructose supports growth only when present at two orders of magnitude higher concentration than glucose, the uptake mechanism for fructose was investigated. The uptake of radioactive-labeled fructose was determined at $10^{-2}$ M in the presence of inulin-*f* as an extracellular marker. Fructose was found to be associated in small quantities with the cell pellet but its rate of association was so rapid that it could not be measured with our experimental technique. As we were not able to measure the rate of uptake of fructose directly, the ability of fructose to compete for the facilitated diffusion of other hexoses was measured. Fructose at $10^{-2}$ M did not affect the uptake of $10^{-6}$ M radioactive-labeled galactose. As presented in Table V, fructose at a concentration of $10^{-1}$ M also does not affect the uptake of the nonutilizable sugar, galactose, but does depress the uptake of $10^{-6}$ M radioactive-labeled glucose by about 70%. As the entry of galactose is not affected, fructose is presumably depressing the apparent glucose uptake by interfering with its intracellular metabolism. In order to properly control this experiment at $10^{-1}$ M

TABLE III: Effect of Triamcinolone Acetonide ($10^{-7}$ M, 3-hr Incubation) on Uptake of Galactose-1-$^{14}C$ in Steroid-Sensitive and Steroid-Resistant Mouse Fibroblasts.[a]

| Cell Type | Expt | Rate of Uptake of Galactose[b] (cpm/μg of Protein N per 5 min) | | Inhibn (%) |
|---|---|---|---|---|
| | | Control | +Steroid | |
| Sensitive | A | 12.72 | 6.96 | 45[c] |
| | B | 13.17 | 7.92 | 40[c] |
| Resistant | A | 9.33 | 8.97 | 4[d] |
| | B | 9.00 | 8.28 | 8[d] |

[a] Replicate 20-ml suspensions of steroid-sensitive or steroid-resistant L cells in Earle's saline containing 1.2 ml of packed cells/suspension were incubated in duplicate with either $10^{-7}$ M triamcinolone acetonide or vehicle. After 3 hr, $10^{-6}$ M galactose-1-$^{14}C$ was added and initial rates of uptake were measured as described in Materials and Methods. [b] Each value in the table represents the average of single determinations from duplicate incubations. [c] Significantly different from controls at $p < 0.01$. [d] Not significantly different from controls.

TABLE IV: Effect of $10^{-7}$ M Triamcinolone Acetonide on Growth of L Cells in Glucose or Fructose.[a]

| Hexose Supplied ($10^{-2}$ M) | Cell Growth[b] (Cells/Culture × $10^{-5}$) | | Inhibn[c] (%) |
|---|---|---|---|
| | Control | +Steroid | |
| Glucose | 44.3 ± 0.44 | 3.3 ± 0.21 | 93 |
| Fructose | 19.2 ± 0.96 | 1.5 ± 0.04 | 92 |
| No hexose | No growth | | |

[a] Replicate cultures of fibroblasts were grown in the presence of triamcinolone acetonide ($10^{-7}$ M) or vehicle, using glucose or fructose as the sole hexose present, as described in Materials and Methods. [b] The figures in the table represent the mean and standard errors of three replicate cultures. [c] Steroid-treated cultures are significantly different from controls at $p < 0.01$.

external hexose concentration, $10^{-1}$ M sucrose, a sugar which does not enter the cell, was added to the control medium such that the osmolality of the medium would be the same as for the samples containing $10^{-1}$ M monosaccharides.

*Effect of Various Unlabeled Hexoses on the Potentiation of the Glucocorticoid-Mediated Inhibition of Galactose Uptake.* Glucose potentiates the inhibition of galactose uptake resulting from steroid treatment (Figure 3), and the glucose must be present during the incubation with steroid in order for this potentiation to be observed (Table II). The ability of two other sugars to affect the steroid-caused inhibition of radioactive-labeled galactose uptake is examined in Table VI. Here it can be seen that, as expected, glucose potentiated the steroid effect. Fructose, a hexose which is metabolized but cannot be demonstrated to interact with the facilitated diffusion mechanism for galactose, did not affect the inhibition of galactose uptake. Deoxyglucose, a hexose which is not metabolized but which does employ the same facilitated diffusion mechanism as galactose, also has no effect on the inhibition of galactose uptake.

TABLE V: Effect of Hexoses on Uptake of $^{14}C$-Labeled Galactose and Glucose.[a]

| Nonradioactive Sugar (0.1 M) | Galactose Uptake[b] (cpm/μg of Protein N per 5 min) | % of Control |
|---|---|---|
| Sucrose | 8.55 | 100 |
| Fructose | 8.52 | 100 |
| Glucose | 0.42 | 5 |
| | **Glucose Uptake[b] (cpm/μg of Protein N per 2 min)** | |
| Sucrose | 11.64 | 100 |
| Fructose | 3.27 | 28 |
| Galactose | 5.13 | 44 |

[a] Replicate 20-ml suspensions of L cells in Earle's saline were incubated for 10 min in the presence of 0.1 M nonradioactive hexose. Radioactive galactose or glucose (each at $10^{-6}$ M) was added and initial rates of uptake were measured as described in Materials and Methods. [b] Figures in the table represent the average of single determinations from duplicate incubations.

TABLE VI: Effect of Triamcinolone Acetonide on Uptake of Galactose-1-$^{14}C$ in the Presence of Glucose, Fructose, or Deoxyglucose.[a]

| Incubn Condn (No. of Expt) | Galactose Uptake[b] (cpm/μg of Protein N per 5 min) | | Inhibn[c] (%) |
|---|---|---|---|
| | Control | +Steroid | |
| No addition (5) | 10.35 ± 0.65 | 7.94 ± 0.62 | 24 |
| +5 mM glucose (5) | 4.51 ± 0.29 | 2.83 ± 0.37 | 38 |
| +10 mM fructose (3) | 9.70 ± 0.67 | 7.09 ± 0.46 | 27 |
| +5 mM deoxyglucose (2) | 2.00 | 1.48 | 26 |

[a] Replicate 20-ml suspensions of L cells in Earle's saline containing the listed concentrations of unlabeled hexoses were incubated for 90 min in the presence or absence of $10^{-7}$ M triamcinolone acetonide as described in Materials and Methods. Initial rates of uptake of galactose-1-$^{14}C$ were measured after the 90-min preincubation as previously described. [b] Figures in the table represent the mean and standard error of the stated number of experiments in parentheses, each carried out in duplicate. [c] Significantly different from controls at $p < 0.01$ (paired comparisons for incubations in each experiment).

## Discussion

Maio and Rickenberg (1962) demonstrated that the uptake of galactose by L cells is carried out by a process which is stereospecific and temperature dependent. It is perhaps difficult to assign the classical term "active transport" to hexose uptake in these cells as free galactose is not concentrated above levels present in the medium. Also, it has not been rigorously demonstrated that glucose and galactose share a single transport process. Both glucose and galactose in high concentration will compete for the uptake of the other sugar (Figure 2), and it has been demonstrated that glucose does not compete for galactose phosphorylation in fibroblasts (Maio and Rickenberg, 1962) as well as in other mammalian cells (Berlin, 1970). It is likely therefore that a hexose-uptake process is shared by these two sugars, although multiple-uptake processes cannot be ruled out.

The hexose-uptake experiments presented in this paper were all carried out at very low hexose concentrations. As the hexose concentrations were not in the physiological range near the $K_m$ of the transport system ($K_m$ glucose $1 \times 10^{-3}$ M and $K_m$ galactose $5 \times 10^{-4}$ M, Maio and Rickenberg, 1962), the inhibition of hexose uptake by glucocorticoids may represent an indirect effect of the steroid on the apparent $K_m$ or it may represent an actual decrease in the maximal velocity of transport. It will be of interest to study the effects of glucocorticoids on the uptake of galactose at increasing concentra-

tions of the sugar, allowing an analysis of this aspect of the problem.

As 2-deoxyglucose, galactose, and glucose are all transferred through the cell membrane and also phosphorylated in the cell, it would be difficult, using these sugars, to investigate the problem of whether the steroid is affecting one process or the other. Fructose, which does not interact with the galactose uptake mechanism (Table V), seemed a useful tool for studying carbohydrate metabolism in these cells as distinct from the carrier-mediated uptake observed with the other hexoses. That fructose, once it enters the cell, is subject to metabolism, is suggested by the fact that the cells will grow on this sugar (chromatographically pure) when it is supplied at a high concentration.

Fructose, unlike glucose, appears to cross the cell membrane by a rapid process of passive diffusion, although the possibility of a separate low-affinity transport system for fructose cannot be completely ruled out. However, the growth of cells which are utilizing fructose as the carbon source is inhibited to the same extent by triamcinolone acetonide as the growth of cells which are utilizing glucose (Table V). This makes it very unlikely that the effect of the steroid in inhibiting cell growth is due to a direct effect of the steroid on the process which mediates the transfer of extracellular glucose to intracellular glucose.

The uptake of nonutilizable sugars (galactose and 2-deoxyglucose) that we have been measuring in this work includes both the process of transfer of extracellular sugar to intracellular sugar and the phosphorylation of the sugar. It is this second process, a process which is common to all of the sugars, which seems most likely to be the part altered by the steroid. A direct way of determining whether the effect of glucocorticoids is on the membrane transfer of hexose or on the subsequent phosphorylation step would be to determine the effect of triamcinolone acetonide on the rate of uptake of 3-*O*-methylglucose. In muscle cells 3-*O*-methylglucose has been demonstrated to be taken up by the same membrane-transfer process as glucose but it is not phosphorylated (Narahara and Özand, 1963). In L cells we found that 3-*O*-methylglucose at high concentrations ($10^{-1}$ M) inhibits the uptake of galactose-$^{14}C$ ($10^{-6}$ M) more than 90%. However in experiments designed to measure the rate of uptake of 3-*O*-methylglucose-$^{14}C$, we found that the trace amount of this sugar which associated with the cells did so essentially instantaneously and it was therefore impossible to measure any rate of uptake.

When cells are incubated in the presence of $5 \times 10^{-3}$ M glucose in addition to the steroid, a marked inhibition of the uptake of radioactive galactose is seen between 30 and 60 min (Figure 4). Also, glucose directly inhibits the uptake of galactose (Figure 2). Under these conditions of measurement, then, control cells are taking up approximately 30% of the radioactive galactose as cultures incubated in the absence of glucose, yet the steroid effect on galactose uptake is enhanced rather than diminished. The basis of this ability of glucose to enhance the steroid-mediated inhibition of galactose uptake is not at all clear. It is a time-dependent phenomenon, however, which appears to require the metabolism of glucose by the cell.

What then is the sequence of events which takes place after L cells are exposed to glucocorticoids? Inhibition of cell growth can be demonstrated readily at 12 hr (Pratt and Aronow, 1966) at which time there is a decrease in the ability of the cell to synthesize both DNA and RNA in a nuclear system (Kemper *et al.*, 1969). By 6 hr marked depression of the rate of uptake of tritium-labeled thymidine into cold acid-insoluble material is evident (Pratt and Aronow, 1966). Between 1 and 2 hr marked depression of the rate of galactose uptake may be seen when cells are incubated in the absence of glucose (Figure 4). When cells are incubated in the presence of glucose, inhibition of accumulation of galactose in the cell is observed between 30 and 60 min (Figure 5). This sequence of responses to steroids seen in L cells is similar to, but more delayed than, the response of thymocytes to glucocorticoids. In thymocytes the earliest effect seen is a decrease in glucose uptake which is evident at 15–20 min (Munck, 1968). This is followed by inhibition of uridine incorporation into cold acid-insoluble form (Makman *et al.*, 1966, 1968; Pratt *et al.*, 1967; Drews, 1969) and amino acids into protein (Young, 1969; Makman *et al.*, 1968; Gabourel and Comstock, 1964). Young (1969) demonstrated that protein synthesis in thymocytes which proceeds in the absence of glucose supplementation of the medium is not inhibited by cortisol. When glucose is provided as substrate there is marked inhibition of amino acid incorporation. In this cell type therefore the later effects on protein synthesis can be related to prior inhibitions of glucose uptake. In lymphosarcoma P1798 cells Rosen *et al.* (1970) have demonstrated that the effect of cortisol in inhibiting thymidine incorporation into DNA is markedly diminished when glucose is not provided in the incubation medium. Thus it would seem that initial inhibition of energy production or utilization may be directly related to later effects on nucleic acid metabolism in these cells, as well as reducing hexose uptake as shown in this study.

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# Estrogen-Induced Synthesis of Histones and a Specific Nonhistone Protein in the Uterus*

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ABSTRACT: Template capacity of uterine chromatin for DNA-dependent RNA polymerase is increased by estradiol given *in vivo*. Removal and replacement of certain histones on DNA is thought to control template capacity. Pulse labeling (30 min *in vivo*) of uterine proteins with [$^{14}$C]amino acids revealed a protein with increased specific activity associated with the arginine-rich histone fraction prepared by Amberlite IRC-50 chromatography from ovariectomized mature rats pulsed 15 min after estradiol. The total amount of the arginine-rich histone fraction in uterus was reduced to 58% of control levels by 15 min after estradiol. The radioactive complex was fractionated on Sephadex G-100, reduced with $\beta$-mercaptoethanol in urea and subjected to polyacrylamide gel electrophoresis. The radioactive protein was not a histone but it was an electrophoretically distinct protein associated with the F3 histone. The protein has a p*I* of 4–5 and its increased synthesis after estradiol is not inhibited by actinomycin D. This acidic nuclear protein is a likely candidate for an intermediate in the estradiol-induced increase in uterine RNA synthesis. It may function by complexing with the F3 histone through its SH groups to remove it or make it less inhibitory to the function of uterine DNA during transcription.

Administration of 17$\beta$-estradiol to either the immature or the ovariectomized mature female rat results in early stimulation of the incorporation of radioactive RNA precursors into uterine RNA followed by an increase in incorporation of labeled amino acids into protein (Ui and Mueller, 1963; Noteboom and Gorski, 1963; Hamilton, 1964; Means and Hamilton, 1966). Sequentially, incorporation of RNA precursors into rRNA appears to occur first followed by incorporation into tRNA and then DNA-like RNA (Billing *et al.*, 1969a; Hamilton *et al.*, 1968). The time course and magnitude of increases in uterine "RNA synthesis" measured *in vivo* remain open to question since the effect of estradiol on the specific activity of the immediate precursors of RNA, the nucleoside triphosphates, at the site of RNA synthesis has not been measured (Joel and Hagerman, 1969; Billing *et al.*, 1969b). The *in vitro* synthesis of RNA from radioactive nucleoside triphosphates by isolated whole uterine nuclei, where the effects of precursor pool dilution should be minimized, is elevated within 0.5 hr after *in vivo* treatment with estradiol (Gorski, 1964; Hamilton *et al.*, 1968). This increase in nuclear RNA synthesis has been shown to be due, at least in part, to the specific activation of the capacity of uterine chromatin to serve as a template for DNA-dependent RNA polymerase (Barker and Warren, 1966; Teng and Hamilton 1968; Church and McCarthy, 1970).

Several studies have shown that annealing of histones to DNA severely restricts the template efficiency of DNA in the DNA-dependent RNA polymerase reaction (Huang and Bonner, 1962; Huang *et al.*, 1964; Spelsberg *et al.*, 1969). Lysine-rich histones were observed to bind more tightly to DNA than the arginine-rich histones and could more effectively inhibit the DNA template capacity. In addition to its affinity for DNA, the arginine-rich histone had the capacity to bind to certain acidic proteins. At least one study suggested that synthesis of the arginine-rich histones in calf endometrium nuclei is associated with periods of enhanced RNA synthesis (Chalkley and Maurer, 1965). They did not determine which of the arginine-rich histones eluted from the Amberlite IRC-50 column (F3 or F2al) was synthesized and further, the possibility that the radioactivity was incorporated into a nonhistone protein with similar ion-exchange properties was not excluded (Stellwagen and Cole, 1968).

This investigation was originally undertaken to determine the effects of 17$\beta$-estradiol on the rate of synthesis of various classes of uterine histones during various phases of the uterine response to estradiol. The finding of a rapidly synthesized, nonhistone protein bound to the F3 arginine-rich histone led to its further preliminary characterization and determination of its possible relationship to the uterine response to estradiol. Since its synthesis is not inhibited by actinomycin D at doses capable of inhibiting 90% of the incorporation of [$^3$H]uridine into RNA, it must be either synthesized by a preexisting mRNA or a mRNA synthesized from actinomycin D insensitive regions of uterine DNA. This property suggests that it may be a specific protein, originally predicted by Mueller *et al.*

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