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GLUCOCORTICOIDS—UPTAKE BY SIMPLE DIFFUSION BY CULTURED REUBER AND NOVIKOFF RAT HEPATOMA CELLS

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Abstract—The initial rate of uptake of [^{3}H]prednisolone by cultured Novikoff and Reuber rat hepatoma cells at 18° was directly proportional to the hormone concentration in the medium between 0.01 μm and 5 mM. Uptake of 0.05 μM prednisolone was not affected by 100 μM dexamethasone or deoxycorticosterone or by depletion of the cells of ATP by preincubation in glucose-free medium containing KCN and iodoacetate. Uptake was also not affected by treatment of the cells with neuraminidase or phospholipase C and no countertransport of prednisolone could be demonstrated. Uptake was also unaffected by a 5000-fold excess of D-glucose in the medium, in spite of the fact that prednisolone acts as a simple competitive inhibitor of D-glucose transport. The results indicate that prednisolone is not taken up by the D-glucose or some glucocorticoid-specific transport system, but rather enters both types of cells by simple diffusion. Net uptake of prednisolone by the glucocorticoid-unresponsive Novikoff cells ceased when the intracellular concentration was 20–100% higher than that in the medium regardless of the medium concentration, indicating some nonspecific binding of the hormone to cellular components. At a concentration of 0.01 μM in the medium the glucocorticoid-responsive Reuber hepatoma cells accumulated two to three times more prednisolone/cell than Novikoff cells. This concentrative effect diminished at higher concentrations, indicating saturation of binding sites.

Glucocorticoids induce a number of effects in mammalian cells. These include the inhibition of growth of mouse lymphoid [1] and L cells [2], the inhibition of D-glucose metabolism in various types of cells [3–8], alterations in the surface membrane of certain hepatoma cells [9] and the induction of various enzymes in these cells and liver [10–15]. An initial step in the action of glucocorticoids is the binding of the hormones to cytoplasmic receptor proteins [16–20]. The hormone resistance of certain mutant lines of mouse L [17] and lymphoma cells [1] and the unresponsiveness to enzyme induction of certain clones of hepatoma cells [20] seem to be related to a decreased binding capacity for the hormone. The mode of entry of glucocorticoids into cells, however, has not been elucidated [21], and the possibility that an unresponsiveness of cells to glucocorticoids may also be regulated at the cell membrane level, therefore, has not been ruled out. Nevertheless, studies on the incorporation of glucocorticoids into whole cells as a measure of their binding to intracellular receptors indicated that the entry of glucocorticoids into cells must be quite rapid. From a series of recent experiments, it has been suggested that a stereospecific transport system is involved in the uptake of triamcinolone acetonide by a line of mouse pituitary tumor propagated in culture [22, 23]. In contrast, our present results show that simple diffusion is the sole process by which prednisolone enters cells of the glucocorticoid-unresponsive Novikoff hepatoma and the Reuber hepatoma in which tyrosine aminotransferase and phosphoenolpyruvate carboxylase are induced by glucocorticoids [11, 12, 14]. Furthermore, the initial rates of uptake are about the same for both types of cells and are not affected by the presence of D-glucose, although prednisolone inhibits D-glucose transport by both types of cells in a competitive manner.

MATERIALS AND METHODS

Novikoff rat hepatoma cells (subline N1S1-67) were propagated in suspension culture [24], and cells in the exponential phase of growth were harvested and suspended to 1×10^7 cells/ml of basal medium 42 (BM42B [25]) or D-glucose-free BM42B (BM42A [26]). Suspensions of cells were supplemented with D-glucose and [^{3}H- 1,2,4] prednisolone (Schwarz–Mann) and unlabeled prednisolone-21-sodium succinate (Sigma) as indicated in the appropriate experiments and incubated on a gyrotory shaker. Duplicate samples of suspension were analyzed for radioactivity in total cell material as follows. The cells were collected by centrifugation, washed once in 5 ml of balanced salt solution (BSS [27]), and suspended in 0.2 ml of 0.5 N trichloroacetic acid. The mixtures were heated at 70° for 30 min and analyzed for radioactivity [24]. Other samples of cell suspension were analyzed for radioactivity in acid-insoluble material [27]. Perchloric acid extracts were prepared from labeled cells and analyzed by ascending paper chromatography with various solvents as described previously [26, 27]. The composition of the solvents was as follows: solvent 9: 79 ml saturated ammonium sulfate, 19 ml of 0.05 M phosphate buffer (pH 6) and 2 ml isopropanol; solvent 28: 30 ml of 1 M ammonium acetate (pH 5) and 70 ml of 95% ethanol; solvent 30: 85 ml *n*-butanol and 15 ml H_2O.

H-35 Reuber rat hepatoma cells [28] grew poorly in suspension and were therefore routinely propagated in monolayer culture in plastic tissue culture

flasks (Falcon Plastics) with surface areas of 25 or 75 cm^2. The growth medium was composed of 90% (v/v) Eagle's basal medium containing four times the normal concentration of vitamins and amino acids, 5% (v/v) fetal calf serum, and 5% (v/v) bovine serum.

Cells of confluent monolayers were dispersed by incubation with trypsin and EDTA [29]. For uptake experiments, cells were propagated in 5-cm Falcon Petri dishes to 2 to 5 × 10^6 cells/dish. Each monolayer was briefly washed with 3 ml BM42A, and 1.5 ml BM42A containing the appropriate concentrations of [^{3}H]prednisolone was added to each plate. After various times of incubation, the medium was poured off duplicate plates and the plates were briefly drained on paper towels. The monolayers were rapidly rinsed four times with 4 ml of cold (4°) BSS, and the plates were drained thoroughly. Then the cells were scraped off the plate in 1 ml H_2O with a rubber policeman and the lysate was analyzed for radioactivity.

Control experiments in which samples without cells were processed indicated that the washing procedure for both types of cells removed over 97 per cent of the extracellular radioactivity. Furthermore, additional rinses of monolayer cultures of H-35 cells removed only insignificant amounts of additional radioactivity from the cells, indicating that little if any intracellular radioactivity was removed by the washing procedure. Duplicate incorporation values did generally not vary more than 10 per cent from the average.

Dexamethasone, deoxycorticosterone, neuraminidase and phospholipase C were purchased from Sigma Chemical Co.

RESULTS AND DISCUSSION

Suspensions of 10^7 Novikoff cells/ml were supplemented with 0.05 μM [^{3}H]prednisolone, and the incorporation of radioactivity into total cell material was determined as a function of time of incubation at 18°. A typical time course of incorporation is shown in Fig. 1A (control). Incorporation was rapid and a maximum intracellular level of radioactivity was attained within about 15 min of incubation. Subsequently, the amount of radioactivity associated with the cells remained approximately constant (not shown). None of the prednisolone was recovered in perchloric acid-insoluble material (not shown). In one preparation of [^{3}H]prednisolone, about 1 per cent of the radioactivity became precipitated in 0.5 N trichloroacetic and perchloric acid, but this precipitation occurred whether or not cells were present and thus probably reflected the precipitation of a labeled contaminant. Chromatographic analysis of acid extracts from 15-min labeled cells with three different solvents (solvents 9, 28 and 30) showed that the intracellular radioactivity was solely associated with prednisolone (not shown). The results in Fig. 1A also show that the time course of incorporation of [^{3}H]prednisolone at 0.05 μM was not affected by the presence

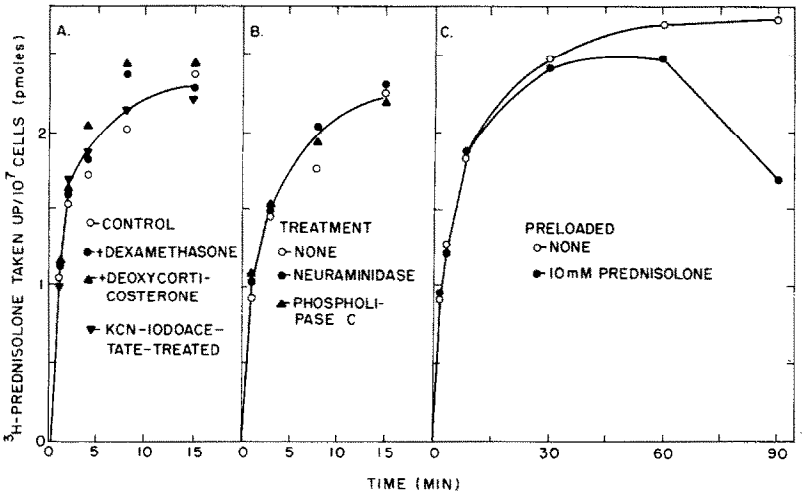

Fig. 1. Effect of various treatments on the incorporation of [^{3}H]prednisolone by NIS1-67 cells. (A) Samples of a suspension of 1 × 10^7 cells/ml of BM42A at 18° were supplemented (zero time) with 0.05 μM [^{3}H]prednisolone (4000 cpm/pmole) and at the same time where indicated with 100 μM dexamethasone or 100 μM deoxycorticosterone. Another sample of suspension was supplemented with 5 mM KCN and 5 mM iodoacetate and incubated at 37° for 10 min and at 18° for 5 min prior to addition of [^{3}H]prednisolone. (B) Samples of a suspension of 1.4 × 10^7 cells/ml of BM42B were supplemented where indicated with 10 μg neuraminidase/ml or 10 μg phospholipase C/ml and incubated at 37° for 20 min and at 18° for 5 min. Then the samples were supplemented (zero time) with [^{3}H]prednisolone as in (A). (C) One sample of a suspension of 1.6 × 10^7 cells/ml in BM42B was supplemented with 10 mM prednisolone; another sample did not receive hormone. Both samples were incubated at 37° for 15 min, then centrifuged and the cells were resuspended (zero time) to the original density in BM42B containing 0.05 μM [^{3}H]prednisolone (4500 cpm/pmole) and pre-equilibrated at 18°. All samples in this figure were incubated at 18° in a gyrotory water bath shaker, and duplicate 1-ml samples were analyzed for radioactivity in total cell material. All points represent averages of the duplicate samples.

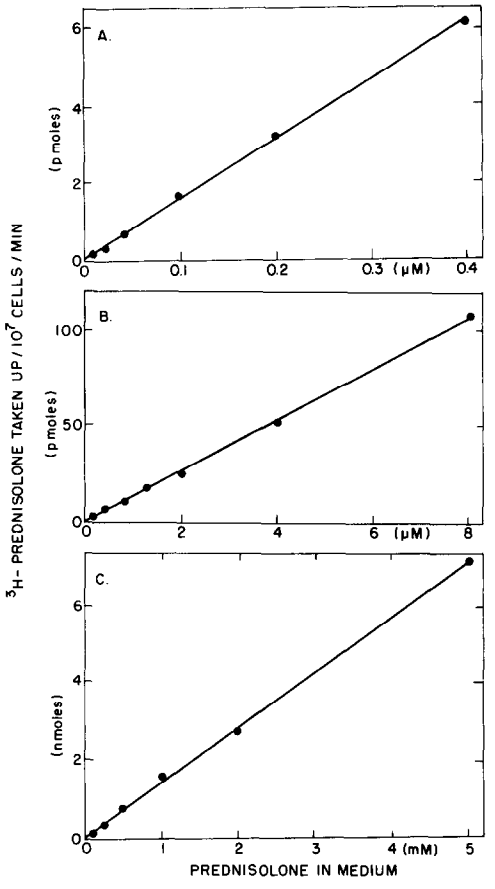

Fig. 2. Initial rates of prednisolone incorporation as a function of prednisolone concentration in the medium. Samples of suspensions of 1×10^7 N1S1-67 cells/ml of BM42A in (B) and (C) or BM42B in (A) were supplemented in (A) with 0.01, 0.02, 0.04, 0.1, 0.2 or 0.4 μM [^{3}H]prednisolone (9000 cpm/pmole) or in (B) with 0.1, 0.2 or 0.4 μM [^{3}H]prednisolone (8000 cpm/pmole) or 0.4, 0.8, 1.4, 2, 4 or 8 μM [^{3}H]prednisolone (500 cpm/pmole) or in (C) with 100,000 cpm [^{3}H]prednisolone/ml plus unlabeled prednisolone to 0.1, 0.2, 0.5, 1, 2 or 5 mM. The suspensions were incubated at 18° and duplicate 1-ml samples were analyzed for radioactivity in total cell material after 1, 3, 5, 10 and 20 min of incubation. The points represent averages of the duplicate 1-min values.

of 20000-fold higher concentrations of other glucocorticoids, such as dexamethasone or deoxycorticosterone in the medium. Depletion of the cells of ATP by incubation of the cells in a glucose-free medium containing 5 mM KCN and 5 mM iodoacetate[30] and in preparation) had also no effect on the time course of prednisolone incorporation (Fig. 1A). Neither did pretreatment of cells with neuraminidase or phospholipase C (Fig. 1B), which was found to markedly inhibit the incorporation of triamcinolone acetonide by cultured mouse pituitary tumor cells [23]. The shapes of the incorporation curves were about the same at various concentrations of prednisolone between 0.01 μM and 5 mM, that is, maximum intracellular levels were attained within about 15 min (not shown). The 1-min values of such time courses of incorporation were taken as estimates of the initial rates of prednisolone uptake. The results in Fig. 2 show that the initial rate of uptake at 18° was directly proportional to the concentration of prednisolone in the medium between 0.01 μM and 5 mM. Thus, prednisolone uptake was not stereospecific or energy dependent, and nonsaturatable at least to a concentration of 5 mM. These results are consistent with the view that prednisolone was taken up by N1S1-67 cells by simple diffusion through the membrane. This conclusion is supported by the finding that countertransport could not be demonstrated for prednisolone (Fig. 1C). When N1S1-67 cells pere preloaded with 10 mM of unlabeled prednisolone and then exposed to 0.05 μM [^{3}H]prednisolone, the initial time course of uptake of radioactivity was about the same as in the control cells which had not been preloaded. Countertransport has been demonstrated to occur in N1S1-67 cells with various substrates that enter these cells by facilitated diffusion [31, 32]. It has been observed that lowering the ATP level in Chinese hamster ovary cells results in an acceleration of the entry of various substrates that seem to enter cells by simple diffusion, suggesting that ATP is involved in establishing a barrier to simple diffusion in animal cells [33]. Our results in Fig. 1A do not support this view for prednisolone uptake in N1S1-67 cells since uptake was the same in normal and ATP-depleted cells.

The experiments illustrated in Fig. 1 and 2 were conducted at 18°, because at this temperature incorporation was slow enough to allow the estimation of initial rates of uptake, whereas at 37° uptake was too rapid for obtaining accurate initial rates (Fig. 3). However, all experiments described so far were also conducted at 37°. The results (not shown) were similar to those at 18°, in that incorporation was not affected

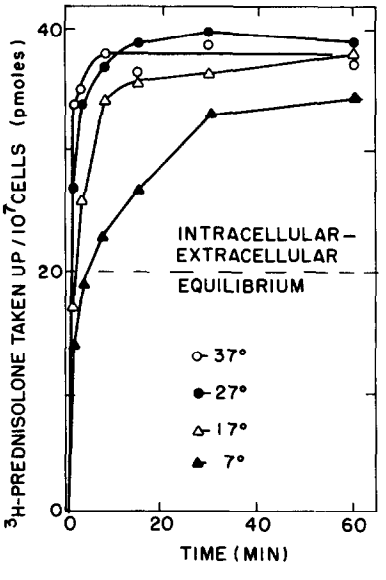

Fig. 3. Effect of temperature on prednisolone incorporation. Samples of N1S1-67 cells were suspended to 1×10^7 cells/ml of BM42B pre-equilibrated at the indicated temperatures. The suspensions were supplemented (zero time) with 1 μM [^{3}H]prednisolone (230 cpm/pmole) incubated at the appropriate temperatures. Duplicate 1-ml samples were analyzed for radioactivity in total cell material. All points represent averages of the duplicate samples.

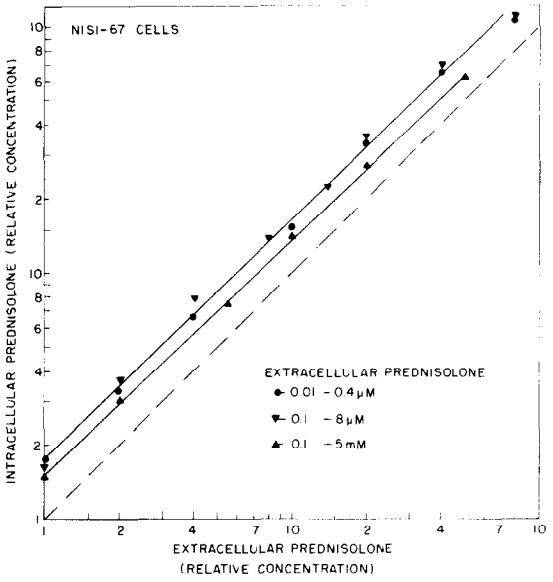

Fig. 4. Maximum concentrations of prednisolone accumulated by N1S1-67 cells as a function of the prednisolone concentration in the medium. The details of the experiment are described in the legend to Fig. 2. All points represent averages of the 20-min incorporation values. The dashed line indicates direct proportionality between intracellular and extracellular concentration.

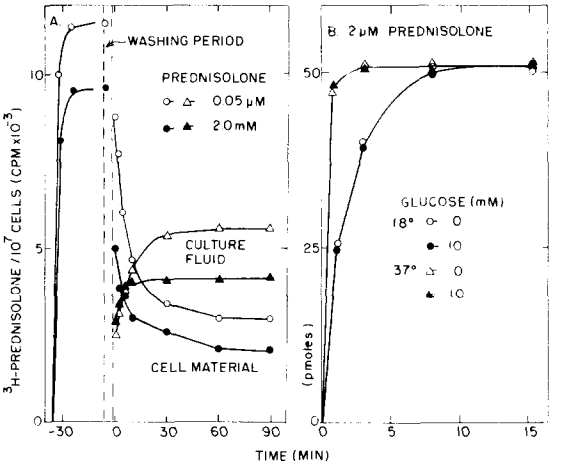

Fig. 5. Release of prednisolone from preloaded cells (A) and effect of D-glucose on prednisolone incorporation (B). (A) Two samples of a suspension of 1×10^7 N1S1-67 cells/ml were supplemented with 0.05 μM [^{3}H]prednisolone (4700 cpm/pmole) and one of them was also supplemented with unlabeled prednisolone to 2 mM. The suspensions were incubated at 37°, and duplicate 1-ml samples were analyzed for radioactivity in total cell material. After 30 min of incubation, the remaining cells were collected by centrifugation, washed once in BM42B, suspended (zero time) to the same density in fresh BM42B, and the suspensions were further incubated at 37°. Duplicate 1-ml samples were centrifuged and the supernatant fluid (culture fluid) and the cell pellet (cell material) were analyzed for radioactivity. All points represent averages of the duplicate samples. (B) Two samples of a suspension of 1×10^7 N1S1-67 cells/ml of BM42A were supplemented (zero time) with 2 μM [^{3}H]prednisolone (450 cpm/pmole) and one of them was also supplemented with 10 mM D-glucose. The suspensions were incubated at 18°, and duplicate 1-ml samples were analyzed for radioactivity in total cell material. All points are averages of the duplicate samples.

by other glucocorticoids, or by treatment with neuraminidase or phospholipase C, or preloading the cells with 10 mM prednisolone, and occurred normally in KCN–iodoacetate-treated cells.

One finding not entirely consistent with the simple diffusion model for prednisolone uptake was that the glucocorticoid accumulated slightly against a concentration gradient regardless of the temperature of incubation (Fig. 3). Based on an average cell volume of about 20 μl/10^7 cells [34], the maximum intracellular concentration was between 20 and 100% higher than the extracellular concentration even when the cells were exposed to millimolar concentrations of prednisolone (Fig. 4). The latter finding suggests that the apparent accumulation of prednisolone against a concentration gradient was not only due to the binding of the hormone to cytoplasmic hormone receptors [16–20]. It seems, in part at least, to reflect a non-specific interaction of prednisolone with cell components, since it was not affected by the presence of high concentrations of other glucocorticoids (Fig. 1A). The nature of this interaction or of the cell components involved is not known, but a similar accumulation against a concentration gradient has been observed with other glucocorticoids in HTC hepatoma cells [1, 35, 36]. Some saturation of these cell components, however, seems to be involved, since the ratio of intracellular to extracellular concentration of prednisolone was lower at a high than at a low concentration of the hormone in the medium (Fig. 4). This difference was most apparent in experiments in which duplicate samples of cell suspension were supplemented with the same amount of [^{3}H]prednisolone (0.05 μM; 400,000 cpm/ml) and one of the samples was also supplemented with 2 mM unlabeled prednisolone. Regularly the amount of radioactivity accumulated by the cells in the latter suspension was between 20 and 30 per cent lower than that accumulated by the cells in the control suspension (Fig. 5A). This difference might have reflected binding to specific receptor sites. Upon washing of the [^{3}H]prednisolone-labeled cells and incubation in fresh medium, a large proportion (about 60 per cent) of the accumulated prednisolone was released from the cells whether the cells had been labeled with 0.05 μM or 2 mM [^{3}H]prednisolone (Fig. 5A). A second wash and incubation in fresh medium resulted in the release of 50 per cent of the residual radioactivity but most of the remainder of the radioactivity (about 15 per cent of the total associated initially with the cells) remained cell bound regardless of how often the cells were washed (not shown). Thus, the interaction of most of the prednisolone with cell components was readily reversible. Because of this interaction of prednisolone with cell components it is not entirely clear whether the effect of temperature on the initial rate of incorporation (1-min values, Fig. 3) solely reflected an effect on uptake or also on binding to cell components. Although accurate values could not be obtained from these data, the Q_{10} for the initial rate of prednisolone incorporation seemed to fall between 1.2 and 1.6 (Fig. 3).

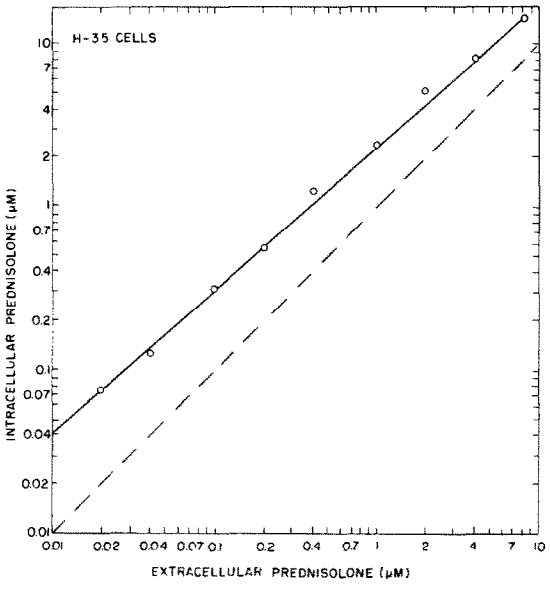

Fig. 6. Maximum intracellular concentration of prednisolone accumulated by H-35 cells as a function of the prednisolone concentration in the medium. The details of the experiment are described in the legend to Fig. 7. All points represent averages of the duplicate 20-min incorporation values. The dashed line indicates direct proportionality between intracellular and extracellular concentration.

Results with H-35 cells were very similar to those obtained with N1S1-67 cells, except that at low concentrations of the hormone (0.01 to 1 μM) H-35 cells accumulated two to three times more prednisolone on a per cell basis than N1S1-67 cells (Fig. 6). Since the overall volume of H-35 cells is approximately the same as that of N1S1-67 cells (about 20 μl/10^7 cells), the ratio of intracellular to extracellular concentration at these low concentrations was even higher in H-35 than in N1S1-67 cells. A slightly increased intracellular accumulation of glucocorticoids at low concentrations has also been reported for another glucocorticoid-sensitive hepatoma cell line, HTC [1, 26, 27], and related to the binding of hormone to cytoplasmic receptors [1]. H-35 cells have been shown to possess a receptor protein similar or identical to that of normal rat liver [37]. Nonspecific binding as observed with N1S1-67 cells, however, also occurred with H-35 cells, since the ratio of intracellular to extracellular concentration was greater than 1 even at millimolar concentrations of the hormone (Fig. 6).

The initial rates of prednisolone uptake (1-min values) by H-35 cells, as by N1S1-67 cells, were directly proportional to hormone concentration (Fig. 7), and the initial rates of uptake were about the same for both types of cells (compare Figs. 1 and 7). For instance, at 8 μM prednisolone in the medium the intial rates of uptake were 110 and 140 pmoles/10^7 cells/min for N1S1-67 and H-35 cells respectively. These results suggest that glucocorticoids enter both types of cells by simple diffusion and that they enter glucocorticoid-responsive and -unresponsive cells equally well. Furthermore, the uptake of prednisolone by either cell type was not affected by the presence of 10 mM D-glucose in the medium either at 18° or 37° (see Fig. 5B for N1S1-67 cells), in spite of the fact that prednisolone has approximately the same affinity for the D-glucose transport system of N1S1-67 cells as the substrate itself [8]. This conclusion was indicated by the finding that the K_i for the competitive inhibition of 2-deoxy-D-glucose transport by prednisolone was about the same as the K_m for 2-deoxy-D-glucose or D-glucose transport (1–2 mM). Additional studies have shown that the D-glucose transport system of H-35 cells is inhibited by prednisolone to a similar extent as that of N1S1-67 cells; the K_i for the competitive inhibition of 2-deoxy-D-glucose transport at 37° was approximately 10 mM. The results indicate that prednisolone interacts with and blocks the D-glucose transport system without being itself transported by the system. The physiologi-

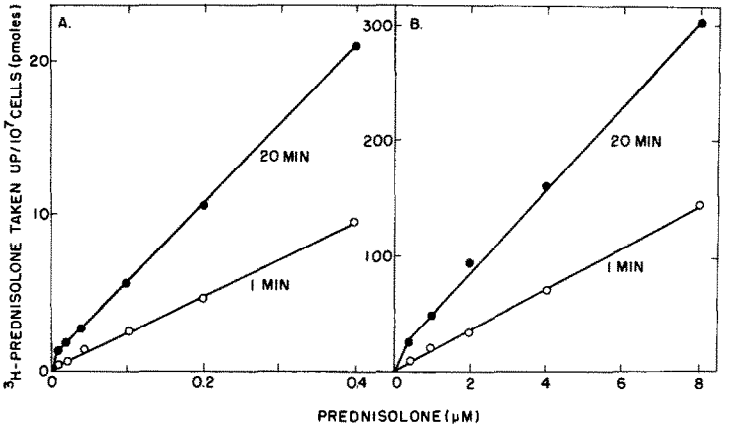

Fig. 7. Uptake of prednisolone by H-35 cells as a function of hormone concentration in the medium. Petri plate cultures (5-cm diameter) of H-35 cells (3.5 × 10^6 cells/plate) were rinsed with cold BM42A and then overlayed with 1.5 ml of 18° BM42A containing (A) 0.01, 0.02, 0.04, 0.1, 0.2 or 0.4 μM [^{3}H]prednisolone (8000 cpm/pmoles) or (B) 0.4, 1, 2, 4 or 8 μM [^{3}H]prednisolone (500 cpm/pmole). The plates were incubated at room temperature, and at 1 and 20 min of incubation duplicate plates were analyzed for radioactivity in total cell material. All points represent averages of duplicate plates.

cal significance of this inhibition of D-glucose transport is uncertain, however, since the effect is apparent only at unphysiologically high concentrations of prednisolone and since glucocorticoid-responsive and -unresponsive cells are equally affected.

Our finding that prednisolone enters, equally well, glucocorticoid-responsive and -unresponsive hepatoma cells and that uptake is by simple diffusion contrasts with the report of Harrison *et al.* [22, 23] that triamcinolone acetonide enters cultured mouse pituitary adenocarcinoma cells by a stereospecific transport system. Their conclusion, however, may not be warranted, since these investigators did not clearly distinguish between glucocorticoid uptake and binding. No initial rates of uptake, saturation kinetics or stereospecificity of uptake were demonstrated. The conclusion of these investigators was largely based on the temperature dependence of incorporation of the hormone by the cells and on differences in the time courses of binding of the substrate by whole cells and cell-free extracts. The Arrhenius plot of whole cell incorporation of triamcinolone acetomide exhibited a temperature transition at 16 [22], but this observation was probably unrelated to glucocorticoid uptake since equilibrium concentrations of hormone were analyzed rather than initial rates of uptake.

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