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KINETICS OF STEROID TRANSPORT THROUGH CELL MEMBRANES: COMPARISON OF THE UPTAKE OF CORTISOL BY ISOLATED RAT LIVER CELLS WITH BINDING OF CORTISOL TO RAT LIVER CYTOSOL

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SUMMARY

Viable rat liver cells suspended in a medium containing cortisol take up the steroid rapidly; the uptake is temperature dependent. After incubation for 1 min at 27°C the uptake is 20 times higher than that observed at 5°C. The activation energy calculated for the uptake of cortisol was 18 kcal between 5° to 20°C and 6 kcal from 20° to 32°C. With increasing concentrations of cortisol two saturable systems were observed, one with an apparent K_M value of 0.2 μM and the other with 2.0 μM. The uptake after 1 min of incubation at 27°C was 9 pmol per mg cell protein. Diffusion of cortisol into the cell was also observed at the steroid concentrations tested. Metabolic inhibitor KCN inhibited the saturable uptake system; pCMB decreased the uptake of cortisol. Binding of cortisol to liver cytosol, carried out under conditions of uptake of cortisol by rat liver cells was more rapid; however, the binding after 1 min of incubation at 27°C was twice that observed at 5°C. The Arrhenius plot was linear from 5° to 32°C; the activation energy was calculated to be 1.2 kcal. The binding was linear from 5 to 10,000 nM cortisol. After incubation for 1 min at 27°C the binding of cortisol to cytosol proteins was 5% of the uptake by liver cells. KCN did not inhibit the binding, whereas pCMB decreased the binding. The results suggest that the uptake of cortisol by liver cells is mediated by carrier proteins in the plasma membrane and that cytosol proteins are not directly involved in this initial uptake process.

INTRODUCTION

Numerous studies on the mechanism of steroid hormone action have shown that the first step involves the binding of the steroid to specific receptor proteins in the cytoplasm of the cell. The steroid–receptor-complex thus formed migrates to the nucleus, a process which has been shown to be dependent on temperature. In the nucleus the final expression of the biological activity of the steroid hormone takes place. The step prior to the binding of the steroid hormones to cytoplasmic proteins, however, is the permeation through the plasma membrane. In this context it has often been assumed that steroid hormones enter the cells of target organs by simple diffusion. Recent investigations in our laboratory however, have shown that viable rat hepatocytes, when suspended in medium containing estrone, estradiol-17β, testosterone, corticosterone or cortisol, take up the steroid rapidly. The uptake shows dependence on temperature and with increasing concentration of the steroids saturation can be observed [1, 2]. Diffusion becomes predominant as the concentration of steroid in the medium is increased.

From the studies on uptake of cortisol by rat hepatocytes using substrate analogs, metabolic inhibitors and sulfhydryl-group blocking agents, we proposed that cortisol is transported into the cell by proteins in the plasma membrane that act as carriers [3].

The question that arises in these type of studies is to what extent the cytoplasmic proteins or receptors contribute directly or indirectly to the transfer of extracellular cortisol into the cell, although it is firmly believed that proteins that bind steroids with high degree of specificity are located exclusively inside the cell [4]. To get some insight into this problem, the binding studies of cortisol to rat liver cytosol were carried out under conditions that were used for uptake of cortisol by the hepatocytes in order to be able to compare uptake by intact cells with the subcellular fraction of the cell. With regard to dependence on temperature, saturability by cortisol and the effects of dexamethasone and KCN, it could be shown that there exist large differences between the process of uptake of cortisol by isolated hepatocytes and the binding of cortisol to cytosol. These results suggest that the proteins in the cytoplasm are not directly involved in the uptake of extracellular steroids, but that carrier proteins in the plasma membrane translocate the extracellular steroid into the cell.

* The studies in this paper are taken from the dissertation of Karl Schulze-Hagen submitted to the Faculty of Medicine of the University of Bonn.

EXPERIMENTAL PROCEDURE

[1,2-^{3}H]-Cortisol (55 Ci/mmol) was obtained from the Radiochemical Centre, Amersham, Bucks, England. The purity was checked before use by t.l.c. Hepes (N-2′-hydroxyethylpiperazine-2-ethane-sulfonic acid) was obtained from Serva, Heidelberg.

The buffers used had the following composition: Hepes I buffer (pH 7.4): 152 mM NaCl, 6.7 mM KCl, 10 mM Hepes, 10 mM NaOH; Hepes II buffer (pH 7.6): 72.4 mM NaCl, 6.72 mM KCl, 100 mM Hepes, 66 mM NaOH; Hepes III buffer (pH 7.4): 143 mM NaCl, 6.72 mM KCl, 1.22 mM $CaCl_2$, 10 mM Hepes, 11 mM KH_2PO_4, 7 mM Na_2SO_4, 13.2 mM $MgCl_2$, 6.6 mM NaOH; Hepes IV (pH 7.4): 152 mM NaCl, 6.72 mM KCl, 1.22 mM $CaCl_2$, 1 mM Hepes, 11 mM KH_2PO_4, 7 mM Na_2SO_4, 13.2 mM $MgCl_2$, 0.6 mM NaOH.

Isolation of liver cells

The procedure followed was that of Berry and Friend[5] and Seglen[6] with slight modifications [3]. In the present investigations all procedures were carried out as described in [3] except for the buffer solutions. The organ was flushed at room temperature (22°C) with 400 ml Hepes I buffer which was continuously gassed with O_2. The flushed liver was perfused subsequently with 68 ml Hepes II buffer which was gassed with O_2 and which contained 35 mg Collagenase II (Boehringer Mannheim, Germany). The perfusion was carried out at a temperature of 28°C for 15 min. At this stage 12 mg $CaCl_2 \cdot 2H_2O$ was added in 2 ml Hepes II buffer and the perfusion was continued for another 15 min. The subsequent working up procedures for obtaining cells were carried out as described previously [3] with the difference, that instead of Krebs–Ringer-phosphate buffer, Hepes II buffer was used. The isolated liver cells were 90 to 95% intact as judged by exclusion of the dye trypan blue. Glucose formed from 10 mM pyruvate was 80 to 115 μmol/g wet weight/30 min. Protein was measured according to Lowry *et al.*[7].

Preparation of cytosol from rat liver

Male Wistar rats weighing from 280 to 320 g were killed by cervical dislocation. The liver was removed and homogenized with 9 parts of Hepes III buffer at 2°C with an Ultra Turrax homogenizer (Janke & Kunkel, Staufen, Germany). The homogenate was centrifuged for 10 min at 25,000 ***g*** and for 30 min at 214,000 ***g***, each time transferring the supernatant to fresh centrifuge tubes. The 214,000 ***g*** supernatant was taken as the cytosol fraction with which binding studies were carried out. The binding capacity of the freshly prepared cytosol was compared to cytosol kept for 1 to 30 days at −20°C; no difference in the binding capacity could be observed between freshly prepared cytosol and cytosol that was frozen.

Measurement of uptake of cortisol by isolated rat liver cells

The procedures for the preparation of samples containing nonlabeled and radioactive cortisol and measurement of uptake of cortisol by the liver cells using the Millipore filtration technique described previously [3] were used. The cells on the glass fiber filter were washed twice with Hepes IV buffer. Counting the radioactivity associated with the cells as well as calculation of the amount of radioactivity taken up by the cells have been described before [3]. The differences observed in the velocity of uptake of cortisol in the present study are due to inherent variations arising from different collagenase preparations and uncontrollable factors during perfusion and isolation of cells in addition to the variations between the rats used. For each experiment cells were freshly isolated from livers of different animals. However, experiments were carried out both in the absence and in the presence of additives mentioned in the text.

Measurement of binding of cortisol by liver cytosol

The procedure for measurement of binding of cortisol was that described by Korenman[8] with slight modifications. The preparation of the samples for binding studies was carried out exactly as that described for uptake by the cells. The incubation was carried out for 50 s at 27°C in order to compare the binding of cortisol to the cytosol with the uptake of cortisol by viable rat liver cells. The incubation was stopped by keeping the tubes in ice-water and adding 0.1 ml of an ice-cold suspension of Norit A charcoal (100 mg/ml) in Hepes III buffer. The tubes were shaken vigorously for 5 s and afterwards centrifuged at 600 ***g*** for 2 min; the supernatants were transferred to new centrifuge tubes by aspiration and centrifuged at 12,000 ***g*** for 10 min. Aliquots of 0.4 ml were mixed with 12 ml Brays' scintillation fluid and counted in a liquid scintillation spectrometer. Blank values were carried out over the entire concentration range of cortisol, except that protein was omitted. The charcoal quantitatively adsorbed cortisol in the incubation medium from 5 to 10,000 nM cortisol. Studies of binding to cytosol were carried out in triplicate or sextuplicate; more than 6000 counts were accumulated to keep the counting error below 1.5%. The coefficient of variation for triplicate determinations was 4.8%, for sextuplicate 6.4%.

RESULTS AND DISCUSSION

Dependence of uptake of cortisol by liver cells and binding of cortisol to cytosol, on protein concentration, time and temperature

Uptake of cortisol at a concentration of 100 nM in the medium by isolated liver cells and the binding of cortisol by cytosol were linear up to 2.5 mg cellular or cytosol protein. The uptake of cortisol by liver cells equivalent to 1 mg of cellular protein was

2.1 pmol per min; binding of cortisol to 1 mg cytosol protein studied under identical incubation conditions was 0.21 pmol per min. The uptake of cortisol by liver cells was temperature dependent (Fig. 1A) in that at 37°C it is linear up to 45 s, at 27°C up to 60 s, at 22°C up to 90 s and at 5°C up to 30 min. After incubation for 1 min at 27°C the uptake is 20 times higher than that observed at 5°C.

The binding of cortisol to cytoplasmic proteins (Fig. 1B) proceeded very rapidly. At all temperatures studied steady states were reached faster with cytoplasmic proteins than with liver cells: however the binding after 1 min of incubation at 27°C was twice that observed at 5°C. Thus the binding of cortisol to cytosol proteins was one tenth when compared with the uptake by liver cells. These results show that the uptake system of cortisol by liver cells is more sensitive to changes in temperature than the binding of cortisol by the cytosol fraction. This effect of temperature was studied in more detail with 100 and 250 nM cortisol, using liver cells and cytosol. As can be seen from the Arrhenius plot (Fig. 2) the uptake of cortisol by liver cells is influenced by increasing temperatures to a greater extent than the binding of cortisol to cytosol. With the liver cells a sharp change in the slope was observed at 20°C; this transition in the velocity of uptake reflects a change in the activation energy. A similar observation was made by Linden *et al.*[9] for the transport of β-glycosides by *E. coli*. They concluded that the transition is the result of a change in the "fluidity" of the membrane. The activation energy calculated for the uptake of cortisol by liver cells from 5° to 20°C was 18 kcal and from 20° to 32°C 6 kcal. The Arrhenius plot for cytosol is linear from 5°C to 32°C; the activation energy was 1.2 kcal, a value which is considerably lower than that observed for uptake of cortisol by liver cells.

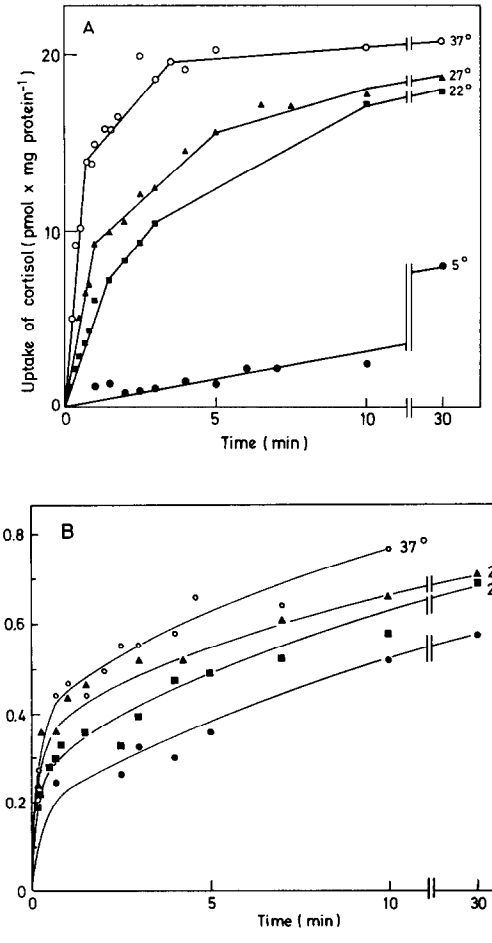

Fig. 1. Dependence of uptake of cortisol by isolated rat liver cells and binding of cortisol to cytosol on time and temperature. The incubation medium was Hepes III buffer, pH 7.4; the cortisol concentration was 250 nM. (A) Cells equivalent to 1 mg of protein/ml (about $6.54 \pm 0.16 \times 10^5$ cells) were used. At the indicated times, aliquots of the incubation medium were pipetted onto Whatman GF/C filter discs attached to a Millipore filter manifold connected to a suction pump. The cells on the filter discs were washed twice with 5 ml of ice-cold Hepes IV buffer, pH 7.4. The radioactivity taken up by the cells was measured as described in the text. Each point in the figure represents the mean of three incubations. (B) Cytosol fraction equivalent to 1 mg of protein/ml was used. At the indicated times 0.1 ml of ice-cold suspension of Norit A charcoal in Hepes III buffer was added. The tubes were shaken for 5 s and centrifuged at 600 *g* for 2 min; the supernatants were centrifuged again at 12,000 *g* for 10 min. The radioactivity bound to the cytoplasmic proteins was measured as mentioned in the text.

Dependence of uptake and binding on the concentration of cortisol

As previously mentioned [3] uptake of cortisol by liver cells is dependent on the concentration of corti-

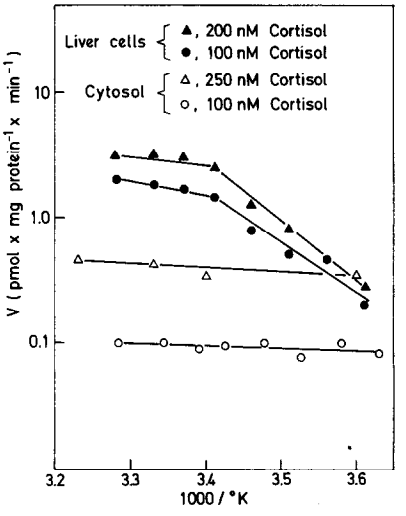

Fig. 2. Arrhenius plot of uptake of cortisol by isolated rat liver cells: ▲——▲, liver cells incubated with 200 nM cortisol and ●——●, 100 nM cortisol; binding of cortisol to cytosol proteins: △——△, cytosol fraction incubated with 250 nM cortisol and ○——○, 100 nM cortisol. Each point is the mean of six incubations. The working up procedures are described under "Experimental Procedure". The straight lines in this and subsequent figures were calculated by the method of least squares with a program for an Olivetti P 102 desk computer.

sol used, in that two saturable systems are evident in addition to diffusion. In the previous investigation [3] Krebs–Ringer-phosphate buffer was used, whereas the present experiments were carried out in Hepes III buffer. In the present studies Hepes III buffer was chosen because the cells were viable for a longer period of time than in the Krebs–Ringer-phosphate buffer. Figure 3A shows that cortisol is taken up by liver cells by saturable processes (an elaboration on the results and the interpretation of the different uptake systems is presented in Ref. [3]). Lineweaver–Burk plots (Figures not shown) obtained from 5 different experiments yielded both a high affinity K_M (average 0.2 μM) and a low affinity K_M value (average 2 μM).

In contrast to the cells, the binding of cortisol to cytosol proteins increases with increasing cortisol concentration and no saturation was attained (Fig. 3B). In these experiments no efforts were made to differentiate between specific and non-specific binding systems in the cytosol; this aspect has been adequately dealt with by other authors [10–15]. In this study the binding by cytoplasmic proteins was measured, since one may argue that these proteins might be responsible for the transport of cortisol into the cell. A comparison of the uptake of cortisol by liver cells with binding to cytoplasmic proteins when plotted according to Scatchard[16] (Fig. 4) shows, that under identical incubation conditions i.e. amount of protein, temperature, time, substrate concentration and ionic composition of the buffer, the liver cells exhibit more than one uptake system while the binding of cortisol by cytoplasmic proteins shows only a single binding component.

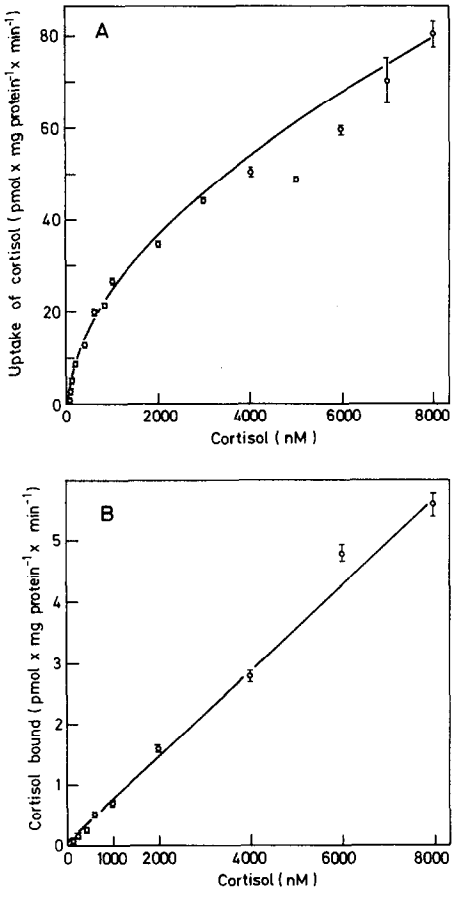

Fig. 3. Initial rates of uptake of cortisol by rat liver cells and binding of cortisol by cytosol proteins. The tubes contained increasing concentrations of nonlabeled cortisol prepared by serial dilution plus a constant amount of [^{3}H]-cortisol (2×10^5 d.p.m.) in Hepes III buffer, pH 7.4. Incubations were started by pipetting 0.5 mg of protein and carried out at 27°C for 50 s. Working up of the samples is described under "Experimental Procedure". Each point is the mean of triplicate determinations and the vertical bars in this and following Figs. indicate one standard deviation. (A) Uptake of cortisol by liver cells; (B) binding of cortisol by cytosol protein.

Influence of dexamethasone on uptake and binding of cortisol

Koblinsky *et al.*[11] reported that Binders A and B in rat liver cytosol bind natural glucocorticoids and in addition Binder G also binds dexamethasone. If binding of dexamethasone to the cortisol-transferring protein in the membrane would take place, then an inhibitory effect of dexamethasone on the transport of cortisol into the cells should be expected. The results in Fig. 5A show that dexamethasone does inhibit the uptake of cortisol in a non-competitive manner. In contrast to this result, the binding of cortisol to cytoplasmic proteins in the presence of 2000 nM dexamethasone showed that dexamethasone did not influence the binding of cortisol to cytoplasmic proteins (Fig. 5B). This result may be used to distinguish between the uptake system of cells and the binding process of cortisol to cytoplasmic proteins.

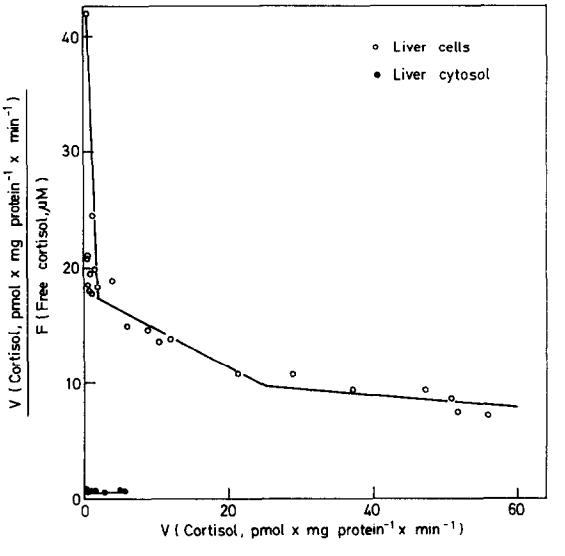

Fig. 4. Scatchard plot of the uptake of cortisol by liver cells and binding of cortisol to liver cytosol proteins. The concentration of cortisol in both cases ranged from 5 to 8000 nM.

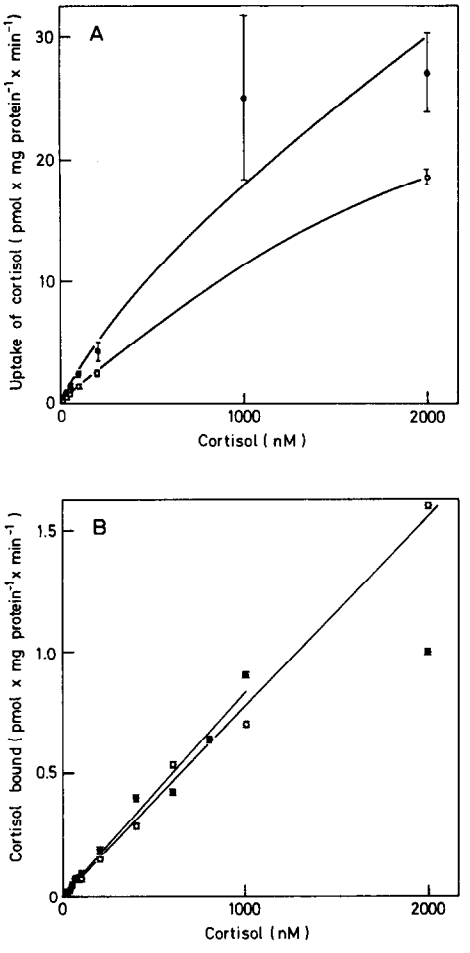

Fig. 5. Initial rates of uptake of cortisol by rat liver cells and of binding of cortisol to cytosol of rat liver in the absence and presence of 2000 nM dexamethasone. The tubes contained increasing concentrations of nonlabeled cortisol and a constant amount of [^{3}H]-cortisol (2×10^5 d.p.m.) in Hepes III buffer, pH 7.4. Incubations were started by pipetting 0.5 mg protein and were carried out at 27°C for 50 s. (A) Uptake of cortisol by liver cells; ●——●, without dexamethasone and ○——○, in the presence of 2000 nM dexamethasone. (B) Binding of cortisol to liver cytosol; ●——●, without dexamethasone and ○——○, in the presence of 2000 nM dexamethasone.

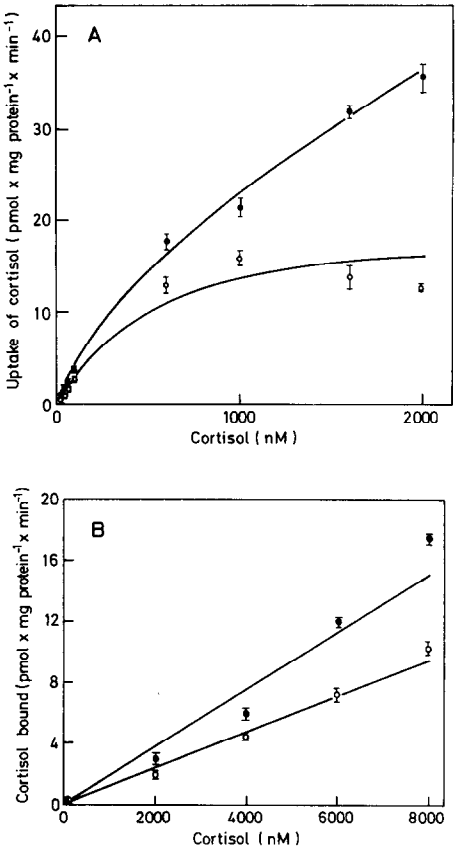

Fig. 6. Effect of pCMB on the initial rates of uptake of cortisol by liver cells and of binding of cortisol to cytosol of rat liver. The liver cells and the cytosol were treated for 2 h at 2°C with 1 mM pCMB. The tubes contained increasing concentrations of nonlabeled cortisol and a constant amount of [^{3}H]-cortisol (2×10^5 d.p.m.) in Hepes III buffer, pH 7.4 and 1 mM pCMB. Incubations were carried out with 0.5 mg protein at 27°C for 50 s. (A) Uptake of cortisol by liver cells; ●——●, without 1 mM pCMB; ○——○, in the presence of 1 mM pCMB. (B) Binding of cortisol to liver cytosol; ●——●, without pCMB and ○——○, in the presence of 1 mM pCMB.

Influence of sulfhydryl reagents

It has been shown that sulfhydryl reagents can inhibit the binding of steroids to receptor proteins [11, 17]. In the present studies 1 mM pCMB partially inhibited the uptake of cortisol by liver cells; the binding of cortisol to cytoplasmic proteins was also reduced (Fig. 6A and B). Thus intact —SH groups are necessary for the uptake of cortisol by liver cells as well as for the binding of cortisol to cytoplasmic proteins.

Influence of metabolic inhibitors

The studies on the influence of metabolic inhibitors such as 2,4-dinitrophenol and KCN on uptake of cortisol by liver cells indicated that metabolic energy may be involved in the uptake process of the cells. Comparative studies on the influence of KCN (1 mM) on liver cells and cytosol showed that uptake of cortisol by cells is inhibitied by KCN (Fig. 7A), whereas binding of cortisol by cytoplasmic proteins was not affected to any significant extent (Fig. 7B).

CONCLUSION

Freshly isolated viable rat liver cells show properties of a living system [18, 19] such as the capability to synthesize glucose from pyruvate and alanine. These viable cells also translocate cortisol from the medium into the cells. It may be speculated that uptake systems studied with liver cells *in vitro* could reflect processes which take place *in vivo*. In the present investigation the uptake of cortisol by viable liver cells has been compared to the binding of this compound by the cytosol fraction. The results show that the transport of cortisol into the cell is mediated by

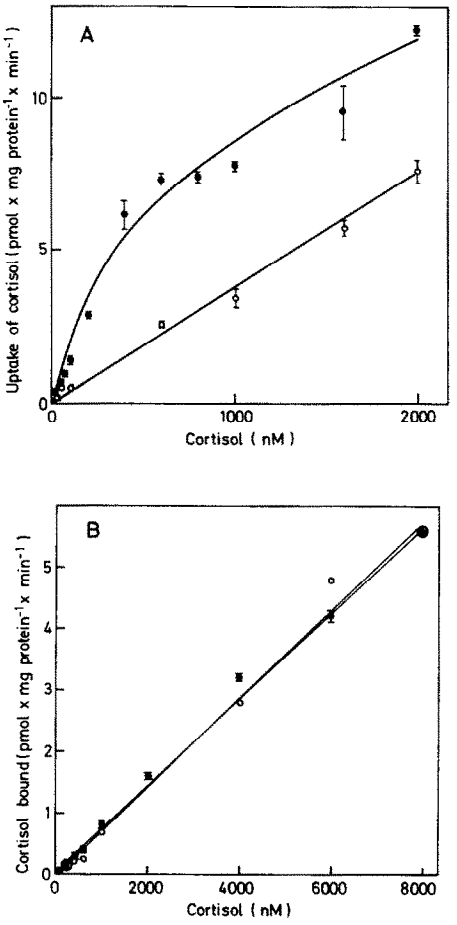

Fig. 7. Effect of KCN on the initial rates of uptake of cortisol by liver cells and of binding of cortisol to cytosol of rat liver. The liver cells and the cytosol were treated for 2 h at 2°C with 2 mM KCN. The tubes contained increasing concentrations of nonlabeled cortisol and a constant amount of [^{3}H]-cortisol (2×10^5 d.p.m.) in Hepes III buffer, pH 7.4 and 2 mM KCN. Incubations were carried out with 0.5 mg protein at 27°C for 50 s. (A) Uptake of cortisol by liver cells; ●——●, without KCN and ○——○, in the presence of 2 mM KCN. (B) Binding of cortisol to liver cytosol; ●——●, without KCN and ○——○, in the presence of 2 mM KCN.

carrier protein(s) located in the membrane and that it is unlikely that cytoplasmic proteins contribute to the transference of cortisol. The biphasic nature of the temperature curve points to changes in the structure of the lipid components in the plasma membrane. Thus the carrier protein seems to be closely associated with the lipid components of the plasma membrane. Binding of cortisol to cytoplasmic proteins at 4°C proceeds at a much faster rate than the uptake by liver cells; thus access of cortisol to cytosol proteins is limited by a membrane barrier.

These results suggest that the plasma membrane functions in the translocation of cortisol from the extracellular space to the inside of the cell and that cytoplasmic proteins are not directly involved in this process.

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DISCUSSION

Liddle. I have just a comment that should please Dr. Rao. His data using hepatocytes resemble very much Dr. Robert Harrison's data using isolated pituitary cells that make ACTH. The process of transport into cell is modified by somewhat different inhibitors in his studies. He finds the process can be inhibited by the enzymes phospholipase and neuraminidase but not by trypsin and pronase.

Rao. Yes, I am familiar with the work of Drs. Harrison, Fairfield and Orth. It somewhat surprises me that proteolytic enzymes did not have any effect on the uptake of glucocorticoid by cells from these tumours, but as you mentioned, it might be due to the different type of cells used by them.

Clark. We have done some imperfect experiments along these lines, what happens at 37°?

Rao. As I said, at 37°C the initial rates of uptake are linear up to 45 sec and these experiments were done at 27°C. The incubations were all done in triplicate or some-

times quadruplicate. To be able to manage the starting and stopping of the incubations one has to have some time and we could easily do this with a time period of 50 sec. If you carry out the incubations at 37°C you have to choose time periods of less than 30 sec, which we found were difficult to manage.

Clark. Yes that's very difficult, but when we tried with uterus and also pituitary cells at 37° and we see no saturable phenomen is lowered.

Rao. I just showed the uptake at 37°C; if one prolongs the incubation time over the 30 sec limit, say till about 4 min, we see that cortisol is being metabolized. We did substrate saturation curves at lower temperatures and were able to detect saturability.