Journal of Steroid Biochemistry, Vol. 10, pp. 573 to 579
Pergamon Press Ltd 1979. Printed in Great Britain

KINETIC STUDIES ON THE MECHANISM OF GLUCOCORTICOID INHIBITION OF HEXOSE TRANSPORT IN RAT THYMOCYTES*

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(*Received* 3 *November* 1978)

SUMMARY

Glucocorticoids decrease uptake of glucose, transport of 3-O-methylglucose and formation from glucose of glucose-6-P in rat thymus cells. The parallel nature of these effects has led to the hypothesis that the principal underlying action of cortisol is to decrease hexose transport. The present studies, utilizing a short pulse technique for measuring transport of radio-labelled 3-O-methylglucose and glucose, support this hypothesis by demonstrating that the effects on transport exhibit the same glucocorticoid specificity as demonstrated earlier for effects on glucose metabolism, and that the time course and magnitude of the cortisol effects on glucose and 3-O-methylglucose transport are almost identical to those previously measured on glucose-6-P. With 3-O-methylglucose the inhibitory effects of cortisol are observed both on influx and efflux. Kinetic measurements on 3-O-methylglucose transport at several temperatures furthermore show that cortisol significantly decreases V_{max} but has only marginal effects on K_M. With a "carrier" model for hexose transport, a decrease in V_{max} can be explained by a decrease in either carrier mobility or number. The following results point to a decrease in effective carrier number: (a) the relative magnitude of the cortisol effect on V_{max} remains approximately the same at temperatures from 23° to 41°C, despite a tenfold increase in V_{max}; (b) the cortisol effect remains intact in cells exposed to anaerobic conditions, which are thought to stimulate uptake by increasing carrier mobility. The postulated decrease in carrier number produced by cortisol is unlikely to be due to a decreased rate of carrier synthesis since the carrier half-life, as estimated by blocking protein synthesis with cycloheximide, far exceeds 2 h, whereas the cortisol effect becomes apparent within 20 min.

Physiological concentrations of glucocorticoids inhibit glucose metabolism by normal and neoplastic lymphoid cells [1]. With rat thymus cells this inhibition has been measured as decreased glucose uptake and decreased generation of glucose-6-P [2]. The latter effect can be detected by means of a 5-min glucose pulse as early as 15–20 min after exposure to 1.0 μM cortisol at 37°C. These and other results have led to the hypothesis that cortisol decreases glucose entry into thymus cells [3]. Rosen *et al.* drew similar conclusions from studies of glucocorticoid inhibition of 2-deoxy-glucose metabolism in lymphosarcoma cells [4]. The ability of cortisol to decrease uptake of 3-O-methylglucose, a hexose that is not metabolized by thymus cells [5], has provided more direct evidence for a glucocorticoid action on sugar entry [6, 7].

The purpose of the present work has been to characterize kinetically the effect of cortisol on glucose and 3-O-methylglucose uptake under various conditions of temperature and metabolic state. Our results suggest that cortisol acts to decrease the number of effective sugar carriers. Some of this work has already been reported briefly [7].

* This work was supported by U.S.P.H.S. Research Grant AM 03535.

† KRB: Krebs–Ringer bicarbonate buffer.

EXPERIMENTAL PROCEDURES

Male CD rats (Charles River Breeding Laboratories) were adrenalectomized 4–14 days prior to use and maintained on 1% NaCl and Labena (Ralston–Purina). Thymus glands were rapidly removed from decapitated rats and prepared in KRB† in equilibrium with 95% O_2–5% CO_2 as described previously [8]. Incubations were carried out in neoprene-stoppered 10-ml Erlenmeyer flasks. KRB alone for controls, and KRB with steroids and metabolic inhibitors in solution, were added to flasks in 10–35 μl volumes. Incubations were begun by adding 0.3–0.5 ml of cell suspension (at cytocrits of 0.1–0.4 ml packed cells per ml cell suspension) to flasks pregassed with 95% O_2–5% CO_2. The flasks were shaken at approx. 40 oscillations per min in a temperature-controlled water bath.

Transport assay. The pulse assay for uptake of radiolabeled glucose and 3-O-methylglucose has been described in detail elsewhere [9]. Briefly, it consists in adding 20 μl of a solution of radiolabeled sugar in KRB to an incubating cell suspension from which, after a fixed interval of incubation, a 20-μl aliquot is removed, immediately cooled and centrifuged through a layer of KRB at 0°C. The supernatant is discarded and the cell pellet is placed directly in scintillation fluid for counting. When several concen-

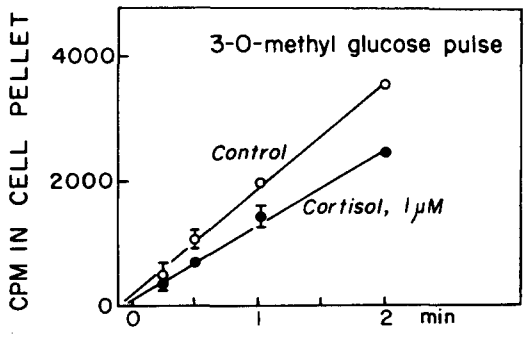

Fig. 1. Early time course of 3-O-methylglucose uptake by transport assay at 37°C. Triplicate aliquots of thymus cell suspensions were incubated for 60 min at 37°C with (●) and without (○) 1 μM cortisol. At 60 min 3-O-[^{14}C]methylglucose was added to give a final concentration of 1 nM. After the indicated times 20-μl aliquots were removed for determination of the radioactivity that entered the cells, as described in Methods. Each point, representing cpm in the cell pellet, is the mean of the three separate incubations. Vertical lines give ±1 SE where these are larger than the symbols.

trations of a radiolabeled sugar in substrate amounts are used, radiolabeled sugar is added to the highest concentration of substrate and lower concentrations obtained by dilution.

For most of the present studies we have subtracted a "blank" value to correct for extracellular contamination of the cell pellet. The blank is determined by incubating cells for 10 s at 0–3°C with the same concentration of sugar used for the assay, then obtaining a cell pellet as usual. The counts are subtracted from the counts of the transport assay pellet. These blank values ranged from 10% of the total count in a cell pellet for the lowest sugar concentration used in a pulse to 50% for the highest. Uptake of 3-O-methylglucose determined by this method is essentially linear over the first 2 min (Fig. 1). The pulse times used for each experiment are given in the figure captions.

Anaerobic conditions. Anaerobiosis was produced by adding 10 μl of a solution of NaCN in water to the cell suspension to give a final concentration of 1 mM. In preliminary experiments we ascertained that this treatment stimulated glucose uptake and decreased ATP, glucose-6-P, and glucocorticoid receptor levels in thymus cells in a similar manner to gassing with 95% N_2–5% CO_2, a procedure used in earlier studies to produce anaerobiosis [2, 10]. NaCN also stimulated uptake of 3-O-methylglucose. This finding is in agreement with other work showing that anaerobiosis produced by argon causes stimulation of 3-O-methylglucose uptake by rat thymocytes [5]. Radiolabeled sugar uptake was measured in the same way as described for aerobic conditions. ATP was assayed by the firefly luciferase method [11].

Materials. D-[U-^{14}C]-glucose (150–250 Ci/mol), D-3-O-[methyl-^{3}H]-methylglucose (4–5 Ci/mmol), and D-3-O-[methyl-^{14}C]methylglucose (20–55 Ci/mol) were obtained from New England Nuclear. Substrate amounts of unlabeled sugars were obtained in Grade A purity from Calbiochem. Cortisol, cortisone, and cortexolene* were obtained in Grade B purity from Calbiochem and recrystallized four times from methanol–hexane. Purity was checked by melting point determination. Cycloheximide, and reagents for the ATP assay were obtained from Sigma.

* Cortexolone: 17,21-dihydroxypregn-4-ene-3, 20-dione.

RESULTS AND DISCUSSION

Glucocorticoid specificity and time course of the cortisol effect on 3-O-methylglucose transport

The results in Fig. 2 show that the effects of the various steroids tested on 3-O-methylglucose transport into thymus cells are in good agreement with previously demonstrated effects on glucose uptake [8, 10]. Cortisol at 1 μM in a 1-h incubation inhibits by about 25% and cortisone is essentially inactive. Cortexolone at 10 μM, though somewhat inhibitory by itself, acts as an anti-glucocorticoid since it clearly reduces the effect of cortisol. These effects in turn can be understood in terms of the affinities of the steroids for glucocorticoid receptors in thymus cells [1, 10].

Figure 3 shows the time courses of inhibition at 37°C by 1 μM cortisol of 3-O-methylglucose and glucose uptake, measured with 2-min pulses of radiolabeled glucose or 3-O-methylglucose. These time courses are almost identical to that found for inhibition by cortisol of glucose-6-P formation using 5-min pulses of substrate amounts of glucose [2]; all are characterized by the abrupt appearance of inhibition after 15–20 min exposure to cortisol. In magnitude the inhibition of 3-O-methylglucose uptake is usually somewhat smaller than that of glucose uptake and glucose-6-P formation.

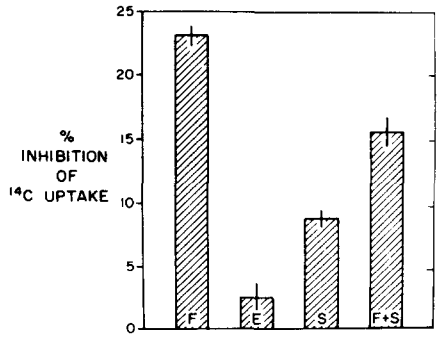

Fig. 2. Effect of cortisol, cortisone, and cortexolone on uptake of 3-O-methyl-glucose after 1 h exposure to hormone. Quadruplicate aliquots of cell suspension were incubated for 1 h at 37°C without steroid or with 1 μM cortisol (F), 1 μM cortisone (E), 10 μM cortexolone (S), and 1 μM cortisol plus 10 μM cortexolone (F + S). At 1 h, 2-min pulse assays with 1 nM 3-O-[^{14}C]methylglucose were performed as described in Methods. Values are the mean percent inhibitions relative to controls. Vertical lines give ±1 SE.

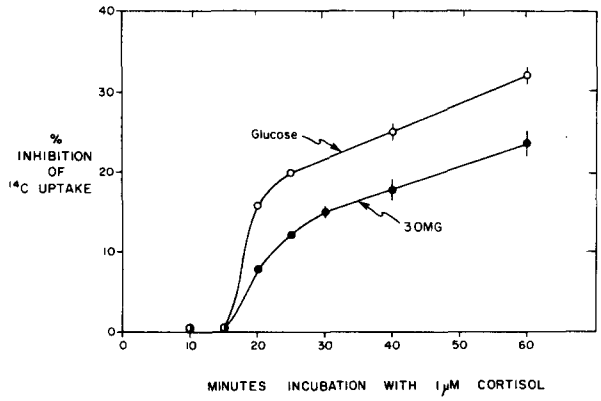

Fig. 3. Time course of cortisol inhibition of 3-O-methylglucose and glucose uptake. Aliquots of thymus cells were incubated at 37°C with or without 1 μM cortisol. At the times indicated, 2-min pulse assays were performed in triplicate with [^{14}C]-glucose or 3-O-[^{14}C]-methylglucose (30MG) at 1 nM. In these experiments the blank correction described in Methods was not applied. Each point is the mean percent inhibition of cortisol-treated aliquots relative to controls of 6–12 incubations with cortisol from several independent experiments. Vertical lines indicate ±1 SE where these are larger than the symbols.

These results provide strong evidence that inhibition of hexose transport as measured by inhibition of 3-O-methylglucose uptake accounts for a major part of the cortisol effect on glucose and glucose-6-P.

Counter-transport of 3-O-methylglucose by glucose; carrier model

Counter-transport of 3-O-methylglucose induced by addition of glucose is demonstrated by the results in Fig. 4, which confirm the observations of Reeves[5] obtained using considerably different methods. Reeves also demonstrated counter-transport under anaerobic conditions. This phenomenon points to the existence in thymus cells of a system for hexose transport consistent with a "carrier" model [12]. It also indicates strongly that glucose and 3-O-methyl glucose are transported by a common carrier, supporting our other evidence that glucose and 3-O-methylglucose can be used interchangeably for measuring the glucocorticoid effect on transport. Similar conclusions regarding hexose transport in thymus cells have also been reached by other experimental routes [5, 13].

The kinetic data presented below will accordingly be interpreted in terms of a simple carrier model [12, 13] consisting of a single mobile carrier that can traverse the membrane, bind sugars at both surfaces and transport sugars in either direction.

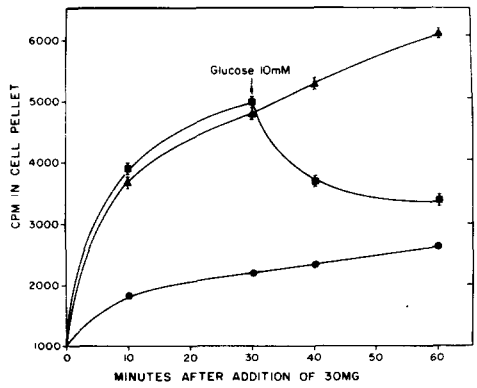

Fig. 4. Counter-transport of 3-O-[^{14}C]methyl-glucose by glucose at 37°C. 3-O-[^{14}C]methyl-glucose was prepared to give a final concentration of 0.1 mM in the incubation medium. A solution of glucose was prepared such that 20 μl added to a flask would produce a concentration of 10 mM. At zero time flasks received either 3-O-[^{14}C]methylglucose alone (■, ▲), or 3-O-[^{14}C]methyl-glucose and glucose (●). After 30 min incubation at 37°C, glucose was added to one group of flasks incubating with 3-O-[^{14}C]-methyl-glucose alone (■). Samples for measurement of [^{14}C] uptake were taken at the times indicated. Each point is the mean of four incubations. Vertical bars give ±1 SE where larger than the symbols.

Kinetics of 3-O-methylglucose transport and cortisol effects at various temperatures; carrier number versus mobility

Figure 5 illustrates the kinetics of 3-O-methylglucose uptake by thymus cells after incubation at 37°C with or without 1 μM cortisol. Figure 6 shows comparable results for 41°C and 23°C. Data are plotted as S/v, the ratio of 3-O-methylglucose concentration (S) to rate of 3-O-methylglucose uptake (v), against S. This plot has been shown to reduce weighting errors incurred in regression analysis when compared to the Lineweaver–Burk double reciprocal plot [14]. The classic kinetic parameters K_M and V_{max} are given, respectively, by the negative intercept on the abscissa and the reciprocal of the slope [14]. These values are collected in Table 1.

Results are given for three pulse temperatures. Those for 37°C show that after 25 min exposure to cortisol, a time soon after the effects of cortisol on 3-O-methylglucose uptake have become manifest, cortisol has reduced V_{max} relative to the control without effecting a change in K_M. By 60 min V_{max} has decreased further, but K_M has decreased as well. We interpret these data as indicating a primary effect of

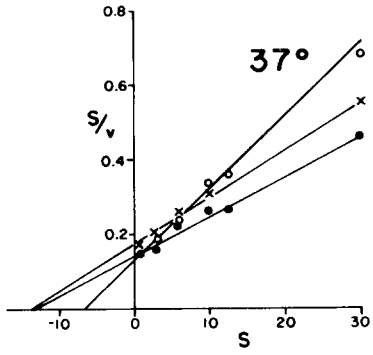

Fig. 5. Kinetic analysis of uptake of 3-O-methylglucose at 37°C by cells incubated with or without cortisol at 37°C. Thymus cells were incubated at 37°C for 25 (×) or 60 min (○, ●) with (○, ×) and without (●) 1 μM cortisol. Radiolabeled 3-O-methylglucose was then added to each flask to give a final concentration S (mM). Uptake was assayed after 2 min as described in Methods, and calculated as v (μmol per h per ml packed cell volume). Straight lines were fitted to the points by least squares and used to calculate K_M (mM) from the negative intercept on the abscissa, and V_{max} (μmol per h per ml packed cell volume) from the reciprocal of the slope [14]. These values are given in Table 1.

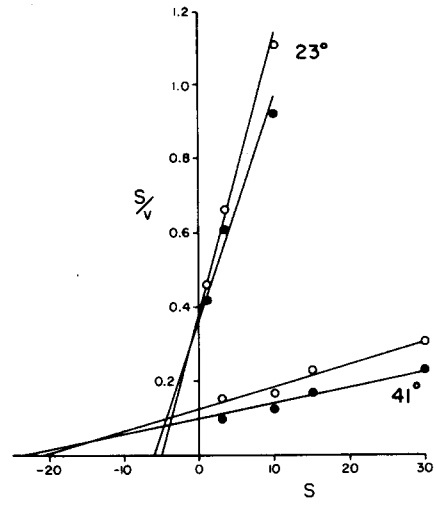

Fig. 6. Kinetic analysis of uptake of 3-O-methylglucose at 41°C and 23°C by cells incubated with or without cortisol at 37°C. Thymus cells were incubated at 37°C for 60 min with (○) and without (●) 1 μM cortisol. The flasks with the cells were transferred to baths at 41°C or 23°C and shaken for 5 min. Radiolabeled 3-O-methylglucose was then added to each flask to give final concentrations (mM). Uptake was assayed after 1 min at 41°C and 2 min at 23°C as described in Methods, and calculated as v (μmol per h per ml packed cell volume). Straight lines were fitted to the points by least squares and used to calculate K_M (mM) from the negative intercept on the abscissa, and V_{max} (μmol per h per ml packed cell volume) from the reciprocal of the slope [14]. These values are given in Table 1.

cortisol on factors controlling V_{max}, with possible secondary effects on K_M. The K_M value of 13.7 ± 1.3 mM for controls compares well with the value of 12.1 ± 2.0 obtained by Reeves[5] using a different technique for preparing cells and different buffers. His values for V_{max} are somewhat lower than ours. Whitesell *et al.*[13], also using different techniques, report a K_M at 35°C of 7.7 mM.

In terms of the carrier model mentioned above, a decrease in V_{max} such as that produced by cortisol is consistent with a decrease either in the mobility of the carriers or in the effective number of carriers. Kinectic methods alone cannot discriminate between these two mechanisms. Comparison of the cortisol effects at different temperatures provides some insight, however.

Referring to the results in Table 1, it can be seen that the cortisol effect on V_{max} remains almost unchanged over the temperature range from 23°C to 41°C despite a 10-fold increase in V_{max}. This observation is most easily understood in terms of a cortisol effect on carrier number, since if cortisol inactivates a given fraction of carriers, the fractional cortisol effect on V_{max} will of necessity remain constant, regardless of temperature, as long as V_{max} is propor-

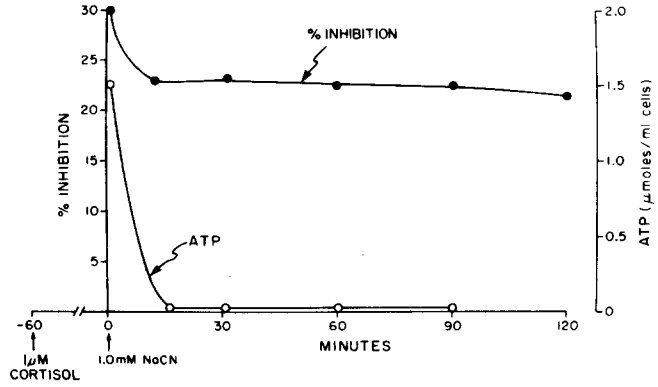

Fig. 7. Effect of anaerobiosis on the cortisol effect initiated under aerobic conditions for 1 h. Aliquots of cell suspension were incubated with or without 1 μM cortisol for 1 h under aerobic conditions at 37°C. At 60 min a NaCN solution was added to give a final concentration of 1 mM. Paired flasks were pulsed at the indicated times with a 5-min pulse of 5.5 mM [^{14}C]-glucose for assay of the cortisol effect in the usual manner. Immediately afterwards aliquots were removed for ATP assay. The points for percent inhibition represent means of duplicate incubations from a representative experiment. ATP levels are the means of duplicate determination of two flasks for each time point.

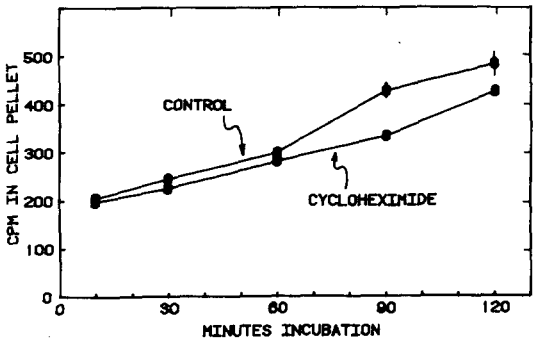

Fig. 9. Effect of cycloheximide on the uptake of 3-O-[^{14}C]methyl-glucose. Thymus cell suspensions were incubated from time zero at 37°C in KRB alone (control) or in KRB containing 10 μM cycloheximide. After the times indicated, duplicate flasks were pulsed for one minute with 3-O-[^{14}C]-methylglucose for assay of 3-O-methylglucose transport as described in Methods. Each point is the mean of two flasks, and represents the amount taken up by the cells in the 1-min pulse after subtraction of a blank. Vertical lines represent ranges where these are larger than the symbols.

Effect of cortisol on efflux of 3-O-methylglucose from thymus cells

The simple carrier model predicts that if cortisol decreases the number carriers in thymus cells, a decrease in 3-O-methylglucose transport must be present on both influx and efflux of the sugar. The rsults in Fig. 8 show that, in accord with this prediction, the rate of efflux of 3-O-methylglucose is lower from cortisol treated cells. This finding is consistent with the hypothesis that cortisol decreases carrier number, but does not exclude an effect on carrier mobility. It takes on added significance in view of the conclusion of Whitesell *et al.*[13] that the carrier system in thymus cells is not symmetrical with respect to influx and efflux, a conclusion that emphasizes the possibility, excluded by our data, that the hormone effect might be expressed on one but not the other of these parameters.

Effect of cycloheximide on 3-O-methylglucose transport

One mechanism by which cortisol could reduce carrier number is by reducing the rate of carrier synthesis. The rapidity of the cortisol effect, however, would require that the carrier have a very short lifespan. To test this possibility we incubated thymus cells for up to 2 h with or without cycloheximide at 10^{-5} M, a concentration known to inhibit more than 90% of protein synthesis [18, 19], and measured 3-O-methylglucose uptake at intervals with 1-min pulses. Figure 9 shows the results. No difference in uptake between cycloheximide-treated and control cells appears until after 60 min, so if carrier synthesis was blocked by cycloheximide and the rate of inactivation was unaltered, the carrier must have a half-life much greater than 1 h. This time is far too long to account for the 15–20 min cortisol effect on transport. (We have no explanation for the increase in transport rate with incubation time shown in Fig. 9, though it is a fairly regular observation).

It thus seems unlikely that cortisol inhibits transport by decreasing carrier synthesis. Taking into account the earlier observations that led us to the hypothesis that glucocorticoids in thymus cells induce the synthesis of a protein that inhibits glucose transport [1, 18], the present results, interpreted within the framework of conventional transport models, suggest that this protein directly or indirectly inactivates a fraction of the glucose "carriers".

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Table 1. Kinetic parameters, V_{max} and K_M, estimated from the results in Fig. 5

Incubations at 37°C					
Cortisol (μM)	Time (min)	Number	Pulse temp. (°C)	V_{max} (μmol per h per ml cell vol.)	K_M (mM)
0	60	28	41	223 (190–261)	22.5
1	60	24	41	172* (152–187)	22.6
0	25, 60	62	37	94 (83–104)	13.7 (11–6)
1	25	25	37	77* (69–88)	13.8 (11–17)
1	60	53	37	51* (43–61)	6.6* (3–10)
0	60	18	23	21.8 (20–23)	6
1	60	17	23	12.7* (12–14)	5

* These values differ significantly ($P < 0.05$) from those obtained without cortisol under the same conditions.

V_{max} and K_M are given as means and 95% confidence limits.

Number of incubations refers to the total number used with the various pulse concentrations S indicated in Fig. 5. Values for 37°C pulses after 25 and 60-min incubations without cortisol appeared indistinguishable and were pooled.

tional to the number of active carriers. No such simple relationship would be expected if the cortisol effect was due primarily to restriction of carrier mobility.

Cortisol effect on hexose uptake under anaerobic conditions

Additional evidence for the cortisol effect being on carrier number rather than mobility emerges from the following studies utilizing anaerobic conditions. Anaerobiosis stimulates transport of glucose and 3-O-methylglucose, and depresses cellular ATP levels in thymus cells [2, 5, 10]. Stimulation of sugar transport in response to anaerobiosis is thought to be due to increased carrier mobility [15]. If this view is correct and the effect of cortisol is to reduce carrier number, then anaerobiosis would not be expected to eliminate the cortisol effect on transport.

Our previous studies have shown that the cortisol effect does not develop when cells are exposed to cortisol under anaerobic conditions [2]. The inactivity of cortisol appears to be due to at least two defects, one at the level of binding to glucocorticoid receptors, and the other at a later step [10]. Up to now, however, the question of whether a cortisol effect, generated initially under aerobic conditions, will persist when cells are subsequently exposed to anaerobic conditions, has not been answered.

The results in Fig. 7 show clearly that the cortisol effect does persist, remaining almost unchanged for up to 2 h under anaerobic conditions. The drop in ATP levels measured during the same experiment demonstrates the effectiveness of the cyanide treatment. For practical reasons the experiment was carried out using glucose rather than 3-O-methylglucose, but as argued above, a glucose pulse probably gives as good a relative measure of hexose transport as a 3-O-methylglucose pulse.

Persistence of the cortisol effect under anaerobic conditions supports the conclusion from the previous experiments that in terms of a simple carrier model, cortisol reduces the effective number of carriers. This conclusion is also in accord with the observation that glucocorticoid inhibition of transport persists in thymus cells in which transport is stimulated with insulin [16].

The findings in Fig. 7 also show that the second defect mentioned above is not at the level of glucose transport or beyond, and thus presumably must lie in some of the intermediate steps of stimulation of mRNA and protein synthesis that have been postulated to transmit the hormonal "message" from the glucocorticoid-receptor complex to the glucose transport system [17, 18].

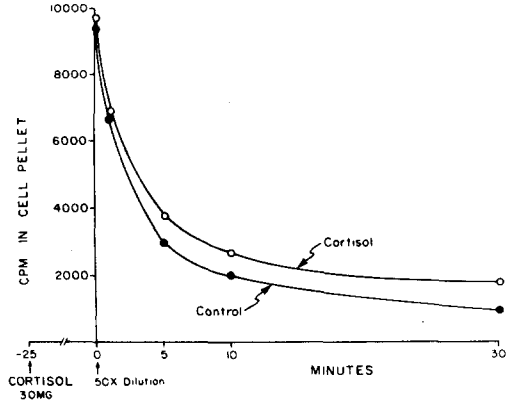

Fig. 8. Effect of cortisol on efflux of 3-O-[^{3}H]-methyl-glucose. Thymus cells were incubated at 37°C with 5.5 mM 3-O-[^{3}H]-methyl-glucose (30MG) for 28 min in the presence of 1 μM cortisol and for 25 min in the case of the control without cortisol. At these times the cell suspensions were diluted 50-fold into KRB at 37°C. Subsequently, 300-μl samples were removed at the intervals indicated on the abscissa and centrifuged to obtain cell pellets that were counted for radioactivity. Each point is the mean of seven determinations. Zero-time points were obtained immediately before dilution by taking triplicate 20-μl samples of the cell suspension and counting the cell pellets after centrifugation. Standard errors are smaller than the size of the symbols.

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