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CORTISOL-INDUCED INHIBITION OF AMINO ACID TRANSPORT IN THYMIC LYMPHOCYTES:

KINETIC PARAMETERS; RELATION TO ATP LEVELS AND PROTEIN SYNTHESIS; AND SPECIFICITY

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SUMMARY

1. α-Aminoisobutyric acid accumulation in rat thymic lymphocytes increased in linear fashion for 10 min and reached an apparent steady state in 60 min. α-Aminoisobutyric acid was transported by concentrative and non-concentrative processes. Cortisol inhibited only the concentrative process. Inhibition was non-competitive, indicating a decrease in the total capacity of the active transport mechanism. The effect of cortisol on α-aminoisobutyric acid transport was evident only after 60–90 min of treatment but preceeded the appearance of reductions in cellular ATP by an additional 60–90 min, suggesting that cortisol inhibition of α-aminoisobutyric acid transport was not caused by the decrease in ATP.

2. Inhibition of protein synthesis with cycloheximide reduced α-aminoisobutyric acid uptake without decreasing cellular ATP, pointing to the participation of labile proteins in the active transport of α-aminoisobutyric acid which are not related to ATP metabolism. Inhibition of α-aminoisobutyric acid transport caused by cortisol and cycloheximide together was no greater than that caused by each agent alone. Moreover, cortisol failed to decrease cellular ATP levels during simultaneous exposure of thymic lymphocytes to cycloheximide. Hence, inhibition of α-aminoisobutyric acid transport and reduction of ATP content are separate manifestations of cortisol action, each arising by a process which depends upon *de novo* protein synthesis.

3. Inhibition of α-aminoisobutyric acid transport by cortisol appeared to be related to its glucocorticoid activity since (a) low concentrations (0.1 μM and 1.0 μM) were inhibitory, yet steroids devoid of glucocorticoid activity (progesterone, cortisone and testosterone) were non-inhibitory at the same concentrations, and (b) 11-deoxycortisol (cortexolone), which is known to impede the binding of glucocorticoids to cellular receptors, appreciably reduced the degree of inhibition caused by cortisol. High concentrations of progesterone and cortisone (10 μM) inhibited α-aminoiso-

Abbreviations: ECW, extracellular trapped water; ICW, total pellet water *minus* extracellular trapped water.

butyric acid transport slightly suggesting that the decrease in α-aminoisobutyric acid uptake caused by 10 μM cortisol might reflect both specific (glucocorticoid-related) and non-specific effects.

INTRODUCTION

Treatment with adrenal glucocorticoids is known to inhibit the transport of certain free amino acids in lymphoid cells[1–4] and in other types of mammalian tissue[5–10]. Three lines of evidence suggest that inhibition arises indirectly; (a) it is delayed in onset and does not disappear rapidly upon cessation of treatment, (b) simultaneous blockade of RNA or of protein synthesis during the initial (inductive) phase of treatment nullifies the cortisol effect, and (c) inhibition is less marked when lymphoid cells are incubated in substrate-free medium. Taken together with the demonstration that cortisol rapidly inhibits glucose uptake and impairs carbohydrate dependent ATP generation in lymphoid cells[11–20], these observations are consistent with the hypothesis that glucocorticoids inhibit amino acid transport by interfering with glucose utilization and ATP production through a prior alteration in RNA and protein synthesis. According to currently held concepts, these events are triggered by the binding of cortisol to glucocorticoid specific cytoplasmic and then nuclear receptors[21].

Mammalian cells are known to possess multiple systems for amino acid transport, including energy requiring, concentrative, carrier-mediated systems, and systems which are neither concentrative, nor energy requiring (non-active), but which may be carrier mediated (see ref. 22 for review). If cortisol inhibition of amino acid transport were secondary to interference with glucose utilization or ATP production, one would predict that (a) cortisol should inhibit active but not non-active amino acid transport, and decrease the total capacity (V) of the active mechanism, (b) simultaneous inhibition of protein synthesis, or blockade of cytoplasmic receptors, should nullify the effects of cortisol on amino acid transport, and (c) decreases in ATP should precede or coincide with decreases in amino acid transport. To test this hypothesis, we have examined the effects of cortisol on the kinetics and energetics of active transport of the non-metabolizable amino acid, α-aminoisobutyric acid in rat thymic lymphocytes. We have compared the effects of cortisol with cycloheximide, a known inhibitor of protein synthesis, and finally we have studied the effects of other steroids on α-aminoisobutyric acid transport in order to determine whether the cortisol inhibition of α-aminoisobutyric acid transport is a specific glucocorticoid-related action.

METHODS AND MATERIALS

Hank's balanced salt solution was obtained from Grand Island Biologicals, radioactively labeled compounds from New England Nuclear, bovine serum albumin (four times recrystallized) from Nutritional Biochemicals, cycloheximide from Upjohn, cortisol, progesterone, testosterone and cortisone of the highest purity from California Corporation for Biochemical Research, and 11-deoxycortisol (cortexolone) and firefly lantern extract (Luciferase) and ATP from Sigma Chemical Co.

Isolation of rat thymic lymphocytes

100–150 g male Sprague–Dawley rats were sacrificed by decapitation, their thymus glands removed, cleaned of extraneous tissue and rinsed in Hank's balanced salt solution. Thymus cells were dispersed with three gentle strokes of a Dounce homogenizer (loose fitting pestle) and passed twice through fine mesh gauze to remove large clumps of cells and connective tissue. Over 95% of the cells so isolated were found to be small lymphocytes by staining characteristics and by phase microscopy.

Amino acid transport

Isolated, washed thymic lymphocytes were suspended in Hank's balanced salt solution containing 0.05% bovine serum albumin at a final concentration of $2 \cdot 10^8$–$2.5 \cdot 10^8$ cells/ml; 400-μl aliquots of this suspension were added to 25 ml polypropylene flasks containing 2 ml of Hank's balanced salt solution. All incubations were performed at 37 °C (unless otherwise specified) with gentle shaking (5–10 cycles/min) in a Dubnoff shaking incubator, in air. Cortisol, other steroids, cycloheximide, and α-aminoisobutyric acid were added at times and in amounts warranted by each experiment. At termination, each cell suspension was transferred into previously chilled 2-ml conical centrifuge tubes ($K_{i\max}$) and centrifuged at $950 \times g$ for 45 s in an International centrifuge at 4 °C. The medium was decanted, the inside walls of the tubes dried with Kimwipes, and the small amount of medium atop the cell pellet removed by gentle blotting with a thin strip of filter paper. The cell pellets were dissolved overnight in 0.3 M KOH at 37 °C, aliquots were transferred to counting vials containing 10 ml of Bray's solution[23], and radioactivity determined by liquid scintillation spectrometry. The degree of quenching was estimated by external standardization, and dpm values calculated. In all experiments, the protein content of the dissolved cell button was estimated by the method of Lowry *et al.*[24].

Estimation of intracellular water content

Replicate treated and untreated cell suspensions were incubated with [^{14}C]-sucrose in place of α-amino[3-^{14}C]isobutyric acid and centrifuged in tared 2-ml conical tubes. The wet weight of the pellet was determined and the cell pellet dried at 80 °C for 18 h. Total pellet water was calculated by subtracting the dry pellet weight from its wet weight. The dry pellet was digested in 0.5 ml of 0.3 M KOH and the radioactivity of 0.1 ml of the digest measured in 10 ml of Bray's solution. Extracellular trapped water (ECW) was calculated by dividing total pellet radioactivity by the radioactivity per 0.1 ml of the incubation medium. ICW (total pellet water *minus* extracellular trapped water) was 65–75% of the total cell weight. Neither steroids nor cycloheximide altered the ECW or ICW after 1–4 h of exposure.

Calculation of intracellular amino acid content and expression of transport data

The intracellular accumulation of labelled amino acid per ml of ICW (A_i, α-aminoisobutyric acid radioactivity/ml of cell water) was calculated from the formula:

$$A_i = \frac{R_t - A_0 \cdot V_e}{V_t - V_e}$$

where R_t equals the net radioactivity of the cell pellet, A_0 the radioactivity/ml of incubation medium, V_e the volume of ECW in ml, and V_t the total pellet water in ml.

Results are expressed either as the distribution ratio (A_i/A_0), where A_i is the radioactivity/ml of cell water and A_0 radioactivity/ml of incubation medium, or as mmoles/l of cell water. The contribution of non-active transport to the accumulation of α-aminoisobutyric acid was estimated according to the method of Akedo and Christensen[25] and kinetic data were adjusted to represent active transport.

Estimation of thymic lymphocyte ATP levels

A 0.5-ml aliquot of each thymic lymphocyte suspension was added to 4.5 ml of distilled water and placed in boiling water for 90 s, immediately chilled on melting ice and frozen in plastic tubes at −20 °C for up to one week before assay. Thymic lymphocyte ATP was measured by a modification of the firefly bioluminescence method[26]. Disodium ATP (Sigma Chemical Co.) over a concentration range of 0.1 to 1 μM was used as standard. 0.1 ml of each thawed thymic lymphocyte lysate, 0.85 ml of Tris–histidine buffer, pH 7.3, containing 80 mM NaCl, 15 mM KCl, 4 mM $MgCl_2$, and 0.05 ml of luciferase previously prepared in distilled water were combined in scintillation counting vials and light emission measured exactly 30 s later in a Nuclear Chicago scintillation spectrometer. All measurements were made in triplicate or quadruplicate.

EXPERIMENTAL RESULTS

Time course of accumulation: effects of cortisol

α-Amino[3-^{14}C]isobutyric acid accumulation in thymic lymphocytes increased rapidly in linear fashion for 10 min and approached an apparent steady state in 60 min at a distribution ratio (A_i/A_0) of 40 (Fig. 1). Treatment with 10 μM cortisol for 4 h markedly diminished the net accumulation of α-aminoisobutyric acid initially as well as at the apparent steady state (Fig. 1). In experiments not shown, we have corroborated the observation of Makman *et al.*[1] that cortisol does not alter the exodus of α-aminoisobutyric acid from thymic lymphocytes.

In order to determine whether cortisol modifies nonactive α-aminoisobutyric acid transport, we examined the accumulation of α-aminoisobutyric acid at extra-

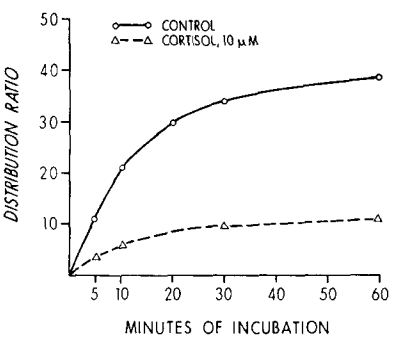

Fig. 1. Thymic lymphocytes were prepared and incubated as described in the text. After 4 h of incubation with or without cortisol, α-amino[3-^{14}C]isobutyric acid (2.7 Ci/mole) was added to a final concentration of 10 μM and the incubation continued for 5–60 min. Hence, the total duration of treatment with cortisol was 245 to 300 min.

cellular α-aminoisobutyric acid concentrations above those which were required to produce a near maximal velocity of uptake (Table I, Experiment I). Accumulation of α-aminoisobutyric acid was non-concentrative at high α-aminoisobutyric acid concentrations, and was not inhibited by cortisol. α-Aminoisobutyric acid accumulation was markedly reduced during incubation of thymic lymphocytes in the absence of extracellular Na^+ and cortisol failed to inhibit Na^+-independent α-aminoisobutyric acid uptake (Table I, Experiment II).

TABLE I

EFFECT OF CORTISOL ON NON-ACTIVE α-AMINOISOBUTYRIC ACID ACCUMULATION

In Expt I, cells were maintained in Hank's balanced salt solution throughout. α-Amino[3-^{14}C]-isobutyric acid (2.7 Ci/mole) was added either 5 min (Expt II) or 2 min (Expt I) before termination. High concentrations were achieved by diluting radioactively labelled α-aminoisobutyric acid with non-labelled α-aminoisobutyric acid before addition to the medium. Each value represents mean of 4 or 5 separate incubation flasks, together with the standard error of the mean (S.E.) In Expt II, thymic lymphocytes were incubated for 3 h in Hank's balanced salt solution with or without 10 μM cortisol, then washed and treated for an additional hour in medium containing 5.6 mM glucose, 10 mM Tris–HCl (pH 7.4), 5 mM KCl, 1 mM $CaCl_2$, 5 mM $MgCl_2$ and either 140 mM NaCl (Na^+) or 140 mM choline chloride (Ch^+).

	A_i/A_0	
	Control	*Cortisol*
Expt I		
25 mM α-aminoisobutyric acid	0.39 ± 0.13	0.39 ± 0.04**
40 mM α-aminoisobutyric acid	0.33 ± 0.01	0.37 ± 0.07**
Expt II		
140 mM Na^+	6.0 ± 0.51	4.0 ± 0.09 (−33%)*
140 mM Ch^+	1.0 ± 0.05	1.1 ± 9.10**

* Differs significantly from control ($P < 0.01$).
** Does not differ significantly from control.

Effects of cortisol on the kinetic parameters of active α-aminoisobutyric acid transport

It is generally held that free amino acids are actively transported from the outer to the inner surface of the plasma membrane by a carrier-mediated process which derives energy from ATP. Cortisol is known to impair glucose uptake and glucose-linked ATP generation in lymphoid cells[11–20] and could therefore inhibit active α-aminoisobutyric acid uptake by limiting the amount of ATP available for transport. Accordingly, cortisol might be expected to decrease the total capacity (V) of the active transport mechanism. Therefore, we have examined the effects of cortisol on kinetic parameters of transport (V, apparent K_m). In these experiments, cells were treated with 0.1, 1.0 or 10 μM cortisol for 4 h and the initial rate of α-aminoisobutyric acid transport (V_0) was calculated from the estimated intracellular accumulation of α-aminoisobutyric acid over a 5-min period. Graphic representation of V_0 according to the method of Lineweaver and Burk, at α-aminoisobutyric acid concentrations

ranging from 50 μM to 5 mM, was compatible with a single transport process having an apparent K_m of 1.0 mM and V of 14.3 mmoles/kg cell water per 5 min. Treatment with 10 μM cortisol for 4 h markedly decreased V (9.3 mmoles/kg cell water per 5 min) but failed to change the apparent K_m (1.1 mM) appreciably, indicating, according to traditional concepts, a decrease in the total capacity of the transport mechanism. To confirm this observation, we performed additional experiments at 500 μM–5 mM α-aminoisobutyric acid and represented the results in an alternate graphic form V_0 *vs* V_0/Cs (Fig. 2), which has been found to give more reliable estimates for apparent K_m and V[27]. These experiments yielded kinetic parameters for α-aminoisobutyric acid transport which were very similar to those obtained with the Lineweaver and Burk representation, and provide additional evidence that cortisol inhibits α-aminoisobutyric acid transport noncompetitively. The same kinetic perturbation developed at two lower concentrations of cortisol (0.1 and 1.0 μM); that is, a reduction in V without significant change in K_m.

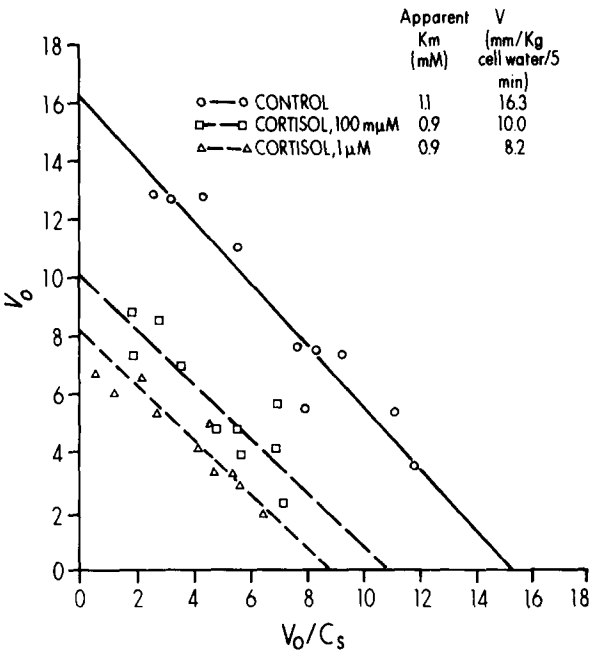

Fig. 2. Conditions of incubation were as described in the text. Cells were treated for 4 h, and α-amino[3-^{14}C]isobutyric acid, together with an appropriate amount of unlabelled α-aminoisobutyric acid, added 5 min before termination. V_0 refers to mmoles of α-aminoisobutyric acid accumulated kg cell water per 5 min *minus* the apparently non-saturable component of uptake. Data represent the means of three separate experiments. Curves were generated from the data by linear regression analysis. Analysis of variance strongly supports the hypothesis that the slopes of the 3 lines are similar ($F=0.01$).

Relationship between the effect of cortisol on α-aminoisobutyric acid transport and ATP content in thymic lymphocytes

The failure of cortisol to inhibit non-concentrative α-aminoisobutyric acid accumulation and the observation that cortisol inhibits active uptake non-competitively are consistent with the concept that cortisol acts by reducing the supply of energy

TABLE II

TIME COURSE OF THE EFFECTS OF CORTISOL (10 μM) ON α-AMINOISOBUTYRIC ACID TRANSPORT AND ON CELL ATP LEVELS

Lymphocytes pooled from the thymus glands of six rats were transferred to incubation flasks and treated with 10 μM cortisol for indicated time periods. Some flasks received α-amino[3-^{14}C]-isobutyric acid 5 min before termination, others to be used for ATP determinations were terminated simultaneously but without prior labelling. Each value represents the mean $\pm$ S.E. of 4–10 separate flasks.

Time (min)	A_i/A_0		*ATP· 10^{-16} moles/cell*	
	Control	*Cortisol*	*Control*	*Cortisol*
60	7.3 $\pm$ 0.3	6.5 $\pm$ 0.2 (−11%)*	1.56 $\pm$ 0.06	1.57 $\pm$ 0.04
90	7.9 $\pm$ 0.2	6.4 $\pm$ 0.2 (−19%)**	1.73 $\pm$ 0.03	1.70 $\pm$ 0.10
120	9.2 $\pm$ 0.6	5.9 $\pm$ 0.2 (−36%)**	1.30 $\pm$ 0.01	1.18 $\pm$ 0.02 (− 8%)**
180	7.8 $\pm$ 0.5	5.1 $\pm$ 0.03 (−35%)**	1.40 $\pm$ 0.04	1.08 $\pm$ 0.02 (−21%)**

* Differs significantly from control ($P < 0.05$).
** Differs significantly from control ($P < 0.01$).

TABLE III

TIME COURSE OF THE EFFECTS OF CORTISOL (1 μM) ON α-AMINOISOBUTYRIC ACID TRANSPORT AND ON CELL ATP LEVELS

Lymphocytes pooled from the thymus glands of five rats were transferred to incubation flasks and treated with 1 μM cortisol for the indicated time periods. Some flasks received α-amino-[3-^{14}C]isobutyric acid for 5 min before termination, others to be used for ATP determinations were terminated simultaneously but without prior labelling. Each value represents the mean $\pm$ S.E. of 6–8 separate flasks.

Time (min)	A_i/A_0		*ATP· 10^{-16} moles/cell*	
	Control	*Cortisol*	*Control*	*Cortisol*
60	7.9 $\pm$ 0.3	7.4 $\pm$ 0.3 (− 6%)	2.72 $\pm$ 0.05	2.67 $\pm$ 0.04
90	8.0 $\pm$ 0.3	6.3 $\pm$ 0.2 (−21%)*	2.65 $\pm$ 0.07	2.70 $\pm$ 0.04
120	7.0 $\pm$ 0.2	5.7 $\pm$ 0.2 (−28%)*	2.44 $\pm$ 0.03	2.38 $\pm$ 0.06 (−3%)
180	8.9 $\pm$ 0.3	5.7 $\pm$ 0.2 (−36%)*	2.34 $\pm$ 0.02	2.13 $\pm$ 0.02 (−9%)*

* Differs significantly from control ($P < 0.01$).

(ATP) for transport. Treatment of thymic lymphocytes with cortisol was found to reduce ATP levels, but significant decreases appeared only after 120 min at 10 μM cortisol (Table II) and 180 min at 1 μM cortisol (Table III). In each case, inhibition of α-aminoisobutyric acid transport preceded the reduction in ATP (Tables II and III).

Role of protein synthesis in cortisol-induced inhibition of transport and reduction in cell ATP

In order to investigate the role of protein synthesis in mediating the effects of

cortisol, we examined α-aminoisobutyric acid transport and ATP levels in thymic lymphocytes treated with cycloheximide and cortisol alone, and with both agents simultaneously. Exposure of thymic lymphocytes to cycloheximide alone for 4 h, at a concentration which blocked protein synthesis (100 μg/ml), caused an appreciable decrease in α-aminoisobutyric acid accumulation but failed to decrease ATP levels (Table IV). ATP levels were slightly (but not to a statistically significant degree) higher in cycloheximide-treated than in untreated cells. Cortisol and cycloheximide together were no more effective in reducing α-aminoisobutyric acid transport than each substance alone (Table IV). Cortisol did not decrease ATP levels in thymic lymphocytes which were treated simultaneously with cycloheximide (Table IV).

TABLE IV

EFFECT OF CORTISOL AND CYCLOHEXIMIDE ON α-AMINOISOBUTYRIC ACID UPTAKE AND CELL ATP LEVELS

Conditions were as described previously. Lymphocytes were treated with cycloheximide (100 μg/ml) previously found to inhibit protein synthesis by more than 95%, and cortisol (10 μM). The duration of treatment was 3 h and of labelling 5 min. Each value represents the mean ± S.E. of 4 or 5 separate flasks.

	A_i/A_0	$ATP \cdot 10^{-16}$ moles/cell
Control	7.5 ± 0.7	1.27 ± 0.08
Cortisol	5.1 ± 0.2 (−31%)*	1.04 ± 0.06 (−23%)**
Cycloheximide	5.4 ± 0.1 (−28%)*	1.36 ± 0.06
Cortisol and cycloheximide	5.3 ± 0.1 (−29%)*	1.37 ± 0.06

* Differs significantly from control ($P < 0.01$).
** Differs significantly from control ($P < 0.05$).

TABLE V

PERSISTENCE OF CORTISOL EFFECT AFTER INCUBATION IN STEROID-FREE MEDIUM

Lymphocytes were incubated for 3 h in the presence and absence of cortisol (10 μM). At that time groups designated control and cortisol were labelled for 5 min with α-aminoisobutyric acid while the cells in the remaining groups were washed and then re-incubated for three additional h in medium free of, or containing cortisol (10 μM) as indicated. At termination, these flasks received a 5-min pulse with α-amino[3-^{14}C]isobutyric acid. Values represent a mean ± S.E. of 4 separate flasks.

	A_i/A_0
Control	8.2 ± 0.4
Cortisol	4.0 ± 0.8 (−51%)*
Control resuspended in control medium	10.3 ± 1.0
Cortisol resuspended in cortisol medium	3.0 ± 0.4 (−68%)*
Cortisol resuspended in control medium	5.1 ± 0.6 (−50%)*

* Differs significantly from control ($P < 0.01$).

Persistence of the cortisol effect

In view of the possibility that cortisol inhibits α-aminoisobutyric acid transport indirectly, by a process which requires continuing protein synthesis, inhibition once established, might not be expected to disappear rapidly when previously treated cells are incubated in cortisol-free medium. In fact, marked inhibition of α-aminoisobutyric acid accumulation was evident in cells which had been treated for 3 h and were then incubated for an additional 3 h in the absence of cortisol (Table V).

Specificity of cortisol inhibition of α-aminoisobutyric acid accumulation

In order to determine whether inhibition of α-aminoisobutyric acid accumulation in thymic lymphocytes was a specific glucocorticoid action of cortisol, we examined the effects of low (0.1 and 1 μM) as well as high (10 μM) concentrations of cortisol and of steriods devoid of glucocorticoid activity on α-aminoisobutyric acid transport. At 1 and 10 μM, cortisol was a more potent inhibitor of α-aminoiso-

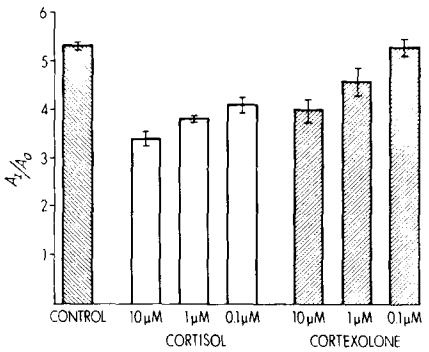

Fig. 3. Conditions of incubation were as described in the text. After 3 h of incubation with or without cortisol (10, 1 and 0.1 μM) or cortexolone (10, 1, and 0.1 μM), α-amino[3-^{14}C]isobutyric acid was added to a final concentration of 10 μM and the incubation continued for 5 min.

TABLE VI

STEROID INHIBITION OF α-AMINOISOBUTYRIC ACID ACCUMULATION

Lymphocytes were treated with the appropriate steroid (10 μM) for 3 h and then labelled for 5 min with α-amino[3-^{14}C]isobutyric acid. Values represent the mean ± S.E. of 10 separate flasks.

	A_i/A_0	% *Inhibition*
Control	5.6 ± 0.18	—
Cortisol (10 μM)	3.7 ± 0.10*	34%
Cortexolone (10 μM)	4.3 ± 0.11*	23%
Progesterone (10 μM)	4.7 ± 0.14**	16%
Cortisone (10 μM)	5.0 ± 0.14***	11%
Testosterone (10 μM)	5.6 ± 0.26	—

* Differs significantly from control ($P < 0.001$).
** Differs significantly from control ($P < 0.005$).
*** Differs significantly from control ($P < 0.02$).

butyric accumulation than its analogue, 11-deoxycortisol (cortexolone), and in contrast with 0.1 μM cortisol, 0.1 μM cortexolone was ineffective (Fig. 3). Neither progesterone, cortisone nor testosterone were inhibitory at 1 μM (data not shown) and were only slightly inhibitory or non-inhibitory at 10 μM (Table VI). Cortexolone has been shown to compete with cortisol for binding to cytoplasmic receptors and thereby block cortisol inhibition of glucose utilization in rat thymus cells[28]. Likewise, we have observed that cortexolone is able to reduce cortisol-induced inhibition of α-aminoisobutyric acid accumulation (Table VII).

TABLE VII

EFFECT OF CORTEXOLONE ON CORTISOL INHIBITION OF α-AMINOISOBUTYRIC ACID UPTAKE

Lymphocytes were treated for 3 h with cortisol (0.1 μM) and/or cortexolone (1.0 μM), and then labelled with α-amino[3-^{14}C]isobutyric acid for 5 min. Values represent the mean ± S.E. of 4 separate flasks.

	A_i/A_0
Control	5.33 ± 0.06
Cortisol (0.1 μM)	4.13 ± 0.14 (−23%)*
Cortexolone (1 μM)	4.55 ± 0.30 (−13%)**
Cortisol and cortexolone	4.83 ± 0.07 (− 9%)***

* Differs significantly from control ($P < 0.001$).
** Differs significantly from cortisol ($P < 0.05$).
*** Differs significantly from cortisol ($P < 0.001$).

DISCUSSION

Our data clearly indicate that concentrative, Na^+-dependent α-aminoisobutyric acid transport in thymic lymphocytes is sensitive to cortisol *in vitro*. Furthermore, non-concentrative α-aminoisobutyric acid accumulation demonstrated at high extracellular α-aminoisobutyric acid concentrations was cortisol insensitive. Thymic lymphocytes also failed to concentrate α-aminoisobutyric acid over a 5-min period when incubated in the absence of extracellular Na^+. Longer exposure to α-aminoisobutyric acid, however, may have revealed concentrative uptake. Therefore, Na^+-independent α-aminoisobutyric acid accumulation, not inhibited by cortisol, may reflect non-concentrative transport or a concentrative process which is separate from and slower than Na^+-dependent transport. Although kinetic studies of α-aminoisobutyric acid transport performed *in vitro* may not represent precisely the interrelation between α-aminoisobutyric acid and the carrier mechanism, the finding that cortisol alters the V but not the apparent K_m, indicates a decrease in the total capacity of the cell for α-aminoisobutyric acid transport, rather than a decrease in the affinity of α-aminoisobutyric acid for carrier. We have reported similar kinetic alterations of α-aminoisobutyric acid transport induced by cortisol in human leukemic lymphocytes[29]. According to currently held concepts of active amino acid transport, decreased total capacity for α-aminoisobutyric acid transport could result from reduced

(a) availability of energy (ATP synthesis by cellular enzymes or transduction by membrane ATPases), (b) quantity of carrier (number of transport sites), or (c) carrier mobility.

Because cortisol rapidly inhibits glucose uptake and the generation of ATP from extracellular substrates in thymic lymphocytes[1–10], we considered the possibility that cortisol inhibited α-aminoisobutyric acid transport by limiting the availability of energy (ATP). Our observation that cortisol-induced inhibition of α-aminoisobutyric acid transport precedes by 60–90 min its effect on thymic lymphocyte ATP levels argues against an important role for ATP depletion in mediating early cortisol inhibition of α-aminoisobutyric acid transport. We cannot exclude the possibility that cortisol rapidly decreases the supply of ATP which resides in a small transport-related compartment. In this regard, results of recently reported studies[30] suggest the presence of a small, plasma membrane-located pool of ATP in rat muscle cells. Furthermore, Makman *et al.*[20] have reported that cortisol reduces the incorporation of $NaH_2{}^{32}PO_4$ into ATP in rat thymic lymphocytes at least as rapidly as it inhibits amino acid transport. However, the possibility that reduced $NaH_2{}^{32}PO_4$ incorporation was caused by decreased phosphate transport rather than ATP formation was not excluded.

We have confirmed previous observations in demonstrating that blockage of protein synthesis with cycloheximide inhibits amino acid transport[4,8,31–33], a finding which suggests that cycloheximide prevents the replacement of labile protein(s) involved in transport. Since cycloheximide did not decrease thymic lymphocyte ATP levels, this putative protein would not appear to be related to ATP generation. The experimental results also confirm previous findings that cortisol and inhibitors of protein synthesis[1,4,8] together cause no greater inhibition of transport than either substance alone. This observation, which indicates that the cortisol effect requires continuing protein synthesis, is consistent with two possible modes of cortisol action; (a) that cortisol selectively inhibits the formation of a labile protein involved in transport and (b) that cortisol, through *de novo* protein synthesis, promotes the formation of a fraudulent transport protein or an inhibitor of transport. The latter possibility is the more likely, however, since cycloheximide which inhibited protein synthesis virtually completely was no more effective than cortisol in reducing α-aminoisobutyric acid transport. Moreover, the fact that cycloheximide prevented the decrease in ATP due to cortisol, yet in itself did not decrease ATP levels, suggests that *de novo* protein synthesis is required for the appearance of this cortisol effect. *De novo* protein synthesis also appears to be involved in cortisol inhibition of α-aminoisobutyric acid transport in human leukemic lymphocytes[29]. Recently, Makman *et al.*[20] have provided evidence that cortisol inhibits other transport processes by a similar mechanism.

The failure of established cortisol inhibition of α-aminoisobutyric acid transport to disappear rapidly after transfer of treated cells to cortisol-free medium is consistent with an indirect mechanism for cortisol action. If the effect of cortisol is mediated by the formation of a new protein, this observation would indicate that its synthesis, once stimulated, does not decline readily, or alternatively, that its rate of turnover is slow (or both). It should be stressed however, that cortisol may persist intracellularly and continue to exert biological effects after transfer.

Thus, cortisol may inhibit α-aminoisobutyric acid transport and decrease ATP

levels by separate mechanisms, each arising from an earlier step which involves *de novo* protein synthesis, and, to extrapolate from the observations of Mosher *et al.*[34] and from those of Makman *et al.*[20], RNA synthesis as well. Depletion of ATP may contribute to already established inhibition of amino acid transport.

At present, it is felt that the specific effects of cortisol are mediated by binding to glucocorticoid-specific cytoplasmic and then nuclear receptors and subsequent rapid stimulation of RNA and protein synthesis[34]. The observations (a) that cortisol inhibits α-aminoisobutyric acid transport at low concentrations (0.1–1.0 μM), and (b) that cortexolone, which is known to compete with cortisol for binding to cytoplasmic receptors, reduces cortisol-induced inhibition of α-aminoisobutyric acid uptake, are indicative of a specific, glucocorticoid-related effect. In addition, in human leukemic lymphocytes, equipotent concentrations of dexamethasone produces similar inhibition of α-aminoisobutyric acid accumulation as cortisol[35]. Cortisol is a more potent inhibitor of α-aminoisobutyric acid transport than either progesterone, cortisone or testosterone, as has been previously reported for inhibition of uridine incorporation into rat thymic cells by Makman *et al.*[1]. For cortisone to exert specific inhibitory effects, it must be converted to cortisol[36]. Since conversion may not occur in isolated thymocytes, the decrease in α-aminoisobutyric acid uptake caused by 10 μM cortisone probably reflects a non-specific effect.

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REFERENCES

1 Makman, M. H., Nakagawa, S. and White, A. (1967) *Rec. Progr. Horm. Res.* 23, 195–227
2 Makman, M. H., Dvorkin, B. and White, A. (1968) *J. Biol. Chem.* 243, 1485–1497
3 Young, D. and Klurfeld, G. (1968) *Fed. Proc.* 27, 626 (Abstr.)
4 Makman, M. H., Nakagawa, S., Dvorkin, B. and White, A. (1970) *J. Biol. Chem.* 245, 2556–2563
5 Wool, I. G. (1960) *Nature* 186, 728
6 Eichorn, J. E., Scully, E., Halkerston, I. D. and Hechter, O. (1961) *Proc. Soc. Exp. Biol. Med.* 106, 153–157
7 Kostyo, J. L. and Schmidt, J. E. (1963) *Am. J. Physiol.* 204, 1031–1038
8 Kostyo, J. L. and Redmond, A. F. (1966) *Endocrinology* 79, 531–540
9 Mohri, T. (1967) *Endocrinology* 81, 454–460
10 Ariyoshi, Y. and Plager, J. E. (1970) *Endocrinology* 86, 996–1003
11 Bartlett, D., Morita, Y. and Munck, A. (1962) *Nature* 196, 897–898
12 Morita, Y. and Munck, A. (1964) *Biochim. Biophys. Acta* 93, 150–157
13 Munck, A. (1965) *Endocrinology* 77, 356–360
14 Kattwinkel, J. and Munck, A. (1966) *Endocrinology* 79, 387–390

15 Munck, A. (1968) *J. Biol. Chem.* 243, 1039–1042
16 Young, D. A. (1969) *J. Biol. Chem.* 244, 2210–2217
17 Rosen, J. M., Fina, J. J., Milholland, R. J. and Rosen, F. (1970) *J. Biol. Chem.* 245, 2074–2080
18 Young, D. A. (1970) *J. Biol. Chem.* 245, 2747–2752
19 Rosen, J. M., Milholland, R. J. and Rosen, F. (1970) *Biochim. Biophys. Acta* 219, 447–454
20 Makman, M. H., Dvorkin, B. and White, A. (1971) *Proc. Natl. Acad. Sci. U.S.* 68, 1269–1273
21 Munck, A., Wira, C., Young, D. A., Mosher, K. M., Hallahan, C. and Bell, P. A. (1972) *J. Steroid Biochem.* 3, 567–578
22 Christensen, H. N. (1967) *Perspect. Biol. Med.* 10, 471–494
23 Bray, G. A. (1960) *Anal. Biochem.* 1, 279–283
24 Lowry, O. H., Rosebrough, N. J., Farr, A. L. and Randall, R. J. (1951) *J. Biol. Chem.* 193, 265–275
25 Akedo, H. and Christensen, H. N. (1962) *J. Biol. Chem.* 237, 118–122
26 Aledort, L. M., Weed, R. I. and Troup, S. B. (1966) *Anal. Biochem.* 17, 268–277
27 Dowd, J. E. and Riggs, D. S. (1965) *J. Biol. Chem.* 240, 863–869
28 Munck, A. and Brinck-Johnson, T. (1968) *J. Biol. Chem.* 5556–5565
29 Baran, D. T., Lichtman, M. A. and Peck, W. A. (1972) *J. Clin. Invest.* 51, 2181–2189
30 Reporter, M. (1972) *Biochem. Biophys. Res. Commun.* 48, 598–604
31 Adamson, L. F., Langeluttig, S. C. and Anast, C. S. (1966) *Biochim. Biophys. Acta* 115, 355–360
32 Elsas, L. J. and Rosenberg, L. E. (1967) *Proc. Natl. Acad. Sci. U.S.* 57, 371–378
33 Elsas, L. J., Albrecht, I. and Rosenberg, L. E. (1968) *J. Biol. Chem.* 243, 1846–1853
34 Mosher, K. M., Young, D. A. and Munck, A. (1971) *J. Biol. Chem.* 246, 654–659
35 Frengley, P., Lichtman, M. A. and Peck, W. A. (1973) *J. Clin. Invest.*, in the press
36 Berliner, D. L. and Ruhmann, A. G. (1966) *Endocrinology* 78, 373–382