Amino Acid Transport in Hepatoma Cell Cultures during Tyrosine Aminotransferase Induction

Abstract. *Alpha-aminoisobutyric acid transport in hepatoma cells in culture was increased by insulin but not by hydrocortisone. Both of these agents induce tyrosine aminotransferase activity in this system. The apparent increase in alpha-aminoisobutyric acid transport and tyrosine aminotransferase activity produced by glucagon is probably caused by insulin contamination. Insulin did not increase transport in this system until after tyrosine aminotransferase activity had reached maximum levels. The mechanisms underlying increased alpha-aminoisobutyric acid transport appear to differ from those for tyrosine aminotransferase induction with hydrocortisone despite their close association in previous whole animal experiments.*

Hormonal induction of the liver enzyme tyrosine aminotransferase (TAT) (L-tyrosine: 2-oxoglutarate aminotransferase, E.C. 2.6.1.5) occurs in the whole animal (*1*) as well as in perfused liver (*2*), fetal liver explants (*3*), and hepatoma cells in culture (*4*). Recent studies with normal and tumor-bearing rats have demonstrated an apparent correlation between TAT activity and the accumulation of nonmetabolizable amino acids in liver and tumor tissues, which suggests a regulatory role of amino acid influx in the control of amino acid metabolizing enzymes (*5, 6, 7*). The transport of one of these nonmetabolized amino acids, α-aminoisobutyric acid (AIB), is mediated by the alanine-preferring system, one of the overlapping transport systems for neutral amino acids (*8*). Its accumulation in rat liver has been shown to be affected by hormones (*5–7, 9*).

In parallel with whole animal studies involving a correlation between TAT induction and AIB transport activity in rat liver following injections of glucagon or theophylline (*10, 11*), we have attempted to carry out studies on the same parameters in the cell culture system in which we originally demonstrated TAT induction [Pitot *et al.* (*4*) and Potter *et al.* (*4*)].

Reuber hepatoma (H-4-II-E) cells were grown as monolayers at 37°C in an atmosphere of 5 percent CO_2 and 95 percent air in 8 to 10 ml of Swim 77 medium (*12*) modified to contain 0.004*M* glutamine and supplemented with 20 percent horse serum and 5 percent fetal calf serum (the growth medium). In order to eliminate effects due to serum or to inducers in serum, the medium was replaced with S77 medium, which lacks serum, or with Hanks solution (*13*) either 18 hours before or at the initiation of the time interval of study, as indicated in Table 1. Hydrocortisone, glucagon, and insulin were added at indicated concentrations at the beginning of the indicated time intervals. Cells were removed by the addition of 0.25 percent trypsin in 0.01*M* citrate (*p*H 7.6) to the culture plates and then suspended in 0.1*M* potassium phosphate buffer (*p*H 8.0) containing 0.001*M* dithiothreitol for TAT assays, which were performed by a method based on that of Lin and Knox (*14*) or by the method of Diamondstone (*15*). After a 1-hour pulse of 0.4 μc of [1-^{14}C]α-AIB (specific activity, 20 mc/mmole) per plate, monolayers were rinsed twice with 5 ml of 0.154*M* sodium chloride, and the cells were then collected in 3.0 ml of saline with rubber policemen. Samples were taken for protein determinations by a method based on that of Lowry *et al.* (*16*). The remaining cell suspension was precipitated with trichloroacetic acid at a final concentration of 13 percent, and radioactivity was determined by liquid scintillation spectrometry on 0.5 ml of the acid soluble fraction. Data are expressed per milligram of protein according to the method of Foster and Pardee (*17*).

In order to examine the time sequence of rises in AIB transport with TAT induction as influenced by hydrocortisone, glucagon, and insulin, we measured the changes hourly in experiments in which the growth medium was replaced with fresh S77 medium 18 hours before the addition of the hormones (Fig. 1). The insulin-mediated rise in transport occurred after a lag of 3 hours, a time at which the

Table 1. Effect of hydrocortisone, glucagon, and insulin on AIB transport and TAT activity in H35 hepatoma cells incubated in S77 medium or Hanks solution. In experiments A and C, measurements were made 6 hours after growth medium was removed from plates and replaced with S77 medium or Hanks solution with additives after two rinses with the indicated media. In experiment B, growth medium was removed and replaced with S77; after a period of 18 hours the medium was replaced with fresh S77 with additives, and the measurements were made 6 hours later. Control plates had medium changed only. Experiment B was carried out with cells grown in glass scintillation vials instead of in plastic petri dishes and TAT was assayed by the Diamondstone method (*15*). α-Aminoisobutyric acid transport is expressed as the mean (disintegrations per minute per milligram of protein per hour) × 10^{-3}, ± standard error ($n = 3$), measured over a 1-hour period from the 5th hour to the 6th hour after the medium was changed. Tyrosine aminotransferase activity is expressed as the mean micromoles of *p*-hydroxyphenylpyruvate per milligram of protein per hour, ± S.E. ($n = 2$ to 4, except for glucagon value, experiment A, which represents a single determination). Hydrocortisone sodium succinate (Solu-Cortef, lot LG-963) was donated by the Upjohn Co. "Glucagon" as the hydrochloride (for injection, lot 1YL68A in experiment A and lot 3DX74D in experiment B) was obtained from Eli Lilly and Co. It may be contaminated with as much as 0.05 percent insulin. At the level of 40 μg of glucagon per milliliter of medium this could amount to 0.020 μg of insulin per milliliter of medium. We have determined that 0.005 μg of insulin per milliliter medium gives a maximum tyrosine transaminase elevation in this system. Glucagon* (purified by chromatography, treated with dithiothreitol, and recrystallized by Dr. W. W. Bromer from starting material similar to "Glucagon" used above, lot 258-1011B-104-1) was provided by Dr. Ronald E. Chance, Eli Lilly and Co. Insulin (glucagon-free, lot PJ-4609) was provided by Dr. W. R. Kirtley, Eli Lilly and Co.

Addition	Final conc.	S77 medium		Hanks solution	
		AIB transport	TAT activity	AIB transport	TAT activity
		Experiment A			
None		1.6 ± 0.1	0.21 ± 0.13	15.8 ± 0.5	0.10 ± 0.04
Hydrocortisone	$10^{-6}M$	1.7 ± .2	2.55 ± .12	12.7 ± .6	1.07 ± .36
Hydrocortisone + "glucagon"	$10^{-6}M$ 40 μg/ml	2.8 ± .2	3.24 ± .00	12.5 ± 1.3	0.47 ± .00
"Glucagon"	40 μg/ml	2.5 ± .2	0.73	10.5 ± 1.7	.38 ± .02
		Experiment B			
None		0.83 ± 0.01	0.71 ± 0.05		
"Glucagon"	40 μg/ml	1.38 ± .04	1.30 ± .03		
"Glucagon"	10 μg/ml	0.90 ± .07	0.93 ± .05		
Glucagon*	40 μg/ml	.91 ± .04	.74 ± .00		
Glucagon*	10 μg/ml	.85 ± .02	.80 ± .03		
		Experiment C			
None		1.8 ± 0.1	0.26 ± 0.01	21.0 ± 0.5	0.12 ± 0.00
Insulin	0.5 μg/ml	3.1 ± .1	.46 ± .01	24.4 ± .7	.32 ± .02

TAT induction approached maximum values.

Figure 1 also shows that data obtained with "glucagon" in this system were very similar to those with insulin. Information received from Kenney (*18*) indicated that the apparent effect of "glucagon" on TAT activity is actually due to insulin contamination. Information obtained from Chance (*19*) verifies that there is indeed sufficient insulin contamination in the standard pharmaceutical glucagon preparation to account for the TAT elevation at the dose level we used. Such preparations were used in both of the experiments involving glucagon shown in Fig. 1 and support the findings by Kenney (*18*). The expected rise in TAT activity after hydrocortisone occurred without a corresponding rise in AIB uptake. In addition, Fig. 1 shows results of experiments in which the effect of the combination of the two hormones was examined. α-Aminoisobutyric acid uptake was also stimulated after a 3- to 4-hour lag in these exepriments, and presumably the effect was due to the insulin contamination in the glucagon preparation. As shown, hydrocortisone alone did not induce an increase in the samples taken at 6 hours. The augmented induction of TAT by combined hormonal treatment as compared with that of a glucocorticoid alone, shown at the 6-hour interval only, has been previously demonstrated in vivo by Wicks *et al.* (*1*) and in cell cultures by Potter *et al.* (*4*). The amount of hydrocortisone used gave a maximal response.

On the basis of Kenney's report (*18*) we obtained a specially purified glucagon preparation from Chance (*19*) and made direct comparisons of various additives. Table 1 lists the results of hormone additions on the transport of AIB and on TAT activity after 6 hours of incubation, the time selected on the basis of the curves shown in Fig. 1. α-Aminoisobutyric acid uptake was increased by insulin and by insulin-contaminated glucagon and its combination with hydrocortisone in S77 medium, which contains natural amino acids, but was not affected by purified glucagon or hydrocortisone alone. The level of AIB uptake in the cells incubated in Hanks solution, which contains no amino acids, was about tenfold greater than the uptake in the medium with amino acids, but no significant changes in transport activity were seen under these conditions. The

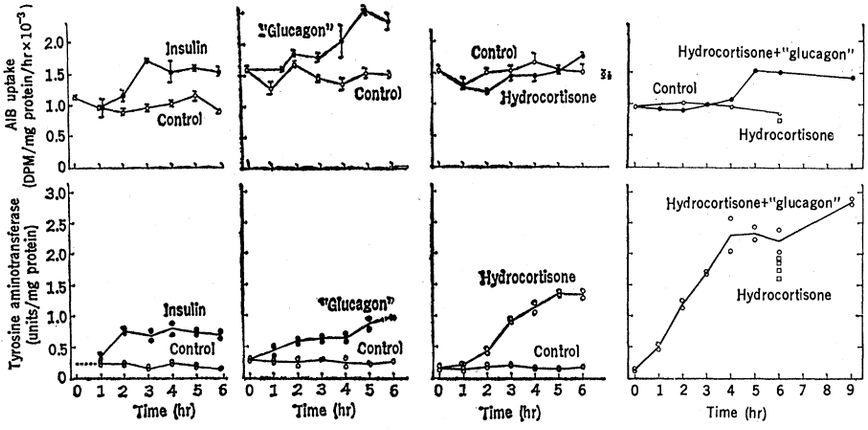

Fig. 1. α-Aminoisobutyric acid uptake and tyrosine aminotransferase activity measured hourly in H35 hepatoma cells cultured in S77 medium. Medium containing serum was removed 18 hours before onset of experiments. Hormones were added at beginning of time course. α-Aminoisobutyric acid uptake is shown as mean ± S.E. of triplicate plates except for hydrocortisone + "glucagon" graph which does not include S.E. Tyrosine aminotransferase activity is expressed as micromoles of *p*-hydroxyphenylpyruvate per milligram of protein per hour with points representing each of duplicate plates. Insulin free of glucagon was used at 0.5 μg/ml. "Glucagon" that contains traces of insulin (see text) was used at 40 μg/ml. Hydrocortisone sodium succinate was used at $10^{-6}M$. "Glucagon" and hydrocortisone sodium succinate in combination were used at the same levels as they were individually. Squares represent TAT activity or AIB uptake 6 hours after addition of hydrocortisone sodium succinate alone.

corresponding results for TAT indicate that insulin, insulin-contaminated glucagon, and hydrocortisone, or their combination, yielded an increase in TAT activity in S77 medium as well as a smaller elevation in Hanks solution. Purified glucagon had no effect on the TAT activity.

The results indicate that AIB transport can be increased by insulin but not by hydrocortisone, although both of these hormones increase enzyme activity. The increased transport requires the presence of natural amino acids in the incubation medium. Apparently the presence of natural amino acids, which compete with the nonmetabolizable amino acids for the transport site, is important in assessing the increase in the level of amino acid transport reflected by AIB uptake. The indicated competition suggests that a different protocol for AIB experiments may be required to give results in this system comparable to those obtained in vivo (*5, 6, 7*). It is assumed that any effect on AIB transport or TAT activity by glucagon in this system is a result of insulin contamination. In vivo results should be reexamined with this possibility in mind. The increase in AIB transport mediated by insulin alone or in combination with hydrocortisone occurs after a lag of 3 to 4 hours, a time at which TAT induction is almost maximal; this indicates that the increase in transport of amino acids by the system accumulating AIB does not occur as a controlling step.

In view of the increased transport of amino acids produced by hydrocortisone in vivo (*5–7, 9*), and in isolated perfused liver (*6, 7, 9*), the lack of increase in AIB transport in this report suggests that hydrocortisone does not directly mediate the changes in amino acid influx in the liver. However, it must be noted that the absence of transport in these cells may reflect a property of the malignant cells cultured, since one of the minimal deviation hepatomas (Morris hepatoma 9618A) studied in vivo by Baril *et al.* (*5*) showed no increase in AIB transport or TAT after administration of hydrocortisone. Chambers *et al.* (*6, 7*) have also shown increased AIB transport by insulin and glucagon but not by epinephrine in the isolated perfused liver; they suggest that the effect of the latter in whole animals may be via glucagon. The enzyme induction mediated by hydrocortisone is thought to occur via increased synthesis of messenger RNA (*4*), whereas the other two hormones probably act at a post-transcriptional level, perhaps by differing mechanisms for the two hormones (*1, 4, 20*). Chambers *et al.* (*6, 7*) could not block the hydrocortisone-increased AIB transport in perfused liver with actinomycin D but blocked some of the increase that was

mediated by insulin. Holt and Oliver (*21*) have suggested that different hormones may induce different forms of TAT, and Wicks (*1, 3*) has presented evidence that the enzyme induction involves cyclic AMP. In whole animal experiments we have obtained TAT induction that closely followed AIB intake when glucagon and theophylline were injected, possibly implicating cyclic AMP in both processes. It appears that the mechanisms underlying the changes in AIB transport can be separated from those underlying TAT induction by hydrocortisone, but complete concordance among cells in culture, in perfused liver, and in whole animals remains to be achieved.

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Charge-Mosaic Membranes: Dialytic Separation of Electrolytes from Nonelectrolytes and Amino Acids

Abstract. *Charge-mosaic membranes were used for dialytic separations of potassium chloride from low-molecular-weight nonelectrolytes and neutral amino acids. The permeability ratio (potassium chloride to uncharged species) ranged from about 6 in the case of methanol to about 86 in that of mannitol. A theoretical model predicts that optimum rates of dialysis should be achieved by dialyzing against salt concentrations other than zero; this prediction was confirmed by experiment. These observations suggest potential applications of mosaics in laboratory separations, industrial processing, and hemodialysis.*

We have described the observation of negative reflection coefficients and high electrolyte permeabilities in charge-mosaic membranes (*1*). The former property suggested the possible use of such membranes for desalination of seawater or brackish water by pressure dialysis (piezodialysis); the latter suggested their application to the separation of electrolytes from other low-molecular-weight species. In this paper we report dialytic separations of KCl from the uncharged organic species methanol, urea, glycine, L-phenylalanine, and D-mannitol.

A charge-mosaic membrane consists of a set of cation and anion exchange elements arranged in parallel, each element constituting a continuous pathway from one bathing solution to another. The high electrolyte permeability attainable with charge-mosaics, as compared with other membranes of tight structure, arises from two properties: (i) the high concentration, typically several molar, of cation in the cation exchanger and anion in the anion exchanger, and (ii) the ease with which the two ions can flow in parallel through their respective pathways, the two flows neutralizing each other electrically. These flows are equivalent to circulating electric currents, as predicted and later verified in model systems by Sollner and co-workers (*2*).

The membranes used in our studies and the method of their preparation have been described (*1*), A single layer of cation and anion exchange beads was embedded in an inert silicone matrix in such a way that each bead communicated with both surfaces of the membrane. The beads were of 8 percent cross-linked polystyrene-based resins.

For dialysis the membrane was mounted in a small Lucite cell; the "dialysand" compartment was filled, after several washings, with 1.3 ml of an aqueous solution containing KCl and labeled organic species. The initial concentration of KCl in all experiments was 0.2 mole/liter; the concentration of the organic species varied as indicated in Table 1. A solution of 0.01*M* KCl flowed from a large reservoir into the "dialysate" compartment; the effluent from this compartment was collected in flasks for analysis. A simple gravity-flow device maintained a constant flow of approximately 5 ml/hour, sufficient to ensure that the solution in the dialysate compartment remained within 10 percent of its nominal KCl concentration (0.01 mole/liter) and practically devoid of bulk and tracer organic solute. Teflon-coated magnetic stirrers provided vigorous stirring near each face of the membrane, and the temperature was controlled at 25.0° ± 0.2°C in an air thermostat. Concentrations of KCl in both compartments were determined from conductance measurements, whereas concentrations of organic solutes were obtained by scintillation counting of the tritium or carbon-14 label. Each experiment was continued for 35 to 50 hours (by which time the KCl concentration in the dialysand had been reduced to roughly one-third of its initial value). Figure 1 shows the time-course of a separation of urea and KCl.

The permeability ω^*_s of each solute, defined as

$$\omega^*_s = \left(\frac{J_s}{\Delta\pi_s}\right)_{\Delta p = 0} \quad (1)$$