EVIDENCE FOR A HEPATIC TRANSPORT SYSTEM NOT RESPONSIVE TO GLUCAGON OR THEOPHYLLINE*

Lester I. Harrison and Halvor N. Christensen

Department of Biological Chemistry, The University of Michigan
Ann Arbor, Michigan 48104

Received February 17, 1971

SUMMARY: In experiments designed to examine which transport systems are responsive to the adenine cyclase system, young rats received isotopically labeled, non-metabolizable amino acid analogs, selected for specificity to known transport systems. After allowing 38 hr for the amino acid to be distributed, glucagon or theophylline were injected intraperitoneally. Two amino acids typically reactive with a Na^+-dependent transport system for neutral amino acids, and one with a system for cationic amino acids, showed sharply elevated hepatic levels, relative to the plasma. Levels for the first group also rose in the diaphragm. A model amino acid typically reactive with a Na^+-insensitive transport system underwent no change in its distribution during 2 to 6 hr.

Glucagon was shown in 1965 by Chambers, Georg and Bass to stimulate the transport of α-aminoisobutyric acid (AIB) into the perfused rat liver[1]. Mallette and Park confirmed this observation and extended it to cycloleucine, alanine and lysine[2,3]. Similar effects on AIB† and cycloleucine transport have since been observed for liver slices as well[4]. Although theophylline did not increase the transport of AIB into rat liver slices[4] or adipose tissue[5], Adamson has recently shown it to have a stimulatory action similar to glucagon on cycloleucine uptake into embryonic chick bone[6]. Both theophylline and glucagon were able to stimulate the uptake of AIB by the liver of the rat *in vivo* (Scott *et al.*[7]). Both of these agents presumably act by increasing the concentration of cyclic AMP.

Cyclic AMP and its artificial dibutyryl derivative produce effects on amino acid uptake resembling those of glucagon and theophylline. Stimulation by cyclic AMP has been shown for the transport of AIB into perfused rat liver[8], into rat liver slices[4], and into fetal rat calvaria and slices of renal cortex[9]. This agent also increased the accumulation of leucine and lysine into the uterus[10], and of cycloleucine into the perfused rat liver[3]. Much lower doses of dibutyryl cyclic AMP sufficed, and a wider range of amino acids has

*Supported in part by grant HD-01233 from National Institutes for Child Health and Human Development, National Institutes of Health, U.S.P.H.S. Glucagon was the gift of Dr. Otto K. Behrens, Eli Lilly and Co.

†The abbreviations used are defined in Table I.

been found to be affected. The following transports have been seen to be stimulated by dibutyryl cyclic AMP: AIB into kidney cortex slices[9], isolated bovine thyroid cells[11], and perfused rat liver[8]; AIB into the liver of the intact rat[7]; AIB, glycine, cycloleucine, tyrosine and p-fluorophenylalanine into embryonic chick bone[6]; AIB and cycloleucine into rat liver slices[4]; leucine and lysine into the uterus[10]; also AIB, glycine and proline into the fetal rat calvarium[9]. Dibutyryl cyclic AMP also increased the incorporation of amino acids into protein in the cited experiments with embryonic chick bone, isolated bovine thyroid cells and the uterus.

No clear structural limit to the range of amino acids that will respond to these agents has so far been defined, especially since apparently all of those tested have responded to dibutyryl cyclic AMP. Amino acid transport is, however, known to be carried out by several apparently distinct agencies or systems as summarized elsewhere[12]. In an effort to discover whether these effects apply only to some or to all of the transport systems, we have explored the influences of glucagon and theophylline on the distribution between plasma and liver (and also between plasma and diaphragm) of certain model amino acids. For this purpose we selected model substrates that have been found to define transport systems in one or another isolated cell (Table I).

Table I. *Identity and features of model amino acids tested.* Four of the five artificial amino acids were examined in carboxyl ^{14}C-labeled form, whereas DCG had been tritiated by the Wilzbach procedure.

Name	Abbreviation	Metabolizable?	Transport system with which it reacts
α-aminoisobutyric acid	AIB	neglig.	Na^+-dependent System *A* in isol.cells which may be broader in kidney and liver
α-(methylamino)-isobutyric acid	MeAIB	neglig.	Same, but more sharply excluded from Systems *ASC*, *L*, *Ly*$^+$ by N-Me group
2-aminonorbornane-2-carboxylic acid	BCH	neglig.	Na^+-independent *L* in isol. animal Branched-chain system in *E. coli* Excluded from *Ly*$^+$
α,α-dicyclopropylglycine	DCG	neglig.	Largely excluded from all known systems in isol.animal cells. Uptake *in vivo* by liver, by brain slices appears to be by System *L*
4-amino-1-guanylpiperidine-4-carboxylic acid	GPA	neglig.	Restricted to transport system for cationic amino acid in tested cases
Taurine, endogenous	tau	sluggish	Restricted to system for *beta* amino acids

METHODS AND RESULTS

We used a schedule described by Potter *et al.*[13] for housing and feeding of rats to limit the effects of diurnal variation. 50-g male rats were housed in individual cages under an inverted lighting schedule: 12 hr of darkness in the daytime, 12 hr of light at night. Food was available for the first 8 hr of darkness; only the person feeding the animals entered the room. On the fourth day of the diet, the rats were injected intraperitoneally with 0.1 mM test amino acid, equivalent to 1 μci per rat, in 2 ml 0.9% NaCl. Food was withheld on the sixth day[7]; 2 ml of 0.9% NaCl with or without glucagon was injected in its place. Two hr later the rat was decapitated, and blood collected. The liver and diaphragm were homogenized in 10 ml of 3% sulfosalicylic acid per gram of tissue, using a little alumina powder in an all-glass homogenizer. Plasma was treated with an equal volume of 6% sulfosalicylic acid. The tissue suspensions were held 5 min at 100° and then centrifuged. 0.2-ml samples of the supernatant solutions were added to a standard phosphor in an alcohol-toluene solution, and the scintillations counted.

Experiments with theophylline preceded those with glucagon; on a perhaps somewhat less ideal schedule, food was made available for the first 4 hr of both light and dark periods, without any change in the injection schedule. Animals receiving only NaCl solution showed very similar distribution ratios for the test amino acids in the two series. The dosages were 10 mg of theophylline or 1 mg of glucagon per 100 g body wt.

Tables II and III compare the results obtained with and without glucagon or theophylline. AIB and MeAIB were accumulated to similar extents by liver, but the second appeared to be concentrated about twice as much as the first by the diaphragm. The responses of the distribution of the two amino acids to

Table II. *Effect of theophylline* (10 mg/100 g body wt) *2 hr after injection on the tissue levels of model amino acids, relative to the plasma level.* The distribution ratio is the ratio cpm per g tissue/cpm per ml plasma. The number of experiments is given in parentheses; the standard deviation, indicated by ±, follows the distribution ratio.

amino acid analog	Liver		Diaphragm	
	Distribution ratio		Distribution ratio	
	saline control	theophylline	saline control	theophylline
AIB	4.07±0.97 (6)	22.6 ±3.70 (4)	10.7 ±2.73 (6)	20.2 ±4.41 (5)
MeAIB	5.15±1.40 (9)	21.3 ±8.01(10)	21.9 ±3.50(10)	45.0 ±8.20(12)
GPA	3.67±0.64 (7)	6.81±0.93 (7)	6.44±1.06 (7)	8.78±1.79 (7)
BCH	1.39±0.46 (6)	1.47±0.17 (6)	2.19±1.06 (7)	2.24±0.47 (6)
DCG	2.48±1.22 (9)	2.03±1.08 (5)	3.44±1.62 (9)	3.54±1.60 (6)

Table III. *Effect of glucagon* (1 mg/100 g body wt[7]) *on the distribution ratios of model amino acids.* Interval 2 hr, except 6 hr for BCH experiments marked by asterisk. The parenthetic number indicates the number of animals. The standard deviation is indicated by ±.

amino acid analog	Liver		Diaphragm	
	Distribution ratio		Distribution ratio	
	saline control	glucagon	saline control	glucagon
AIB	9.56±2.70 (6)	48.9 ±3.29 (7)	8.76±1.30 (6)	20.8 ±1.94 (6)
MeAIB	9.45±2.78(11)	47.0 ±6.90(13)	18.6 ±2.91 (7)	32.9 ±5.58(10)
GPA	5.58±1.11 (8)	9.32±0.78 (7)	3.07±0.38 (8)	3.56±0.93 (7)
BCH	1.67±0.21 (7)	1.63±0.44 (8)	1.69±0.27 (8)	1.63±0.46 (7)
BCH*	1.42±0.17 (4)	1.45±0.15 (4)	1.66±0.14 (4)	1.64±0.10 (4)

Table IV. *Relative distribution ratios for the glucagon and theophylline experiments.* The figures reported in this table show by what factor the tissue level has been increased by theophylline or glucagon, compared with saline-injected controls, each tissue level of radioactivity having first been normalized by dividing it by the plasma level. Interval in each case, 2 hr.

amino acid analog	Liver				Diaphragm			
	theophylline	P	glucagon	P	theophylline	P	glucagon	P
AIB	5.55	<0.001	5.11	<0.001	1.90	0.025	2.38	<0.001
MeAIB	4.13	0.01	4.97	<0.001	2.06	<0.001	1.77	0.01
GPA	1.86	0.005	1.67	<0.001	1.36	0.20	1.16	0.50
BCH	1.06	0.90	0.98	0.90	1.02	0.90	0.96	0.90
DCG	0.82	0.70			1.03	0.90		

glucagon and theophylline were similar. The arginine analog, GPA†, was not concentrated as intensely by the tissues studied, although hepatic accumulation was intensified by glucagon or theophylline. DCG† distribution failed to respond to theophylline (Table II). 2-Aminonorbornane-2-carboxylic acid was at an apparent concentration in the tissues about 65% higher than that of the plasma, corresponding to calculated distribution ratios between the cellular and extracellular compartments in the range 2.5 to 2.8. No effect on the distribution was seen under the experimental conditions, whether observations were made 2 or 6 hr after injection of glucagon. Relatively high concentrations of glucagon did not stimulate the uptake of BCH by the intact diaphragm (Table V).

Phenylalanine, methionine and leucine were found much more effective inhibitors of BCH uptake by the intact, isolated diaphragm than were AIB, alanine or serine (Table V). BCH uptake and inhibition of uptake were not affected by omission of Na^+ from the medium.

Table V. *Percent inhibition of the uptake of the norbornyl amino acid by the intact, isolated diaphragm.* Uptake of BCH-^{14}C by isolated, intact diaphragms[17] of 50- to 100-g male rats was observed during 1 hr at 37° in 40 ml of Krebs-Ringer bicarbonate medium containing 10 mM glucose and 0.012 to 0.039 M thiosulfate for the determination of extracellular space. Where amino acids were present at 200 mM, [Na^+] was only 24 mN, [Na^+] having been found to have little effect on BCH uptake. Extraction of the tissue and calculation approximately followed an earlier procedure[18]. The experiments in the two columns were made by different observers at an interval of 2 years. The K_m of BCH for uptake appeared to be about 13 mM.

Inhibitory amino acid	Uptake, mmoles/kg cell water of 0.5 mM BCH at 40 mM inhibitor*	of 1 mM BCH at 200 mM inhibitor
no inhibitor	0.423	0.50
phenylalanine	0.233	0.13
methionine	0.258	—
leucine	0.206	0.15
alanine	—	0.36
serine	—	0.45
AIB	0.347	—

*Simultaneously, glucagon at 3 mg per 40 ml of medium was without significant effect (+10 and -9%) on uptake of BCH (0.1 and 0.5 mM) by the isolated rat diaphragm.

DISCUSSION

Previous efforts to discriminate more than one hepatic transport system for neutral amino acids have been rather unsuccessful. We have found it difficult to retain typical uphill transport for preparations of liver cells or tissue *in vitro*. We were not successful in maintaining levels of 2-aminonorbornane-2-carboxylic acid in the intact rat high enough to lower significantly the hepatic levels of neutral amino acids by competition for transport[14]. Such effects were readily obtained in two hours with AIB and its N-methyl derivative, but unfortunately most of the endogenous neutral amino acids appeared to be influenced indiscriminately[15]. One source of that behavior may be the gradual appearance of secondary effects after the inhibition of a single transport system. Another difficulty is equally troublesome: although we believe that various tissues have much the same set of transport systems for amino acids, as reviewed elsewhere[12], in some occurrences the scope of reactivities of these systems appears to be much broader than in others, so that more severe tests will be needed to uncover heterogeneity of the kind so far described in detail for a number of tissues. Our results on hepatic accumulation of AIB compare very closely with those of Scott *et al.*[7]. Although plasma and liver levels of endogenous taurine were measured on an amino acid analyzer, no clear pattern has emerged from the data.

We are severely restricted in the methods that can be used for the liver

to show that the uptake of BCH does indeed occur by System *L*. For example, demonstration of a Na^+-independent hepatic transport presents severe technical difficulties. Uptake of the norbornane amino acid by isolated intact diaphragm has been shown insensitive also to the addition of insulin[14]. The uptake of DCG by brain slices is inhibited by methionine, leucine, or the norbornane amino acid[16], and its entry into the liver of the intact rat is inhibited by methionine[15]. Hence the slow transport of DCG may well occur by the same system as for the norbornane amino acid. Presumably it did not serve as intended as a control substrate, lacking mediated transport into the two tissues tested.

Our experiments *in vivo* show that of the artificial model amino acid substrates tested, only the one characteristic of the Na^+-independent transport system which we have called System *L*, namely the norbornane amino acid, fails to have its level in the liver and the diaphragm increased by administration of glucagon or theophylline. This test is not necessarily comprehensive, since no model substrate specific to System *ASC* is available.

The experiments described here indicate that the intact isolated diaphragm has a transport system serving for 2-aminonorbornane-2-carboxylic acid which is inhibitable by System *L* substrates. Dr. Thomas R. Riggs of this department has also obtained evidence for the distinction between Na^+-sensitive and Na^+-independent transport of neutral amino acids into the isolated diaphragm[17].

REFERENCES

1. J. W. Chambers, R. H. Georg and A. D. Bass, *Mol. Pharmacol.* 1, 66 (1965).
2. L. E. Mallette and C. R. Park, *J. Biol. Chem.*, 244, 5713 (1969).
3. L. E. Mallette, J. H. Exton and C. R. Park, *J. Biol. Chem.*, 244, 5724 (1969).
4. J. K. Tews, N. A. Woodcock, and A. E. Harper, *J. Biol. Chem.*, 245, 3026 (1970).
5. V. F. V. Brunchhausen, *Z. Physiol. Chem.*. 349, 1437 (1968).
6. L. F. Adamson, *Biochim. et Biophys. Acta*, 201, 446 (1970).
7. E. F. Scott, R. D. Reynolds, H. C. Pitot and V. R. Potter, *Life Sciences*, 9, 2, 1133 (1970).
8. A. D. Bass, J. W. Chambers and R. H. Georg, *Endocrin.*, 87, 366 (1970).
9. J. M. Phang, S. J. Downing and I. W. Weiss, *Biochim. et Biophys. Acta*, 211, 605 (1970).
10. D. M. Griffin and C. M. Szego, *Life Sciences*, 7, 1017 (1968).
11. B. Wilson, E. Raghupathy, T. Tonone and Winton-Tong, *Endocrin.*, 83, 877 (1968).
12. H. N. Christensen, *Advances in Enzymology*, 32, 1 (1969).
13. V. R. Potter, E. F. Baril, M. Watanabe and E. D. Whittle, *Federation Proc.*, 27, 1238 (1968).

14. H. N. Christensen and A. M. Cullen, *J. Biol. Chem.*, 244, 1521 (1969).

15. H. N. Christensen and A. M. Cullen, *Biochim. et Biophys. Acta*, 150, 237 (1968).

16. P. Fleming and H. N. Christensen, result documented by H. N. Christensen in *Advances in Biochemical Psycho-pharmacology*, Vol. 4, E. Costa and M. S. Ebadi, eds., Raven Press, New York, 1971.

17. T. R. Riggs, personal communication.

18. H. Akedo and H. N. Christensen, *J. Biol. Chem.*, 237, 118 (1962).