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CHARACTERISTICS OF A TRANSPORT SYSTEM SERVING FOR THE TRANSFER OF HISTIDINE INTO S37 ASCITES TUMOR CELLS

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SUMMARY

1. S37 ascites tumor cells accumulate the amino acid histidine by 2 distinguishable processes. One of these has been characterized in an earlier publication (*Biochim. Biophys. Acta*, 203 (1970) 457).

2. The second system possesses kinetic parameters such that it could be mistaken for a "non-saturable" uptake process. However, the flux associated with this system may exceed that which might be attributable to "non-saturable" uptake, and competition for uptake by this site is shown to occur.

3. The high *c* system described in this article resembles the "A" system described by Oxender and Christensen (*J. Biol. Chem.*, 238 (1963) 3686), in its specificity.

4. The high *c* system does not appear to function in exchange of amino acids.

5. Na^+ stimulates uptake by the high *c* system, but is not required for transfer of histidine to occur by the high *c* system. Comparison of uptake in the presence of various alkali metal ions discloses a strong field sequence for interaction of the ions with the transport process.

6. Concentrative uptake by the high *c* system is dependent upon the availability of metabolic energy.

7. Trans inhibition of the high *c* system is shown to occur.

INTRODUCTION

It has been suggested[1] that uptake of neutral amino acids into ascites tumor cells occurs by at least two distinct transport systems of overlapping specificities. A commonly used approach to the elucidation of the properties of a given transport system is to study transport with competitive inhibitors supposedly specific to other systems present[2]. As an alternative approach, a substrate was chosen whose kinetic parameters for uptake by two systems differed so greatly that a biphasic Lineweaver–Burk plot was obtained[3]. Our intention was to determine differential properties of two transport systems without the use of high concentrations of competitive inhibitors by observing the occurrence or non-occurrence of a biphasic Lineweaver–Burk plot under various circumstances. This approach did serve to indicate that only one of the two neutral amino acid transporting systems served for the efflux of histidine by an exchange process, an expected result[3]. However, the occurrence of a biphasic plot

for histidine uptake from a Na^+-free medium was an unexpected result in view of the findings of Christensen[4]. The present work represents an attempt to further characterize the transport system for histidine uptake into S37 ascites tumor cells exhibiting the higher K_m value. In referring to the properties of the two observed transport systems, we shall find it convenient to refer to the system with the lower K_m value as the low *c* system in that its action is most noticeable at lower concentrations; the system with the higher K_m value may similarly be referred to as the high *c* system.

MATERIALS AND METHODS

Procedures utilized were generally similar to those described previously[3,5]. The conditions for termination of incubations and extraction of cells have been modified slightly and are now as follows. Incubation is terminated by pouring the incubation medium containing the cells (3 ml) into a centrifuge tube containing 8 ml of Krebs–Ringer phosphate medium chilled in ice. Centrifugation is for one minute in a model HN-S centrifuge (International Equipment Company) equipped with a No. 809 head. Under our conditions, approx. 30 s is required to attain a speed of 3700 rev./min, corresponding to 1940 × *g*. The supernatant solution is removed by siphoning, and extracellular radioactivity entrained in the pellet is removed by resuspending the pellet in 8 ml of chilled Krebs–Ringer phosphate medium and repeating the centrifugation. After siphoning off the second supernatant, a third 1-min centrifugation is used for the final packing of the cell pellet. After removal of excess moisture, the cells are measured by wet weight. Following this, the cell pellet is extracted with 5 ml of 100 % ethanol for a period in excess of 30 min. A 0.5-ml aliquot is taken for liquid scintillation counting.

A dioxane-free liquid scintillator has been utilized that has proven quite useful for counting small aqueous or ethanolic samples of ^{14}C- or ^{3}H-labeled amino acids or sugars. This scintillator may be considered as a slight modification of a scintillator proposed by Patterson and Greene[6]. It forms stable solutions with small aqueous or ethanolic samples (less than 1 ml), yields good counting efficiencies (as high as 46 % with ^{3}H), and is less costly than commercially-prepared scintillators. The solvent for this scintillator consists of one part ethanol, two parts Triton X-100, and four parts toluene, by volume. The scintillator is prepared by adding 100 g naphthalene, 0.3 g POPOP (1,4-bis-2-[5-phenyloxazolyl]benzene), and 10 g PPO (2,5-diphenyloxazole) to each liter of the mixed solvent. It is necessary to stir the scintillator overnight to obtain solution of the POPOP. The constituent materials for this liquid scintillator have been obtained from the following sources: PPO and POPOP from Nuclear Equipment Chemical Corporation, 165 Marine St., Farmingdale, N.Y., 11735. Reagent grade naphthalene and reagent grade toluene from J.T. Baker Chemical Co., Phillipsburg, N.J. Triton X-100 from Packard Instrument Co., Downers Grove, Ill. Absolute ethanol from U.S. Industrial Chemicals Co., New York, N.Y.

RESULTS

Hypothetical transport systems

Uptake of ^{14}C- or ^{3}H-labeled histidine over a broad concentration range into S37 ascites tumor cells has consistently shown a biphasic Lineweaver–Burk[7] plot,

as has been indicated previously[3]. A Lineweaver–Burk plot of this general shape may represent the combined action of two saturable transport systems, or it may alternately represent the net effect of one saturable uptake process and one non-saturable uptake process, as is illustrated in Table I and Fig. 1 below. In that it is apparently difficult to distinguish between a non-saturable uptake, which could be attributable to diffusion, and a saturable transport system with a high K_m value, information in addition to the biphasic plot previously observed is needed to characterize the high c transport system.

Inhibition of uptake in the presence of competitors

One line of evidence supporting the existence of a specific transport system is the action of structural analogs in reducing uptake by that system. Data indicating

TABLE I

HYPOTHETICAL UPTAKE OF A SOLUTE BY DIFFERENT TRANSPORT MECHANISMS

c_0 is the extracellular concentration of the transferred solute, J_1 and J_2 are both saturable fluxes: $J_1 = 0.2\ c_0/(0.1 + c_0)$ and $J_2 = 2\ c_0/(10 + c_0)$. J_1' is also a saturable flux: $J_1' = 0.22\ c_0/(0.1 + c_0)$ but $J_2' = 0.13\ c_0$. The sum $J_1 + J_2$ is intended to represent an observed uptake with 2 saturable transport processes operating over a short period of time, whereas $J_1' + J_2'$ represents the uptake over a short period of time if only one of two transfer processes is saturable.

c_0	J_1	J_2	$J_1 + J_2$	J_1'	J_2'	$J_1' + J_2'$
0.100	0.100	0.0198	0.120	0.110	0.013	0.123
0.125	0.111	0.0247	0.136	0.122	0.016	0.138
0.167	0.125	0.0328	0.158	0.138	0.022	0.160
0.250	0.143	0.0487	0.192	0.157	0.033	0.190
0.500	0.167	0.0954	0.262	0.183	0.065	0.248
1.000	0.182	0.182	0.364	0.200	0.130	0.330
2.000	0.190	0.333	0.523	0.209	0.260	0.469
2.500	0.192	0.400	0.592	0.211	0.325	0.536
3.333	0.194	0.500	0.694	0.213	0.434	0.647
5.000	0.196	0.667	0.863	0.216	0.650	0.866
10.000	0.198	1.000	1.198	0.218	1.300	1.518

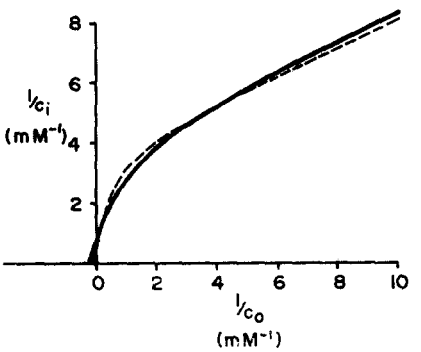

Fig. 1. Uptake of a solute by different hypothetical transport mechanisms. The uptake of solute by the different hypothetical systems specified in Table I is shown here in double reciprocal plots of the form due to Lineweaver and Burk[6]. The solid line represents the action of two saturable systems whereas the dashed line represents that of one saturable system and one non-saturable system.

TABLE II

COMPARISON OF DIFFERENT TRANSPORT SYSTEMS ON THE BASIS OF INHIBITION BY COMPETITORS

Competing amino acid	*Inhibition (%) of*			
	*High c**	*Low c***	*"A"****	*"L"§*
L-Alanine	61	7	73	10
L-Proline	54	−1		
L-Methionine	53	63	62	49
α-Aminoisobutyric acid	52	2	44	3
1-Aminocyclopentane carboxylic acid	48	45	51	40
L-Tryptophan	39	84	20	73
Glycine	35	4	26	0
L-Leucine	32	74	41	71
L-Phenylalanine	31	81	24	66
L-Valine	21	57	22	24
L-Threonine	17		39	11
D-Alanine	10			
L-Lysine	6	6		

* % Inhibition of 15 mM histidine uptake at 37 °C for 2 min into S37 cells by a 15 mM competitor.

** % Inhibition of 0.1 mM histidine uptake at 20 °C for 3 min into S37 cells by 3 mM competitor[3].

*** % Inhibition of 1 mM alanine uptake at 37 °C into Ehrlich ascites tumor cells by a 5 mM competitor[1].

§ % Inhibition of 1 mM leucine uptake at 37 °C into Ehrlich ascites tumor cells by a 5 mM competitor[1].

such inhibitions is contained in Table II. One point to note is that the amino acids proline, α-aminoisobutyric acid and glycine, which do not compete with histidine for uptake by the low *c* system, are very significant inhibitors for histidine uptake by the high *c* system.

Exchange efflux

As reported previously[3], incubation of cells previously loaded with labeled histidine in media containing varying concentrations of unlabeled histidine elicits an accelerated efflux of the labeled histidine from the cells. However, when a double-reciprocal plot is constructed of the stimulation of efflux of labeled histidine as a function of unlabeled histidine concentration in the medium, only a single straight line is found to occur. The K_m for this process resembles the K_m for uptake by the low *c* transport site[3]. This suggests that the high *c* site is not active in the process of efflux by exchange.

Kinetic parameters of the high c transport system

More than two dozen replications of the measurement of the uptake of histidine over a relatively broad concentration range have invariably yielded a biphasic plot of the general form shown in Fig. 1. However, there has been variability shown in the location of the breaking point of the curve, and in the apparent K_m values which

one might calculate by extrapolation of the 2 limbs of the curve. Attempts to fit experimental uptake data to terms of a Michaelis–Menten type have indicated that although the kinetic parameters for histidine uptake by these two transport systems are disparate enough to yield clearly biphasic double-reciprocal plots, the contribution of one transport system in the concentration range in which the other system is dominant is still appreciable enough to provide an impediment to easy calculation of accurate kinetic parameters. The variation in the precise configuration of the biphasic plot may perhaps be associated with variation of the activity in one or both transport systems from one day to the next. For determination of the K_m value of the high c system, an experiment has been selected in which the low c system appeared to be relatively minimal in its activity. The internal concentration, c_i, found after 5 min incubation at 37 °C may be approximated by

$$\frac{0.27\ c_0}{c_0 + 0.10} + \frac{19.3\ c_0}{c_0 + 10.0}$$

where c_0 is the external concentration of labeled histidine in mM. As the data contained in Table III indicates, this fitting achieves accuracy to within 5 % of the observed result for all but one data point. The K_m suggested for the high c system is then about 10 mM. Many experiments in which the low c transport system shows greater activity would suggest a somewhat lower value for this parameter, as low as 5 mM in many cases. However, because of the effect of the low c system in obscuring the precise value of the kinetic parameters of the high c system, it is considered that the result in a case in which the low c system was relatively inactive probably is yielding a more accurate true value for the high c system's K_m. It may also be noted that uptake is concentrative at all points except the highest concentration studied, a result incompatible with transfer by passive diffusion. Further, suppose that we are considering the c_i measured at 5 min to represent uptake by a non-saturable

TABLE III

APPROXIMATION OF HISTIDINE UPTAKE INTO S37 ASCITES TUMOR CELLS BY TWO TERMS OF MICHAELIS–MENTEN FORM

c_0 is the extracellular concentration of labeled histidine and c_i the intracellular concentration following a 5-min incubation at 37 °C. J_1 is a hypothetical flux equal to $0.27\ c_0/(c_0 + 0.10)$ and J_2 a hypothetical flux equal to $19.3\ c_0/(c_0 + 10.0)$. The discrepancy between the sum of the 2 hypothetical fluxes and the experimental result c_i is used to calculate the % error of the approximation at each concentration.

c_0	c_i	J_1	J_2	$J_1 + J_2$	*Error* (%)
0.10	0.33	0.135	0.191	0.326	−1
0.125	0.39	0.150	0.238	0.388	0
0.167	0.49	0.169	0.317	0.486	−1
0.25	0.62	0.193	0.471	0.664	+7
0.50	1.13	0.225	0.920	1.145	+1
1.00	2.09	0.246	1.75	2.00	−4
2.00	3.49	0.257	3.22	3.48	0
4.00	5.67	0.264	5.52	5.78	+2
8.00	9.01	0.267	8.59	8.86	−2
16.00	11.99	0.270	11.88	12.15	+1

process and a saturable one. The data gathered at 5 min would be a conservative estimate of initial velocity[8]. Let us consider the contribution labeled as J_2 in Table III to be replaced by a non-saturable process. Since the uptake is concentrative and J_2 exceeds c_0 throughout most of the range, let us estimate our parameter k_D, for non-saturable uptake conservatively as 0.20. ($J_2 = k_D \times c_0$; $k_D = 1.00/5 = 0.20$). This k_D would be at least 10 times as large as the upper limit calculated for non-saturable transfer previously[3]. Returning to the Michaelis–Menten representation of the "high c" system, dividing 19.3 by 5 would give us a value of 3.9 for the maximum velocity, V, shown by the "high c" system. Other experiments have given variable results for this parameter, some as high as 10.

Influence of sodium on the high c system

As may be seen from Table IV, uptake of 15 mM histidine is more rapid initially and attains higher levels at longer times in the presence of sodium than in the presence of other ions. This stimulation of histidine uptake by sodium has led Christensen[4] to characterize the uptake by his "A" system in Ehrlich ascites cells as "Na^+-requiring".

TABLE IV

TIME COURSE OF UPTAKE OF 15 mM [^{3}H]HISTIDINE IN THE PRESENCE OF VARIOUS CATIONS

The figures given in the table are mM concentrations found in the cells at the indicated times. Incubations were at 37 °C. All media contained 10 mM glucose initially, and 20 mM potassium phosphate buffer, pH 7.4. Cations were present as the chloride, and the media labeled "none" contained sucrose in place of any of these salts.

Principal cation	*1 min*	*2 min*	*5 min*	*30 min*	*50 min*	*70 min*
Li^+	2.12	3.04	4.06	7.33	8.21	8.62
Na^+	3.71	5.96	10.6	29.6	35.8	36.1
K^+	1.41	1.85	2.40	4.60	5.21	4.47
None	1.40	1.95	2.72	5.19	5.16	6.75

However, as has been suggested previously[3], both systems appear to take part in the uptake of histidine into S37 cells from sodium-free media, although at somewhat reduced rates. The uptake of histidine from sodium-free media yields biphasic curves of the form of Fig. 1.

Extending experiments in which Na^+ was replaced by different alkali metal ions to include Cs^+ and Rb^+ as well as Li^+ and K^+, the average c_i value found for uptake at 20 °C for 3 min in the presence of the various ions was: Na^+, 4.3; Li^+, 3.0; K^+, 2.2; Rb^+, 2.3; Cs^+, 2.3. The sequence for stimulation of histidine uptake by the high c system thus appears to be: $Na^+ > Li^+ > K^+$, Rb^+, Cs^+; uptake from media including the latter 3 ions being identical within experimental error. This sequence would agree with sequence 10 of the 11 selectivity sequences commonly seen for the interaction of alkali metal cations with various minerals, resins and glass electrodes[9].

Effect of metabolic inhibitors

Incubation of 15 mM [^{3}H]histidine in a Krebs–Ringer phosphate buffer at 37 °C for 1 h yielded intracellular concentrations averaging 57.1 mM. Inclusion of 2 mM

iodoacetate and 0.5 mM 2,4-dinitrophenol in such an incubation led to an intracellular concentration of 23.7 mM. Alternatively, inclusion of 10 mM 2-deoxyglucose and 5 mM NaCN led to an intracellular [^{3}H]histidine concentration of 12.0 mM. 0.1 mM ouabain led to an intracellular concentration of 52.0 mM, a very slight inhibition.

Effect of preincubation of S37 cells with various amino acids

As recorded in Table V, preincubation of S37 cells led, in most cases, to either a negligible effect upon subsequent labeled histidine uptake, or to a slight stimulation of uptake. In two cases, preincubation with α-aminoisobutyric acid or tryptophan, an appreciable decrement in uptake, or *trans* inhibition, was observed.

TABLE V

UPTAKE OF 15 mM [^{3}H]HISTIDINE INTO S37 CELLS PREVIOUSLY INCUBATED WITH VARIOUS AMINO ACIDS

First incubations were for a period of 1 h at 37 °C in the presence of 10 mM amino acid. Second incubations were conducted for 3 min at 37 °C with 15 mM labeled histidine present. The increase or decrease in the magnitude of the uptake observed when S37 cells which had been preincubated without amino acid were used for comparison is converted to a per cent of the uptake observed in that control condition. Positive numbers indicate that preincubation with a given amino acid led to augmentation of [^{3}H]histidine uptake, whereas negative numbers denote a diminished uptake. The figures reported represent averages of the results obtained on 4 separate occasions.

Pre-loaded amino acid	*Inhibition (−) or stimulation (+)* (%)
α-Aminoisobutyric acid	−35
Tryptophan	−11
Proline	− 4
Alanine	+ 3
Glycine	+13
Phenylalanine	+20
Valine	+21
1-Aminocyclopentane carboxylic acid	+22
Leucine	+32
Methionine	+33
Histidine	+46

DISCUSSION

The similarity of specificity for competitors leads one to suggest that the high *c* system is the same as that described by Oxender and Christensen[2] as their "A" system. As has been noted, the present results lead to a somewhat different characterization of the operation of this transport system.

While the high *c* system is unable to generate the customary large concentration gradients when inhibitors of energy-yielding glycolysis and oxidative phosphorylation are included, inclusion of ouabain caused only a very slight inhibition in the capability of the S37 cell to generate a concentration gradient of histidine. This is in agreement with the earlier work of Paine and Heinz[10] and would argue against the type of transport mechanism suggested by Crane[11] in which Na^+ is cotransported with either a

sugar or amino acid molecule, the Na^+ gradient providing the immediate basis for sugar or amino acid concentration. Various authors[4,12] have favored such a model for amino acid transport in ascites tumor cells. Na^+ in the extracellular medium obviously does have a stimulating effect upon amino acid transport (Table IV), however it has previously been demonstrated that the high *c* system may transfer histidine in the absence of Na^+ so that obligatory coupling does not exist. Potashner and Johnstone[13] have demonstrated a much greater dependence of amino acid uptake on cellular ATP than on a Na^+ gradient; they have further suggested that it is the presence of Na^+ in the extracellular medium rather than the existence of a Na^+ gradient which stimulates amino acid transport. The high *c* system may serve to raise intracellular amino acid concentrations above an equilibrium level by an as yet unspecified linkage to cellular metabolism, whereas the low *c* system may serve to broaden the intracellular mixture of amino acids present by exchange processes.

Although many of the amino acids listed in Table V had an apparent stimulatory effect on 14 mM histidine uptake, estimates of V from exchange experiments indicate, that the degree of stimulation observed may be due to the action of the low *c* system as an exchanger. The particularly significant feature of this data is not then, any suggestion of exchange uptake, but rather of the inhibition engendered by intracellular α-aminoisobutyric acid and tryptophan. This effect, trans inhibition, has been observed for transport in various cells. Pall and Kelly[14,15] found that a variety of amino acids could produce trans inhibition of amino acid uptake systems in *N. crassa*. Heinz *et al.*[16] found that either α-aminoisobutyric acid or 1-aminocyclopentane carboxylic acid could elicit trans inhibition of amino acid transport into *S. hydrogenans*. Belkhode and Scholefield[17] found 1-aminocyclopentane carboxylic acid to be an effective trans inhibitor of amino acid transport into Ehrlich and Novikoff ascites tumor cells. It is noteworthy that tryptophan has a high degree of affinity for the low *c* exchanging system; thus the estimate of trans inhibition given in Table V is an underestimation of the effect of tryptophan in inhibiting the high *c* system.

Amino acid concentrations are elevated in rapidly dividing tissues[18–20], and amino acids have been shown to stimulate DNA replication[21,22] and rRNA synthesis[23,24]. Trans inhibition could serve as one factor in the control of cellular growth and division. It may be of some interest that the essential amino acid tryptophan is concentrated effectively by the central nervous system[25,26], a non-dividing tissue, and is normally present at very constant levels in plasma[27].

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