BBA 75900

REGULATION OF AMINO ACID TRANSPORT IN CHICK EMBRYO HEART CELLS

I. ADAPTIVE SYSTEM OF MEDIATION FOR NEUTRAL AMINO ACIDS

G. C. GAZZOLA, R. FRANCHI, V. SAIBENE, P. RONCHI AND G. G. GUIDOTTI

Istituto di Patologia Generale, Università di Milano, Centro per lo Studio della Patologia Cellulare del C.N.R., Via Mangiagalli 31, 20133 Milano, and Istituto di Patologia Generale, Università di Cagliari, Via Porcell 4, 09100 Cagliari (Italy)

(Received November 8th, 1971)

SUMMARY

The regulation of amino acid transport systems in chick embryo heart cells has been studied. Experiments were designed to investigate activity and kinetics of transport for analogue and natural amino acids as a function of time under various *in vitro* conditions (active and inhibited protein and RNA synthesis, amino acid dependence, transinhibition).

Conclusions based on the proposed experimental approach include the following:

1. The transport activity of a group of neutral amino acids (corresponding to those ascribed to the A mediation by Christensen and co-workers, *J. Biol. Chem.*, 238(1963)3686; *Adv. Enzymol.*, 32(1969)1) increases with time when cells or intact hearts are incubated in the absence of added amino acids. This increase is abolished in the presence of puromycin, cycloheximide and actinomycin D.

2. The increase in activity of the A transport system (investigated by aminoisobutyrate and proline as representative amino acids) is prevented by the addition to the incubation medium of amino acids assigned to the same system of mediation on the basis of competition studies. It is not substantially altered by the addition of amino acids pertaining to the L system of mediation or basic amino acids.

3. Transinhibition does not appear of major importance in the regulation of the activity of the A transport system.

4. Kinetic analyses of aminoisobutyrate uptake in which the transport of the analogue by the A and L systems of mediation could be separated stress further that a time-dependent regulation is effective for the sole A transport system (affecting the maximal velocity without substantial changes in K_m) and provide a comprehensive explanation for previously reported changes with time and intracellular substrate concentration of the kinetic parameters governing the uptake of the analogue[9].

5. Provisional mechanisms for the observed adaptive regulation of the activity of the A transport system for neutral amino acids have been considered.

INTRODUCTION

In the chick embryo heart, amino acids are transported into the cardiac cells by saturable processes of mediation[1-3]. Progress has been made toward delineating properties and activity of transport systems for various analogue and natural amino acids[3,4]. Little is known, however, about the mechanisms by which such systems are regulated.

Recent reports describe experiments showing that the intracellular concentration of amino acids can influence the inward transport of these molecules in bacteria, fungi and mammalian cells either by transinhibition[5-7] or by control mechanisms effective on protein synthesis[7,8]. In previous studies with chick embryo hearts[9], the evaluation of the kinetic constants for α-aminoisobutyric acid transport in short-term nonsteady state experiments and in long-term equilibrium experiments provided values indicating time-dependent changes in the systems involved; moreover, the observed decrease of α-aminoisobutyrate uptake by hearts when the tissue was preloaded with the analogue[9] suggested the occurrence of an amino acid-dependent control of this process.

The present investigation has been designed to explore the mechanisms responsible for the regulation of amino acid transport systems in chick embryo heart cells. We studied the changes with time of the activity of analogue and natural amino acid transport: (a) in the absence and in the presence of inhibitors of protein and RNA synthesis; (b) in the absence and in the presence of individual amino acids (competitive and non-competitive for the uptake of the molecule under study). We also analysed the kinetics of amino acid transport before and after prolonged incubation periods under different experimental conditions.

Evidence is presented showing that a time-dependent adaptive system for the transport of a group of neutral amino acids (corresponding to those assigned to the A mediation by Christensen and co-workers[10,11]) is operative in chick embryo heart cells. This system is subject to regulation by its substrate molecules.

MATERIALS AND METHODS

α-Amino-[1-^{14}C]isobutyric acid, L-[U-^{14}C]alanine, L-[*ring*-2-^{14}C]histidine, L-[U-^{14}C]leucine, L-[^{35}S]methionine and L-[U-^{14}C]valine were obtained from the Radiochemical Centre, Amersham, Bucks, England. 1-Aminocyclopentane [1-^{14}C]carboxylic acid, [1-^{14}C]glycine, L-[U-^{14}C]lysine, L-[U-^{14}C]phenylalanine, L-[U-^{14}C]-proline, L-[U-^{14}C]serine, L-[U-^{14}C]threonine and [*methoxy*-^{3}H]inulin came from New England Nuclear Chemicals, Dreieichenhain, West Germany. Each compound was diluted as appropriate with unlabelled material.

The procedures of heart dissection from 5- and 7-day-old chick embryos and isolation of cells from hearts by collagenase treatment have been described previously in detail[1-3]. Incubations were carried out in silicone-treated glass vessels at 37.5 °C under continuous mild stirring[2] in an atmosphere of $O_2 + CO_2$ (95:5, by vol.); the basic incubation medium was Krebs–Ringer bicarbonate buffer containing 8 mM glucose. Under these conditions cardiac cells and intact hearts are viable for several hours[4,12]. Additions to the medium (amino acids, inhibitors, *etc.*) are specified in Results.

Amino acid uptake was measured by transferring samples of cell suspension (or intact hearts) into conical flasks containing Krebs–Ringer bicarbonate buffer supplemented with 8 mM glucose and the appropriate additions (labelled amino acids, inhibitors). In some experiments (as described in Results) the cells were washed with fresh medium before being transferred. The flasks, placed in a Dubnoff metabolic shaker at 37.5 °C, were incubated for 5 min (cell suspensions) or 20 min (hearts) in an atmosphere of $O_2 + CO_2$ (95:5, by vol.); under these conditions, results were initial velocities (*cf.* refs 1 and 3).

The means for determining intracellular accumulation and chemical identification of amino acids, for evaluating the proper corrections to be introduced and for estimating the kinetic constants of the transport processes in isolated cardiac cells were as described by Guidotti *et al.*[1,3,13].

The procedures for studying [^{14}C]leucine incorporation into protein of isolated cardiac cells were as described previously[2,13]. Tissue α-amino nitrogen concentrations were determined with a ninhydrin colorimetric technique[14] from protein-free supernatants of cells extracted by boiling in 3% sulphosalicylic acid.

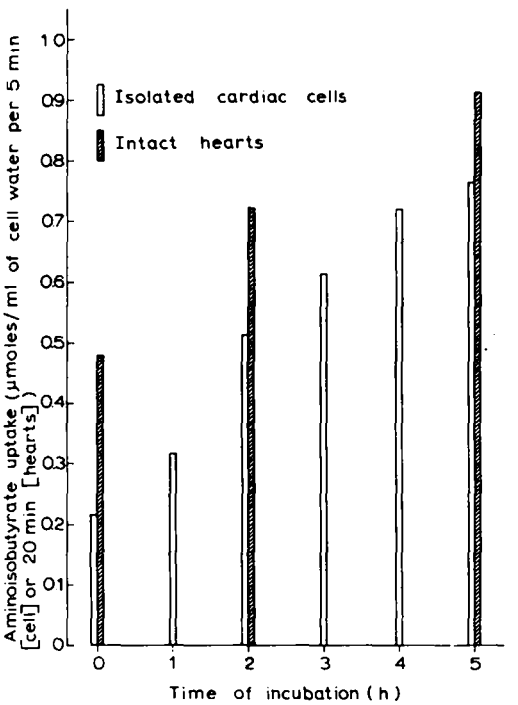

Fig. 1. α-Aminoisobutyric acid uptake by isolated cardiac cells and intact chick embryo hearts: changes in transport activity with time. Cardiac cell suspensions obtained from 7-day-old chick embryo hearts or intact hearts from 5-day-old embryos were incubated for 5 h in Krebs–Ringer bicarbonate buffer, pH 7.4, containing 8 mM glucose. Incubation was at 37.5 °C in an atmosphere of $O_2 + CO_2$ (95:5, by vol.). Amino acid uptake (initial velocity) was measured by transferring samples of cell suspension (10^7 cells) or intact hearts (approx. 1 mg of tissue protein) into flasks containing the same medium supplemented with α-amino-[1-^{14}C]isobutyric acid (0.1 mM, final concn) and incubating for 5 min (isolated cells) or 20 min (intact hearts). Results of six separate determinations were averaged.

RESULTS

General properties

When cardiac cell suspensions were incubated in a Krebs–Ringer bicarbonate with the sole addition of glucose, the activity of amino acid transport, as measured by aminoisobutyrate uptake in experiments of 5-min duration (initial velocity), increased with time up tc 5 h; the same result has been obtained with intact hearts incubated under comparable experimental conditions (Fig. 1). The addition of bovine albumin (3 mg/ml) or a complete mixture of amino acids during the procedure of isolation of the cardiac cells by collagenase treatment did not affect the subsequent rate of change of the phenomenon. Increased transport activities with time have been also detected for a number of naturally occurring amino acids including glycine, alanine, serine, proline and for the analogue cycloleucine. Only minor changes occurred with methionine, threonine, phenylalanine and histidine. Definite decrements took place with leucine, valine and lysine (Table I). In the course of similar experiments (cell suspensions incubated for 3 h in Krebs–Ringer buffer) the intracellular concentration of free α-amino nitrogen and the initial rate of total protein synthesis (as measured by the incorporation of labelled leucine into protein) of isolated cardiac cells did not change substantially (Fig. 2).

Effects of inhibitors

The addition of puromycin or cycloheximide to the incubation medium at concentrations sufficient to abolish protein synthesis almost completely, prevented

TABLE I

AMINO ACID UPTAKE BY ISOLATED CARDIAC CELLS: CHANGES IN TRANSPORT ACTIVITY WITH TIME

Cardiac cell suspensions were incubated for 3 h under experimental conditions identical to those described in the legend of Fig. 1. Amino acid uptake (initial velocity) was measured by transferring samples of cell suspension (10^7 cells) into flasks containing Krebs–Ringer bicarbonate supplemented with 8 mM glucose and the ^{14}C-labelled amino acid (0.1 mM, final concn) under study and incubating for 5 min at 37.5 °C. The values are means of 2–4 separate determinations.

Amino acid	*Uptake (μmoles/ml of cell water in 5 min)*		
	Incubation time (h)		*Difference*
	0	*3*	(%)
Glycine	0.24	0.55	+129
L-Alanine	0.97	1.88	+ 94
L-Serine	0.75	1.61	+115
L-Proline	0.39	1.36	+249
L-Methionine	0.31	0.34	+ 10
L-Threonine	0.54	0.43	− 20
L-Phenylalanine	0.20	0.17	− 15
L-Valine	0.27	0.17	− 37
L-Leucine	0.37	0.26	− 30
L-Histidine	0.51	0.46	− 10
L-Lysine	0.52	0.30	− 42
α-Aminoisobutyric acid	0.23	0.63	+174
1-Aminocyclopentanecarboxylic acid (cycloleucine)	0.66	1.06	+ 61

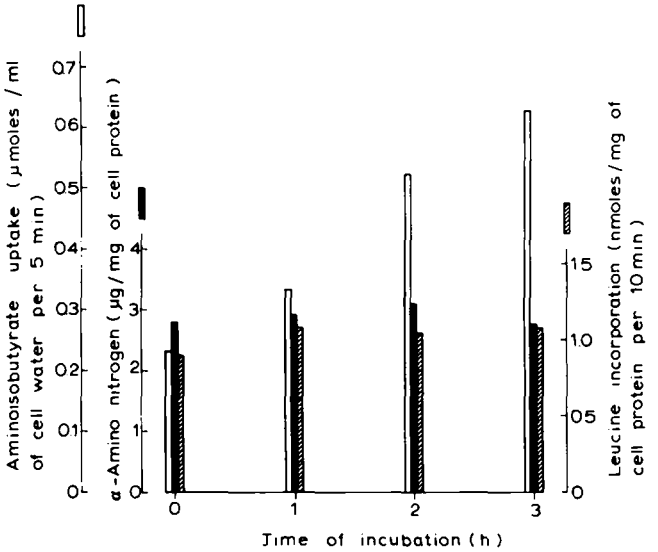

Fig. 2. Intracellular concentration of α-amino nitrogen of free amino acids and initial rate of total protein synthesis in isolated cardiac cells during the incubation in Krebs–Ringer buffer. Incubation period: 3 h; conditions as described in the legend of Fig. 1. Cells were washed with Krebs–Ringer buffer to remove extracellular amino acids before amino nitrogen determination. Amino acid incorporation into protein was measured by transferring samples of cell suspension into flasks containing Krebs–Ringer bicarbonate buffer supplemented with L-[^{14}C]leucine (0.1 mM, final concn) and incubating for 10 min at 37.5 °C. Results of three separate determinations were averaged. The values of aminoisobutyrate uptake (initial velocity, see Fig. 1) showing increase of amino acid transport activity with time during the incubation have been introduced for convenient reference.

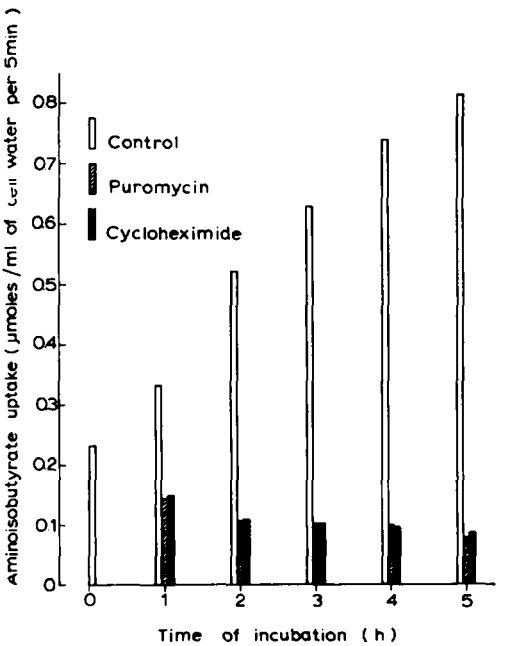

Fig. 3. Effects of puromycin and cycloheximide on the activity of α-aminoisobutyric acid transport into isolated cardiac cells as a function of time. Incubations and assay of aminoisobutyrate uptake were as described in the legend of Fig. 1. Concentrations of inhibitors: puromycin, 50 μg/ml; cycloheximide, 5 μg/ml. Results of two separate determinations were averaged.

the increase with time of the activity of aminoisobutyrate transport into isolated cardiac cells and actually depressed its rate of uptake progressively (Fig. 3). The same result has been obtained after addition of actinomycin D at concentrations ranging between 1 and 10 μg/ml (Fig. 4). After 5 h of incubation in the presence of

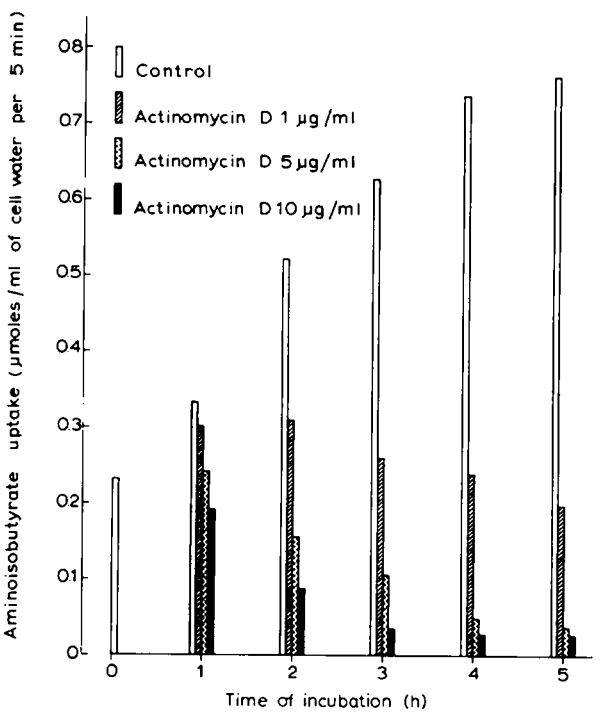

Fig. 4. Effects of increasing concentrations of actinomycin D on the activity of α-aminoisobutyric acid transport into isolated cardiac cells as a function of time. Incubations and assay of aminoisobutyrate uptake were as described in the legend of Fig. 1. Results of two separate determinations were averaged.

puromycin or cycloheximide, the ratio of amino acid concentration between intracellular and extracellular water in experiments of 5-min duration approached unity; concentrations of actinomycin D as high as 5 and 10 μg/ml of incubation medium lowered this ratio even further. Calculations based on the apparent diffusion constant (K_D) for aminoisobutyrate in isolated cardiac cells in the presence of the inhibitors (see later) showed, however, that in all cases the ratios attained in 5-min incubations were higher than those expected for free diffusion indicating that the inhibitors were unable to suppress completely the mediated transport of the analogue within the experimental period.

The reported effects on amino acid transport were not secondary to nonspecific cell injury. In cardiac cell suspensions incubated in the presence of puromycin, cycloheximide or actinomycin D as described, oxygen consumption and oxidative phosphorylation, as monitored by polarographic methods (Oxygraph, Gilson Medical Electronics), were not different from control values and were maintained at a constant rate for several hours.

Competitive effects

The inhibition by several amino acids at a 5-mM concentration of the uptakes of 0.1 mM aminoisobutyrate, L-proline, L-phenylalanine and cycloleucine was investigated. Table II shows that these amino acids respond differently to the various additions and that the interactions correspond to those reported by Oxender and Christensen[10] for amino acid uptake by Ehrlich ascites tumour cells. The affinities of neutral amino acids clustered into two groups, one including such amino acids as glycine, alanine, serine, proline and the analogue α-aminoisobutyric acid (alanine-preferring or A system)[10,11]; the other, such amino acids as leucine, valine and phenylalanine (leucine-preferring or L system)[10,11]; methionine and cycloleucine had intermediate affinities (*cf.* ref. 10). A definite overlap between the groups was observed. The low values for lysine interactions with all the amino acids tested suggest that this basic amino acid is transported by a separate system of mediation (*cf.* ref. 15).

Remarkably, the amino acids assigned to the A system were the same which exhibited an increase with time of their transport activity in isolated cardiac cells incubated in Krebs–Ringer buffer (Table I).

Amino acid dependence

Isolated cardiac cells, incubated in Krebs–Ringer bicarbonate buffer, showed a rapid increase with time of the activity of the transport system for a group of amino acids (Fig. 1, Table I) corresponding to the A system of mediation[11] (Table II). Aminoisobutyrate and proline were selected as representative amino acids of this

TABLE II

COMPARATIVE INHIBITORY ACTION OF VARIOUS AMINO ACIDS ON UPTAKE OF RADIOACTIVE α-AMINOISOBUTYRIC ACID, PROLINE, PHENYLALANINE AND 1-AMINOCYCLOPENTANECARBOXYLIC ACID (CYCLOLEUCINE)

Uptake was studied with isolated cardiac cells, suspended in Krebs–Ringer bicarbonate buffer containing 8 mM glucose, in 5-min experiments (temperature 37.5 °C; atmosphere $O_2 + CO_2$ (95:5, v/v)). The four test labelled amino acids were present in turn at 0.1 mM concn. and the unlabelled inhibitor at 5 mM, except for the amino acid mixture which was composed by sixteen 0.5 mM amino acids. Values calculated on the average of 2–4 separate determinations.

Unlabelled amino acid	*Inhibition (%) of uptake of*			
	Aminoisobutyrate	*L-Proline*	*L-Phenylalanine*	*Cycloleucine*
Glycine	40	51	16	33
L-Alanine	82	75	29	—
L-Serine	77	66	33	70
L-Proline	74	—	15	—
L-Methionine	89	79	46	—
L-Phenylalanine	18	30	—	69
L-Valine	30	24	55	—
L-Leucine	27	39	60	77
L-Tryptophan	36	32	72	—
L-Lysine	0	3	23	28
α-Aminoisobutyric acid	—	59	11	—
Cycloleucine	—	65	39	—
Amino acid mixture	77	—	—	—

TABLE III

UPTAKE OF α-AMINOISOBUTYRIC ACID AND L-PROLINE BY ISOLATED CARDIAC CELLS: CHANGES IN TRANSPORT ACTIVITY UPON INCUBATION IN THE PRESENCE OF AMINO ACIDS

Cardiac cell suspensions were incubated for 3 h in Krebs–Ringer bicarbonate buffer containing 8 mM glucose, in the presence and absence of individual amino acids at 5 mM concn. Incubation was at 37.5 °C in an atmosphere of $O_2 + CO_2$ (95:5, v/v). Aminoisobutyrate or proline uptake (initial velocity) was measured at the beginning and at the end of incubation, on samples of cell suspension (10^7 cells) which were washed with Krebs–Ringer buffer (to remove the amino acid when present in the incubation medium), transferred into flasks containing the same medium supplemented with 0.1 mM α-amino-[1-^{14}C]isobutyric acid or L-[^{14}C]proline and incubated for 5 min at 37.5 °C. The values are means of 2–4 separate determinations. Data in μmoles/ml of cell water in 5 min.

Amino acid added	*Aminoisobutyrate uptake (μmoles/ml cell water in 5 min)*		*L-Proline uptake (μmoles/ml cell water in 5 min)*	
	Incubation time (h):		*Incubation time (h):*	
	0	*3*	*0*	*3*
None	0.25	0.69	0.36	1.17
α-Aminoisobutyric acid	—	—	0.32	0.27
Glycine	0.21	0.18	0.37	0.35
L-Alanine	0.18	0.15	—	—
L-Serine	0.18	0.12	0.34	0.25
L-Proline	0.17	0.14	—	—
L-Methionine	0.18	0.18	—	—
L-Leucine	0.25	0.45	0.36	0.97
L-Phenylalanine	0.26	0.71	0.37	1.29
L-Lysine	0.23	0.56	0.39	1.50

group. The addition of individual amino acids to the medium during the incubation period (3 h) had a profound and selective effect on the rate of change in activity of this transport system. Activity was measured at the beginning and at the end of incubation (after removal of the added amino acid) by aminoisobutyrate or proline uptake in 5-min experiments (initial velocities). Using aminoisobutyrate as tracer (Table III), the increase of activity of the system was completely blocked when glycine, alanine, serine, proline and methionine were present during the incubation and was not or little affected by the addition of phenylalanine, lysine and leucine. Using proline as tracer, the increase of activity of the system was abolished when glycine, serine and aminoisobutyrate were added to the medium during the incubation and was not substantially altered by the addition of phenylalanine, lysine and leucine. These results show that the inhibition of the increase (and actually a depression) in activity of the transport system for the amino acids selected as representative of the A mediation is obtained with amino acids which have been assigned to the same group on the basis of competition studies (Table II). The inhibitory effect of methionine is likely to reflect its strongly mixed affinity for both the A and L systems of mediation of neutral amino acids.

Transinhibition

As shown in Table III the increase in activity of the A transport system is abolished by the addition to incubation medium of amino acids pertaining to the same mediation. One possible mechanism which would interfere at this level would

TABLE IV

EFFECTS OF CYCLOHEXIMIDE AND ALANINE ADDITION ON AMINOISOBUTYRATE UPTAKE AND FREE α-AMINO NITROGEN CONCENTRATION IN ISOLATED CARDIAC CELLS

Cardiac cell suspensions were incubated for 2 h in Krebs–Ringer bicarbonate buffer containing 8 mM glucose. Cycloheximide (5 μg/ml, final concn) or L-alanine (10 mM, final concn) were then added and the incubation was continued for 1 h. Uptake (initial velocity) of aminoisobutyrate (0.1 mM) was measured as described in Table III. α-Amino nitrogen was determined on samples of cell suspension which were washed with Krebs–Ringer buffer to remove extracellular amino acids. The time course of alanine uptake was followed by adding L-[^{14}C]alanine (10 mM final concn; 50 μCi/mmole). The values are means of three determinations.

Incubation time (h)	*Aminoisobutyrate uptake (μmoles/ml of cell water in 5-min experiments)*			*α-Amino nitrogen (μg/mg of cell protein)*			*Alanine uptake (μmoles/ml of cell water)*
0	0.22			2.8			—
2	0.50			3.1			—
Additions at 2 h:	*none*	*cycloheximide (5 μg/ml)*	*alanine (10 mM)*	*none*	*cycloheximide (5 μg/ml)*	*alanine (10 mM)*	*[^{14}C]alanine (10 mM)*
2.5	0.59	0.54	0.51	2.9	3.1	6.6	19
3	0.67	0.50	0.40	2.7	3.2	7.5	30

be if the amino acid transported and accumulated intracellularly during the incubation period were to depress the uptake of the tracer amino acid by which the activity of the transport system is measured. Such inhibition by internal amino acids has been called transinhibition[5,7]. In order to search for possible transinhibition in our experimental model, cardiac cell suspensions were incubated for 2 h in Krebs–Ringer buffer (thus enhancing the activity of the A transport system) and for 1 additional hour in the absence and in the presence of cycloheximide (to suppress protein synthesis) or 10 mM L-alanine. Activity of the A transport system, measured by aminoisobutyrate uptake in 5-min experiments (initial velocity) and free α-amino nitrogen concentration were assayed on samples of cell suspension which were washed with Krebs–Ringer buffer to remove amino acids present extracellularly. The time course of alanine uptake was followed in parallel experiments by radioactivity measurements after chromatographic separation of the labelled amino acid[2,13]. It was found (Table IV) that the addition of cycloheximide prevented the increase in activity of aminoisobutyrate transport under conditions in which the intracellular concentration of free α-amino nitrogen was essentially normal. The addition of alanine had a similar effect within 30 min of incubation and depressed transport activity only slightly after 1 h, though its intracellular concentration rose rapidly to very high levels (and total α-amino nitrogen concentration more than doubled in 30 min). In a different experiment cardiac cell suspensions were incubated with cycloheximide (to suppress protein synthesis) up to 2.5 h in the presence and absence of glycine or phenylalanine as representative amino acids for the A and L mediation respectively. The activity of the A transport system was measured, after removal of added glycine or phenylalanine, by aminoisobutyrate uptake in 5-min experiments (initial velocity). Table V shows that cycloheximide depressed transport activity; this inhibition was slightly enhanced in the presence of added amino acids but was of the same order of magni-

TABLE V

α-AMINOISOBUTYRIC ACID UPTAKE BY ISOLATED CARDIAC CELLS INCUBATED IN THE PRESENCE OF CYCLOHEXIMIDE AND AMINO ACIDS

Cardiac cell suspensions were incubated for 1.5 and 2.5 h in Krebs-Ringer bicarbonate buffer containing 8 mM glucose, in the presence and absence of cycloheximide (5 μg/ml) and amino acids (glycine or phenylalanine, 2 mM). Incubation was at 37.5 °C in an atmosphere of $O_2 + CO_2$ (95:5, v/v). Aminoisobutyrate uptake (initial velocity) was measured as described in Table III. Values calculated on the average of two separate determinations.

Additions to the incubation medium	*Aminoisobutyrate uptake in 5-min experiments (% of level in the absence of additions)*	
	Incubation time (h):	
	1.5	*2.5*
None	100	100
Cycloheximide	36	27
Cycloheximide + glycine	29	22
Cycloheximide + phenylalanine	30	21

tude with both glycine and phenylalanine. A comparison of the effects of cycloheximide under different experimental conditions (see Fig. 3, Tables IV and V) indicates that this inhibitor is more efficient in depressing aminoisobutyrate uptake when added before any increase of transport activity has occurred. All these results suggest that, although the occurrence of transinhibition cannot be excluded, it is unlikely that this mechanism is of major importance to explain the inhibition of activity of the A transport system upon addition of amino acids transported by the same agency. Instead, a regulation on the basis of the availability of active transport molecules is favoured.

Kinetic aspects

The experimental conditions of this study were such that the rate of intracellular accumulation of the labelled amino acid (aminoisobutyrate) by isolated cardiac cells was linear over the entire incubation period (5 min) at all the external concentrations used. The extrapolation to infinite concentration[16] of the data from experiments with high aminoisobutyrate concentrations in the medium (40–100 mM) provided a rate constant for apparent diffusion* (K_D) of 0.016 min^{-1}. This value was not substantially altered after 3 h of incubation of the cells in Krebs–Ringer bicarbonate buffer. When the medium contained puromycin (50 μg/ml), cycloheximide (5 μg/ml) or actinomycin D (10 μg/ml) and the incubation lasted 4 h, the values of K_D increased to 0.020–0.022 min^{-1}. Rate constants of 0.006 min^{-1} for apparent diffusion of aminoisobutyrate have been obtained with intact chick embryo hearts in previous experiments[1,9].

Initial uptake velocities of aminoisobutyrate over a 0.1–12.8 mM range of analogue concentrations were determined at the beginning and at the end of a 3-h incubation period in Ringer bicarbonate buffer (supplemented with 8 mM glucose); in some experiments 5 mM glycine was present during the incubation period (3 h)

* The term 'diffusion' is used here to describe a process apparently not subject to saturation, with the usual reservations as to its meaning[1,17].

and was removed before the measurement of aminoisobutyrate uptake. In additional experiments L-phenylalanine in excess (phenylalanine/aminoisobutyrate concentration ratio, 5:1), added during the measurement of aminoisobutyrate uptake, served to minimize the entry of the analogue by the L system. Uptake values were corrected for the non-saturable component of transport[16,17] and analysed by graphical transformations[18] and by computer methods[3].

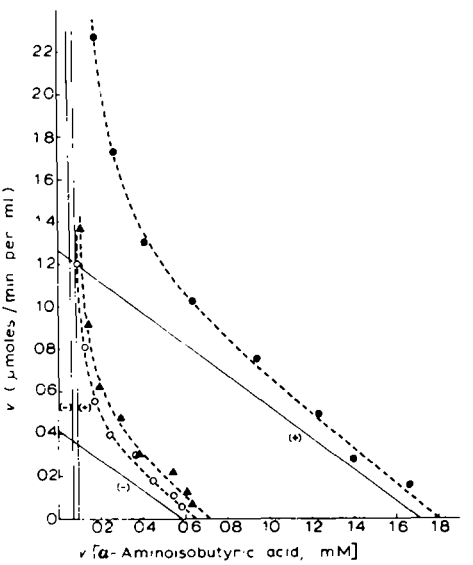

Fig. 5. Initial velocities (v) of α-aminoisobutyric acid uptake (saturable process) by isolated cardiac cells plotted against the ratio of velocity to medium concentration. The data points are means of 2–3 experiments at the beginning (○) and at the end of a 3-h incubation period in the absence (●) and in the presence (▲) of 5 mM glycine. Broken lines (total aminoisobutyrate transport) are drawn according to the fitting of the data obtained by digital-computer methods[3]. Continuous lines represent single transport systems at the beginning (−) and at the end (+) of the incubation period (in the absence of added glycine) as derived from values of V and K_m (see Table VI) of two overlapping components separated by computer analysis. Lines defining single transport systems obtained from experiments at the end of the incubation period in the presence of glycine have been omitted to avoid confusion.

Fig. 5 shows the plot of v *versus* $v/[S]$ for aminoisobutyrate uptake by the saturable process at the beginning and at the end of a 3-h incubation period in Ringer bicarbonate buffer in the absence and in the presence of added glycine. At high external concentrations of the analogue the initial velocities of uptake tended to increase more rapidly than was expected for a single component obeying Michaelis–Menten kinetics under all the experimental conditions adopted. This result suggested the occurrence of more than one agency for aminoisobutyrate transport, presumably reflecting the entry of the analogue by the A and L systems of mediation. Two independent overlapping components were then separated by programming a digital computer to read out the total transport velocity from the summation of independent Michaelis–Menten expressions[3]. These components (subsequently called System I and II according to their primary operational range at low and high substrate concentration respectively) were defined by the values of V and K_m reported in Table VI.

TABLE VI

UPTAKE OF α-AMINOISOBUTYRIC ACID BY ISOLATED CARDIAC CELLS: CHANGES WITH TIME OF THE KINETIC CONSTANTS (SATURABLE COMPONENTS)

Values calculated from the curves presented in Figs 5 and 6 by using computer methods (see text). The two components of aminoisobutyrate uptake have arbitrarily been numbered I (low-concentration system) and II (high-concentration system) for convenient reference. Owing to the small number of observations at high substrate concentration, the precision in estimating the kinetic parameters in this region is rather poor; to obtain potentially realistic solutions, the condition $0 < K_m \leqslant 100$ mM for System II has been imposed in the computing procedure.

Addition to incubation medium (Krebs–Ringer bicarbonate)	*Transport system*	*Incubation time (h):*			
		0		*3*	
		K_m *(mM)*	V *(μmoles/min per ml)*	K_m *(mM)*	V *(μmoles/min per ml)*
None	I	0.72	0.42	0.74	1.27
	II	100	6.97	100	9.39
Glycine (5 mM)	I	—	—	0.76	0.49
	II	—	—	100	7.87
None (but phenylalanine added during uptake measurement)	I	0.69	0.35	0.67	1.13

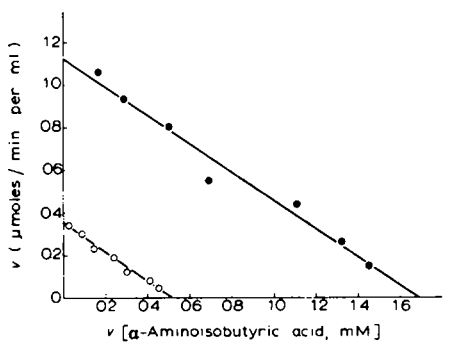

Fig. 6. Plot of v against $v/[S]$ for α-aminoisobutyric acid uptake (saturable component) by isolated cardiac cells, measured in the presence of L-phenylalanine in excess at the beginning (○) and at the end (●) of a 3-h incubation period. Curves are derived by the method of the least squares.

The incubation period in plain Ringer buffer did not alter K_m and markedly increased V of System I. The increase of V was prevented by the presence of 5 mM glycine during the incubation period. No substantial modifications were induced by the incubation period (in the absence and in the presence of glycine) on V characterizing System II. The fact that the latter component was not detectable when the measurements were carried out in the presence of sufficient phenylalanine to prevent aminoisobutyrate uptake by the L system[19] (Fig. 6) suggests that System I can be identified with the A mediation. Therefore, the results described in this paragraph indicate that the regulation of the activity of amino acid transport by the A system occurs through a mechanism affecting the maximal velocity, without substantial changes in substrate concentration for half-maximal transport velocity (K_m).

DISCUSSION

This study has shown that in the chick embryo heart a time-dependent adaptive system for the transport of a number of neutral amino acids is operative, which is subject to regulation by substrate molecules for which it is competent: the activity of transport increases in the absence of added pertinent amino acids and decreases in their presence (Table III).

The amino acids involved in this regulation correspond to those assigned to the A system of mediation by Oxender and Christensen[10] (*cf.* Tables I and II). Neutral amino acids transported by the L system[10] do not participate in this control mechanism (Tables I and III). The recently identified ASC system (typical substrates: alanine, serine, cysteine and threonine)[11,19] also appears to behave independently as indicated by the lack of time-dependent increase of transport activity using threonine (Table I) and by the enhancement of activity using aminoisobutyrate and glycine (which show little or no uptake attributable to the ASC system)[19]; no attempts have been made, however, to discriminate further between A and ASC systems using specific substrates such as α-(methylamino)isobutyric acid and *N*-methylalanine[11,19].

The properties of the described regulation of the activity of the A transport system (slow rate of change and persistence in time, action on the maximal velocity of transport without substantial changes in substrate concentration for half-maximal transport velocity, Figs 1 and 5, Table VI), the failure to explain the regulatory effects in terms of inhibition by internal amino acids (transinhibition[5,7], Tables IV and V) and the suppression of the regulation (with progressive decrease of transport activity) in the presence of inhibitors of protein and RNA synthesis (Figs 3 and 4) suggest that the mechanism involved affects the availability of a protein or proteins needed for the transport process. In turn, an increased availability of transport carriers (or sites) might result from either *de novo* protein synthesis or activation of formerly inactive molecules. A definite increment of total protein synthesis (as measured by the incorporation of amino acid into heart protein) has not been detected under conditions in which the activity of the A transport system increased with time (Fig. 2). However, this might simply reflect the extreme specificity of the synthetic process. In this context, the observation that cycloheximide becomes less effective in depressing subsequent amino acid uptake when added after the occurrence of a substantial enhancement of transport activity (*cf.* Fig. 3 and Table IV) suggests that, under this condition, an increase in the average half-life of the population of carrier molecules has occurred as a result of the contribution of newly synthesized transport proteins. It will be noticed that the increase of transport activity for the natural amino acids of the A mediation (represented by L-proline in the actual experiment, Table III) is prevented by the presence of the non-utilizable analogue aminoisobutyrate (which shares the same transport system). This result might be interpreted to indicate that the mechanism of regulation utilizes the intact amino acid molecule rather than metabolic derivatives. Recent reports of amino acid-dependent regulation of RNA synthesis[20] and of selective sensitivity of an A-type transport system in rat intestine upon treatment with actinomycin D[21] might render feasible the concept that a repression–derepression mechanism by amino acids acting at the transcription level would control transport activity. However, a regulation at a different site (activation–inactivation of transport molecules requiring active translation for

maintenance) cannot be excluded on the basis of the present experiments. In this respect, it is of interest that the amino acids which undergo the described regulation of transport activity (A system) correspond closely to those whose uptake is stimulated by insulin in muscle tissues[4,22] and that this hormonal stimulation of amino acid uptake by isolated cardiac cells remains effective for a few hours under conditions of inhibited protein and RNA synthesis[23]. Similar difficulties in defining the nature of control mechanisms for inducible amino acid transport systems have been encountered in *Neurospora crassa*[24].

Previous calculations of the kinetic constants for uptake of aminoisobutyrate by the chick embryo heart in short-term nonsteady state experiments[1] yielded values of V and K_m which were higher than those calculated from long-term equilibrium experiments[4,9]; moreover, measurements of rates of approach to equilibrium at increasing concentrations of aminoisobutyrate indicated that the higher the external concentration of the analogue the faster the equilibrium was reached[9]. The tentative explanation offered was that the parameters governing the saturable process of aminoisobutyrate transport changed during the course of uptake, the accumulated amino acid progressively interfering with further operation of a carrier[9]. The results of the kinetic experiments reported in this paper provide a more comprehensive interpretation. We have shown that aminoisobutyrate enters the cell by at least two systems of mediation, the A and the L system (Figs 5 and 6) which can be separated and analysed independently. During the course of incubation, the activity of the A transport system increases (Table III) so that a progressively larger fraction of amino acid is transported by this mediation which is characterized by lower values of V and K_m (Table VI). Hence, the overall values of the kinetic constants, as formerly measured without previous separation of the two overlapping components[9], appear to decrease during incubation as a result of an insufficient discrimination. Finally, since the inhibition of the increase in activity of the A transport system is likely to be a function of the concentration of added amino acids taken up by the same agency of mediation, the equilibrium condition will be attained earlier at high than at low external amino acid concentration.

Preliminary experiments indicate that changes with time of the activity of amino acid transport occur also with such biological preparations as isolated rat diaphragms and mouse embryo hearts. In the light of this findings many concepts on the regulation of amino acid transport in muscle tissue should be re-examined.

ACKNOWLEDGEMENTS

This investigation was supported by Grant AM-05290 from the National Institutes of Health, U.S. Public Health Service, Bethesda, Md,. U.S.A.

REFERENCES

1 G. G. Guidotti, A. F. Borghetti, G. Gaja, L. Loreti, G. Ragnotti and P. P. Foà, *Biochem. J.*, 107 (1968) 565.
2 G. G. Guidotti, B. Lüneburg and A. F. Borghetti, *Biochem. J.*, 114 (1969) 97.
3 G. G. Guidotti, A. F. Borghetti, B. Lüneburg and G. C. Gazzola, *Biochem. J.*, 122 (1971) 409.
4 G. G. Guidotti, in P. P. Foà, *The Action of Hormones, Genes to Population*, C. C. Thomas, Springfield, 1971, p. 181.
5 K. Ring and E. Heinz, *Biochem. Z.*, 344 (1966) 446.

6 M. L. Belkhode and P. G. Scholefield, *Biochim. Biophys. Acta*, 173 (1969) 290.
7 M. L. Pall, *Biochim. Biophys. Acta*, 233 (1971) 201.
8 J. M. Phang, G. A. M. Finerman, B. Singh, L. E. Rosenberg and M. Berman, *Biochim. Biophys. Acta*, 230 (1971) 146.
9 K. L. Manchester, G. G. Guidotti, A. F. Borghetti and B. Lüneburg, *Biochim. Biophys. Acta*, 241 (1971) 226.
10 D. L. Oxender and H. N. Christensen, *J. Biol. Chem.*, 238 (1963) 3686.
11 H. N. Christensen, *Adv. Enzymol.*, 32 (1969) 1.
12 G. G. Guidotti, *Atti Soc. It. Patol.*, 10 (1967) 483.
13 G. G. Guidotti, G. Gaja, L. Loreti, G. Ragnotti, D. A. Rottenberg and A. F. Borghetti, *Biochem. J.*, 107 (1968) 575.
14 L. J. Fisher, S. L. Bunting and L. E. Rosenberg, *Clin. Chem.*, 9 (1963) 573.
15 H. N. Christensen, M. E. Handlogten and E. L. Thomas, *Proc. Natl. Acad. Sci. U.S.*, 63 (1969) 948.
16 H. Akedo and H. N. Christensen, *J. Biol. Chem.*, 237 (1962) 118.
17 H. N. Christensen and M. Liang, *J. Biol. Chem.*, 241 (1966) 5542.
18 J. E. Dowd and D. S. Riggs, *J. Biol. Chem.*, 240 (1965) 863.
19 H. N. Christensen, M. Liang and E. G. Archer, *J. Biol. Chem.*, 242 (1967) 5237.
20 M. E. Smulson and J. Thomas, *J. Biol. Chem.*, 244 (1969) 5309.
21 C. Yamada, A. J. Clark and M. E. Swendseid, *Science*, 158 (1967) 129.
22 K. L. Manchester, in G. Litwack, *Biochemical Actions of Hormones*, Vol. 1, Academic Press, New York, 1970, p. 267.
23 G. C. Gazzola, R. Franchi, P. Ronchi, E. Saibene and G. G. Guidotti, *7th Annu. Meet. E.A.S.D., Southampton, 1971*, Abstr. No. 68.
24 L. Wolfinbarger, Jr and A. Gib DeBusk, *Biochim. Biophys. Acta*, 241 (1971) 677.