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ACTIVE TRANSPORT OF AMINO ACIDS INTO BONE CELLS

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SUMMARY

The transport of the model amino acid α-aminoisobutyric acid (AIB) into bone cells isolated mechanically from metaphyses of 40-day-old rats has been examined. Net transfer of AIB into bone cells against a concentration gradient was observed. This process was temperature and energy dependent, thus fulfilling the criteria for "active" transport. Intact cell membranes were required. Glucose concentration in the medium, attempts to manipulate the intracellular electrolyte composition by means of leaching and variations of external calcium, magnesium and phosphate had no effect on this system. Two-thirds of the transport of AIB into bone cells was dependent on the concentration of sodium and potassium in the external medium. It is therefore suggested that two different mechanisms are involved, one independent and the other dependent on ambient sodium and potassium concentrations. The fact that the latter shares a group of properties with the cation-dependent ATPase system points to a possible relation between these two mechanisms. The experiments reported here suggested the possibility that this active transport step might represent a site of control in the biosynthesis of collagen and other proteins in this tissue.

INTRODUCTION

The uptake of amino acids into bone cells has long been of interest to students of collagen biosynthesis, since one of the main tasks of these cells is to synthesize this specialized protein. Early investigations of the problem permitted us to report evidence of the existence of active transport of the model amino acid α-aminoisobutyric acid (AIB) into bone cells isolated mechanically from their calcified matrix and the dependence of the process on oxygen and temperature as well as its competitive inhibition by glycine[1].

Since then, FINERMAN AND ROSENBERG[2] and ADAMSON, LANGELUTTIG AND ANAST[3] have reported similar observations mainly in embryonic bone. Their obser-

Abbreviation: AIB, α-aminoisobutyric acid.

vations include kinetic analyses of competitive inhibition among different amino acids, the dependence of transport on the presence of sodium in the medium and its sensitivity to a variety of metabolic inhibitors, notably ouabain. The data reported below confirm and extend these results. They indicate that similar phenomena take place in cells isolated from the metaphysis of young adult rats studied in suspension—a point of importance since embryonic cells do not have necessarily all the same properties as adult cells[4]. Emphasis in this report is on the need for electrolytes (particularly sodium and potassium) in the transport system under study. Magnesium was studied because of its intimate relation to the former two ions in transport systems, and effects of calcium and phosphate were sought because the concentration of these ions in the plasma is controlled by parathyroid hormone which in turn seems to affect various cellular transport mechanisms[5–7] as well as bone resorption.

MATERIALS AND METHODS

Forty-day-old male albino rats of the Charles River strain were used. Bone cells were isolated from metaphyseal bone as previously described[8] and cells were resuspended in Krebs–Ringer bicarbonate buffer containing glucose, 11 mM. The electrolyte composition of the medium was modified for various experiments as indicated, choline being substituted for other cations when necessary. [1-^{14}C]AIB* was added in a concentration of 0.05 mM to the cell suspension (0.5 ml in a sealed 10-ml erlenmeyer flask). Incubations were generally for 60 min in a Dubnoff metabolic shaker (100 cycles/min) at 37° and pH 7.4 under a gas phase of 95% O_2/5% CO_2. In experiments in which the incubation lasted only 10 min, the concentration of AIB was increased 4-fold to 0.2 mM. When cellular uptake was studied as a function of concentration, AIB in the medium was varied between 0.05 and 0.5 mM as indicated.

Incubations were stopped by rapid chilling and subsequent steps were performed at 0–2°. The contents of the flasks were transferred into long-stem Kolmer–Brown tubes and centrifuged at 1450 × *g* for 15 min. The supernatant was decanted and an aliquot removed for measurement of radioactivity (extracellular counts). The tubes were then thoroughly dried inside with cotton on a wooden applicator. After determining the wet weight, 1 ml of distilled water was added and the tubes were placed in a boiling-water bath for 5 min. After cooling, the tubes were again centrifuged at 1450 × *g* for 15 min, the supernatant decanted and used for measurement of the radioactivity of the cellular phase. The precipitate was subsequently dried in an oven at 105° and after 16 h the dry weight was determined. The difference between the wet and dry weights was considered to equal the tissue water. Samples were prepared for radioactivity measurement by suspending 0.1 ml in 1.5 ml hyamine and 10 ml scintillation fluid. They were counted in a Packard Tri-Carb liquid-scintillation counter (efficiency 54% for ^{14}C). All counts were corrected for quenching determined by internal standardization. Results are expressed as distribution ratios, that is counts/min per ml tissue water, divided by counts/min per ml extracellular fluid, or as μmoles AIB taken up per ml tissue water per 1 h.

* Secured from New England Nuclear Corp., Boston, Mass. Specific activity 4 mC/mmole.

RESULTS

Requirements for AIB transport: Data bearing on certain fundamental aspects of AIB transfer into bone cells are summarized in Figs. 1a–d. The intracellular concentration of AIB increased linearly with time reaching a maximum of almost 10-fold the extracellular concentration at 30–45 min (Fig. 1a). This level was maintained up to 90 min, the end of the time period studied suggesting that it represented the steady-state distribution of this amino acid analog between bone cells and incubation medium under these conditions.

The effect of temperature is illustrated in Fig. 1b. No concentration was achieved at 0.5°. A modest concentrating ability appeared at room temperature (21°) and a clear-cut increase of 3–4-fold over this level was noted at 37°.

The absence of any effect of medium glucose concentration on the AIB concentration differences achieved is shown in Fig. 1c. Equal intracellular concentrations were achieved in the absence of glucose and in the presence of concentrations up to 500 mg% indicating that adequate stores of metabolizable substrate for energy production sufficient for this transport was present in the cells.

Adequate O_2 tension in the gas phase plays a role in the transport mechanism also as shown in Fig. 1d. In the absence of O_2, AIB concentration was reduced to about 50% of that achieved in its presence. There was no significant difference in the concentration ratio of AIB achieved between 21% and 95% O_2.

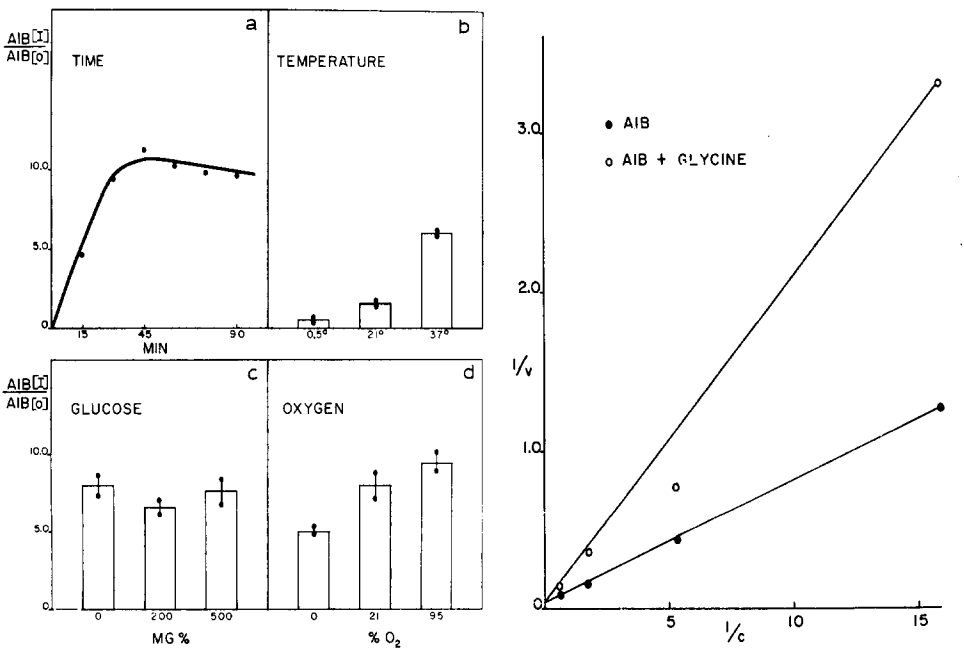

Fig. 1. Effects of time, temperature and substrate availability on AIB transport. Transport is expressed as the ratio of concentration of AIB inside (AIB[I]) and outside (AIB[O]) the cells.

Fig. 2. Effects of concentration of AIB and glycine on AIB transport. Reciprocals of the uptake of AIB in μmole/h are plotted on the ordinate while reciprocals of AIB concentration in the medium in mmole/l are plotted on the abscissa. Solid circles indicate AIB alone; open circles experiments in which 10 mM glycine was present.

Effects of the concentration of AIB and of glycine: Variations in the concentration of AIB in the external medium resulted in the exhibition of saturation kinetics by the transport mechanism. These are illustrated in the form of a Lineweaver–Burk plot in Fig. 2 The effect of glycine in 10 mM concentration is shown. The resulting inhibition of uptake of AIB can be seen to possess kinetics characteristic of competitive inhibition and similar to those which have been shown by others in other systems[10,11].

Cell integrity: Although the cell preparations used—when viewed by light microscopy—consisted essentially of intact mononuclear cells, it was critical to know that the cell membrane remained functionally intact as well. In order to test this, the cells were either ruptured by internal cavitation with N_2 gas[12] or lysed in distilled water. Both procedures abolished AIB transport. The distribution ratio using damaged cells was 1.4 ± 0.1 (4 determinations), whereas the control cells concentrated AIB to a distribution ratio of 8.6 ± 0.9 (4 determinations). This observation confirmed the integrity of the cell preparations used and indicated that they were adequate for studies of amino acid transport. Moreover, they suggested the cell plasma membrane as the probable site of transport rather than the membranes of intracellular organelles since the former method (internal cavitation) was found to preserve intracellular organelles while the latter (lysis in distilled water) does not.

Leaching: One of the steps in the cell isolation procedure required that the cells be suspended for 60 min at 2° in a large volume of fluid containing 0.15 M NaCl, 0.0015 M Na_2HPO_4 and 0.0015 M $CaCl_2$. Since leaching of potassium from cells in exchange for sodium or other electrolytes has been reported under these conditions[13–15], it was important to determine whether the uptake of AIB was affected by such treatment. No significant differences in AIB uptake upon subsequent incubation in the usual medium were detected when cells were leached for periods ranging from 30 to 120 min, nor were differences seen when cells were suspended in the following media: 0.15 M NaCl, Krebs–Ringer bicarbonate buffer or a medium described by KLEINZELLER[16] for the incubation of minced tissue and which contains 42.5 mM sodium, 34 mM potassium, 8.5 mM magnesium and 20 mM phosphate.

These experiments were extended further to test whether the AIB flux rate was affected by leaching cells for 90 min in a medium containing 0.153 M potassium. During this period, the cells presumably not only leach but also swell[13,17,18]. It was therefore important to know whether either process affected the initial flux rate. As

TABLE I

THE EFFECT OF INTRACELLULAR ELECTROLYTE COMPOSITION ON AIB FLUX

Cells leached for 90 min in 0.15 M NaCl or 0.15 M KCl were subsequently incubated at 37° or 0° for 10 min in Krebs–Ringer bicarbonate buffer. AIB concentration 0.2 mM.

Leaching medium	*Incubation temp.*	*AIB flux (J = μmoles AIB per ml tissue water per h)*	*S.D.*	*Distribution ratio after 10 min*
Na	37°	2.035	± 0.084	1.91
K	37°	2.010	± 0.051	1.85
Na or K	0°	0.772	± 0.047	0.74

can be seen from Table I, the AIB flux as calculated from a 10-min incubation in standard medium was identical for cells leached in either potassium or sodium media. It was interesting that the distribution ratios were almost 2 at 37° but below 1 at 0°. It should be noted that the higher values for AIB transport in Table I compared to those in Table II were observed because flux rate rather than uptake was measured and also because higher initial AIB concentrations were required to yield comparable numbers of counts in these shorter incubations. On the basis of these results it was concluded that leaching of intracellular ions and readjustment of intracellular composition did not contribute significantly to AIB transport into these cells.

The effects of magnesium, calcium and phosphate: Effects of magnesium, calcium and phosphate concentration on bone cell AIB transport were also investigated because of their relation to cation transport and membrane integrity in general and their possible relation to the control of bone matrix synthesis in particular. No effects were observed when the concentrations of these ions alone and in concert were varied from zero to many fold their normal physiological levels.

Sodium and potassium dependence of active transport: Active transport of many substrates into a variety of tissue cells has been shown to depend in part at least on the presence of sodium and in some cases potassium in the system. Similar effects appear to be present in the adult rat bone cells studied here. When cells were incubated in media from which either the sodium or potassium was omitted (the difference being made up by choline), AIB uptake was reduced to the same extent (−70%) compared to control incubations in which both cations were present at their physiological level (Table II).

TABLE II

AIB UPTAKE AS A FUNCTION OF THE CONCENTRATION OF Na AND K IN THE MEDIUM

Cells were incubated in media in which choline substituted for either K or Na or with both ions present at physiological levels. AIB concentration was 0.05 mM. Transport was reduced to the same extent when either Na or K was deleted. The sum of the uptake under these two conditions did not account for the transport when cells were placed under physiological conditions. Thus Na and K have potentiating rather than additive effects.

Incubation medium		*AIB uptake (μmole AIB per ml tissue water per h)*	*S.D.*	*Distribution ratio after 60 min*
Na	*K*			
146	0	0.110	± 0.004	2.8
0	4	0.111	± 0.012	2.9
146	4	0.322	± 0.016	9.6

The fact that active AIB uptake was reduced but not totally abolished in the absence of sodium or potassium suggests that 2 different mechanisms are involved in this process, one cation dependent, the other not —a conclusion also reached by FINERMAN AND ROSENBERG[2] on the basis of observations related to changes in sodium concentration alone. Moreover, the data in Table II indicate that sodium and potassium have to be present together to assure maximal transport activity, and that they have a potentiating effect on each other rather than an additive one. In fact, the sum of the AIB uptake in the two cases where either ion was absent cannot

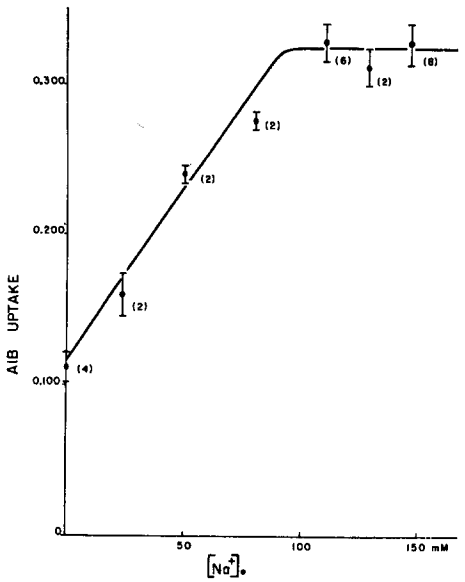

Fig. 3. AIB uptake (μmole AIB per ml tissue water per h) as a function of Na concentration in the medium. Incubation was at 37° for 1 h. Na was substituted by choline. Isomolarity was kept constant at 0.154 M. The vertical bars represent one standard deviation above and below the point. Numbers in parentheses indicate the number of observations at each point in this and subsequent figures.

account for the uptake in the presence of both at their physiological concentration meaning that both are needed simultaneously in order to activate the cation-dependent portion of AIB transport. Since it has been shown that these two cations are linked together in their own transport system, the data suggest that the two transport mechanisms may be related.

To test this hypothesis further, AIB uptake was studied as a function of varying concentrations (0–150 mM) of either cation in the presence of physiological concentrations of the other. It was found that maximal AIB transport activity was obtained when sodium concentration was at or above 110 mM (Fig. 3). Below this AIB uptake was linearly related to increasing sodium concentration. It was thus possible to estimate the sodium concentration which permits half-maximal activity of the cation dependent portion of AIB transport to be 46 mM.

The effect of changing potassium concentration is shown in Figs. 4 and 5. This ion had a biphasic effect with maximal activation of AIB uptake at 4 mM—the physiological concentration. Between zero concentration and this peak, increasing concentrations of potassium stimulated AIB uptake. Again, it was possible to assess the concentration of potassium which elicited half-maximal activity of the cation-dependent AIB transport system. From the expanded plot of AIB uptake as a function of low potassium concentrations (Fig. 5) it was estimated to be 1.3 mM.

Concentrations of potassium higher than physiological on the other hand progressively inhibited AIB uptake. When potassium concentrations above 36 mM were used, sodium concentrations had to be reduced in order to maintain isomolarity. The fact that cells tend to swell[13,17,18] under these conditions may have been respon-

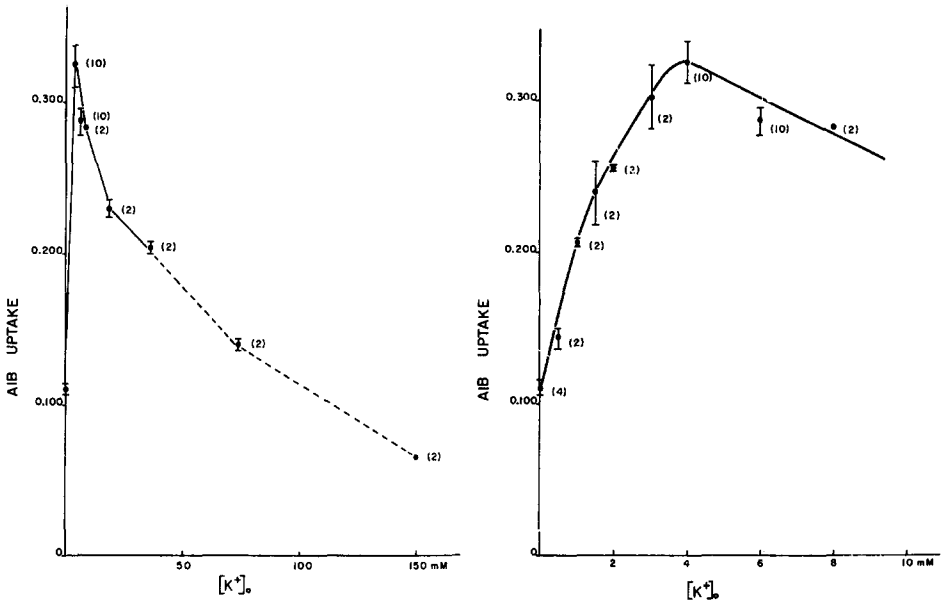

Fig. 4. AIB uptake (μmole AIB per ml tissue water per h) as a function of K concentrations in the medium. Conditions are the same as in Fig. 3. The area of low concentrations is plotted in Fig. 5 on an expanded scale. If K was present in concentrations higher than 36 mM, Na concentrations had to be reduced accordingly. Under these conditions cells tend to swell. Therefore the connection between points representing AIB uptake at these high K concentrations are drawn as dotted lines.

Fig. 5. AIB uptake as a function of low K concentration in the medium. Conditions are the same as in Fig. 4.

sible for the reduction of active transport to below the level assumed to be the baseline of the cation-dependent portion of the process.

On the basis of these data it appears that the cation-dependent portion of AIB transport requires the presence of both sodium and potassium and that either cation alone is ineffective. Moreover, the activating effect of potassium outside the membrane is higher than that of sodium, since the concentration required for half-maximal activity was 1.3 mM, whereas the corresponding sodium concentration was 46 mM.

It is noteworthy that these features of AIB transport are remarkably similar to those which characterize both ATPase activity and the active transport system for monovalent cations in a variety of tissues[14,19]. A possible relationship of AIB transport to these systems has been suggested in kidney on the basis of a similarity of sodium dependence alone[17]. This idea is further strengthened by the data presented here which indicate a quantitative similarity of the potassium dependence as well. Whether the two systems are indeed analogous or whether some similar but not identical system is involved remains to be determined.

DISCUSSION

The existence of an active transport system for AIB and other amino acids in the bone cells of young adult animals and embryos now seems clear from the data

which have been reported from this and other laboratories[1-3]. Not only has it been shown that this model amino acid as well as proline, hydroxyproline and others are all accumulated by bone cells against a concentration gradient, but the data indicate that this process depends upon the availability of metabolic energy in the cells as evidenced by its temperature and O_2 dependence.

The fact that these characteristics of amino acid transport can be demonstrated as well in such different preparations as suspensions of bone cells isolated by mechanical means from their calcified matrix and whole fragments of embryonic bone provides welcome confirmation of their presence in all bone cells and suggests that either system can be used to study the characteristics of the cellular mechanisms involved.

The further dependence, shown in the experiments reported above, of $^2/_3$ of the total transport activity on the ambient concentrations of sodium and potassium was of considerable interest since it suggested that two transport systems exist for AIB—one sensitive to medium alkaline earth ion concentrations, the other not. Moreover, the apparent similarity of the properties of this transport to those of the cation-dependent ATPase system found in other cells[14,19] suggests that some relationship between the two may exist. Further work will be needed, however, to answer these questions.

While the significance in terms of skeletal physiology of this partial cation dependence is not clear at present, other characteristics of the transport system seem more directly related. It was important to note, for example, that changing the ambient concentrations of such important skeletal ions as Mg^{2+}, PO_4^{3-}, and Ca^{2+} was without effect thus rendering a direct effect of the extracellular concentration and availability of these ions on the rates of new bone matrix synthesis questionable. The apparent competition between glycine and AIB for a common carrier, on the other hand, may reflect a potential or actual site for the control of protein synthesis in this tissue. Whether transmembrane amino acid transport constitutes an important hormonally controlled rate-limiting step in protein biosynthesis in bone as is suggested by experiments in other tissues[20] remains to be determined. The data reported here suggest that suspensions of bone cells such as those used in these experiments constitute a suitable and convenient system for the study of this important question.

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