BBA 75276

AMINO ACID TRANSPORT IN ADULT DIAPHYSEAL BONE CONTRAST WITH AMINO ACID TRANSPORT MECHANISMS IN FETAL MEMBRANOUS BONE

THEODORE J. HAHN, SYLVIA J. DOWNING AND JAMES M. PHANG

Metabolism Branch, National Cancer Institute, National Institutes of Health, Bethesda, Md. 20014 (U.S.A.)

(Received January 6th, 1969)

SUMMARY

1. This report describes a system for *in vitro* study of amino acid transport in intact sections of adult rat diaphyseal bone.

2. Kinetic analysis of amino acid transport indicates a common transport mechanism for neutral amino and imino acids in adult diaphyseal bone. This is in contrast to evidence for separate transport mechanisms for neutral amino and imino acids in fetal rat calvaria.

3. Removal of Na^+ from the incubation medium decreases but does not abolish transport of both neutral amino and imino acids in adult rat diaphysis.

4. The implications of differences in transport mechanisms between fetal membranous and adult diaphyseal bone are discussed.

INTRODUCTION

The transport of amino acids into bone cells affords a potential regulatory mechanism for protein synthesis in bone[1]. However, data on membrane transport of amino acids in bone is still limited, despite extensive studies of *in vitro* metabolism of osseous tissue in a variety of preparations[2–6].

To date, detailed studies of amino acid transport in intact bone have primarily been limited to fetal tissues. FINERMAN AND ROSENBERG[7] have presented kinetic evidence for the existence of two mechanisms for membrane transport of amino acids in fetal rat calvaria *in vitro*. Their data indicate that in fetal membranous bone, neutral amino acids are transported by mediated processes that differ, at least in part, from those for imino acids. On the other hand, ADAMSON AND INGBAR[8] working with embryonic chick pelvic bone have suggested that in fetal trabecular bone, neutral amino and imino acids may be transported by similar processes.

The present investigations were undertaken to study the mechanisms by which amino acids are transported into cells in adult diaphyseal bone *in vitro*. Special attention was paid to transport kinetics for neutral amino and imino acids in order to compare transport mechanisms in adult long bone with those reported for fetal membranous and trabecular bone. It was also our purpose to develop a system for analysis

of *in vitro* membrane transport in relatively intact adult long bone. It was felt that such an *in vitro* model which preserves the normal relationships between bone cells and matrix would be well suited to the evaluation of effects of hormonal and other biochemical agents in bone.

The present investigations indicate that, in contrast to fetal membranous bone, adult diaphyseal bone has quite similar membrane transport mechanisms for neutral amino and imino acids.

METHODS

6 week old female Sprague–Dawley rats weighing 100–110 g were killed by stunning and decapitation. Both tibias were rapidly dissected out and the surfaces scraped with a scalpel blade to remove adherent periosteum and soft tissues. The tibias were then bisected longitudinally and the bone marrow completely removed by scraping with the point of a scalpel and washing in cold Krebs–Ringer buffer. Sections of diaphysis were obtained by making transverse cuts across the hemitibias employing a cutting form and scalpel. 2 uniform sections of diaphysis, 6 mm in length and starting 6 mm distal to the proximal epiphyseal plate, were obtained from each tibia. Each section weighed 8–10 mg. Both sections from a single tibia were used in each incubation flask. In inhibition studies each animal was used as its own control, with the sections from one tibia in the control flask and those from the other tibia in the inhibitor flask. Fetal rat calvaria were prepared by the method of FINERMAN AND ROSENBERG[7].

Tissues were preincubated for 30–40 min in either Krebs–Ringer bicarbonate buffer or in the appropriate sodium-free buffer. Incubations were performed in 2 ml of the appropriate buffer, pH 7.4, under a gas phase of 95 % O_2–5 % CO_2 in a Dubnoff metabolic shaker at 37°. Each flask contained a total of 0.4 μC of radioactivity. At the end of the incubation period, the bone was removed and briefly rinsed twice in 5 ml of 0.9 % NaCl, blotted dry with filter paper, and weighed to the nearest 0.1 mg on a microtorsion balance. The tissue was then placed in 2 ml of distilled water and the tissue amino acid pools equilibrated with the distilled water by boiling for 10 min. After the tubes were cooled and centrifuged, 0.2-ml aliquots of both the tissue extracts and media were placed in counting vials and counted in a Nuclear Chicago Mark I liquid scintillation counter according to previously described techniques[9]. Sufficient counts were obtained to reduce counting error for each sample to less than 2 %.

Total tissue water was determined by the difference in tissue weight after blotting and after drying for 24 h at 105°. [^{14}C]Sucrose spaces were determined by the method of ROSENBERG *et al.*[9].

Free label recovery from tissue extracts was determined by thin-layer chromatography utilizing a chloroform–butanol–methanol–conc. NH_4OH (50:30:15:7, by vol.) solvent system in a silica gel medium (Gelman type SG). Radioactivity of the chromatograms was analyzed by cutting the gel into 1 cm × 2 cm strips and counting the strips individually in 10 ml of a 1:25 dilution of Liquifluor (Nuclear Chicago) in a liquid scintillation counter. When α-aminoisobutyric acid was used as the incubation substrate, a single peak of radioactivity at the R_F of α-aminoisobutyric acid was found. However, when naturally occurring amino acids were used, a radioactive peak was found at the origin as well as at the R_F appropriate to the amino

acid. The magnitude of the origin peak varied with the substrate amino acid and incubation time, but in all cases was less than 8 % of total tissue radioactivity. Correction was made for nonspecific adsorption of free amino acid to substances at the origin by adding high specific activity labeled amino acid to certain samples before spotting and then observing the percent of free amino acid bound to the origin. The total tissue extract counts were then corrected by subtracting an amount determined by the percentage of label remaining at the origin of the chromatogram as counts in substances other than free amino acid.

To control for possible adsorption of amino acids onto tissue surfaces, bone sections were killed by lyophilizing and then incubated for 150 min under the appropriate conditions. Tissue counts in excess of those which would be contained in a volume of medium equal to the total tissue water space were assumed to represent adsorption. Adsorbed counts were subtracted from total tissue counts before calculating distribution ratios. Adsorption was corrected for in this manner for all incubation conditions studied. Distribution ratios (intracellular concentration of amino acid/extracellular concentration of amino acid) were calculated according to the method of ROSENBERG *et al.*[10].

MATERIALS

α-Amino[1-^{14}C]isobutyric acid (specific activity, 48 mC/mmole), uniformly labeled L-[^{14}C]proline (specific activity, 185 mC/mmole), and [^{14}C]sucrose (specific activity, 280 mC/mmole) were obtained from Volk. L-4-[^{3}H]hydroxyproline (specific activity, 177 mC/mmole), uniformly labeled L-[^{14}C]alanine (specific activity, 125 mC per mmole) and L-[^{14}C]serine (specific activity, 118 mC/mmole) were obtained from New England Nuclear. α-Aminoisobutyric acid, L-proline, hydroxy-L-proline, L-glycine, L-alanine, and L-serine were obtained from Calbiochem.

RESULTS

Tissue water spaces

Total tissue water content of the tibia diaphysis sections averaged 16.4 ± 0.2 % after 30 min of incubation in Krebs–Ringer bicarbonate buffer. Water content of the fetal rat calvaria was 58.9 ± 0.8 % following a 30-min incubation. Total water values did not change when $Tris^+$ was substituted for Na^+ in the incubation medium. Extracellular fluid space of the tibia sections determined with [^{14}C]sucrose following a 30-min incubation in Krebs–Ringer bicarbonate buffer was 6.4 ± 1.0 %. Sucrose space values did not change when $Tris^+$ was substituted for Na^+ in the medium. Total water and sucrose spaces did not change significantly between 30 and 120 min incubation.

Uptake of amino acids by tibia diaphysis

Tibia sections were demonstrated to accumulate the following amino acids against a concentration gradient: α-aminoisobutyric acid, L-alanine, L-serine, L-proline, and hydroxy-L-proline (Table I). The time course of uptake for α-aminoisobutyric acid and L-proline in the tibia diaphysis sections is shown in Fig. 1. During the initial 30 min, uptake was essentially linear with time for both α-aminoisobutyric acid and L-proline. Steady state conditions were approximated after 180 min for both.

Effect of metabolic inhibitors on α-aminoisobutyric acid uptake

To establish the dependence of amino acid uptake upon active cellular metabolism in this system, the uptake of α-aminoisobutyric acid was studied under conditions known to inhibit cellular metabolism (Table II). At 4° uptake was virtually absent.

TABLE I

UPTAKE OF AMINO ACIDS BY TIBIA DIAPHYSIS

Tissues were incubated for 30 min in Krebs–Ringer bicarbonate buffer, pH 7.4, at 37°. Amino acid concentration was 0.07 mM throughout.

Amino acid	*Distribution ratio* (± *S.D.*)
α-Aminoisobutyric acid	2.47 ± 0.15
L-Alanine	2.66 ± 0.20
L-Serine	3.40 ± 0.60
L-Proline	1.91 ± 0.12
Hydroxy-L-proline	2.41 ± 0.43

TABLE II

INHIBITION OF α-AMINOISOBUTYRIC ACID UPTAKE BY METABOLIC INHIBITORS

Duration of incubation was 30 min. α-Aminoisobutyric acid concentration was 0.07 mM throughout. All values were significantly different from those for controls ($P<0.01$).

Condition	*Distribution ratio* (± *S.D.*)	
	Control	*Treated*
Incubation at 4°	2.35 ± 0.21	0.13 ± 0.03
Anaerobic incubation (95% N_2–5% CO_2)	2.35 ± 0.21	1.63 ± 0.08
Sodium fluoride (10^{-2} M)	2.27 ± 0.24	1.50 ± 0.15
Sodium cyanide (10^{-2} M)	2.27 ± 0.24	0.85 ± 0.29
Iodoacetamide (10^{-2} M)	2.27 ± 0.24	0.78 ± 0.05

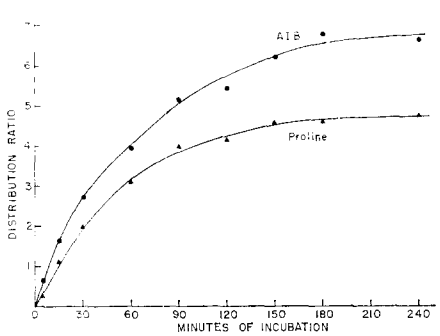

Fig. 1. Time course of amino acid uptake in tibia diaphysis. Incubation was carried out at 37° in Krebs–Ringer bicarbonate buffer, pH 7.4. Initial amino acid concentration was 0.07 mM. AIB, α-aminoisobutyric acid.

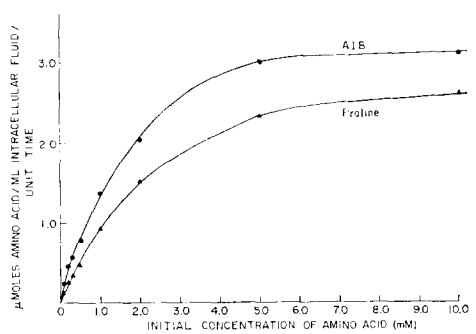

Fig. 2. Effect of initial medium concentration on uptake of α-aminoisobutyric acid (AIB) and L-proline by tibia diaphysis. Duration of incubation was 30 min for both AIB and proline. Final intracellular amino acid concentrations were corrected for diffusion to obtain intracellular concentration attributable to mediated transport. Values represent mean of 4 determinations.

Under anaerobic conditions (95% N_2, 5% CO_2) uptake was moderately depressed. Iodoacetamide proved a very potent inhibitor of α-aminoisobutyric acid uptake, while the effects of fluoride and cyanide were definite but less marked.

Analysis of membrane transport kinetics

Tibia sections were incubated with α-aminoisobutyric acid or L-proline over a 100-fold concentration range, from 0.10 to 10.0 mM concentration of amino acid. At the highest media concentrations, the intracellular amino acid concentrations reached a plateau (Fig. 2), suggesting saturation of the membrane transport mechanism. Assuming a saturable transport system, data were analyzed using Michaelis–Menten kinetic analysis in the form

$$v = \frac{V \cdot c_s}{K_m + c_s}$$

where v is the initial reaction velocity (in this case μmoles amino acid transported/ml intracellular fluid per unit time); V is the theoretical maximum initial velocity; c_s is the amino acid concentration in the medium; and K_m is the Michaelis constant defined as the amino acid concentration required to produce half-maximal transport velocity. Uptake data was corrected for diffusion by the method of AKEDO AND CHRISTENSEN[11]. Values for V and K_m were derived by plotting data according to the Lineweaver–Burk transformation of the Michaelis–Menten equation.

Transport inhibition by neutral and imino acids

Transport inhibition by mutual competition between amino acids was studied over a 100-fold concentration range, with the amino acid under study present either alone or in media containing a constant concentration of another amino acid. Control and inhibitor flasks were paired so that each animal served as its own control. Inhibition of neutral amino acid transport by another neutral amino acid was studied

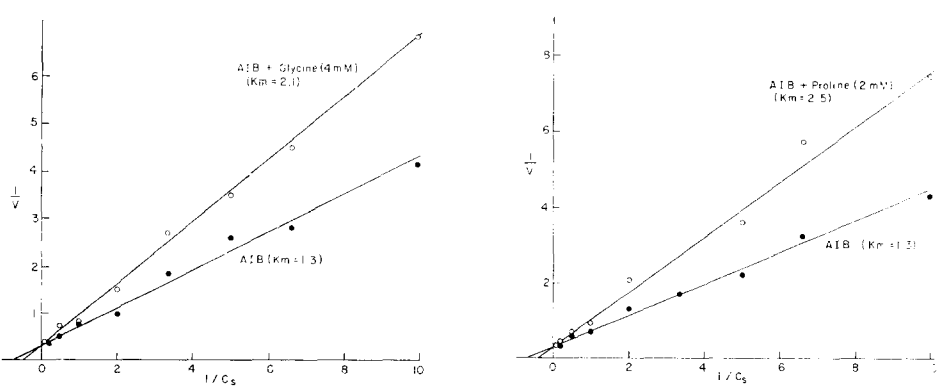

Fig. 3. Effect of glycine (4 mM) on saturation kinetics for α-aminoisobutyric acid (AIB). v denotes micromoles of substrate transported by mediated mechanism in unit time. c_s is initial medium substrate concentration (mM). Duration of incubation was 30 min. Values represent mean of 4 determinations. A common V is interpreted to indicate competitive inhibition.

Fig. 4. Effect of L-proline (2 mM) on saturation kinetics for α-aminoisobutyric acid (AIB). v and c_s are as defined in Fig. 3. Duration of incubation was 30 min. A common V is interpreted to indicate competitive inhibition.

initially. α-Aminoisobutyric acid transport appeared to be competitively inhibited by glycine, since the presence of a 4 mM concentration of glycine in the medium resulted in an increase in the K_m for α-aminoisobutyric acid while V remained unchanged (Fig. 3). This finding would be consistent with the hypothesis that neutral amino acids

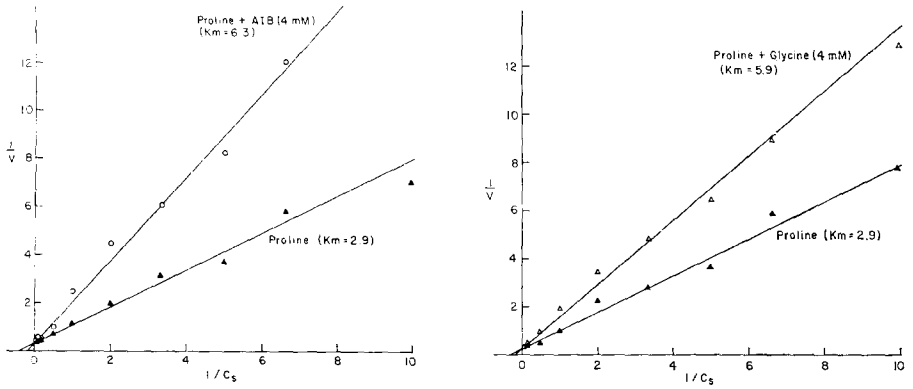

Fig. 5. Effect of α-aminoisobutyric acid (AIB) (4 mM) on saturation kinetics for L-proline. v and c_s are as defined in Fig. 3. Duration of incubation was 30 min. A common V is interpreted to indicate competitive inhibition.

Fig. 6. Effect of glycine (4 mM) on saturation kinetics for L-proline. v and c_s are as defined in Fig. 3. Duration of incubation was 30 min. A common V is interpreted to indicate competitive inhibition.

share a common membrane transport mechanism in this system. However, α-aminoisobutyric acid transport also appeared to be competitively inhibited by an imino acid, L-proline (Fig. 4). To investigate this latter finding further, the inhibition of L-proline uptake by neutral amino acids was studied. It was found that L-proline uptake was competitively inhibited both by α-aminoisobutyric acid (Fig. 5) and by glycine (Fig. 6). In addition, comparison of the transport kinetics of α-aminoisobutyric acid and of L-proline in the absence of competing amino acids demonstrates that they have an identical V but slightly differing K_m's (Fig. 7). The foregoing, then, strongly suggests that in the adult tibia diaphysis, neutral amino and imino acids share a common transport mechanism or mechanisms for which they have different binding affinities.

The results of kinetic analysis of amino acid transport in adult tibia diaphysis differ from the results of similar studies in fetal rat calvaria reported by FINERMAN AND ROSENBERG[7]. These authors demonstrated noncompetitive inhibition between neutral amino and imino acids which they interpreted to indicate that in fetal membranous bone the imino acids are transported by a unique transport mechanism in addition to one which they share with neutral amino acids. It would appear, then, that the membrane transport mechanisms for neutral amino and imino acids in adult diaphysis differ from those in fetal calvaria.

To eliminate the possibility that variations in procedural and analytic techniques between the two studies were responsible for the apparent difference between transport kinetics in fetal membranous and adult diaphyseal bone, we repeated FINERMAN AND ROSENBERG's experiments in fetal rat calvaria. The inhibition of L-proline uptake by glycine in the fetal calvaria system was clearly noncompetitive

as indicated by control and inhibitor plots which intersect at a common K_m but indicate different values for V (Fig. 8).

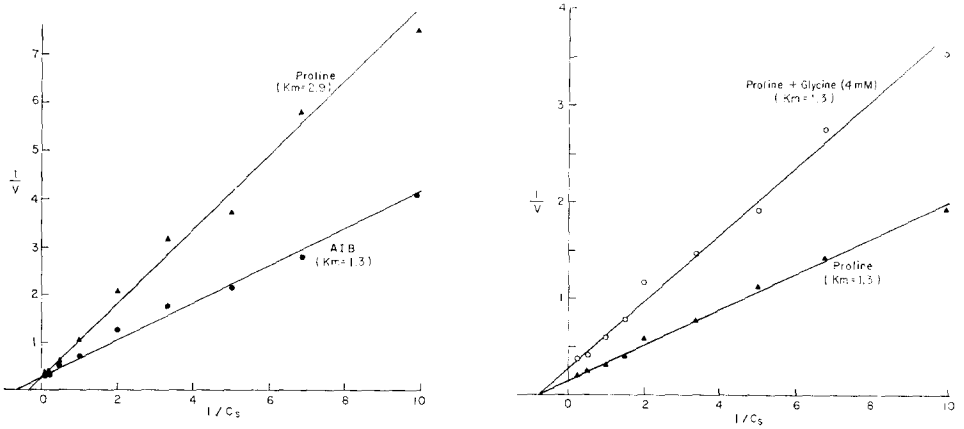

Fig. 7. Comparison of saturation kinetics for α-aminoisobutyric acid (AIB) and L-proline in absence of competing amino acids. v and c_s are as defined in Fig. 3. Duration of incubation was 30 min.

Fig. 8. Fetal calvaria. Effect of glycine (4 mM) on saturation kinetics for α-aminoisobutyric acid (AIB). v and c_s are as defined in Fig. 3. Duration of incubation was 15 min. A common K_m is interpreted to indicate noncompetitive inhibition.

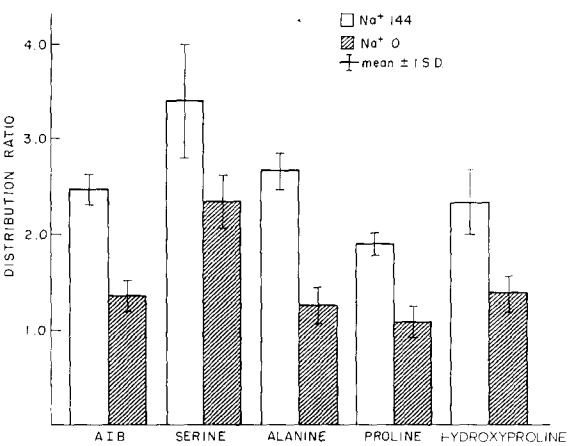

Fig. 9. Effect of deletion of Na^+ from medium on uptake of amino acids in tibia diaphysis. Na^+ 144 indicates standard Krebs–Ringer bicarbonate buffer. Na^+ 0 indicates a buffer (pH 7.4) in which $Tris^+$ was substituted for Na^+. AIB = α-aminoisobutyric acid. Values represent the mean of at least 5 determinations. Duration of incubation was 30 min.

Effect of sodium removal

It has been established that maintenance of Na^+–K^+ gradient across cell membranes is required for concentrative transport of amino acids in a variety of tissues[12]. In addition, it has been demonstrated in kidney cortex slices[13] and fetal calvaria[7] *in vitro*, that removal of extracellular Na^+ selectively abolishes transport of certain groups of amino acids. Accordingly, an attempt was made to distinguish transport

mechanisms for neutral and imino acids in tibia diaphysis by evaluating the effect of deleting Na^+ from the incubation medium.

Substitution of $Tris^+$ for Na^+ in the medium decreased 30-min uptake values for a variety of amino and imino acids to a similar degree (Fig. 9). In no case did active uptake cease. To further evaluate this observation, the effect of Na^+ deletion on the transport kinetics of α-aminoisobutyric acid and proline was investigated. Removal of Na^+ from the medium resulted in a marked decrease in the V for α-aminoisobutyric acid transport, but K_m was not significantly changed (Fig. 10). Similar results were obtained with proline (Fig. 11). Both α-aminoisobutyric acid and proline transport were mediated by a saturable process in the absence of Na^+, as indicated by the continued adherence of the uptake data to Michaelis–Menten kinetics. This again is in contrast to the transport system in fetal membranous bone where deletion of Na^+ from the incubation medium totally abolishes active transport of proline[7].

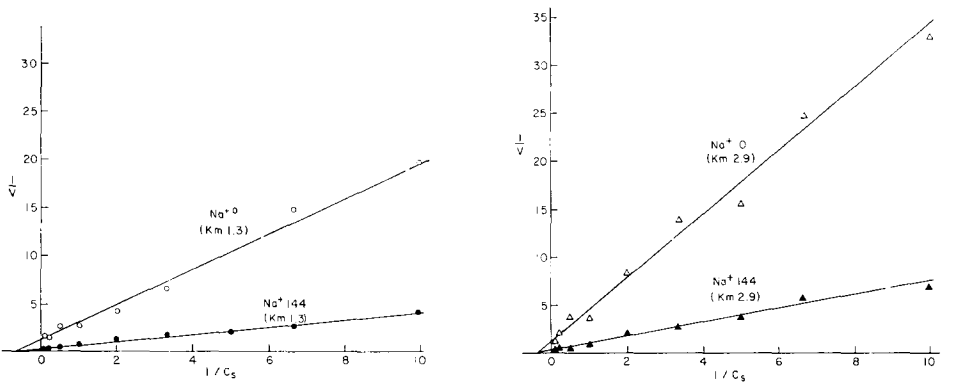

Fig. 10. Effect of deletion of Na^+ from the medium on saturation kinetics for α-aminoisobutyric acid. Na^+ 144 indicates standard Krebs–Ringer bicarbonate buffer. Na^+ o indicates a buffer (pH 7.4) in which $Tris^+$ was substituted for Na^+. v and c_s are as defined in Fig. 3. Duration of incubation was 30 min.

Fig. 11. Effect of deletion of Na^+ from the medium on saturation kinetics for L-proline. v and c_s are as defined in Fig. 3. Duration of incubation was 30 min.

DISCUSSION

The tibia diaphysis preparation described in this report was developed to study amino acid transport in intact adult bone. It was felt that an adult animal system would permit evaluation of the effects of endocrine gland ablation and other modes of *in vivo* manipulation not feasible in previously studied fetal animals. In addition, it was of interest to compare amino acid transport characteristics in intact adult long bone with those reported for fetal membranous and trabecular bone. Sprague–Dawley rat tibia was chosen for our study because of its convenient size, and diaphyseal sections were used to eliminate as much as possible the bone marrow contamination inherent in metaphyseal preparations[2]. Relatively intact tissue sections were used so as to preserve normal structural relationships.

The present studies have demonstrated an energy-requiring saturable mechanism for amino acid transport in adult rat tibia diaphysis. In mediated transport across cell membranes in this tissue neutral amino and imino acids inhibit one another

competitively, suggesting that these two groups of amino acids share a common transport mechanism. This hypothesis is strengthened by the observation that transport kinetics for α-aminoisobutyric acid and L-proline exhibited the same V but different K_m's, a finding compatible with the concept that α-aminoisobutyric acid and L-proline share a common transport site or sites for which they have different binding affinities.

Elimination of Na^+ from the incubation medium did not permit differentiation between transport mechanisms for neutral and imino acids in adult diaphyseal bone. For both α-aminoisobutyric acid and proline transport, Na^+ deletion decreased the transport V without altering K_m. The fact that Na^+ deletion decreased the V for α-aminoisobutyric acid and proline transport to a similar degree again supports the concept of a common transport mechanism. The lack of significant change in the apparent K_m in response to removal of Na^+ from the medium suggests that the presence of extracellular Na^+ may not be a critical factor in determining affinity of amino acids for the membrane transport mechanism in adult diaphyseal bone.

Partial but not complete inhibition of amino acid transport produced by removal of extracellular Na^+ may be interpreted in at least two possible ways. One either can assume that there is a single transport mechanism which requires the presence of extracellular Na^+ to operate at maximum efficiency, or can invoke the presence of two systems for mediated transport, one Na^+ independent and one requiring the presence of extracellular Na^+. Our present data does not allow us to choose between these possibilities.

The contrast between transport mechanisms in fetal membranous and adult diaphyseal bone is of interest. On the basis of our data, it can be concluded that the totally Na^+ dependent transport mechanism for imino acids found in fetal membranous bone[7] is not present in adult diaphyseal bone. In contrast, membrane transport of neutral amino and imino acids in adult diaphyseal bone appears to be mediated by a common mechanism or mechanisms only partially dependent on the presence of extracellular Na^+.

The physiologic significance of this observation is uncertain. It has been reported[8] that α-aminoisobutyric acid and proline appear to be transported by similar mechanisms in embryonic chick pelvic bone. Thus, it is possible that the differences in transport mechanism between adult diaphysis and fetal calvarium may reflect a tissue difference between membranous and trabecular bone. Additional support for this view is provided by the observation that membranous and trabecular bone show different patterns of protein synthesis in response to hypophysectomy[14]. An alternate hypothesis is that the difference in transport mechanisms is due to differences in degree of tissue maturation or hormonal environment. In this regard, ELSAS *et al.*[15] have recently demonstrated age dependent differences in the inulin responsiveness of membrane transport mechanisms for α-aminoisobutyric acid in rat diaphragm *in vitro*. These authors suggest that the difference could be explained either by a decreased turnover rate of transport protein or by the loss of a direct effect of insulin on the carrier mechanism in older animals. Conceivably, then, age related qualitative changes in membrane transport mechanisms could account for the difference between fetal calvarium and adult tibia diaphysis. On the basis of our present data, it is not possible to choose between these alternate explanations.

A direct test, of course, would be to compare transport mechanisms in adult

calvaria with those in fetal calvaria. However, to date it has not been possible to obtain reproducible sections of adult calvaria sufficiently free of marrow contamination to allow definitive studies. Further investigations into the nature and significance of differences in membrane transport mechanisms among various osseous tissues are currently under way in this laboratory.

ACKNOWLEDGMENT

This investigation was supported in part by National Institutes of Health Fellowship 1-F3-CA-39 622-01.

REFERENCES

1 G. A. M. FINERMAN, S. DOWNING AND L. E. ROSENBERG, *Biochim. Biophys. Acta*, 135 (1967) 1008.
2 A. BORLE, N. NICHOLS AND G. NICHOLS, JR., *J. Biol. Chem.*, 235 (1960) 1206.
3 W. H. DAUGHADAY AND I. K. MARIZ, *J. Lab. Clin. Med.*, 59 (1962) 741.
4 D. J. PROCKOP, O. PETTENGILL AND H. HOLTZEN, *Biochim. Biophys. Acta*, 83 (1964) 189.
5 W. A. PECK, S. J. BIRGE, JR. AND S. A. FEDAK, *Science*, 146 (1964) 1476.
6 C. C. JOHNSTON, JR. AND W. P. DEISS, JR., *Endocrinology*, 76 (1965) 198.
7 G. A. M. FINERMAN AND L. E. ROSENBERG, *J. Biol. Chem.*, 241 (1966) 1487.
8 L. F. ADAMSON AND S. INGBAR, *J. Biol. Chem.*, 242 (1967) 2646.
9 L. E. ROSENBERG, S. J. DOWNING AND S. SEGAL, *Am. J. Physiol.*, 202 (1962) 800.
10 L. E. ROSENBERG, A. BLAIR AND S. SEGAL, *Biochim. Biophys. Acta*, 54 (1961) 479.
11 H. AKEDO AND H. N. CHRISTENSEN, *J. Biol. Chem.*, 237 (1962) 118.
12 L. E. HOKIN AND M. R. HOKIN, *Ann. Rev. Biochem.*, 32 (1963) 553.
13 M. FOX, T. SAMUEL, L. ROSENBERG AND S. SEGAL, *Biochim. Biophys. Acta*, 79 (1964) 167.
14 W. A. PECK AND T. R. DIRKSEN, *Clin. Orthopaed.*, 48 (1966) 243.
15 L. J. ELSAS, I. ALBRECHT AND L. E. ROSENBERG, *J. Biol. Chem.*, 243 (1968) 1846.