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AMINO ACID TRANSPORT IN BONE

II. REGULATION OF COLLAGEN SYNTHESIS BY PERTURBATION OF PROLINE TRANSPORT

GERALD A. M. FINERMAN, SYLVIA DOWNING AND LEON E. ROSENBERG*

Metabolism Branch, National Cancer Institute, National Institutes of Health, Bethesda, Md. and The Department of Medicine, Yale University School of Medicine, New Haven, Conn. (U.S.A.)*

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SUMMARY

1. This report describes a system for the determination of tissue free proline, proline incorporation into protein and hydroxyproline formation in fetal rat calvaria.

2. The magnitude of the tissue free proline pool, and the rates of proline incorporation into net protein and collagen varied directly with extracellular proline concentrations less than 0.15 mM. Above 0.15 mM, protein and collagen synthesis were unaffected by further increments in the medium concentration of proline.

3. Addition of α-aminoisobutyric acid, hydroxyproline and L-azetidine-2-carboxylic acid to the incubation medium decreased the intracellular free proline and the rates of proline incorporation into protein and collagen.

4. We conclude that alterations in intracellular proline concentration produced either by varying extracellular proline concentration or by inhibiting the uptake of proline into the intracellular pool, may contribute to the regulation of collagen synthesis.

INTRODUCTION

Active transport systems for amino acids have been documented in a host of microbial and mammalian systems. These studies have emphasized chemical and stereospecificity of the transport processes, their dependence on metabolic energy, and the effects of hormones and vitamins[1]. With few exceptions, however, earlier reports have not concentrated on the critical relationship between the entry of amino acids into cells and their subsequent incorporation into specific proteins. In a previous report, we described the features of the transport processes for neutral amino and imino acids in fetal rat calvaria[2]. The present study extends these observations to investigate the role of alterations in proline transport and the free intracellular proline pool on subsequent collagen synthesis in this tissue. The results indicate that

* Address of requests for reprints.

transport of proline influences collagen synthesis markedly, thus providing an additional mechanism for regulation of this vital phenomenon.

METHODS

Calvaria from full-term rat fetuses were obtained and prepared for incubation as described previously[2]. All studies were carried out under steady-state conditions. The calvaria were preincubated in bicarbonate buffer containing unlabelled L-proline for 180 min prior to addition of tracer L-[^{14}C]proline. Tissues were then incubated for 1–120 min and the experiments terminated as described previously[2]. Tissues were then rinsed in saline, blotted, weighed on a torsion balance and homogenized in 95% ethanol–5% 0.1 M HCl solution. The resulting precipitate was washed 4 times. The acidic ethanol supernatants resulting from the 4 washes were combined, dried in air, and dissolved in 1 ml of distilled water. Small samples of this solution were chromatographed on paper using a descending phenol–water (100:39, w/v) *plus* 8-hydroxyquinolone (0.04%) system, which adequately separate proline from hydroxyproline. The chromatograms were counted in a Vanguard strip counter and only a single radio active peak with the appropriate R_F for proline was found. Experiments in which [^{14}C]proline was added to intact calvaria immediately prior to homogenization and extraction indicated that more than 98% of the initial radioactivity was recovered by this procedure, even in the presence of 100-fold excess of unlabelled proline. Separate aliquots (0.2 ml) of this solution, assumed to represent the free tissue proline pool were prepared for liquid-scintillation counting as described previously[2] as were 0.2-ml aliquots of the incubation medium.

The washed precipitate was suspended in 5 ml of 6 M HCl in a teflon capped screw top test tube and hydrolyzed for 20 h at 100–110°. The HCl was evaporated, residue dissolved in 200 μl of distilled water and an aliquot taken for paper chromatography in the descending phenol–water–hydroxyquinolone system described above. The chromatogram was dried and cut into 1-cm squares which were placed in liquid-

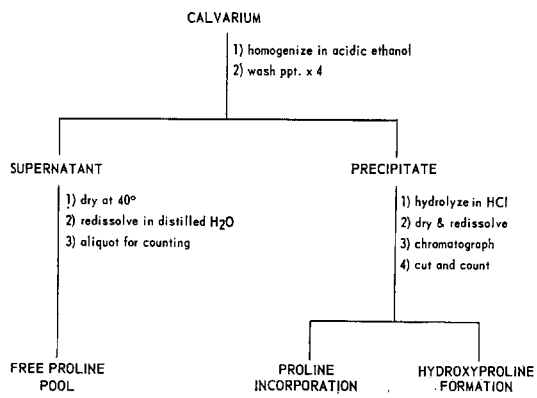


Fig. 1. Schema for analysis of tissue homogenate from fetal rat calvaria. The 'free proline pool' represents the acidic ethanol-soluble [^{14}C]proline. The 'proline incorporation' represents [^{14}C]-proline incorporated into collagen and non-collagen protein. 'Hydroxyproline formation' represents the [^{14}C]hydroxyproline content of protein and provides an estimate of new collagen formation.

scintillation counting vials containing a 2,5-diphenyloxazole–1,4-bis-(5-phenyloxazolyl-2)-benzene phosphor obtained commercially (Liquiflor, New England Nuclear Corp.). The radioactivity migrating with the appropriate R_F value for proline was identified by appropriate standards and taken as a measure of incorporation of proline into total tissue proteins. The radioactive hydroxyproline, identified similarly, was taken as a measure of new collagen synthesis[3]. Fig. 1 summarizes the scheme used in preparing these various tissue fractions.

The total isotopic recovery from the free tissue proline pool, the proline incorporated into tissue protein, and the proline converted to hydroxyproline in protein (collagen synthesis) was calculated and expressed as the fraction of the initial medium radioactivity per 100 mg of tissue wet weight. These values for the two protein fractions are analogous to specific activity determinations since the proline and hydroxyproline content of tissue proteins were noted to be directly proportional to tissue weight.

Chemical determinations of proline were performed by the method of SUMMER AND ROSZEL[4].

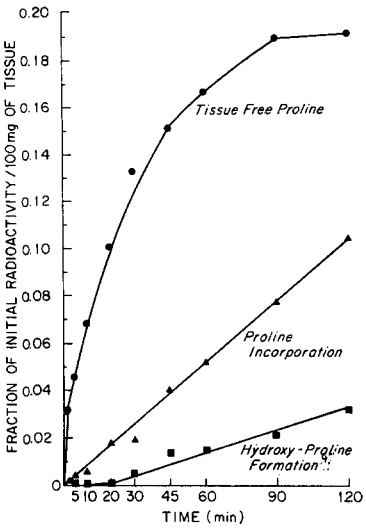
Uniformly labelled [^{14}C]proline (specific activity 188 mC/mmole) was obtained from New England Nuclear Corp. Unlabelled proline, hydroxyproline and α-aminoisobutyric acid were obtained from California Biochemical Corp. L-Amino acids were used throughout.

RESULTS

Fig. 2 presents results from a representative experiment in which the kinetics of [^{14}C]proline uptake into the free tissue pool, proline incorporation into protein and hydroxyproline formation were measured. Tissues were preincubated for 3 h with 0.14 mM proline to achieve steady-state conditions. The uptake of [^{14}C]proline into the free amino acid pool was maximal during the first 15 min and reached a plateau after 90 min. The incorporation of proline into total tissue proteins was linear throughout the 120-min study. Collagen synthesis as measured by hydroxyproline in collagen occurs on the ribosome after incorporation of the proline into peptides[5,6]. The lag noted for hydroxyproline formation in our experiments could reflect such a ribosomal event.

The influence of variations in incubation medium proline concentration on steady-state values for tissue free proline, proline incorporation into total protein and hydroxyproline formation are presented in Fig. 3. Tissue free proline increased throughout the range of concentrations used. These findings are consistent with our previous observations which indicated that the uptake mechanism for proline failed to show saturation below 5 mM proline concentrations. In contrast, proline incorporation into total protein reached a plateau when the medium proline concentration exceeded 0.15 mM. These findings suggest that at extracellular concentrations below 0.15 mM, the rate of total protein and collagen synthesis was dependent on the transport of proline into the cell. Above this concentration, protein and collagen synthesis were unaffected by further increments in free proline pool size, suggesting that under these conditions, the rate-limiting step in protein synthesis was beyond the uptake process.

The above results showed that extracellular fluid proline concentrations in-



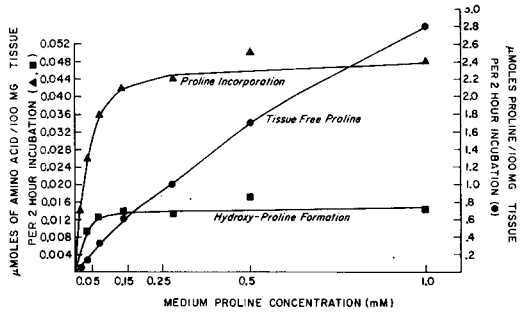
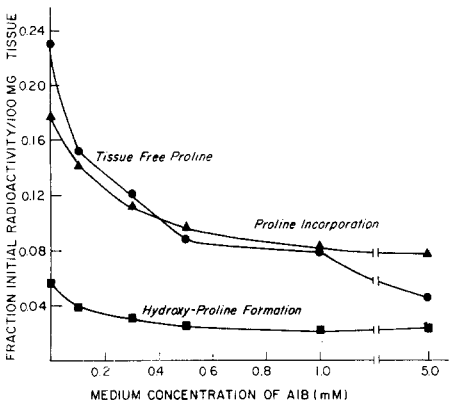


Fig. 2. Steady-state time curve of [^{14}C]proline uptake into tissue free proline pool, incorporation into total tissue protein and formation of hydroxyproline in collagen. Tissue was preincubated for 180 min in 0.14 mM proline prior to the addition of carrier-free [^{14}C]proline. Data is presented as the fraction of initial medium radioactivity per 100 mg of tissue and represents mean of duplicate values. Hydroxyproline formation was negligible during the first 20 min.

Fig. 3. Effect of medium proline concentration upon the steady-state uptake of [^{14}C]proline into tissue free proline pool, incorporation into total tissue protein and formation of hydroxyproline. Tissues were preincubated for 180 min in varying proline concentrations prior to the addition of carrier-free [^{14}C]proline and were then incubated for 120 min. Values for tissue free proline are presented on the right ordinate while those for proline incorporation and hydroxyproline formation are shown on the left ordinate. Values represent the mean of at least duplicate determinations.

fluenced the tissue free proline pool and, at least for concentrations below 0.15 mM, collagen synthesis. Earlier studies from this laboratory[2] have shown that other amino acids (*i.e.*, α-aminoisobutyric acid and hydroxyproline) compete with proline for uptake into the free amino acid pool, while dipeptides such as glycyl-proline, prolyl-glycine, glycyl-hydroxyproline and hydroxyprolyl-glycine did not influence proline uptake when added to the incubation medium*. Therefore, it seemed important to determine if these competing amino acids also served to regulate the rate of collagen synthesis. Chemical determinations of proline in the tissue pool showed that incubation of fetal rat calvaria in a buffer containing 0.14 mM proline and 1.0 mM α-aminoisobutyric acid resulted in a 30% decrease in free proline when compared to tissue incubated in 0.14 mM proline alone. The data in Fig. 4 demonstrate the effect of increasing medium concentrations of α-aminoisobutyric acid on the fraction of initial [^{14}C]proline radioactivity found in tissue free proline, total protein and collagen. For these experiments tissues were preincubated in 0.14 mM proline and varying α-aminoisobutyric acid concentrations for 3 h prior to the addition of carrier-free [^{14}C]proline and the incubation carried out for 2 h. As the concentration of α-aminoisobutyric acid in the medium was increased, there was a decrease in tissue free proline, incorporated proline and newly formed hydroxyproline. Since α-aminoiso-

* G. A. M. FINERMAN AND L. E. ROSENBERG, unpublished observations.



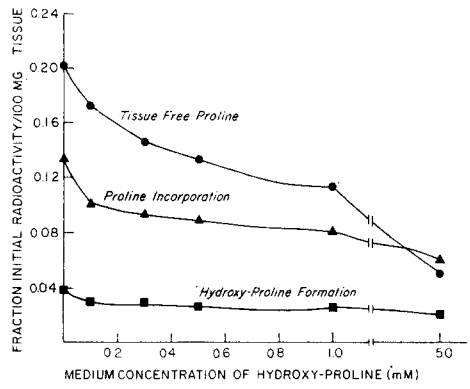


Fig. 4. Effect of varying medium concentrations of α-aminoisobutyric acid (AIB) upon the steady-state uptake of [^{14}C]proline into tissue free proline pool, incorporation into total tissue protein and formation of hydroxyproline. Tissues were preincubated for 180 min in 0.14 mM proline and varying concentrations of AIB prior to the addition of carrier-free [^{14}C]proline and were then incubated for 120 min. Data is presented as the fraction of initial medium radioactivity per 100 mg of tissue and represents mean of duplicate values.

Fig. 5. Effect of varying medium concentrations of hydroxyproline upon the steady-state uptake of [^{14}C]proline into tissue free proline pool, incorporation into total tissue protein and formation of hydroxyproline. Tissues were preincubated for 180 min in 0.14 mM proline and varying concentrations of hydroxyproline prior to the addition of carrier-free [^{14}C]proline and were then incubated for 120 min. Data is presented as fraction of initial medium radioactivity per 100 mg of tissue and represents mean of duplicate values.

butyric acid has been shown in numerous studies to influence transport mechanisms without modifying intracellular processes, it seems likely that this non-metabolized amino acid reduced collagen synthesis secondarily by reducing proline uptake. Similar findings were noted when increasing concentrations of hydroxyproline were substituted for α-aminoisobutyric acid in the incubation medium (Fig. 5). These studies indicate that any decrement in the free proline pool produced by α-aminoisobutyric acid or hydroxyproline was also reflected in a decrement in intracellular utilization. This suggests that either part or all of the free proline pool regulates synthesis of collagen and other tissue proteins.

TABLE I

EFFECT OF SECOND AMINO ACID ON KINETICS OF STEADY-STATE PROLINE UPTAKE AND INCORPORATION

Calvaria were preincubated with L-proline (0.14 mM)and a second amino acid (1 mM) for 180 min prior to addition of carrier-free D-[^{14}C]proline. Incubation was then continued for 120 min. All values represent mean of at least two separate determinations.

Second amino acid	*Inhibition (%)*		
	Uptake	*Proline incorporation*	*Hydroxy-proline formation*
α-Aminoisobutyric acid	66.4	54.2	58.9
L-Hydroxyproline	44.4	39.3	33.3
L-Azetidine-2-carboxylic acid	53.9	47.8	68.2

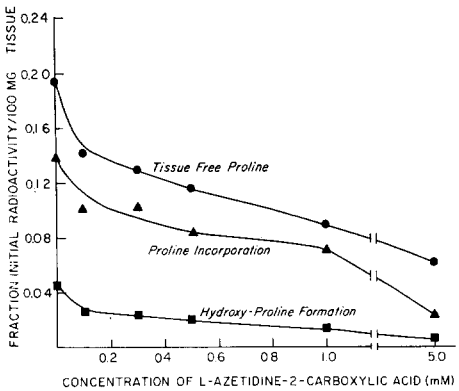


Fig. 6. Effect of varying medium concentrations of L-azetidine-2-carboxylic acid upon the steady-state uptake of [^{14}C]proline into tissue free proline pool, incorporation into total tissue protein and formation of hydroxyproline. Tissues were preincubated for 100 min in 0.14 mM proline and varying concentrations of L-azetidine-2-carboxylic acid prior to the addition of carrier-free [^{14}C]proline and were then incubated for 120 min. Data is presented as fraction of initial medium radioactivity per 100 mg of tissue and represents mean of duplicate values.

Slightly different results were obtained using L-azetidine-2-carboxylic acid. This amino acid is the 4-carbon-ring analog of proline and in contrast to α-amino-isobutyric acid or hydroxyproline, can be incorporated into protein. Again there is decreased tissue free proline, proline incorporation and hydroxyproline formation. The data presented in Fig. 6 reveal that as the medium concentration of L-azetidine-2-carboxylic acid increased, appearance of [^{14}C]proline in the free pool, total protein pool and collagen pool decreased. However, as shown in Table I, collagen synthesis was inhibited more than total protein synthesis. This was not seen with α-amino-isobutyric and hydroxyproline and suggests that L-azetidine-2-carboxylic acid inhibits collagen synthesis both by reducing the size of the precursor proline pool and by competing directly with proline for incorporation into collagen.

DISCUSSION

The kinetics of the metabolic parameters followed in this study are noteworthy. As anticipated uptake of proline into the free intracellular pool was curvilinear with time while the plot of incorporation of proline into total tissue protein was linear. These results agree with previous findings in single cell systems[7–9] and mammalian tissues. KIPNIS[10], HALVORSON[11], ROSENBERG[12], and their co-workers have argued that the linear kinetics of protein incorporation indicate compartmentation of the intracellular free amino acid pool since an initial lag in the rate of incorporation would be expected if equilibration of the tracer amino acid with the total intracellular pool was a necessary precedant to protein synthesis. We hoped to resolve this significant question by following the rate of synthesis of a specific protein, collagen, rather than relying, as have past workers, on total trichloroacetic acid-precipitable protein. The kinetics of collagen synthesis in this system did, indeed, show an early lag followed by a linear plot. This lag, however, could reflect either equilibration of the tracer proline with the intracellular pool or the necessity of a finite

time period to hydroxylate proline after ribosomal peptide linkage has been accomplished. Thus the kinetics of collagen synthesis, alone, do not resolve the question of intracellular pool compartmentation.

The present findings do indicate, however, that below 0.15 mM, alterations in medium proline concentration are reflected in changes in collagen and net protein synthesis. From these results alone, one could not determine whether the changes in protein synthesis were produced by modifying the extracellular proline pool or, alternatively, the intracellular pool. The experiments with α-aminoisobutyric acid and hydroxyproline indicate that it is the intracellular pool which is responsible for the changes in protein synthesis, since these compounds were noted to reduce the rates of uptake of proline into the free pool and into protein while having no effect on the extracellular pool size. Thus, we can say that the free intracellular proline pool size influenced synthesis of collagen and total protein and, hence, that whatever is responsible for the linear kinetics of total protein synthesis does not represent total dissociation between the free amino acid pool and the 'pre-protein' pool. The above remarks, however, do not mean that the free amino acid pool is necessarily homogeneous. The data in Fig. 7 offer a comparison between the inhibitory effects of α-aminoisobutyric acid on proline uptake *versus* incorporation. It is noteworthy that α-aminoisobutyric acid reproducibly inhibited uptake more than incorporation, meaning either that the rate-limiting step in protein and collagen synthesis is beyond

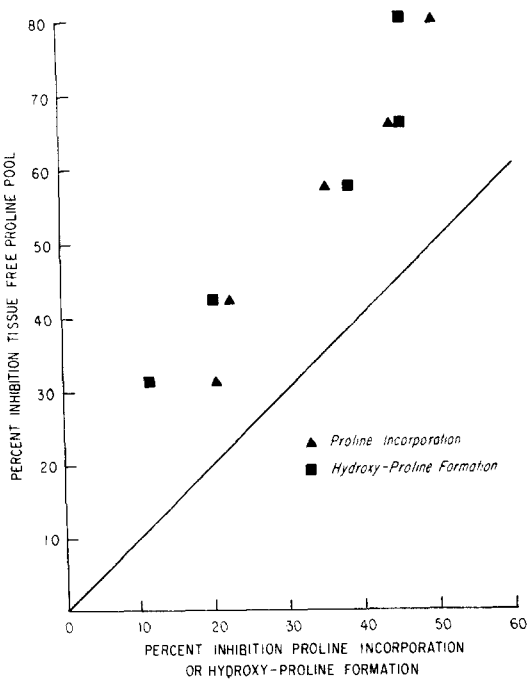


Fig. 7. Comparison of inhibitory effects of α-aminoisobutyric acid (AIB) on uptake of proline into the free tissue pool (ordinate) *versus* incorporation into total protein or collagen (abscissa). Values are derived from data shown in Fig. 3 and are presented as per cent inhibition of control. Note that uptake is inhibited more than incorporation at each point shown, as indicated by points lying above 45° axis (solid line).

the uptake mechanism or alternatively that only a portion of the free proline pool acts as a protein precursor fraction. The complex kinetics of this system are being studied currently using a multicompartment, computer analysis.

At medium proline concentrations above 0.15 mM, proline incorporation into total protein and collagen becomes constant. STEIN AND MOORE[13] have analyzed the free amino acids in human plasma and found the fasting concentration of proline to be about 0.2 mM. No similar results are available for fetal rats but it seems likely that the circulating free proline concentration in plasma is close to that found necessary for maximal collagen synthesis. It must be remembered, however, that other amino acids can decrease the intracellular free proline concentration by competing for transport and thereby decrease proline incorporation into protein and hydroxyproline formation. In this regard, HURYCH AND CHVAPIL[14] have shown, in studies with chick embryo skin slices, that the medium concentration of free hydroxyproline increased during incubation, presumably due to breakdown of newly formed soluble collagen. Thus, cells rapidly synthesizing collagen may have a form of negative feedback similar to that demonstrated in the studies reported presently.

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