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Source of amino acids for tRNA acylation in growing chicks

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Summary. Specific radioactivity in three amino acid compartments was examined in broiler chicks following a flooding dose of leucine or phenylalanine. In general, specific radioactivity of leucine and phenylalanine in deproteinated plasma (SA_e) and tissue (SA_i) compartments, exceeded that in acylated-tRNA (SA_t). In most tissues, SA_e and SA_i rapidly reached a similar peak level by 5 min followed by a slow decline for the next 30 minutes. Many tissues (eg. GI tract, liver, skin, and thigh) failed to maintain equilibrium between SA_e and SA_i over time. More metabolically active tissues, such as GI and liver had the greatest differences between these compartments. The difference between SA_e and SA_i for both leucine and phenylalanine were due to SA_i decreasing faster than SA_e, indicating dilution with unlabelled amino acids from proteolysis. Plasma and tissue specific radioactivity overestimated tRNA specific radioactivity by as much as 5 and 2.8 fold using leucine or 2.7 and 1.4 fold using phenylalanine, respectively. These data suggest that intracellular compartmentation of protein metabolism and the coupling of protein degradation and synthesis occur, in vivo.

Keywords: Amino acids – Protein synthesis – tRNA charging – Amino acid metabolism

Introduction

Protein accretion, the net result of the antagonistic processes of protein synthesis and degradation, is a primary determinant of growth. Small changes in synthesis and degradation can result in large changes in protein accretion. Experimentally, fractional protein synthesis rate is determined from the relationship between specific radioactivity (SA) of an amino acid in protein and specific radioactivity

of that free amino acid in the precursor pool for protein synthesis following injection of radiolabeled amino acid (Zak et al., 1979). However, the immediate precursor of protein synthesis, aminoacylated tRNA, is seldom used in the calculation due to the difficulty involved in aminoacyl-tRNA isolation and technical problems associated with quantifying specific radioactivity of this small amino acid pool. Current methodology typically substitutes alternative free amino acid pools as the precursors for protein synthesis on the assumption that amino acid pools rapidly equilibrate (Schaefer and Scott, 1993).

The loading dose or flooding dose technique for measuring rate of protein synthesis was originally used by Henshaw et al. (1971) in an effort to stabilize the specific radioactivity of a tracer amino acid in the tissue pool over a 20 minute period. By injecting 75 mmoles of radiolabeled lysine to 100-g rats they demonstrated that SA in plasma, muscle and liver was in steady state by 10 minutes post-injection and that the condition held during the next 10 minutes. The loading dose method is currently used in an attempt to eliminate the need to determine the SA in tRNA (SA_t) for the purpose of estimating rate of protein synthesis. This method takes advantage of the observation that increasing extracellular amino acid concentration of the tracer amino acid decreases differences between the SA in the extracellular pool (SA_e) and the intracellular pool (SA_i; Bernier et al., 1986; Garlick et al., 1980; McNurlan et al., 1979). Assuming that tRNA is acylated with amino acids from one or both of these pools, SA_e, SA_i, and SA_t should be equal.

Direct measurement of SA_t in livers perfused with a loading dose level of tracer amino acid (Khairallah and Mortimore, 1976), in lungs of rats infused with a high concentration of tracer amino acid (Kelly et al., 1984), and hearts perfused with a high level of tracer amino acid (McKee et al., 1978) demonstrated the validity of assuming that such conditions would force SA_t to be equal to SA_e and/or SA_i. However, work in cell culture using smooth muscle (Opsahl and Ehrhart, 1987), normal and dystrophic skeletal muscle (Schneibel and Young, 1984), rat epitrochlearis muscle (Stirewalt and Low, 1983), osteoblasts (Hall and Yee, 1989), pulmonary macrophages (Airhart et al., 1981; Hammer and Rannels, 1981), and a chicken macrophage cell line (Barnes et al., 1992) demonstrated that SA_t failed to equilibrate with SA_e or SA_i despite flooding conditions. These data suggest that tRNA may be charged from amino acid pools other than the general cytosolic or extracellular pools. In fact Barnes et al., (1992) presented data suggesting that tRNA in a chicken macrophage line is charged directly by amino acids which are products of protein degradation and which do not enter the general pool of cytosolic amino acids.

Despite considerable evidence of compartmentation of tracer amino acids across various pools both in vitro or in tissue perfusion, few comprehensive studies have been reported in vivo. Smith et al. (1991) demonstrated that neither a tracer nor a flooding dose of valine could eliminate the contribution of proteolysis to tRNA acylation in the brain of rats. They found that the contribution of protein degradation to tRNA acylation was 63 and 23% with a tracer and flooding dose, respectively. Additionally, the impact of physiological state on amino acid compartmentation is not clear. The objective of the present research was to examine in vivo the relationship between amino acid compart-

ments over time in a variety of tissues of a growing chick after a single injection of a loading dose of radiolabeled amino acid.

Materials and methods

Experiment 1

Eleven male Hubbard x Hubbard broiler chicks fed a commercial chick starter ad libitum were raised to 7 days of age and an average weight of 102 g. Each chick was injected intracardially with 1.0 ml 0.8% saline containing 300 μCi ^{3}H- leucine (L-3,4,5-^{3}H, New England Nuclear, 153 Ci/mmol) and 100 mmol unlabeled leucine. Three chicks were bled by cardiac puncture and sacrificed by cervical dislocation at 5 minutes and four each at 10 and 30 minutes post-injection. Liver, heart, pectoralis, and thigh muscles (Illiotibilias and Illiofibularis) were removed and frozen using aluminum clamps cooled in liquid nitrogen. Prior to freezing, the heart was flushed with 3.0 ml of phosphate buffered saline. Tissues were typically frozen within 1 minute of cervical dislocation.

Blood was placed on ice and plasma obtained by centrifugation at 1000 x g for 30 minutes. Plasma proteins were precipitated by addition of an equal volume addition of 16% trichloroacetic acid (TCA) followed by centrifugation at 10,000 x g for 10 minutes. The resulting supernatant represented the extracellular pool of amino acids. A 0.5 gram aliquot of frozen tissue was homogenized for 30 seconds in 1.0 ml of water using a Polytron homogenizer (Brinkman Instruments, Westbury, NY) equipped with a PTA-10 probe. Protein was precipitated by addition of 1.0 ml 16% TCA and centrifugation. The resulting supernatant represented intracellular amino acids.

Amino acids representing aminoacyl-tRNA were obtained following isolation and alkaline hydrolysis of aminoacyl-tRNA (Barnes et al., 1992). Briefly, approximately 0.5 g of finely minced frozen tissue was homogenized in 2.0 ml 100 mM sodium acetate (pH 5.5), 1.0% (w/vol) SDS, and 1.0 mM EDTA (NaAc buffer), using a Polytron homogenizer equipped with a PTA-7 probe. Nucleic acids were isolated by phenol:chloroform:isoamyl alcohol (25:24:1) extraction and ethanol precipitation. Amino acids were hydrolyzed from tRNA by adjusting the pH to 10 with 0.5 M NaOH, and incubating at 41°C for one hour. This method results in little introduction of labeled or unlabeled amino acids arising from contaminates in the isolation procedure to tRNA derived amino acids (Barnes et al., 1992).

Amino acid concentrations were determined using phenylisothiocyanate (PITC) derivitization, reverse phase HPLC separation, and detection of the phenylthiocarbamyl amino acids at 254 nm (Bidlingmeyer et al., 1984). Radioactivity associated with the leucine peak was measured using a Packard LS1600 liquid scintillation counter.

Experiment 2

16 male Hubbard x Hubbard chicks fed a commercial chick starter were raised to 12 days of age and an average weight of 125 g. In an effort to control variation due to feed consumption and insure that chicks were in a fed state, feed was removed 6 hours prior to and then returned 2 hours prior to isotope injections.

Chicks were injected intracardially with 1.0 ml 0.8 % saline solution containing 400 μCi ^{3}H-phenylalanine (ring -2,3,4,5,6-^{3}H, New England Nuclear, 100 Ci/mmol) and 100 mM unlabeled phenylalanine. Five minutes after injection 4 chicks were bled by cardiac puncture and sacrificed by cervical dislocation. Six birds each were bled and sacrificed at 10 and 30 minutes post-injection. Samples of thigh, pectoralis, gastrointestinal tract (GI), liver, heart, and skin were collected and frozen with aluminum clamps cooled in liquid nitrogen. The heart and GI were flushed with 6 and 10 ml of 0.8% saline, respectively, prior to freezing. Extracellular, intracellular and aminoacyl-tRNA amino acid compartments were isolated as previously described.

Results

Experiment 1

Leucine SA_e, SA_i and SA_t peaked by 5 minutes post injection and declined rapidly from 5 to 10 minutes relative to the change between 10 and 30 minutes (figure 1). Time influenced ($P < .01$) specific radioactivity (SA) of leucine in all tissues, as did amino acid pool, although not to the same degree in thigh ($P = .07$; Table 1). The interaction between time and amino acid pool was not significant in any of the four tissues. There was no difference between SA_e and SA_i in any of the 4 tissues at 5 minutes post-injection, although SA_t was lower ($P < .05$) than SA_e and SA_i in heart and liver (figure 1). At 30 minutes post-injection, SA_i in all tissues was decreased ($P < .05$) relative to SA_e but was similar to SA_t.

The average of SA over time was influenced ($P < .05$) by pool (Fig. 1). In both heart and liver SA_e was greater than SA_i, which was greater than SA_t with SA_t, being 38 and 36% of SA_i, and 26 and 20% of SA_e, in heart and liver, respectively (Fig. 1). Pectoralis SA_i and SA_t were similar but lower ($P < .05$) than SA_e. In the thigh SA_i did not differ from either SA_e or SA_t, although SA_e was significantly greater than SA_t (Fig. 1).

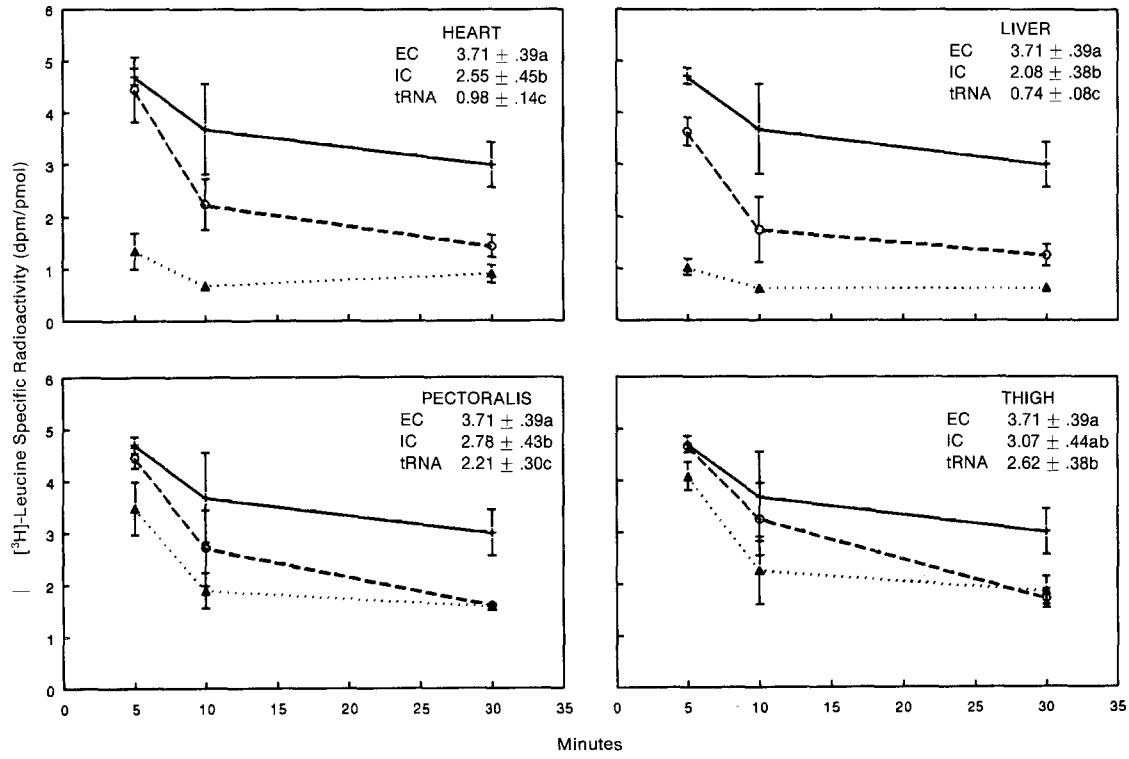

Fig. 1. Changes in extracellular (*EC*; —), intracellular (*IC*; - - -), and aminoacyl-tRNA (*tRNA*; ·····) specific radioactivity (dpm/pmol leucine) following injection of a flooding dose of ^{3}H-leucine. Each point represents the mean ± SE of 3 chicks (5 min) or 4 chicks (10 and 30 min). Values represent the average specific activity between 5 and 30 min for each compartment. Values with different letters are statistically different ($p < .05$)

Table 1. Analysis of variance, mean squares, and (F probability) of effects of time and amino acid pool upon leucine specific radioactivity in selected tissues of the growing chick

Source of variation	Degrees of freedom	Tissue			
		Heart	Liver	Pectoralis	Thigh
Time*	2	7.950 (<.01)	5.944 (<.01)	12.084 (<.01)	13.477 (<.01)
Pool*	2	20.607 (<.01)	24.918 (<.01)	6.027 (<.01)	3.018 (=.07)
Time × Pool	4	1.488 (=.15)	0.928 (=.31)	0.403 (=.77)	0.571 (=.69)
Error	24	0.800	0.726	0.882	1.012

* Specific radioactivity was measured at 5, 10 and 30 minutes in plasma (SA_e), tissue (SA_i), and aminoacylated-tRNA (SA_t).

Experiment 2

The effect of time on phenylalanine SA was significant in all tissues (Table 2). Similarly, SA was influenced by the amino acid pool in all tissues except heart. The interaction of time and pool was significant for GI, liver and thigh. The most likely explanation for this interaction is the lack of change in SA_e over time relative to SA_i and SA_t which fell over time. The SA_e did not change with time in any tissue. In contrast, GI SA_i was decreased ($P < .05$) at 10 min as compared to 5 min and SA_i at 30 min was decreased ($P < .05$) relative to that at 10 minutes. GI SA_t at 5 and 10 minutes was similar but lower ($P < .05$) at 30 min as compared to the two previous time points. Liver SA_t was not influenced ($P > .05$) by time while SA_i at 30 min was lower ($P < .05$) as compared to SA_i at 5 and 10 min. The effect of time on thigh SA_i and SA_t was similar to that of liver SA_i with both thigh SA_i and SA_t at 30 min being decreased relative to the respective pools at 5 and 10 minutes. Both heart and pectoralis SA_i were unaffected by time while SA_t at 30 min in both tissues was decreased relative to SA_t at 5 and 10 minutes. Skin SA_t followed the same pattern as heart and pectoralis SA_t while skin SA_i at 30 min was decreased relative to that at 5 min, with the 10 min value not differing from either that at 5 min or 30 minutes. Thus for all tissues, even though SA_e was not influenced ($P > .05$) by time, SA_i and/or SA_t decreased with time post injection.

Averaged over time phenylalanine SA_i in GI, liver, skin and thigh was lower as compared to SA_e of the respective tissue (Fig. 2). Averaged over time SA_t was lower than SA_e in all tissues except heart. Phenylalanine SA_t in GI and liver was lower than SA_i. Skin SA_t was greater than SA_i while pectoralis SA_t was similar to SA_i. In heart there was no effect of amino acid pool on SA averaged over time.

Discussion

Accurate determination of the specific radioactivity in the appropriate amino acid pool is required to estimate rates of protein synthesis from incorporation of labeled amino acids into protein. In vivo administration of a flooding dose of amino acid typically results in free amino acid specific radioactivity of plasma and tissue pools rapidly reaching a peak followed by a slow decline for the next

Table 2. Analysis of variance, mean squares, and (F probability) of the effects of time and amino acid pool upon phenylalanine specific radioactivity in selected tissues of the growing chick

Source of variation	df*	Tissue					
		GI	Heart	Liver	Pectoralis	Skin	Thigh
Time**	2	16.79 (<.01)	8.091 (<.01)	9.49 (<.01)	6.24 (<.01)	9.85 (<.01)	25.11 (<.01)
Pool**	2	57.59 (<.01)	0.058 (=.96)	18.3 (<.01)	4.10 (=.04)	24.33 (<.01)	9.57 (<.01)
T × P	4	3.15 (=.01)	1.63 (=.35)	3.35 (=.04)	1.95 (=.17)	1.69 (=.13)	4.61 (=.01)
Error	39	0.89	1.434	1.176	1.153	.833	1.261

* Degrees of freedom = df with error df as 39 with the exception of heart, skin and thigh which are 38, 38 and 35, respectively; *GI* Gastrointestinal tract; ** Specific radioactivity was measured at 5, 10 and 30 minutes after injection with the radiolabeled amino acid and was determined in plasma (SA_e), tissue (SA_i), and aminoacyl-tRNA (SA_t).

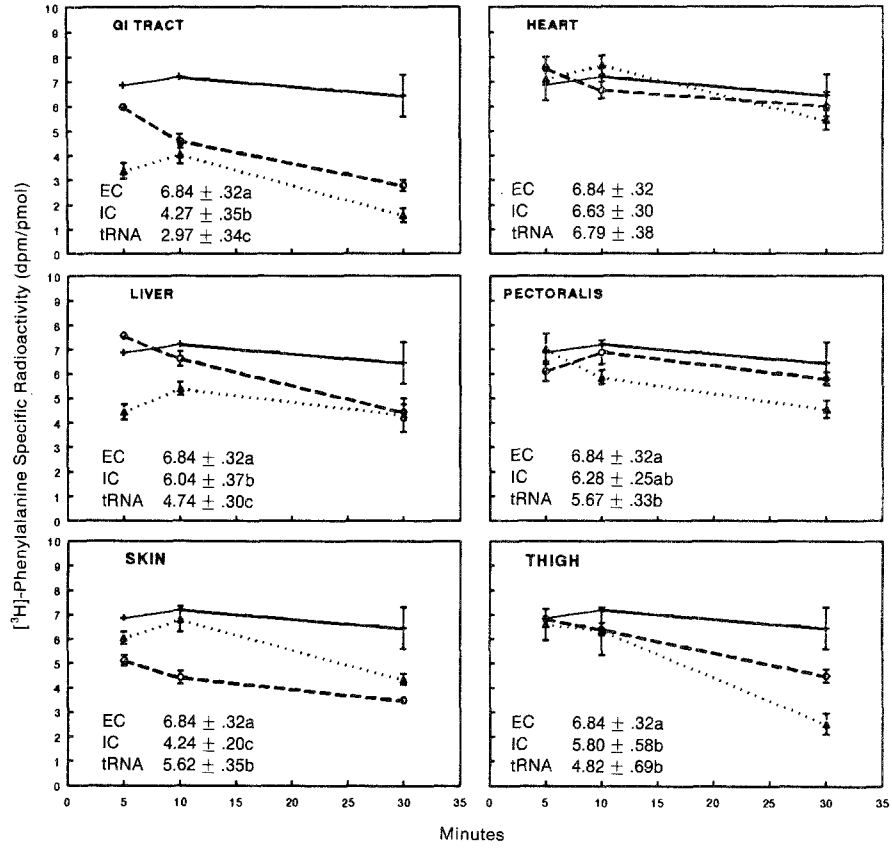

Fig. 2. Effect of a ^{3}H-phenylalanine flooding dose injection on extracellular (*EC*; —), intracellular (*IC*; - - -), and aminoacyl-tRNA (*tRNA*; · · · · ·) specific radioactivity (dpm/pmol phenylalanine) in fed chicks. Each point is the least squares mean ± SE of 4 (5 min) or 6 birds (10 and 30 min). Values represent the average specific activity between 5 and 30 min for each compartment. Values with different letters are statistically different ($p < .05$)

30 minutes (Fig. 1 and McNurlan et al., 1979). This relationship between SA_e and SA_i is observed for a variety of amino acids (Airhart et al., 1974; Garlick et al., 1980; Obled et al., 1989). Amino acids with small pool sizes, such as phenylalanine, decrease at a slower rate (Fig. 2 and Obled et al., 1989). The relationship between the SA_e and SA_i was dependent on amino acid and tissue examined in the current study as well as those of others (Garlick et al., 1980; Obled et al., 1989).

In most tissues, the difference between SA_i and SA_e increased with time. The observed decline in SA_i suggests a decreased contribution of labeled extracellular amino acids to the intracellular pool and/or an increase in unlabeled amino acids contributed by proteolysis. One mechanism which may explain this apparent influence of time on SA_i is the saturation or regulation of amino acid transport into the cells by the sudden expansion of the intracellular pool size. Inhibition of transport by high intracellular amino acid concentration could limit pool equilibration with time. Without influx of amino acids from plasma, SA_i would decline due to the release of unlabeled amino acid from protein degradation. This may explain why leucine SA_i decreased more rapidly than SA_e and why

phenylalanine SA_i decreased while SA_e remained constant. It is important to note that initial equilibrium is reached due to an initial influx of label and expansion of the intracellular amino acid pool. Supporting this argument is the small increase in tissue amino acid concentration measured compared with large changes observed in the plasma pool. Tissue and plasma leucine concentrations were approximately 5–8 moles leucine/g of tissue and 4.5 moles/ml, respectively compared to previously reported basal levels of 2.5 moles leu/g muscle and 0.23 mole/ml (Klasing et al., 1984), representing a 2–3-fold expansion of the tissue-free amino acid pool compared with the 20 fold expansion in plasma-free amino acids.

The divergence of SA_i and SA_e over time may also be explained by changes in protein synthesis or degradation induced by the flooding dose. Increased protein degradation or decreased protein synthesis might result in a decrease in SA_i over time due to the accumulation of amino acids from proteolysis. However, leucine inhibits protein degradation and stimulates protein synthesis in heart, skeletal muscle (Tischler et al., 1982), and liver (Seglen et al., 1980), and a flooding dose of phenylalanine has not generally been reported to have significant effects on protein synthesis or degradation in normal animals (Garlick et al., 1980; Southorn et al., 1992).

Rates of amino acid exchange across the membrane need not change for SA_i to diverge from SA_e. Channeling of amino acids to specific processes within the cell could explain this observation. If amino acids at the higher SA entering the cell from the plasma are partitioned toward catabolism, SA_i could decrease as observed in the present experiment. Schneible et al. (1981) examined the relative contributions of different leucine pools in cultured skeletal muscle to leucine oxidation and protein synthesis. These authors also found that up to 99% of leucine oxidized in the muscle originated from extracellular sources and the amount channeled to catabolism was dependent on amino acid concentration suggesting that the cell can differentiate between amino acid sources. In the same study, 60% of the leucine for protein synthesis was derived from protein degradation with little change in the partitioning of leucine when the intracellular leucine pool was flooded.

In the present study, a flooding dose of labeled leucine or phenylalanine in growing broiler chicks did not result in equilibration between SA_e, SA_i and SA_t in most tissues. Indeed, SA_t was significantly less than SA_e and often less than SA_i. Our in vivo data support a growing number of in vitro reports where SA_t is not similar to that of SA_e or SA_i. (Airhart et al., 1981; Barnes et al., 1992; Hammer and Rannels, 1981; Opsahl and Ehrhart, 1987; Schneibel and Young, 1984; Stirewalt and Low, 1983). Previous in vivo experiments have resulted in variable results. Smith et al., (1991) demonstrated that neither a tracer nor a flooding dose of valine could eliminate the contribution of proteolysis to tRNA acylation in the brain of rats. They found that the contribution of protein degradation to tRNA acylation was 63 and 23% with a tracer and flooding dose, respectively. Also in the brain of rats given a loading dose of ^{14}C-leucine, Sun et al. (1992) estimated the extent of leucine recycling from protein degradation to be near 50%. Chikenji et al. (1983) found that SA_t was lower than SA_i in the thigh muscle and skin of rats infused with a tracer dose of lysine in one of two

separate experiments; although the lack of replication and statistical analysis confounds interpretation. Watt et al. (1991) found that SA_t was between SA_e or SA_i in the muscle and liver of mature rats infused with a tracer dose of ^{13}C-leucine. If the difference between the results of Watt et al. and this current study was due to species, growth rate, or methodology is not clear; although the relatively long period of labeling may have decreased the contribution of short lived proteins to the observed tRNA acylation values in the study by Watt et al.

The difference between SA_t and the other free amino acid pools may indicate aminoacylation is from a distinct amino acid pool which is slow to equilibrate with plasma or intracellular amino acid pools. The major source of unlabeled amino acid is previously synthesized protein and tRNA charging can utilize a significant portion of the amino acids originating from proteolysis (Barnes et al., 1992; Schneible et al., 1981). Our data suggests that acyl-tRNA is partially charged from a distinct amino acid pool supplied from proteolysis and that the relationship between aminoacyl-tRNA and its precursor pool of amino acids is dynamic and varies with tissues. In the liver and GI, SA_i and SA_t are slow to equilibrate while in the pectoralis, heart, and thigh they are initially equivalent and either remain as such or diverge as in the thigh. We have observed that GI, liver and macrophages have high rates of protein turnover relative to muscle, suggesting that the difference between SA_t and SA_i increases with rate of turnover. The skin may be a special case in that the SA_t was consistently intermediate to SA_e and SA_i. Given the large contribution of extracellular protein to the total protein of this tissue, it is likely that protein degradation would contribute less amino acids to the intracellular pool used to acylate tRNA.

Association of aminoacyl-tRNA with the machinery of protein degradation or compartmentation of lysosomes has not been demonstrated. Evidence for the compartmentation of protein biosynthesis is more convincing. Aminoacyl-tRNA and tRNA synthetases are found in aggregates, and these aggregates have been shown to associate with ribosomes (Moline et al., 1974) and the cytoskeleton (Mirande et al., 1985). Sivaram and Deutscher (1990) recently reported the existence of two forms of arginyl-tRNA synthetase and suggest the two forms serve distinct purposes and compartments. One tRNA is used in protein synthesis, the other in postranslational modification of proteins. Thus, compartmentation of protein synthetic machinery supports the possibility that amino acids are derived from specific compartments. Further evidence of compartmentation of protein synthesis has been reported by Negrutskii and Deutscher (1991). They showed, in intact cells, that while exogenously supplied free amino acids were used for protein synthesis, aminoacyl-tRNAs introduced into the cytosol by electroporation were poor precursors. In contrast both sources work equally well in cell free systems. This data suggested that the structural organization of cells prevented the use of exogenous tRNA.

In our in vivo measurements of tissue amino acid specific activities (SA_i in this study), we were unable to distinguish between true cytoplasmic amino acids and amino acids present in the interstitial space. In order to establish that SA_t is less than cytosolic amino acids and provide support for the channeling of amino acids from protein degradation to RNA acylation, a correction for the contribution of interstitial amino acids must be made. To the extent that intersti-

tial specific radioactivity is similar to SA_e, tissue amino acid specific activity is an over estimation of cytosolic amino acid specific activity. Assuming interstitial space is 20% of the wet weight (Hider et al., 1971) and that the specific radioactivity of the interstitial space equals SA_e, the over estimation of the specific activity of cytosolic amino acids due to interstitial amino acids is sufficient to explain the difference between the specific activity of tissue amino acids and SA_t in some tissues. This methodological consideration can not explain the disparity between phenylalanine SA_i and SA_t in GI and liver at 5 minutes and skin at 10 minutes or between leucine SA_i and SA_t in the heart and liver at all time points. Because the specific activity of interstitial amino acids is probably intermediate to that in plasma and in the cytoplasm, the true cytosolic specific activity is presumably higher than SA_t in other tissues as well. Consequently, this study provides in vivo evidence for tRNA charging from an unlabeled amino acid source, presumably protein degradation, in a variety of tissues.

Failure to measure SA_t for the calculation of protein synthetic rates results in underestimation of protein synthesis in most tissues. With leucine as the tracer, using SA_t to calculate protein synthesis would result in estimates of up to 5 times greater than those calculated using SA_e (heart, figure 1). With phenylalanine as the tracer, a 2.7 fold higher rate would be obtained in the GI using SA_t compared to SA_e. Estimates of whole body protein synthetic rates would not be as greatly biased by failure to measure SA_t because of the large contribution of skeletal muscle, a tissue with little divergence between SA_t and SA_e and of skin, a tissue where SA_t is intermediate to SA_e and SA_i.

It is evident from the growing literature describing compartmentation of amino acids that the limitations of a single precursor single product model of protein synthesis must be resolved if quantitative rates of synthesis are to be obtained. Currently there are few data adequately defining the relationship between extracellular, intracellular, and tRNA amino acid pools in vivo. We have demonstrated here that; 1) in some tissues, a significant proportion of leucine and phenylalanine from protein degradation is recycled back into synthesis without mixing with amino acids from extracellular sources, 2) after administration of a flooding dose of amino acid, specific radioactivity associated with aminoacyl-tRNA decreases with time and is not necessarily equivalent to SA_e or SA_i, and 3) conditions under which SA_i is representative of SA_t vary with time post-injection and tissue, indicating the importance of direct measurement of SA_t for calculations of protein synthetic rates.

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