Biochem. J. (1977) **162**, 257–266
Printed in Great Britain

Compartmentation of Free Amino Acids for Protein Biosynthesis

INFLUENCE OF DIURNAL CHANGES IN HEPATIC AMINO ACID CONCENTRATIONS ON THE COMPOSITION OF THE PRECURSOR POOL CHARGING AMINOACYL-TRANSFER RIBONUCLEIC ACID

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(*Received* 30 *June* 1976)

To investigate further the mechanisms by which amino acids are segregated for protein biosynthesis, the distribution of a pulse of [^{3}H]valine was monitored in hepatic amino acid pools at seven intervals in the diurnal cycle of meal-fed rats. Although each condition was characterized by a unique balance between intracellular and extracellular valine, in every case the specific radioactivity of valyl-tRNA at steady state was higher than that of intracellular valine but below the extracellular value. Further, the specific radioactivity of the valyl-tRNA could be accurately predicted if extracellular and intracellular valine were combined in proportions specified by the transmembrane concentration gradient. These observations not only substantiate our earlier conclusions that the amino acids used for protein synthesis do not originate exclusively from either the intracellular or extracellular pools, but also strengthen our theory that the membrane transport system is the physical basis for such compartmentation. On the basis of these data we present a method for measuring the specific radioactivity of the precursor pool for protein biosynthesis in cases where the actual isolation of the aminoacyl-tRNA is not technically feasible, and also suggest a theoretical basis for interpreting the unequal distribution of both total and [^{3}H]valine between intracellular and extracellular fluids.

We have previously reported that in livers of pulse-labelled rats the valine associated with tRNA *in vivo* was intermediate in specific radioactivity compared with valine in the intracellular or extracellular pools (Airhart *et al.*, 1974). From this observation we concluded that neither the extracellular nor the intracellular amino acid pool could be the sole precursor for the charging of valyl-tRNA and proposed that both pools contribute to a restricted compartment which funnels amino acids to protein biosynthesis. Since amino acids in the process of exchange between tissues and blood are also derived from both intracellular and extracellular sources, we suggested that the cell-membrane system may serve as the physical basis for such compartmentation. This theory is supported by the observation that the relative contributions of the two sources to the specific radioactivity of the tRNA-bound valine were not equal, but instead reflected the concentration gradient across the cell membrane.

The above experiments were performed on meal-fed rats at a single post-absorptive time in their diurnal cycle. If the correlations between membrane transport and the source of amino acids for protein synthesis has a functional basis, then alterations in the concentration gradient should produce proportional changes in the specific radioactivity of the valyl-tRNA. In the present paper we provide evidence that it is possible to use the membrane model of amino acid compartmentation to predict mathematically the specific radioactivity of valyl-tRNA throughout the diurnal cycle where the concentration gradients between the extracellular and intracellular compartments are widely divergent.

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Experimental

Male rats of the Lewis strain, obtained from Microbiological Associates, Bethesda, MD, U.S.A., were housed under controlled lighting conditions (dark period 17:30h to 05:30h). Powdered Purina Rat Chow mixed 1:1 with a 3% (w/v) agar gel was given once daily at 18:00h and the uneaten portion removed at 21:00h. Water was given *ad libitum*. The rats were about 8 weeks old at the time of death and had been maintained on the feeding schedule for approx. 2 weeks.

Rats were anaesthetized with Nembutal (5mg/

0.5ml per 100g body wt.) and 15min later injected intraperitoneally with [2,3-^{3}H]valine (31.2Ci/mmol) (Amersham/Searle, Chicago, IL, U.S.A.) at a dosage of 33μCi/0.3ml per 100g body wt. At selected times after isotope administration, the abdomen of the anaesthetized rat was opened by a ventral-midline incision and blood was drawn from the portal vein immediately proximal to the liver. The thoracic cavity was then exposed and blood was drawn from the left ventricle. Samples of approximately 1ml were collected in dry test tubes, previously coated with heparin (10000i.u./ml) and quickly spun in a clinical centrifuge at 100g for 2min. The resulting plasma was immediately mixed with 5vol. of ice-cold 12% (w/v) trichloroacetic acid and, after standing 1h on ice, deproteinized by centrifugation at 730g for 1min. The supernatants were frozen at −20°C and later analysed for free valine content and radioactivity with a Beckman 120C amino acid analyser and an LS-100 scintillation counter respectively as described earlier (Airhart *et al.*, 1973).

Directly after cardiac blood was drawn, the liver was excised, passed twice through 200ml washes of 0.17M-NaCl, wrapped in preweighed aluminum foil, and immediately frozen between two blocks of solid CO_2. Livers were stored at −80°C until the time of assay, when they were homogenized in 3vol. of buffer (0.05M-cacodylate, 0.17M-NaCl, 0.33M-sucrose, pH6.0) with a Willems Polytron homogenizer. Samples of homogenate were immediately taken into an equal volume of ice-cold 20% (w/v) trichloroacetic acid and processed as described for plasma. The remaining homogenate was used to isolate aminoacyl-tRNA by a technique previously described (Wallyn *et al.*, 1974).

The specific radioactivity and content of intracellular valine was calculated by subtracting from total homogenate values the extracellular contribution (in d.p.m. and nmol) as determined from the $^{36}Cl^-$ space of the liver (Airhart *et al.*, 1974). The extracellular components used in this correction were derived from our calculation of liver blood, a composite of portal and arterial values in proportions that varied with the physiological status of the rat. For the first 5h after the meal, portal and cardiac plasmas were combined in a ratio of 4:1 (v/v); at all other times the ratio of 7:3 was used to calculate the composition of the extracellular fluid (see the Discussion section for rationale of proportions used). In all cases, measurements were made after intraperitoneally injected $Na^{36}Cl$ had been circulating for at least 20min, which is the time we found necessary for the radioisotope to be evenly distributed throughout the extracellular water of the rat. Liver intracellular water was calculated from the difference between the measured values for $^{36}Cl^-$ space and the total liver water (70%); plasma was assumed to be 94% (v/v) water.

Evaluation of methods

The reliability of our measurements depended on monitoring the precise physiological status of hepatic amino acid pools. Therefore various controls were run to assure that our procedures did not significantly alter the homoeostasis of the free valine pools in plasma or liver.

Effect of anaesthesia or surgery. Anaesthesia was necessary, since it was technically impossible to sample portal blood in animals that had been decapitated or stunned by a blow on the head. To determine if the anaesthesia affected the concentration or specific radioactivity of plasma and intracellular valine, comparisons were made between rats killed by decapitation, with and without Nembutal. In both cases, neck blood was collected. Cervical plasma served as an adequate correction to convert homogenate valine into intracellular valine, since the systemic blood concentration of valine closely approximates to that of liver blood in the post-absorptive rat (Airhart *et al.*, 1974). Nembutal showed no significant effect on the concentration of valine in plasma or liver cells. Further, in the process of sampling portal and cardiac blood, the abdominal cavity was open about 40s and the thoracic cavity about 15s before the liver was actually excised. However, if blood was obtained from the external jugular vein of anaesthetized rats before opening the abdominal cavity, it was possible to excise the liver without the 40s delay. This modification did not alter either plasma or intracellular liver valine. From these studies we concluded that the valine pools of liver and blood obtained by our routine procedures were not measurably influenced by our manipulation of the animal.

Plasma versus whole blood. Extracellular valine was assayed as a routine in plasma rather than in whole blood or blood cells. Since other investigators have suggested that blood cells are significantly involved in the transport of amino acids between organs and that a direct exchange may exist between haemocyte and tissue amino acids (Elwyn *et al.*, 1968; Felig, 1975), we were concerned that measurements made on plasma alone were not adequate to fully describe extracellular amino acids. However, neither the concentration nor the specific radioactivity of valine differed significantly among the various fractions of heparinized blood in either the absorptive or post-absorptive rat (Table 1, part A).

Use of heparin versus citrate as an anti-coagulant for plasma. Samples prepared from freshly drawn blood mixed 1:1 with 0.2M-sodium citrate as an anticoagulant had an elevated concentration of free valine when compared with plasma from heparinized blood (Table 1, parts A and D). The 20% increase in valine content in citrated plasma occurred without any significant alteration in specific radioactivity. Since the citrate did not alter the valine content or

Table 1. *Stability of plasma valine under various conditions of plasma preparation*

At 5min after intraperitoneal injection of [^{3}H]valine (10μCi/0.3ml per 100g body weight), portal blood was drawn from five anaesthetized rats into pre-heparinized tubes and combined for analysis. Plasma was obtained by centrifugation; trichloroacetic acid was added as described in the text. The blood-cell valine content was calculated by subtraction. The hematocrit was assumed to be 50%; plasma water content, 94%; cellular water content, 65%. Rats were meal-fed from 18:00 to 21:00h; post-absorptive = 10:00h; absorptive = 23:00h. Values are means (±S.E.M.) for six determinations. Only asterisked (*) values were significantly different ($P<0.01$) from control values, as judged by the *t* test for unpaired variables. Treatment A (Control): blood centrifuged immediately; fractions treated with trichloroacetic acid as rapidly as possible. Treatment B: blood stored 1h at 4°C, then spun and treated with trichloroacetic acid. Treatment C: blood spun immediately, plasma frozen and stored at −20°C for 2 weeks before trichloroacetic acid treatment. Treatment D: blood diluted with an equal volume of 0.2M-sodium citrate, then processed as for treatment A.

		Valine content and specific radioactivity					
		Post-absorptive			Absorptive		
Treatment	Fraction	(nmol/ml of blood)	(μM)	(d.p.m./nmol)	(nmol/ml of blood)	(μM)	(d.p.m./nmol)
A	Whole blood	148±1	185	2490±10	240±3	300	788±10
	Plasma	87±2	185	2490±50	138±1	294	776±14
	Cells	61±2	188		102±1	314	
B	Whole blood	170±4*		2102±18*	241±2		778± 2
	Plasma	98±1*		2141±20*	138±1		773± 9
	Cells	72±1*			103±1		
C	Plasma	87±1		2491±41	134±2		776±15
D	Whole blood	151±3		2524±11	239±5		784± 1
	Plasma	104±1*		2518±33	166±2*		784± 7
	Cells	47±1*			73±2*		

specific radioactivity of whole blood, it must, therefore, be assumed that the valine was leached from the haemocytes after dilution of the blood with citrate. This distortion was avoided by the use of precoated heparinized tubes which prevented coagulation without diluting the plasma before its isolation.

Isolation and freezing of plasma. Blood, once obtained, had to be centrifuged, and cells and plasma separated immediately. Even on ice, short-term delays in centrifugation led to increases in plasma valine content. This was especially noted in post-absorptive rats, where delays not only increased the plasma-valine content, but were accompanied by a 13% rise in whole-blood valine and a proportional decrease in specific radioactivity (Table 1, part B). This suggests an input of unlabelled valine arising from protein degradation that was avoided by the above mentioned precautionary measures. Further, it was often more convenient to freeze plasma samples immediately after centrifugation and carry out the acid precipitation at a later date. Such a variation in technique did not alter the results (Table 1, part C).

Rinsing the livers. In our initial attempts to monitor hepatic valine, we froze livers immediately after excision, unwilling to risk the delay involved in a wash step and confident that our $^{36}Cl^-$ correction would compensate for the extra blood adhering to the liver. This procedure not only resulted in data that were highly variable, but more critically, in absorptive animals, the specific radioactivity of liver valine often exceeded that of the portal plasma. This suggested that a small amount of [^{3}H]valine from the intraperitoneal injection can remain on the outside of the unrinsed liver and artificially contribute to the homogenate specific radioactivity. To determine if this was the case, livers were quickly swirled through two beakers of 0.17M-NaCl and the valine compositions of the wash solutions were examined (Table 2). Since the valine content of the washes comprised less than 2% of total liver valine, it is unlikely that the rinsing disturbed the internal valine pools. However, on the basis of the $^{36}Cl^-$ content of the washes, we calculated that 0.2ml of the blood had been rinsed from each liver, but 76% of the ^{3}H associated with the unwashed liver could not be accounted for by the Cl^- correction. Further, the specific radioactivity of the valine in the first wash was 20 times greater than that of portal blood, precluding that it could have been derived from any tissue compartment. Since the specific radioactivity of valine from the unwashed livers overestimated that of the washed liver by 18%, it was concluded that by 5min after radioisotope administration as much as 0.6% of the [^{3}H]valine injected must still be adhering to the surface of the liver. Two rapid rinses in 0.17M-NaCl were found to be sufficient to eliminate the problem. Controls have already been described which indicated that washing the liver does not alter the specific radioactivity of the tRNA-associated valine (Wallyn *et al.*, 1974). Hence all homogenate and intracellular data reported in the

Table 2. *Valine removed from rat liver by brief* 0.17M-NaCl *washes*

At 15min after injection of $Na^{36}Cl$ (1μCi/0.3ml per rat), anaesthetized absorptive rats were injected intraperitoneally with [^{3}H]valine (22μCi/0.2ml per 100g body weight). After 5min, bloods were drawn and the livers excised and washed twice as described in text. Values that are 'Cl-corrected' have been adjusted for blood content in the same manner as homogenate values were converted into intracellular valine (see under 'Evaluation of methods'). The values for 'unwashed liver' were reconstructed mathematically from the data of lines (3) and (4).

	Valine sp. radioactivity or concentration		
	$10^{-3}\times$ radioactivity (d.p.m./liver)	(nmol/ liver)	(d.p.m./ nmol)
(1) Portal plasma	—	—	2250
(2) Unwashed liver	2080	887.0	2350
(3) Washed liver	1630	818.2	1990
(4) Total wash	450	68.8	6540
(5) Total wash (Cl-corrected)	340	16.5	20600
(6) Wash no. 1 (Cl-corrected)	298	7.4	40300

present and the previous paper (Airhart *et al.*, 1974) were determined on washed livers.

Freezing the tissues and sampling the homogenates. It must be stressed that the rapidity of freezing the tissues and sampling the homogenates is of extreme importance so as to minimize proteolytic activity and to prevent any significant expansion of the intracellular amino acid pools (Schurr *et al.*, 1950). This was found to be especially important in post-absorptive rats, where homogenates left standing on ice for 30min or centrifuged for the same length of time resulted in an approximately 30% increase in intracellular valine content.

Results and Discussion

The influx of amino acids from the gastrointestinal tract and the peripheral tissues over the diurnal cycle would be expected to alter the distribution of valine within the extracellular and intracellular compartments of the liver. Data previously accumulated from meal-fed rats during the post-absorptive state had suggested a precise correlation between the specific radioactivity of the valyl-tRNA and the concentration gradient across the hepatocyte membrane (Airhart *et al.*, 1974). If this relationship was to persist as valine concentrations changed, we could quantitatively assess the relative contribution of extracellular and intracellular amino acid pools for

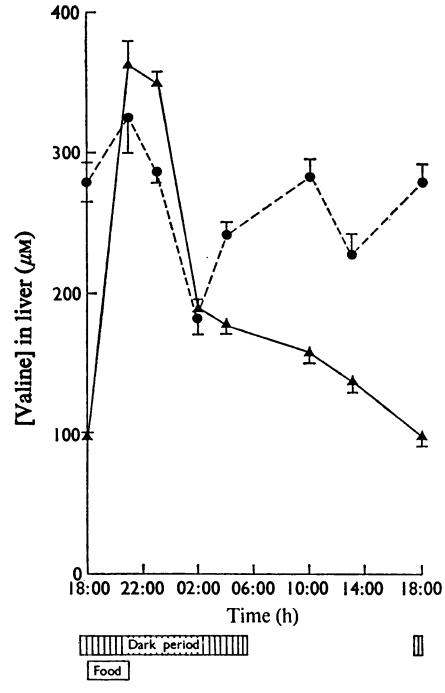

Fig. 1. *Diurnal variations in the concentration of hepatic extracellular and intracellular valine*

Samples were obtained and analysed by methods described or referenced in the text from rats trained to feed from 18:00 to 21:00h. ▲, Hepatic extracellular free valine (μM); ●, hepatic intracellular free valine (μM); the vertical bars indicate the S.E.M.

hepatic protein synthesis during the entire diurnal cycle.

Hepatic valine concentrations in meal-fed rats varied considerably over a 24h period (Fig. 1). Extracellular valine exhibited the most dramatic fluctuations, rising almost 400% during the 3h feeding period (18:00–21:00h). This increase was sustained for several hours, but fell abruptly between 23:00h and 02:00h and thereafter declined gradually to return to the 18:00h minimum value. For the first few hours after the meal, intracellular valine concentrations were actually below the extracellular value. However, by 02:00h the amino acid was equally distributed, and for the ensuing 16h of the diurnal cycle the liver cell concentrated valine to 1.4-fold above the extracellular value at 04:00h and almost threefold by 18:00h.

Some insight into the source and organization of these hepatic valine pools can be derived from examining their relative radioisotope contents under

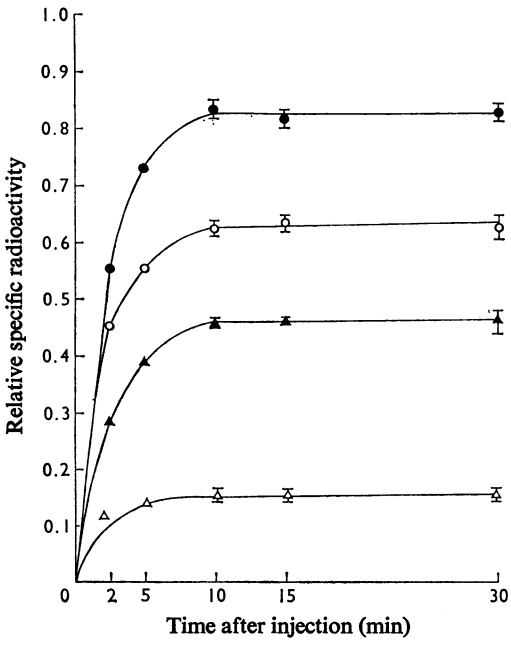

Fig. 2. *Relative specific radioactivities of tRNA-bound and intracellular valine in livers of absorptive and post-absorptive rats*

Specific radioactivities of intracellular free valine (○, △) and tRNA-bound valine (●, ▲) are expressed as a fraction of the extracellular value in absorptive (23:00h, ○, ●) and post-absorptive (10:00h, △, ▲) rats. Animals were trained to feed from 18:00 to 21:00h. Administration of label and isolation and analysis of tissue samples are described or referenced in the text. Vertical bars indicate the S.E.M.

steady-state conditions. To obtain such data, it was necessary to determine the time required for a radioactive tracer to accumulate within the various intracellular pools such that inward flow of label into each compartment was equalled by outflow. The kinetics of hepatic distribution of intraperitoneally injected [^{3}H]valine are illustrated in Fig. 2, where the specific radioactivity of the intracellular and valyl-tRNA pools of both absorptive (23:00h) and post-absorptive (10:00h) rats are plotted as a fraction of the specific radioactivity of extracellular valine. Although the relative specific radioactivities of the two pools were significantly higher in the absorptive state, the timing of the uptake was similar: by 10min after label administration the ^{3}H content of the cellular pools had reached a steady state, which remained unchanged over 30min (Fig. 2).

In this manner steady-state measurements of the specific radioactivities for free intracellular and tRNA-bound valine were determined at various times during the diurnal cycle (Table 3). Just before the meal (18:00h), the specific radioactivity of intracellular free valine was only 20% of the extracellular value and that of valyl-tRNA was 40%. However, within 3h the extracellular specific radioactivity had fallen to one-third of its pre-meal maximum, whereas the intracellular specific radioactivity increased by 50%. This resulted in dramatic alterations in steady-state conditions at 21:00h such that the intracellular specific radioactivity was now 78% and the valyl-tRNA 90% of the extracellular value. By 02:00h the specific radioactivity of valyl-tRNA reached a maximum, but as the major influx of amino acids from the meal subsided (Fig. 1) there was a concomitant rise in the specific radioactivity of the extracellular valine; thus at peak specific radioactivity, the valyl-tRNA actually fell to 64% of the extracellular value. From 04:00h to 18:00h the extracellular specific radioactivity gradually increased. During this period, intracellular valine was relatively unlabelled with specific radioactivities ranging from 15 to 24% of the extracellular values, whereas the relative specific radioactivity of valyl-tRNA hovered around 50%.

The intracellular and extracellular data in Table 3 were derived from the content and specific radioactivities of valine in portal and cardiac plasma and in homogenized liver (Table 4). This calculation required certain assumptions about the relative arterial and portal contributions to hepatic blood supply. Although this information is not available for the rat, experiments on various mammals suggest that the arterial proportion is approx. 30% in healthy non-absorptive livers (Greenway & Stark, 1971); we therefore have used this value from 04:00h to 18:00h. However, because arterial flows as high as 40% have been reported, the data for 18:00h were analysed by using both values (Table 3). Since portal circulation increases during absorption (Elwyn *et al.*, 1968), a ratio of 80% portal:20% cardiac was assumed for the first 5h after the meal. Again, arterial contributions as low as 10% have been reported in absorptive animals; thus the data for 21:00h were also calculated on this basis. Uncertainty as to the precise time for changing from absorptive to post-absorptive ratios prompted us to use both values at 16:00h. At no time did such manipulations of the portal/cardiac proportions produce significant changes in the intracellular or extracellular parameters (Table 3).

Certain general conclusions about the specific radioactivity of valyl-tRNA can be developed from the data of Table 3. Valine intended for protein synthesis possessed a unique steady-state specific radioactivity significantly different from that of the two other hepatic pools at all times during the diurnal cycle. The specific radioactivity of the valyl-tRNA was intermediate, being higher than the intracellular pool value but always lower than the extracellular values. Further, during the absorptive hours

Table 3. *Hepatic concentrations and specific radioactivities of intracellular and extracellular free valine across the diurnal cycle of the meal-fed rat and the use of these values to predict the specific radioactivity of valyl-tRNA*

At various times of the diurnal cycle, rats trained to feed between 18:00 and 21:00h were injected intraperitoneally with [^{3}H]valine. After 10 or 15min, portal and cardiac blood were sampled and the livers removed. The specific radioactivity (d.p.m./nmol) of valine from aminoacyl-tRNA (tRNA) was measured and the concentration (μM) and specific radioactivity of free hepatic extracellular (EC) and intracellular (IC) valine were calculated by methods described in the Experimental section. ^{3}H in the intracellular and tRNA pools had attained a steady state relative to extracellular valine. The notation x/y at the top of each column refers to the percentages of portal (x) and cardiac (y) plasma assumed in calculating the composition of the extracellular fluid. The method for predicting the specific radioactivity of valyl-tRNA is given in the text. Values are means (±S.E.M.) for the numbers of determinations given in parentheses at the bottom of each column.

Time ...	18:00h		21:00h		23:00h	02:00h	04:00h		10:00h	13:00h
x/y ...	70/30	60/40	90/10	80/20	80/20	80/20	80/20	70/30	70/30	70/30
(*a*) Concentration ratios										
IC/EC	2.86 ±0.15	3.03 ±0.14	0.85 ±0.04	0.87 ±0.04	0.82 ±0.01	0.98 ±0.06	1.40 ±0.03	1.41 ±0.03	1.80 ±0.07	1.65 ±0.07
$\frac{EC}{EC+IC}$	0.27 ±0.01	0.26 ±0.01	0.54 ±0.01	0.53 ±0.01	0.55 ±0.00	0.51 ±0.02	0.42 ±0.01	0.42 ±0.01	0.36 ±0.01	0.38 ±0.01
(*b*) Sp. radioactivity										
EC	6609 ±186	6256 ±178	2426 ±96	2416 ±95	3801 ±96	5694 ±105	5033 ±268	4830 ±255	5020 ±162	4955 ±259
IC	1182 ±65	1257 ±63	1856 ±90	1832 ±90	2374 ±74	1765 ±160	1072 ±47	1170 ±57	766 ±37	887 ±55
(*c*) Aminoacyl-tRNA sp. radioactivity										
Observed	2689 ±108		2169 ±67		3123 ±86	3661 ±117	2741 ±182		2294 ±62	2373 ±115
Predicted	2643 ±76	2540 ±62	2144 ±78	2124 ±79	3141 ±101	3730 ±102	2736 ±131	2698 ±138	2310 ±91	2444 ±138
	(13–14)		(13–14)		(11–16)	(7)	(7)		(12–15)	(13)

(18:00h–02:00h) when valine was more concentrated outside the hepatocytes (Fig. 1), the specific radioactivity of the tRNA fluctuated in concert with the changing extracellular pattern (Fig. 3). However, this correspondence shifted during the post-absorptive hours (04:00–18:00h); as valine became more concentrated within the cells, the changes in the tRNA specific radioactivities more closely paralleled those of the intracellular compartment (Fig. 3).

These observations are entirely consistent with a model for amino acid compartmentation proposed previously (Airhart *et al.*, 1974). We postulated that, as amino acids rapidly exchange across the cell membrane, a pool of transient amino acids exists within the membrane such that the tRNA synthetases can activate directly from this restricted pool (Fig. 4). Moreover, since the rate of transport of valine is concentration-dependent (Oxender & Christensen, 1963) and the transport system is not saturated at physiological concentrations (Mortimore *et al.*, 1972; Pardridge & Jefferson, 1975; Khairallah & Mortimore, 1976), we propose that the fractional composition of this small but labile amino acid pool could be proportional to the concentration gradient across the membrane. For example, if the extracellular free valine concentration was twice the intracellular value, the aminoacyl-tRNA would be charged with twice as many valine molecules derived from the extracellular fluid as from the intracellular pool. Since label is entering the cell from the extracellular compartment, the steady-state specific radioactivity of the valyl-tRNA in this case would be closer to the extracellular value than to the intracellular.

The above relationship can be expressed as an equation in which the specific radioactivity (S.A.) of the valyl-tRNA can be predicted from measured values of the specific radioactivities and the molar concentrations of the extracellular [EC] and intracellular [IC] valine:

$$S.A._{tRNA} = S.A._{EC}\left(\frac{[EC]}{[EC]+[IC]}\right) + S.A._{IC}\left(\frac{[IC]}{[EC]+[IC]}\right)$$

By using the above equation, we calculated the specific radioactivities of valyl-tRNA throughout the diurnal cycle for comparison with values determined experimentally (Table 3*c*). In the absorptive state,

Table 4. *Concentrations and specific radioactivities of free valine in portal and cardiac plasma and in homogenized liver at selected times during the diurnal cycle of meal-fed rats*

For experimental design, see the legend to Table 1. Portal = free valine of portal plasma; Cardiac = free valine of cardiac plasma; Homog. = free valine of homogenized liver; $^{36}Cl^-$ space = plasma equivalent of $^{36}Cl^-$ in homogenized liver (ml/100g of liver). Values are means (±S.E.M.) for the numbers of determinations given in parentheses at the bottom of each column.

Time ...	18:00h	21:00h	23:00h	02:00h	04:00h	10:00h	13:00h
	Concentration (μM)						
Portal	96 ±4	382 ±19	359 ±8	195 ±9	185 ±6	157 ±7	139 ±6
Cardiac	101 ±4	288 ±16	303 ±7	165 ±8	168 ±4	141 ±9	141 ±6
Homog.	210 ±10	333 ±21	316 ±11	183 ±9	225 ±7	219 ±11	190 ±8
	Sp. radioactivity (d.p.m./nmol)						
Portal	7753 ±251	2436 ±97	3995 ±117	6013 ±93	5348 ±337	5890 ±217	5908 ±317
Cardiac	4102 ±162	2308 ±104	3095 ±130	4208 ±275	3320 ±220	3166 ±70	2768 ±140
Homog.	2249 ±57	2049 ±79	2927 ±82	3139 ±142	2157 ±101	1929 ±132	2038 ±115
$^{36}Cl^-$ space	30.4 ±0.4	23.3 ±0.6	24.2 ±0.6	24.4 ±0.5	25.3 ±0.6	26.9 ±0.4	28.0 ±0.3
	(13–14)	(13–14)	(11–16)	(7)	(7)	(11–12)	(12–13)

where an influx of amino acids from the meal increased the extracellular valine concentration (Fig. 1), the model predicted that the specific radioactivity of the valyl-tRNA would reflect a membrane pool containing a proportionally greater contribution of extracellular amino acids. For example, at 23:00h, the concentration gradient suggested a ratio of 55% extracellular to 45% intracellular valine (Table 3*a*). By using these fractions and the steady-state specific radioactivities of Table 3(*b*), we calculated a specific radioactivity of 3141±101 for the valyl-tRNA, an excellent approximation to the observed value of 3123±86d.p.m./nmol (Table 3*c*). By contrast, at 10:00h, where the intracellular valine concentration exceeded that found in the extracellular fluid by almost 2:1, the tRNA specific radioactivity was calculated to be 2310±91 d.p.m./nmol (36% extracellular and 64% intracellular), which compared favourably with the observed value of 2294±62. A maximum intracellular contribution, 73%, was inferred from the elevated intracellular concentration just before the meal. Again the predicted tRNA specific radioactivity closely matched the experimentally determined value. At this time, as well as at 21:00 and 4:00h, several permutations of the portal to cardiac ratios were used to calculate the composition of hepatic extracellular fluid; these manipulations did not significantly alter our ability to predict the specific radioactivity of the tRNA (Table 3).

In addition to experiments on intact rats, data on hepatic amino acid concentrations and steady-state specific radioactivities were also available for perfused liver (Table 5). Under these conditions extracellular valine concentrations mimicked those of the early absorptive period *in vivo*, but intracellular valine was also increased and relatively unlabelled (47% of the extracellular specific radioactivity). Such experiments also indicate that in using the proportions specified by the transmembrane gradient, close agreement was obtained between calculated and experimentally measured values for the specific radioactivity of the valyl-tRNA (Table 5). In fact, at no time during the diurnal cycle or in perfused liver preparations did the concentration gradient err by more than 5% in predicting the specific radioactivity of the tRNA.

These correlations suggest that the changes in the valine gradient across the cell membrane are accompanied by exact parallel alterations in the composition of the pool available to the tRNA synthetases, and thus strongly implicate the transport system as an intermediate in the initial steps of protein biosynthesis. Data obtained from rat liver studies have suggested that the capacity for neutral amino acid flux far exceeds physiological demands (Mortimore *et al.*, 1972; Pardridge & Jefferson, 1975), and that a 12-fold increase in the extracellular concentration of valine does not make the availability of other amino acids rate-limiting for protein biosynthesis (Khairallah & Mortimore, 1976). Thus it was not unexpected that the transport system itself would respond so precisely

to changes in the extracellular and intracellular amino acid concentrations.

The partitioning of valine between extracellular and intracellular pools also suggests that protein biosynthesis cannot be proceeding from a homogeneous intracellular pool. Since protein synthesis is the only significant metabolic pathway for valine in rat liver, and the transport systems are far from saturated (Mortimore *et al.*, 1972), one would expect that the concentration of valine should rapidly equilibrate across the membrane. However, the data in Table 3(*a*) clearly indicate that concentration gradients for valine do exist between hepatocytes and the surrounding fluids. The transport of branched-chain amino acids in mammalian systems is generally thought to proceed not by active transport but by facilitative diffusion, a non-concentrative process (Christensen, 1975). Finding no evidence for active transport of valine in rat liver slices, Crawhall & Davis (1971) postulated that protein degradation must be maintaining the elevated intracellular concentrations. However, to accept this explanation, one must assume that, on a diurnal basis, protein breakdown must exceed synthesis for 14 out of the 24h. This is unlikely in a tissue that must not only maintain its own protein mass but also secrete a considerable portion of its synthetic products as plasma proteins. Proposing a compartmentation of intracellular amino acids such that the excess of valine is sequestered in some organelle merely shifts the burden from one membrane system to another and creates a new and steeper

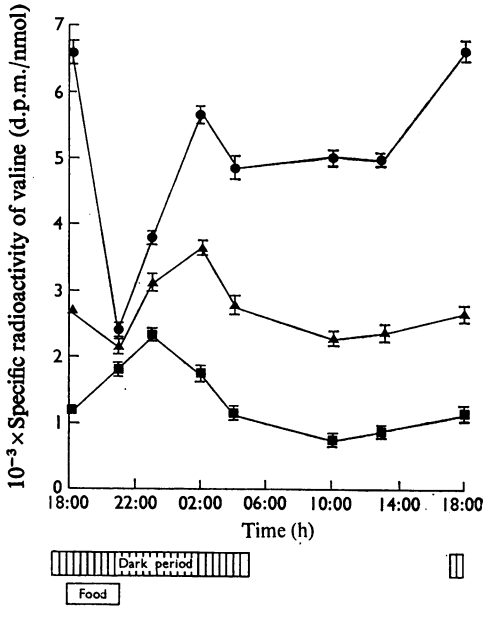

Fig. 3. *Diurnal variations in the specific radioactivities of hepatic intracellular, extracellular and tRNA-bound valine*
For experimental design, see the legend to Table 1. Specific radioactivities (d.p.m./nmol): intracellular free valine (■); tRNA-bound valine (▲); extracellular free valine (●). Vertical bars indicate the S.E.M.

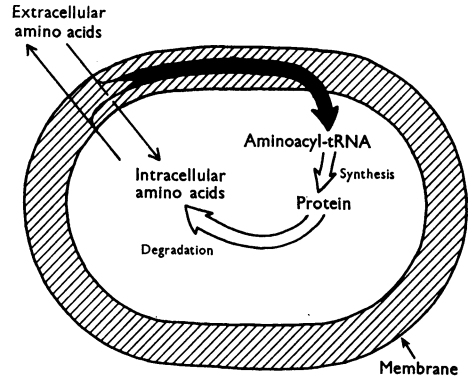

Fig. 4. *Model for compartmentation of amino acids for protein synthesis within the transport system of the cell membrane*

Table 5. *Concentrations and specific radioactivities of intracellular and extracellular free valine in perfused rat liver, and the use of these values to predict the specific radioactivity of valyl-tRNA*
Data were calculated from Khairallah & Mortimore (1976). Livers from rat donors fed *ad libitum* were cyclically perfused *in situ* by a technique previously described (Mortimore *et al.*, 1972). Label (8.4μCi of [1-^{14}C]valine) was added for 15min to the perfusate 60min after initiating perfusion. Samples were obtained and analysed as described in the Experimental section. The method for calculating the specific radioactivity of valyl-tRNA is described in the text. Values are means (±S.E.M.) for six determinations.

			Valyl-tRNA	
	Extracellular	Intracellular	Observed	Predicted
Valine (μM)	374±12	528±18	—	—
Valine sp. radioactivity (d.p.m./nmol)	1011±61	477±33	690±42	698±32
Calculated contribution to tRNA (%)	41	59	—	—

gradient from organelle to cytoplasmic fluid. The model illustrated in Fig. 4 provides an alternative explanation. According to our theory, entry of amino acids into the transport system would be proportional to their concentrations at the membrane surfaces, yet within the membrane some amino acids are removed for protein synthesis. Consequently the expected equilibration is subverted by a metabolic trap. Indeed, valine will be concentrated within the cell whenever protein degradation exceeds only that fraction of protein synthesis supplied by the inward transport system. Under these conditions, a high intracellular valine concentration may co-exist with low plasma concentrations and a net positive nitrogen balance without recourse to active transport or any unique permeability barriers within the cell.

Not only did the concentrative ability of hepatocytes vary considerably during the diurnal cycle, but there were also substantial changes in the extent to which extracellular ^3H was diluted intracellularly. Because intracellular valine is derived from two sources, protein degradation and the extracellular fluid, this dilution must be directly related to the rates of valine flow into the intracellular compartment from these two sources. Gan & Jeffay (1967) viewed the ratio of intracellular to extracellular specific radioactivity as a relative index of internal protein degradation; that is, a lowering of the ratio would indicate an increased flow of unlabelled amino acids arising from protein breakdown. Immediately after the meal (21:00h), the intracellular steady-state specific radioactivity was 76% of the extracellular value (Table 3), indicating that for every ten valine molecules entering the intracellular pool from the extracellular fluid, three were derived from internal protein breakdown. By contrast, the specific radioactivity ratio of 15% at 10:00h translates to a flow of 56 valine molecules arising from degradation for every ten molecules entering the intracellular compartment from outside the cell. The dramatic decrease in the relative intracellular specific radioactivity in the post-absorptive livers suggests that the rate of protein breakdown is substantially greater than it was in the absorptive state. Garlick *et al.* (1973) have concluded from other evidence that post-absorptive rats exhibit enhanced protein catabolism. We have also reported preliminary evidence that supports the view that intracellular protein degradation is indeed suppressed in the absorptive animal (Khairallah *et al.*, 1976). However, inspection of Fig. 4 reveals that there could be two other means of lowering the specific radioactivity ratio without necessarily affecting intracellular protein degradation. For example, an increased rate of protein synthesis could trap more of the incoming valine before it mixed with the intracellular pool, and, since the extracellular fluid is the source of the label, the intracellular specific radioactivity would decrease. Alternatively, a decrease in the concentration of extracellular valine or in the rate of transport itself would also diminish the flow of valine into the intracellular compartment. We must therefore refrain from conclusions about rates of protein degradation based solely on specific-radioactivity ratios until reliable measurements of rates of protein synthesis and amino acid transport *in vivo* are also considered.

In conclusion, we have found that the composition of the pool of valine active in protein synthesis in rat liver has a simple and direct relationship to the concentration gradient across the cell membrane, so that the specific radioactivity of the valyl-tRNA responds quantitatively to relative changes in valine concentration. Although we are at present unable to determine if the valine is actually bound to the transport carrier or exists in a small pool immediately adjacent to the internal surface of the membrane (Khairallah & Mortimore, 1976), the present data strengthen our conviction that there is a functional relationship between the membrane-transport system and the activation of amino acids for protein synthesis. These observations also provide a method for measuring the specific radioactivity of the precursor pool for protein synthesis when actual isolation of the aminoacyl-tRNA is not feasible. The unique and predictable specific radioactivity of valyl-tRNA at every period during the diurnal cycle and perfusion reaffirms our previous data on post-absorptive rats and again prompts us to caution that significant errors in radioisotopic measurements of protein biosynthesis can be introduced by assuming the specific radioactivity of either intracellular or extracellular amino acids as being that of the correct precursor pool.

Several investigators have suggested that the tRNA itself may be compartmentalized so that some tRNA species charge directly from the general cell pool whereas others charge from some membrane-associated compartment (Ilan & Singer, 1975; Fern & Garlick, 1976; Hod & Hershko, 1976). The present data are readily compatible with such compartmentation, but we feel that the alternative suggestions would have less predictive value than the model proposed here.

We deeply appreciate the technical and moral support of Ms. Janet Woodcock and Ms. Sue Ann Wartell. These persons, as well as Dr. A. Phillips and Dr. K. Doeg offered helpful suggestions in the preparation of this manuscript. This work was supported by grants from the National Institute of Health (AM 15919) and The University of Connecticut Research Foundation.

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