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The Specific Radioactivity of the Tissue Free Amino Acid Pool as a Basis for Measuring the Rate of Protein Synthesis in the Rat *in vivo*

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1. Rats were infused *in vivo* with [U-^{14}C]glycine for periods of 2–6h, during which time the specific radioactivity of the free glycine in plasma and tissue approached a constant value. 2. Free serine also became labelled. The ratio of specific radioactivity of serine to that of glycine in the protein of liver, kidney, brain, jejunum, heart, diaphragm and gastrocnemius muscle was closer to the ratio in the free amino acid pool of the tissue than that of the plasma. 3. The kinetics of incorporation of [^{14}C]glycine and [^{14}C]serine into the protein of gastrocnemius muscle further suggested that the plasma free amino acids were not the immediate precursors of protein. 4. Infusion of rats with [U-^{14}C]serine resulted in labelling of free glycine. The ratio of specific radioactivity of glycine to serine in the protein of liver, kidney, brain, jejunum and heart again suggested incorporation from a pool similar to the free amino acid pool of the tissue. 5. Rates of tissue protein synthesis calculated from the incorporation into protein of both radioactive glycine and serine, either infused or derived, were very similar when the precursor specific radioactivity was taken to be that in the total free amino acids of the tissue. Except for gastrocnemius muscle and diaphragm during the infusion of radioactive serine, the rates of tissue protein synthesis calculated from the specific radioactivity of the free glycine and serine in plasma differed markedly.

One of the major problems in determining the rate of protein synthesis by incorporation of a labelled amino acid is the measurement of the specific radioactivity of the amino acid immediately before its incorporation into protein. If in an experimental system the free amino acid achieved a uniform specific radioactivity throughout, the problem would cease to exist because the specific radioactivity of the immediate precursor of protein would be equal to that in any free amino acid pool within the system. However, there is strong evidence for the existence of effective compartmentation. For instance, when rats are continuously infused with a labelled amino acid, although an isotopic steady state (plateau) is reached within a comparatively short space of time, the free amino acid does not achieve the same specific radioactivity throughout the body. The plateau specific radioactivity of the intracellular pool of liver, for example, has been shown to be as much as 50% lower than that of plasma (Gan & Jeffay, 1967; Waterlow & Stephen, 1968; Garlick & Marshall, 1972), and in brain it has been shown to be about 70% lower (Seta *et al.*, 1973). The problem would be of less importance if some knowledge of the location of the precursor pool for protein synthesis was known, but although this area has received a great deal of attention in recent years there is still controversy over whether amino acids are incorporated after prior equilibration with a homogeneous intracellular pool, from a discrete intracellular pool, or directly from an extracellular or intramembrane source (Kipnis *et al.*, 1961; Hendler, 1962; Rosenberg *et al.*, 1963; Manchester & Wool, 1963; Guidotti *et al.*, 1964; Hider *et al.*, 1969, 1971; Righetti *et al.*, 1971; Morgan *et al.*, 1971; Mortimore *et al.*, 1972; Adamson *et al.*, 1972; Winkler, 1972; van Venrooij *et al.*, 1972, 1973; Li *et al.*, 1973).

The most direct method of assessing the precursor specific radioactivity would be to measure the specific radioactivity of the amino acid moiety of aminoacyl-tRNA, but as the total tRNA pool size in heart, for instance, is less than 0.1% of the total tissue weight (Earl & Korner, 1966; Wool *et al.*, 1968) and the half-life for aminoacyl-tRNA is in the region of 1–3 s (Manchester, 1970), there are obvious practical difficulties involved.

We have therefore adopted a more indirect approach in which we have assumed that the ratio of the specific radioactivities of two amino acids in protein will reflect the ratio of the specific radioactivities of the same two amino acids in the precursor pool.

Glycine and serine are interconvertible in some tissues through the action of serine hydroxymethyltransferase (EC 2.1.2.1) and the glycine cleavage system (Yoshida & Kikuchi, 1972). As a consequence

infusion of a radioactive form of one gives rise to appreciable labelling of the other. In tissues where this interconversion takes place the specific-radioactivity ratio of glycine and serine will therefore be different from that in the plasma. In other tissues differences in the rates of cellular uptake of these two amino acids (Henriques *et al.*, 1955; Shank & Aprison, 1970; Guidotti *et al.*, 1971) would serve to produce a different specific-radioactivity ratio in the tissue from that in the plasma.

Consequently we have measured the ratio of specific radioactivities of glycine and serine in the protein of various tissues and compared it with the ratio in two experimentally measurable pools of free amino acids, namely the plasma and the total tissue pool. These pools were chosen since it is not practical *in vivo* to measure the specific radioactivity in discrete pools, such as the extracellular fluid, because of the length of time involved in the preparation of the tissue for such measurements and the relatively high rates of amino acid flux across the cell membrane.

In order to interpret the ratio in protein correctly, it is important that the ratio of the free amino acids in plasma and tissue should remain constant. In a preliminary experiment (Fern & Garlick, 1973) we showed that after 2h of infusion of [U-^{14}C]glycine the ratio in both plasma and tissue free amino acid pools remained relatively constant.

We have also compared the calculated rates of protein synthesis based on the specific radioactivity of free glycine and free serine in the plasma with those based on the specific radioactivity of free glycine and free serine in the tissues. If free amino acid pools in either plasma or tissue reflect the specific radioactivity of the protein precursor then the rates of synthesis calculated from the incorporation of glycine and serine should be identical.

Experimental

[U-^{14}C]Glycine (10mCi/mmol) and [U-^{14}C]serine (10mCi/mmol) were obtained from The Radiochemical Centre, Amersham, Bucks., U.K. Chemicals for use in the automated amino acid analyser were all of AnalaR grade, from BDH Chemicals Ltd., Poole, Dorset, U.K. Female hooded rats were purchased from Animal Suppliers (London) Ltd., London N.12, U.K.

Three separate experiments were carried out, the details of which are shown in Table 1. The rats at the time of infusion had a mean body weight of 100g and

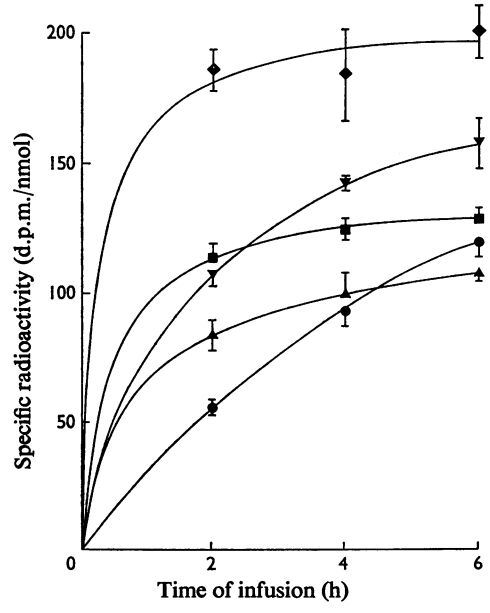

Fig. 1. *Time-course of the change in specific radioactivity of free glycine in total tissue and plasma pools (Expt. 1)*

Rats were continuously infused for 2, 4 or 6h with [U-^{14}C]-glycine. At the end of the infusion samples of tissue and plasma were taken, deproteinized with 3% sulphosalicylic acid and the supernatants analysed. The specific radioactivity of free glycine was determined by ion-exchange chromatography by using an automated amino acid analyser fitted with a column effluent stream splitter. Radioactivity was measured by liquid-scintillation counting. Results are the means of four animals at each time-point (±S.E.M.). Specific radioactivity of free glycine in plasma (♦), liver (▲), heart (■), diaphragm (▼) and gastrocnemius (●) is shown.

Table 1. *Details of experimental protocol*

Expt. no.	Infusion label	Infusion time (h)	Radioactivity (μCi/ml of infusate)	No. of rats	Tissues studied
1	[U-^{14}C]Glycine	2	25.0	4	Liver, heart, gastrocnemius, diaphragm, plasma
		4	12.5	4	
		6	8.3	4	
2	[U-^{14}C]Glycine	2	25.0	2	Kidney, brain, jejunum, plasma
		6	8.3	2	
3	[U-^{14}C]Serine	2	25.0	2	As in Expts. 1 and 2
		6	8.3	2	

a growth rate of 3%/day. Each rat was infused with a total of 25μCi of [U-^{14}C]glycine or [U-^{14}C]serine, dissolved in 0.9% NaCl containing 10mM-carrier amino acid. The rate of infusion was 0.478ml/h. The infusion technique was similar to that of Garlick & Marshall (1972). At the end of the infusion the rat was killed by decapitation, the blood collected and the plasma separated. Immediately the tissues were removed, washed free of blood in ice-cold 0.9% NaCl and homogenized in 3% (w/v) sulphosalicylic acid. The entire heart, brain, diaphragm, kidney and gastrocnemius muscle and 0.5g of liver were taken. The jejunal sample (1.5g) was taken 15cm from the pylorus after the contents of the lumen had been washed out with 0.9% NaCl. In all cases the tissue free amino acid pool was taken to be the supernatant after homogenization and precipitation of protein with 3% sulphosalicylic acid. No correction was made for free glycine and serine in either the extracellular fluid or in any residual blood within the tissue. The protein pool was taken to be the precipitate from the above after a process of lipid extraction as described by Hider *et al.* (1971). The specific radioactivities of the free and protein-bound amino acids were determined by ion-exchange chromatography on a Locarte amino acid analyser fitted with a column effluent stream splitter as described previously (Fern & Garlick, 1973).

Results and Discussion

During the constant infusion of [U-^{14}C]glycine (Expt. 1) the specific radioactivity of free glycine had risen to a plateau at approx. 2 h (Fig. 1). This value was maintained to the end of the infusion. Similar results have been obtained with a number of different amino acids (Gan & Jeffay, 1967; Waterlow & Stephen, 1968; Garlick & Marshall, 1972; Seta *et al.*, 1973). In liver and heart there was also little increase in the specific radioactivity of free glycine between 2 and 6h, but in diaphragm and gastrocnemius muscle there was a continued rise throughout the infusion period. In gastrocnemius the rise is particularly slow because of the slow turnover of skeletal-muscle protein and the large tissue pool of free glycine. The specific radioactivity in the tissues always remained lower than in the plasma (Aub & Waterlow, 1970). In liver the plateau value was 55% of that of the plasma, in heart it was 65%, and although in gastrocnemius muscle and diaphragm the specific radioactivity was still rising the plateau value was calculated to be approx. 80% in both cases.

By contrast, the specific radioactivity of free serine derived from the infused [U-^{14}C]glycine was much higher in liver, its major site of synthesis, than in plasma. Heart, diaphragm and gastrocnemius were similar to plasma (Fig. 2). In no tissue had the specific radioactivity of serine fully reached a plateau value.

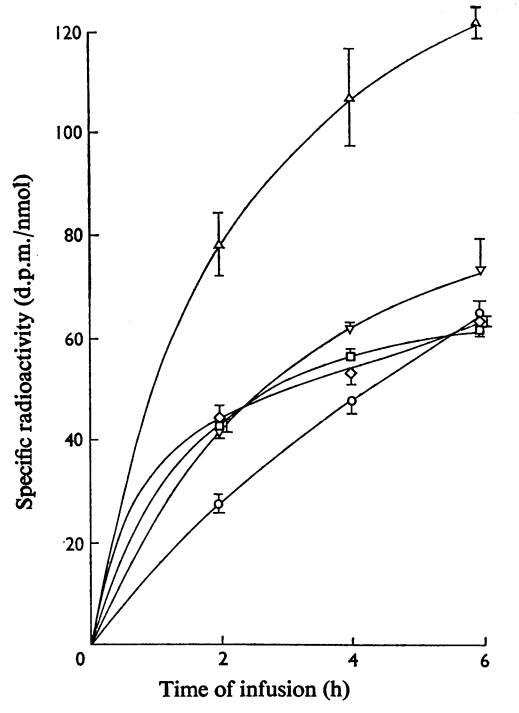

Fig. 2. *Time-course of the change in specific radioactivity of free serine in total tissue and plasma pools (Expt. 1)*

Rats were continuously infused for 2, 4 or 6h with [U-^{14}C]-glycine. Specific radioactivity of free serine was determined by automated amino acid analysis and liquid-scintillation counting. Results are the means of four animals at each time-point (±S.E.M.). Specific radioactivity of free serine in plasma (◇), liver (△), heart (□), diaphragm (▽) and gastrocnemius (○) is shown.

In liver the specific radioactivity of serine (Fig. 2) rose to above that of glycine (Fig. 1), its precursor. Generally speaking it is not possible for a product to have a higher specific radioactivity than its precursor, but in this situation the explanation lies in the fact that serine is a C_3 whereas glycine is a C_2 amino acid. Serine is formed by the addition of a carbon atom, via methylenetetrahydrofolate, to a glycine molecule (Yoshida & Kikuchi, 1972). If glycine is uniformly labelled and the additional carbon is unlabelled, both amino acids will have the same specific radioactivity at equilibrium. However, if the additional carbon atom is also labelled then the specific radioactivity of serine will be higher. The maximum theoretical ratio of the specific radioactivity of serine to glycine is therefore 1.5. In our system C-3 of serine is gradually becoming labelled because glycine also supplies the methylene moiety of methylenetetrahydrofolate.

The rates of incorporation of [^{14}C]glycine and

[^{14}C]serine into protein of the various tissues are shown in Figs. 3 and 4. Incorporation of both amino acids into liver protein was linear with respect to time, without any apparent initial lag period. Similarly the incorporation into heart and diaphragm protein was also linear but there was evidence of a short lag period of 25–45min. Incorporation into gastrocnemius protein, however, remained non-linear throughout the 6h of infusion.

Table 2 shows the ratio of specific radioactivity of serine to that of glycine in protein and in sulphosalicylic acid supernatants of plasma and tissue. The ratio for free amino acids in every tissue was appreciably higher than that in the plasma, owing either to the conversion of glycine into serine in some tissues, such as the liver (Yoshida & Kikuchi, 1972) and to a lesser extent in the kidney (Sato *et al.*, 1969; Pitts *et al.*, 1970), or to a proportionally higher rate of cellular uptake of serine (see Table 3). The ratio in the various proteins was also much higher than that in the plasma and was much nearer to that of their respective tissue pools, indicating that the specific radioactivity of the precursor pool is not represented by that of the mixed venous and arterial plasma sample. As we did not measure the ratio of serine to glycine in arterial, venous or hepatic-portal plasma separately, we cannot say to what extent the ratios in plasma will vary with the different sites of sampling. The results of Wolff & Bergman (1972) in sheep suggest that such differences are small.

It was not intended that the present study should determine the kinetics of incorporation. However, Figs. 1–4, although constructed from measurements at only three time-points, provide useful additional evidence that glycine and serine at the site of protein synthesis do not have the same specific radioactivity as in plasma. If one assumes that free amino acids in

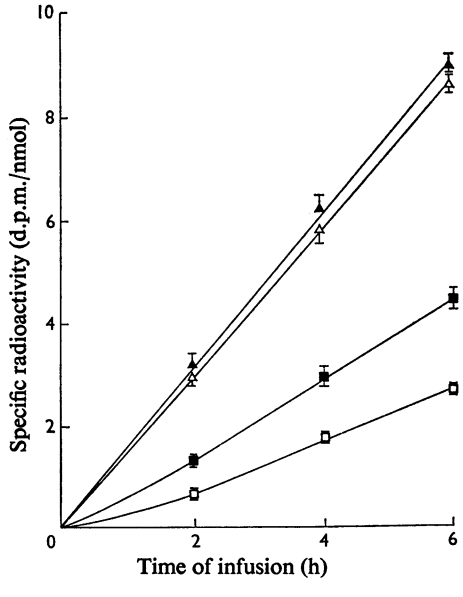

Fig. 3. *Time-course of the change in specific radioactivity of protein-bound glycine and serine in liver and heart* (*Expt.* 1)

Rats were continuously infused for 2, 4 or 6h with [U-^{14}C]-glycine. At the end of the infusion samples of the tissues were taken. The protein was precipitated with 3% sulphosalicyclic acid, lipid extracted and hydrolysed for 24h in constant-boiling HCl. Specific radioactivity was determined by ion-exchange chromatography and liquid-scintillation counting. Results are the means of four animals at each time-point (±S.E.M.). Specific radioactivity of liver protein-bound glycine (▲), liver protein-bound serine (△), heart protein-bound glycine (■) and heart protein-bound serine (□) is shown.

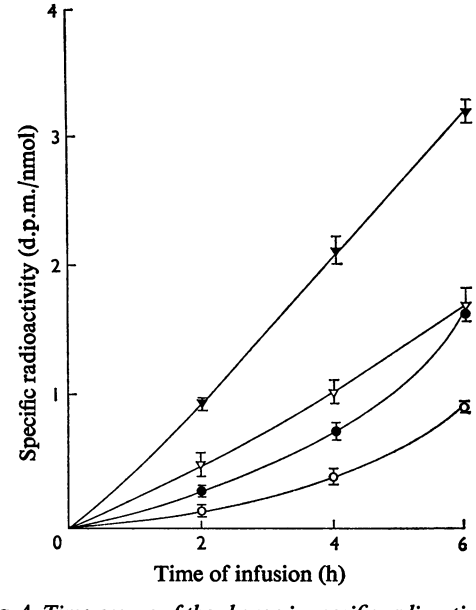

Fig. 4. *Time-course of the change in specific radioactivity of protein-bound glycine and serine in diaphragm and gastrocnemius* (*Expt.* 1)

Rats were continuously infused for 2, 4 or 6h with [U-^{14}C]-glycine. Protein from tissue samples taken at the end of the infusion was precipitated with 3% sulphosalicylic acid. After lipid extraction the protein was hydrolysed for 24h at 105°C in constant-boiling HCl. Specific radioactivity was determined by ion-exchange chromatography and liquid-scintillation counting. Results are the means of four animals at each time (±S.E.M.). Specific radioactivity of diaphragm protein-bound glycine (▼), diaphragm protein-bound serine (▽), gastrocnemius protein-bound glycine (●) and gastrocnemius protein-bound serine (○) is shown.

plasma were the precursors for protein synthesis the incorporation into protein should be linear, because the specific radioactivity of free glycine in plasma rises by only 10% between the second and sixth hour of infusion and that of serine by 40%. If on the other hand the assumption is made that the tissue free pool is the immediate precursor then, taking the extreme example of gastrocnemius muscle, the incorporation of both amino acids would have to follow curvilinear kinetics because the specific radioactivity of free glycine in the tissue pool rises by 120% in this same period and that of serine by 130%. In Fig. 4 the graphs showing the incorporation into protein of gastrocnemius are better explained by a model that proposes that the tissue free pool is the precursor for protein.

Table 2. *Serine/glycine specific-radioactivity ratios (Expts. 1 and 2)*

Rats were continuously infused for 2 and 6h with [U-^{14}C]-glycine. Specific radioactivities of plasma free, tissue free and tissue protein-bound glycine and serine were determined by ion-exchange chromatography and liquid-scintillation counting.

	Serine/glycine specific-radioactiviy ratio			
	2h Infusion		6h Infusion	
Tissue	Free	Protein	Free	Protein
Plasma	0.245		0.304	
Liver	1.052	0.938	1.108	0.952
Heart	0.387	0.486	0.487	0.609
Diaphragm	0.396	0.472	0.464	0.533
Gastrocnemius	0.518	0.486	0.530	0.573
Kidney	0.600	0.566	0.700	0.628
Brain	0.982	0.790	1.108	1.044
Jejunum	0.457	0.603	0.706	0.702

In the other tissues it is not possible to say with certainty that the rate of increase of the specific radioactivity of protein is more consistent with incorporation from one or other of the free pools, because the changes in the specific radioactivities of the free amino acids over the 4h period were much smaller than those seen in the gastrocnemius muscle. In heart and liver, for instance, the changes in the specific radioactivity of free glycine and serine were very similar to those seen in the plasma, being in the order of 15 and 30% for glycine and 40 and 50% for serine respectively. Even in diaphragm the changes in specific radioactivity of free glycine (55%) and serine (75%) were not totally different from that in the plasma.

The results of the constant infusion of [U-^{14}C]-serine (Expt. 3) are given in Table 3. At both 2 and 6h the specific radioactivity of serine in diaphragm, gastrocnemius muscle and heart was very similar to that in plasma. It has been observed by a number of workers that the specific radioactivity of the infused amino acid in the plasma is always higher than that in the tissues because of the release of unlabelled amino acids from the degradation of tissue protein (Fig. 1; Gan & Jeffay, 1967; Waterlow & Stephen, 1968; Garlick & Marshall, 1972; Seta *et al.*, 1973). The transport of labelled serine from the plasma into these tissues must therefore be extremely rapid to overcome the diluting effect of proteolysis. In practical terms it means that the rate of protein synthesis calculated from the specific radioactivity of free serine in either plasma or tissue is nearly the same and is not likely to differ appreciably from the true rate (see Table 4).

The specific radioactivity of glycine derived from the infused [U-^{14}C]serine was lower in plasma than in all tissues, with the exception of gastrocnemius muscle and brain at both 2 and 6h. The specific radioactivity of glycine in gastrocnemius muscle was

Table 3. *Glycine/serine specific-radioactivity ratios (Expt. 3)*

Rats were continuously infused for 2 and 6h with [U-^{14}C]serine. Specific radioactivities of plasma free, tissue free and tissue protein-bound glycine and serine were determined by ion-exchange chromatography and liquid-scintillation counting.

	Specific radioactivity of free pool (d.p.m./nmol)				Glycine/serine specific-radioactivity ratio			
	Serine		Glycine		2h		6h	
Tissue	2h	6h	2h	6h	Free	Protein	Free	Protein
Plasma	65.2	90.5	13.4	23.8	0.206		0.263	
Liver	30.5	42.4	16.4	27.5	0.538	0.431	0.648	0.532
Heart	71.9	79.3	21.7	33.0	0.302	0.225	0.416	0.327
Diaphragm	70.3	88.6	16.2	29.8	0.235	0.209	0.336	0.258
Gastrocnemius	55.7	86.9	9.6	21.9	0.171	0.178	0.254	0.221
Kidney	58.9	73.3	16.7	33.5	0.285	0.313	0.457	0.355
Brain	12.7	17.8	5.7	7.6	0.375	0.336	0.426	0.483
Jejunum	40.2	75.7	13.6	33.6	0.339	0.297	0.450	0.418

slightly lower than in plasma, but brain only achieved 30% of the plasma value.

Table 3 also shows the ratio of the specific radioactivity of glycine to that of serine in the plasma and tissue pools and in tissue protein after the infusion of [U-^{14}C]serine. With the exception of gastrocnemius, the ratio in plasma always remained lower than that in the tissues. In the protein, the ratio was also generally lower than in the corresponding tissue pool, but in liver, kidney, jejunum and brain it remained much nearer to the ratio in the tissue than in the plasma. With gastrocnemius muscle also the ratio in protein was more closely related to the ratio in the tissue free pool, although in this case the differences between the three pools were small.

In contrast with the results from the infusion of glycine (Table 2) the ratios of the specific radioactivity of glycine to that of serine in the protein in heart and diaphragm were more similar to the ratio in plasma. However, when the ratio in the free amino acids continues to rise throughout the infusion the ratio in protein will always be lower than that of its precursor. In heart the ratio was higher than that in plasma and because of this free glycine and serine in the plasma could not have been the immediate precursors of protein. It was not possible to make such a distinction in the diaphragm, as the ratio in protein was almost identical with the ratio in plasma. However, with heart, diaphragm and gastrocnemius muscle one of the reasons why the comparison of ratios does not clearly indicate which experimental pool more closely represents the precursor is that there is very little difference in the specific radioactivity of both serine and glycine in either pool. In diaphragm, for example, the specific radioactivity of free serine after 6h of infusion was only 4% less than that in plasma, whereas in liver, kidney, jejunum and brain it was 17–80% lower. This similarity in specific radioactivities is reflected in the calculated rates of protein synthesis (Table 4).

Table 4 shows the fractional rate of protein synthesis for all the tissues calculated from the increase in specific radioactivity of each amino acid in the protein between the second and sixth hour of infusion (Seta *et al.*, 1973). The specific radioactivity of the precursor pool was taken to be that of the average value of the free amino acid in either the tissue or in plasma over the same time-period. In more rapidly synthesizing tissues (e.g. liver and jejunum) this method will slightly underestimate the true rate because it does not allow for the radioisotope lost from protein turnover. If the results of infusions of [U-^{14}C]glycine and [U-^{14}C]serine are taken separately, it is evident that when the specific radioactivity of the free amino acid in plasma is taken to be that of the precursor, the estimated rates of protein synthesis differ very much more than if the value for the tissue pool is used. Theoretically, when calculations of the rate of protein synthesis are based on the specific radioactivity of the true precursor pool the calculated rate derived from one amino acid should not differ from that derived from any other amino acid. In this case it appears inherently unlikely that the plasma can therefore be the precursor. In contrast the estimated rates of pro-

Table 4. *Estimated fractional rate of tissue protein synthesis*

Rats were continuously infused for 2 and 6h with either [U-^{14}C]glycine or [U-^{14}C]serine. The rates of protein synthesis were calculated from the increase in specific radioactivity of both glycine and serine in the protein over the 4h period (Seta *et al.*, 1973). The specific radioactivity of the precursor was taken to be that of the mean value of the free amino acid in either the tissue or the plasma. Synthesis rates calculated from Expt. 1 are obtained from four infusions at each time-point. Those from Expts. 2 and 3 are calculated from two infusions at each time-point.

			Estimated rate of protein synthesis (%/day)			
		Precursor site ...	Tissue		Plasma	
Tissue	Expt. no.	Amino acid infused	Glycine	Serine	Glycine	Serine
Liver	1	Glycine	35.9	33.1	18.0	60.6
	3	Serine	37.8	38.8	46.2	19.1
Heart	1	Glycine	15.3	23.0	9.6	22.3
	3	Serine	14.8	14.8	22.3	15.1
Diaphragm	1	Glycine	9.6	12.6	6.7	13.3
	3	Serine	12.4	13.0	15.8	13.8
Gastrocnemius	1	Glycine	9.2	10.5	4.2	8.9
	3	Serine	7.4	6.9	6.5	6.6
Kidney	2	Glycine	39.9	35.3	20.3	50.2
	3	Serine	29.2	29.1	40.6	25.9
Brain	2	Glycine	18.7	19.7	1.2	5.8
	3	Serine	26.0	19.3	8.8	1.3
Jejunum	2	Glycine	59.1	60.8	36.0	91.8
	3	Serine	134.0	118.6	176.6	92.1

tein synthesis based on the tissue specific radioactivity of glycine or serine, infused or derived, are very similar indeed. The three exceptions are heart, diaphragm and gastrocnemius during the infusion of [U-^{14}C]serine, when there is little to choose between either amino acid or either precursor site. Reasons for this have been discussed above. In addition, in all the tissues except the jejunum, synthesis rates calculated from the specific radioactivity of the tissue pool were similar irrespective of which amino acid was infused. These rates are in agreement with several published values also calculated from the specific radioactivity of the tissue free amino acid pool after constant infusion of a labelled amino acid. The average rate for liver (36.4%/day) differs little from those reported when glycine (Garlick, 1972) or tyrosine (Garlick, 1972; Garlick *et al.*, 1973) were used. The synthesis rate for gastrocnemius muscle (8.5%/day) is in reasonable agreement with those estimated for 100g female rats by using lysine (Waterlow & Stephen, 1968) and also with the rate calculated from an infusion of tyrosine (Garlick *et al.*, 1973) and glycine (Garlick, 1969). The mean value for brain (20.9%/day) is almost identical with that of Seta *et al.* (1973), using glycine, and the mean value for diaphragm (11.8%/day) is similar to that of Turner & Garlick (1974) calculated from a tyrosine infusion. There are few published rates of synthesis for kidney, heart or jejunum, but those of Millward & Garlick (1972) are in the same region as our results.

Although the present study has done little to clarify any understanding of the exact location of the precursor pool, it has confirmed our earlier belief that during a continuous infusion of a labelled amino acid *in vivo* the specific radioactivity at the site of protein synthesis is very similar to that of the free amino acid pool of the whole tissue. Consequently, the specific radioactivity of this pool can be used as a reasonable basis for calculating the rate of protein synthesis *in vivo*.

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