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A Comparison of Methods for the Measurement of Protein Turnover *in vivo*

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Steady-state rates of turnover of two single proteins were measured *in vivo* by two independent methods. The fractional rate of synthesis of liver ornithine aminotransferase, measured by a continuous infusion of L-[2,6-^{3}H]tyrosine, was 0.42 day^{-1}, whereas in the same animals the fractional rate of degradation measured by loss of radioactivity from amino acids labelled via [^{14}C]bicarbonate was 0.40 day^{-1}. The agreement between methods confirms the reliability of each method for the study of hepatic protein turnover. In contrast, [^{14}C]bicarbonate-labelled amino acids are extensively reutilized in muscle, and are therefore unsuitable for measuring rates of muscle protein breakdown.

Studies of protein turnover *in vivo* can be made by measuring either the fractional rate of protein synthesis or the fractional rate of degradation, or both (for review, see Waterlow *et al.*, 1978). All methods in current use involve various assumptions and limitations. For this reason, Schimke (1970) has suggested that, whenever possible, more than one method should be used when absolute rates are to be determined.

Rates of protein breakdown are usually determined from the loss of radioactivity from protein previously labelled by the injection of a single dose of radioisotopic tracer. In most cases, however, radioactive amino acids derived from protein breakdown persist in the precursor pool and are reutilized for protein synthesis. Such reutilization results in apparent degradation rates which underestimate the 'true' rates of breakdown (e.g. Poole, 1971; Swick & Ip, 1974).

The rate of protein synthesis can be estimated from the uptake of a labelled amino acid into protein when the label is administered continuously, either by intravenous infusion (Garlick *et al.*, 1973) or by inclusion in the diet (Harney *et al.*, 1976). The primary difficulty with this method has been uncertainty about the specific radioactivity of the amino acid incorporated into protein. There is now evidence that, in liver and muscle, tRNA may be charged by amino acids from both the extracellular and intracellular pools (Vidrich *et al.*, 1977; Martin *et al.*, 1977; McKee *et al.*, 1978). Because the specific radioactivity of aminoacyl-tRNA is difficult to measure, it is conventional to assume that the precursor specific radioactivity is equal to that of the trichloroacetic acid-soluble extract of the tissue of interest. The errors introduced by this assumption can lead to either underestimates or overestimates of synthesis rates, but are usually less serious than errors caused by reutilization of radioisotope in decay measurements.

If available methods are to be considered reliable, independent measurements of rates of protein degradation and synthesis, if done in the same animals, should give identical results. To test this, simultaneous measurements were made of the rates of synthesis and of degradation of two pure proteins: ornithine aminotransferase in liver and aldolase in muscle. Rates of protein degradation were measured by the loss of radioactivity after an injection of [^{14}C]bicarbonate, and rates of protein synthesis were measured by the continuous infusion of L-[2,6-^{3}H]tyrosine. [^{14}C]Bicarbonate has been suggested as the tracer of choice for the study of hepatic protein turnover (Swick & Ip, 1974) and has been used to measure rates of muscle protein turnover as well (Perry, 1974; Millward, 1970; Swick & Song, 1974; Hoover-Plow & Clifford, 1978). Our results demonstrate that, for the liver protein, [^{14}C]bicarbonate yields a rate of degradation equal to the rate of synthesis. In contrast, amino acids labelled in this manner may be highly reutilized in muscle, and [^{14}C]bicarbonate cannot be used to obtain accurate rates of degradation in this tissue.

Materials and Methods

Radioisotopes

$NaH^{14}CO_3$ (59Ci/mmol) and L-[2,6-^{3}H]tyrosine (30Ci/mmol) were obtained from Amersham/Searle.

Synthesis and degradation of ornithine aminotransferase (*EC* 2.6.1.13)

Male Holtzman rats weighing 180–200g were kept in a room with a 12h-light/12h-dark cycle and fed on a 60%-(w/w)-casein diet *ad libitum* for 5 days before the injection of radioisotope. This period

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was shown to be adequate to induce the enzyme to a new higher steady-state activity (Chee & Swick, 1976). Each rat was given an intraperitoneal injection of 800μCi of $NaH^{14}CO_3$, and groups of four rats were killed at 1, 3 and 5 days after the injection. At 6h before each rat was killed it was given a continuous infusion of L-[2,6-^{3}H]tyrosine (10μCi/ml) at a rate of 0.6ml/h as described by Garlick *et al.* (1975). The liver was removed as the infusion continued, and 1g of liver was homogenized in 10% (w/v) trichloroacetic acid. Ornithine aminotransferase was isolated from the remaining liver as described previously (Chee & Swick, 1976), and the fractional degradation rate (k_d) was determined from the loss of total ^{14}C radioactivity (Chee & Swick, 1976). The specific radioactivity of protein-bound tyrosine and of free tyrosine was determined as described by Garlick *et al.* (1973). The fractional rate of synthesis (k_s) of ornithine aminotransferase was calculated as described by Garlick *et al.* (1973), eqn. (7). Because the value of λ_p in this equation needs only to be approximate (Garlick, 1978), λ_p was taken to be 80 days^{-1}. Although this value of λ_p was determined for L-[U-^{14}C]tyrosine (Garlick *et al.*, 1973), it has been observed in our laboratory that the infusion of L-[2,6-^{3}H]tyrosine results in values for liver protein synthesis which are 10–15% lower than those obtained when L-[U-^{14}C]tyrosine is infused (S. L. Augustine & T. L. Burk, unpublished work).

Synthesis and degradation of muscle fructose bisphosphate aldolase (*EC* 4.1.2.13)

Male Holtzman rats weighing 275–300g were kept in a room with a 12h-light/12h-dark cycle and trained for 2 weeks to eat a powdered 20%-(w/w)-casein diet from 09:00 to 12:00h daily (beginning of the dark cycle). For 5 days before the synthesis measurement, each group was fed on an L-amino acid diet in agar-gel form (Harney *et al.*, 1976). Each rat received an intraperitoneal injection of 1.2mCi of $NaH^{14}CO_3$, and groups of four to five rats were killed at 4, 11 and 20 days after the injection. At 6h before each rat was killed it was given 10g of the agar-gel/amino acid diet containing 250μCi of L-[2,6-^{3}H]tyrosine, beginning at 09:00h. Rats were killed by decapitation and the combined leg muscles were removed and minced on ice. Approx. 1g of muscle was frozen immediately in liquid nitrogen. Aldolase was isolated from the remaining muscle by the method of Ting *et al.* (1971), with some modifications. The specific radioactivity of protein-bound tyrosine and of free tyrosine was determined as described above.

The fractional rate of degradation of aldolase was estimated only from the decay in specific radioactivity because the rats in this study were growing very slowly, less than 1% per day. Under steady-state conditions, the decay in specific radioactivity will be equal to the decay in total radioactivity of protein. The fractional rate of synthesis of mixed muscle proteins was calculated as described by Garlick *et al.* (1973), eqn. (8), and was determined to be 0.040 day^{-1}. R was taken to be 454, an average of the values reported by Garlick *et al.* (1973). These values were used to calculate the fractional rate of synthesis of aldolase (k_e) as described by Waterlow *et al.* (1978), eqn. 10.13. In all experiments, radioactivity was determined as described by Augustine & Swick (1979).

Results and Discussion

A comparison was made of two independent methods of measuring the rate of turnover of individual proteins *in vivo*. The fractional rate of degradation of ornithine aminotransferase, calculated from the decay in total radioactivity in amino acids labelled via [^{14}C]bicarbonate, was similar to the rate of synthesis given by the incorporation of [^{3}H]tyrosine (Table 1). This indicates a minimal reutilization of the ^{14}C-labelled amino acids in rat liver. The agreement between the two methods in the liver also suggests that the specific radioactivity of the total free amino acid may be an appropriate value for the calculation of rates of liver protein synthesis by the continuous-infusion technique. A similar fractional degradation rate was obtained

Table 1. *Fractional rates of synthesis and degradation of liver ornithine aminotransferase and muscle aldolase in the rat*
Rates of synthesis and degradation of the pure proteins were measured in the same animals. For ornithine aminotransferase, decay was measured over 1–5 days after the injection of [^{14}C]bicarbonate; for aldolase, decay was measured over 4–20 days after the injection. Values are expressed as $k_d \pm$ standard error of the slope, and k_s or $k_e \pm$ standard error of the mean (n = 12–13). Abbreviation: n.d., not determined.

Method	Ornithine aminotransferase k_s or k_d	Aldolase k_e or k_d
Continuous infusion of L-[2,6-^{3}H]tyrosine	0.42 ± 0.02 day^{-1}	0.051 ± 0.004 day^{-1}
Decay in specific radioactivity of protein labelled with $NaH^{14}CO_3$	0.45 ± 0.03 day^{-1}	0.022 ± 0.008 day^{-1}
Decay in total radioactivity of protein labelled with $NaH^{14}CO_3$	0.40 ± 0.05 day^{-1}	n.d.

in rats fed on diets containing only 12% (w/w) casein (Chee & Swick, 1976), indicating that there was no 'chase' effect of the high-protein diet.

[^{14}C]Bicarbonate has been used in a number of studies of protein turnover in the liver (Swick & Ip, 1974; Augustine & Swick, 1979; Conde & Scornik, 1976, 1977; McGowan *et al.*, 1979), as well as in the brain (Chee & Dahl, 1977, 1978; Dahl & Weibel, 1979). Several precautions must be taken, however, for the results to be considered valid. For a single protein, changes in specific or total radioactivity should be measured over at least one half-life (Waterlow *et al.*, 1978). For a comparison of mean turnover rates of mixtures of proteins, measurements over three half-lives, or the time taken for the specific radioactivity of the mixture to fall to 10% of its maximum value, may provide a reasonable estimate of the true mean turnover rate (Garlick *et al.*, 1976). However, for the measurement of short-term changes in liver protein breakdown, the loss of label over a period as short as 24h after the injection of $NaH^{14}CO_3$ can be used, with the understanding that the rates of degradation over a short period will be strongly biased by proteins with rapid rates of turnover (e.g. Augustine & Swick, 1979; Conde & Scornik, 1976, 1977).

In contrast with the results in liver, the rate of degradation of rat muscle aldolase, calculated from the decay in specific radioactivity with [^{14}C]bicarbonate as the tracer, appeared to be less than half as rapid as the rate of synthesis (Table 1), although the animals in this study were growing very slowly. Millward (1970) suggested that the decay in protein-bound glutamate and aspartate, labelled *in vivo* with bicarbonate, may give the most reasonable rates of degradation in muscle. In the experiment shown in Table 1, glutamate and aspartate were isolated from muscle aldolase by the method of Partridge (1949), determined by the ninhydrin method (Spies, 1957), and counted for ^{14}C radioactivity. The decay in bound glutamate from days 11 to 20 gave a degradation rate of 0.042 day^{-1}, which is closer to the rate of synthesis. However, there was no significant decay from day 4 to day 11, perhaps because of the additional error introduced by the isolation and quantification of the amino acids. Nor were the values obtained for aspartate useful. It appears that a decay period of at least two half-lives (28 days) would be necessary to obtain any rates of degradation by this method. Swick & Song (1974) compared the rate of degradation of aldolase calculated from the loss of specific radioactivity in protein or from the loss of specific radioactivity in glutamate of aldolase for 40 days after the injection of $NaH^{14}CO_3$. The decay in total specific radioactivity yielded a fractional rate of 0.036 day^{-1}, whereas the decay in glutamate specific radioactivity gave a value of 0.063. The results demonstrate that the decay in total radioactivity derived from CO_2 fixation is unsuitable for the measurement of muscle protein breakdown in rats. The high degree of reutilization in muscle may be due in part to extensive recycling of labelled arginine (Millward, 1970), probably derived from visceral tissues. We have calculated that the re-incorporation into muscle protein of 1% of the labelled amino acids derived from protein breakdown in liver of rapidly growing chicks, for example, is sufficient to prevent a fall in total muscle radioactivity (M. L. MacDonald, unpublished work).

Although labelled glutamate (or aspartate) may give reasonable rates of degradation in muscle under basal conditions, the long decay periods required are unacceptable for the study of most treatments of interest, such as developmental changes or nutritional or hormonal shifts. Furthermore, these amino acids may also be reutilized in muscle, especially during dietary deprivation and refeeding (Waterlow *et al.*, 1978; Young *et al.*, 1971; Nettleton & Hegsted, 1973). An additional problem to be considered when loss of label in muscle is measured is the possibility that the turnover of myofibrillar proteins is non-random. If so, the interpretation of decay curves may be complicated when different treatments are being compared (Millward *et al.*, 1975). At this time, no known radioisotopic technique gives satisfactory rates of degradation in muscle under all conditions. A practical approach is to estimate rates of degradation indirectly by subtracting the rate of accumulation of the protein(s) of interest from the rate of synthesis measured by a continuous infusion (e.g. Waterlow *et al.*, 1978; Millward *et al.*, 1975; Laurent *et al.*, 1978) or a massive dose of a labelled amino acid (Conde & Scornik, 1977).

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