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Equilibration of leucine between the plasma compartment and leucyl-tRNA in the heart, and turnover of cardiac myosin heavy chain

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By 30min continuous infusion of [^{3}H]leucine into rats, the specific radioactivities of plasma leucine and tissue-free and tRNA-bound leucine in heart were equal. The specific radioactivity of leucyl-tRNA in heart therefore follows a time course identical with that of plasma leucine soon after the start of infusion. The half-life of cardiac myosin heavy chain (5.5 days) was the same as that reported by other investigators who used the pulse-labelling protocol.

Several investigators have emphasized that precise calculation of the protein-turnover rate in experiments with labelled amino acids requires determination of the specific radioactivity of the protein precursor, aminoacyl-tRNA (Martin *et al.*, 1977; Vidrich *et al.*, 1977; McKee *et al.*, 1978). Because it is difficult and sometimes impractical to determine the specific radioactivity of the aminoacyl-tRNA in tissues *in vivo*, amino acids in the tissue free pool have generally been used as the source of amino acids for protein synthesis (Garlick *et al.*, 1976; Nicholas *et al.*, 1977; Laurent *et al.*, 1978; Everett *et al.*, 1979). An error in the calculated rate of protein turnover can therefore be expected, depending on the magnitude of the difference between the specific radioactivity of the tissue-free and tRNA-bound amino acid. This difference depends on the tissue and on the particular labelled amino acid administered.

We have therefore investigated the relationship *in vivo* between the specific radioactivities of extracellular and intracellular leucine, and of leucyl-tRNA, in the heart during continuous infusion of [^{3}H]leucine. This relationship was also investigated during rapid cardiac growth, since it may change significantly when the intracellular amino acid pool is enlarged. The half-life of cardiac myosin heavy chain, calculated by the infusion procedure with leucyl-tRNA used as the precursor, was compared with the value obtained when the pulse-labelling protocol and the same precursor were used. Such a comparison between the two protocols for determining the protein half-life has not been made previously.

Experimental

Constant infusion

Female Sprague–Dawley rats were maintained with alternating 12h periods of light and darkness. They were supplied with food and water *ad libitum*. Under chloral hydrate (28mg/100g) anaesthesia a jugular vein was catheterized (Weeks & Davis, 1964), and 2 days later the unrestrained rats received infusions through the catheter with a solution in 0.9% NaCl of L-[3,4-^{3}H]leucine (59.0Ci/mmol; Amersham Corp.) containing heparin (10 units/ml) and carrier leucine (0.1mM), at a rate of 0.8ml (0.8mCi)/h. After 1h of infusion, hearts were processed and ground to a powder under liquid N_2 as in the procedure of Martin *et al.* (1977).

Extraction of RNA

Each ground heart was transferred from liquid-N_2 storage into 4ml of 0.2M-sodium acetate buffer, pH4.0 (Allen *et al.*, 1969), and 3ml of water-saturated phenol and homogenized with an MSE blade homogenizer. The RNA was precipitated from the aqueous phase at −20°C by addition of 2.5vol. of ethanol containing potassium acetate (1.2%, w/v) and was further processed by the method of Allen *et al.* (1969).

Determination of leucine specific radioactivity

A modification of the dansyl (5-dimethylaminonaphthalene-1-sulphonyl) chloride method (Briel & Neuhoff, 1972) was used as follows.

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Leucine from tRNA. Washed RNA was dissolved in 0.5ml of 50mM-Na_2CO_3 buffer (pH 10.0) and incubated at 37°C for 90min for deacylation of tRNA. Nucleic acids were precipitated overnight at −20°C by the addition of 2.5vol. of ethanol. After centrifugation (10000*g* for 10min), the supernatant was dried under N_2 and 100μl of water and 100μl of 2mM-[*N-methyl*-^{14}C]dansyl chloride (Amersham Corp.) in acetone were added. The specific radioactivity of the latter was adjusted with unlabelled dansyl chloride (Pierce Chemical Co.) to about 8d.p.m./pmol, which was approximately the specific radioactivity expected for free and tRNA-bound leucine. After incubation at 37°C, 2ml of acetone was added and the precipitate was removed by centrifugation at 10000*g* for 10min. The supernatant was dried under N_2 and the residue was redissolved in 30μl of acetone for chromatography.

Leucine from plasma and tissue-free pool. Plasma was deproteinized with 5% (w/v) trichloroacetic acid and passed over a 0.5ml column of Dowex AG 50W-X4 resin (Bio-Rad) for removal of acid. Amino acids were eluted with 25% (v/v) NH_3, and the eluate was evaporated to dryness. The residue was dissolved in 50mM-Na_2CO_3 buffer (pH 10.0), and a 10μl sample (containing about 500pmol of leucine) was incubated with 10μl of 2mM-[^{14}C]-dansyl chloride (8d.p.m./pmol) for 30min at 37°C in preparation for chromatography. The specific radioactivity of leucine in the supernatant remaining after the first ethanol precipitation in the extraction of RNA was determined by the procedures for plasma. In one experiment, after infusion of four rats, one half of the heart from each animal was homogenized in ice-cold 5% (w/v) trichloroacetic acid, and after centrifugation the supernatant was assayed for leucine specific radioactivity as described for plasma.

Leucine in protein. Myosin heavy chain was isolated electrophoretically on 5% polyacrylamide gel and hydrolysed in 6M-HCl (Martin *et al.*, 1977). Amino acids were recovered and dried as described for plasma and redissolved in 0.7ml of water; of this solution, 0.5ml was taken for radioactivity determination and the remainder for assay of the leucine concentration by isotope dilution (Airhart *et al.*, 1979).

Chromatography. Chromatograms were developed in tanks on 15cm × 15cm polyamide sheets (Cheng Chin Trading Co.), in the first dimension in water/formic acid (100:3, v/v) and in the second dimension in benzene/acetic acid (9:1, v/v). Dansyl-leucine was separated from other dansylated amino acids, notably dansyl-isoleucine, by identification with standards seen under u.v. light. The dansyl-leucine spot was cut from the polyamide sheet and placed into 10ml of 4.75% NCS (Amersham Corp.) and 4% Liquifluor (New England Nuclear) in toluene for radioactivity determination. The efficiencies of ^{3}H and ^{14}C counting were about 40 and 60% respectively, with 14% spill-over of ^{14}C radioactivity into the ^{3}H channel. From the known specific radioactivity of [^{14}C]dansyl chloride and the ^{3}H/^{14}C ratio of dansyl-leucine, the specific radioactivity of [^{3}H]leucine was determined. An identical ratio was obtained when the dansyl-leucine was eluted from the polyamide sheet (McKee *et al.*, 1978). We determined the specific radioactivity of the [^{14}C]dansyl chloride accurately by using a complete amino acid standard (Pierce Chemical Co.) containing a trace of high-specific-radioactivity [^{3}H]leucine.

As a check of these procedures, eight rats received infusions with about 0.7mCi of [^{3}H]leucine for 1h, and the resulting specific radioactivity of free leucine in the heart, determined by competitive aminoacylation of tRNA (Zak *et al.*, 1978), was 89 ± 0.4 (S.E.M.) d.p.m./pmol, compared with 9.0 ± 0.4 d.p.m./pmol obtained with the dansylation procedure.

Calculation of protein half-life

The fractional rate of synthesis (k_s) of cardiac myosin heavy chain was calculated from the specific radioactivities of the protein and precursor (leucyl-tRNA) after 1h infusion of [^{3}H]leucine, with the equations outlined in detail by Waterlow *et al.* (1978). The half-life in the steady state is then given by $\ln 2/k_s$.

Results and discussion

Equilibration of leucine in plasma and heart during constant infusion of [^{3}H]leucine

The specific radioactivity of plasma leucine increased exponentially, with a first-order rate constant averaging 84 ± 12 (S.E.M.) day^{-1} after infusion of seven rats for 1h. Representative results from four infusions are shown in Fig. 1. The specific radioactivity of leucyl-tRNA in heart muscle of normal rats and of rats after 2 days constriction of the ascending aorta (Nair *et al.*, 1968) was the same as that of plasma leucine after 30 and 60min infusion (Table 1). Thus the specific radioactivity of leucyl-tRNA in heart follows a time course similar to, if not identical with, that of plasma leucine very soon after the start of infusion both in the steady state and during rapid cardiac growth. This finding is consistent with previous studies showing that the specific radioactivity of leucine is the same in the plasma and tRNA compartments 5min after pulse injection of [^{3}H]leucine, when the plasma specific radioactivity is changing very rapidly (Martin *et al.*, 1977).

In Table 1, the specific radioactivity of leucyl-tRNA is compared with that of plasma leucine from blood remaining in the chest cavity after the heart was removed. The results confirmed that the specific radioactivity of plasma leucine obtained immediately before and after death was the same (Expt. 2).

Leucine in the free amino acid pool of heart muscle was also analysed, since the specific radioactivity of amino acids in this pool has generally been used for the precursor in the calculation of the protein-turnover rate. In the present experiments, total free leucine in the heart was taken to be that in the supernatant after ethanol precipitation of RNA from phenol-extracted heart homogenate. This enabled us to use the small amount of available tissue for both RNA extraction and isolation of leucine from the free pool. This method for extracting free amino acids from heart could be unsuitable, however, since the use of different extraction procedures has been shown to influence the amount of amino acid recovered (Manchester, 1980). Therefore, the value of the amino acid specific radioactivity may be in error if, for example, subcellular amino acid pools are not in rapid equilibrium, as appears to be the case in the liver (Fern & Garlick, 1976; Ward & Mortimore, 1978). We therefore determined the specific radioactivity of leucine in the supernatant after homogenizing the heart in ice-cold 5% (w/v) trichloroacetic acid, because this method for extracting amino acids from the tissue free pool has been used most often by other investigators. The specific radioactivity of leucine in the trichloroacetic acid-soluble fraction of the heart was found to be the same as that in the ethanolic supernatant in the RNA extraction after 1h infusion (Expt. 3, Table 1). At least with these two extraction procedures, there is no evidence, in the cytoplasm of the heart, for any significant leucine pool that is not in complete and rapid equilibrium with the extracellular and other intracellular compartments, including the leucyl-tRNA pool.

In summary, these experiments demonstrate that, during continuous infusion of [^{3}H]leucine, the specific radioactivity of the protein precursor (leucyl-tRNA) in the heart can be represented accurately by leucine in either the plasma or the

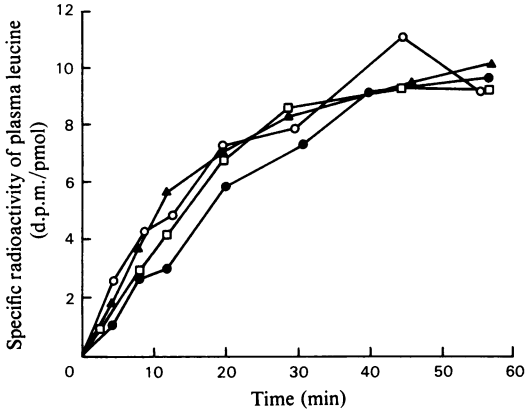

Fig. 1. *Specific radioactivity of plasma leucine during infusion of [^{3}H]leucine into four rats*
Rats were infused via a catheter implanted in a jugular vein 2 days previously. Blood samples (25–50 μl) were taken from the severed tip of the tail. The results shown are for rats with body weights averaging 187 ± 3 g, infused at a rate of 0.78 ml/h with a 0.9% NaCl solution of [^{3}H]leucine (1 mCi/ml) which contained carrier leucine (0.1 mM) and heparin (10 units/ml). The results for each rat are shown by a different symbol.

Table 1. *Specific radioactivity of leucine in plasma and heart after intravenous infusion of [^{3}H]leucine into rats*
Chest blood was sampled immediately after removal of the heart, whereas tail blood was taken just before death. The free pool is represented by amino acids remaining in the supernatant after (1) ethanol precipitation of nucleic acids after their extraction from heart homogenized in 4 ml of 0.2 M-sodium acetate buffer (pH 4.0) and 3 ml of distilled phenol and (2) homogenization of the heart in ice-cold 5% (w/v) trichloroacetic acid immediately after death. Results from Expts. 1, 2, and 3 were for normal rats; those from Expt. 4 were for rats after 2 days constriction of the ascending aorta, in which the heart weight increased by an average of 23% over that of sham-operated controls (results not shown). Values are given as means ± S.E.M., with the numbers of animals in parentheses. Within each experiment, there was no significant difference in the leucine specific radioactivities from any source.

Expt. no.	Rat weight (g)	Infusion time (min)	Radioactivity infused (μCi/min)	Plasma leucine (d.p.m./pmol) Chest blood	Plasma leucine (d.p.m./pmol) Tail blood	Heart free-pool leucine (d.p.m./pmol) Phenol	Heart free-pool leucine (d.p.m./pmol) Trichloroacetic acid	Heart leucyl-tRNA (d.p.m./pmol)
1	222 ± 2 (4)	30	23	19.5 ± 1.1		19.1 ± 0.8		19.2 ± 0.8
2	207 ± 4 (4)	60	14	10.6 ± 0.5	10.4 ± 0.7	10.9 ± 0.6		11.0 ± 0.5
3	192 ± 4 (3)	60	12	10.3 ± 0.2		9.9 ± 0.3	10.4 ± 0.4	
4	194 ± 2 (4)	60	12	9.4 ± 0.4		9.7 ± 0.7		9.1 ± 0.9

trichloroacetic acid-soluble fraction of this tissue in the steady state and during cardiac growth. This is not necessarily the case with all amino acids, since the specific radioactivity of glycine (Wyborny *et al.*, 1978), proline (Laurent *et al.*, 1978) and tyrosine (Everett *et al.*, 1979) in the acid-soluble pool of the myocardium has been shown to be less, usually by between 10 and 20%, than that of the plasma after infusion of these labelled amino acids.

McKee *et al.* (1978) reported that, in isolated perfused hearts, equilibration of phenylalanine between the extracellular and intracellular compartments occurs within minutes. They also noted that the specific radioactivities of perfusate and tissue-free phenylalanine and phenylalanyl-tRNA reach parity in the presence of higher than physiological (5-fold) concentrations of phenylalanine in the perfusate. In the present experiments, complete equilibration of leucine in plasma and heart pools occurred at normal plasma concentrations of leucine.

Half-life of cardiac myosin heavy chain

For this calculation, the specific radioactivity of leucyl-tRNA was assumed to follow a time course identical with that of plasma leucine, with a first-order rate constant of 84 day^{-1}. The half-life of myosin heavy chain was then calculated to be 5.5 ± 0.2 (mean $\pm$ S.E.M., $n = 7$) days in the steady-state myocardium. This value is the same as the 5.4 days reported by Martin *et al.* (1977), who made a full analysis of the precursor (leucyl-tRNA) and product specific radioactivities after pulse injection of [^{3}H]leucine. Thus the infusion and pulse-labelling protocols yield the same protein half-lives when aminoacyl-tRNA is used as the precursor. The choice of protocol then depends on the requirement of the experiments; this has been reviewed elsewhere (Waterlow *et al.*, 1978; Everett & Zak, 1980).

A relatively short infusion time of 1 h was adopted in these experiments, compared with the 6 h which has usually been employed by others. Both short and long infusions will yield the same values for the protein half-life if the rate of rise to plateau of the precursor specific radioactivity is known with some certainty and, ideally, for each animal infused. More commonly, however, an average value of this parameter is adopted for all animals. It is therefore desirable to increase the infusion time to minimize any error in the calculated rate of protein turnover which results from differences among animals in the time course of the rise to plateau of the precursor specific radioactivity. Zak *et al.* (1979) made a theoretical assessment of this error for infusions of 1 h and 6 h duration. Only when the rate of rise to plateau of the precursor specific radioactivity in a given animal is very much greater than the assumed average (82 day^{-1} in the present experiments) can the error in the calculated turnover rate be decreased by an increase in the infusion period. In sampling plasma throughout the infusion, we found no evidence for a large variation in the value of this parameter among individual animals (Fig. 1), and thus it was unnecessary to extend the infusion beyond 1 h. The shorter infusion is preferable in that it decreases the possible effects of stress on the animal during the experiment and the effects of diurnal changes in the animal's protein metabolism on measurements of protein turnover in the heart.

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