Synthesis of Guinea-Pig Cardiac Myosin as Measured by Constant Infusion

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An equation was derived from which the turnover time of individual muscle proteins could be calculated from measurements made at a single time interval in individual animals after initiation of constant intravenous infusion of labelled amino acid. The calculation requires only the specific radioactivities of the amino acid in plasma, in the intracellular fluid and in the protein under study. Pool sizes were not required. When the equation was applied to adult guinea-pig cardiac myosin, the average turnover time was 16 ± 1 days.

The constant- or continuous-infusion approach has been used to calculate the turnover of mixed proteins in muscle (Waterlow & Stephen, 1968; Garlick, 1969; Garlick *et al.*, 1973; Pain & Garlick, 1974; Millward *et al.*, 1975, 1976). The formulation as originally developed by Waterlow & Stephen (1968) and as later modified by Garlick *et al.* (1973) is limited to mixed protein. The present paper describes further modifications in the formulation so that the constant-infusion technique can be used to calculate the turnover of individual muscle proteins. The modified equation was used to calculate the turnover of cardiac myosin in the adult guinea pig.

Methods

Infusion procedure

Male guinea pigs (Rich-Glo Lab Animals, El Campo, TX, U.S.A.) weighing 500–900 g were fed on commercial guinea-pig chow (Ralston Purina Co., St. Louis, MO, U.S.A.) *ad libitum* until 8–12 h before cannula implantation, at which time the chow was withdrawn. Venous cannulation was performed by inserting polyethylene tubing (Intramedic 7411; Clay Adams Co., Parsippany, NJ, U.S.A.) containing heparin [1000 USP units/ml] into the right jugular vein while the animal was anaesthetized with 0.4 ml of Innovar-Vet (fentanyl citrate, 0.4 mg/ml; droperidol, 20 mg/ml; Pitman-Moore, Washington Crossing, NJ, U.S.A.)/kg. The cannula was threaded subcutaneously and brought out behind the right ear. On the next day the infusion was performed while the animals were restrained in a fabricated Plexiglass apparatus designed such that the width was adjustable and the bottom was open. During the infusion the animals had limited movement back and forth, but were unable to turn around.

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The infusing solution, which consisted of [$1\text{-}^{14}\text{C}$]glycine (100 $\mu\text{Ci/ml}$; 16 $\mu\text{mol/ml}$; New England Nuclear Corp., Boston, MA, U.S.A.) in 0.9% NaCl, was infused at 0.5 ml/h with a Unita infusion pump (VWR Scientific, Houston, TX, U.S.A.) for 6, 8 or 10 h. At the end of the infusion period, the animals were killed with a sharp blow to the back of the head. Blood samples were withdrawn either from the heart at the time of death or by cardiopuncture through the chest. The hearts were excised and flushed with cold water by forcing the water from a wash bottle through the aorta.

Free glycine

Free glycine was extracted from ventricular and plasma samples with 1% picric acid and was passed through a column (1 cm $\times$ 2 cm) of Dowex 2 resin (8X; chloride form) to remove the picric acid. The free amino acid were washed from the Dowex column with 0.02 M-HCl and the liquid was removed by flash-evaporation. The residue, which contained the free glycine, was dissolved in 0.2 M-sodium citrate, pH 2.2, for amino acid analysis and measurement of radioactivity.

Glycine in myosin

Myosin was isolated from ventricular tissue by fractionation with $(\text{NH}_4)_2\text{SO}_4$ in the presence of LiCl (Luchi *et al.*, 1965). Guinea-pig cardiac myosin appears homogeneous when examined by Sephadex G-200 column chromatography and ultracentrifugation (Goodkind *et al.*, 1974); however, it may contain a small amount of actin, as evidenced by sodium dodecyl sulphate/polyacrylamide-gel electrophoresis (Goodkind *et al.*, 1974). The concentration of purified myosin was measured from its u.v. absorption at 280 nm ($A_{1\text{cm}}^{1\%} = 5.1$ in 0.4 M-KCl/borate, pH 6.8) after correction for Rayleigh scatter-

ing. Purified myosin was precipitated by dilution with 12 parts (v/v) of cold water, then dissolved in 6M-HCl and hydrolysed for 24h at 110°C. The liquid was removed by flash-evaporation and the residue was dissolved in 0.2M-sodium citrate, pH2.2, for amino acid analysis and measurement of radioactivity associated with glycine.

Glycine analysis and radioactivity

Glycine was separated from the other amino acids on a Beckman model 120C amino acid analyser (Beckman Instruments, Palo Alto, CA, U.S.A.) by using 0.2M-sodium citrate, pH3.25, and Beckman AA-15 resin (0.9cm × 53cm column). The buffer and ninhydrin solution flow rates were maintained at 68 and 34ml/h respectively. Another sample was separated on the amino acid-analyser column under identical conditions and collected into 3.4ml fractions with a Buchler Fractomat (Buchler Instruments, Fort Lee, NJ, U.S.A.) for determination of radioactivity. The amount of radioactivity was determined with a Packard Tri-Carb liquid-scintillation spectrometer by using 0.5ml of column effluent in 15ml of 'cocktail' containing 4g of PPO (2,5-diphenyloxazole), 0.05g of POPOP [1,4-bis-(5-phenyloxazol-2-yl)benzene] and 100ml of Biosolv BBS-3 (Beckman Instruments, Fullerton, CA, U.S.A.) per litre of toluene. The counting efficiency was approx. 75%.

Development of equations for individual muscle proteins

During constant intravenous infusion of labelled amino acid, the specific radioactivity of the amino acid in plasma rapidly rises to a plateau value that is maintained for several hours (Gan & Jeffay, 1967; Waterlow & Stephen, 1967; Aub & Waterlow, 1970). The specific radioactivity of the intracellular amino acid also reaches a plateau; however, it is usually approached more slowly and is represented mathematically as:

$$S_i = S_{i, \max.} (1 - e^{-kt}) \quad (1)$$

where S_i and $S_{i, \max.}$ are the specific radioactivities of the intracellular amino acid at time t and at the plateau respectively, and k is the fractional (first-order) rate constant for the exit of the amino acid from the intracellular pool. Since the specific radioactivity of the amino acid in plasma rapidly approaches a plateau, the specific radioactivity of the amino acid in plasma can be treated as a constant, and the maximal specific radioactivity of the amino acid in the intracellular pool, $S_{i, \max.}$, can be determined by the fraction of the amino acid derived from each source (Zilversmit, 1960; Garlick *et al.*, 1973), thus:

$$S_{i, \max.} = RS_p \quad (2)$$

where R is the ratio of the rate of entry of the amino acid from plasma to the total rate of exit of the amino acid from the intracellular pool and S_p is the specific radioactivity of the amino acid in plasma at plateau. By substituting eqn. (2) into eqn. (1), it becomes:

$$S_i = RS_p(1 - e^{-kt}) \quad (3)$$

For the purpose of the present study it was assumed that R was equal to 1, thus making:

$$S_i/S_p = 1 - e^{-kt} \quad (4)$$

The value of k was evaluated at any time t by measuring the specific radioactivities of the amino acid in plasma and the free intracellular pool.

If a steady state is assumed, the rate of synthesis of a particular protein will be equal to the rate of degradation, i.e.:

$$r_s - r_d = k_1 q = k_2 Q \quad (5)$$

where r_s is the flux of the amino acid into the protein, r_d is the flux of the amino acid out of the protein, k_1 is the fractional (first-order) rate constant for incorporation of the amino acid into the protein, k_2 is the fractional rate constant for release of the amino acid from the protein, q is the amount of the amino acid in the free intracellular pool (amount/g of tissue) and Q is the amount of the amino acid in the protein (amount/g of tissue). By considering the flux of labelled amino acid into the protein and limiting the infusion period to several hours so that the labelled amino acid derived from the protein can be neglected, the change in the amount of labelled amino acid in the protein is:

$$dQ^*/dt = r_s S_i \quad (6)$$

where Q^* is the amount of labelled amino acid in the protein (radioactivity/g of tissue). Dividing eqn. (6) by the amount of amino acid in the protein gives:

$$d(Q^*/Q)/dt = dS_Q/dt = r_s S_i/Q \quad (7)$$

where S_Q is the specific activity (amount of radioactivity/amount of amino acid) of the amino acid in the protein.

From eqn. (5), it is evident that r_s/Q is equal to k_2 , and therefore substitution into eqn. (4) gives:

$$dS_Q/dt = k_2 S_i \quad (8)$$

Substitution of the value of S_i from eqn. (1) into eqn. (8) produces:

$$dS_Q/dt = k_2 S_{i, \max.} (1 - e^{-kt}) \quad (9)$$

and on integration becomes:

$$S_Q/k_2 S_{i, \max.} = [kt - (1 - e^{-kt})]/k \quad (10)$$

Substituting the value of $S_{i, \max}$ from eqn. (1) into eqn. (10) and rearranging, one obtains:

$$k_2 = \frac{S_0/S_i}{\frac{t}{(1-e^{-kt})} - \frac{1}{k}} \quad (11)$$

By designating the reciprocals of $(1-e^{-kt})$ and k as P and E respectively, eqn. (11) simplifies to:

$$k_2 = \frac{S_0/S_i}{(Pt-E)} \quad (12)$$

The specific radioactivities of the amino acid in the protein, S_0 , in the free intracellular pool, S_i , and in the free plasma pool, S_p , are determined experimentally. The values of P and E are calculated from the specific radioactivities S_i and S_p by using eqn. (4). The turnover was expressed as the turnover time, which is equal to the reciprocal of k_2 . The mean turnover time was expressed $\pm$ S.E.M. ($n = 5$).

Results and Discussion

The original formulation used to calculate fractional rate constants for synthesis of muscle protein after constant infusion was developed by Waterlow & Stephen (1968) and was later modified by Garlick *et al.* (1973). The present formulation differs from the previous ones in that it can be applied to individual muscle proteins and instead of pool sizes it requires the specific radioactivity of the free amino acid in plasma, which is readily obtained experimentally.

In the present study [$1-^{14}\text{C}$]glycine was used as the infusing amino acid. The specific radioactivity of free glycine in the plasma approached a plateau value within 2 h after initiation of the infusion (Fig. 1). Other investigators (Gan & Jeffay, 1967; Waterlow & Stephen, 1967, 1968; Garlick & Marshall, 1972; Garlick *et al.*, 1973; Seta *et al.*, 1973) have obtained similar results with different labelled amino acids and different animals. These results thus justify the assumption that the specific activity of the amino acid in plasma rapidly reaches a plateau value during constant infusion.

For the purposes of the present study the ratio of the rate of entry of glycine from plasma to the total rate of exit of glycine from the intracellular pool, R , was ascribed a value of 1. This assumption appears reasonable, since R is also equal to $S_{i, \max}/S_p$ (see eqn. 2) and S_i/S_p was 0.9 after 10 h (Table 1). Pain & Garlick (1974) also obtained a value near 1 for heart muscle when labelled tyrosine was infused into the rat. It thus appears that the amount of free intracellular amino acid derived endogenously in the ventricular tissue is negligible compared with the amount of amino acid entering and leaving the tissue per unit time.

Re-utilization of labelled glycine derived from ventricular protein did not appear to be a problem in

the present study. The specific radioactivity of glycine obtained from cardiac myosin was always less than 2% of the specific radioactivity of the free intracellular glycine (Table 1). Even if one assumed that the specific radioactivity of protein glycine was 5% of the specific radioactivity of the free intracellular glycine, that each gram of tissue contained only 20 times as much protein glycine as free intracellular glycine, and the fractional turnover rate of the protein was 1%/h, then the amount of labelled glycine derived from protein would still be only about 1%/h.

It was assumed that the specific radioactivity of free tissue glycine did not differ from the immediate precursor of protein synthesis. Naturally, compartmentalization of free amino acids (Mortimore *et al.*, 1972) would invalidate this assumption. The assumption is consistent, however, with the incorporation of labelled amino acid into protein during heart perfusion (Morgan *et al.*, 1971). Also, use of the specific radioactivity of the free intracellular amino acid appears to give a better estimate of the

Fig. 1. Effect of infusion period on the specific radioactivity of free glycine in plasma

Blood samples were withdrawn by cardiopuncture at various times after initiation of infusion with [$1-^{14}\text{C}$]glycine into the jugular vein of the guinea pig, and the amount of free glycine in plasma and the amount of radioactivity associated with glycine were determined (see the Methods section).

Table 1. Specific radioactivities of glycine

Values expressed as decimals are ratios. All other values are d.p.m./μmol of glycine. S_p = specific radioactivity of free glycine in plasma. S_i = specific radioactivity of free glycine in ventricular tissue. S_0 = specific radioactivity of glycine in cardiac myosin.

Expt. no.	Infusion period (h)	S_p	S_i	S_0	S_i/S_p	S_0/S_i
1	6	640 000	303 000	2500	0.47	0.008
2	8	241 000	195 000	2900	0.81	0.015
3	8	305 000	185 000	2700	0.61	0.014
4	8	375 000	351 000	4000	0.94	0.011
5	10	319 000	284 000	4700	0.89	0.016

Table 2. Fractional rates and turnover time for cardiac myosin synthesis

The fractional rate constants and turnover times were calculated from the data in Table 1. k = fractional rate constant for exit of glycine from the intracellular pool. k_2 = fractional rate constant for release of glycine from myosin. Turnover time = $1/k_2$.

Expt. no.	Infusion period (h)	k (days ⁻¹)	k_2 (days ⁻¹)	Turnover time (days)
1	6	2.54	0.060	16.7
2	8	4.83	0.072	13.9
3	8	2.75	0.076	13.2
4	8	7.93	0.049	20.4
5	10	5.29	0.059	16.9

rate of protein synthesis than use of the specific radioactivity of the amino acid in plasma (Fern & Garlick, 1973).

The turnover of protein can be expressed as (1) a fractional turnover rate, which is the first-order rate constant for degradation, (2) a turnover time, which is the time required for the replacement of the quantity of protein present in the compartment under study and is also equal to the reciprocal of the first-order rate constant for degradation, or (3) a half-life, which is the time required for one-half of the original protein in the compartment under study to be replaced, and is equal to the turnover time divided by 1.44 when a steady state exists (Zilversmit, 1960). For comparative purposes the data of other investigators were converted into turnover times.

The mean turnover time for adult guinea-pig cardiac myosin was 16 ± 1 days (Table 2). The turnover times calculated after 6 and 10 h did not differ substantially from those calculated after 8 h. Probably the closest system *in vitro* to that of constant infusion *in vivo* is organ perfusion. Our calculations, based on the data of Schreiber *et al.* (1970), collected from the perfusion of baby-guinea-pig heart for 2 h, yielded a turnover time of 12 days, assuming a lysine content of $870 \mu\text{mol/g}$ of myosin (Thyrum *et al.*, 1970) and a myosin content of 35 g/kg of myocardial tissue (Katz, 1970). The lower turnover time with the perfused heart may be due to an age-dependency in muscle protein turnover. In the rat (Waterlow & Stephen, 1968; Millward *et al.*, 1975) and in the mouse (Srivastava & Chaudhary, 1969) the turnover time of total muscle protein has been shown to increase with age.

Similar turnover times have also been calculated for rabbit and rat cardiac myosin. Turnover times of 11 and 17 days have been calculated from pulse-labelling results obtained after injection of [³H]-lysine into the rabbit (Kimata & Morkin, 1971) and [³H]leucine into the rat (Rabinowitz, 1973; Wikman-Coffelt *et al.*, 1973) respectively. A slightly

higher value, 21 days, was obtained with perfusion of the rat heart (Morgan *et al.*, 1971).

Our formulation developed for the calculation of protein turnover of individual muscle proteins after constant intravenous infusion yielded a reasonable result when applied to adult guinea-pig cardiac myosin. It is believed that our formulation used in conjunction with the constant infusion technique will have wide applicability in measuring the turnover of other muscle proteins. Further, the relatively close agreement in the turnover time of guinea-pig cardiac myosin to those of rabbit and rat cardiac myosin may indicate that re-utilization of labelled amino acid may not be a problem in heart tissue.

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