Synthesis Rates of Protein in the Langendorff-Perfused Rat Heart in the Presence and Absence of Insulin, and in the Working Heart

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(Received 4 October 1972)

The synthesis rates of total heart protein and of sarcoplasmic and myofibrillar protein fractions have been determined by perfusion of isolated rat hearts with [^{14}C]tyrosine at constant specific radioactivity. In hearts perfused without insulin, both myofibrillar and sarcoplasmic proteins were synthesized at a fractional rate of 10–11% per day. This corresponds to a half-life for synthesis of about 7 days. The effect of added insulin was to increase the rate of heart-protein synthesis to a half-life of 3–4 days. With hearts perfused via the left atrium and performing external work, there was a rise in the specific radioactivity of intracellular free tyrosine, and the half-life for synthesis of proteins was 3–4 days. The extent of labelling of individual myofibrillar proteins was estimated after polyacrylamide-gel electrophoresis of solubilized myofibrils in the presence of sodium dodecyl sulphate. No particular protein showed an unusually high or low specific radioactivity after labelling in perfusion. Insulin caused a general increase in labelling of all the proteins analysed.

Insulin is known to stimulate the incorporation of amino acids into protein. The effect is small relative to insulin action on carbohydrate metabolism and it is independent of the presence of glucose (Chain & Sender, 1973). To evaluate the physiological significance of this effect, we decided to measure the turnover rate of protein in the perfused rat heart under conditions in which the insulin effect was observed. Little is known of the extent to which cardiac muscle participates in the dynamic state of body protein. The continual degradation and resynthesis of a mechanically active organ, and the extent to which physical activity affects protein synthesis, are of great interest.

The measurement of turnover rates of protein is markedly influenced by the recycling of labelled amino acids and also by problems associated with measurement of the specific radioactivity of rapidly changing precursor pools after the single injection of labelled amino acid (Koch, 1962). These difficulties may be circumvented *in vivo* by using a continuous infusion of labelled amino acid, which serves to maintain the precursor pool at constant specific radioactivity (Loftfield & Harris, 1956; Gan & Jeffay, 1967; Waterlow & Stephen, 1968).

The present paper reports the use of this approach to measure rates of synthesis of heart protein *in vitro* in the isolated perfused organ, where the specific radioactivity of the precursor amino acid in the perfusate may similarly be held constant. The effects of hormonal stimulation by insulin on the rate of protein synthesis and of the performance of external mechanical work by the heart have been studied. To

compare the rates of synthesis of individual myofibrillar proteins to see if differences could be detected, a study was also made of the labelling of the different contractile proteins separated by polyacrylamide-gel electrophoresis.

Experimental

Materials

L-Tyrosine decarboxylase (type I from *Streptococcus faecalis*), tyramine hydrochloride and sodium dodecyl sulphate were from Sigma (London) Chemical Co. Ltd. (London S.W.6, U.K.). Other chemicals (purest grade available) were from British Drug Houses Ltd. (Poole, Dorset, U.K.) or from the source indicated in the preceding paper (Chain & Sender, 1973). D- $[\text{}^3\text{H}]$ Sorbitol (200 mCi/mmol) and L- $[\text{}^{14}\text{C}]$ tyrosine hydrochloride (12.9 mCi/mmol) were obtained from The Radiochemical Centre (Amersham, Bucks., U.K.).

Methods

Perfusion. Hearts were from male albino rats of the Sprague-Dawley strain, weighing 280–320 g. They had free access to water and a stock laboratory diet (Thompson rat cubes; Pilsbury, Birmingham, U.K.). Langendorff recirculation perfusions (perfusate volume 10 ml) were performed as described in the preceding paper (Chain & Sender, 1973). Recirculation perfusions with a perfusate volume of 50 ml were done by the method of Neely *et al.* (1967) ('working' hearts),

with the modifications of Chain *et al.* (1969). The perfusion medium was bicarbonate-buffered salt solution (Krebs & Henseleit, 1932) equilibrated with $O_2 + CO_2$ (95:5) at 37°C. This medium contained glucose (5 mM) and natural (L) amino acids at approximately the usual concentrations found in rat serum: alanine, 0.45 mM; lysine, 0.35 mM; glycine, threonine and proline, 0.25 mM; arginine, glutamate, glutamine, leucine, serine and valine, 0.20 mM; methionine, histidine, isoleucine, phenylalanine, tryptophan and tyrosine, 0.10 mM; aspartate, asparagine and cysteine, 0.05 mM (Mallett *et al.*, 1969; Scharff & Wool, 1965). Other additions are given in the text, figures or tables.

Preparation of heart-protein fractions and myofibrils. The heart-protein fractions were prepared as described in the preceding paper (Chain & Sender, 1973). Part of the tissue was homogenized in 10% (w/v) trichloroacetic acid, 3 ml/g, in a Vortex model 404 homogenizer, for estimation of the specific radioactivity of intracellular tyrosine and of total heart protein. The remainder of the heart was used for the preparation of myofibrils, which were purified from contaminating sarcoplasmic proteins as described by Sender (1971). The myofibrils were precipitated with 10% (w/v) trichloroacetic acid for the measurement of specific radioactivity, or dissolved in sodium dodecyl sulphate for electrophoresis (see below).

The first supernatant fraction obtained after centrifuging the heart myofibrillar homogenate was treated with an equal volume of 20% (w/v) trichloroacetic acid to precipitate the sarcoplasmic proteins. Radioactivity deriving from free amino acids was removed from trichloroacetic acid-precipitated proteins by the method of Wool & Krahll (1959).

Measurement of tyrosine specific radioactivity. The specific radioactivity of tyrosine in the perfusion and intracellular fluids and in hydrolysed protein fractions was measured as described by Garlick & Marshall (1972).

Extracellular fluid space in perfused rat hearts. Hearts were perfused under the appropriate conditions with perfusion fluid containing D-[1- 3H]-sorbitol (2.5 μ Ci/ml) and carrier sorbitol (1 mg/ml) for the measurement of the extracellular fluid space (Morgan *et al.*, 1961). The dilution of [3H]sorbitol was also used as a measure of the circulating volume. Control perfusions done without hearts to check on possible losses of fluid by evaporation gave no indication of increase in concentration of [3H]sorbitol.

Calculation of synthesis rate of protein. The rate of incorporation of radioactivity from [^{14}C]tyrosine into protein is given by:

$$dR_b/dt = vS_p$$

where R_b = total radioactivity incorporated (μ Ci/heart), v = rate of incorporation of total ([^{12}C]tyro-

sine + [^{14}C]tyrosine) (mmol/day per heart) and S_p = specific radioactivity (μ Ci/mmol) of the immediate precursor for protein synthesis (free tyrosine). It is assumed that the amount of label lost by subsequent degradation of newly synthesized protein is negligible during a 1 h perfusion.

If Q is the amount of tyrosine in heart protein (μ mol/heart), then:

$$dS_b/dt = vS_p/Q$$

(S_b = specific radioactivity of protein tyrosine as μ Ci/mmol). If S_p , v and Q are assumed to remain constant during the experiment, we can solve this differential equation with the condition $S_b = 0$ when $t = 0$:

$$S_b/S_p = vt/Q$$

But v/Q is the fraction of the total protein tyrosine pool that is synthesized per day, and is equal to the fractional rate of protein synthesis, k_s (days $^{-1}$).

Solubilization and gel electrophoresis of heart myofibrillar proteins. Analytical gel electrophoresis of solubilized myofibrillar proteins on polyacrylamide gels containing sodium dodecyl sulphate, gel densitometry and the identification of some of the protein bands were done as described previously (Sender, 1971).

Determination of radioactivity of myofibrillar proteins in polyacrylamide gels. Myofibrils were prepared from hearts that had been perfused for 1 h with 3.1 μ Ci of the ^{14}C -labelled amino acid mixture. Approx. 1 mg of solubilized myofibrillar protein was subjected to electrophoresis for 2–3 h at 18 mA/gel, constant current, in a large-diameter polyacrylamide gel (9.6 mm $\times$ 100 mm). These gels were identical in composition with the smaller analytical gels used, and contained 0.1% sodium dodecyl sulphate, 5 M-urea and 4% Cyanogum 41 (British Drug Houses Ltd.) in 0.1 M-sodium phosphate (pH 7.1). The gels were stained, frozen in aluminium troughs over solid CO_2 and cut transversely into 1 mm slices with a gel-slicer (Mickle Laboratory Engineering Co., Gomshall, Surrey, U.K.). The slices were then dissolved for liquid-scintillation spectrometry (Tishler & Epstein, 1968). Counting efficiency was 70–80% (channels-ratio method) and the recovery of the total radioactivity applied to the gel was 90–105%. There was no detectable difference between the weights of different gel slices, and the reproducibility of the method was very good when gels were run in triplicate.

Results

Specific radioactivity of free tyrosine in the perfusate and the heart

Fig. 1 demonstrates that the specific radioactivity of intracellular free tyrosine very rapidly rises to a plateau value in the perfused heart, and it remains at

Fig. 1. Uptake and incorporation into protein of [¹⁴C]tyrosine by the isolated perfused rat heart

Hearts were perfused (Langendorff preparation; 100cmH₂O aortic pressure) with 5mM-glucose, a full amino acid complement and 100μM-[¹⁴C]tyrosine. The specific radioactivities of intracellular (●), extracellular (perfusate; ○) and protein-bound (▲) tyrosine were measured in hearts perfused for the times indicated. The time-course of the incorporation of [¹⁴C]tyrosine under identical conditions but in the presence of added insulin (5munits/ml) is also shown (Δ). The specific radioactivities of intra- and extra-cellular free tyrosine in these hearts were not significantly different from those of the insulin-free controls (see Table 1). Each point represents the mean of at least three separate perfusions.

this value for the duration of a 1h perfusion. The intracellular specific radioactivities have been corrected for a heart extracellular (sorbitol) space of 210±18 μl/g wet wt., which was not affected by insulin (in working hearts the value was 486±23 μl/g wet wt.). There is no decline in perfusate specific radioactivity over this time.

The results presented in Table 1 demonstrate that, whereas in the Langendorff heart preparation perfused either with or without insulin, the specific radioactivity of the free intracellular tyrosine reaches a value of about 70 % of that in the perfusate, when the heart performs external mechanical work the intra- and extra-cellular specific radioactivities of free tyrosine become very nearly equal (the intracellular specific radioactivity being 95 % of that in the perfusate).

Incorporation of [¹⁴C]tyrosine into heart protein

Fig. 1 shows that the incorporation of [¹⁴C]-tyrosine into total heart protein, both in the presence and absence of insulin, proceeds at a linear rate with respect to time for the 1h perfusion period.

Insulin stimulates [¹⁴C]tyrosine incorporation into both protein fractions of the Langendorff perfused heart preparation by about 60%. The performance of external mechanical work leads to a more pronounced increase of [¹⁴C]tyrosine incorporation (Table 1), but preliminary studies indicated that no further significant increase occurred when insulin was added to the working heart.

Table 1. Effect of added insulin and of the performance of external work on the uptake and incorporation of [¹⁴C]tyrosine into myofibrillar, sarcoplasmic and total heart proteins by the isolated perfused rat heart

All hearts were pre-perfused for 15min and then perfused under the conditions specified (Langendorff preparation: 60mmHg aortic pressure; working hearts: 20cmH₂O left atrial pressure) by recirculation for 1h with 5mM-glucose as substrate and a 'normal' amino acid complement (100μM-tyrosine, with the final measured perfusate specific radioactivity indicated as the extracellular value). The recirculation volume was 10ml for Langendorff-perfused hearts, and 50ml in the working hearts. *Differences between these means are significant (*P* <0.05).

Perfusion conditions (no. of hearts)	Sp. radioactivity of free tyrosine (μCi/mmol)		Sp. radioactivity ratio (S _i /S _e)	Sp. radioactivity of protein-bound tyrosine (S _b) (μCi/h per mmol)		
	Extracellular (S _e)	Intracellular (S _i)		Total	Myofibrillar	Sarcoplasmic
Langendorff (8)	2017 ± 54	1431 ± 41	0.71 ± 0.025*	6.15 ± 0.36	6.56 ± 0.37	6.10 ± 0.22
Langendorff + insulin (5munits/ml) (8)	1981 ± 72	1387 ± 56	0.70 ± 0.031	10.92 ± 0.45	8.59 ± 0.25	10.30 ± 0.33
Working (6)	1876 ± 102	1782 ± 87	0.95 ± 0.056*	14.27 ± 0.56	13.85 ± 0.48	10.84 ± 0.45

Estimation of turnover rates of heart-protein fractions

The finding of a rapid rise of intracellular tyrosine specific radioactivity to a plateau value, and the constancy of this value during the perfusion period, permitted the simple equation derived in the Experimental section to be used to calculate the synthesis rates for heart-protein fractions under the various perfusion conditions (Table 2).

In the working heart and in the Langendorff heart preparation perfused with insulin, 19% of the total heart protein is turned over per day, corresponding to a half-life of approx. 4 days. The turnover rates of myofibrillar and sarcoplasmic proteins did not differ markedly, the latter being higher in the presence of insulin, and the former higher in working hearts.

Incorporation of radioactivity from ^{14}C -labelled amino acids into individual myofibrillar proteins and effect of insulin

To compare the relative turnover rates of the individual proteins within the myofibril, hearts were perfused in the presence and absence of insulin with ^{14}C -labelled amino acids, and the myofibrillar proteins were separated for liquid-scintillation counting by electrophoresis on large-size polyacrylamide gels. Fig. 2 presents typical results from these experiments. The amount of ^{14}C incorporated was proportional to the quantity of protein present. No protein could be detected with a particularly high specific radioactivity. Insulin caused an overall increase in specific radioactivity, and did not preferentially increase the labelling of any particular protein. No correlation could be detected between the molecular weight of the protein, as indicated by the distance migrated along the gel from the origin (Shapiro *et al.*, 1967),

and the amount of ^{14}C -labelled amino acid incorporated.

Discussion

The specific radioactivity of tyrosine in the perfusion fluid did not fall significantly during the course of a 1 h perfusion, and it was therefore unnecessary to add extra label to the perfusate in the course of the experiment. This indicates that the dilution of ^{14}C label by non-radioactive tyrosine liberated from the breakdown of heart protein during the perfusion is small in relation to the total perfusate tyrosine pool. As accurate specific radioactivity measurements alone were required for the calculation of protein-synthesis rates, quantitative extraction of tyrosine was not achieved in the assay. We thus do not have direct information on the total amounts of free tyrosine in the intracellular fluid and in the perfusate.

The rapid increase of intracellular free tyrosine specific radioactivity to a plateau indicates that tyrosine is rapidly transported across the myocardial cell membrane and mixes with a relatively small pool of intracellular free tyrosine. The magnitude of the plateau intracellular specific radioactivity of free tyrosine (in comparison with that of the perfusate) then represents the balance between uptake of the amino acid from the extracellular fluid and dilution of the intracellular labelled amino acid by unlabelled tyrosine from protein breakdown (Gan & Jeffay, 1967). Pathways of tyrosine metabolism other than protein synthesis and breakdown are probably quantitatively unimportant in muscle (Guroff & Udenfriend, 1960). Insulin did not affect the perfusate/intracellular specific radioactivity ratio (Table 1). This would imply

Table 2. Effect of insulin and of the performance of external mechanical work on the rate constants (k_s) and half-lives ($t_{\frac{1}{2}}$) for synthesis of myofibrillar, sarcoplasmic and total protein in the isolated rat heart perfused at constant specific radioactivity with ^{14}C tyrosine

Hearts were perfused for 1 h ($t = 4.166 \times 10^{-2}$ days) with ^{14}C tyrosine at a specific radioactivity of approx. $2000 \mu\text{Ci}/\text{mmol}$, as described in the legend to Table 1. There was no significant change in perfusate specific radioactivity during the course of perfusion, and the measured variation was $\pm 6\%$. Turnover rates were calculated as discussed in the text from intracellular free (S_1) and protein-bound (S_b) tyrosine specific radioactivities by using the relationships $k_s = (S_b/S_1)/t$ and $t_{\frac{1}{2}} = \ln 2/k_s$. The results are expressed as means $\pm$ S.E.M.

Perfusion conditions (no. of hearts)	Total heart protein		Myofibrillar protein		Sarcoplasmic protein	
	k_s	$t_{\frac{1}{2}}$ (days)	k_s	$t_{\frac{1}{2}}$ (days)	k_s	$t_{\frac{1}{2}}$ (days)
Langendorff-perfused (12)	0.103 ± 0.007	6.72 ± 0.43	0.110 ± 0.015	6.30 ± 0.93	0.102 ± 0.009	6.77 ± 0.60
Langendorff-perfused + insulin (5 munits/ml) (8)	0.189 ± 0.022	3.67 ± 0.58	0.149 ± 0.013	4.66 ± 0.45	0.179 ± 0.025	3.88 ± 0.57
Working (7)	0.192 ± 0.010	3.61 ± 0.28	0.187 ± 0.016	3.71 ± 0.33	0.146 ± 0.013	4.75 ± 0.38

Fig. 2. Pattern of radioactive labelling of myofibrillar proteins from hearts perfused in the presence of insulin (—) and of control hearts (----)

Perfusion conditions: Langendorff recirculation (1 h) with 5 mM-glucose $\pm$ insulin (5 munits/ml) and 3.1 μ Ci of 14 C-labelled amino acid mixture. After perfusion, myofibrils were prepared and solubilized. Approx. 1 mg of the solubilized protein (specific radioactivity about 1300 pCi/mg for controls and 2600 pCi/mg for insulin-treated) was subjected to electrophoresis on 4% polyacrylamide gels (9.6 mm $\times$ 100 mm) containing 0.1% (w/v) sodium dodecyl sulphate; then

either that insulin did not inhibit proteolysis, as it does in the perfused rat liver (Mortimore & Mondon, 1970), or that there was a balancing change in uptake of [14 C]tyrosine from the perfusate. In the working heart the intracellular and extracellular specific radioactivities became nearly equal. Once again we do not have the information on total tyrosine concentration that is necessary to distinguish between an effect on the rate of protein breakdown and on the membrane transport of tyrosine. The latter could be a result of the increased coronary circulation in the working heart.

For the purposes of the calculation of synthetic rates of heart protein it has been assumed that the total intracellular amino acid pool represents the precursor for protein synthesis. There is evidence for compartmentation of the intracellular pool of free amino acids in the intact rat diaphragm (Kipnis *et al.*, 1961) and the experiments of Hider *et al.* (1971) suggest that in their preparation the immediate precursor for protein synthesis is in more rapid equilibrium with the extracellular space than is the total intracellular pool. In the perfused rat heart, however, Morgan *et al.* (1971a) report kinetics of incorporation of labelled amino acids indicating that the intracellular pool is on the pathway to protein synthesis. If the precursor pool were situated extracellularly, the magnitude of the error in the calculation of the synthesis rates in the Langendorff heart preparations would be 20–30%. In the working heart, where there is hardly any difference in specific radioactivity between perfusate and intracellular fluid, the site of the precursor pool does not significantly affect the calculations.

Morgan *et al.* (1971a) have calculated, from the [14 C]phenylalanine incorporation rates of Langendorff-perfused rat hearts in the absence of insulin, what is in effect a turnover time of 14.3 days. This would correspond to a fractional synthesis rate of 0.07 days $^{-1}$ ($t_{1/2}$ 9.9 days), somewhat lower than that found in the present study. These authors have also calculated the degradation rate of heart protein from the liberation of unlabelled phenylalanine over a 3 h perfusion, and find this to be almost twice the rate of synthesis. This would indicate an accelerated rate of protein breakdown or decreased synthesis, to explain the deviation from the equilibrium that must obtain *in vivo*.

The synthesis rate for heart protein in the unanaesthetized rat *in vivo* as measured by constant

gels were stained, frozen and cut transversely into 1 mm slices. These were dissolved and the radioactivity was counted. The results presented are from two representative gels. A gel scan, in which some of the peaks are identified, is aligned with the radioactivity histograms.

infusion with [^{14}C]tyrosine was found to be 3–4 days (Millward & Garlick, 1973). The very close agreement with the value found in the working heart would indicate that during an hour's perfusion there is no significant fall in protein synthesis rate in the heart.

The half-life of rat skeletal muscle protein is about 6 days (Waterlow & Stephen, 1968; Garlick, 1969). Cardiac muscle thus turns over about twice as rapidly as does skeletal muscle. The working heart preparation showed an increased rate of protein synthesis as compared with the Langendorff heart preparation. Schreiber *et al.* (1966) have previously demonstrated an increased incorporation of [^{14}C]lysine by guinea-pig hearts perfused at increased pressures. It is possible to speculate that the differences in turnover rates between skeletal and cardiac muscle are a result of the different work loads of the two tissues; there is no information on the mechanism by which the mechanical stimulus affects the protein-synthetic machinery or whether it may be the result of improved supply of metabolites to the tissue.

Very little is known about the mechanism of turnover of the myofibril. The results of the present experiments in which incorporation was measured into myofibrillar protein bands in polyacrylamide gels would indicate a homogeneity in synthesis rates of myofibrillar proteins, consistent with the myofibril being synthesized and broken down as a unit. Dreyfus *et al.* (1960), on the basis of a finding of constant specific radioactivity of [^{14}C]glycine in myosin for 30 days, suggested that the myofibril might not be degraded in a random fashion, but might have a definite life-span like that of haemoglobin. Millward (1970) and Goldberg (1969) found, in contrast with this, exponential decay of muscle protein. Both these workers used fairly crude muscle fractions where the first-order breakdown of highly labelled sarcoplasmic proteins may have masked the non-random breakdown of myofibrillar protein. The present experiments do not provide any information on the kinetics of myofibrillar breakdown, and the question of a possible life-span for the myofibril must remain an open one. This consideration would not affect the validity of the model used for the present calculations, as we have assumed that the return of labelled amino acid from the breakdown of radioactive protein is negligible.

Insulin stimulates incorporation of radioactive amino acids into different muscle fractions (Goldstein & Reddy, 1967) and into the different sarcoplasmic proteins of muscle separated by gel electrophoresis (Kurihara & Wool, 1968). We have extended this study to the range of myofibrillar proteins, confirming the apparent non-specificity of this insulin effect in terms of the proteins synthesized. This is consistent with current views, which place the probable site of insulin action at the stimulation of ribosomal translation of preformed mRNA by enhanced

efficiency of initiation of protein synthesis (Morgan *et al.*, 1971b).

The authors thank the Medical Research Council for financial support and Professor Sir Ernst Chain, F.R.S., for his enthusiastic encouragement and advice.

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