Protein synthesis in skin and bone of the young rat

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1. Fractional rates of protein synthesis in tissues of young growing rats were estimated by injection of flooding amounts (1·5 mmol/kg body-weight) of [^{3}H]phenylalanine. Rates of 63·6%/d and 90·4%/d respectively were obtained in the skin and bone (tibia) of fed animals. These rates were comparable to that in liver (86·3%/d) but considerably higher than in muscle (16·9%/d).

2. Absolute amounts of protein synthesized in tissues of fed rats were estimated. Together the skin and bones accounted for 25% of whole-body synthesis, a value similar to the contribution of liver (15%) and muscle (25%).

3. In fed rats the ratio, RNA:protein in skin and bone was lower than in liver, but much higher than in muscle. However, the amounts of protein synthesized per unit RNA in skin and bone were higher than in both liver and muscle.

4. After 2 d of starvation the fractional rates of protein synthesis in skin and bone fell by 26% and 31% respectively. This was greater than the fall in liver (17%) but less than in muscle (66%). In bone the fall in synthesis was accompanied by decreases in both RNA:protein and synthesis per unit RNA, but in skin there was a fall in RNA:protein which was partially countered by an increase in the rate of synthesis per unit RNA.

In the laboratory rat, skin and bones account for 30 and 2% respectively of total body nitrogen (V. R. Preedy, unpublished observation; Waterlow & Stephen, 1966). However, despite their combined contribution to the whole body, very little is known about protein metabolism in these tissues. By contrast, the regulation of protein turnover in tissues such as liver or skeletal muscle is comparatively well documented (Waterlow *et al.* 1978). This paper describes the measurement of the rates of protein synthesis in skin and bone, their contribution to whole-body protein metabolism, and their response to nutritional stress (2 d starvation). A similar approach was made some 17 years ago by Waterlow & Stephen (1966). By injecting a tracer dose of labelled amino acid into young growing rats they found that as much as 45% of the radioactivity was contained in skin and another tissue of the evicerated carcass. However, they only measured incorporation of label into these tissues, and were not able to calculate actual rates of protein synthesis. Values for the rate of synthesis in mammalian skin (Lobley *et al.* 1978; Simon *et al.* 1978; Davis *et al.* 1981) obtained by constant infusion of labelled amino acids (Waterlow & Stephen, 1968; Garlick *et al.* 1973) may not be correct because of the difficulty of measuring the specific radioactivity of the free amino acid at the site of protein synthesis. The difference between the specific radioactivity of the free amino acid in plasma and in tissue homogenates was quite large. This would have introduced uncertainty about the true synthesis rate of the tissue, since it is not known which of these two specific activities is more appropriate for the calculation (Waterlow *et al.* 1978). In the present study, therefore, we used a flooding dose of labelled amino acid (McNurlan *et al.* 1979; Garlick *et al.* 1980) to minimize the difference between tissue and plasma specific radioactivities during the period of incorporation of label into protein. A preliminary account of these results has been published (Preedy & Waterlow, 1981).

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MATERIALS AND METHODS

L-[4-^{3}H]phenylalanine (25 Ci/mmol) was purchased from Amersham International Ltd (Amersham, Bucks.). Phenylalanine decarboxylase (*EC* 4.1.1.53), β-phenylethylamine and pyridoxal phosphate were purchased from Sigma (London) Chemical Co. (Poole, Dorset) and all other chemicals were obtained from BDH Chemicals (Poole, Dorset).

Male Cobs Wistar rats were obtained from Charles River (Margate, Kent) and maintained on a standard cubed diet containing 230 g crude protein (N × 6·25)/kg (Oxoid Ltd, Basingstoke, Hants.) until they weighed between 130–140 g. They were housed in a humidified, temperature-controlled room on a 12 h light–12 h dark cycle. Fed animals were allowed free access to food and water up to and including the day on which they were injected with [^{3}H]phenylalanine. Starved animals had their food removed 48 h before injection but were allowed free access to water.

Rates of protein synthesis were measured by the method of Garlick *et al.* (1980). Animals were immobilized by wrapping in a towel, a needle was inserted into a lateral tail vein and 1·5 mmol [^{3}H]phenylalanine/kg body-weight (approximately 0·2 μCi/μmol) were injected. After 2 or 10 min animals were decapitated, blood was collected in heparinized tubes and tissues were rapidly removed and plunged into liquid N_2. The skin covering the entire hind-legs was removed and the tibia and gastrocnemius muscle were dissected out. A portion of the liver was also taken. Samples were stored at −20° until analysis.

For the analysis of skin and bone, tissues were crudely fragmented on dry-ice, then 0·2–1·0 g portions were precipitated in 3 ml cold (0–4°) perchloric acid (20 g/l) and homogenized with a Polytron ultrasonic tissue homogenizer (The Northern Medica Supply Co. Ltd, Hull). Analysis of liver and skeletal muscle was similar to that of skin and bone, except that the tissue was finely powdered between two aluminium blocks before precipitation with 3 ml $HClO_4$ (20 g/l). All subsequent steps were performed in the cold (0–4°). The acid supernatant fraction was removed and neutralized with saturated tripotassium citrate for the determination of the specific radioactivity of free phenylalanine in tissue homogenates (S_i). The protein pellets were washed three times with 10–15 ml $HClO_4$ (20 g/l) and the pellet suspended in 10 ml 0·3 M-sodium hydroxide and incubated at 37° for 1 h. Those tubes containing skin were then centrifuged (3000 ***g***, 4°, 20 min) and the fraction soluble in 0·3 M-NaOH taken for the successive steps. For all tissues 1 ml portions of the NaOH solutions were taken for measurement of protein by the method of Lowry *et al.* (1951) as modified by Munro & Fleck (1969), with bovine serum albumin as a standard. The remainder was then precipitated with 2 ml $HClO_4$ (200 g/l) and the RNA determined by measurement of the absorbance at 260 and 232 nm in the acid supernatant fraction as described by Munro & Fleck (1969). In order to verify the values in skin, RNA was also determined by the orcinol method (Munro & Fleck, 1969). The protein pellets were then washed three times with large volumes (10–15 ml) $HClO_4$ (20 g/l) before hydrolysing at 110° in 6 M-hydrochloric acid for 20 h. After removal of HCl by evaporation the hydrolysate was used for determination of the specific radioactivity of [^{3}H]phenylalanine in the tissue protein (S_B). Samples (0·1 ml) of plasma were precipitated in 3 ml $HClO_4$ (20 g/l). The acid supernatant fraction was neutralized with saturated tripotassium citrate before measurement of the specific radioactivity of free phenylalanine in plasma (S_p).

The method for determining S_i, S_p and S_B, based on the enzymic conversion of phenylalanine to β-phenylethylamine with phenylalanine decarboxylase, is described by Garlick *et al.* (1980). After extraction of PEA into dilute sulphuric acid, radioactivity was measured by liquid-scintillation spectrometry and β-phenylethylamine by a fluorimetric assay (Garlick *et al.* 1980).

Table 1. *Specific radioactivities of [^3H]phenylalanine at the end of the labelling period and fractional rates of protein synthesis in skin, bone, liver and skeletal muscle of fed and 48 h-starved rats*†

(Values are means with their standard errors)

Treatment Group	Tissue	Specific radioactivity of phenylalanine (disintegrations/min per nmol)				Fractional synthesis rate (%/d)	
		Protein (S_B)		Free (S_i)			
		Mean	SE	Mean	SE	Mean	SE
Fed	Skin	1·73	0·04	357	5	63·6	1·7
	Bone	2·26	0·04	316	3	90·4	2·3
	Liver	2·45	0·14	352	17	86·3	5·6
	Muscle	0·49	0·02	371	3	16·9	0·6
Starved	Skin	1·23	0·03	361	11	47·1***	1·9
	Bone	1·58	0·07	334	6	62·0***	3·4
	Liver	20·2	0·08	342	7	71·8*	2·9
	Muscle	0·15	0·01	352	11	5·8***	0·3

Differences between fed and 48 h-starved rats were significant: * $P < 0·05$, *** $P < 0·001$

† Rats were injected with [^{3}H]phenylalanine as described on p. 518. At recorded intervals tissues were rapidly dissected out and plunged into liquid N_2. The specific radioactivity of phenylalanine was measured in tissues removed at the end of the labelling period, which was between 10·42 and 10·75 min after injection of the isotope. At the end of the labelling period the specific radioactivities in plasma were 416±4 (*n* 6) and 413±6 (*n* 6) disintegrations/min per nmol in fed and 48 h-starved rats respectively.

Fractional rates of protein synthesis (k_s, i.e. the percentage of tissue protein synthesized daily) were calculated from the following formula:

$$k_s = \frac{S_B \times 100}{\bar{S}_i \times t},$$

where t is the incorporation time in days and $\bar{S}_i$ is the mean specific radioactivity of free phenylalanine in the tissue during the incorporation period. The mean values were calculated from the values obtained by killing animals at 2 min (four rats) and 10 min (six rats) as described previously (Garlick *et al.* 1980).

Values are given as means with their standard errors for six observations. The statistical significances of the differences between fed and 48 h-starved animals were assessed by the Student's *t* test.

RESULTS

Table 1 shows the specific radioactivities of [^{3}H]phenylalanine in the protein and acid-soluble fractions of skin, tibia, liver and gastrocnemius muscle at the end of the 10 min labelling period. In skin we measured the specific radioactivity of that protein fraction which was soluble in dilute alkali (after incubation at 37° for 1 h). As Waterlow & Stephen (1966) showed, this soluble fraction contains approximately 15–20% of the total skin N. The remainder includes mainly collagen, keratin and elastin. As no radioactivity was found in the insoluble fraction 10 min after the injection of labelled phenylalanine, we concluded that it did not have a measureable turnover rate, and no further measurements were made on this fraction. The following results, therefore, refer to that fraction of skin protein that is

Table 2. *RNA:protein and synthesis per unit RNA in skin, bone, liver and skeletal muscle of fed and 48 h-starved rats*†

(Values are means with their standard errors)

Treatment group	Tissue	RNA:protein (mg/g)		Protein synthesis (mg/mg RNA per d)	
		Mean	SE	Mean	SE
Fed	Skin	21·2	0·7	30·2	1·4
	Bone	36·4	1·6	24·9	0·9
	Liver	49·7	1·3	17·5	0·7
	Muscle	10·2	0·3	16·7	0·8
Starved	Skin	11·4***	0·7	41·8**	3·0
	Bone	29·0**	1·1	21·4*	0·8
	Liver	49·9	1·7	14·5*	0·7
	Muscle	6·9***	0·3	8·5***	0·6

Differences between fed and 48 h-starved rats were significant: * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

† RNA and protein concentrations were estimated in the samples removed at the end of the labelling period. The values for synthesis per unit RNA were calculated by dividing the fractional synthesis rates by RNA:protein.

soluble in dilute alkali. We ascertained that the other three tissues contained negligible amounts of insoluble protein (in 0·3 M-NaOH) and so the entire protein fraction of each tissue was analysed.

Table 1 also shows the calculated fractional rates of protein synthesis (i.e. the percentage of the tissue protein pool synthesized daily). In skin of fed animals the rate calculated from the specific radioactivity in the protein (S_B) and the tissue homogenate (S_i) was 63·6%/d, compared with 90·4%/d in the tibia. These values are similar to those in liver (86·3%/d) but considerably higher than those in gastrocnemius muscle (16·9%/d). Although starvation reduced protein synthesis in all four tissues, Table 1 shows that the rates in skin and bone were still between those in liver and skeletal muscle.

Because of uncertainty regarding the specific activity of the free amino acid at the site of protein synthesis, fractional rates of synthesis were also calculated from the specific radioactivity of free phenylalanine in the plasma (values not shown). This gave slightly lower estimates of synthesis rates but the over-all picture was the same. Values for skin and bone were similar to those in liver and higher than those in muscle of both fed and starved rats. A 2 d period of starvation caused a reduction in synthesis in both skin and bone of approximately 30% compared with decreases of approximately 20% in liver and 70% in muscle, when calculated by either method. Consequently, only those values calculated from the specific radioactivity of free phenylalanine in the tissue homogenates will be discussed further.

Table 2 shows the values for the ratio, RNA:protein in the tissues of fed and starved animals. Since most of the RNA in tissues is believed to be ribosomal, this has been taken as a measure of the number of ribosomes in the tissue, i.e. the amount of protein synthetic apparatus (Henshaw *et al.* 1971). It can be seen that these values approximately parallel the rates of protein synthesis shown in Table 1. However, when rates of synthesis per mg RNA were calculated by dividing the fractional rates of protein synthesis by RNA:protein, differences between tissues became apparent. The rate of synthesis per mg RNA in both skin and bone of fed rats was higher than in liver and muscle. After 2 d of starvation there was a small reduction in synthesis per mg RNA in bones, a similar decrease in liver and a much larger decrease in muscle. By contrast, in skin this value was increased by starvation.

DISCUSSION

The rate of protein synthesis that we have observed in skin is considerably higher than previously reported by Lobley *et al.* (1978), Simon *et al.* (1978) and Davis *et al.* (1981), who made measurements by constant infusion of labelled amino acids (Waterlow & Stephen, 1968; Garlick *et al.* 1973). With the constant-infusion technique the label is given as a tracer dose and under certain circumstances questionable results can be obtained because of compartmentation of the free amino acid pool (McNurlan & Garlick, 1981). By contrast, the large dose technique was adopted in order to minimize this potential problem (Henshaw *et al.* 1971; McNurlan *et al.* 1979). Also, in the other studies of skin the rate of synthesis was expressed as a fraction of the total protein, whilst our results are given as fractions of the soluble (in 0·3 M-NaOH) protein. During 10 min there was no incorporation of label into the insoluble fraction so that rates expressed as fractions of total protein would be lower than those given here. Indeed, if the soluble protein comprised approximately 20% of total skin protein, as suggested by Waterlow & Stephen (1966), and the insoluble component had a similar proportion of phenylalanine to the soluble component, then the fractional rate of synthesis of total protein would be as low as 13%/d. This value compares with that of 21%/d obtained in lean Zucker rates by Lobley *et al.* (1978).

The rate of synthesis in the tibia is also very high in relation to that in other tissues. As far as we are aware, values for this tissue have not been published previously, but the high rate is not unexpected. The total protein of the tibia was analysed, including the marrow. Therefore, protein synthesis in this tissue must not only account for growth and endogenous breakdown of bone proteins, but also for the production of erythrocytes. Erythrocyte production would be expected to be particularly active in immature rats.

The estimated contributions of skin and bone to protein synthesis in the whole-body are shown in Table 3. The fractional rates of protein synthesis from Table 1 were multiplied by the protein content of each tissue to obtain estimates of the amount (g) of protein synthesized per day. These values were then expressed as proportions of synthesis in the whole body on the assumption that the fractional rate of whole-body synthesis was the same as that reported previously (McNurlan & Garlick, 1980). These estimates are only approximate, since the whole-body rate of synthesis was not measured in the present experiment. It was also assumed that the skin from the leg and the tibia could be taken as representative of the total skin and total skeleton respectively. Despite the fact that bones are only a small component of the rat by weight they account for approximately 8% of whole-body protein synthesis. Similarly, skin accounts for another 18% of whole-body synthesis. Together, skin and bones have similar contributions to that of liver (15%) and skeletal muscle (25%). These tissues, along with the gastrointestinal tract (McNurlan & Garlick, 1980) are therefore the major contributors to whole-body protein turnover.

It has been argued (Millward *et al.* 1981) that the rate of protein synthesis per mg RNA is the same in all tissues of the rat, but the values for skin and bone do not confirm this. Although RNA:protein in fed rats was highest in liver, the fractional rate of synthesis was highest in bone, implying that synthesis per mg RNA, or per ribosome, was also higher in the bone. In skin, the rate of synthesis per mg RNA was even higher. Similar variations were observed by Henshaw *et al.* (1971), who found that the rate of synthesis per unit RNA in liver was three times greater than in brain and five times greater than in testis. Scornik (1975) also noted that brain and testis incorporated less label per unit RNA after a massive injection of [^{14}C]leucine. However, neither of these workers made measurements on skin or bone. We are unable to explain why these tissues should be different. The measurement of RNA concentration from the absorbance at 260 nm was not specifically designed for skin. It is possible that the correction for peptides (absorbance at 232 nm) was inappropriate to this tissue, with its high concentration of proteins with atypical structure (e.g. collagen).

Table 3. *Comparison of fractional and absolute rates of protein synthesis in tissues and their contribution to whole-body protein metabolism in fed rats*†

Tissue	Soluble protein (% of whole body)	Fractional synthesis rate (%/d)	Absolute synthesis rate	
			g/d per kg rat	% whole-body synthesis
Skin	9·7	63·6	7·9	18
Bone	2·8	90·4	3·2	8
Liver	5·9	86·3	6·5	15
Muscle	49·6	16·9	10·7	25

† The percentage contribution of each tissue to the total body soluble protein (in 0·3 M-sodium hydroxide at 37° for 1 h) was based on our own values for whole rat, skin, bone and liver weights, and their protein concentrations. The proportion of muscle in the rat was obtained from Miller (1969). Fractional synthesis rates are from Table 1. Absolute synthesis rates, defined as the amount of protein synthesized by each tissue/d per kg rat, were obtained by multiplying the soluble protein content of each tissue by its fractional synthesis rate. It was assumed that the rate of whole-body protein synthesis was 33·6%/d (McNurlan & Garlick, 1980).

However, when the measurements of RNA in skin were repeated by reaction with orcinol (Munro & Fleck, 1969) the values for synthesis per mg RNA remained higher than in muscle. Although orcinol gave somewhat higher concentrations of RNA in skin than the u.v. absorbance method, with muscle a similar difference between methods was obtained. Another potential explanation lies in the implicit assumption, when rates of synthesis are expressed per mg RNA, that all, or most, of the RNA is present as ribosomes. Although this was not confirmed in the present study, it has been suggested that in tissues as diverse as muscle (Young, 1970), liver, Hela cells and hepatoma (Henshaw *et al.* 1971), this assumption is essentially correct. It is therefore unlikely that this could explain the higher values for synthesis per mg RNA in skin and bone.

The fractional rate of protein synthesis in skin and bone was reduced by 2 d of starvation. This response was more pronounced than that in liver but less than in muscle. In bone and muscle the over-all decrease in protein synthesis with starvation was brought about partly by a loss of RNA and partly by a decrease in synthesis per mg RNA, while in liver the change in synthesis resulted entirely from a change in synthesis per mg RNA. In skin of starved rats the increase in synthesis per mg RNA appeared to partially compensate for a larger loss of RNA from the tissue. Perhaps such a 'compensatory adaptation' was an endeavour to maintain the synthesis of certain proteins as near as possible to that occurring in the fed animal. An atypical response of skin to nutritional stress has been reported previously. Cabek *et al.* (1963) showed that in rats given a restricted amount of high-protein diet, the protein contents of liver and skeletal muscle were reduced, but the protein content of the fur was increased. Dawson & Milne (1978) gave rats a protein-free diet after a period of adaptation to a low-protein diet and showed that the collagen content of the skin continued to increase. Preferential production of such proteins in the skin could take place either at the expense of other proteins from skin or from other tissues.

We conclude that in the study of body-protein metabolism, skin and bone have been much neglected tissues since, in the young laboratory rat, they account for as much as 25% of whole-body protein synthesis. Both the rate of protein synthesis and the RNA content alter in response to starvation. We therefore suggest that future examination of mammalian whole-body metabolism should include these tissues.

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