Rates of collagen synthesis in lung, skin and muscle obtained *in vivo* by a simplified method using [^3H]proline

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(Received 25 March 1982/Accepted 7 June 1982)

Methods for measurement of rates of collagen synthesis *in vivo* have thus far been technically difficult and often subject to quite large errors. In this paper a simplified method is described for obtaining synthesis rates of collagen and non-collagen proteins, for tissues of rabbits. This involves an intravenous injection of [^3H]proline, administered with a large dose of unlabelled proline, and measurement of the specific radioactivity of proline and hydroxyproline in body tissues up to 3 h later. The specific radioactivity of [^3H]proline in plasma and the tissue free pools rises rapidly to a plateau value which is maintained for at least 2 h, when the specific radioactivity of the type I collagen precursors, isolated from the skin, was similar to that of the plasma and tissue-free pool. Furthermore, over this period, the increase in the specific radioactivity of proline in collagen and non-collagen protein was linear with respect to time. These results suggest that the large dose of proline floods the precursor pools for protein synthesis, and that this effect can be maintained for quite long periods of time. Such kinetics greatly simplified the method for obtaining collagen synthesis rates *in vivo*, which were calculated for lung, heart, skin and skeletal muscle, and shown to be quite rapid, ranging between about 3 and 10%/day. The lung was a particularly metabolically active tissue, with synthesis rates of about 10%/day for collagen and 35%/day for total non-collagen proteins, indicating rapid turnover of both intracellular and extracellular proteins of this tissue.

Collagen, the most abundant protein in vertebrate animals, is found in the extracellular matrix and basement membrane of almost all body tissues. Although the biochemical reactions for synthesis and degradation of this protein are known in some detail (see reviews by Prockop *et al.*, 1979*a,b*), very little is known about the overall rates of these processes. This is of interest, since in a variety of pathological conditions, such as atherosclerosis, liver cirrhosis, pulmonary fibrosis, skin keloids and rheumatoid arthritis, there is evidence for increased collagen contents of the affected tissues, and for this reason evidence has been sought to explain these diseases on the basis of alterations in the rates of collagen synthesis or degradation.

Most studies of collagen have indicated that it is either metabolically inert or has extremely slow rates of synthesis and degradation (Neuberger & Slack, 1953; Thompson & Ballou, 1956; Gerber *et al.*, 1960; Metivier *et al.*, 1978; Nissen *et al.*, 1978). However, these experiments were performed by

using decay methods, where animals were injected with isotopically labelled amino acids and subsequent loss of radioactivity from the collagen pool was measured. Such methods are now known to be subject to error owing to amino acid reutilization, a problem encountered with various amino acids, including proline (Jackson & Heininger, 1975), which has been used because the specific radioactivity of this imino acid in collagen can be obtained from the hydroxyproline specific radioactivity. Although there will be no reutilization of hydroxyproline, there will be reutilization of proline before its post-translational hydroxylation. Reutilization may be eliminated to some extent by 'chasing' with large amounts of unlabelled proline (Nissen *et al.*, 1978), but this approach appears to be only partially effective (Laurent & Sparrow, 1977). Furthermore, the methods are time-consuming, and require long periods of time (i.e. over 20 days), making them inappropriate for studies of short-term changes in collagen metabolism.

Rates of collagen synthesis have also been obtained *in vivo* by measuring incorporation of labelled proline into collagen, as hydroxyproline, during a period of several hours (Laurent *et al.*, 1978b; Palmer *et al.*, 1980). However, these methods were technically difficult to perform and involved rather complex mathematical analyses, with quite major assumptions about the kinetics of the precursor pool for protein synthesis. Palmer *et al.* (1980) approached the latter problem by measuring the specific radioactivity of collagen precursors, which had been shown previously to equilibrate rapidly with the precursor pool for collagen synthesis (Robins, 1979). This seems valid, but the isolation of collagen precursors requires large amounts of tissue and is therefore inappropriate for studies of small organs or tissues from small animals.

In the present paper, a method is described that allows simultaneous measurement of collagen and non-collagen synthesis rates. It involves an intravenous injection of [^3H]proline, administered with a large amount of unlabelled proline, and measurement of the specific radioactivity of proline in protein and free pools 1.5–3 h after injection. This approach requires only small amounts of tissue, and may be more suitable than previously used methods.

Experimental

Animals

Experiments were performed on male New Zealand white rabbits (aged 100–130 days, weight 2.0–2.5 kg) obtained from Cork Farm, Chilham, Kent, U.K. Animals were individually caged with free access to food and water.

Experimental protocol

Changes in plasma [^3H]proline specific radioactivity were examined over a period of about 5 h in two rabbits. To accomplish this, the carotid artery was cannulated with Teflon tubing of external diameter 0.95 mm (Silastic; Dow Corning, Midland, MI, U.S.A.), which was previously softened in toluene and attached to flexible nylon tubing of the same diameter (Portex Ltd., Hythe, Kent, U.K.). The latter was taken under the skin to emerge on the dorsal side of the rabbit's neck. The following day, 5 ml samples of blood were taken at various times after the intravenous injection of L-[5- ^3H]proline (1.0 mCi/kg; 10–20 Ci/mmol; Amersham International, Amersham, Bucks., U.K.), administered

with unlabelled proline [about 2.31 M in sterile saline (0.9% NaCl), dose 7 mmol/kg].

In a further experiment, nine rabbits received intravenous injections (0.8 mCi/kg body wt.) of L-[5- ^3H]proline administered with unlabelled L-proline as described above. Animals were then killed in groups of three, after about 15, 90 and 180 min. Just before being killed, animals were anaesthetized with 27 mg of sodium pentobarbitone (May and Baker, Dagenham, Essex, U.K.)/kg; the chest was opened and blood sampled from the left ventricle. Tissues were then removed, placed on a glass plate over ice, and minced before freezing in liquid nitrogen. The blood was centrifuged at 4°C, plasma isolated and protein precipitated with trichloroacetic acid (final concn. 5%, w/v). This mixture was then centrifuged (2000 g for 15 min) and the supernatant stored at –20°C before analysis.

Extraction of collagen precursors

Type I and type III collagen precursors were isolated from skin of rabbits, which received injections of [^3H]proline as described above. The skin was removed after 2 h and treated as described by Robins (1979), before isolation of type I and type III procollagens (Byers *et al.*, 1974). The purity of the samples was assessed by polyacrylamide-gel electrophoresis as described previously (Laurent *et al.*, 1981a), except that a running-gel concentration of 6% (w/v) acrylamide was used.

Determination of proline and hydroxyproline specific radioactivities

There were based on the procedure of Peterkofsky & Prockop (1962), with several modifications (Laurent *et al.*, 1982). To obtain the specific radioactivity of proline in the 'tissue-free pool' and protein, about 500 mg of previously minced tissues was homogenized in 5% (w/v) trichloroacetic acid. After centrifugation at 4000 g for 10 min, the supernatant from this procedure, plus two further pellet washes, was adjusted to pH 8–9 with KOH and oxidized directly by chloramine-T, as it was found to be unnecessary to remove the trichloroacetic acid before this procedure.

Calculation of collagen contents

Tissue collagen and the ratio of collagen to non-collagen proteins were calculated by previously described methods (Laurent *et al.*, 1978b, 1981b). Assuming that proline represents 4% of non-collagen proteins and 13.6% of collagen:

$$\frac{\text{collagen}}{\text{non-collagen protein}} = \frac{0.30 \times \text{total tissue hydroxyproline content}}{\text{total proline content} - (1.1 \times \text{total hydroxyproline content})}$$

The value of 1.1 represents the ratio of proline to hydroxyproline in collagen and was obtained from the amino acid analysis of purified lung collagens (Laurent *et al.*, 1981a). This value differs slightly between tissues, owing to variation in the relative amounts of the different collagen types. However, estimates of the contents of type I and III collagen in skeletal muscle, heart, lung and skin suggest that type III collagen represents 17%, 19%, 30% and 19% respectively of the total amount of these two most abundant collagens (Laurent *et al.*, 1981b; J. M. E. Kirk and G. J. Laurent, unpublished work). This variation caused an alteration in the proline/hydroxyproline ratio of less than 5%, so that a value of 1.1 was used for all tissues.

Calculation of protein-synthesis rates

The protein-synthesis rates (K_s) were calculated as described previously (McNurlan *et al.*, 1979) by using the formula:

$$K_s = \frac{S_B}{S_A t}$$

where S_B is the specific radioactivity of the protein fraction (either collagen or non-collagen protein), S_A is the estimate of the proline specific radioactivity in

Implicit in these methods is the assumption that, during the period of measurement, the loss of radioactivity from proteins owing to degradation is insignificant compared with the amount incorporated. This assumption is necessary to derive the above formula from the expression $dS_B/dt = K_s \cdot (S_A - S_B)$ where it must be assumed that $\int_0^t K_s S_B$ is insignificant compared with $\int_0^t K_s S_A$, so that the former term may be neglected. The relative magnitudes of these integrals can be estimated from the areas under the curves showing changes in specific radioactivity with time for non-collagen protein and the tissue-free pool (see Fig. 2). On this basis, the synthesis rate obtained for muscle was underestimated by an insignificant amount (less than 0.5%); in the lung, the tissue with the most rapid synthesis rate, this error increased slightly, but was still less than 2.5%. It was assumed for these calculations that protein degradation is random. Although there is extensive evidence for this for many tissue proteins, it is quite likely not the case for collagen, where a significant error may be introduced because of degradation of newly synthesized protein. This phenomenon is discussed in full below.

The mean specific radioactivity of non-collagen proteins was calculated as described previously (Laurent *et al.*, 1978a) by using the formula:

$$\frac{\text{Specific radioactivity of non-collagen protein (d.p.m./}\mu\text{mol)} = \text{total radioactivity in proline (d.p.m.)} - [1.1 \times \text{total radioactivity in hydroxyproline (d.p.m.)}]}{\text{total amount of hydroxyproline } (\mu\text{mol}) - [1.1 \times \text{total amount of hydroxyproline } (\mu\text{mol})]}$$

the precursor pool for protein synthesis (either the tissue-free pool or plasma) and t is time (days) after injection. K_s is a fractional rate constant for protein synthesis and can be expressed as a percentage per day by multiplying by 100.

For calculations of collagen-synthesis rates, S_B was taken to equal the specific radioactivity of hydroxyproline in the trichloroacetic acid-insoluble material. The only other tissue protein containing significant amounts of hydroxyproline is elastin. In the tissues studied here, this protein has the highest concentration in lung, where it represents one-half to one-third that of the collagen content (Pierce & Hocott, 1960; Laurent *et al.*, 1981b). Furthermore, the hydroxyproline content of elastin is about one-fifth that of collagen (Starcher & Galione, 1976; John & Thomas, 1972; Laurent *et al.*, 1981a), so this protein must contain less than 10% of the total tissue hydroxyproline. Thus, if elastin is to influence markedly the rates obtained here, it must have a synthesis rate several times more rapid than that of collagen, and this would seem unlikely in the light of previous studies suggesting that both proteins have similar rates of metabolism (Pierce *et al.*, 1967; Kao *et al.*, 1961).

The parameters in this equation were obtained as described previously (Laurent *et al.*, 1981a,b). In addition, to determine the total radioactivity and amounts of tissue proline it was necessary to obtain the efficiency of extraction of proline, which was accomplished by including tracer amounts of [^{14}C]proline for each tissue.

Results

Specific radioactivities of [^3H]proline in the plasma after an intravenous injection with and without unlabelled proline

Fig. 1(a) shows the changes in the plasma proline specific radioactivity after an intravenous injection of tracer amounts of the imino acid. There was a rapid decrease, particularly during the first 30 min, and after 5 h the specific radioactivity was less than 1% of that observed after 5 min. This decrease reflects the rapidity with which plasma and tissue-free amino acids equilibrate, a phenomenon reported previously for a variety of amino acids (see, e.g., Henriques *et al.*, 1955; Lajtha *et al.*, 1957; Haider & Tarver, 1969; Arnal *et al.*, 1971).

Fig. 1. Changes in plasma proline specific radioactivity (●) and concentration (○) after injection of $[^3\text{H}]$ proline. The tracer was administered alone (a) or with a large dose (7 mmol/kg) of unlabelled proline (b). For full details see the text.

In contrast, when a large dose of unlabelled proline was administered with the $[^3\text{H}]$ proline the specific radioactivity was relatively constant, particularly during the first 2–3 h (Fig. 1b). After this time, there appeared to be a more rapid decline in the specific radioactivity, until by 5 h it was 66% of that observed at 5 min. Also shown in Fig. 1(b) are the changes in concentration of free proline in the plasma, compared with normal values in Fig. 1(a). After 5 min, the concentration was about 100 times normal; this difference diminished thereafter, although at 5 h the proline concentration was still more than 10 times normal.

To confirm that a plateau specific radioactivity is maintained over the first 2–3 h, a further animal was injected with $[^3\text{H}]$ proline plus unlabelled proline and blood samples were taken at five times between 5 and 150 min after injection. The results were very similar to those shown in Fig. 1(b). The specific radioactivity of plasma proline decreased by about 10%, with values of 1.23×10^5 , 1.18×10^5 , 1.15×10^5 , 1.13×10^5 and 1.1×10^5 d.p.m./ μmol at 5, 30, 60, 90 and 150 min after injection. Similarly, the proline concentration was maintained above that before injection ($0.21 \mu\text{mol/ml}$), with values of 19.1, 7.8, 5.7, 4.5 and $3.4 \mu\text{mol/ml}$ at the above times.

Specific radioactivity of proline in the tissue-free pool and proteins after $[^3\text{H}]$ proline injection

Fig. 2 compares the specific radioactivities of proline in the tissue-free pool, collagen and non-collagen proteins of skeletal muscle at about 20, 90 and 180 min after injection. The tissue-free-pool specific radioactivities at these times were not significantly different. Furthermore, during this

Fig. 2. Specific radioactivity of proline in tissue-free pool (▲), non-collagen protein (○) and collagen (●) after an intravenous injection of $[^3\text{H}]$ proline with a large dose of unlabelled proline.

The specific radioactivity of $[^3\text{H}]$ proline in collagen is obtained by measuring the hydroxyl- $[^3\text{H}]$ proline specific radioactivity. Values shown are means for three animals, with bars representing S.E.M.

period, the change in specific radioactivity for collagen and non-collagen proteins was essentially linear with respect to time.

Table 1 shows the corresponding tissue-free-pool specific radioactivities in lung, skin and heart, although in these tissues measurements were only made at two times, 90 and 180 min after injection. Again, there was no significant change in the specific radioactivity between these times. Also shown in Table 1 are the plasma proline specific radioactivities, which were higher than those in the tissues. The ratio of the tissue-free pool to plasma specific radioactivities ranged from about 0.70 for muscle and 0.95 for skin and heart. Furthermore, the specific radioactivity of the injected solution (2.67×10^5 d.p.m./ μmol) was almost identical with that of the plasma at 90 min after injection, and after 180 min the difference was still only 6%.

To obtain information on the specific radioactivity of proline in the precursor pool for proline synthesis, collagen precursor proteins were isolated from skin 2 h after injection of $[^3\text{H}]$ proline. The purity of these preparations was assessed by polyacrylamide-gel electrophoresis, which separated the precursor collagens for both type I and III collagen from the mature $\alpha 1$ and $\alpha 2$ chains (Fig. 3), with a pattern similar to that described previously (Byers *et al.*, 1974; Robins, 1979). Determination of the relative amounts of protein in the various bands

Table 1. *Specific radioactivity of [³H]proline in plasma and the free pools of various tissues 90 and 180 min after an injection of [³H]proline with a large dose of unlabelled proline*

Values are means ± S.E.M. for three animals.

Time after injection (min) ...	10 ⁻⁵ × Sp. radioactivity (dpm/μmol)	
	90	180
Plasma	2.70 ± 0.16	2.15 ± 0.06
Lung	1.99 ± 0.06	1.93 ± 0.07
Heart	2.56 ± 0.22	2.39 ± 0.03
Skin	2.42 ± 1.10	2.39 ± 0.20
Skeletal muscle	1.86 ± 0.11	1.81 ± 0.01

by densitometry indicated that about 95% of the total collagen was present as procollagen molecules or their intermediates for both collagen types.

Table 2 compares the specific radioactivities of hydroxyproline in the precursor collagens with that of proline in the tissue-free pool and plasma. The mean value for the specific radioactivity of hydroxyproline in the type I collagen precursors was intermediate between that of the plasma and the tissue-free pool. However, there was quite a large variation in the relationship between the specific radioactivities of these pools, with the ratio of the collagen-precursor specific radioactivity to that in the tissue-free pool varying from 0.81 to 1.55 and that of the precursor to plasma from 0.69 to 0.99.

Collagen and non-collagen-protein synthesis rates

Table 3 shows the fractional rates of collagen and non-collagen-protein synthesis calculated from the specific radioactivity of proline in both the tissue-free pool and plasma. There was no difference in the collagen synthesis rates for skeletal muscle, skin and heart, but the rate for lung was significantly greater than that in the other tissues (*P* < 0.025). For all tissues the non-collagen synthesis rates were 3–5 times greater than for collagen (*P* < 0.001).

Differences between synthesis rates in tissues also occurred for non-collagen proteins. The rate for muscle was lower than that in the other tissues (*P* < 0.01), and the value for skin was slightly lower than that for heart (*P* < 0.05). Lung proteins were again synthesized at the most rapid rates, which were higher than for all other tissues (*P* < 0.01). When the synthesis rates were calculated from the plasma-free pool specific radioactivities, the synthesis rates showed the same pattern as described above, although the rates were consistently lower.

Table 4 shows the collagen contents of the various tissues as well as the relative contributions of collagen and non-collagen protein to total protein synthesis in these tissues. The contents of skeletal

Fig. 3. *Polyacrylamide-gel electrophoresis of collagen and collagen precursors prepared from rabbit skin*

Type III (track *a*) and type I (track *b*) collagens were prepared by partial solubilization with pepsin and differential precipitation in NaCl (Chung & Miller, 1974). The collagen precursors for type I (track *c*) and type III (track *d*) collagens were prepared as described in the Experimental section. Samples were reduced with mercaptoethanol before electrophoresis, which was performed as described previously (Laurent *et al.*, 1981*a*). Staining of proteins was with Coomassie Blue R-250. The identification of the various precursor collagens is similar to that used previously (Byers *et al.*, 1974; Robins, 1979) for both the procollagens (designated 'pro') and the intermediate collagen precursors (designated 'p'). The latter contain either the *N*- or *C*-terminal extension peptides, but not both. It is uncertain why the precursor collagens are indistinct and in the case of proα1 and proα2 appear to be present as doublets. One possible reason for this may be the presence of underhydroxylated peptides.

muscle, heart and lung were all similar, with collagen representing 0.7–0.9% of the wet weight and 6–7% of the total protein content. Furthermore, for these tissues, collagen synthesis represented less than 3% of the total non-collagen protein synthesis. However, for skin, where collagen represents about 33% of the total proteins, the contribution was about 15%.

Table 2. *Specific radioactivity of [³H]proline in collagen precursor and free pools after injection of a 'flooding' dose of [³H]proline*

Individual values are given for five animals as well as the means and the S.E.M. values. The values for type I and type III collagens are obtained from the hydroxyproline specific radioactivity measured as described in the text.

Animal	10 ⁻⁴ × Sp. radioactivity (d.p.m./μmol)		
	Type I collagen precursors	Tissue-free pool	Plasma
R52	18.47	15.14	22.70
R53	18.40	11.86	19.29
R55	13.24	16.26	19.20
R56	21.31	16.70	22.50
R37	20.02	18.80	20.20
Mean	18.29	15.75	20.78
S.E.M.	1.37	1.14	0.76

Discussion

Validity of the method used

The administration of large amounts of amino acids, given with the radiolabelled tracer, has been used previously to measure rates of total protein synthesis (Henshaw *et al.*, 1971; Scornick, 1974; Dunlop *et al.*, 1975; McNurlan *et al.*, 1979; Garlick *et al.*, 1980), but not collagen synthesis. A major criterion in the choice of an amino acid for such studies is that, after injection, the plasma and tissue-free-pool specific radioactivities either remain constant or follow simple kinetics. In this study, proline was chosen as the tracer, and because this amino acid has not been used previously, experiments were required to establish its suitability.

Administration of [³H]proline with a large dose of unlabelled proline (i.e. 7.0 mmol/kg body wt.)

Table 3. *Synthesis rates of collagen and non-collagen proteins in tissues of rabbits*

Rates are calculated by using the specific radioactivity of [³H]proline in both the tissue-free pool (TFP) and plasma as an estimate of the specific radioactivity of [³H]proline in the precursor pool for protein synthesis. Values are means ± S.E.M. (numbers of animals shown in parentheses) obtained from rabbits killed at various times between 90 and 180 min. Animals were injected with [³H]proline administered with a large amount of unlabelled proline (7 mmol/kg), and synthesis rates were calculated as detailed in the Experimental section.

	Synthesis rate (%/day)			
	Collagen		Non-collagen proteins	
	TFP	Plasma	TFP	Plasma
Lung (12)	9.5 ± 1.4	7.7 ± 0.9	34.6 ± 1.8	29.1 ± 1.5
Skeletal muscle (gastrocnemius) (9)	3.3 ± 0.3	2.7 ± 0.4	11.1 ± 0.8	8.7 ± 0.8
Skin (9)	4.0 ± 0.6	3.2 ± 0.4	16.1 ± 2.3	14.5 ± 2.8
Heart (9)	4.7 ± 0.9	4.2 ± 0.8	22.2 ± 1.7	21.3 ± 1.3

Table 4. *Collagen contents and relative amounts of synthesis of collagen and non-collagen proteins in tissues of young rabbits*

The collagen content and collagen/non-collagen protein (NCP) ratios are calculated as described in the Experimental section. The latter is used in the calculation of the ratio for the total amounts of collagen and non-collagen protein synthesis (final column). These values are derived from the product of the ratios of the fractional synthesis rates, calculated by using the [³H]proline specific radioactivity of the tissue-free pool (Table 3) and the ratio of the contents:

$$\left[\text{i.e. } \frac{k_s(\text{collagen})}{k_s(\text{NCP})} \times \frac{\text{collagen}}{\text{NCP}} (\text{column 3}) \right]$$

The ratio collagen/total protein is derived from the ratio collagen/NCP by using the formula: collagen/total protein = 1/[1 + (NCP/collagen)]. All values are means ± S.E.M., with the numbers of observations given in parentheses.

Tissue	Collagen content (mg/g wet wt.)	Collagen	Collagen	Total collagen synthesis
		NCP	total protein	total NCP synthesis
Lung (12)	8.4 ± 0.2	0.072 ± 0.004	0.067 ± 0.004	0.022 ± 0.003
Skeletal muscle (gastrocnemius) (9)	8.6 ± 1.1	0.076 ± 0.009	0.071 ± 0.008	0.022 ± 0.004
Skin (9)	50.6 ± 5.5	0.493 ± 0.111	0.330 ± 0.075	0.148 ± 0.016
Heart (left ventricle) (9)	7.0 ± 0.5	0.068 ± 0.006	0.064 ± 0.006	0.014 ± 0.02

resulted in plasma and tissue-free-pool specific radioactivities which rapidly reached a maximum, which was maintained for 2–3 h. Furthermore, after injection, the protein specific radioactivities increased linearly with respect to time. Such kinetics greatly simplified the calculations of protein-synthesis rates, which can be performed by measuring the free-pool and protein specific radioactivities at only one time after injection.

In previous studies using other amino acids, the rate of decrease of specific radioactivity appeared to be more rapid. For this reason, protein-synthesis rates were determined over shorter periods of time (e.g. 10 min) and required measurements of tissue specific radioactivities at two different times (Henshaw *et al.*, 1971; McNurlan *et al.*, 1979; Garlick *et al.*, 1980). This approach is technically less convenient, and for proteins such as collagen, with relatively low rates of synthesis, large amounts of tracer must be given to ensure incorporation of measurable amounts of radioactivity.

On the basis of these experiments, proline was shown to be suitable for studies of protein metabolism particularly for collagen. However, implicit in the methods used are two major assumptions: first, that the high dose of proline is sufficient to eliminate the existence of tissue pools with widely differing specific radioactivities, and second, that the high dose of unlabelled proline does not influence the synthesis rates of either collagen or non-collagen proteins.

The results obtained in this study indicate that the first assumption is valid. When the high dose of proline was given, the tissue-free-pool and plasma specific radioactivities were similar and quite close to that of the injected solution, even after 3 h. This contrasts with the studies where no flooding dose of proline was administered, where the tissue-free-pool specific radioactivities were often less than one-half of that in the plasma (Laurent *et al.*, 1978a; Robins, 1979; Palmer *et al.*, 1980). Also, the specific radioactivity of the type I collagen precursors in skin were at least as great as that for the tissue-free pool. Type I procollagen is known to be converted into collagen very rapidly. Robins (1979) suggested a half-life *in vivo* of less than 26 min for procollagen from rabbit skin. On this basis, the specific radioactivity of type I procollagen at 2 h should be very close to its plateau value and should represent the specific radioactivity of the precursor pool for synthesis of this collagen type.

The data here suggest that the true precursor-pool specific radioactivity may be intermediate between that of the plasma and the tissue-free pool. On this basis, synthesis rates which were calculated from the specific radioactivities of both these pools (see Table 3) should represent the minimum and maximum possible values respectively. These values

varied by less than 5% for heart, but the difference was 25% in skeletal muscle. This variation was much less than that seen in studies using only tracer amounts of proline, where the synthesis rates derived from the tissue-free-pool specific radioactivity were about one-third of those obtained by using the type I collagen precursors (Palmer *et al.*, 1980).

Studies of whole-organ protein metabolism in other laboratories also indicate that the first assumption is valid. The effect of giving high doses of amino acids with the tracer has been investigated in isolated perfused heart (McKee *et al.*, 1978), liver (Khairallah & Mortimore, 1976) and lung (Watkins & Rannels, 1980). In all these studies, where low or physiological concentrations of amino acids were used, the intracellular and extracellular (perfusate) pool specific radioactivities were markedly different, with an intermediate value for the aminoacyl-tRNA specific radioactivity. However, with the administration of high doses of the amino acids, this difference was eliminated, making the specific radioactivity in these pools equal. Thus, as was the case for the present study *in vivo*, a flooding of amino acid pools appears to have been achieved by administering large amounts of the amino acid.

In contrast, evidence from studies of isolated cells has suggested that large doses of amino acids may not always exert flooding effects. This was the conclusion of studies with both fibroblasts (Hildebran *et al.*, 1981) and macrophages (Hammer & Rannels, 1981), where the aminoacyl-tRNA specific radioactivity was less than that of either the extracellular or the intracellular pool, and this difference could not be altered by increased concentrations of these amino acids in the extracellular pool. These results suggest that tRNA in the cell is preferentially acylated by amino acids of low specific radioactivity, and that this pool cannot be 'flooded' by high concentrations of amino acid. Clearly, there is a difference between the results of studies *in vivo* or with whole organs and those conducted *in vitro* on isolated cells. The reason for this difference is at present unresolved, but may reflect a fundamental difference between kinetics of the two systems, or differences in the methods employed.

The second assumption of the method used here is that the administration of a large amount of unlabelled proline does not by itself affect the protein-synthesis rate. To my knowledge, there are no studies *in vivo* examining the effect of above-normal proline concentrations on protein-synthesis rates. Such experiments have been conducted *in vitro*, but the results are controversial. Changes in lung proline concentrations did not affect total protein-synthesis rates for human lung fibroblasts in culture (Hildebran *et al.*, 1981). Nor did they affect collagen-synthesis rates in chick or mouse fibroblasts and human epithelial cells (Baich *et al.*, 1980).

Furthermore, investigations of the effects of amino acid on rates of protein synthesis and degradation have also been conducted with muscle preparations *in vitro*, where only the branched-chain amino acids were shown to have any stimulatory effects (Fulks *et al.*, 1975; Buse & Reid, 1975). In contrast, several studies of rat liver slices have indicated that an increased concentration of proline correlates with an increased rate of collagen synthesis (Työppönen *et al.*, 1980; Rojkind & Diaz De Lyon, 1970; Dunn *et al.*, 1977), and Finerman *et al.* (1967) reported a similar relationship for foetal rat calvaria, although only up to a proline concentration of 0.15 mM, less than that found in plasma of the animals studied here. Clearly, there are differences in the conclusions of the studies *in vitro*, possibly because of variations in the different preparations with respect to substrate supply, hormonal support etc. For this reason, it may be useful to conduct experiments *in vivo* to assess this phenomenon.

Rates of non-collagen-protein synthesis in skeletal muscle, heart, skin and lung

The synthesis rates ranged from about 10%/day for skeletal muscle to about 35%/day for lung. I am aware of three previous studies of protein-synthesis rates in rabbit tissues, although in each case the animals were at a different stage of development from those used in this study. Two studies of fully grown adult animals, using decay methods (Signoret *et al.*, 1973) or the continuous infusion method (Nicholas *et al.*, 1977), gave rates of about 2%/day for total muscle proteins. More recently, a study of young rabbits (weighing up to 1.4 kg) indicated a mean rate of total protein synthesis of about 5%/day (Palmer *et al.*, 1980), a value about one-half that obtained in the present study using older animals (all weighing at least 2 kg). The latter result is surprising, given that muscle protein-synthesis rates have been shown previously to decrease with age (Millward *et al.*, 1975) rather than to increase.

Such a discrepancy may have arisen from differences in the values assumed for precursor pool specific radioactivity. In the study of Palmer *et al.* (1980), the tissue-free-pool specific radioactivity was used, although type I collagen precursors in skin had a specific radioactivity less than one-half of this value. If the latter was employed, the calculated rate of synthesis would have more than doubled, and been similar to the value obtained in the present study. Furthermore, in calculating total protein-synthesis rates, Palmer *et al.* (1980) made no allowance for the [^3H]proline in collagen. Since collagen contains a disproportionate amount of this imino acid, with a lower specific radioactivity than proline in the non-collagen protein (about five times lower in the present study), neglecting such a correction may give rates which are underestimates.

The rates obtained for skin and lung are of interest, since relatively little is available on the turnover of proteins in these tissues. Rapid rates for skin have also been noted in rats by Preedy & Garlick (1981). This is important, since skin, like muscle, constitutes a large proportion of the total protein mass of animals, and for this reason must be a major contributor to total protein turnover. The rate obtained for lung is similar to that obtained *in vivo* for rats by Garlick *et al.* (1980), but more rapid than that observed in young growing pigs (Garlick *et al.*, 1976). Studies on perfused lungs have produced discrepant results, with rates ranging from 14%/day (Rannels *et al.*, 1979) to 72%/day (Chiang *et al.*, 1979) for adult rats. The extremely rapid rates for lung protein metabolism are consistent with the currently recognized role of the lung as a metabolically active organ for other substrates such as lipids and carbohydrates (Tierney, 1974; Tierney & Levy, 1976; Mason, 1976), and demonstrate the inadequacy of the traditional view of lung as an inert tissue which functions simply as a vehicle for gas exchange.

Collagen synthesis rates: most rapid in lung

Measurements of collagen synthesis rates by uptake techniques have several problems which arise because of complexities in the kinetics of collagen turnover (see discussion by Laurent *et al.*, 1978b). Most importantly, the value obtained will underestimate the true rate of collagen synthesis by an amount proportional to the amount of collagen wastage (i.e. the proportion of newly synthesized collagen that is degraded within the period of measurement). Studies *in vitro* have suggested that as much as one-third of newly synthesized procollagen may be degraded within minutes of synthesis (Bienkowski *et al.*, 1978a,b). If this was the case, the rates obtained here would underestimate the true synthesis rate by about 30%.

Despite these problems, the rates shown here are in general more rapid than those reported previously. For example, in experiments using decay methods, Gerber *et al.* (1960) and Nissen *et al.* (1978) reported rates of about 1%/day for skin collagen in rats, and Metivier *et al.* (1978) reported a rate of 2%/day for the rapidly synthesized pool in rat lung, values about one-fifth of those observed here. This discrepancy is almost certainly caused by reutilization, a problem arising in decay studies with most amino acids, including proline, which was used in the above studies (Jackson & Heininger, 1975).

Lower rates of synthesis were also obtained in two studies based on the uptake of labelled proline (Bradley *et al.*, 1974; Laurent *et al.*, 1978b). Studying tissue slices of rabbit lung, Bradley *et al.* (1974) obtained proline incorporation rates of about 0.8 nmol of hydroxyproline formed/h per mg of DNA, for

rabbits of about the same age as those used here. Assuming a collagen content of 11 mg/g wet wt., and a DNA concentration of 4.4 mg/g wet wt. (Laurent *et al.*, 1981a), this is equivalent to a rate of 0.8%/day, about one-tenth of the value obtained in the present study. This discrepancy may have arisen owing to uncertainties in the measurement of precursor pool specific radioactivities, or perhaps because the conditions *in vitro* were not optimal for protein synthesis. Whatever the cause, this difference should be borne in mind when interpreting studies of lung collagen metabolism in diseases where the '*in vitro*' method has been used (Phan *et al.*, 1980; Clarke *et al.*, 1980; Zuckerman *et al.*, 1980; Reiser & Last, 1981; McCullough *et al.*, 1978).

Applying the continuous-infusion method, Laurent *et al.* (1978b) obtained rates *in vivo* of less than 1%/day for various muscle types in adult fowls. However, this study assumed that the precursor pool specific radioactivity equalled that of the tissue-free pool. This is most probably incorrect, given the observation by Robins (1979), using the same technique, that collagen precursors in skin did not reach a specific radioactivity more than 37% of that of the tissue-free pool. On the basis of these results, Palmer *et al.* (1980), have used the continuous-infusion technique and calculated collagen synthesis rates, using the specific radioactivity of the collagen precursors as an estimate of the specific radioactivity of the precursor pool. They obtained rates which ranged from 2.1 to 9.2%/day, for muscles of young rabbits. These values were in general higher than those obtained here, but this is not surprising, since the present study used older rabbits and collagen synthesis rates are known to decrease with age (Bradley *et al.*, 1974).

Finally, the collagen synthesis rates obtained for lung were 2–3 times those observed in the other tissues, a difference similar to that seen for non-collagen proteins. These rapid rates do not represent increased protein content because of tissue growth. The lung weight has stabilized in animals of the age used here, and there are no further changes in collagen or non-collagen-protein contents (Bradley *et al.*, 1974; G. J. Laurent, unpublished work). Thus the rates obtained represent collagen turnover, both synthesis and degradation, and demonstrate the dynamic nature of the lung with respect to the metabolism of both intracellular and extracellular proteins.

This work was supported by a grant from the Medical Research Council of Great Britain. I thank Mr. Robin J. McNulty for his excellent assistance. I am also grateful to Miss Jan Foster for typing this manuscript.

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