Metabolism of Rabbit Skin Collagen

DIFFERENCES IN THE APPARENT TURNOVER RATES OF TYPE-I- AND TYPE-III-COLLAGEN PRECURSORS DETERMINED BY CONSTANT INTRAVENOUS INFUSION OF LABELLED AMINO ACIDS

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Growing rabbits were infused for up to 10h with labelled proline, tyrosine and leucine to achieve plateau conditions within body free pools: for [^3H]proline infusion, blood free-proline specific radioactivity remained constant after about 1h. For individual animals, type-I- and type-III-collagen precursors were isolated by precipitation with $(\text{NH}_4)_2\text{SO}_4$ and DEAE-cellulose chromatography. Experiments where ^3H - and ^{14}C -labelled proline and tyrosine were infused concurrently for different periods of time showed that type I procollagen reached plateau specific radioactivity within 3h and 90% of the plateau value after 2h infusion, corresponding to a calculated apparent $t_{1/2}$ of less than 26min. Plateau values for type I procollagen were taken as precursor amino acid pool specific radioactivities. The type-III-collagen-precursor fractions consistently showed lower rates of label incorporation and, by assuming that both type I and type III collagens are synthesized from the same amino acid pools, kinetic analysis revealed an apparent $t_{1/2}$ for the isolated type-III-collagen precursors of 3.9h. For proline, there were large variations between animals in the ratio between the precursor pool for collagen synthesis and the skin homogenate free pool (0.31 ± 0.13 , mean $\pm$ s.d.), so that collagen-synthesis rates based solely on total tissue free-pool values for proline are subject to large and inconsistent errors.

The biosynthetic precursor of collagen, procollagen, contains globular extension peptides at both ends of the molecule (for reviews see Prockop *et al.*, 1976; Fessler & Fessler, 1978). For type I collagen, these extensions are removed before fibrillogenesis by at least two proteinases in a process that gives rise to a number of collagen-precursor intermediates (Davidson *et al.*, 1975, 1977; for nomenclature see Martin *et al.*, 1975). Of the genetically distinct forms of collagen now known to exist (see Miller, 1976), skin comprises mainly type I and type III collagens, the latter predominating in foetal tissue, whereas it is a relatively minor constituent of adult skin (Epstein, 1974; Sykes *et al.*, 1976). Analyses of neutral-salt extracts of skin from a number of species have revealed, in addition to type-I-collagen precursors, the presence of high-molecular-weight forms of type III collagen (Byers *et al.*, 1974; Lenaers & Lapière, 1975; Timpl *et al.*, 1975; Anesey *et al.*, 1975; Smith *et al.*, 1977), suggesting that this type of collagen undergoes extracellular processing by a similar mechanism.

The technique of constant intravenous infusion of labelled amino acids (Waterlow & Stephen, 1967; Gan & Jeffay, 1967) aims to achieve constant or plateau specific radioactivities of the amino acid within body free pools so that measurement of these

values, together with protein-bound amino acid specific radioactivities at the end of the infusion period, allows, with certain assumptions, calculation of the rates of protein synthesis (Garlick *et al.*, 1973). As with other methods of radioactive-label administration, however, a major problem is to define the specific radioactivity of the amino acid pool used for protein synthesis. This pool is not necessarily equivalent to the total free pool isolated from the tissue, since the latter represents only the average value for a number of intracellular and extracellular compartments (see Waterlow *et al.*, 1978).

Attempts to overcome this problem directly have been made either by isolating the relevant aminoacyl-tRNA (Khairallah & Mortimore, 1976; Vidrich *et al.*, 1977; Regier & Kafatos, 1977; McKee *et al.*, 1978) or by determining the specific radioactivity of nascent polypeptides (Ilan & Singer, 1975), but these techniques are often impracticable for certain mammalian tissues.

If the turnover of type-I-collagen precursors *in vivo* is rapid, as indicated by studies *in vitro* (Bellamy & Bornstein, 1971; Morris *et al.*, 1975), then the specific radioactivity of amino acids in these proteins should approach a plateau value during the course of an infusion, thus providing a true reflection of the specific radioactivity in the amino acid pools used for collagen

synthesis. Infusion experiments were therefore undertaken with the primary aim of determining absolute rates of collagen synthesis. In achieving the preliminary objective of determining whether collagen precursors did in fact attain a constant specific activity during the infusions, large differences in apparent turnover rates between type-I- and type-III-collagen precursors were observed and the present paper reports the kinetic results obtained.

Experimenta

Materials

L-[5-³H]Proline (sp. radioactivity 12Ci/mmol), L-[side chain-2,3-³H]tyrosine (sp. radioactivity 20Ci/mmol), L-[4,5-³H]leucine (sp. radioactivity 30Ci/mmol), L-[U-¹⁴C]proline (sp. radioactivity 300Ci/mol) and L-[U-¹⁴C]tyrosine (sp. radioactivity 400Ci/mol) were obtained from The Radiochemical Centre, Amersham, Bucks., U.K. Scintillation fluid (NE 260) was purchased from Nuclear Enterprises, Edinburgh, Scotland, U.K.

Methods

Rabbit infusions. Growing New Zealand White rabbits (800–1400g) were used. Radioactive amino acids in sterile 0.9% NaCl were infused through a peripheral ear vein (Nicholas *et al.*, 1977). The use of a coiled flexible connection to the Slow Infusion Apparatus (Scientific and Research Instruments, Croydon, Surrey, U.K.) allowed the animals normal movement during the periods of infusion; food and water were given *ad libitum*. The infusions, normally at 2.05ml/h, were continued for various times up to 10h. For the administration of different radioactive amino acids concurrently for various time periods, changing the infusion solutions, contained in matched 5ml syringes, resulted in negligible interruption of flow (<30s), and allowance was made for the flow-time through the flexible tubing. To expedite subsequent procedures, dorsal fur was removed with clippers immediately before stopping the infusion and killing the animal by cervical dislocation.

Isolation of collagen precursors. The skin was removed and scraped clean of fat and adhering tissue on a cooled glass plate, all subsequent extraction procedures also being carried out at 4°C. The tissue (40–60g) was minced with scissors into extraction buffer (250ml), which contained 150mM-NaCl, 50mM-Tris/HCl (pH 7.5), 25mM-EDTA, 10mM-N-ethylmaleimide, 10mM-iodoacetate, 0.2mM-2-aminopropionitrile, and approx. 0.2mM-phenylmethanesulphonyl fluoride, the latter compound being added periodically during the isolation procedure as a stock solution in ethanol, owing to its relative instability in aqueous

solution. After homogenization for 10s by a Polytron (Northern Media Supply Co., Hull, U.K.), the tissue was centrifuged at 40000g for 10min and a small portion of the supernatant was removed for free-pool determination. The remainder was filtered through glass wool and collagen precursors were isolated essentially by the method developed by Byers *et al.* (1974). Briefly, (NH₄)₂SO₄ solution was added to 20% saturation and, after stirring overnight, the precipitated protein was centrifuged, washed with 20% (w/v) NaCl (2 × 50ml) and then stirred for 1h in extraction buffer (150ml) made 0.5M with respect to NaCl. Undissolved material was centrifuged and collagenous proteins in the supernatant were precipitated by the addition of solid NaCl to 20% (w/v). After centrifugation, the residue was redissolved in 200mM-NaCl/50mM-Tris/HCl (pH 7.5), and passed through a DEAE-cellulose column (16cm² × 25cm) equilibrated with the same buffer to remove acidic macromolecules. Unretarded material was rechromatographed on a DEAE-cellulose column (2.5cm² × 10cm) previously equilibrated with 20mM-NaCl/30mM-Tris/HCl/2mM-EDTA/2mM-urea (pH 7.6): collagen precursors were eluted by application of a linear gradient from 20mM- to 200mM-NaCl over a total volume of 600ml. Relevant fractions were pooled, dialysed against four changes (3 litres) of water and freeze-dried.

In some experiments, the residue from the 0.15M-NaCl extract was stirred with 0.5M-NaCl extraction buffer for 24h and, after centrifugation, collagenous proteins in the supernatant were precipitated by the addition of NaCl to a final concentration of 1.8M. The precipitate was redissolved and chromatographed on DEAE-cellulose as described previously.

Free-pool determination. For determination of the specific radioactivity of the free pool of amino acids in skin, a portion (10ml) of the homogenate supernatant, cooled on ice, was mixed with 2.5M-HClO₄ (2ml). After 10min, the precipitated protein was centrifuged down and perchlorate in the supernatant was then precipitated by the addition of 4M-KOH to pH 4. The supernatant was fractionated on a Locarte analyser and the specific radioactivity of amino acids in the relevant pooled fractions was determined. The tissue-homogenization step was always completed within 30min of killing the animal, but to check for possible dilution of the free pool during this time by unlabelled amino acids from endogenous sources, portions of freshly removed skin were frozen directly in liquid N₂. Subsequent determination of the specific radioactivity of the homogenate pools of free ³H-labelled proline and tyrosine in these samples agreed to within 5% of the values obtained by the standard procedure.

Analytical techniques. Sodium dodecyl sulphate/polyacrylamide-gel electrophoresis in Tris/borate buffer, pH 8.6, was carried out in an apparatus based

on a horizontal flat-bed design (B. C. Sykes, personal communication). Densitometry was performed by using a Zeineh laser scanner (Crump Scientific Products, Rayleigh, Essex, U.K.). For preparative work, the required bands were located by staining guide strips with Coomassie Blue and were then excised, heated at 100°C for 10 min in sodium dodecyl sulphate gel buffer containing 1% 2-mercaptoethanol, and packed into tubes (0.6 cm² × 15 cm) closed at one end with a no. 2 sinter. Electrophoresis of the material out of the gel into a dialysis sac placed over the sintered end of the tube was carried out at 300 V for 3 h in an apparatus similar to that described by Stephens (1975).

Amino acid analyses were carried out by using a Locarte analyser and radioactivity measurements with NE 260 scintillation fluid were made in a Beckman LS-345 counter.

Results

Characterization of precursors

The elution pattern of skin collagen precursors on DEAE-cellulose showed an initial peak of unadsorbed material and two peaks eluting at higher ionic strength. Preliminary characterization of these components together with an indication of their purity was achieved by sodium dodecyl-sulphate/polyacrylamide-gel electrophoresis (Fig. 1), which showed that the first of these peaks contained type-I-collagen precursors and that the second characteristically asymmetric peak contained precursors of type III collagen. Confirmation of the identity of these collagen isomorphs was obtained by the release of α -chains from the precursors by limited cleavage with pepsin and by characteristic peptide patterns after cleavage with CNBr (results not shown). The removal of extension peptides at either end of procollagen leads to a number of intermediates that co-chromatograph on DEAE-cellulose, but that are evident in the gel patterns (Fig. 1). The yield of type-I-collagen precursors was similar to that of type-III-collagen precursors in each of the animals, amounting to approx. 50 μ g/g wet wt. of tissue.

The unadsorbed material from the DEAE-cellulose chromatography contained type-I-collagen monomers, but no type III collagen was detected in this fraction by using an interrupted electrophoresis technique (Sykes *et al.*, 1976). In the collagenous proteins subsequently extracted from skin with 0.5 M-NaCl, however, type III monomers were detected after DEAE-cellulose chromatography of the fraction that precipitated at 1.8 M-NaCl. Also this chromatographic step yielded only very small amounts (<0.2 mg) of type-III-collagen precursors, indicating that the initial extraction with 0.15 M-NaCl was virtually complete.

Fig. 1. Sodium dodecyl sulphate/polyacrylamide-gel electrophoresis of collagen precursors

The Figures show the results of electrophoresis in a 6% acrylamide gel of collagen-precursor fractions isolated by DEAE-cellulose chromatography. The densitometer traces of gels stained with Coomassie Blue are of samples reduced with 1% (v/v) 2-mercaptoethanol (full lines) and unreduced (broken lines). (a) shows type-I-collagen-precursor fraction where intermediates containing N-terminal extension peptides (p_N - α chains) were identified by the lack of interchain disulphide bonding (Byers *et al.*, 1975); (b) shows type-III-collagen-precursor fraction where the intermediate, p - α 1(III), was not fully characterized.

Constant-infusion experiments

The results of an experiment to determine the rate of attainment of isotopic equilibrium during an infusion of ³H-labelled proline are shown in Fig. 2. The specific radioactivity of blood free proline reached a plateau value shortly after 1 h with a calculated first-order-rate constant of 2.5 h⁻¹. Similar rates of increase to a plateau value were previously determined in rabbits for radioactively labelled tyrosine (Nicholas *et al.*, 1977).

The results of an infusion of radioactively labelled proline and tyrosine for different periods of time to determine the rate of increase in specific radioactivity in procollagen are shown in Table 1. For both amino acids, the specific-radioactivity ratio between type-I-collagen precursors and the free amino acid pool is similar at 3 and 6 h, indicating that the type-I-precursor pool has reached its plateau specific radioactivity within 3 h. This result can be expressed

in terms of the similarity in the $^3\text{H}/^{14}\text{C}$ ratio between the free pool and type-I-collagen-precursor fraction (Table 1). By contrast, the specific radioactivities of type-III-collagen precursors were lower than those of the type I fraction and the higher $^3\text{H}/^{14}\text{C}$ values, together with the increase in the ratio of specific radioactivity of type-III-collagen precursors to the specific radioactivity of type-I-collagen precursors (Table 1), confirm that this fraction has not reached its maximum value. Nevertheless the similarity in the results for proline and tyrosine and the close agreement in specific radioactivities for proline and

hydroxyproline in the proteins (Table 1) provide some indication of the purity of the isolated components and confirm the validity of using amino acids other than proline for these experiments.

In a series of infusions carried out for periods longer than 3 h, specific radioactivities for type-III-collagen precursors lower than those for the type-I-precursor fraction (assumed to be at a plateau) were consistently recorded (Table 2). There was considerable variation, however, in the ratio for proline between the type-I-precursor pool and the homogenate free pool: this was not associated with any gross differences in the size of the extracted pool. Further kinetic data were obtained by using a multiple-infusion schedule, the results of which are shown in Table 3. There was an increase in the specific radioactivity ratio between type-III- and type-I-collagen precursors throughout the 10 h period of infusion. The $^3\text{H}/^{14}\text{C}$ ratio for tyrosine in the type-I-collagen-precursor fraction and the free pool were similar (Table 3), indicating the attainment of a plateau value in the protein by 4 h. Thus, of the specific radioactivities for the type-I-collagen-precursor fraction in Table 3, only that for ^{14}C -labelled proline (2 h infusion) does not represent a plateau value, as reflected in the high $^3\text{H}/^{14}\text{C}$ ratio for hydroxyproline compared with the free pool of proline. Examination of the type-I-collagen-precursor fraction from this animal by sodium dodecyl sulphate/polyacrylamide-gel electrophoresis showed that, in contrast with the normal pattern (Fig. 2), a higher proportion of lower-molecular-weight precursors was present, particularly those containing the *N*-terminal

Fig. 2. Specific radioactivities of rabbit blood free proline during constant infusion of $[^3\text{H}]$ proline

$[^3\text{H}]$ Proline was infused through an ear vein at $180\mu\text{Ci/h}$ and specific radioactivities (S_p) were measured in blood sampled from the other ear. The rise to a plateau specific radioactivity ($S_{p(\text{max.})}$) approximated to $S_p = S_{p(\text{max.})}(1 - e^{-k_p t})$, where the rate constant $k_p = 2.5\text{ h}^{-1}$.

Table 1. Rabbit infusion with labelled proline and tyrosine

The radioactively labelled compounds, contained in two infusion solutions, were given at a constant rate for the times indicated so that the animal was receiving all four radioactively labelled amino acids at the end of the experiment. Specific radioactivities in the skin homogenate free pool and isolated collagen precursors were determined as described in the Experimental section.

Radioactively labelled amino acid	Infusion time (h)	Specific radioactivity (d.p.m./nmol)			Ratio of specific radioactivities		
		Skin free pool (SFP)	Type-I-collagen precursors (S_I)	Type-III-collagen precursors (S_{III})	S_I/SFP	S_{III}/SFP	S_{III}/S_I
$[^3\text{H}]$ Proline	6	244	(Hyp) 90.2	(Hyp) 62.9	0.37	0.26	0.69
			(Pro) 85.7	(Pro) 60.2	0.35	0.25	0.70
$[^{14}\text{C}]$ Proline	3	51.8	(Hyp) 18.3	(Hyp) 9.34	0.35	0.18	0.51
			(Pro) 16.8	(Pro) 8.46	0.32	0.16	0.50
$[^3\text{H}]$ Tyrosine	6	442	349	228	0.79	0.52	0.65
$[^{14}\text{C}]$ Tyrosine	3	109	79.7	40.9	0.73	0.38	0.51
$^3\text{H}/^{14}\text{C}$ ratio							
		SFP	S_I	S_{III}			
Hyp		—	4.93	6.74			
Pro		4.71	5.10	7.11			
Tyr		4.06	4.38	5.58			

Table 2. *Constant infusions of [³H]proline into rabbits*

After infusions of [³H]proline for the times indicated, specific radioactivities of free proline in deproteinized extracts of whole blood and skin homogenate, and of hydroxyproline in collagen precursors isolated from skin were determined.

Infusion time (h)	Specific radioactivity (d.p.m./nmol)				S_{III}/S_I ratio
	Whole blood	Skin free pool	Type-I-collagen precursors (S_I)	Type-III-collagen precursors (S_{III})	
5	957	189	88.7	58.1	0.66
6	580	230	68.6	41.7	0.61
8	995	308	52.9	40.0	0.76
10	543	264	134	109	0.81

Table 3. *Multiple infusion of radioactively labelled amino acids into a growing rabbit*

The amino acids, contained in five infusion solutions, were given at a constant rate for the time indicated so that the animal was receiving all five labelled amino acids at the end of the experiment. For further details, see the Experimental section.

		Specific radioactivity (d.p.m./nmol)			
Labelled amino acid	Infusion time (h)	Skin free pool	Type-I-collagen precursors (S_I)	Type-III-collagen precursors (S_{III})	S_{III}/S_I ratio
[^{14}C]Proline	2	190	17.6	8.11	0.46
[^{14}C]Tyrosine	4	205	157	84.5	0.54
[^3H]Leucine	6	224	230	146	0.64
[^3H]Proline	8	829	138	103	0.75
[^3H]Tyrosine	10	1221	1002	835	0.83

$^3\text{H}/^{14}\text{C}$ ratio			
	Skin free pool	S_I	S_{III}
Pro	4.36	7.85	12.67
Tyr	5.96	6.36	9.87

Table 4. *Fractionation of type-I-collagen precursors by preparative electrophoresis*

Type-I-collagen precursors (1.4 mg) from a multiple-infusion experiment were subjected to preparative electrophoresis without reduction in a 3.5% acrylamide gel. Procollagen and intermediates containing only *N*-terminal extension peptides ($p_N\alpha 1$ chains) were isolated and their specific radioactivities were determined as described in the Experimental section.

	Specific radioactivity (d.p.m./nmol)		
	Type-I-collagen-precursor fraction*	Type I procollagen	$p_N\alpha 1$
[³ H]Hydroxyproline	138	145	151
[¹⁴ C]Hydroxyproline	17.6	29.3	14.5
³ H/ ¹⁴ C ratio	7.85	4.95	10.41

* Data from Table 3.

extension peptides. After isolation of procollagen and an intermediate by preparative electrophoresis, determination of specific radioactivities showed that type I procollagen approached its plateau value within 2 h, as shown by the similarity in the ³H/¹⁴C ratio to that in the free pool (Table 4). The observed ¹⁴C specific radioactivities of the intermediate contributed to the lower specific radioactivity of the type-I-precursor fraction as a whole.

Discussion

The results show that there are large differences in the rates at which types I and III procollagen of rabbit skin are processed *in vivo*. For type I procollagen, the infusion times used allow only an approximate value for the turnover time to be calculated from the rate of increase to the plateau value for specific radioactivity. Thus, as type I procollagen attained 90% of its plateau value after

2 h infusion (Table 4), the calculated change with time of infusion (t) in the specific radioactivity of procollagen (S_I) in terms of its plateau value ($S_{I(\max.)}$) can be expressed by the equation:

$$S_I = S_{I(\max.)} (1 + 1.81e^{-2.5t} - 2.8e^{-1.6t})$$

where the first exponential term accounts for the rate of increase to the plateau value in the free pool of proline in skin, which was assumed to be the same as that determined for plasma ($k_p = 2.5 \text{ h}^{-1}$; Fig. 3). The first-order rate constant for type I procollagen (1.6 h^{-1}) corresponds to a half-life for the protein of 26 min, an estimate that must be regarded as a maximum value, since the rate of increase in the tissue free pool certainly cannot exceed that in the blood. Similar rates of procollagen turnover were observed for calvarial bone *in vitro* (Bellamy & Bornstein, 1971; Morris *et al.*, 1975; Davidson *et al.*, 1975). Because much of the isolated procollagen originates from intracellular pools, as evidenced by the increased amounts extracted under similar conditions after treatment with inhibitors of secretion (Smith *et al.*, 1977), the calculated turnover time includes that required for secretion of procollagen. From evidence for a number of different tissues, a secretion time of 10–20 min can be inferred (Dehm & Prockop, 1972; Vuust & Piez, 1972; Weinstock & Leblond, 1974), although the kinetics may be more complex (Kao *et al.*, 1977). The results are therefore consistent with the view that processing of type I procollagen commences very soon after secretion from the cell and possibly at the cell surface. Although in bone a scheme for the processing of type I procollagen involving initial removal of the *N*-terminal extensions was elucidated (Davidson *et al.*, 1975, 1977), the presence in some skin extracts of chains with *N*-terminal extensions having lower specific radioactivities than procollagen (Table 4) indicates that this mechanism does not operate in all tissues. The kinetics of removal of the *N*-terminal extensions may be important in view of the suggestion that the excised peptides may act by negative feedback to control collagen-synthesis rates (Hörlein *et al.*, 1978).

Processing of type III procollagen

Since type I procollagen approaches constant specific radioactivity within 2 h of infusion (Table 4), plateau conditions, where unproven, were assumed for longer periods of infusion. These values are therefore equal to the specific radioactivity of the amino acid pools used for collagen synthesis. Fibroblasts have been shown to synthesize both type I and type III collagen simultaneously (Gay *et al.*, 1976) and it is a reasonable assumption therefore that synthesis of the two collagen types utilizes the same amino acid pools. The specific radioactivities of type I procollagen therefore correspond to the maximum

values for type III procollagen. The data approximately obey first-order kinetics (Fig. 3) with an apparent half-life of the 0.15 M-NaCl-soluble type-III-collagen-precursor fraction of 3.9 h. The virtual absence of precursor molecules in subsequent extracts suggests that this value is representative of the total pool of type-III-collagen precursors in skin.

The slower apparent turnover of type-III-collagen precursors is consistent with the relatively large amounts isolated despite the predominance of type I collagen in the tissue: similar characteristics were reported for extracts from young rat skin (Lenaers & Lapière, 1975; Smith *et al.*, 1977). Differences in the rates of processing of type-I- and type-III-collagen precursors were also noted in cultured fibroblasts, which apparently lacked a proteinase that converts type-III-collagen-precursor intermediates into α -chains (Goldberg, 1977). The isolation of type III collagen without extension peptides by prolonged extraction is in agreement with the results for rat and bovine skin (Byers *et al.*, 1974; Timpl *et al.*, 1975; Smith *et al.*, 1977) and the indication that conversion into this form occurs, at least partially, *in vivo* is supported by specific-radioactivity measurements (S. P. Robins, unpublished work). The slower pro-

Fig. 3. Labelling kinetics of collagen precursors. Data in Tables 1–3 for specific radioactivities of type-III-collagen precursors (S_{III}) relative to those of type-I-collagen precursors (S_I) at various times of infusion (t) were fitted to the equation $S_{III} = S_{III(\max.)} (1 - e^{-kt})$, where $S_{III(\max.)}$ is assumed to be equal to S_I at the plateau and k is a first-order rate constant: the value for S_I at 2 h is taken from Table 4. The plot of $-\ln(1 - S_{III}/S_I)$ against t shows values for infusions of proline (○), tyrosine or leucine (□, ■), where the open symbols refer to data from a single animal (Tables 3 and 4). Regression analysis for all the points gave a line passing through the origin with gradient $k = 0.181 \pm 0.025 \text{ h}^{-1}$ (mean $\pm$ s.d.) corresponding to a half-life $[(\ln 2)/k]$ of the type-III-collagen-precursor fraction of 3.9 h.

cessing time of type-III-collagen precursors may explain the results of immunofluorescence studies of human skin that showed that, whereas antibodies to type-I-collagen precursors stained only a thin subepidermal layer, the staining for precursors to type-III-collagen was indistinguishable from that using antibodies to the helical region (Nowack *et al.*, 1976; Meigel *et al.*, 1977). The close association of type-III-collagen precursors with the fibres together with their slower apparent turnover suggests that removal of extension peptides may have some important regulatory function for fibrillogenesis.

Amino acid precursor pools

The use of pro-proteins with rapid turnover for determining precursor amino acid pool specific radioactivity has advantages over alternative methods in that it involves isolation of well defined products rather than polypeptide fragments from ribosome 'stripping' and the results are less liable to methodological problems associated with the isolation of aminoacyl-tRNA. The precursor pool utilized for secreted proteins, however, may not be typical of the whole tissue (Ilan & Singer, 1975; Fern & Garlick, 1976) and the results are strictly only applicable to the single protein.

The mean ratio ($\pm$ S.D.) for tyrosine specific radioactivity between the precursor amino acid pool (defined by type I procollagen) and the skin homogenate was 0.77 ± 0.03 ($n = 5$); this ratio for leucine was 1.03. Although the results for tyrosine could possibly be explained in terms of the contribution of interstitial fluid to the homogenate free pool (i.e. the precursor pool may be identical with the total intracellular free pool), the result for leucine is more consistent with models involving contributions to the amino acid precursor pool from both intracellular and extracellular sources (Airhart *et al.*, 1974; Hod & Hershko, 1976; Vidrich *et al.*, 1977). For the non-essential amino acid, proline, the precursor to homogenate free-pool ratio was much lower (0.31 ± 0.13 ; $n = 8$) and was more variable than that for tyrosine, despite the fact that the results for proline incorporated the same series of experiments. Evidently, the contribution of (labelled) extracellular proline to the precursor pool for collagen synthesis is minor relative to that supplied intracellularly from protein breakdown and synthesis of the amino acid *de novo*, and specific radioactivities at steady state showed that as little as 5% of the precursor pool may be in equilibrium with free proline in blood (see Table 2). This is in contrast with the model proposed for calvarial bone in which proline entering the cell was considered to equilibrate first with the protein precursor pool (Phang *et al.*, 1971). More recently, the importance of synthesis *de novo* was demonstrated for xiphoid

cartilage in culture in which about 20% of the proline incorporated into protein appeared to be synthesized from ornithine even in the presence of adequate exogenous proline (Smith & Phang, 1978), and synthesis from glutamate may be a more important pathway in some tissues (Shen & Strecker, 1975). Thus the variability in the precursor pool is probably a result of changes in the relative contribution of proline from intracellular and extracellular sources. The results indicate, therefore, that in estimating collagen-synthesis rates by incorporation of labelled hydroxyproline both *in vivo* and *in vitro*, a knowledge of the precursor pool of proline is essential and measurements based on the tissue homogenate free pool may incur large and inconsistent errors.

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