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Synthesis and degradation rates of collagens *in vivo* in whole skin of rats, studied with $^{18}O_2$ labelling

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Rates of synthesis and degradation *in vivo* of collagens in 0.5 M-acetic acid-soluble and -insoluble extracts from skins of three growing rats were determined by using a labelling procedure involving exposure of the animals to an atmosphere of $^{18}O_2$ for 36 h. For comparison, rats also received injections of [^{2}H]proline. Serial skin biopsies were taken at frequent intervals over 392 days. Enrichment of ^{18}O and ^2H in the hydroxyproline of the collagen fractions was determined by gas chromatography–mass spectrometry. Changes in size of the soluble and insoluble collagen pools were considered in the evaluation of isotope kinetic data. The insoluble collagen fraction showed no degradation. The efflux (mean ± S.D., expressed as μmol of hydroxyproline) from the soluble collagen pool was estimated to be 59.9 ± 1.9 per day from the ^{18}O data, and 25.5 ± 7.5 per day from the ^2H results. The finding indicates significant reutilization of ^2H-radiolabelled proline for hydroxyproline synthesis. From these isotope data and estimates of size of the collagen pools it was determined that 55% of the collagen disappearing from the soluble pool was due to maturation into insoluble collagens and 45% of the disappearance was a result of actual degradation of soluble collagen. These results confirm the utility of $^{18}O_2$ as a non-reutilizable label for studies of collagen turnover *in vivo*.

INTRODUCTION

Collagen accounts for about one-third of body protein (Harkness *et al.*, 1958; Picou *et al.*, 1966), and alterations in the synthesis of collagen and regulation of collagen fibre formation occur in a number of disease states, including oesteogenesis imperfecta, the Marfan Syndrome and the Menke's steely-hair syndrome (Prockop & Kivirikko, 1984). It therefore seems important to have reliable estimates of rates of synthesis and breakdown of collagen *in vivo* in various tissues and organs and of the effects of factors such as hormones and various stressful stimuli on these rates. Although early investigations suggested that collagen is relatively inert (Neuberger *et al.*, 1951; Thompson & Ballou, 1956; Nissen *et al.*, 1978), it is now clear that some collagen pools, notably the soluble collagens, are more labile than others, especially in comparison with the insoluble collagens (Laurent, 1982; Bienkowski, 1984). However, accurate estimation of rates of turnover of collagens *in vivo*, as obtained by analysis of decay of radioactivity after administration of a labelled amino acid, is complicated due by significant reutilization of the labelled amino acid. To overcome this problem, Jackson & Heininger (1974, 1975) proposed the novel approach of labelling collagen *in vivo* with $^{18}O_2$. Molecular O_2 is used in the prolyl hydroxylase (EC 1.14.11.2) reaction, responsible for the post-translational hydroxylation of peptide-bound proline. Their preliminary investigation in a single rat suggested that this method could provide a reliable estimate of collagen turnover. Hence we have undertaken an initial study designed to evaluate further the method of $^{18}O_2$ labelling of hydroxyproline and to compare the turnover of soluble and insoluble collagens *in vivo* in rat skin. This study was carried out to establish the labelling and analytical conditions necessary to explore the effects of nutritionally induced growth retardation on collagen synthesis and turnover in the skin, particularly because the entire skin might account for as much as 25% of whole-body protein synthesis (Preedy *et al.*, 1983).

METHODS

Three weanling male Sprague–Dawley rats (mean wt. 49 g and age 25 days; Charles River Breeding Laboratories, Kingston, NY, U.S.A.) were given *ad libitum* an adequate diet containing 18% lactalbumin (Young *et al.*, 1971) and placed in a metabolic chamber (Molnar *et al.*, 1986). After a 12 h adjustment period, the animals were then exposed to $^{18}O_2$ (63 atom% excess; 24 litres; Cambridge Isotopes, Woburn, MA, U.S.A.) for 36 h in the closed environment, with the atmosphere being approx 20% O_2 and 80% N_2.

Immediately on removal from the chamber, a single skin biopsy of approx. 100 mg wet wt. of tissue was obtained from each animal. This was designated as the zero-time sample. During the following 22 h, each animal was labelled also with L-[$^2H_7$]proline (12 mg/g body wt.; 96 atom% excess; Cambridge Isotopes) via 12 subcutaneous injections given about every 2 h. A second biopsy of skin was obtained at the end of this period and then another one 18 h later. Subsequent biopsies were obtained at consecutive 5-day intervals until day 92, when the biopsy interval was increased. The final biopsy was taken at 392 days.

Abbreviation used: g.c.–m.s., gas chromatography–mass spectrometry.

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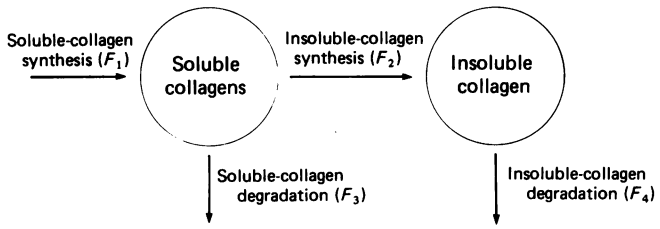
The procedure for obtaining samples of skin involved giving the rats a halothane anaesthetic, local removal of hair by shaving and depilatory and removing a section of skin about 0.5 cm^2 or 100 mg wet wt. The skin was closed with interrupted nylon sutures (Ethicon, Somerville, NJ, U.S.A.). Excess subcutaneous tissue was removed from the skin samples, and they were finely minced and freeze-dried. The samples were subsequently defatted with chloroform/methanol (2:1, v/v) (Folch *et al.*, 1957) and then extracted with 0.5 M-acetic acid (1 ml/mg of sample) for 24 h at 4 °C (Swann & Sotman, 1980). After centrifugation at 90000 *g* for 30 min, the pellet was retained as the insoluble collagen fraction. The supernatant was adjusted to 2.5 M with respect to NaCl (Trelstad *et al.*, 1976), stirred for 24 h at 4 °C and centrifuged as above. The pellet was then collected for analysis as the soluble collagen fraction. Both preparations of soluble and insoluble collagens were freeze-dried, hydrolysed in 6 M-HCl at 100 °C for 24 h, and then the free amino acids were converted into their *N*-trifluoroacetyl amino acid propyl esters (Adams, 1974).

G.c.–m.s. analysis was performed with a Hewlett–Packard 5985 system. G.c. separaton of amino acids was performed on a 1.8 m column of 3% OV17 on 100/120 Supelcoport (Supelco, Bellefonte, PA, U.S.A.) with injection-port temperature of 220 °C, an initial column temperature of 170 °C and with temperature programmed to change at 15 °C/min. Carrier gas flow rate was 25 ml/min. Isotopic (^{18}O and ^{2}H) enrichment of the hydroxyproline was accomplished by using electron-impact ionization and selected ion monitoring, with examination of the nominal 182/184 mass ratio for the $^{18}O_2$ label and the 164/169 mass ratio for ^{2}H label in hydroxyproline. These ions were chosen since they avoided the potential complications of enrichment in the carboxy group.

Estimates of collagen pool size were obtained from analysis of whole skins (excluding head, feet and tail) of 92 unlabelled rats killed at various ages that had been fed and housed as for the three rats given the isotopic labels. Skin samples were processed as for the biopsies used to separate soluble and insoluble collagen. Hydroxyproline concentration in the hydrolysed samples was determined with a Beckman 119C amino acid analyser. An extensive description of these pool-size data is not presented here, but a summary of the data is used in the present paper to predict the size of the skin collagen pools for each time used for measurement of isotope enrichment in biopsy samples obtained from the labelled rats. Briefly, predictive equations, based on animal age, for soluble and insoluble collagen pool size were obtained by using the IMSL subroutine ZXSSQ for the Marquardt–Levenberg algorithm for non-linear least squares (Marquardt, 1963). For the soluble collagen pool the equation used was: pool size (μmol of hydroxyproline) $= 0.4\times10^{-3}A^3-0.390A^2+86.9A-1800$. The insoluble collagen pool size (μmol of hydroxyproline) $= 0.26\times10^{-2}A^3+0.793A^2-26.5A+272$. In both equations A = age of rat in days. Total label in the entire skin collagen fraction was calculated from the product of pool size and enrichment of hydroxyproline with the ^{2}H and ^{18}O labels.

Kinetic analysis of the isotope data was based on a two-pool model with a undirectional precursor–product relationship (Scheme 1). Assumptions of this model include homogeneous mixing of the label, first-order



Scheme 1. Two-pool model used to assess rates of collagen synthesis and degradation *in vivo*

F_1 = rate of soluble-collagen synthesis; F_2 = rate of insoluble-collagen synthesis (or maturation of soluble collagen); F_3 = rate of soluble-collagen degradation; F_4 = rate of insoluble-collagen degradation.

reactions and no additional input of label into the soluble pool after a given point in time ($t = 0$). The following differential equations were derived from principles of conservation of mass [where: * = tracer quantity, M_S^* = total tracer in soluble pool (μmol), C_S^* = concentration of tracer in soluble pool (mol% excess), V_S = volume of soluble pool (μmol), F = flux (μmol/day) and F_S = total flux leaving soluble pool (F_2+F_3)]:

$$M_S^* = C_S^* V_S \quad (1)$$

After tracer administration, assuming no further input into pool, then:

$$dM_S^*/dt = -(F_2+F_3)C_S^* \quad (2)$$

Thus:

$$\frac{dM_S^*}{M_S^*} = -\frac{(F_2+F_3)dt}{V_S(t)} \quad (3)$$

Therefore:

$$M_S^*(t) = M_S^*(0)\exp[-(F_2+F_3)\int_0^t V_S^{-1}(u)du] \quad (4)$$

Upon inspection of the data plots of total label for the soluble pool (see the Results and discussion section), it was apparent that the assumption of no further influx of label at the end of the 36 h $^{18}O_2$-labelling period into the collagen fractions being measured was not valid. There was apparently a 'precursor' pool for the soluble pool that continued to supply label into the soluble pool for some time after the end of the labelling period. On the basis of the available data, it was not possible to model this precursor pool with acceptable reliability, and thus a simplified approach was taken by leaving out the initial data points for that period. Thus all kinetic modelling of the soluble-pool data began at 7 days after rats had been exposed to $^{18}O_2$.

For the insoluble pool, the situation is more complex. Again, inspection of the data (see the Results and discussion section) indicated that there was a continual transfer of label from the precursor soluble pool for several months and that the simplifying assumptions that had been made for the soluble pool did not apply to the insoluble pool. Hence estimation of soluble-collagen synthesis rate was based on the change in the size of the collagen pool and the assumption that there was no degradation of insoluble collagen, as discussed below.

The various flux rates (Scheme 1) were determined in a sequential fashion. First, soluble-pool total efflux ($F_S = F_2+F_3$) was determined from analysis of the

isotope decay curves for the soluble pool. It was then possible to estimate F_1 (the rate of synthesis of soluble collagen), F_2 (the rate of synthesis of insoluble collagen) and F_3 (the rate of soluble collagen degradation) from mass balance and from the estimate for F_S. F_1 was determined as the sum of the efflux from the soluble pool (F_S) plus the mass accretion of soluble collagen (μmol of hydroxyproline) during the period of study. F_2 was estimated on the basis of the assumption of no degradation of the insoluble pool (see the Results and discussion section). Hence F_3 was calculated as the difference between F_S and F_2.

Fractional turnover rates were estimated from the mean pool size for the period of study. Thus mean pool size was determined by solving the definite integral for the estimate of soluble-collagen pool size for the period of study and dividing by the number of days. It must be recognized that in the non-steady state for the healthy animals in this study the fractional turnover rate determined strictly applied at only a short period in time (when actual pool size equalled the mean pool size). This expression of turnover is used here only for the purpose of comparison of our data with those in the literature.

Analysis of the decay curves was by means of weighted non-linear least squares, by the algorithm of Marquardt (1963). The weights for least-squares fitting were taken to be inversely proportional to the variance of the measurements of isotopic enrichment. Judged by visual comparison with the experimental data, their overall fit was good; but the analysis of the residuals suggested some small systematic deviations. It could not be determined if these small deviations were due to inadequacies in the kinetic model or if they were due to other sources. A further criterion used to gauge the adequacy of the least-squares analysis was the magnitude of the coefficient of variation of the parameter estimates. The coefficient of variations for the soluble-collagen efflux estimates from the fitted curve was 6.4% with the $^{18}O_2$ label and 10.4% with the [$^2H_7$]proline label.

RESULTS AND DISCUSSION

Fig. 1 presents an example of the change in isotopic enrichment of hydroxyproline obtained with a single rat for each of the two labels given and collagen fractions analysed. For the soluble and insoluble collagen fractions, a precursor–product relationship (Zilversmit *et al.*, 1943) is evidence, as indicated by the following: (i) peak isotopic enrichment in the precursor pool (soluble collagen) preceded the peak in the product, (ii) peak enrichment in the product (insoluble collagen) was equal to that in the precursor pool at the cross-over point, and (iii) the enrichment of insoluble collagen remained higher than that for soluble collagen.

Because the labelled rats were growing, or in the non-steady state, such curves (Fig. 1) cannot be readily analysed to determine turnover rates, since a decrease in isotopic enrichment may arise simply from continued synthesis of unlabelled collagen. Hence, by using the data for pool size obtained from analysis of the total hydroxyproline in skin soluble and insoluble collagen, a predictive equation for pool size was obtained (J. A. Molnar, unpublished work). From this equation the soluble and insoluble hydroxyproline collagen pool sizes for labelled animals were estimated for the day when the skin biopsy was obtained. From the product of pool size and isotopic enrichment of hydroxyproline in the two

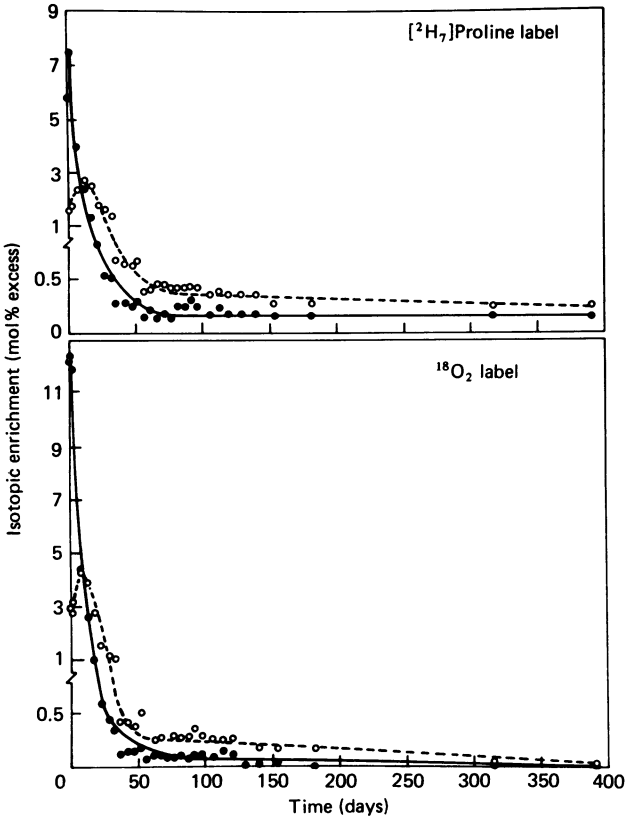


Fig. 1. Isotopic enrichment decay curve for soluble (●) and insoluble (○) collagen proteins from rat skin

Data are plotted as mol% excess in hydroxyproline at various times after exposure *in vivo* to $^{18}O_2$ was discontinued (day 0) for a single animal that received both [$^2H_7$]proline and $^{18}O_2$.

collagen fractions the amount of label in the total skins for each pool was determined and the results from these estimations were depicted in Fig. 2. These plots represent loss of label via removal from the pool and are not affected by new synthesis of unlabelled collagen. These findings show that although significant removal of soluble collagen occurred, regardless of the label used, the insoluble collagen fraction did not show a measurable loss of the label; if anything, the amount of label in the insoluble collagen pool tended to increase with time. Thus it is likely that there was a slow infusion of label from the precursor, or soluble, pool throughout the period of study. Nevertheless, from these observations it seems reasonable to conclude that there was little, and more probably no, degradation of the insoluble collagen fraction during the period of observation.

The rate of loss of total label (^2H or ^{18}O) from the soluble pool was determined as described above, and the results are given in Table 1. Thus mean efflux from the soluble pool, expressed in μmol of hydroxyproline/day, was 59.0 with the $^{18}O_2$ label and 25.5 with the [$^2H_7$]proline label. Assuming a mean soluble collagen pool size of approx. 3000 μmol for these three rats (J. A. Molnar, unpublished work), this efflux corresponds to mean fractional rates (day^{-1}) of 0.0196 and 0.00833 respectively for the ^{18}O and ^2H labels. In either case, it is apparent that the [$^2H_7$]proline label gave a turnover rate less than one-half of that determined with

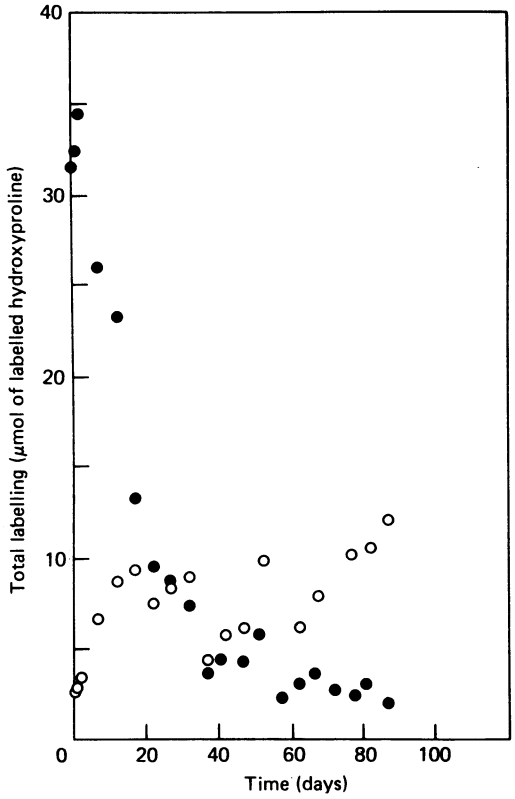


Fig. 2. Total isotope ($^{18}O_2$) present in soluble (●) and insoluble (○) collagen pools of skin as a function of time after termination of exposure to $^{18}O_2$ *in vitro*

The data displayed here represent the product of hydroxyproline enrichment and the estimated pool size (see the Methods section) for the entire skin for each time point. Thus the decline in total enrichment is dependent on loss of labelled molecules only.

the ^{18}O label, a difference consistent with that found by Jackson & Heininger (1975). There is therefore a significant reutilization of labelled proline and, in consequence, an underestimate of the rate of removal and possibly of degradation (Waterlow *et al.*, 1978).

Since the efflux of collagen molecules, as well as the net increase of hydroxyproline in the total soluble collagen pool, are known, an estimate of the rate of synthesis of soluble collagen was made, and was found to be equivalent to 92.0 μmol of hydroxyproline/day (Table 1). From the data of Piez *et al.* (1963), this is equivalent to 95 mg of soluble collagen/day.

Our data (see Table 1) can also be used to estimate the rates of degradation of collagen in the soluble fraction (F_3; Scheme 1) and maturation (F_2; Scheme 1) of the soluble collagens (conversion into insoluble collagen). Thus, assuming no degradation of insoluble collagen (Fig. 2) it may be determined from the prediction equations (see the Methods section) that between 32 and 210 days of age there was an accumulation of 5750 μmol of hydroxyproline in the insoluble-collagen pool of the entire skin. During this same period there was a daily efflux of 59.0 μmol of hydroxyproline from the soluble-collagen pool, or a total of 10500 μmol of hydroxyproline for this entire period. Thus 55% of the efflux from the soluble-collagen pool was associated with a conversion into insoluble collagen, and the remainder, or 45% of the efflux, can be attributed to true degradation of the soluble collagens.

It should be noted that the initial data points for the soluble-collagen curve (Fig. 2) suggest that there was a continued input of label for a brief period after removal of rats from the $^{18}O_2$ environment. This input function apparently derives from a precursor to the soluble-collagen fraction as isolated by our procedure. In studies where a reutilizable label is used, this input could arise due to the label being first released from within other body and tissue protein pools and subsequently

Table 1. Efflux, synthesis and degradation of collagens in acetic acid-soluble and -insoluble fractions of whole skins of growing rats, measured with $^{18}O_2$ and [$^2H_7$]proline labels

All values are μmol of hydroxyproline/day, except for the fractional-rate values. Data are based on analysis of isotopic data gathered from days 32 to 210.

Parameter	Rat no. 1	Rat no. 2	Rat no. 3	Mean ± S.D.
Soluble collagens				
Efflux:				
With $^{18}O_2$ label	59.2	60.8	57.0	59.0 ± 1.9
With [^{2}H]proline label	33.0	25.4	18.1	25.5 ± 7.5
Fractional rate (day^{-1})*				
With $^{18}O_2$ label	0.0197	0.0200	0.0190	$0.0196 \pm 5 \times 10^{-4}$
With [^{2}H]proline label	0.0110	0.0085	0.0060	$0.0083 \pm 2.5 \times 10^{-3}$
Synthesis†	92.2	93.7	90.0	92 ± 1.9
Degradation‡	26.9	28.5	24.7	26.7 ± 1.9
Insoluble collagen				
Synthesis§				32.3
Degradation				0

* Assumes mean pool size of 300 μmol of hydroxyproline.

† Based on $^{18}O_2$ label and assumes maximum pool size of 3860 μmol (from prediction equations of J. A. Molnar (unpublished work; also see the Methods section). Synthesis = Efflux + net accumulation of soluble collagens.

‡ Derived from $^{18}O_2$ data. Degradation = Efflux − change in pool size of insoluble collagen.

§ Equivalent to maturation rate of soluble collagen (see F_2 in Scheme 1). Only a mean value is given here, since the accumulation of insoluble collagen was based on a single prediction equation with all rats being of the same age.

re-incorporated into collagen. However, in the present investigation, the $^{18}O_2$ label cannot be recycled in this way. Jackson & Heininger (1975) did not observe this input function, perhaps because they began sampling the skin collagen pool 7 days after completion of their initial labelling period. Also, our data (Fig. 2) suggest that the input function does not apparently last for longer than about 7 days. It is likely that the procedure of acetic acid extraction and salt precipitation as used in the present study removed a precursor pool with a relatively rapid turnover and which was depleted of label within a few days after the rats were removed from the $^{18}O_2$-labelling chamber. Indeed, it is implied and suggested by a number of investigators that much of the precursor pool of collagen is degraded before coming 'mature' collagen (Sodek, 1977; Nimni *et al.*, 1967; Jackson & Bentley, 1960).

A further point should be made here concerning the evaluation of isotope decay data for assessment of collagen turnover. We found that a single exponential equation adequately described the efflux of label from the soluble collagen present in the skins of our rats. However, Sodek (1977) found a complex decay curve, and used the steepest part of the curve to estimate the rate of collagen turnover. Furthermore, Ohuchi & Tsurufuji (1970) and Nimni & Bavetta (1964) also found complex curves, which they considered represented pools with different half-lives. A similar complex curve has been described by Lindstedt & Prockop (1961), on the basis of examination of the labelling pattern of urinary hydroxyproline. Whereas Ohuchi & Tsurufujii (1970) and Nimni & Bavetta (1964) interpreted their findings to represent the result of a change in collagen turnover rate with advancing age of animals, Lindstedt & Prockop (1961) suggested that their observations reflected the different turnover rates of 'soluble' and 'insoluble' collagens. Differences in analysis of the decay data and in the interpretation of decay kinetics, such as these, also contribute to the variations reported in the literature for rates of collagen turnover.

The present methodology also allowed calculation of rates of synthesis *in vivo* as well as of degradation. Soluble-collagen synthesis rates were estimated to be equivalent to 92.0 ± 1.86 μmol of hydroxyproline/day (Table 1). This corresponds to a mean fractional synthesis rate of approx. 3.1%. Laurent *et al.* (1978), using a continuous 6 h intravenous infusion of [^{14}C]proline, estimated the fractional rate of collagen synthesis in skeletal muscles of the adult fowl to be 0.59%/day. Palmer *et al.* (1980) reported fractional synthesis rates of 2.1–9.2%/day for rabbit muscle collagens. Although the present study is not strictly comparable, owing to species and tissue differences, estimates of collagen turnover of similar magnitude to those found in our investigation were obtained in these earlier studies performed *in vivo* involving application of a continuous isotope-infusion procedure.

In conclusion, we confirm that for precise estimation of collagen turnover *in vivo*, especially for the determination of collagen degradation, by using an isotope-decay kinetic procedure, in the skin it is necessary to label hydroxyproline with the non-reutilizable stable isotope $^{18}O_2$. Other probes such as [^{14}C]- or [^{2}H]-proline are extensively reutilized, leading to inaccurate estimates of soluble-collagen turnover. In view of the feasibility of the $^{18}O_2$ procedure for labelling collagen *in vivo* as demonstrated in the present study and previously by Jackson & Heininger (1975), it should now be possible to explore the quantitative effects of various factors as malnutrition, hormones, exercise, aging and injury on the processing and turnover of collagens *in vivo* in various tissues.

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