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Gas chromatography–mass spectrometry determination of $^{18}O_2$ in ^{18}O-labelled 4-hydroxyproline for measurement of collagen synthesis and intracellular degradation

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ABSTRACT

The use of gas chromatography–mass spectrometry (GC–MS) and $^{18}O_2$, a stable isotope which is incorporated into collagen during the post-translational conversion of proline to hydroxyproline, offers the potential advantages of high levels of sensitivity and specificity as compared to other techniques for measuring rates of collagen synthesis and degradation *in vitro* and *in vivo*. Trifluoracetylation and methanol esterification of hydroxyproline yields two derivatives of hydroxyproline: N,O-trifluoroacetyl methyl 4-hydroxy-L-proline (N,O-TFA-Hyp) and N-trifluoroacetyl methyl 4-hydroxy-L-proline (N-TFA-Hyp). In the past, N-TFA-Hyp, which yields the $^{16}O/^{18}O$-containing *m/z* 182/184 ion pair $[M - COOH_3]^{+\cdot}$ when analyzed by electron impact ionization GC–MS, has been proposed for analysis of ^{18}O-enriched collagen. Although N,O-TFA-Hyp can be converted to N-TFA-Hyp by solvolysis, we find that this leads to degradation of the chromatography in GC–MS and demonstrate here that this extra chemical step is unnecessary if the *m/z* 278/280 ion pair (representing the $[M - COOCH_3]^{+\cdot}$ fragment) is measured by selected ion monitoring. By labelling fibroblasts in culture with $^{18}O_2$, a

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sample of isotope-enriched collagen was obtained which was used to calibrate the GC–MS over the range 0.5–49% atom percent enrichment (APE). The greater sensitivity of $^{18}O_2$ *versus* [^{15}N]proline for labelling newly synthesized collagen was demonstrated by the finding of a ten-fold higher enrichment in the former isotope when administered to cell cultures at the same precursor APE. Thus, the approach described herein permits the determination of total hydroxyproline and APE on the same sample avoiding additional processing steps while maintaining the quality of chromatography and the sensitivity of detection. Measurement of absolute rates of both collagen synthesis and intracellular degradation of newly synthesized collagen in cell cultures is thus possible. Preliminary results comparing collagen metabolism in pairs of fibroblasts from hypertrophic scars and normal skin in post-burn patients are presented.

INTRODUCTION

Collagen is an extracellular protein accounting for nearly 25% of total body protein, and its excessive production is a major component of diverse pathologic processes such as pulmonary fibrosis, hepatic cirrhosis, dermal keloids and hypertrophic scarring [1]. Measurement of the rates of collagen synthesis and degradation *in vivo* has been made using the stable isotope of oxygen, $^{18}O_2$, as a precursor for synthesis of hydroxyproline via peptide-bound prolyl hydroxylation by the enzyme proline hydroxylase (prolyl-glycyl-peptide,2-oxoglutarate:oxygen oxidoreductase, 4-hydroxylating, EC 1.14.11.2) [2]. As a tracer for collagen metabolism, $^{18}O_2$ offers the advantages of a non-recycling label uniquely incorporated at a post-translational level [3], for which the precursor pool size is small and rapidly turning over and whose product 4-hydroxy-L-proline (Hyp) is almost unique to collagen and critical for the formation of a stable triple helix [4].

Although measurement of $^{18}O_2$-labelled Hyp turnover as an index of collagen metabolism requires relatively expensive stable isotopes and sophisticated equipment (gas chromatography–mass spectrometry, GC–MS), such technology affords very high sensitivity and a broad dynamic range coupled with a high degree of specificity [5]. As we have previously described [5], two derivatives are formed when Hyp is prepared for GC–MS analysis using the trifluoroacetylation/methylesterification procedure. These are N,O-bis(trifluoroacetyl)-4-hydroxy-L-proline methyl ester (N,O-TFA-Hyp) and N-trifluoroacetyl-4-hydroxy-L-proline methyl ester (N-TFA-Hyp), each with its own chromatographic and fragmentation characteristics on GC–MS [5]. GC–MS analysis of the N-TFA–Hyp results in a large peak ([M − $COOCH_3$]$^{+\cdot}$) containing the ^{18}O-labelled 4-hydroxyl group with *m/z* 182 or 184 as the principal ion. However, similar analysis of N,O-TFA-Hyp results in a base peak of *m/z* 164, which unfortunately does not include the 4-hydroxyl group necessary for kinetic studies of collagen metabolism. Experience has shown the N,O-TFA-Hyp derivative to be the predominant species formed after esterification with methanol and acetylation with trifluoroacetyl anhydride (TFA) [5]. Despite previous reports, it does not yield the *m/z* 182 (or 184) ion which we and previous investigators have employed for measurement of $^{18}O_2$ incorporated into Hyp [2,3,5].

Thus we propose the use of an alternative fragmentation species of N,O-TFA-Hyp characterized by the principal ion *m/z* 278 (or 280): [M − $COOH_3$]$^{+\cdot}$. This method allows determination of absolute concentration of Hyp as well as the measurement of enrichment of $^{18}O_2$ using selected ion monitoring (SIM) and GC–MS of the single derivatization species, N,O-TFA-Hyp. This approach also eliminates the need for extra steps to convert N,O-TFA-Hyp to N-TFA-Hyp in which losses occur, and the quality of chromatography is reduced, possibly due to binding of the underivatized polar hydroxyl group to the stationary phase of the capillary columns used for GC–MS analysis despite previous reports [6]. Additionally, we describe calibration of the method using ^{18}O-labelled Hyp derived from collagen synthesis in a cell culture system and compare the results to a proline tracer, [^{15}N]proline. Preliminary results using this stable-isotope GC–MS technology for collagen synthesis and intracellular degradation in hypertrophic scar and normal fibroblast pairs are presented.

EXPERIMENTAL

Materials

5-Hydroxy-L-pipecolic acid hydrochloride (5-OH-PA), L-pipecolic acid, *trans*-4-hydroxy-L-proline (Hyp) and Amberlite IRA-93 ion-exchange resin were obtained from Sigma (St. Louis, MO, USA), TFA was from Pierce (Rockford, IL, USA), methanol (99.9 mol %) and ethyl acetate were from Fisher Scientific (Nepean, Canada) and acetyl chloride and 2,2-dimethoxypropane were from Aldrich (Milwaukee, WI, USA). Type I collagen standard was obtained from Johnson and Johnson (Boston, MA, USA). All solvents were of analytical grade. Esterification reagent (methanol–HCl, 4:1 molar ratio) was prepared by adding one volume of acetyl chloride dropwise to two volumes methanol at 4°C. Water was removed from Amberlite IRA-93 resin by washing repeatedly with absolute methanol followed by drying in a centrifugal evaporator (Speed-Vac, Savant Instruments, Farmington, NY, USA). $^{18}O_2$ gas (62.47% ^{18}O) was obtained from Cambridge Isotopes (Cambridge, MA, USA) and [^{15}N]proline (99% abundance) was obtained from Merck Sharp and Dohme (Montreal, Canada).

Preparation of ^{18}O-labelled hydroxyproline

Sited-matched, paired hypertrophic scar and normal skin specimens were obtained under local anesthesia from two thermally injured patients after informed consent. Primary cultures were established by growing the cells on plastic flasks (Costar, Cambridge, MA, USA) in Dulbecco's modified Eagle's medium (DMEM), supplemented with 10% fetal calf serum (FCS), 50 U/ml penicillin, 50 μg/ml streptomycin, 5 μg/ml chlorotetracycline hydrochloride and 25 U/ml mycostatin [7]. All explants were kept under 95% air–5% CO_2 and fed twice a week. After five to six weeks nearly confluent cultures of fibroblasts were obtained and then the primary cultures were passed by trypsinization with 0.25% trypsin in phosphate-buffered saline (PBS) solution, and the secondary cultures were maintained in the same medium. Cells were harvested from a confluent growth of six 75-cm^2 flasks (approximately $3.6 \cdot 10^6$ cells) and transferred into a ten-layered cell factory (Nunc, Denmark). Incubation was carried out for a further seven days at 37°C in 5% CO_2–94.9% air at 97% humidity after which time approximately 80% of the surface of the cell factory was covered with a confluent growth of fibroblasts.

Labelling of fibroblasts with $^{18}O_2$

Twenty-four hours prior to labelling of the fibroblasts with $^{18}O_2$, the culture medium was replaced with 1 l of freshly prepared DMEM containing 10% FCS, 50 μg/μl ascorbic acid, 50 μg/μl β-aminoproprionitrile and 0.1 m*M* proline. At 0 h on the day of experiment the gas phase within the cell factory was replaced with 5% CO_2–23% O_2 [62.47% atom percent enrichment (APE) ^{18}O]–72% N_2 by gently blowing the mixture through sterile filters at one end of the cell factory. Gas samples (20 ml) were removed from the cell factory with gas tight syringes and placed in sterile non-siliconized uncoated empty vacutainers (Becton Dickinson Vacutainer Systems, Rutherford, NJ, USA) for measurement of the fractional concentration of oxygen (FiO_2) and $^{18}O_2$ precursor analysis. A 10-ml aliquot of media was withdrawn into sterile syringes through 0.22-μm filters (Millipore, Bedford, MA, USA) and stored in 10-ml screw-capped sterile containers for collagen extraction for serial measurement of Hyp stable-isotope enrichment. Both gas and media samples were withdrawn at 2.0, 4.0, 8.0, 12.0, 20.0 and 24.0 h after the replacement of media and atmosphere with stable isotope-labelled precursors. After 24 h the remaining media were removed (1000 ml) and stored frozen (−70°C) for collagen extraction. In separate experiments, double labelling of cultured fibroblasts was performed with [^{15}N]proline at a final calculated enrichment of approximately 60% APE and total final proline concentration of 0.1 m*M* in the medium, in addition to $^{18}O_2$ as described above.

Measurement of [^{15}N]proline precursor concentrations in medium

Proteins were precipitated from 1-ml medium samples with 0.4 ml of 20% sulfosalicylic acid and removed by centrifuging for 15 min at 10 000 *g*. The supernatants were applied to small ion-exchange columns (Dowex AG-50W-X8, 200–400 mesh) and the amino acids eluted with 3 *M* ammonium hydroxyde [8], dried on a rotary centrifugal evaporator and stored at 4°C until derivatization as described below.

Extraction of collagen

For the large-scale preparation, labelled collagen in the medium was precipitated at 4°C by adding solid ammonium sulfate to a final concentration of 20% and stirred overnight. Precipitated proteins were collected by centrifugation at 12 000 *g* for 30 min, washed with cold 20% ammonium sulfate solution and recollected by centrifugation. The precipitate was stirred in 0.1 *M* acetic acid overnight at 4°C. Undissolved material was removed by centrifugation at 16 000 *g* for 1 h, resuspended in 0.1 *M* acetic acid overnight and recentrifuged. Both supernatants were combined, dialyzed against four changes of water at 4°C over 48 h and freeze-dried.

For the serial analysis of labelled collagen secreted into the media at different times after exposure to $^{18}O_2$, 2-ml medium samples were treated with 4 ml of absolute ethanol for 2 h at 4°C before centrifuging at 2000 *g* for 10 min. Precipitates were dried and processed for hydrolysis and derivatization prior to GC–MS analysis.

Measurement of collagen synthesis and intracellular degradation

Measurement of exact cellular rates of synthesis and intracellular degradation was performed using normal and hypertrophic dermal fibroblast pairs from two different patients at confluence in six-well plates. Before labelling, the media from each well were aspirated, washed once with 1 ml of PBS and replaced with 2 ml of DMEM supplemented with 10% dialyzed FCS, 50 μg/μl ascorbic acid, 50 μg/μl β-aminoproprionitrile and 0.1 m*M* proline. The fibroblasts were exposed to an atmosphere of $^{18}O_2$ (22% FiO_2–5% CO_2–73% N_2) in stainless-steel anaerobic cylinders (Torbal Model AJ3, Baxter Diagnostics, Mississauga, Canada) using four plates in each cylinder at 37°C for 48 h. $^{18}O_2$ and FiO_2 measurements were made immediately after exchanging room air with $^{18}O_2$ and at the end of 48 h exposure. The media and one wash with 1.0 ml of 0.1 *M* acetic acid were aspirated from each of five wells. The cells were harvested by treatment with 0.25% trypsin and 0.02% EDTA for 5 min at 37°C and an aliquot counted using a Coulter Counter (Coulter Electronics, Hialeah, FL, USA). The remaining cells were subjected to three cycles of freezing to −70°C and thawing at 37°C to lyse the cells for measurement of cell-associated collagen. Media and cell lysates were stored at −70°C for ethanol extraction at a later time. Ethanol (67%) extraction of the samples was used to separate high-molecular-mass collagenous and other proteins in the precipitate from low-molecular-mass di- and tripeptides or amino acids in the supernatant as described previously by Bienkowski *et al.* [9]. This extraction allowed measurement of [^{18}O]Hyp in collagen to determine synthetic rates and in low-molecular-mass material containing Hyp to determine rates of intracellular degradation.

Processing collagen for GC–MS analysis

Collagen hydrolysates were prepared as previously described [5,10]. Collagen calibration curves were prepared from a stock solution of Hyp (1.0 μg per μl 0.1 *M* HCl) with sample dilutions made in triplicate in a range from 0.5 to 1000 ng/μl with 5-OH-PA (for the N,O-TFA-Hyp derivative) or pipecolic acid (for the N-TFA-Hyp derivative) as internal standards [5]. Preparation of the N,O-TFA methyl and N-TFA methyl derivatives of Hyp were performed as described earlier [5,6].

GC–MS analysis

GC–MS was performed using a Hewlett-Packard (HP) (Palo Alto, CA, USA) 5890 gas chromatograph and a HP5970 mass-selective detector controlled by a HP5970 Chemstation or a VG

7070E mass spectrometer (VG Analytical, Manchester, UK). Elemental analysis of the derivatives was obtained by high-resolution mass measurement on a VG 7070E mass spectrometer using GC–MS or on a Kratos MS 50 mass spectrometer (Kratos, Manchester, UK) using a direct probe.

Splitless injections of 1.0-μl samples from sealed vials with inserts (Kimble, Vineland, NJ, USA) were made via the HP7673A automatic sampler fitted with a 10-μl Hamilton fixed-needle syringe (Hewlett Packard) onto a 15 m × 0.25 mm I.D. DB-1 (methyl silicone) column, 0.25 μm film thickness (J&W Scientific, Folsom, CA, USA). Calibration of the GC–MS instrument was performed daily using the usertune function of the HP5970 mass-selective detector where the internal standard was perfluorotributylamine, and the calibration curves were adjusted to masses 69, 131, and 219. Chromatographic conditions were as follows: initial temperature, 50°C, 40°C/min from 50 to 175°C, 50°C/min from 175 to 255°C, holding for 1.0 min; injector temperature, 250°C; transfer line temperature, 280°C. Prepurified helium (99.996%) (Linde-Union Carbide, Toronto, Canada) from a cylinder fitted with a high-capacity gas purifier and an OMI-1 indicating purifier (Supelco, Oakville, Canada) was used as a carrier gas at a total flow-rate of 30 ml/min (measured) and a column head pressure of 30 kPa (4 p.s.i.) allowing a volumetric flow-rate through the column of 1.1 ml/min (calculated).

Electron impact (EI) ionization (70 eV) was used with the mass spectrometer in the scanning mode or the selected ion monitoring (SIM) mode detecting ions specific to each of the Hyp derivatives (*m/z* 182, 184 for N-TFA-methyl-Hyp labelled with $^{18}O_2$ and *m/z* 278, 279, 280, 281 for N,O-TFA-methyl-Hyp labelled with [^{15}N]proline and $^{18}O_2$), proline (*m/z* 166, 167 for the N-TFA-methyl derivative of proline), hydroxypipecolic acid (*m/z* 178) and pipecolic acid (*m/z* 180). Peak integration was performed using the software settings of the HP MS Chemstation where peak width was 0.020 arbitrary MS units and threshold was 18 arbitrary MS units. Dwell time in SIM was 100 ms allowing 0.86–1.21 scans/s across the peaks selected. Calibration curves were constructed through a range of Hyp (0.5–1000 ng) and hydrolyzed collagen (0.5–5000 ng). The expressed value for the peak-area ratio was obtained by dividing the Hyp peak area (retention time 1.970 min) by the peak areas of the internal standard, 5-OH-PA (retention time 2.255 min) as previously described [5].

$^{18}O_2$ gas samples were analyzed within 24 h by split injections (split ratio of 100) of 25 μl of gas from each time point sampled via 100-μl pressure lock gas syringes (Dynatech Precision Sampling Corporation, Baton Rouge, LA, USA) onto a HP 5890 gas chromatograph and a HP5970 mass-selective detector controlled by a HP5970 Chemstation equipped with a porous-layer open-tubular fused-silica column (25 m × 0.32 mm I.D., film thickness 30 μm) coated with molsieve 5A (Chrompack, Middelburg, Netherlands). Chromatographic conditions were as follows: initial temperature, 30°C held isothermal throughout the injection; injection port temperature, 50°C, transfer line temperature, 80°C; and column head pressure, 60 kPa (8.5 p.s.i.). SIM of O_2 gas samples was performed by EI at 70 eV monitoring the ions at *m/z* 32, 34, 36 as described above. Samples were run in triplicate and were usually reproducible within 5%, otherwise repeat triplicate analysis was performed.

Calculation of isotopic enrichment and protein turnover

As described previously [5], the enrichment of the stable isotope $^{18}O_2$ in Hyp is determined by SIM of the [M + 0] and the [M + 2] ions corresponding to the nominal mass pairs of 280/278 and 182/180 for the $[M - COOCH_3]^{+\cdot}$ ion. These enrichments as measured on the EI GC–MS system are corrected for the background contribution to the [M + 2] mass species which is made by the natural abundance of isotopic contributions from all atoms in the $[M - COOCH_3]^{+\cdot}$ molecular fragment arising from fragmentation in the mass spectrometer from each distinct parent molecular species for the N,O-TFA-Hyp and the N-TFA-Hyp forms of the TFA derivative of Hyp. The background en-

richment, determined for Hyp in collagen extracted from tissue culture medium samples prior to labelling, was 0.0073 ± 0.0011 (n = 12) for m/z 184, very similar to that found by others [2,3] and 0.0086 ± 0.0036 (n = 12) for m/z 280. The APE for the mth sample was calculated by the formula:

$$APE_m = 100\ (R_m - R_0)/(1 + (R_m - R_0))$$

where R_m is the ratio of isotope over the non-isotopic species of the labelled sample and R_0 is the ratio of the [M + 2] species over the [M + 0] species in the background ion specific for each form of the TFA-Hyp derivative analyzed in the SIM mode by EI GC–MS [11,12]. For analysis of the precursor enrichment of $^{18}O_2$, gas samples injected on the GC–MS system were monitored for the ions m/z 32, 34, 36 and the total enrichment of the $^{18}O_2$ precursor was calculated as above except that the APE expressed is the sum of the APE calculated for the [M + 4] peak and one half the [M + 2] peak which have either a 100 or 50% chance, respectively, of incorporation into Hyp. The [M + 2] peak as measured is an impure tracer consisting of one atom of ^{16}O and one atom of ^{18}O whereas the [M + 4] peak contains two atoms of ^{18}O, either of which can take part in the ordered ter–ter dioxygenase mechanism of reaction well characterized for the enzyme, proline hydroxylase [13,14].

The fractional synthetic rate of collagen (FS) was calculated from the equation:

$$FS = APE_m/(APE_p \times t)$$

where APE_p is the average enrichment of the precursor over the time, t (hours) corresponding to the duration of collagen synthesis occurring in an $^{18}O_2$-enriched atmosphere yielding ^{18}O-enriched Hyp in the mth sample [15]. For six-well plate experiments in which both the APE and the concentration of Hyp in the medium are measured, the absolute rate of collagen production (R) per fibroblast is calculated by:

$$R = (FS \times M)/C$$

where M is the concentration of both labelled and unlabelled Hyp at time t and C is the number of fibroblasts in the culture.

RESULTS

Chromatography of N,O-TFA-methyl- and N-TFA-methyl-Hyp

As illustrated in Fig. 1a, the diacyl derivative, N,O-TFA-methyl-Hyp, has a short retention time (1.989 min) in GC in the total ion scan mode of the GC–MS (Fig. 1b) and contains the characteristic ions (m/z 252, 164, 69) illustrated in Fig. 1a. The structure of the molecular ion of this derivative is illustrated in Fig. 1b (inset) , but is not present in appreciable amounts after EI ionization (Fig. 1a). However, by high-resolution MS using the VG 7070E mass spectrometer, the molecular ion was found to contain two $COCF_3$ groups (337.0377 measured, 337.0385 theoretical). Using SIM, it is also seen to yield the m/z 278 ion ($[M - COOCH_3]^{+\bullet}$) (Fig. 1c) which contains the potentially ^{18}O-labelled hydroxyl group, as confirmed by elemental analysis (278.0253 measured, 278.0254 theoretical) (inset Fig. 1d). This ion was formed at about one sixth the abundance of the base peak (m/z 164), which lacks the hydroxyl group (Fig. 1c) [5]. By selectively monitoring only the ions characteristic for Hyp (m/z 164, 278), much improved and acceptable chromatography can be achieved of the N,O-TFA-Hyp derivative in contrast to the total ion current mode (Fig. 1a), despite two orders of magnitude reduction in signal intensity with SIM (Fig. 1d) and minimal sample "clean-up".

The N-TFA-methyl-Hyp derivative emerges with a later retention time (4.205 min) and the potentially ^{18}O-labelled hydroxyl group resides in the m/z 182 ion fragment ($[M - COOCH_3]^{+\bullet}$), which is the base peak of the chromatogram, as confirmed by high-resolution MS (182.0426 measured, 182.0429 theoretical) (Fig. 2).

SIM of the ion envelope, m/z 278/279/280 from an ^{18}O-unlabelled sample of Hyp allows integration of the area under the curve of the ion containing the ^{18}O isotope for quantitation as illustrated in Fig. 3. Thus, the ability to avoid monitoring the total ion current by SIM of only the ions of the amino acid of interest (N,O-TFA-Hyp) minimizes errors encountered with amino acids which tend to coelute due to the specificity

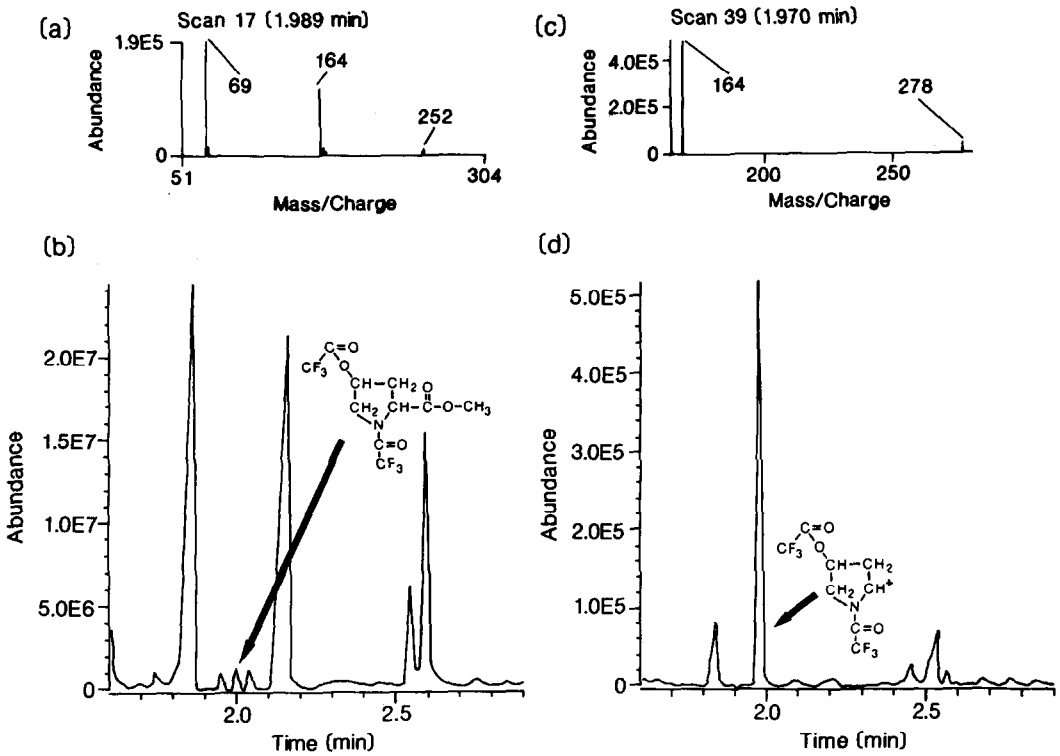

Fig. 1. Fused-silica capillary GC–MS analysis of N,O-TFA methyl esters of ethanol-extracted proteins from media obtained from the cell factory. (a) Mass spectrum of the peak eluting at 1.989 min. (b) Total ion chromatogram of extracted protein hydrolysate. (c) Selected ion monitoring of the peak eluting at 1.970 min for the ions *m/z* 164 and 278. (d) Ion chromatogram of the *m/z* 164 and 278 ions of the sample in (a) using SIM.

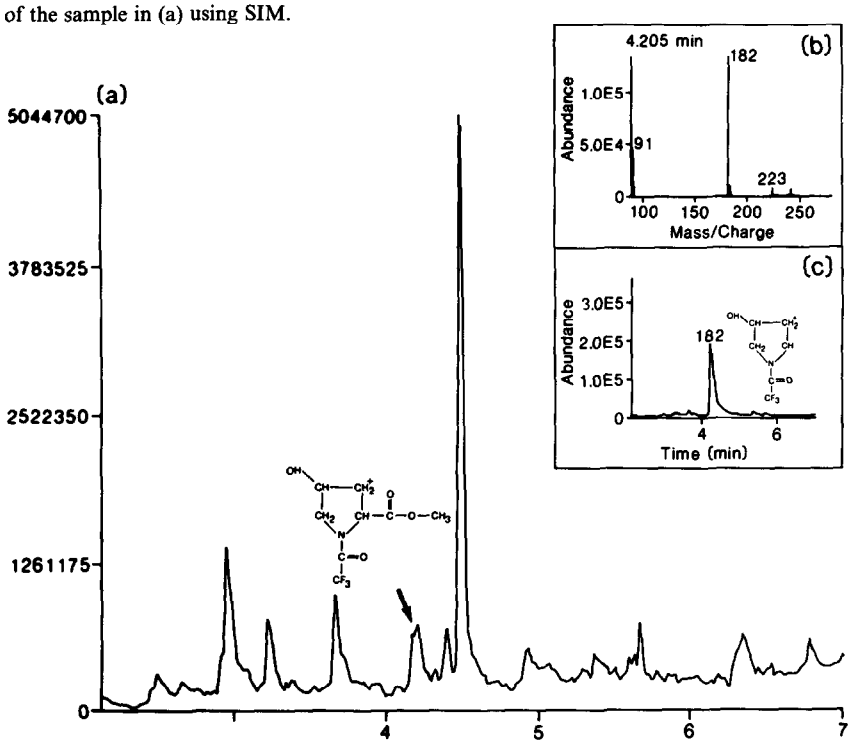

Fig. 2. Fused-silica capillary GC–MS analysis of N-TFA methyl esters of a collagen standard following solvolysis with methanol–Amberlite. (a) Total ion chromatogram. (b) Mass spectrum of the peak eluting at 4.205 min. (C) Selected ion monitoring of the *m/z* 182 ion.

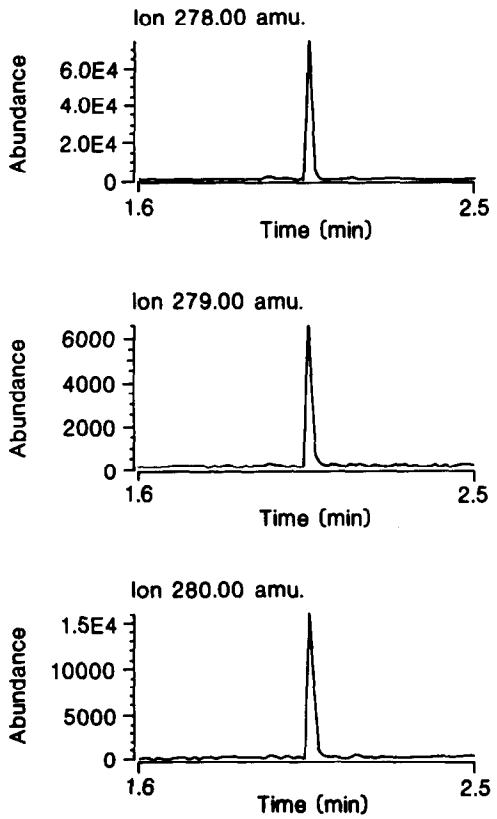

Fig. 3. Selected ion monitoring of the *m/z* ion group 278/279/280 of an ^{18}O-labelled Hyp sample extracted from media *in vitro*, as described earlier.

of the fragmentation profile unique to each amino acid and improves the chromatography considerably particularly for samples containing considerable cellular debris (*e.g.* cell lysates) despite minimal preliminary clean-up procedures. This allows more accurate integration as shown in Fig. 1d *versus* Fig. 1b for total Hyp quantitation [5] and in Fig. 3 for quantitation of ^{18}O-labelled Hyp.

Calibration of GC with ^{18}O-labelled hydroxyproline

GC–MS analysis of collagen synthesized in an $^{18}O_2$ atmosphere was employed to generate hydroxyproline maximally labelled with ^{18}O. After extraction of labelled collagen from the medium and derivatization of hydrolyzed amino acids as described above, a calibration curve was established for the GC–MS detection of the stable isotope through a range of isotopic enrichment. These samples were prepared by serial dilution of the maximally labelled standard which was examined for Hyp concentration and ^{18}O enrichment before dilution with unlabelled hydroxyproline. Determination of the enrichment of isotopically labelled Hyp was reproducible (intra-assay coefficient of variation average 4.5%) and linear through a range of 0–49% APE (the upper limit of our ability to synthesize ^{18}O-labelled collagen), with a tight correlation ($y = 0.26573 + 0.96178x$, $r^2 = 0.999$). The intra-assay coefficient of variation was 5.33% at low enrichments (0.50% APE, $n = 6$) and 1.48% at maximal enrichments of ^{18}O in Hyp (49% APE, $n = 6$).

Correlation of measurements of m/z 182/184 and m/z 278/280 ions

Serially diluted samples of labelled Hyp were split and derivatized as described above to yield the monoacetylated species (N-TFA-methyl-Hyp) or the diacylated species (N,O-TFA-methyl-Hyp). By SIM of the *m/z* 278/280 ion pair from N,O-TFA-methyl-Hyp ($[M - COOCH_3]^{+\cdot}$) and the *m/z* 182/184 ion pair from N-TFA-methyl-Hyp ($[M - COOCH_3]^{+\cdot}$), very good agreement was found between the relative enrichments determined with either method through the range of stable-isotope enrichment 0–15% APE ($y = -0.24683 + 0.97490x$, $r^2 = 0.999$) which were examined.

Measurements of [^{15}N]proline and $^{18}O_2$ precursor levels

By administering [^{15}N]proline and $^{18}O_2$ together to fibroblasts in the cell factory, newly synthesized collagen was labelled at both translational and post-translational steps. Precursor enrichments for each substrate (necessary information for measurements of rates of collagen synthesis) are depicted in Fig. 4. As illustrated, the fractional concentration of ambient oxygen in the culture system and the precursor enrichments for $^{18}O_2$ gas remained relatively constant for the duration of the experiment, as reflected in the low slope of the fitted line. However, the pre-

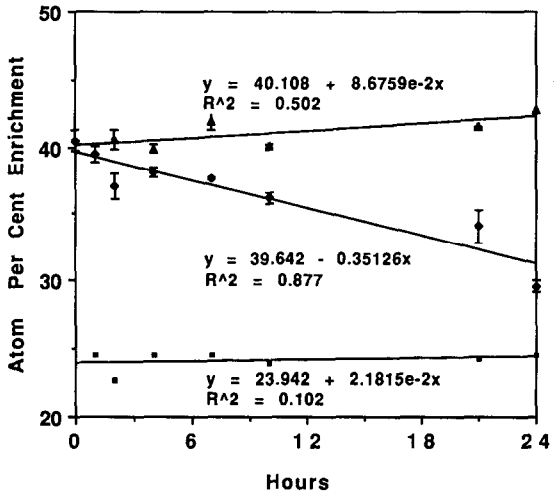

Fig. 4. Analysis of fractional concentration of ambient oxygen and the precursor enrichments in the fibroblast cell factory for $^{18}O_2$ and [^{15}N]proline over 24 h in culture (see text for details). The triangular symbols represent $^{18}O_2$ enrichment (upper line), the diamond symbols represent [^{15}N]proline (middle line), and the small squares represent the fractional oxygen concentration (lower line) within the cell factory.

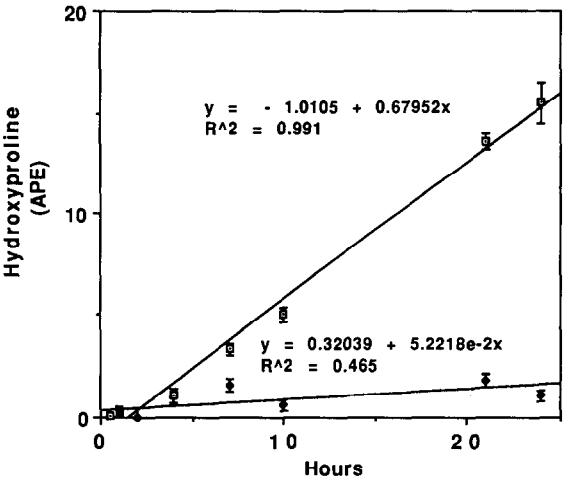

Fig. 5. Rates of incorporation of [^{15}N]proline and $^{18}O_2$ gas into Hyp in 67% ethanol-extracted collagen from tissue culture media over 24 h in normal human fibroblasts *in vitro*. The open squares represent $^{18}O_2$ enrichment (upper line) and the diamond symbols represent [^{15}N]proline in Hyp (lower line).

cursor APE for the proline tracer, [^{15}N]proline, declined in an approximately linear fashion from an original level of 39.9 to 30.1% APE after 24 h.

Measurement of collagen synthetic rates with [^{15}N]proline and $^{18}O_2$

Collagen synthesized *in vitro* demonstrates a rapid incorporation of ^{18}O with a linear increase in enrichment over the duration of the experiment (Fig. 5). Similarly, [^{15}N]proline is incorporated into collagen but the level after 24 h is much lower than that of $^{18}O_2$ (1.09 ± 0.45 *versus* 15.11 ± 1.74% APE) and the linearity of measurement of collagen synthesis using this isotope is much poorer (r^2 = 0.465 *versus* 0.991). This presumably reflects the unique metabolism of each labelled precursor [3,16].

Collagen synthesis and intracellular degradation in hypertrophic and normal dermal fibroblasts

As illustrated in Table I, this methodology allows meaurement of Hyp concentrations in the fibroblast culture medium from six-well plates and also the enrichment of Hyp containing ^{18}O. The product of these two variables is divided by the average precursor enrichment of $^{18}O_2$ and corrected for cell number to yield the amount of newly synthesized Hyp per cell during the 24-h period of isotope exposure. Collagen synthetic rates measured by this method are significantly higher for each hypertrophic fibroblast strain as compared to its site-matched, normal dermal fibroblast control in each of two patients. Rates of intracellular degradation for both normal and hypertrophic fibroblasts varied between 7.9 and 18.5% of the total collagen synthesized during the labelling period and were again significantly higher in the hypertrophic strain in each of the two fibroblast pairs (Table II). These rates of intracellular degradation as measured are of the same order but somewhat lower than that previously published using other methods [8,17].

DISCUSSION

Since the original use of $^{18}O_2$ by Prockop *et al.* [18] to demonstrate the role of molecular oxygen in hydroxylation of peptide-bound proline, the

TABLE I

RATES OF COLLAGEN SYNTHESIS IN FIBROBLASTS FROM SITE-MATCHED NORMAL SKIN (N) AND HYPERTROPHIC SCAR TISSUE (HTS) FROM TWO PATIENTS

Each value is the mean of five wells with the S.D. in parentheses.

Strain	Total Hyp content (pg/cell)	Hyp APE (%)	$^{18}O_2$ APE (%)	Synthesis (pg/cell/24 h)
Patient 1				
N	4.22 (0.12)	13.11 (0.18)	39.32	1.41 (0.02)
HTS	8.81 (1.07)	14.46 (0.40)	39.32	3.24 (0.13)[a]
Patient 2				
N	5.93 (2.02)	28.08 (1.51)	34.12	4.88 (0.49)
HTS	19.89 (1.71)	25.78 (2.26)	34.12	15.03 (0.59)[a]

[a] $p < 0.001$ by paired t-test.

incorporation of one atom of molecular oxygen in Hyp formation and the stability it imparts to newly secreted collagen has been better understood [19]. Proline 4-hydroxylase, a dioxygenase enzyme, incorporates molecular oxygen into collagen by a ter–ter mechanism wherein ordered binding and release of substrates and cofactors from the enzyme occurs [13,14]. The enzyme catalyzes the formation of a reactive intermediate in which oxygen is activated by an Fe^{2+}–α-ketoglutarate complex leading to hydroxylation of the nascent collagen peptide chain, wherein ascorbate is not consumed stoichiometrically but instead functions to reduce Fe^{3+} to Fe^{2+} to reactivate the enzyme [14].

Jackson and Heininger [3] and more recently Molnar *et al.* [2], employed $^{18}O_2$ and the TFA derivative of Hyp to demonstrate the efficiency of recycling of proline tracers from catabolized collagen *in vivo* and the much more rapid turnover of collagen measured using $^{18}O_2$ labelling for decay analysis. In these studies, open-tubular

TABLE II

COLLAGEN DEGRADATION IN SITE-MATCHED NORMAL (N) AND HYPERTROPHIC SCAR (HTS) FIBROBLAST PAIRS FROM TWO PATIENTS

Each value is the mean of five wells with the S.D. in parentheses.

Strain	Hyp (pg/cell)	Hyp APE (%)	Degradation (pg/cell/24 h)	% of synthesis[a]
Pair 1				
N	1.82 (0.09)	7.01 (0.68)	0.32 (0.01)	18.5
HTS	2.85 (0.75)	7.64 (0.65)	0.55 (0.07)[b]	14.5
Pair 2				
N	3.60 (0.25)	17.32 (0.97)	0.55 (0.05)	10.1
HTS	9.24 (1.97)	16.16 (2.89)	1.29 (0.15)[b]	7.9

[a] % synthesis is the amount of intracellular degradation divided by the sum of synthesis and degradation for each strain.
[b] $p < 0.001$ by paired t-test.

packed columns were used for Hyp analysis by GC–MS monitoring of the *m/z* 182/184 ion pair of the N-TFA-Hyp derivative. However, the greater abundance of the derivative N,O-TFA-Hyp was not apparent until recently [5]. As described [5], conversion of N,O-TFA-Hyp to N-TFA-Hyp is possible but losses occur and the quality of the chromatography is poorer when compared to the N,O-TFA-Hyp derivative form, likely due to the exposed hydroxyl group of N-TFA-Hyp which delays elution. Thus, the recognition of the less abundant but larger ion derived from N,O-TFA, $[M - COOCH_3]^{+\cdot}$ (*m/z* 278), which contains the hydroxyl group, avoids the need for additional preparative steps, improves the sensitivity and quality of the chromatography, and allows simultaneous quantitation of total Hyp and measurement of $^{18}O_2$ uptake.

As described, very similar measurements of APE of $^{18}O_2$ in Hyp were obtained using either form of the TFA derivative of Hyp. Similarly, serial dilution curves of the $^{18}O_2$-labelled collagen standard demonstrate a linear response in EI GC–MS analysis of ^{18}O in Hyp over a range from 0.50 to 49% APE. Although concern for "spill over" of measurements from one mass to the adjacent mass has been raised by others in stable-isotope analysis, particularly when very high enrichments are achieved [12,20–22], we do not find this to be significant. Thus, our data support the validity of using APE as an expression of stable-isotope enrichment in Hyp, as originally proposed by Schoenheimer and Rittenberg [23]. Many studies using stable isotopes of amino acids infused into the plasma of humans assume that the mass of stable-isotope tracer is negligible relative to the endogenous production of the tracer, leading to small errors in calculating protein turnover rates. Correction of these errors requires extensive mathematical manipulation of the data [24]. $^{18}O_2$ tracer studies *in vitro* and *in vivo* avoid this problem because the tracer is not biochemically identical to the tracee and direct incorporation of a non-recycled tracer into protein product is analyzed [3], avoiding the assumptions inherent in indirect measurements based on plasma flux of tracers at theoretical steady states [25].

Comparison of results of analysis of precursor enrichment for $^{18}O_2$ gas and [^{15}N]proline demonstrates an additional advantage of $^{18}O_2$ for the kinetic analysis of collagen metabolism. Although the fractional concentration of ambient oxygen (0.24) and the APE of $^{18}O_2$ within the oxygen atmosphere remained relatively constant during the 24 h of the experiment (Fig. 4), an apparently linear decay of [^{15}N]proline tracer occurred in the medium. As no exogenous proline was added during the course of the experiment this likely represents dilution of the [^{15}N]proline label by unlabelled proline, the most probable source of which is endogenous synthesis by the fibroblasts in the culture system [26]. Rose *et al.* [27] have demonstrated *in vivo* that under conditions of adequate energy and alternative amino acid supply, proline is a non-essential amino acid.

Its synthesis occurs from either ornithine or glutamate through the common intermediate proline-5-carboxylate, before reduction by proline-5-carboxylate reductase leads to proline formation in an energy-dependent process [26]. Both pathways appear sensitive to substrate and product feedback regulation [16,26]. Fibroblasts may therefore release unlabelled proline into the medium in substantial amounts. This endogenous proline would also dilute the appearance of proline tracers intracellularly, at the site of translation of mRNA to form the α-chains of collagen [25]. In the past, attempts to overcome compartmentalization of amino acid tracers for protein metabolism has led many investigators to employ large "flooding" doses of proline so that extracellular precursor enrichments more closely resemble the intracellular enrichment or specific activity close to the site of collagen synthesis [15,28]. Unfortunately, exogenous proline has been demonstrated to have both inhibitory and stimulatory effects on collagen synthesis depending on the experimental system [15,16,29]. Such potential experimentally induced changes in measurement of collagen kinetics are avoided by the use of $^{18}O_2$ tracers and GC–MS technology.

As illustrated in Fig. 5, there is a marked discrepancy in apparent collagen fractional synthetic rates depending on the form of the tracer ex-

amined. Despite the limitations inherent in proline tracers as discussed, $^{18}O_2$ is a much more sensitive tracer for collagen metabolism. By employing endogenous enzymes *in situ* as intracellular probes, Jones [30] has demonstrated that plasma membranes are not a significant permeability barrier to O_2. Similarly, Erickson *et al.* [31] have concluded that no significant O_2 gradient exists between the extracellular space and the endoplasmic reticulum, the site of hydroxylation of proline in collagen. Thus, the increased sensitivity of $^{18}O_2$ for collagen synthesis measurements likely arises from rapid diffusion into cells, a relatively small and homogeneous intracellular precursor pool, and the post-translational insertion of the label into nascent pro-α-chains just prior to formation of the triple helix and secretion out of the cell.

Bienkowski and co-workers [9,17] have demonstrated that a significant proportion of newly synthesized collagen undergoes intracellular degradation. However, investigations of this process using radioisotopes are difficult due to possible contamination of proline with Hyp and/or spontaneous hydroxylation of proline, limitations in chromatography and possible loss of radiolabel during hydrolysis and processing [17]. Using $^{18}O_2$ methodology with SIM and EI GC–MS of Hyp and the internal standard 5-OH-PA, our preliminary data show an intracellular degradation rate varying between 10.1 and 18.5% for normal fibroblasts (Table II). Insignificant amounts of spontaneous hydroxylation of free proline occurred in the media of wells not containing fibroblasts which were exposed in parallel with fibroblasts *in vitro* to an $^{18}O_2$ atmosphere as described earlier (data not shown). As discussed by Laurent [15,28], this intracellular degradation process may cause an underestimation of total collagen synthesis, although its biologic importance is as yet incompletely understood.

As pointed out by Cohen and Mast [32], the $^{18}O_2$ methodology can potentially be applied in innovative ways to the study of disorders of fibroblast metabolism, not only *in vitro*, but *in vivo* with relative ease on a benchtop mass spectrometer. In the human situation, the disadvantages of the expense and complexity in the stable isotope and GC–MS technology are offset by the safety of stable isotopes, which offer the potential for the investigation of fibroproliferative disorders such as hypertrophic scarring, where no appropriate animal model exists.

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