Prolyl-tRNA-based rates of protein and collagen synthesis in human lung fibroblasts

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Knowledge of the dynamics of collagen turnover requires information regarding rates of synthesis of this group of connective-tissue proteins. The relationship of various amino acid pools to the tRNA precursor pool used for protein synthesis is known to vary between different cell types and tissues, even for essential amino acids. We studied extracellular, intracellular and tRNA-proline pools in cultured human lung IMR-90 fibroblasts to determine the relationship between them as candidate proline precursor pools for total protein and collagen synthesis. Time-course experiments showed that the three proline pools attained distinctly different steady-state specific radioactivities (extracellular > intracellular > tRNA) at the extracellular proline concentration of 0.2 mM. The kinetics of radioisotope incorporation into cell protein and collagenase-digestible protein indicated that the intracellular free proline pool could not be used reliably as a precursor for calculating synthetic rates. However, tRNA-proline behaved isotopically as if it were the precursor and provided synthesis rates 2–3-fold higher than those calculated by using either free proline pool. The incorporation of labelled lysine and leucine was constant over a wide range of extracellular proline concentrations. Fractional rates of protein synthesis based on tRNA-amino acid were the same with [³H]phenylalanine as with [³H]proline. The specific radioactivity of cell-associated hydroxyproline reached a steady-state value 8–10 h after radioisotope administration which matched the mean tRNA-proline specific radioactivity, suggesting that tRNA-proline is not isotopically compartmentalized. A model of cellular proline-pool relationships is presented and discussed.

Interest in the turnover of connective-tissue compartments of mammalian tissues has led to a number of studies of collagen synthesis in intact animals (Jackson & Heininger, 1975; Sodek, 1977; Laurent *et al.*, 1978; Robins, 1979), isolated organs (Reeds *et al.*, 1980), tissue fragments (Bradley *et al.*, 1974, 1975; Collins & Jones, 1978; Phan *et al.*, 1980; Fulmer *et al.*, 1980) and cultured connective-tissue cells such as fibroblasts (Dehm & Prockop, 1971; Peterkofsky, 1972; Faris *et al.*, 1978; Uitto *et al.*, 1979; Breul *et al.*, 1980). Our particular interest has been in connective-tissue turnover of the lung. The focus of our studies has been the cultured lung fibroblast, the cell type which synthesizes the predominant interstitial collagens (types I and III) of the organ and which is considered to play a central role in pulmonary connective-tissue diseases such as fibrosis.

Cellular amino acid pools and their functional relationship to the amino acid pool immediately

available to aminoacyl-tRNA synthetase have been studied in a number of tissues and cell types (Van Venrooij *et al.*, 1974; Airhart *et al.*, 1974, 1981; Regier & Kafatos, 1977; Vidrich *et al.*, 1977; McKee *et al.*, 1978; Watkins & Rannels, 1980). It is clear from those studies that amino acids acylated to tRNA are derived from both external and internal sources, whose relative contributions can change under a variety of experimental conditions. Because of these complexities, the accurate measurement of absolute protein-synthetic rates by isotopic-tracer techniques at least requires the direct analysis of the specific radioactivity of amino acid acylated to tRNA.

Until recently these measurements in tissues and cells have been technically difficult, owing to the small size of the tRNA amino acid pool and its rapid turnover (Lewis & Ames, 1972). It is now possible to measure routinely the specific radioactivity of a particular tRNA amino acid derived from a cell

mass of less than 1 mg of protein, by using a new ultramicro-method developed in this laboratory (Airhart *et al.*, 1979).

Previous studies of amino acid pools have been confined largely to analyses of pool relationships for essential amino acids. Because proline is synthesized *de novo* by mammalian cells, we expected that its cellular pool relationships would be complex. In this study we report that proline used for the biosynthesis of protein and collagen in human lung fibroblasts is largely derived from a non-expandable endogenous cellular source. We also document that absolute rates of synthesis based on non-tRNA proline pools are subject to very large errors in the rate determinations. Finally, a model is presented which describes proline pool relationships in these cells.

A preliminary report of part of this work has been presented at the Annual Meeting of the American Thoracic Society, Washington, D.C., May 1980 (Hildebran *et al.*, 1980).

Materials and methods

L-[5-³H]Proline (14–27 Ci/mmol), L-[2,6-³H]-phenylalanine (57 Ci/mmol), L-[4,5-³H]lysine (25 Ci/mmol), L-[U-¹⁴C]leucine (342 mCi/mmol) and [*N*-methyl-¹⁴C]dansyl (5-dimethylaminonaphthalene-1-sulphonyl) chloride (43.9 mCi/mmol) were obtained from Amersham Corp. (Arlington Heights, IL, U.S.A.).

Cell culture

IMR-90 human foetal lung fibroblasts (Institute for Medical Research, Camden, NJ, U.S.A.) and CHO-K1 cells (kindly provided by Dr. Joan Moehring, Department of Medical Microbiology, University of Vermont), mycoplasma-free, were seeded into 60 mm plastic tissue-culture dishes (Lux, Miles Laboratories, Naperville, IL, U.S.A.) at a density of 3×10^5 – 5×10^5 cells/dish. Cells were grown in Eagle's minimal essential medium (Microbiological Associates, Walkerville, MD, U.S.A.) (7 ml/dish) supplemented with 10% foetal bovine serum and L-glutamine (2 mM). Medium for CHO-K1 cells was supplemented with L-proline (0.2 mM). Cultures were incubated at 37°C in a humidified atmosphere of room air with 5% CO₂ (Forma Incubator model 3314, Marietta, OH, U.S.A.). The cultures were confluent 5–7 days after seeding and received at this time a supplement of ascorbic acid (final concn. 0.1 mM). Isotope-labelling experiments were begun 24 h later. In this study confluency was defined as a monolayer covering the entire surface of the culture dish as judged by phase-contrast microscopy. We found that confluent 60 mm culture dishes contained 2.5×10^6 – 3.0×10^6 cells per dish. All studies were conducted with fibroblasts in the mid-range of their

lifespan *in vitro* (population doubling level = 20–30). Rates of protein and collagen synthesis were normalized to mg of protein content of the washed cell layer (Lowry *et al.*, 1951). Fibroblast cultures analysed at 24 h after confluency consistently contained 400–500 µg of protein (Lowry) per 60 mm dish, with bovine serum albumin as a standard.

Isotope-labelling protocol

The medium of confluent cultures was changed 3 h before the introduction of [³H]amino acid, and consisted of: Eagle's medium, supplemented with 10% foetal bovine serum; 0.1 mM-ascorbic acid; 25 mM-Hepes [4-(2-hydroxyethyl)-1-piperazine-ethanesulphonic acid] buffer, pH 7.4; glutamine (final concn. 2 mM); and unlabelled amino acids at the same concentration used during the radioisotope incorporation. The medium was again changed at the start of the labelling period (2 ml/dish) and was identical with the previous one except that it contained [³H]amino acid. Cultures were analysed and processed as described below at specified times after the introduction of [³H]amino acid.

Analysis of radioactivity in the tRNA-bound pool, free amino pools, protein and collagen

Details of the methods used to isolate different amino acid samples have been described (Airhart *et al.*, 1979). We have modified the rinsing of residual extracellular medium from cell layers to be analysed for tRNA amino acids by substituting ice-cold 165 mM-sodium acetate (pH 5) for ice-cold phosphate-buffered saline (pH 7.4). Stopping cellular metabolic reactions at pH 5 affords greater protection to the ester bond of aminoacyl-tRNA (Andrulis & Arfin, 1979). The washed cell layer to be analysed for tRNA amino acid specific radioactivity was solubilized in 1% sodium dodecyl sulphate buffered with 0.05 M-cacodylate, pH 6.8. The solubilized cell layer was deproteinized by the addition of 88% (v/v) freshly distilled phenol and centrifuged at 10000 g for 15 min at 4°C. Nucleic acids were precipitated from the upper aqueous phase by the addition of 2.5 vol. of ice-cold 95% (v/v) ethanol. Amino acids bound to tRNA were released by the addition of 0.12 M-KOH at 37°C, after which nucleic acids were reprecipitated by the addition of 0.5 M-HCl.

Separate sets of cultures were processed to measure intracellular free amino acid pools and total incorporation of [³H]amino acid into cellular protein and collagen. At the conclusion of the labelling period, the extracellular medium was rapidly aspirated from the cell layer. The dish was immediately dipped through a series of five beakers of ice-cold phosphate-buffered saline to remove residual medium from the cell layer. No radioactivity was detected in the fibroblast intracellular

amino acid pool after a 1–2 s exposure to medium containing [^3H]proline by using this wash procedure.

Ice-cold 10% (w/v) trichloroacetic acid was added to the washed cell layer and the cells were then scraped from the culture dish with a rubber policeman and transferred to a silicone-treated 13 mm $\times$ 100 mm glass tube with a silicone-treated Pasteur pipette. After 1 h on ice the samples were centrifuged at low speed (1500 g, MSE model GT-2 instrument) at 4°C for 10 min, and the supernatant containing free acid-soluble amino acids was removed with a silicone-treated Pasteur pipette. This sample was designated the intracellular pool, and the specific radioactivity of the amino acids was determined as described below. The trichloroacetic acid precipitate was washed three times with ice-cold 5% trichloroacetic acid, dispersed in a bath sonicator (Blackstone Ultrasonics, Sheffield, PA, U.S.A.; model CT-.5,SS-OC) to create a fine suspension and separated into three aliquots. One aliquot of the cell layer was collected on glass-fibre filters (Millipore Corp., Bedford, MA, U.S.A.), and transferred to a scintillation vial containing 0.5 ml of NCS tissue solubilizer (Amersham Corp.). The capped vials were heated to 55°C for 3 h, cooled to room temperature, and 10 ml of Econofluor (New England Nuclear, Boston, MA, U.S.A.) was added to each vial. Radioactivity was determined by scintillation spectrometry. The counting efficiency as determined by an external standard averaged 40%.

The second aliquot of acid-precipitated cell layer was prepared for acid hydrolysis by low-speed centrifugation at 4°C. The supernatant was discarded and residual trichloroacetic acid removed from the pellet by one wash with ice-cold 95% ethanol. The ethanol supernatant was discarded after centrifugation and residual ethanol evaporated under a stream of nitrogen. Hydrolysis was carried out for 18 h in 6 M-HCl (Ultrax; Baker Chemicals, Philadelphia, PA, U.S.A.) at 110°C under vacuum in reusable hydrolysis tubes (MRA Corp., Clearwater, FL, U.S.A.). A sample of hydrolysate was removed and evaporated to dryness in a Speed-Vac Concentrator (Savant Instruments, Hicksville, NY, U.S.A.). The specific radioactivity of [^3H]amino acids in the hydrolysate was determined as described below.

The third aliquot of acid-precipitated cell layer was prepared for digestion with bacterial collagenase (Form III; Advance Biofactures Corp., Lynbrook, NY, U.S.A.) by low-speed centrifugation at 4°C. The supernatant was discarded and residual trichloroacetic acid removed from the pellet by one wash with ice-cold 95% ethanol. After centrifugation the ethanol supernatant was discarded and residual ethanol evaporated under a stream of nitrogen. The washed pellet was resuspended in 0.1 ml of collagenase buffer solution (2.5 mM-CaCl₂/

12.5 mM-*N*-ethylmaleimide/0.3 M-Tris, pH 7.6) with a bath sonicator and then heat-inactivated at 100°C for 10 min. The remainder of the assay followed the method of Peterkofsky & Diegelmann (1971). Incubations were carried out with 25 units (1 unit solubilizes 1 μmol of leucine equivalent/24 h at 37°C) of bacterial collagenase for 3 h in a shaking water bath at 37°C. Protein not digested by collagenase was precipitated at 4°C with 10% trichloroacetic acid. Radioactivity was measured by the same method used above to determine total radioactivity incorporated into protein. Each assay included a positive control substrate, [^3H]proline-labelled rat skin collagen, prepared as described by Newman & Cutroneo (1978), and a negative control, [^3H]leucine-labelled chick skeletal-muscle protein, to ensure that the collagenase retained both activity and specificity. The radioactivity (d.p.m.) of collagenase-digestible cell protein per mg of 'Lowry' protein was used in the calculation of collagen-synthesis rate.

The amount of [^3H]protein secreted by the cell layer was determined by precipitating a sample of the extracellular medium with trichloroacetic acid (final concn. 10%). The acid precipitate was washed and analysed by the same techniques used above in the measurement of total [^3H]proline incorporated into cell protein.

Ultramicro-method of amino acid analysis

Analysis of the specific radioactivity of [^3H]amino acids isolated from the intracellular free pool, extracellular free pool, aminoacyl-tRNA and cell-protein hydrolysate of cultured fibroblasts was carried out by the ultramicro-method described by Airhart *et al.* (1979). [^3H]Amino acids reacted with [^{14}C]dansyl chloride (Dns-Cl) of known specific radioactivity, the dansyl derivatives were separated by two-dimensional ascending t.l.c. and the appropriate spots cut from the polyamide t.l.c. plate. ^3H and ^{14}C were measured in a liquid-scintillation counter (Packard Tri-Carb, model 3320; average counting efficiency with external standards was 37% for ^3H , 40% for ^{14}C , with 14% spill into the ^3H channel). Specific radioactivity of the [^3H]amino acid was calculated by the formula:

$$SA_{aa} = \frac{^3\text{H (d.p.m.)}}{^{14}\text{C (d.p.m.)}} \cdot SA_{\text{Dns-Cl}} \cdot K$$

where SA = specific radioactivity (d.p.m./pmol), K = mol of dansyl group bound/mol of amino acid, and aa = amino acid.

Aside from the advantages of greater speed and economy compared with amino acid analysers, the sensitivity of this ultramicro dansyl method permits the specific radioactivity determination of 1–2 pmol of amino acid.

Calculations

The rate of protein synthesis (total and collagenase-digestible) was calculated by the following equation:

$$R = \frac{\Delta I}{P} \quad (1)$$

where R = rate of total protein or collagenase-digestible-protein synthesis (pmol of amino acid incorporated/h per mg of Lowry protein); ΔI = [^3H]amino acid incorporated into total cellular protein or collagenase-digestible protein between 1 and 2 h of labelling (d.p.m./mg of 'Lowry' protein); P = mean tRNA-amino acid precursor specific radioactivity between 1 and 2 h of labelling (d.p.m./pmol of amino acid).

The fractional rate of protein synthesis was calculated from the following equation:

$$R_f = \frac{\Delta H}{P} \quad (2)$$

where R_f = fractional rate of protein synthesis (a dimension-less fraction per h); ΔH = change in specific radioactivity of a [^3H]amino acid in the cell protein hydrolysate between 1 and 2 h of labelling (d.p.m./h per pmol).

Results

The first objective of this study was to define the relationship of the various proline pools of IMR-90 fibroblasts. Cells were labelled with [^3H]proline at a physiological extracellular proline concentration (0.2 mM), and the specific radioactivities of free extracellular and intracellular proline, the tRNA proline and the cell protein hydrolysate proline were measured over a 4 h period (Fig. 1).

The extracellular proline specific radioactivity decreased at the rate of 1.5%/h during the 4 h labelling period. Thus it appears that these cultured lung fibroblasts are net producers of proline. In each of five subsequent experiments the extracellular proline specific radioactivity decreased at a mean rate ($\pm$ S.D.) of $2.6 \pm 1.1\%$ /h. This fractional rate corresponds to an absolute release of 10.4 nmol of proline/h per dish, or a net proline production by IMR-90 cells of 3.8 nmol/h per 10^6 cells. Kruse & Miedema (1965) reported a net production of 2 nmol of proline/h per 10^6 cells for WI-38 fibroblasts.

The specific radioactivity of cell-associated protein-bound proline was negligible for the initial 15–30 min, before rising to achieve a linear rate of increase by 1 h. The specific radioactivity of intracellular free proline continued to rise over the first 2 h of labelling, reaching a plateau value at 2 h of

Fig. 1. Time-course of specific radioactivities for proline pools in IMR-90 human lung fibroblasts

[^3H]Proline (200 μCi /dish) was added to cultures at zero time (arrow). The proline concentration in the extracellular medium was 0.2 mM. The means of duplicate or triplicate determinations from three separate representative experiments are presented, with data normalized to account for differences in specific radioactivity of proline in the extracellular medium. Extracellular free ($\circ$), intracellular free ($\square$) and tRNA ($\triangle$) proline specific radioactivity plots refer to the left-hand ordinate. The cell-protein ($\bullet$) proline specific-radioactivity plot refers to the right-hand ordinate. The extracellular, tRNA (1–4 h data points) and cell-protein proline plots were determined by least-squares linear regression. The intracellular proline curve was drawn by hand, as was the tRNA curve between 30 and 60 min.

approx. 68% of the extracellular proline specific radioactivity. By contrast, the specific radioactivity of tRNA-bound proline reached a distinctly different plateau value between 30 and 60 min and thereafter appeared to decline slightly, as had been seen with extracellular free proline specific radioactivity. The mean tRNA-proline specific radioactivity was approx. 24% of the extracellular proline specific radioactivity for the next 3 h.

It is significant that the specific radioactivity of the intracellular proline pool did not reach a plateau until well after incorporation of proline into protein was linear. The correspondence between equilibration of the tRNA pool and establishment of linear rates of proline incorporation was much better, but not perfect, at very early labelling times (30–60 min). Further and closer scrutiny of events at these times is necessary to establish whether this discrepancy is apparent or real. The specific radioactivity of tRNA-bound proline reached plateau values between 30 and 60 min, during which time incorporation into protein became linear. Thus the intracellular proline pool did not behave kinetically

as a precursor for proline incorporation into protein, but the tRNA-proline pool appeared to do so.

A separate 24 h labelling experiment with [³H]-proline confirmed the findings presented in Fig. 1 (results not shown). Specifically, the intracellular and tRNA-bound proline pools maintained different steady-state specific radioactivities. After 24 h of labelling with [³H]proline the specific radioactivity of tRNA-bound proline was still less than 25% of the extracellular specific radioactivity, whereas the specific radioactivity of intracellular free proline was nearly equal to that of the extracellular medium.

We next examined the proline pool relationships over a wide range of proline concentrations. This technique, referred to as 'pool expansion' or 'flooding', has been successfully employed to bring tRNA-bound amino acid to the same specific radioactivity as that in the extracellular pool in studies with liver (Khairallah & Mortimore, 1976), heart (McKee *et al.*, 1978) and lung (Watkins & Rannels, 1980). Results of these experiments are shown in Fig. 2. The intracellular/extracellular and tRNA/extracellular specific-radioactivity ratios were constant at concentrations of extracellular proline between 0.2 and 5 mM, but decreased at concentrations lower than 0.2 mM.

Fig. 2. Steady-state proline pool specific radioactivities in IMR-90 human lung fibroblasts as a function of extracellular proline concentration

Cultures were labelled with [³H]proline for 2 h in the presence of a wide range of extracellular free proline concentrations as described in the Materials and methods section. Intracellular free (□) and tRNA (△) proline specific radioactivities were normalized to the extracellular free (○) proline specific radioactivity and plotted as dimensionless ratios. Each point represents the mean of duplicate or triplicate determinations. Curves were drawn by hand. The mean ± s.d. for *n* experiments is shown for the intracellular free and tRNA proline pools at 0.2 mM-proline.

Thus the steady-state specific radioactivities of intracellular free and tRNA-bound proline remained distinctly different from that of extracellular free proline and from each other at all concentrations of proline studied. Subsequent synthesis-rate experiments were carried out with medium containing 0.2 mM-proline, since the tRNA-bound extracellular proline ratio was relatively constant over the range 0.2–5.0 mM extracellular proline and because this represents a physiological concentration of the amino acid.

A separate experiment was performed to determine if protein synthesis was altered over the proline concentration range that we studied. Triplicate fibroblast cultures were labelled with [³H]lysine and [¹⁴C]leucine for 1 h at a variety of extracellular proline concentrations, and incorporation of each labelled amino acid into protein as well as their relative incorporation (³H/¹⁴C ratio) was determined (Table 1). These parameters were not affected by extracellular free proline concentration, and hence we concluded the rate of protein synthesis had not been altered.

Rates of cellular protein (collagen plus non-collagen) and collagen synthesis based on three proline pools (extracellular, intracellular and tRNA-bound) were determined in five experiments in which the extracellular proline concentration was 0.2 mM (Table 2). We chose in all these experiments to measure rates of protein and collagen synthesis between 1 and 2 h after the introduction of the [³H]proline, because the incorporation of [³H]-proline into protein was linear, tRNA-proline specific radioactivity was at a plateau value, and secretion of [³H]protein into the medium was less than 5% of the total cell-associated [³H]protein

Table 1. Incorporation of [³H]lysine and [¹⁴C]leucine into cellular protein of IMR-90 human lung fibroblasts as a function of the extracellular proline concentration
Confluent cultures were labelled with [³H]lysine and [¹⁴C]leucine for 1 h over a 100-fold range of extracellular proline concentration. Total incorporation per dish of the two radioisotopes into cellular protein was measured by collecting trichloroacetic acid-insoluble material on glass-fibre filters as described in the Materials and methods section. Each value is the mean (± s.d.) of triplicate determinations.

Proline concn. (mM)	[³ H]Lysine incorporated (d.p.m./dish)	[¹⁴ C]Leucine incorporated (d.p.m./dish)	³ H/ ¹⁴ C ratio
0.05	4001 ± 210	10 541 ± 422	0.380
0.2	3265 ± 251	8257 ± 1077	0.395
1.0	3915 ± 89	10 277 ± 904	0.381
2.0	3918 ± 65	10 401 ± 339	0.377
5.0	3642 ± 325	9272 ± 748	0.393

(results not shown). The tRNA-proline-based rate was approx. 3.8 times that based on extracellular proline and 2.5 times that based on intracellular free proline specific radioactivities.

We conducted additional studies with the essential amino acid phenylalanine for comparative purposes and to determine if the same rate of protein synthesis could be calculated. Results of these experiments are shown in Table 3. Phenylalanine, at an extracellular concentration of 0.5 mM, demonstrated an intracellular/extracellular ratio of near unity, indicating complete isotopic equilibrium. However, the tRNA/extracellular ratio of 0.36 indicates that under steady-state conditions nearly two-thirds of the phenylalanine charged to tRNA was not derived from the intracellular free amino acid pool. The fractional rates of protein synthesis

based on tRNA proline and phenylalanine, however, were very similar.

Separate experiments using the CHO-K1 proline-auxotrophic cell line and [^3H]proline were also conducted to test the hypothesis that prollyl-tRNA of cells lacking the pathway for proline synthesis *de novo* would receive a higher proportion of proline from the extracellular medium. At an extracellular free proline concentration of 2.0 mM, the extracellular, intracellular and tRNA proline pools of CHO-K1 cells were completely equilibrated. Equilibration of all proline pools in CHO-K1 cells indicates that an artifact owing to analytical techniques is an unlikely explanation of the tRNA-proline specific-radioactivity measurements in IMR-90 cells. The fractional rate of synthesis in CHO-K1 cells, based on tRNA-proline, was 0.041/h.

The interpretation of synthesis-rate measurements from studies with tRNA-bound labelled amino acid requires the isotopic homogeneity of the tRNA-bound amino acid. Procollagen, the intracellular precursor protein of extracellular collagen, turns over rapidly, owing to secretion (Kao *et al.*, 1977; Peterkofsky, 1972) and intracellular degradation of newly synthesized procollagen (Bienkowski *et al.*, 1978a,b). Hydroxyproline is a good marker for collagen, since it is present in very few other proteins (Bradley *et al.*, 1974). Therefore it is reasonable to expect that the specific radioactivity of cell-protein hydroxyproline would rapidly attain a steady-state value or plateau value equal to that of the nascent procollagen chains. Booth *et al.* (1980) have demonstrated that cellular non-diffusible [^3H]-hydroxyproline radioactivity reaches a plateau between 8 and 12 h in confluent human skin fibroblasts.

Fibroblast cultures were labelled with [^3H]proline at an extracellular concentration of 0.2 mM for up to 24 h. The cell protein (hydrolysate) was then

Table 2. Rates of protein and collagen synthesis in IMR-90 human lung fibroblasts

Cultures were labelled for 1 and 2 h with [^3H]proline as described in the Materials and methods section at an extracellular proline concentration of 0.2 mM. The calculated rates (mean $\pm$ S.D.) were measured in five experiments and are based on the mean tRNA, extracellular and intracellular proline pool specific radioactivities as described in the Materials and methods section (see eqn. 1).

Proline pool used as precursor	Rate of synthesis (pmol of proline/h per mg of protein)	
	Collagen + non-collagen cellular protein synthesis	Cellular collagen synthesis
tRNA proline	11010 $\pm$ 970	3140 $\pm$ 530
Extracellular free proline	2860 $\pm$ 250	820 $\pm$ 140
Intracellular free proline	4340 $\pm$ 380	1240 $\pm$ 210

Table 3. Amino acid pool relationships and fractional rates of protein (collagen + non-collagen) synthesis based on the tRNA-amino acid precursor

Cultures of IMR-90 human lung fibroblasts and CHO-K1 cells were labelled with various [^3H]amino acids as described in the Materials and methods section for 1 and 2 h. Ratios of steady-state amino acid pool specific radioactivities were calculated between 1 and 2 h of labelling. The fractional rates of protein synthesis ($\pm$ S.D.) were calculated as described in the Materials and methods section (see eqn. 2). Numbers of separate experiments are shown in parentheses, but both values are listed where experiments were done twice. Duplicate or triplicate determinations were made in each experiment.

Cell line	Extracellular amino acid concn. (mM)	Specific-radioactivity ratio		Fractional rate based on tRNA amino acid (h^{-1})
		Intracellular	tRNA	
IMR-90	Proline (0.2)	Extracellular	Extracellular	
		0.66 $\pm$ 0.11 (8)	0.26 $\pm$ 0.05 (7)	0.054 $\pm$ 0.018 (6)
	Phenylalanine (0.5)	0.97 $\pm$ 0.03 (3)	0.35, 0.37	0.052, 0.067
CHO-K1	Proline (2.0)	1.00	0.96	0.041

Fig. 3. Independent verification of the steady-state tRNA-proline specific radioactivity by comparison with the plateau specific radioactivity for hydroxyproline in cellular protein

Cultures of IMR-90 human lung fibroblasts were labelled with [^3H]proline as described in the Materials and methods section for up to 24 h. The extracellular proline concentration was 0.2 mM. Hydroxyproline specific radioactivity (●) in cellular protein was measured in duplicate or triplicate for each point. The corresponding curve was drawn by hand for points between 0 and 8 h, and the plateau value (240 d.p.m./pmol) represents the mean of five points between 7 and 24 h. The mean tRNA-proline specific radioactivity (260 d.p.m./pmol) is indicated by the arrow on the ordinate. The stippled region indicates ± 1 s.d. of the tRNA-proline measurements. Data shown are from seven experiments, with all points normalized to an extracellular proline specific radioactivity of 1000 d.p.m./pmol.

analysed at various times for the specific radioactivity of hydroxyproline. Our results are presented in Fig. 3, with all data normalized to an extracellular proline specific radioactivity equal to 1000 d.p.m./pmol. The specific radioactivity of cell-protein hydroxyproline attained a plateau value at 8–10 h and was the same as tRNA-proline specific-radioactivity measurements. We also observed in this experiment that cell-protein proline specific radioactivity also attained a plateau value which closely matched the tRNA-proline specific radioactivity (results not shown).

Discussion

The purpose of this study was to define the relationship between cellular proline pools in IMR-90 human lung fibroblasts, to establish methods for accurate determination of rates of protein and collagen synthesis. Our most important conclusion is that analyses of tRNA-bound proline are essential to avoid substantial errors in determining synthesis rates in this cell line.

A number of our observations illustrate the complexity of proline precursor-pool relationships, as well as indicate the validity of using the general tRNA-proline pool as a legitimate precursor for measuring synthesis. (a) At a physiological concentration of extracellular proline (0.2 mM), the intracellular free and tRNA-proline pools attain distinctly different steady-state specific radioactivities, both substantially below the specific radioactivity of free proline in the extracellular medium. (b) One cannot force isotopic equilibration of the intracellular or the tRNA-proline pools with extracellular free proline by raising the concentration of proline in the extracellular medium (to 5 mM) (Fig. 2). (c) Large differences in synthesis rates based on the intracellular free proline and tRNA-proline occur at all extracellular proline concentrations (Fig. 2). (d) The intracellular free proline pool does not behave as an appropriate precursor pool for protein synthesis, on the basis of kinetic data (Fig. 1). (e) Rates of synthesis measured with labelled proline and phenylalanine are the same when both are based on the specific radioactivity of the appropriate tRNA-bound amino acid. (f) The steady-state specific radioactivity of protein-bound hydroxyproline, representing newly synthesized collagen, matches steady-state tRNA-proline specific radioactivity (Fig. 3); similarly, the specific radioactivity of proline in total protein approaches a plateau value equal to tRNA-proline specific radioactivity after 24 h of labelling.

Our conclusions regarding lung fibroblasts are in fundamental agreement with previous studies of protein synthesis in which specific radioactivity of tRNA-bound amino acid was measured. These studies represent a diversity of cell types and tissues: HeLa cells (Van Venrooij *et al.*, 1974), sea-urchin embryos (Regier & Kafatos, 1977), rat liver *in vivo* (Airhart *et al.*, 1974; Vidrich *et al.*, 1977), rat heart *in vivo* or perfused (Martin *et al.*, 1977; McKee *et al.*, 1978), perfused lung (Watkins & Rannels, 1980), alveolar macrophages (Airhart *et al.*, 1981) and cultured skeletal-muscle cells (Schneible *et al.*, 1981). In all cases, the general tRNA-bound amino acid pool appears a legitimate precursor for protein synthesis, whereas the extracellular and intracellular free amino acid pools can be used only when all pools, including the tRNA pool, are in or can be expanded to isotopic equilibrium. The above studies involved use of essential amino acids. We expected that added complexities would be revealed when the non-essential amino acid proline was studied in similar fashion.

Indeed, previous work utilizing labelled proline as a precursor for protein synthesis predicted, indirectly, our conclusion that only prolyl-tRNA is an appropriate precursor. It has been shown for muscle (Kipnis *et al.*, 1961), HeLa cells (Robertson &

Wheatley, 1979) and calvaria (Phang *et al.*, 1971; Finerman *et al.*, 1967) that the intracellular proline pool does not equilibrate isotopically until well after incorporation of radioactive proline into protein is linear. Robins (1979) measured the specific radioactivity of several amino acids in skin procollagens *in vivo* after their radioisotopic content had reached steady state, and concluded that the tissue free proline pool was not a satisfactory precursor. Finally, Smith & Phang (1978) have shown ornithine to be the precursor for a substantial fraction of proline incorporated into protein by cartilage, regardless of the extracellular concentration of proline.

The rate of synthesis of total protein and collagen that we report for IMR-90 cells is 3–10-fold higher than those previously reported for cultured lung fibroblasts (Breul *et al.*, 1980) or for minces of freshly isolated lung tissue (Bradley *et al.*, 1974). The discrepancy is most easily explained by the use in those studies of the free intracellular or tissue proline pool as radioisotopic precursor. An added complication in those studies was the use of very low extracellular proline concentrations (0.02–0.08 mM), where the specific radioactivity of tRNA-proline would be affected substantially by proline output (see the Results section, and Smith & Phang, 1978). Our own preliminary studies of HFL-1 cells confirm these impressions (J. N. Hildebran & R. B. Low, unpublished work). In the present paper we have validated the tRNA-proline precursor for protein synthesis by using the approach of Mortimore *et al.* (1972), in which we altered bulk flow of proline from various sources under conditions in which rates of protein synthesis were not altered. Prior justification for using the intracellular free proline pool as precursor in fibroblasts (Breul *et al.*, 1980), on the basis of this approach, is difficult to interpret, because the proline content of the intracellular pool was not altered in those studies.

It is possible that the tRNA-proline used for collagen synthesis has a specific radioactivity different from that of total prolyl-tRNA, because, for example, only one isoaccepting species is used for collagen, and this species is charged from a unique radioisotopic source (Carpousis *et al.*, 1977; Drabkin & Lukens, 1978). Our results, showing equivalence of tRNA-proline specific radioactivity with that of protein-bound proline or hydroxyproline when the latter two have reached steady-state radioactivity, indicate that the general tRNA-proline pool can be used. Nonetheless, studies of nascent chains and distinct isoaccepting species of prolyl-tRNA will be required to resolve this issue completely.

Wheatley & Inglis (1980) have proposed an intracellular amino acid-perfusion-system model for protein synthesis in which the intracellular free

amino acid pool does not contribute to the precursor pool for protein synthesis. The findings of our study fit this model, but in addition point to a dominant endogenous source of proline which is functionally tightly linked to the charging of tRNA (Fig. 4). We have not distinguished between slowly and rapidly equilibrating intracellular pools of amino acids, as have Wheatley & Inglis (1980). The source of endogenous proline could be from synthesis *de novo* and/or proteolysis. Smith & Phang (1978) have demonstrated in rat xiphoid cartilage that a constant proportion of total proline incorporation into protein is derived from ornithine over a wide range of extracellular proline concentrations. Our data show that, even at very high extracellular concentrations of proline (i.e. 5.0 mM), the protein-

Fig. 4. Model of proline pools and their relationship to protein synthesis in IMR-90 human lung fibroblasts. Extracellular free proline associates with a receptor site (RS) on or near the plasma membrane. Nearly all RS-proline complexes dissociate and the free proline enters the intracellular pool. A small fraction of the RS-proline complexes are functionally coupled to the site of acylation, prolyl-tRNA synthetase. The major contributor to precursor proline molecules, however, is the endogenous source of proline, as indicated by the relative size of the arrows to the site of acylation. Proline derived from these two sources (internal and external) now acylated to the appropriate tRNA is incorporated into protein. The endogenous source of proline (synthesis *de novo* and/or proteolysis) is the secondary contributor of proline molecules to the intracellular free proline pool, as indicated by relative size of the arrows. The intracellular free proline pool exchanges with the extracellular free pool, and is a positive net contributor of proline to the external medium.

synthetic precursor pool is not in isotopic equilibrium with the external medium. Both of these observations suggest that newly synthesized proline may be 'sheltered' by compartmentation and functionally linked very closely to the acylation of tRNA.

Protein degradation could contribute substantial amounts of free proline to the cell in our studies (see Bienkowski *et al.*, 1978a,b), especially since the cells were approaching the stationary phase, during which rates of synthesis and degradation are balanced. It should be recalled, in this regard, that a significant proportion of phenylalanine used to charge tRNA can come from an endogenous source (Table 3), which must be degradation, since synthesis of phenylalanine *de novo* does not occur. Our findings, using CHO-K1 cells, a proline-auxotrophic line, suggest that if proline from proteolysis is a source of proline for acylation to tRNA, it does not reside in a 'sheltered' cellular compartment. This may also be true of other amino acids, because we have found that the leucine pool used to charge tRNA in IMR-90 fibroblasts can be expanded to equilibrate with both the intracellular and extracellular pools (J. N. Hildebran, unpublished work).

The complexity of the pathways from which the intracellular free proline pool receives proline would suggest a high degree of flexibility in the source of cellular proline. Indeed, we found the intracellular/extracellular steady-state proline specific-radioactivity ratio to be far more variable than that in the tRNA pool (see Fig. 1). It has been shown in liver that valine intracellular/extracellular and tRNA-bound/extracellular ratios change as a function of plasma valine concentration, in opposite directions in some cases (Vidrich *et al.*, 1977). Robins (1979) also noted a large variation in the ratio of skin-homogenate free proline to the proline precursor pool for rabbit skin procollagen synthesis. Finally, the finding of Breul *et al.* (1980) that the contribution of proline from the extracellular to the intracellular pool changes 2–3-fold as fibroblasts shift from exponential growth to the stationary phase indicates a high degree of variability for proline flux into the intracellular pool.

In summary, direct measurement of the obligatory precursor for protein synthesis, tRNA-proline, appears to be required for the accurate determination of synthesis rates in cultured lung fibroblasts. Our findings indicate that cellular amino acid-pool relationships are complex, and that proline acylated to tRNA is derived from cellular sources very different from the general intracellular pool of free amino acids as conventionally isolated.

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