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Synthesis of Proline and Hydroxyproline in Human Lung (WI-38) Fibroblasts

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Human lung fibroblasts (WI-38) in late exponential phase of growth, in stationary phase after confluency was reached, and at high or low number of population doublings were used to investigate the synthesis of proline and hydroxyproline from glutamate or arginine. Glutamate was from two to five times as effective a precursor as arginine; glutamine did not seem to be involved in these metabolic pathways. Accumulation of protein-bound hydroxyproline in cell layers was observed only after confluency. Confluent cells synthesized more proline from glutamate than did cells in late exponential growth. Conversion of glutamate into intracellular free proline was conducted also to a greater extent in confluent cells at a high number of population doublings. Conversion of glutamate into proline or hydroxyproline in cell-layer protein was not affected significantly by the number of population doublings. Less total protein as well as less hydroxyproline accumulated with cells at a high number of population doublings.

Proline is a non-essential amino acid in animals (Womack & Rose, 1947) and can be synthesized from either arginine or glutamate (Roloff *et al.*, 1940; Womack & Rose, 1947; Sallach *et al.*, 1951; Stetten, 1951; Depocas & Bouthillier, 1951). The relative contributions of these two precursors to proline synthesis in various tissues have not been explored thoroughly but some data are available. In mammalian cell cultures which are grown in media lacking proline, the major precursor of proline appeared to be glutamate or glutamine as judged by nutritional experiments and data with radioactively labelled compounds (Kruse, 1961; Eagle & Piez, 1962; Kao & Puck, 1967). However, osteoblasts in tissue culture were unable to use glutamate for the synthesis of hydroxyproline in collagen (Smith & Jackson, 1959), and arginine and ornithine were better precursors than glutamate for free proline, collagen proline or hydroxyproline in chick-embryo legs (Zinker & Rojkind, 1972). Normal rat liver preparations also incorporated very little glutamate into proline whereas cirrhotic rat liver showed a 100-fold increase in this incorporation (Rojkind & Diaz de Leon, 1970).

These diverse results in determining the metabolic precursors of proline indicate that the relative contributions of glutamate and arginine to proline synthesis is variable according to the tissue examined or to the metabolic state of the tissue. It is conceivable, for example, that collagen-forming cells compared with other cells utilize one precursor better than another. The possibility that collagen and non-collagenous proline might arise from different precursor amino acid pools was rejected by Phang *et al.* (1971). Nevertheless, data in the literature suggest that the amount of free proline available may be a limiting factor in the synthesis of collagen (Finerman *et al.*, 1967; Rojkind & Diaz de Leon, 1970; Kershenobich *et al.*, 1970; Phang *et al.*, 1971).

To obtain additional information on these questions WI-38 fibroblasts were used to examine the relative contributions of glutamate and arginine to proline and hydroxyproline synthesis. These cells accumulate substantial amounts of collagen only after confluency is reached and have been much studied as models for the aging process. The present paper provides data on the synthesis of proline and hydroxyproline according to the stage of the growth curve and the number of population doublings achieved.

Experimental

Materials

Incubation media (BME-Gibco G-13) were obtained from Grand Island Biological Co., Grand Island, N.Y., U.S.A.; Hepes* buffer was from Sigma Chemical Co., St. Louis, Mo., U.S.A.; penicillin and streptomycin were from Pfizer, New York, N.Y., U.S.A.; Sephadex was from Pharmacia Fine Chemicals, Uppsala, Sweden; ion-exchange

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* Abbreviation: Hepes, 2-(*N*-2-hydroxyethylpiperazin-*N'*-yl)ethanesulphonic acid.

resins were from Bio-Rad Laboratories, Richmond, Calif., U.S.A. All other reagents and chemicals were of high purity obtained from commercial sources. Aqueous solutions were prepared with water that was deionized and twice-distilled.

Cell cultures

WI-38 fibroblasts of various 'passage number' were obtained from the laboratory of Dr. L. Hayflick, Stanford University, Calif., U.S.A. The cells were maintained as a routine at 37°C in Eagle's basal medium (BME-Gibco G-13) (Eagle, 1955) containing 25mM-Hepes buffer, pH7.2, 10% (v/v) foetal calf serum, 100μg of streptomycin per ml and 100i.u. of penicillin per ml. The cells were subcultured, after reaching confluency, by a slight modification of the method of Hayflick (1965) by using a 1:4 split ratio. For the experiments described below, cells at late exponential phase or at confluency were incubated with fresh medium as above except that the foetal calf serum was freed of amino acids by passage through Sephadex (Piez *et al.*, 1960).

Experimental incubations and preparation of fractions for amino acid analysis

Ascorbic acid, $FeSO_4$ and labelled amino acid were added as described in the Results section and incubations continued for 24h. The medium was then drained off. The residual cells in the flask were chilled in ice and washed with successive portions of 10ml and 5ml each of ice-cold Hanks balanced salt solution which was free of Ca^{2+}, Mg^{2+} and Phenol Red (Grand Island Biological Co.). The combined medium and washings were treated with trichloroacetic acid to a final concentration of 10% and precipitated protein was removed by centrifugation (3000rev./min for 15min). The cells in the flask were harvested with 10–15ml of ice-cold 10% (w/v) trichloroacetic acid by using a rubber 'policeman'. The suspension in trichloroacetic acid was centrifuged (3000rev./min for 15min) to separate the suspension into trichloroacetic acid-soluble and -insoluble components. This procedure is essentially that described by Eagle *et al.* (1961) and was shown to retain protein in the residual cell pellet. The trichloroacetic acid-soluble fractions were used for determination of intracellular amino acid concentrations. The trichloroacetic acid was removed by repeated extraction with about 3vol. of ether each time. After the third or fourth extraction the aqueous layer was essentially free of acid. The trichloroacetic acid-insoluble precipitate was washed twice with 95% (v/v) ethanol containing 1% (w/v) potassium acetate, twice with ethanol–ether (3:1, v/v) and twice with ether, by the procedure of Munro & Downie (1964). The residual material was dried in a vacuum desiccator and designated cellular protein. The protein was hydrolysed by heating with 6M-HCl for 24h at 110°C in a sealed ampoule. The HCl was then removed by evaporation *in vacuo* at 45°C. The residue was dissolved in water.

Determination of amino acids

Proline and hydroxyproline. Suitable portions of the solutions containing these amino acids (trichloroacetic acid extract or protein hydrolysate) were applied to a column (1cm×15cm) of Dowex AG 50W (X8; H^+ form; 200–400 mesh). The column was eluted with 1.5M-HCl. The fractions containing hydroxyproline and proline were collected and combined separately. The eluate volumes which contained these amino acids were identified by colorimetric procedures (Troll & Lindsley, 1955; Bergman & Loxley, 1963), and the elution procedure was standardized so that the elution volumes containing these amino acids were reproducible for all experiments. The eluates were evaporated to dryness *in vacuo* at 45°C and the residues dissolved in water. The resultant solution containing hydroxyproline was applied to a column (1cm×10cm) of Dowex AG 1 (X8; acetate form; 200–400 mesh) and eluted with water.

Proline content of the appropriate solution eluted from Dowex AG 50W (X8) was determined by the procedure of Troll & Lindsley (1955) except that the Permutit treatment was found to be unnecessary with this partially purified product. Proline radioactivity was determined after applying a known amount of the proline-containing fraction to a thin layer of cellulose and separating it from contaminants with a solvent system of ethanol–chloroform–17% (v/v) NH_3 (17:17:6, by vol.). The cellulose area containing the proline was removed by aspiration, eluted with water and radioactivity determined. Standard solutions of proline of known radioactivity treated in a similar manner were reproducibly determined with a recovery of 85%. Analysed samples from experimental incubations were corrected for this recovery.

Hydroxyproline content of the appropriate solution collected from Dowex AG 1 (X8) was determined by procedure A of Bergman & Loxley (1963). Hydroxyproline radioactivity was determined after separation from other contaminants by t.l.c. as for proline, but by using aq. 77% (v/v) ethanol as developing solvent.

Other amino acids. Glutamate was separated by applying suitable portions of solutions, adjusted to pH6–7, to a column (1cm×10cm) of Dowex AG 1 (X8; acetate form; 200–400mesh), followed by elution with 50ml of water to remove basic and neutral amino acids. The glutamate was eluted with 0.05M-acetic acid, and quantitatively determined by the ninhydrin reaction (Rosen, 1957). No other

amino acid was present in this fraction as determined by t.l.c.

Glutamine was determined by first hydrolysing it to glutamate by heating with 2M-HCl at 110C° for 2h. The hydrolysate was evaporated *in vacuo* at 45°C and glutamate separated and determined as described above. Correction was made for the glutamate present before hydrolysis.

Arginine was determined by applying suitable portions of solutions to a column (1cm×5cm) of Dowex AG 50W (X8; H^+ form; 200–400mesh), followed by 90ml of 1.5M-HCl to remove ornithine as well as acidic and neutral amino acids. The arginine was then eluted with 4M-HCl (25–30ml), and the eluate evaporated to dryness *in vacuo* at 45°C. The residue was dissolved in water and arginine determined by a modified Sakaguchi reaction (Gilboe & Williams, 1956).

Radioactive substrates

DL-[5-^{14}C]Glutamic acid and L-[U-^{14}C]arginine were obtained from New England Nuclear Corp. (Boston, Mass., U.S.A.) or Amersham/Searle Corp. (Arlington Heights, Ill., U.S.A.) and purified as follows. Glutamic acid was purified as described by Niwaguchi *et al.* (1965). Arginine was purified by using a column of Dowex AG 50W (X8) as described above. The solution of arginine obtained in this way was then applied to a preparative t.l.c. plate (0.5mm cellulose, Machery–Nagel Co., Düren, Germany) and developed with the solvent system acetone–pyridine–conc. NH_3 (sp.gr. 0.90)–water (6:9:1:4, by vol.). The area containing arginine was collected and eluted with water.

Other methods

Protein was determined by the method of Lowry *et al.* (1951) with crystalline bovine serum albumin obtained from Reheis Chemical Co., Chicago, Ill. U.S.A., as a standard. Radioactivity determinations were conducted with portions dissolved in Bray's (1960) solvent in vials and measured in a liquid-scintillation spectrometer. Counting efficiencies, estimated by the use of internal standards, were 80±5%.

Cell counting

Cells were removed from the bottle by treatment with a solution of 0.05% trypsin, 1:250 (Difco Laboratories, Detroit, Mich., U.S.A.), and 0.02% EDTA and suspended in incubation medium (BME-Gibco G-13). The suspension was appropriately diluted in 0.2% Trypan Blue in 0.9% NaCl, and cell number and viability were determined with the aid of a Speirs–Levy eosinophil counter.

Results

Utilization of glutamate and arginine, and conversion into proline in cells at late exponential phase

WI-38 cells after 23 and 43 population doublings respectively were inoculated into glass Blake bottles after a 1:4 split and growth was monitored until near-confluency was reached. This required about 4 days or 5–6 days for cells after 23 or 43 doublings respectively. The medium was removed and the adherent cells were rinsed twice with 50ml portions of Earle's balanced salt solution (Gibco K-11, Grand Island Biological Co.). Fresh medium (70ml) was then added, containing 10% amino acid-free foetal calf serum and 0.1mM-ascorbic acid with DL-[5-^{14}C]-glutamic acid or L-[U-^{14}C]arginine as indicated in Table 1. Incubations conducted in duplicate were continued for 24h at 37°C. Another Blake bottle of cells treated in the same manner but without labelled amino acid was used for counting the number of cells.

Glutamate is not a good substitute for glutamine in the nutrition of cultured cells (Levintow & Eagle, 1961), but is nevertheless metabolized by a variety of cells (Levintow *et al.*, 1957; Salzman *et al.*, 1958; Kitos & Waymouth, 1966) including WI-38 fibroblasts (Griffiths, 1970). These experiments provided further substantiation for the relatively rapid consumption of glutamate. Although 91% of the total radioactivity added as DL-[5-^{14}C]glutamate was recovered in the medium after incubation, if the assumption is made that only the L form was metabolized, about 60% and 45% of radioactive L-glutamate was consumed during the 24h of incubation in cells which had reached 23 and 43 population doublings respectively (Table 1). Calculations of utilization of glutamate in terms of concentration changes cannot be made owing to the degradation of glutamine to glutamate during the 24h of incubation as well as an unknown extent of turnover with cellular glutamate. Thus the concentration of L-glutamate in the medium initially was determined to be 0.083 and 0.087μmol/ml in cells after 23 and 43 population doublings respectively which increased to 0.40 and 0.57μmol/ml respectively after 24h of incubation. The specific radioactivity changes of the glutamate in the media, shown in Table 1, reflect this dilution.

L-[U-^{14}C]Arginine was also utilized, but it was not metabolized to any greater extent than was glutamate (Table 1). The dilution of the specific radioactivities of arginine during the 24h of incubation, which are the same for cells after 23 and 43 population doublings, indicates that there was some turnover with cellular sources of unlabelled arginine. At the same time net utilization of arginine did occur. During the incubation with cells at 23 population doublings, arginine concentration decreased from 0.083 to 0.057μmol/ml, and in cells at 43 population doublings, the decrease was from 0.085 to 0.067μmol/ml.

Table 1. *Utilization of* L-*glutamate and* L-*arginine in media of cells approaching confluency*

Incubation conditions and treatment of samples have been described in the text. All values are averages for duplicate incubations. Agreement between duplicates was 5% or less. When DL-[5-^{14}C]glutamate was the substrate, the recoveries of radioactivity shown are calculated for L-glutamate assuming that D-glutamate was not metabolized. In these experiments the average number of cells per bottle initially and after 24h of incubation were 22.0×10^6 and 24.8×10^6 respectively.

		$10^{-6}\times$Radioactivity in substrate			
	No. of population doublings ...	23		43	
Substrate	Time of sampling (h)	(c.p.m./bottle)	(c.p.m./μmol)	(c.p.m./bottle)	(c.p.m./μmol)
DL-[5-^{14}C]Glutamate	0	14.98	2.58	13.44	2.21
	24	6.18	0.13	7.43	0.21
L-[U-^{14}C]Arginine	0	11.76	2.03	12.25	2.06
	24	6.56	1.64	7.60	1.64

Table 2. *Incorporation of carbon from* [^{14}C]*glutamate or from* [^{14}C]*arginine into intracellular free and protein-bound proline in cells approaching confluency*

Data refer to analyses made on the cell extracts prepared after the 24h experimental incubations described in Table 1. See legend to Table 1 for further details.

	No. of population doublings ...	23		43	
	Precursor ...	DL-[5-^{14}C]-Glutamate	L-[U-^{14}C]-Arginine	DL-[5-^{14}C]-Glutamate	L-[U-^{14}C]-Arginine
Total free proline (μmol)		0.105	0.122	0.106	0.127
Free proline (μmol/mg of cell protein)		0.011	0.012	0.011	0.009
Total free proline (c.p.m.)		15200	7100	13900	4600
$10^{-6}\times$Free proline (c.p.m./μmol)		0.147	0.058	0.133	0.057
Total protein-bound proline (c.p.m.)		153118	56565	127609	46739
Protein-bound proline (μmol/mg of cell protein)		0.303	0.325	0.286	0.273
$10^{-6}\times$Protein-bound proline (c.p.m./μmol)		0.0533	0.0176	0.0489	0.0204

The intracellular content of proline both free and in protein was quite similar in cells after 23 and 43 population doublings (Table 2). At least twice as much proline was derived from glutamate as from arginine, both in the cellular pool of free proline and in protein-bound proline. The calculations for the percentage conversion of either glutamate or arginine into proline are impossible to make accurately since during the 24h incubation the specific radioactivities of both precursors were altered, especially for L-glutamate. However, calculations of minimum conversions indicate that no less than 6% of the free intracellular proline or 2% of the protein-bound proline was synthesized from glutamate during this period. The corresponding values for synthesis from arginine are 3 and 1% respectively. There was little or no difference between 'young' (23 population doublings) and 'old' (43 population doublings) cells in this regard. Glutamate may be used to a greater degree than the data in Table 2 would grossly suggest, inasmuch as the dilution of radioactive glutamate during incubation was so much greater than the corresponding dilution of arginine (see Table 1). Hydroxyproline could not be determined in these experiments; it was barely detectable by colorimetric procedures applied to solutions of cell-protein hydrolysates.

Utilization of glutamate and arginine in cells after confluency

Judged by the content of hydroxyproline, very little collagen was accumulated before confluency. Therefore WI-38 cells at low and high number of population doublings were incubated in Blake bottles

Table 3. *Utilization of* L-*glutamate and* L-*arginine in media of cells after confluency*

The number of cells per bottle were 59×10^6 and 25×10^6 for cells after 26 and 47 population doublings (Expt. 1). The corresponding counts for Expt. 2 were not determined. Other conditions were as described for Table 1.

			$10^{-6} \times$ Radioactivity in substrate							
			Expt. 1				Expt. 2			
			26		47		20		51	
Substrate	No. of population doublings ...	Time of sampling (h)	(c.p.m./bottle)	(c.p.m./μmol)	(c.p.m./bottle)	(c.p.m./μmol)	(c.p.m./bottle)	(c.p.m./μmol)	(c.p.m./bottle)	(c.p.m./μmol)
DL-[5-^{14}C]Glutamate		0	9.40	0.74	8.76	0.91	12.3	1.41	12.3	1.41
		24	2.56	0.108	4.02	0.172	5.14	0.380	3.86	0.272
L-[U-^{14}C]Arginine		0	7.50	1.32	8.03	1.42	11.64	1.78	11.64	1.78
		24	3.49	0.95	4.37	0.97	4.66	1.24	6.47	1.44

and maintained until confluency was attained. At this time, the medium was replaced with one containing 0.01 mM-ascorbic acid. The ascorbic acid was replenished every day for 3 days during which period the cells were confluent. On the fourth day the medium was replaced, as described previously, with one containing the radioactive amino acid, amino acid-free foetal calf serum, 0.1 mM-ascorbate and 0.25 μM-$FeSO_4$. Incubations in duplicate were continued for 24h.

The utilization of radioactive glutamate ranged from 55 to 73% in both 'young' (20 or 26 population doublings) and 'old' (47 or 51 population doublings) cells (Table 3), values somewhat higher than with cells near confluency. The recovery of total ^{14}C of glutamate added was also slightly less, in the range 83–86%. The concentration of free glutamate increased during incubation but only by about 80% as compared with the 5–6-fold increase observed with cells near confluency. This lesser increase in glutamate concentration was reflected in the somewhat lesser dilution of specific radioactivities of labelled glutamate in the medium.

Table 4 compares the utilization of glutamate and of arginine by cells before and after confluency. As noted above, the decreases in specific radioactivities of the precursors in the media demonstrated that these precursors were being diluted by endogenous or exogenous replenishment from other sources. Thus the calculations were based on decrease of total radioactivity, not on concentration changes. They indicate, as might be expected, that cells in exponential phase of growth metabolized both glutamate and arginine at a greater rate than cells that were confluent. Arginine seemed to be metabolized at about the same rate by cells at either a high or low number of population doublings. No consistent pattern in this regard was seen for glutamate utilization, reflecting probably the greater metabolic activity of this amino acid.

Intracellular free amino acids and protein-bound proline and hydroxyproline derived from glutamate and arginine in cells after confluency

Table 5 presents data obtained for intracellular glutamate, glutamine and arginine. Neither the concentration nor the specific radioactivity of any particular amino acid varied greatly between the different experiments or for cells at different numbers of population doublings. These observations are in contrast with the corresponding data for those amino acids in the media after 24h incubation. For example, the specific radioactivity in the medium was almost four times as high in Expt. 2 (20 doublings) as in Expt. 1 (26 doublings) (Table 3). The specific radioactivity of glutamate intracellularly also seemed to be somewhat lower generally

Table 4. *Glutamate and arginine utilization in non-confluent and confluent cells*

The data were obtained from the experiments of Tables 1 and 3. Low no. of population doublings refers to 26 or less; high no. of population doublings refers to 43 or more, as presented in Tables 1 and 3.

	Cell protein (average mg per flask)		10^{-6} × Glutamate disappeared (c.p.m./mg of protein)		10^{-6} × Arginine disappeared (c.p.m./mg of protein)	
Experiment	Low no. of population doublings	High no. of population doublings	Low no. of population doublings	High no. of population doublings	Low no. of population doublings	High no. of population doublings
Non-confluent cells	9.45 9.75	9.10 9.00	0.931	0.660	0.533	0.517
Confluent cells Expt. 1	17.56 18.68	13.40 14.60	0.389	0.354	0.215	0.251
Confluent cells Expt. 2	22.87 26.00	15.30 15.30	0.313	0.552	0.268	0.337

than the final specific radioactivity of this amino acid in the external media. With arginine, as might be expected from its relatively less active metabolism, the specific radioactivities in the external media and intracellular pool did not differ appreciably. These results suggest that the rapid metabolism of glutamate within the cell did not permit good equilibration with glutamate in the media, especially when the latter was also diluted by deamidination of unlabelled glutamine. The specific radioactivity of glutamine intracellularly was quite low, in confirmation of the knowledge that the rate of glutamine synthesis is low in cells in tissue culture. Also noteworthy is the low specific radioactivity of glutamate derived from labelled arginine. This specific radioactivity was indeed much lower than that of the intracellular proline derived from arginine (Table 6) and suggests that oxidation of glutamic-γ-semialdehyde to glutamate did not proceed well in these cells under these conditions.

The data on conversion of labelled glutamate or arginine into intracellular free proline are shown in Table 6. In these experiments as in the experiments with cells before confluency (Table 2), the content of free proline was practically the same in cells of a high or low number of population doublings (column 3, Table 6). However this concentration was about 2.5 times as high as in cells harvested before confluency was reached. These higher concentrations of proline partly account for the lower specific radioactivities of the proline in these cells after confluency (column 4, Table 6 compared with line 4, Table 2), in spite of the fact that the total conversion of radioactive glutamate, at least, was somewhat greater (column 5, Table 6). The conversion of glutamate into proline as judged by total c.p.m. of proline was 2–5 times greater than the corresponding conversion of arginine. The incorporation of ^{14}C from glutamate into proline seems also somewhat higher in cells at a higher number of population doublings.

Table 7 presents data on the amounts and radioactivities of proline obtained from the protein hydrolysates. The content of proline in all the experiments was about the same. The specific radioactivities of the radioactive proline residues synthesized from each precursor were also very close except perhaps in the second experiment with labelled glutamate as precursor. These latter results thus stand in contrast with those obtained for free intracellular proline which were presented in Table 6.

The conversion of glutamate into protein-bound proline as judged by total radioactivity in proline was 3–7-fold greater than the corresponding conversion of arginine.

The parallel data for hydroxyproline, which is not found in free form in these experiments, are presented in Table 8. The specific radioactivities of hydroxyproline in the experiments with cells at a lower number of population doublings were higher than the corresponding specific radioactivities of the free intracellular proline, which also indicates a relatively rapid turnover of free proline in these cells. The data of Table 8 demonstrate, as expected, that hydroxyproline is derived chiefly from glutamate. Further, the specific radioactivities of hydroxyproline in all experiments with glutamate as precursor was rather constant, as noted with proline incorporation (Table 8 and Table 7). The hydroxyproline concentrations in all experiments except for the one at 26 population doublings (Expt. 1, Table 8) were also relatively close, as was seen for proline.

Discussion

Quantitative data have been obtained for the utilization of glutamate and arginine and their

Table 5. *Intracellular free amino acids derived from glutamate or arginine in cells after confluency*

The data were obtained from the experiments reported in Table 3 and all conditions were described there.

Labelled precursor	Amino acid determined	Expt. 1 (after 26 population doublings)		Expt. 2 (after 20 population doublings)		Expt. 1 (after 47 population doublings)		Expt. 2 (after 51 population doublings)	
		Amino acid (μmol/mg of protein)	$10^{-6}\times$ Radioactivity (c.p.m./μmol)	Amino acid (μmol/mg of protein)	$10^{-6}\times$ Radioactivity (c.p.m./μmol)	Amino acid (μmol/mg of protein)	$10^{-6}\times$ Radioactivity (c.p.m./μmol)	Amino acid (μmol/mg of protein)	$10^{-6}\times$ Radioactivity (c.p.m./μmol)
DL-[5-^{14}C]Glutamate	Glutamate	0.220	0.107	0.153	0.113	0.167	0.149	0.158	0.141
	Glutamine	0.191	0.0059	0.176	0.0066	0.175	0.0036	0.207	0.0056
L-[U-^{14}C]Arginine	Glutamate	0.194	0.0021	0.213	0.0014	0.178	0.0023	0.286	0.0014
	Arginine	0.0029	1.62	0.00306	1.50	0.00267	1.14	0.00314	1.25

conversion into proline and hydroxyproline by WI-38 fibroblasts. These data demonstrate that glutamate is a better precursor for proline and hydroxyproline than is arginine. These conclusions apply to cells which had not yet reached confluency and with cells 3–4 days after confluency. The latter did accumulate collagen as judged by hydroxyproline content; the former did not.

It can be concluded also that glutamine is probably not a significant precursor for proline in these cells. If the assumption is made that glutamate had to be first converted into glutamine and mixed with the large pool of unlabelled glutamine present in the media, the resulting specific radioactivity of this glutamine would be considerably lower than the determined specific radioactivities of either free or protein-bound proline, or of hydroxyproline. The specific radioactivity of intracellular glutamine was also extremely low, ruling out the possibility that glutamate was converted into glutamine intracellularly, and that this relatively small pool was the precursor for proline.

The concentration of protein-bound proline was very similar in all experiments, in cells after confluency, as well as in cells before confluency. The concentration of intracellular free proline was higher in cells after confluency and about the same in the two sets of experiments conducted with cells after confluency.

The specific radioactivities of hydroxyproline derived from glutamate were rather similar in both sets of experiments and with cells at either low or high number of population doublings (Table 8). These results thus parallel the corresponding data for proline in total protein (Table 7). In contrast there are differences in the specific radioactivity of the intracellular free proline derived from glutamate with cells of low or high number of population doublings (Table 6). Thus it is obvious that there are differences in the rates of proline biosynthesis from glutamate or of the turnover of free proline according to the number of population doublings of the cells, and that these differences are not reflected in protein-bound proline or hydroxyproline. Further investigation is required to provide an explanation for these findings.

Table 8 suggests that the content of cellular hydroxyproline per mg of total protein was the same in cells of either high or low population-doubling number, though Expt. 1 with cells at 26 population doublings was not in good agreement with the other experiments. In a separate series of 13 experiments to check the hydroxyproline content in cells of different population-doubling number the values obtained were in good agreement with most of the data of Table 8.

There appeared to be a rough correlation between the total cellular growth in flasks of cells after

Table 6. *Intracellular free proline derived from glutamate or arginine in cells after confluency*

Conditions were as described in Tables 3 and 5.

		Proline			
Expt.	Labelled precursor	No. of doublings	μmol/mg of protein	10^{-6} × Sp. radioactivity (c.p.m./μmol)	Total radioactivity (c.p.m./bottle)
1	DL-[5-^{14}C]Glutamate	26	0.026	0.0426	19600
		47	0.028	0.0646	23900
	L-[U-^{14}C]Arginine	26	0.025	0.0182	8600
		47	0.029	0.016	6950
2	DL-[5-^{14}C]Glutamate	20	0.025	0.0341	19350
		51	0.025	0.0817	31600
	L-[U-^{14}C]Arginine	20	0.023	0.007	3950
		51	0.028	0.013	5550

Table 7. *Protein-incorporated proline derived from glutamate or arginine in cells after confluency*

Conditions were as described for Tables 3 and 5.

		Proline			
Expt.	Labelled precursor	No. of doublings	μmol/mg of protein	10^{-6} × Sp. radioactivity (c.p.m./μmol)	Total radioactivity (c.p.m./bottle)
1	DL-[5-^{14}C]Glutamate	26	0.43	0.0325	245000
		47	0.42	0.0328	185000
	L-[U-^{14}C]Arginine	26	0.44	0.00810	66580
		47	0.44	0.00550	35330
2	DL-[5-^{14}C]Glutamate	20	0.46	0.0224	235652
		51	0.38	0.0334	194188
	L-[U-^{14}C]Arginine	20	0.43	0.00497	55564
		51	0.39	0.00436	26016

Table 8. *Protein-incorporated hydroxyproline derived from glutamate or arginine in cells after confluency*

Conditions were as described for Tables 3 and 5.

			Hydroxyproline			
			μmol		10^{-6} × Radioactivity	
Expt.	Labelled precursor	No. of doublings	per mg of protein	Total	(c.p.m./μmol)	Total c.p.m.
1	DL-[5-^{14}C]Glutamate	26	0.00475	0.083	0.054	0.00440
		47	0.0129	0.173	0.041	0.00709
	L-[U-^{14}C]Arginine	26	0.00445	0.083	0.027	0.00224
		47	0.0108	0.158	0.013	0.00205
2	DL-[5-^{14}C]Glutamate	20	0.0161	0.368	0.058	0.0213
		51	0.0153	0.234	0.064	0.0150
	L-[U-^{14}C]Arginine	20	0.0108	0.281	0.016	0.00478
		51	0.0163	0.249	0.0065	0.00162

confluency and the metabolic disappearance of either glutamate or arginine. This is indicated in Table 4 by the values for utilization per mg of protein, which do not differ too greatly for high or low number of population doublings or between Expts. 1 and 2. It is possible that utilization of exogenous substrate is related more to population density than to 'age' of cells.

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