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ENZYMES OF THE γ-GLUTAMYL CYCLE IN 'AGING' WI-38 FIBROBLASTS AND IN HeLa S_3 CELLS

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Summary

γ-Glutamyltransferase ((5-glutamyl)-peptide:amino-acid 5-glutamyltransferase, EC 2.3.2.2) activity of WI-38 fibroblasts decreased only slightly in relation to a constant amount of cell-associated protein as the cells were carried in culture serially from middle to late passage numbers leading toward senescence, e.g., from population doubling level 27 through 41. Also, when the enzyme activity was expressed on the basis of a unit number of cells or unit amount of DNA, little change occurred over that range of PDLs. As the culture approached 'phase-out', the transferase activity rose sharply regardless of how the activity was expressed. The possibility is considered that the large increase in activity could be a reflection of a significant increase in size of cells and therefore changes in the membranes where the transferase is located.

The occurrence of other enzymes of the 'γ-glutamyl cycle' in WI-38 and HeLa S_3 cells also was demonstrated. These included γ-glutamylcyclotransferase ((γ-L-glutamyl)-L-amino-acid γ-glutamyltransferase (cyclizing), EC 2.3.2.4) and 5-oxoprolinase, whose activities showed no large increase comparable to that of the γ-glutamyltransferase, as the culture approached 'phase-out'.

Introduction

Orlowski and Meister [1] have proposed a "γ-glutamyl cycle" as a possible means of transport of amino acids into certain cells. Whether or not the several enzymes in that scheme indeed operate physiologically as a cycle in the various cells of the mammalian organism, no question exists regarding their presence

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Abbreviation, PDL. Starting from primary cultures, human fetal lung diploid fibroblasts undergo 50 ± 10 population doublings before the culture dies. The "population doubling level" (PDL) of a culture describes the 'age' of the cells in terms of number of doublings the population has undergone to reach a given point in the life of the culture.

[2]. Indeed, γ-glutamyltransferase ((5-glutamyl)peptide:amino-acid 5-glutamyltransferase, EC 2.3.2.2) has been shown to occur in various cultured mammalian cells as well as in transformed cells [3,4]. Another enzyme in the cycle, γ-glutamylcyclotransferase ((γ-L-glutamyl)-L-amino-acid γ-glutamyltransferase (cyclizing), EC 2.3.2.4), has been studied for many years [5—8]. Orlowski and Meister [8] developed an assay for that enzyme using synthetic L-γ-glutamyl-L-γ-glutamyl-*p*-nitroanilide as a substrate. Several investigators [5,9,10] have provided some evidence that L-γ-glutamyl-L-γ-glutamine, formed from L-glutamine by the action of γ-glutamyltransferase, can also be used as a substrate for assay of the cyclotransferase.

In this report we summarize studies on γ-glutamyltransferase, γ-glutamylcyclotransferase and 5-oxoprolinase activities of WI-38 cells, human diploid fibroblasts derived from fetal lung. This cell strain has a finite life span and has been used as a model for studies of aging in vitro [11,12]. It has the property that the rate of mitosis is higher in young cells, decreases with increasing cell population doubling levels, becomes very slow at the stage called Phase III, then finally ceases. The last stage is called "phase-out" [13].

Experiments

Materials and Methods

Purified rat kidney γ-glutamyltransferase was prepared as described previously [14]. L-γ-glutamyl-L-glutamate and L-γ-glutamyl-L-glutamine were obtained from Calbiochem. Inc.; ATP, phospho*enol*pyruvate and pyruvate kinase from Boehringer-Mannheim Biochemicals; 5-oxo-[^{14}C]proline and ^{14}C-labelled amino acids from New England Nuclear. High voltage paper electrophoresis was performed in a Savant apparatus, using 3MM Whatman paper at pH 3.5, 40 V/cm^2 for 2 h. The cells used in this study were grown and processed according to the procedures described in another paper [3]. For determination of γ-glutamylcyclotransferase and 5-oxoprolinase activities, however, only freshly harvested cells were used. Other chemical and enzymatic methods used have been described in another paper [3].

Assay for γ-glutamyltransferase

The procedure was based on that of Orlowski and Meister [15]. Enzymatic activity was expressed as nmoles of substrate utilized. γ-Glutamyltransferase activity of WI-38 cells was independent of cell density tested at 3 levels; thus in this study cells were harvested at confluency except in the late passage cells at PDL 43a and 43b. In those instances, the cultures reached only about one-third of confluency.

Preparation of γ-[^{14}C]glutamylglutamine

L-[^{14}C]glutamine (10 μCi/0.49 μmol) was diluted 100-fold with unlabeled L-glutamine. Contaminants were eliminated by use of a Dowex 1 X (formate) column. The purified, labeled L-glutamine was dissolved to a final concentration of 10 μmol per ml in a buffer of 0.1 M Tris/0.01 M $MgCl_2$ at pH 9.0. The solution was transferred to an ultrafiltration chamber with an XM50 filter and incubated with purified kidney γ-glutamyltransferase (2 units per ml) for 10

min at 37°C, then the mixture was ultrafiltered. The γ-[^{14}C]glutamylglutamine in the filtrate was purified by chromatography on a Dowex 1 x 8 column or by preparative paper electrophoresis, or both. By either procedure, using 50 μmol of labeled glutamine, the yield of dipeptide was 10.2 μmol (21%) with a specific activity of $1.12 \cdot 10^6$ dpm per mole. Purity of labeled glutamine, formation of synthesized ^{14}C-labelled dipeptide, and unreacted L-[^{14}C]glutamine were determined employing paper electrophoresis as shown in panels A, B, C and D respectively of Fig. 1. The radioactive dipeptide was then diluted with unlabeled L-γ-glutamyl-L-γ-glutamine to a specific activity of $2.9 \cdot 10^4$ dpm per mol. The dipeptide was purified further by chromatography on Dowex 1 x 8 and its ninhydrin reactivity estimated as 72% color yield by comparison with norleucine. After hydrolysis with 6 M HCl, the color equivalent with ninhydrin increased 2.6-fold.

Preparation and assay of γ-glutamylcyclotransferase

The method of preparation was modified from procedures already described [5,16]. The cell pellet obtained from culture medium was sonicated in 0.05 M Tris buffer at pH 7.8, containing 0.005 M 2-mercaptoethanol and 0.15 M KCl. The mixture was centrifuged, and the precipitate washed twice. The supernate plus washings was adjusted to pH 4.2 and the precipitate removed by centrifugation. The supernate was then adjusted to pH 7.8 and saturated with ammonium sulfate. When the preparation was carried out with HeLa S_3 cells, an intermediate precipitate obtained with ammonium sulfate at 50% saturation was discarded, and the supernate then saturated with ammonium sulfate. In either case, the centrifuged precipitate was dissolved in Tris buffer at pH 7.8. The solution was heated at 57°C for 5 min, cooled and then centrifuged. The supernate was dialyzed against Tris buffer at pH 7.8 and assayed using [^{14}C]-glutamylglutamine as substrate.

For the assay, enzyme solution (0.1 ml) was added to 0.1 ml of a solution containing 4 μmol of γ-[^{14}C]glutamylglutamine ($1.15 \cdot 10^5$ dpm) in 0.05 M Tris buffer of pH 7.8. The mixture was incubated at 37°C, the reaction stopped by heating for 3 min at 90°C, and the contents analyzed both by column chromatography and paper electrophoresis. For chromatography, 50—100 μl of the incubation mixture was applied to a Dowex 1 × 8 (formate) column. The [^{14}C]glutamine enzymatically released was eluted with water, unreacted radiolabeled dipeptide with 0.1 M formic acid, and 5-oxo[^{14}C]proline with 1 M formic acid. The fractions were concentrated and radioactivity determined by liquid scintillation counting. Another portion of the reaction mixture was subjected to paper electrophoresis and radiochromatogram scanning.

5-Oxoprolinase preparation and assay

The 5-oxoprolinase preparation from cultured cells and the assay procedure were based on that of Strömme and Eldjarn [17], employing purified 5-oxo-[^{14}C]proline as substrate. In each experiment, a total of $7.5 \cdot 10^5$ cpm per mol 5-oxoproline was used. Enzyme activity was expressed as either nmol of [^{14}C]glutamate formed per min per mg of cell-associataed protein or per 10^6 cells.

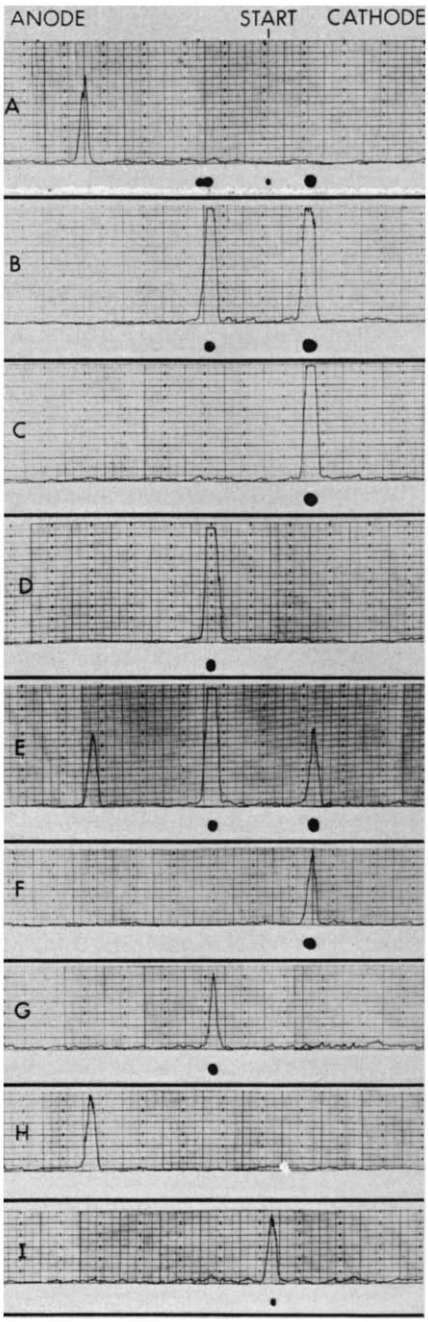

Fig. 1. High voltage paper electrophoresis at pH 3.5 for experiments concerning formation of [^{14}C]-glutamylglutamine by γ-glutamyltransferase, and for determination of γ-glutamylcyclotransferase activity of WI-38 cells. Lettered panels indicate paper strips with location of various pertinent compounds determined by radiochromatogram scanning (shown as peaks in graph) and reaction with ninhydrin (shown as spots below graphs). Panel A: standard amino acids, reading from left to right are, 5-oxo[^{14}C]proline shown as a peak, and ninhydrin spots for L-γ-glutamyl-L-glutamate, L-γ-glutamyl-L-γ-glutamine, L-glutamate and L-glutamine respectively. Panel B; compounds found in an ultrafiltrate of the reaction mixture of purified rat kidney γ-glutamyltransferase and L-[^{14}C]glutamine; left to right, peaks and spots show

Results

γ-Glutamyltransferase in WI-38 cells

In our first studies of γ-glutamyltransferase activity in WI-38 cells, different starter cultures of designated PDL, obtained from the laboratory of Dr. Leonard Hayflick, were used. With that experimental design, no correlation was found between transferase activity and the different population doubling levels of the cultures. The average enzymatic activity from population doubling level 17 to before phase-out was 0.33 nmol/mg of cell-associated protein or 0.13 nmol per 10^6 cells or 16.5 nmol per mg of DNA. However, when a single starter culture of WI-38 cells, obtained from the same source, was carried in successive passage, there was some relation between population doubling level and enzymatic activity. The results of one of four experiments done are shown in Table I and Fig. 2. In that experiment the enzymatic activity, expressed in terms of unit cell number, did not change significantly from population doubling level 25 to 41, but did change in Phase III cells at population doubling level 43. In contrast, expressed in terms of activity per mg of cell-associated protein, activity increased somewhat in population doubling level 27 compared with population doubling level 23 (probably as the culture became adapted from its frozen state), and activity of subsequent sub-cultures of cells appeared to decrease gradually until population doubling level 41 was reached. One should note that the amount of protein associated with the cells increased with late population doubling level; and the increase became larger at the stage just before Phase III and even greater in cells in late Phase III. As the culture approached 'phase-out', the enzymatic activity increased sharply; at that stage one could observe a large increase in cell size. The same trend of change in γ-glutamyltransferase activity of WI-38 cells in relation to population doubling level was observed in four separate experiments; however, the actual population doubling level number at which Phase III was reached, using different starter cultures, ranged from population doubling level 38 to 56.

Recently we obtained starter cultures of IMR-90 human fetal lung fibroblasts from Dr. Warren Nichols of the Institute for Medical Research, Camden, N.J. In preliminary studies carrying a single starter culture of IMR-90 cells through successive passage into Phase III, the pattern of expression of γ-glutamyltransferase activity was found to follow that shown by WI-38 cells as summarized above.

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the product of transpeptidation, [^{14}C]glutamylglutamine and unreacted L-[^{14}C]glutamine respectively. Panel C: peak and spot showing unreacted L-[^{14}C]glutamine in the ultrafiltrate of Panel B separated when the ultrafiltrate was applied to a Dowex 1 column and eluted with water (see text). Panel D: peak and spot showing the product of transpeptidation, [^{14}C]glutamylglutamine, eluted from Dowex 1 column with 0.1 formic acid after application of the ultrafiltrate of Panel B. Panel E: resolution of reaction mixture after action of γ-glutamylcyclotransferase of WI-38 cells on γ-[^{14}C]glutamylglutamine; from left to right, first peak shows the product, 5-oxo[^{14}C]proline, second peak and spot below show [^{14}C]-glutamylglutamine that remained unreacted, and third peak and spot below show [^{14}C]glutamine formed in concert with the 5-oxo[^{14}C]proline. The reaction mixture of E was then applied to a Dowex 1 column and eluted respectively with water (Panel F), 0.1 M formic acid (Panel G), and 1 M formic acid (Panel H). In Panel F the peak and spot show the enzymatically released L-[^{14}C]glutamine; in Panel G the peak and spot show the recovered unreacted [^{14}C]glutamylglutamine; and in Panel H the peak shows the product, 5-oxo[^{14}C]proline. Panel I: Acid hydrolysate of 5-oxo[^{14}C]proline formed as in E and isolated. Peak and spot show L-glutamate that resulted from hydrolysis.

TABLE I

γ-GLUTAMYLTRANSFERASE ACTIVITY OF WI-38 FIBROBLASTS

See text for detailed methods. Each value represents the average of 3 determinations. WI-38 fibroblasts, Lot No. CXXX-1, population doubling level 23, were used as a starter culture; all other cells were obtained from those cells by serial subculture. In all cases except 43a and 43b, the cells achieved confluency in T-75 flasks, were harvested and assayed. Cells labeled population doubling level 43a were prepared by seeding medium with $7 \cdot 10^5$ population doubling level 43 cells, maintained and then harvested after 3 weeks; at that time the culture was not confluent but contained $5.3 \cdot 10^5$ cells. Cells labeled population doubling level 43b were prepared similarly from $7 \cdot 10^5$ cells of population doubling level 43, maintained and then harvested after 6 weeks; at that time, again, the culture was not confluent, but contained $5.9 \cdot 10^5$ cells.

PDL *	No. of cells $\times 10^{-6}$	Cell-associated protein (mg per 10^6 cells)	Enzyme activity (nmol/min of *p*-nitroaniline released)	
			per 10^6 cells	per mg cell-associated protein
23	9.2	0.26	0.05	0.19
25	11.0	0.34	0.12	0.34
27	13.7	0.34	0.14	0.40
29	10.0	0.42	0.15	0.36
31	10.0	0.44	0.12	0.28
33	11.0	0.44	0.13	0.29
37	7.5	0.54	0.15	0.27
39	6.0	0.76	0.18	0.23
41	4.2	0.88	0.18	0.20
43	3.0	1.16	0.46	0.40
43a	0.5	1.91	1.89	0.99
43b	0.6	1.66	3.47	2.09

* Population doubling level.

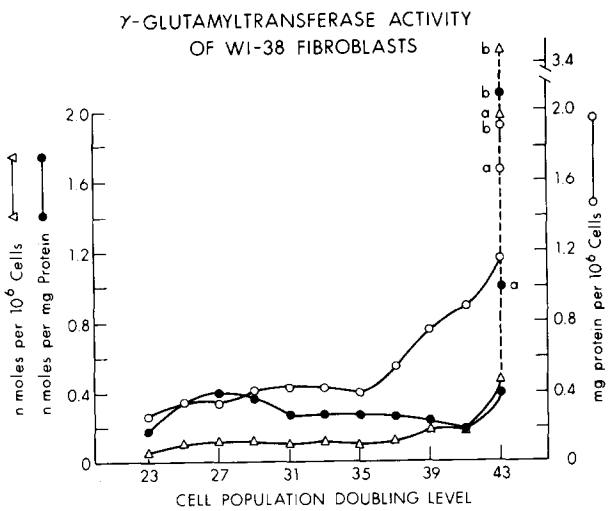

Fig. 2. γ-Glutamyltransferase activity of WI-38 fibroblasts of serial population doubling levels. See footnote to Table I for explanation of cultures at population doubling levels 43, 43a and 43b.

Assay of γ-glutamylcyclotransferase by use of [^{14}C]glutamylglutamine as substrate

Results obtained with this highly sensitive assay are shown in Fig. 1. In Panels E, F, G and H of the figure one may observe representation of the products of the enzymatic reaction, [^{14}C]glutamine and 5-oxo[^{14}C]proline. When the product identified as 5-oxoproline was hydrolyzed with acid, [^{14}C]glutamate was produced as shown in Panel I. In the assay, disappearance of [^{14}C]-

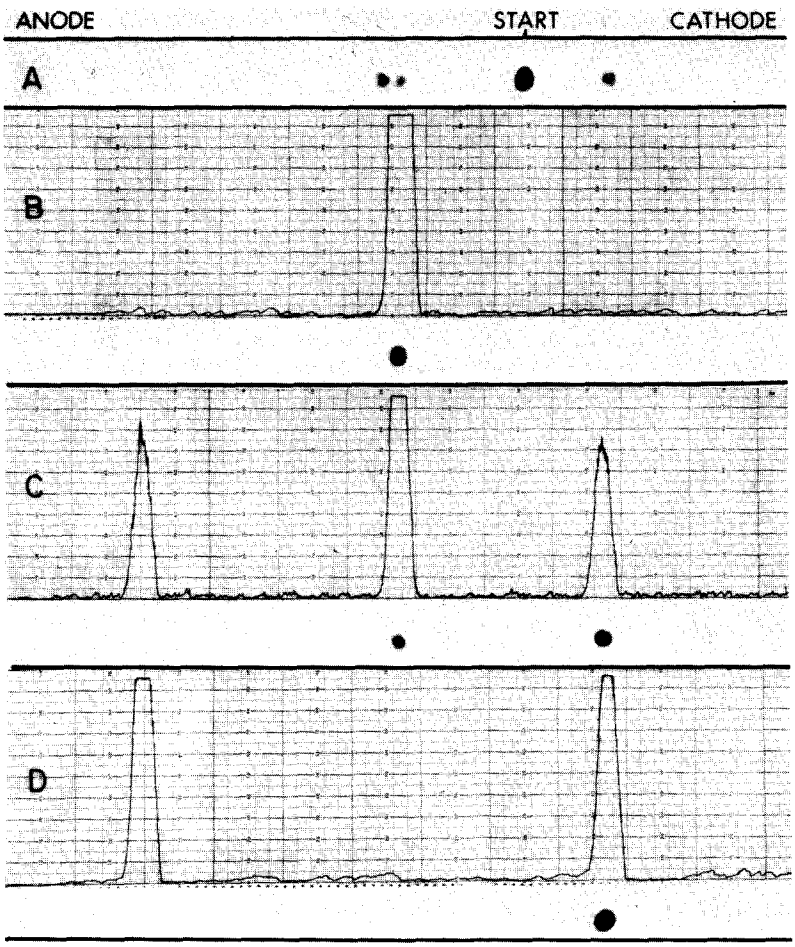

Fig. 3. High voltage paper electrophoresis for demonstration of stoichiometric conversion of γ-[^{14}C]-glutamylglutamine by the γ-glutamylcyclotransferase of HeLa cells. The enzyme (570 μg) was incubated with 0.8 μmol of γ-[^{14}C]glutamylglutamine at 37°C as described in the text. The lettered panels indicate paper strips with location of various pertinent compounds determined by radiochromatogram scanning and reaction spots with ninhydrin. Panel A: Standard amino acids, spots show left to right, L-γ-glutamyl-L-glutamate, L-γ-glutamyl-L-glutamine, L-glutamate and L-glutamine. Panel B: Reaction mixture at zero time incubation. The peak is unreacted [^{14}C]glutamylglutamine. Panel C: Reaction mixture after incubation for 50 min. From left to right, first peak is the product, 5-oxo[^{14}C]proline, the second peak and spot below represent recovered unreacted [^{14}C]glutamylglutamine, and the third peak and spot below represent the second product, [^{14}C]glutamine. Panel D: Shows the reaction mixture after incubation for 100 min. Left to right, the first peak shows 5-oxo[^{14}C]proline, and the second peak and spot below show a stoichiometric amount of the second product, [^{14}C]glutamine.

TABLE II

THE γ-GLUTAMYLCYCLOTRANSFERASE ACTIVITIES OF VARIOUS CELLS MEASURED IN PARTIALLY PURIFIED PREPARATIONS WHICH HAVE REACTED WITH γ-[^{14}C]GLUTAMYLGLUTAMINE TO FORM 5-OXO[^{14}C]PROLINE

See text for detailed methods. Activity is expressed as nmoles of 5-oxo[^{14}C]proline produced in a standard assay system with γ-[^{14}C]glutamylglutamine as substrate. Each value represents the average of 3 determinations; (a) WI-38 fibroblasts, Lot No. CXXVIII, were employed; cell cultures, as harvested from T-75 flasks, were confluent at population doubling level 26 and 35, but not in the older cells of population doubling level 53. (b) HeLa S_3 cells were harvested from suspension culture.

Kind of cell used	No. of cells used in preparation $\times 10^{-6}$	Cell-associated protein (mg per 10^6 cells)	Protein isolated with enzyme activity (mg per 10^6 cells)	Enzyme activity	
				per 10^6 cells	per mg cell-associated protein
WI-38 (PDL 26)	6.20	0.26	0.014	0.114	0.44
WI-38 (PDL 35)	3.25	0.48	0.018	0.108	0.22
WI-38 (PDL 53)	5.50	1.40	0.028	0.124	0.09
HeLa S_3	800.00	0.23	0.002	0.049	0.21

glutamylglutamine was stoichiometric with appearance of 5-oxo[^{14}C]proline. This is shown in Fig. 1, Panel E, for WI-38 cells, and in Fig. 3 for HeLa S_3 cells. The enzymatic activity was found to be constant with time until 60% of substrate had disappeared.

Assay of γ-glutamylcyclotransferase in WI-38 cells of different population doubling levels

WI-38 cells of 3 different population doubling levels, 2 intermediate and 1 late passage, were studied. The preparations were then assayed with radioactive dipeptide. Results are shown in Table II together with results obtained with an enzyme preparation made from HeLa S_3 cells. One may note that WI-38 cells of PDL 53 (Phase III cells in culture still dividing but on the threshold of phase-out) exhibited the expected increase in cell-associated protein. However the enzyme activity, expressed as the amount of 5-oxoproline formed per min per 10^6 cells, was only slightly higher than that observed with cells of population doubling level 26. This was not true if the activity was referred to a unit amount of cell-associated protein.

For assay purposes the enzyme has to be purified partially, so that the results presented here are to be taken as qualitative because the purification procedures might cause some losses in one cell population as compared with another. But the methods are sufficiently reliable to allow the conclusion that major changes in γ-glutamylcyclotransferase activity per 10^6 cells did not occur with age of the cells in culture.

5-Oxoprolinase activity of WI-38 cells

The enzymatic activity of WI-38 cells with population doubling levels of 26, 35 and 53 was found to be 0.10 ± 0.003 nmol of [^{14}C]glutamate formed per min per 10^6 cells. However, when the activity was expressed on the basis of nmoles [^{14}C]glutamate formed per min per mg cell-associated protein, for population doubling level 26 the figure was 0.021, for population doubling

level 35 it was 0.018 and for population doubling level 53 it was 0.010. That of course seems to reflect the greater amount of protein associated with the cell layer of the later passage cells. In any event, the magnitude of activity for this enzyme in WI-38 cells was close to values reported by other workers using skin fibroblasts [17,18].

Discussion

When WI-38 human fetal lung fibroblasts of a single starter culture were carried through successive doublings from population doubling level 23 through phase-out of the culture, activity of the membrane-associated γ-glutamyltransferase per unit number of cells showed no large change until population doubling level 43 was reached, and thereafter increased sharply until phase-out. In contrast, the activities of two other enzymes of the proposed γ-glutamyl cycle, located within the cell, did not show the same large increase. Since cells in Phase III and in 'phase-out' increase greatly in size, one must consider that the change in activity of the membrane-associated enzyme may be secondary to changes in architecture or topology of the membrane. Because of the observed change in cell size, and because cells in different phases of the life of the culture vary in contents of protein, one must first consider the best way of expressing the activity of a membrane-associated enzyme.

Cell layers of fibroblasts obtained from their culture media always have some cell-associated collagen not removed by washing. The amount of remaining protein may vary with culture and harvesting conditions as well as with the phase in the life of the culture. Thus use of amount of 'cell protein' as a basis of reference for various analytical entities, including enzyme activities, is complicated. Furthermore, because the degree of hydroxylation of proline residues in collagen may also vary, the determined 'cell protein' cannot simply be rectified by an independent determination of hydroxyproline contents as a measure of amount of collagen. Accordingly a better reference for enzymatic activity is a unit number of cells backed by a determination of DNA contents to establish the average state of ploidy of the cells in culture. That in fact is the way the γ-glutamyltransferase activities are finally expressed in these studies.

The importance of cell size relative to changes of enzyme activity with age must also be considered. Bowman et al. [19], supplementing other studies [20,21], showed that WI-38 cells in monolayer have increased numbers of large cells associated with age-related increase of a subpopulation that does not synthesize DNA. Schneider and Fowlkes [22], on the basis of measured DNA contents and cell volumes, concluded that senescent cells have increased sizes in both G1 and G2 + M cells in logarithmic growth as well as at confluency. They thought the shift to larger cell volumes occurring during the life span of WI-38 cells may be secondary to an increased number of slow or non-dividing cells as noted previously [23]. They did not rule out a remote possibility that a change in the cell membrane could be primary to both loss of replicative capacity and increase in cell volume.

In those connections, the data shown in Table I can be used to estimate a relative 'size' of cell becoming confluent in the constant area of a T-75 flask. Thus, compared to an 'average' cell and its extracellular concomitants at population

doubling level 27, another 'average' cell at population doubling level 37 roughly accounted for twice the area, at population doubling level 43 about 3 times, and at population doubling level 43a and 43b about 6 times the area. The cell-associated protein did not change significantly until population doubling level 39 was reached, and thereafter increased so that at population doubling level 43 it was about 3 times that at population doubling level 27.

The consequence of these changes may be inferred from Table I and Fig. 2, namely that a single cell approaching phase-out at population doubling level 43 was larger and also had greater γ-glutamyltransferase activity. The increased activity need not indicate synthesis of more enzyme but instead might reflect a distortion of the cell membrane causing greater accessibility of the enzyme to the external substrate. With whole cells, one should recall, assays are performed with a synthetic substrate placed in the incubation medium. On the other hand the 'natural' co-substrate for the enzyme is intracellular glutathione, and perhaps activity measured as transfer of a glutamyl group from reduced glutathione to an amino acid would not show increase with enlargement of the cells as they approach phase-out. In that connection, Zelazo and Orlowski [24] reported that purified sheep kidney γ-glutamyltransferase showed different reactivities when glutathione was used in place of the synthetic substrate.

Finally, one should emphasize that changes in enzyme activity with age of cells in culture can only be compared if the cells used are from a single starter culture carried through successive doublings and the population doubling level at phase-out determined. If two different starter cultures are used their baseline activities might differ considerably, and the progress of change in enzymatic activity could differ even more if the two cultures were destined to phase out at different population doubling level.

Acknowledgements

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References

1 Orlowski, M. and Meister, A. (1970) Proc. Natl. Acad. Sci. U.S. 67, 1248—1255
2 Meister, A. and Tate, S. (1976) Annu. Rev. Biochem. 45, 559—604
3 Takahashi, S., Seifter, S. and Rifas, L. (1974) Fed. Proc. 33, 957; (1977) In Vitro, in the press
4 Novogrodsky, A., Tate, S. and Meister, A. (1976) Proc. Natl. Acad. Sci. U.S. 73, 2414—2418
5 Kakimoto, Y., Kanazawa, A. and Sano, I. (1967) Biochem. Biophys. Acta 132, 472—480
6 Foder, P.J., Miller, A. and Waelsch, H. (1953) J. Biol. Chem. 202, 551—565
7 Connell, G.E. and Hanes, C.S. (1956) Nature 177, 377—378
8 Orlowski, M. and Meister, A. (1969) Biochemistry 8, 1048—1055
9 Szewczuk, A. and Baranowski, T. (1963) Biochem. Z. 338, 317—329
10 Orlowski, M. and Meister, A. (1965) J. Biol. Chem. 240, 338—347
11 Hayflick, L. (1965) Exp. Cell Res. 37, 614—636
12 Hay, R.J. (1967) Adv. Gerontol. Res. 2, 121—158
13 Hayflick, L. and Moorhead, P.S. (1961) Exp. Cell Res. 25, 585—621
14 Takahashi, S., Pollack, J. and Seifter, S. (1974) Biochem. Biophys. Acta 371, 71—75

15 Orlowski, M. and Meister, A. (1970) Methods Enzymol. 17A, 883—889
16 Orlowski, M. and Meister, A. (1970) Methods Enzymol. 17A, 863—868
17 Strömme, J.H. and Eldjarn, L. (1972) J. Clin. Lab. Invest. 29, 335—342
18 Wellner, V.P., Sekura, R., Meister, A. and Larsson, A. (1974) Proc. Natl. Acad. Sci. U.S. 71, 2505—2509
19 Bowman, P.D., Meek, R.L. and Daniel, C.W. (1975) Exp. Cell Res. 93, 184—190
20 Simons, J.W.I.M. (1970) Aging in Cell and Tissue Culture (Holeckova, E. and Christofalo, V.J., eds.), p. 25, Plenum Press, New York
21 Macieira-Coelho, A. (1970) Aging in Cell and Tissue Culture (E. Holeckova, E. and Cristofalo, V.J., eds.), p. 121, Plenum Press, New York
22 Schneider, E.L. and Fowlkes, B.J. (1976) Exp. Cell Res. 98, 298—302
23 Absher, P.M., Absher, R.G. and Barnes, W.D., (1974) Exp. Cell Res. 88, 95—104
24 Zelazo, P. and Orlowski, M. (1976) Eur. J. Biochem. 61, 147—155