Correction. In the article "Pyrroline-5-carboxylate synthase activity in mammalian cells" by Robert J. Smith, Sylvia J. Downing, James M. Phang, Ronna F. Lodato, and Thomas T. Aoki, which appeared in the September 1980 issue of Proc. Natl. Acad. Sci. USA (77, 5221-5225), the authors request that the following corrections be noted. On page 5221 under the subheading "Formation of proline from glutamic acid in cell homogenates" and in the legend to Fig. 2, the units of all assay components listed as nmol should be changed to μmol. Thus, the assay mixture contained 0.75 μmol of L-[U-14C]glutamic acid (2.7 μCi/μmol; 1 Ci = 3.7 × 10^10 becquerels), 1.25 μmol of ATP, an ATP-regenerating system (3.626 μmol of creatine phosphate and 2 units of creatine phosphokinase), 0.1 μmol of NADPH, an NADPH-regenerating system (2.5 μmol of DL-isocitrate and 0.9 unit of isocitrate dehydrogenase), 6.25 μmol of MgCl₂, 0.25 μmol of 2-mercaptoethanol, and 0.1 M potassium phosphate buffer (pH 7.4) in a final volume of 0.2 ml.

Correction. In the article "Somatic cell genetic analysis of regulation of expression of 3-hydroxy-3-methylglutaryl-coenzyme A reductase" by Michael Sinensky, Susan Armagast, Gail Mueller, and Robert Torget, which appeared in the November 1980 issue of Proc. Natl. Acad. Sci. USA (77, 6621-6623), an error occurring in the Proceedings editorial office appeared on p. 6621. The second sentence in the Abstract should read "They are (i) a recessive mutant with abnormally high enzyme activity, apparently defective in degradation of the enzyme; (ii) another, phenotypically a mevalonate auxotroph that maintains permanently repressed levels of enzyme activity; and (iii) recessive, constitutive mutants."
Pyrroline-5-carboxylate synthase activity in mammalian cells
(glutamic acid/proline)

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ABSTRACT Although glutamic acid is known to be a precursor for proline biosynthesis, the enzymatic conversion of glutamic acid to pyrroline-5-carboxylic acid, the immediate precursor of proline, has not been demonstrated in cell-free systems. By providing appropriate concentrations of ATP and NADPH and blocking further metabolism of pyrroline-5-carboxylic acid, we have developed a method for measuring the formation of pyrroline-5-carboxylic acid from glutamic acid in homogenates of mammalian cells. We have designated this activity pyrroline-5-carboxylate synthase. To confirm that our assay is a valid measure of the initial step in proline biosynthesis from glutamic acid, we have compared two mutant lines of Chinese hamster ovary cells. Proline prototrophic cells, which can synthesize proline from glutamic acid, have easily measurable pyrroline-5-carboxylate synthase activity (5.97 nmol of pyrroline-5-carboxylic acid per hr per mg of homogenate protein). In contrast, proline auxotrophic cells, which are unable to synthesize proline from glutamic acid, have no detectable pyrroline-5-carboxylate synthase activity.

L-Proline can be synthesized in mammalian cells from either L-ornithine or L-glutamic acid. The presumed mechanisms of proline formation are illustrated in Fig. 1. The enzymes that catalyze the conversion of ornithine to pyrroline-5-carboxylic acid (ornithine aminotransferase, L-ornithine-2-oxo-acid aminotransferase, EC 2.6.1.13) and the subsequent reduction of pyrroline-5-carboxylic acid to proline [pyrroline-5-carboxylic reductase, L-proline:NAD(P)⁺ 5-oxodihydrogenase, EC 1.5.1.12] have been characterized and extensively studied (1). Results from radiotopic tracer studies in intact animals (2-4) and nutritional studies in cultured cells (unpublished results) are consistent with the pathway leading from glutamic acid to pyrroline-5-carboxylate acid (P5C) shown in Fig. 1. However, direct formation of P5C by this enzyme or enzyme complex, which we have designated P5C synthase, has not been demonstrated in mammalian cells.

By providing appropriate concentrations of cofactors, we have developed a method for measuring the specific activity of P5C synthase in cell-free homogenates of mammalian cells. The determination of P5C synthase activity in cell-free homogenates of mammalian cells is made possible by specifically blocking further metabolism of P5C. We have verified that the assay provides a valid measure of the P5C synthase pathway by comparing two Chinese hamster ovary cell lines that differ from each other only in the presence or absence of the pathway from glutamic acid to P5C.

MATERIALS AND METHODS

Materials. L-[U-¹⁴C]Glutamic acid and L-[U-¹⁴C]proline were obtained from New England Nuclear. AG 50W cation-exchange resins were from Bio-Rad. Disposal polypropylene chromatography columns were from Kontes. Tissue culture media and dishes were obtained from Microbiological Associates. 2-Mercaptoethanol was from Eastman. a-Aminobenzaldehyde (OAB) and all other chemicals were obtained from Sigma. L-P5C and L-[U-¹⁴C]P5C were prepared from ornithine by enzymatic synthesis as described (5).

Cultured Cells. The C9 and R25 cell lines were originally isolated by David Valle and James M. Phang (Metabolism Branch, National Cancer Institute, Bethesda, MD) from the proline-auxotrophic Chinese hamster ovary cell line CHO-K1 described by Kao and Puck (6). The line designated C9 was cloned from CHO-K1 cells by a dilution technique in microtiter plates in a medium supplemented with 0.1 mM proline. Like the parent CHO-K1 cells, the C9 line is proline auxotrophic, requiring proline supplementation of the medium for survival. The R25 line was selected by plating CHO-K1 cells in medium containing 2 mM glutamine, but lacking proline or ornithine, thus favoring cells that had acquired the capacity to form proline from glutamate. Under these selective conditions, rare colonies of growing cells formed. One colony was isolated and designated R25. For subsequent studies, the cells were maintained in monolayer by repeated subculturing in Eagle's minimal essential medium supplemented with 10% (vol/vol) fetal bovine serum and, for C9 cells, with Eagle's nonessential amino acids.

Preparation of Cell Homogenates. Cell suspensions were prepared by controlled incubation of monolayers in 0.25% trypsin, and the cells were plated in the above media in 100-mm petri dishes (1.2-2.5 × 10⁵ cells per dish). After incubation for 16-48 hr, the medium was removed and the cells were gently scraped into ice-cold Dulbecco's phosphate-buffered saline with a rubber spatula. The cells were centrifuged for 10 min at 1000 g, resuspended in cold phosphate-buffered saline, centrifuged, and finally suspended in a small volume of 0.1 M potassium phosphate buffer (pH 7.4). Cells were disrupted by freezing and thawing twice or by sonicating for 40 sec in a Heat Systems Sonifier equipped with a Cup Horn. Protein concentration of cell homogenates was determined by the method of Lowry et al. (7), with bovine insulin as a standard.

Characterization of Cellular Metabolic Pathways. Specific activities of P5C reductase, ornithine aminotransferase, P5C dehydrogenase, and proline oxidase in C9 and R25 homogenates were measured with radiotopic assays as described (8-11). The rate of conversion of glutamic acid to proline in intact cells was determined by a described method (12). In brief, confluent monolayers of cells were incubated with medium containing L-[U-¹⁴C]glutamic acid, and the rate of incorporation of [¹⁴C]proline into cellular protein was quantified.

Formation of Proline from Glutamic Acid in Cell Homogenates. The assay mixture contained 0.75 nmol of L-[U-¹⁴C]glutamic acid (2.7 µCi/nmol; 1 Ci = 3.7 × 10¹² becquerels).

Abbreviations: P5C, pyrroline-5-carboxylic acid; OAB, a-aminobenzaldehyde.
The R25 cells were chosen for the initial studies because they had the capacity for proline formation from glutamic acid in intact cells and thus were expected to have P5C synthase activity. By using cell-free homogenates of R25 cells, we first looked for the formation of proline from glutamic acid. The R25 homogenates catalyzed proline formation at the rate of 10.03 nmol/hr per mg of protein (Fig. 2A). The identity of product proline was confirmed by its comigration with pure L-[14C]proline on 3-ml columns of AG50W (200–400 mesh) cation-exchange resin (Fig. 2B). Under these conditions, proline was separated from substrate glutamic acid as well as from α-ketoglutarate, glutamine, and P5C.

Because proline formation from glutamate presumably involved P5C as an intermediate (see Fig. 1), we next looked for P5C formation by terminating the reaction with 6 M HCl containing 30 mg of OAB per ml. Δ1-Pyrroline compounds react specifically with OAB (15). Previous studies by Phang et al. (11) had also shown that the dihydroquinazolinium product of OAB and P5C bound to AG50W under acid conditions. By use of a 1-ml bed volume column of AG50W (100–200 mesh), labeled glutamic acid, α-ketoglutarate, glutamine, and proline could be eluted with 2 M HCl and the condensation product of OAB and [14C]P5C could subsequently be eluted as a sharp peak with 2 M NaOH (Fig. 3A). The elution profile after reaction of pure L-[14C]P5C with OAB is shown in Fig. 3B. The second milliliter of 2 M NaOH reproducibly contained 25% of the applied [14C]P5C, thus allowing us to collect a single milliliter of eluent and to calculate the total amount of P5C present.

**RESULTS**

**FIG. 1.** Pathways of proline biosynthesis and degradation in mammalian cells. The enzymes are: (1) pyrroline-5-carboxylate (P5C) synthase; (2) P5C dehydrogenase; (3) ornithine aminotransferase; (4) P5C reductase; and (5) proline oxidase. Step 6 is spontaneous.

1.25 nmol of ATP, an ATP-regenerating system (3.626 nmol of creatine phosphate and 2 units of creatine phosphokinase), 0.1 nmol of NADPH, an NADPH-regenerating system (2.5 nmol of DL-isocitrate and 0.9 unit of isocitrate dehydrogenase), 6.25 nmol of MgCl2, 0.25 nmol of 2-mercaptoethanol, and 0.1 M potassium phosphate buffer (pH 7.4) in a final volume of 0.2 ml. The reaction was started by addition of 0.05 ml of cell homogenate (200–400 μg of protein) and the mixture was incubated at 37°C in a Dubnoff shaker. All samples were assayed in triplicate, and blanks either lacking cell homogenate or containing homogenate denatured with HCl were included in each assay.

The reaction was terminated by addition of 0.05 ml of 6 M HCl. After centrifugation for 5 min at 2000 × g, 0.2-ml aliquots of the supernatants were placed on 3-ml bed volume columns of AG50W cation-exchange resin (200–400 mesh, 8% cross-linked, hydrogen form) and eluted with 1 M HCl. The fraction of the effluent containing purified proline was collected and the amount of [14C]proline was determined by liquid scintillation spectrometry.

**P5C Formation from Glutamic Acid in Cell Homogenates (P5C Synthase Activity).** The reaction mixture was identical to that described for measuring proline formation, except that it also contained 0.125 mg of OAB and 50 μmol of L-proline. After incubation at 37°C, the reaction was stopped by adding 0.05 ml of 6 M HCl containing 30 mg of OAB per ml. The samples were incubated for an additional 5 min at 37°C to promote optimal PSC-OAB binding and centrifuged for 5 min at 2000 × g. The supernatants were placed on 1-ml bed volume columns of AG50W (100–200 mesh, 8% crosslinked, hydrogen form). After elution with 16 ml of 2 M HCl, the OAB–P5C condensation product was eluted as a sharp peak with 2 M NaOH and quantified by liquid scintillation spectrometry.
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**FIG. 3**. (A) Fractionation of reaction products after incubation of R25 cell homogenate (397 µg of protein) with the mixture described in the legend to Fig. 2 plus 0.125 mg of OAB and 50 nM of L-proline. After incubation for 60 min at 37°C, the reaction was terminated with 0.05 ml of 6 M HCl containing 30 mg of OAB per ml and the mixture was centrifuged. The supernatant was placed on a 1-ml bed volume column of AG50W (100–200 mesh) and eluted first with 16 ml of 2 M HCl and then with 2 M NaOH. (B) Fractionation of a standard P5C–OAB complex. The dihydroquinazolinium product of P5C and OAB was formed by incubating 0.050 µCi of pure L-[14C]P5C in 1 M HCl containing 5 mg of OAB per ml for 5 min at 37°C. The condensate was then applied to a 1-ml bed volume column of AG50W (100–200 mesh) and eluted first with 2 M HCl and then with 2 M NaOH.

When homogenates of R25 cells were incubated with L-[14C]glutamic acid as described above and treated with OAB in 6 M HCl, only 1.75 nmol of P5C product per hr per mg of protein was recovered. Because proline was formed at a much higher rate (i.e., 10.03 nmol/hr per mg of protein), we reasoned that most of the P5C formed from glutamic acid must have been reduced to proline. Therefore, we attempted to block the further metabolism of P5C (Table 1). Addition of OAB (0.5 mg/ml) during the incubation, which forms a complex with P5C, increased P5C formation to 4.20 nmol/hr per mg of protein and simultaneously decreased proline formation by 19%. Presumably, OAB complexed with P5C, making it unavailable to P5C reductase. On addition of L-proline (0.2 M), which inhibits P5C reductase in extracts of cultured cells (14), formation of neither proline nor P5C was detectable. When OAB (0.5 mg/ml) and L-proline (0.2 M) were present simultaneously, however, P5C formation could be measured at 3.21 nmol/hr per mg of protein, whereas proline was barely measurable at 0.28 nmol/hr per mg of protein. We used this final assay system with 0.5 mg of OAB per ml and 0.2 M proline for subsequent studies of P5C synthase activity.

Although the concurrent increase in recoverable P5C and decrease in proline on addition of OAB and proline suggested that we were measuring an enzyme in the proline biosynthetic pathway, we sought further proof that our assay was a valid measure of P5C synthase activity by comparing activities in homogenates from the C9 and R25 cell lines. The two lines have similar P5C reductase and P5C dehydrogenase activities and undetectable ornithine aminotransferase and proline oxidase (Table 2). The P5C synthase pathway was first assessed by incubating intact monolayers of cells with medium containing

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### Table 1. Effects of OAB and proline on conversion of glutamic acid to P5C and proline

<table>
<thead>
<tr>
<th>Additions</th>
<th>P5C formation</th>
<th>Proline formation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>1.73</td>
<td>10.03</td>
</tr>
<tr>
<td>OAB (0.5 mg/ml)</td>
<td>4.20</td>
<td>8.52</td>
</tr>
<tr>
<td>Proline (0.2 M)</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>OAB (0.5 mg/ml) + proline (0.2 M)</td>
<td>3.21</td>
<td>0.28</td>
</tr>
</tbody>
</table>

OAB or proline (or both) were added to the reaction mixture prior to incubation with R25 cell homogenate. The rates of P5C and proline formation were then determined. Data represent means of two experiments and are given as nmol/hr per mg of protein.

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### Table 2. Activities of proline pathway enzymes in Chinese hamster ovary cell mutants

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Specific activity, nmol/hr per mg of protein</th>
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<tbody>
<tr>
<td>P5C reductase</td>
<td>633 ± 50</td>
</tr>
<tr>
<td>Ornithine aminotransferase</td>
<td>Undetectable</td>
</tr>
<tr>
<td>P5C dehydrogenase</td>
<td>27.6 ± 0.6</td>
</tr>
<tr>
<td>Proline oxidase</td>
<td>Undetectable</td>
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</tbody>
</table>

Enzyme specific activities in homogenates of C9 and R25 cells were determined with sensitive radioisotopic assays as described (8–11). Data represent mean ± SEM of at least four determinations on two separate cell harvests.

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**FIG. 4**. Incorporation into protein of [14C]glutamate and [14C]proline derived from [14C]-labeled glutamate in C9 (●) and R25 (○) Chinese hamster ovary cell mutants. Intact monolayers of cells were incubated with glutamine-free Eagle's minimal essential medium containing 0.1 mM L-glutamic acid and L-[U-14C]glutamic acid at 0.8 µCi/ml. After 60 min, the monolayers were rinsed thoroughly and scraped from the dishes. Trichloroacetic acid was added to a final concentration of 5% (wt/vol), and the samples were centrifuged for 15 min at 2000 × g. The protein pellet was washed several times with 5% trichloroacetic acid and then hydrolyzed by heating for 18 hr at 110°C in 6 M HCl. The hydrolysate was lyophilized, dissolved in a small volume of H2O, placed on a 3-ml bed volume column of AG50W (200–400 mesh) cation-exchange resin, and eluted with 1 M HCl. Fractions of the effluent were collected and 14C was determined by liquid scintillation spectrometry.
As shown in Fig. 4, the C9 cell line was unable to incorporate proline derived from glutamate, whereas the R25 line readily incorporated glutamate-derived proline. Both lines incorporated [14C]glutamic acid itself into protein, thereby excluding a transport defect in the C9 cells.

We concluded that the C9 line cannot synthesize P5C from either ornithine or glutamic acid, although it can convert P5C to proline. The R25 line has specifically acquired the capacity to form P5C from glutamic acid. There are no other known differences between the two cell types.

In agreement with the studies in intact cells, P5C synthase activity was undetectable in cell-free homogenates of C9 and present at 5.97 nmol/hr per mg of protein in homogenates of R25 cells. Thus, P5C synthase activity measured with our assay correlates with the capacity for P5C formation from glutamic acid in intact cells.

Further characterization of P5C synthase activity in R25 cells showed that P5C formation increased linearly for up to 120 min of incubation after an initial lag period of approximately 10 min (Fig. 5A). The lag could be reduced to less than 5 min by preincubating the cell homogenate for 60 min at 37°C (data not shown). Product formation also increased linearly as the amount of added cell homogenate protein was increased from 100 to 400 μg per assay (Fig. 5B).

The reaction required inorganic phosphate and was maximal at a phosphate concentration of 0.1 M. There was no activity with either Tris-HCl or Hepes buffer in the absence of phosphate. NADPH, ATP, and their respective regenerating systems were required for optimal activity (Table 3). The formation of product P5C increased linearly as the concentration of glutamic acid was increased from 0.03 to 100 mM (Fig. 6). Although most of the above experimental data were derived with cells disrupted by repeat freezing and thawing, we determined the activity at different glutamic acid concentrations with freeze-thawed and sonicated preparations and found that P5C formation increased linearly as glutamic acid concentration increased with either procedure. Thus, it is unlikely that the lack of saturation with glutamic acid results from its limited access to subcellular particles generated during freeze-thawing.

**DISCUSSION**

Three studies published 20–30 years ago established that radioisotopically labeled glutamic acid is converted to proline in intact animals (2–4). By using *Escherichia coli* mutants, Vogel and Davis (15) demonstrated that P5C is an intermediate between glutamic acid and proline (Fig. 1). The reduction of P5C to proline by P5C reductase has subsequently been characterized in mammalian cells (16), but the conversion of glutamic acid to P5C by homogenates of mammalian cells has not been reported.

Ross et al. (17) recently described the formation of ornithine from glutamic acid, catalyzed by homogenates of rat small intestine epithelium in the presence of ATP and NADPH. Although the net catalytic activity was low and variable (maximum of approximately 0.2 nmol/hr per mg of protein by our calculation), ornithine presumably was formed via the intermediate P5C. Although P5C and proline formation were not measured, Ross’s study is the only published report in which tissue homogenates have been shown to catalyze the formation from glutamic acid of any other component of this interrelated group of pathways (i.e., P5C, proline, or ornithine).

By using L-[14C] glutamic acid as substrate and by blocking further metabolism of P5C, we have developed a method for...
measuring the formation of P5C from glutamic acid. The identity of the $[^{14}C]$ P5C product was confirmed by its comigration with pure L-$[^{14}C]$ P5C on cation-exchange resin column chromatography. To verify that this enzyme activity represents a segment of the proline biosynthetic pathway, we compared P5C formation in homogenates from two mutant Chinese hamster ovary cell lines. Based on radioisotopic tracer studies with intact cells growing in monolayer culture, the R25 line has the capacity for proline formation from glutamic acid, whereas the C9 line cannot convert glutamic acid to proline. Because the activity of P5C reductase is similar in both lines, we conclude that the C9 line cannot convert glutamic acid to P5C. When we measured P5C formation from glutamic acid in Chinese hamster ovary cell homogenates with our radioisotopic method, the results were consistent with the observations in intact cells. Homogenates from the C9 line did not form P5C whereas the R25 line catalyzed P5C formation at a rate of 5.97 nmol/hr per mg of protein. We have designated this enzyme (or enzyme complex) P5C synthase.

Although P5C synthase activity in R25 cells could be measured without blocking further P5C metabolism, the activity was increased more than 2-fold by adding OAB during the reaction (Table 1). The addition of 0.2 M proline to the assay along with OAB decreased P5C formation by 25% but blocked proline formation entirely. Therefore, with the combination of OAB and 0.2 M proline, measurement of P5C synthase activity should not be affected by changes in the activity of P5C reductase. Interestingly, P5C and proline formation are both completely blocked when proline is added to the reaction in the absence of OAB. It may be that both P5C synthase and P5C reductase are sensitive to proline feedback inhibition in cultured Chinese hamster ovary cells and that OAB somehow prevents the inhibition of P5C synthase by proline.

P5C formation catalyzed by homogenates of R25 cells increases linearly as incubation time or amount of cell homogenate is increased. Glutamic acid does not saturate the enzyme under these conditions, but more careful assessment of substrate affinity will require purified or partially purified enzyme. The requirements of P5C synthase activity for ATP and NADPH are consistent with the two-step reaction sequence originally proposed by Strecker (18) (Fig. 7). The labile $\gamma$-glutamyl phosphate intermediate would be expected to require tightly linked phosphorylation and reduction activities, which might explain why the activity of the pathway has previously been elusive. The success of our method most probably depends upon the concurrent provision of regenerating systems for both ATP and NADPH. Preliminary work with other cell types (data not shown) indicates that the pathway can be measured in other cultured cells.

The radioisotopic assay is specific and sensitive enough for measurement of P5C synthase activity in small quantities of tissue. It should enable us to characterize the activity and regulation of this previously unstudied enzyme in differentiated mammalian tissues.

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