Proline Biosynthesis by Cell-Free Extracts of *Escherichia coli* and Potential Errors Arising from the Use of a Bioradiological Assay Procedure

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1. The growth of *Escherichia coli* proline auxotrophs on medium containing L-proline (50 µg/ml) induces catabolic enzymes. A bioradiological assay system for proline, using *proB* cells of *E. coli*, might give erroneous results owing to proline catabolism by the proline auxotrophs on which the assay depends. 2. Differential utilization of proline and 1-pyrroline-5-carboxylate by the *proB* cells for the synthesis of protein, and failure of the method to distinguish between these two possible products of the proline-biosynthetic enzymes, might also give rise to error. 3. The proline-dependent incorporation of [¹⁴C]phenylalanine into the protein of proline-starved *proB* auxotrophs was to some degree directly influenced by the presence of crude cell extract from *E. coli*, even though this was not supplied with substrate and cofactors, and could thus not itself synthesize proline. 4. The kinetics of proline biosynthesis by cell-free extracts were linear and biphasic, only the last phase being affected by the concentrations of substrate and extract. This phenomenon is not understood. 5. Proline biosynthesis is inhibited, not only by high concentrations of ATP, but also by aspartate, glycine, alanine and serine, aspartate having the greatest effect. 6. Attempts at complementation *in vitro* between extracts of proline auxotrophic mutants were not successful, suggesting the possibility that strain X680 (*proA*) and/or X278 (*proB*) may be a double mutant. 7. The enzymes of proline biosynthesis are co-eluted from a column of Bio-Gel A1.5M in a position corresponding to a mol.wt. of 350000. 8. Comparisons between rates of proline biosynthesis *in vivo* and *in vitro* were made.

Proline biosynthesis by a cell-free extract of *Escherichia coli* when glutamate, ATP and NADPH are provided has been demonstrated by Gamper & Moses (1974). It was shown that the specific activity of this process decreased on dilution of the extract, although the loss of activity was recovered when the extract was concentrated by ultrafiltration. It was concluded that the system involved a dissociable enzyme complex, thus supporting the original proposal by Baich (1969) that the first two enzymes of the proline-biosynthetic pathway are aggregated to protect an unstable intermediate, probably γ -glutamyl phosphate.

As a result of the work to extend the studies by Gamper & Moses (1974) a simplified extract preparation procedure and assay for proline biosynthesis are here described, together with a detailed examination of the unexpected kinetics found. Experiments relating to the reliability of the assay system are also reported.

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Experimental

Organisms and culture media

The *E. coli* strains used in this study were the same as those used by Gamper & Moses (1974). The strains X210, X680, X278 and X277 possessed the common genotype F⁻, *thi*, *str*, *leu*, *thr*, *T1*, 5, 6, λ , *lac y* and were *pro*⁺, *proA*, *proB* or *proC* respectively (from R. Curtiss, III).

All strains were stored at 4°C as stab or slant cultures on nutrient agar supplemented with amino acids as required at concentrations of 1.0 mg of each/mg. Strains were also maintained on minimal medium 63 (Pardee & Prestidge, 1961), containing D-glucose (0.2%, w/v) and threonine and leucine at 200 µg of each/ml. Where necessary proline (200 µg/ml) was also added. Subculturing was carried out once a week and all strains were periodically checked for retention of proline auxotrophy. When reversion occurred (particularly common with X277) a fresh line was prepared from a stock stab or slant culture.

Growth experiments

Overnight cultures in the medium to be used subsequently were diluted at the start of the period of measured growth to give a suspension containing 8.25×10^7 – 13.2×10^7 cells/ml ($A_{650} = 0.05$ – 0.08). The cultures were shaken vigorously at 37°C , growth being recorded by measurement of A_{650} against a water blank, by using a CE272 linear-readout spectrophotometer (Cecil Instruments, Cambridge, U.K.). All readings were corrected for the non-linearity of the instrument response at A_{650} values greater than 1.0.

Amino acid analysis of cell protein

Liquid cultures of bacterial cells (12.5 ml, $A_{650} = 1.0$) were centrifuged for 10 min at 4°C and $15000g$, and the pellets washed with water, resuspended in 70% (v/v) ethanol (5 ml/pellet) and incubated at 45°C for 30 min to remove all small molecules (Roberts *et al.*, 1955). The cell residues were centrifuged again, resuspended in 6M-HCl (1.0 ml/sample), and heated at 105°C for 8 h in sealed tubes. After subsequent evaporation to dryness by a stream of air, the residues were dissolved in water (0.5 ml). Samples were chromatographed on Whatman no. 4 paper in one dimension with 'semi-stench' solvent (Crowley *et al.*, 1963), together with authentic amino acid markers (10 μg /spot), which were located with ninhydrin. Radioactive amino acids were located by excising a strip of chromatography paper in the direction of the solvent flow, from 2.5 cm before the origin to 2.5 cm beyond the solvent front, the strip then being divided into 19 consecutive pieces at 2.5 cm intervals. The amount of ^3H present in each section was determined by solubilizing the sample with 1.0 ml of Soluene 350 (Packard Instruments, Reading, Berks., U.K.)/scintillation fluid [toluene containing 0.4% (w/v) 2,5-diphenyloxazole and 0.05% (w/v) 1,4-bis-(5-phenyloxazol-2-yl)benzene] (1:1, v/v), together with 0.5 ml of water, and incubation at 37°C for 1 h. Scintillation fluid (9.0 ml) was then added and the radioactivity counted with a Packard Tri-Carb model 3320 liquid-scintillation counter optimized for ^3H (A. Finkler, personal communication). Quench correction was by the channels-ratio method.

Bioradiological assay of proline

Strain X278 was grown overnight on medium 63 containing glycerol (0.32%, w/v). Glutamate replaced $(\text{NH}_4)_2\text{SO}_4$ as the N source, and proline, threonine and leucine were included at 200 μg of each/ml. The cells were washed by centrifugation as above with the same medium, but omitting glutamate and proline, resuspended again in the absence

of glutamate and proline to $A_{650} = 4.5$ (7.43×10^9 cells/ml) and incubated at 37°C , with rotary shaking, for 4 h to starve them of proline. Before use the suspension was readjusted, if necessary, to $A_{650} = 4.5$.

The proline assay system was adapted from Gamper & Moses (1974) and contained, in a total volume of 1.2 ml: 39 μM -L-[U- ^{14}C]phenylalanine (1.8 $\mu\text{Ci}/\mu\text{mol}$); 50 mM-L-glutamate (sodium salt); 25 mM-MgCl₂; proline sample, 0–10 μg /ml; cell suspension, 0.4 ml. All the compounds were dissolved in medium 63 containing glycerol, threonine and leucine. The cells were added last, after which the reaction vessels (25 ml McCartney bottles) were vigorously shaken at 37°C for at least 1 h. At intervals, 0.1 ml samples were withdrawn from each vessel and spotted on filter-paper discs (Whatman 3MM, 24 mm diameter). Alternatively, after 1 h, three 0.1 ml samples per vessel were taken. Once the liquid had completely entered the paper (a period of a few seconds) the discs were dropped into aq. 5% (w/v) trichloroacetic acid at room temperature (approx. 20°C), thus killing the cells, fixing them to the paper and removing all [^{14}C]phenylalanine not incorporated into protein. After 45 min the discs were transferred to a second wash of trichloroacetic acid, followed by two washes of acetone/ethanol (1:1, v/v) and finally into acetone. They were then air-dried (H. Tristram, personal communication).

For scintillation counting, discs were placed individually into vials containing 5.0 ml of the scintillation fluid described above. All discs lay flat on the bottoms of the vials and the radioactivity was counted by a Packard Tri-Carb model 3320 liquid-scintillation counter optimized for ^{14}C . Counting efficiency was about 60%. The heterogeneous nature of the system precluded correction for quenching; results were thus expressed as c.p.m.

Bioradiological assay of proline biosynthesis

The standard assay system, modified from that of Gamper & Moses (1974), contained, in a total volume of 1.2 ml: 39 μM -L-[U- ^{14}C]phenylalanine (1.8 $\mu\text{Ci}/\mu\text{mol}$); 50 mM-L-glutamate (sodium salt); 25 mM-MgCl₂; 5 mM-ATP; 0.4 mM-NADPH; X278 cell suspension, 0.4 ml; enzyme sample (approx. 1.5–9.0 mg of protein/ml), 0.3 ml. All components other than the living proline auxotrophs and the enzymes of the cell-free extract were dissolved in medium 63 lacking $(\text{NH}_4)_2\text{SO}_4$, containing glycerol (0.32%, w/v), and mixed to give a stock solution from which 0.5 ml samples were dispensed into McCartney bottle reaction vessels. Cell suspension was added before putting the bottles into a water bath at 37°C . Immediately thereafter the assays were started by adding the cell-free extract and aerated by vigorous shaking.

Every reaction vessel with the 'complete' assay mixture was accompanied by one lacking glutamate, ATP and NADPH. This 'control' vessel permitted measurement of ^{14}C uptake into the trichloroacetic acid-insoluble material of the assay cells due to the release of proline by the proteinases in the cell-free extract. For every set of 'complete' and 'control' assay vessels there was a 'blank' vessel from which all the cofactors and the enzymes were omitted: this allowed correction for ^{14}C incorporation due to release of proline by proteinases or from any other source within the starved cells.

Samples were removed as described above for determination of ^{14}C incorporation. The first sample was taken immediately after the enzymes were added to the reaction mixture and thereafter at 5–10 min intervals over a 1 h period, one sample being taken from each reaction vessel at each time.

Synthesis and assay of 1-pyrroline-5-carboxylate

Pyrroline-5-carboxylate was chemically synthesized by the method of Williams & Frank (1975) and assayed in a mixture containing: trichloroacetic acid (10%, w/v, in ethanol), 0.5 ml; *o*-aminobenzaldehyde (0.125%, w/v, in ethanol), 0.25 ml; pyrroline-5-carboxylate sample, 0.25 ml. The A_{440} was recorded after 30 min at room temperature, with water replacing the sample in the blank, and the concentration of pyrroline-5-carboxylate calculated from the molar absorption coefficient of $2940\text{M}^{-1}\cdot\text{cm}^{-1}$ (Williams & Frank, 1975).

Preparation of cell-free extract of E. coli

E. coli X210 starter cultures were grown overnight (15 h) at 37°C on 50 ml of medium 63 containing glucose (0.2%, w/v), threonine and leucine ($200\mu\text{g}$ of each/ml). Main cultures were inoculated with 150 ml of starter/litre of the same medium, which was subdivided into 500 ml flasks (230 ml/flask) and incubated at 37°C with vigorous rotary shaking until the A_{650} had reached 0.5. Harvesting was by centrifugation for 10 min at $23000g$; the cells were washed by centrifugation with 0.1 M-Tris/HCl buffer, pH 7.4, containing 6 mM-2-mercaptoethanol, and resuspended in the same (5 ml of buffer/litre of original culture). Cellular disruption was by a French pressure cell (Aminco, Silver Springs, MD, U.S.A.) at approx. 2.4 MPa (two passages). Intact cells and debris were removed by centrifugation at $38000g$ for 1 h (all operations after cell harvesting being performed at 4°C). The extract was desalted by passage through a Sephadex G-25 column (1.6 cm $\times$ 9.5 cm) equilibrated with the Tris/HCl buffer and used within 24 h of preparation.

Ultrafiltration of crude cell-free extract

Concentration of protein solutions was effected by ultrafiltration in an Amicon model 12 stirred cell (Amicon, Lexington, MA, U.S.A.) fitted with a UM-10 filter of 2.5 cm diameter. The internal pressure of the cell was about 24 kPa, giving an optimal flow rate of 6.0 ml/h. All operations were carried out at 4°C .

Gel chromatography

A column (1.3 cm $\times$ 58 cm) of Bio-Gel A1.5M was used, equilibrated with 0.1 M-Tris/HCl buffer, pH 7.4, containing 6 mM-2-mercaptoethanol. All runs were in the downward direction at a flow rate of 7–9 ml/h at 4°C , protein elution being monitored by a flow monitor at 280 nm while 2.1 ml fractions were collected (Uvicord II, model 8300; L.K.B. Instruments, Croydon, Surrey, U.K.).

Cell-free extract (8 ml) was concentrated 5-fold by ultrafiltration, 1.0 ml being applied to the drained surface of the gel bed. To lower the viscosity deoxyribonuclease (0.5 mg, 1767 Kunitz units/mg of protein) was added before the ultrafiltration began.

The void volumes of the columns were determined by the passage of 1.0 ml of Blue Dextran (0.03%, w/v) and the columns calibrated by applying protein standards of known molecular weights: horse heart cytochrome *c* (12400) (Margoliash, 1962); bovine erythrocyte carbonic anhydrase (30000) (Peterman & Hakata, 1942); ovalbumin grade V (45000) (Warner, 1954); bovine serum albumin (67000) (Phelps & Putman, 1960); creatine kinase (80000) (Dawson *et al.*, 1967); rabbit muscle aldolase (149000) (Taylor & Lowry, 1956); rabbit muscle pyruvate kinase (235000) (Kayne, 1973); bovine fibrinogen, fraction I (390000) (von Ende *et al.*, 1958); bovine thyroglobulin, type I (670000) (Edelhoch, 1960). Sufficient protein was applied in each case (approx. 4.0 mg) to be detected in the effluent by the u.v. monitor.

Protein determinations

Protein was determined by the method of Lowry *et al.* (1951), with bovine serum albumin (fraction V) as the standard.

Sources of reagents

Amino acids, trichloroacetic acid and scintillation-grade toluene were supplied by BDH, Poole, Dorset, U.K. 2,5-Diphenyloxazole and 1,4-bis-(5-phenyloxazol-2-yl)benzene were from Koch-Light Laboratories, Colnbrook, Bucks., U.K. All cofactors and protein standards were from Sigma (London) Chemical Co., London S.W.6, U.K., except aldolase, which was from Boehringer Corp. (London) Ltd.,

London W.5, U.K. Inorganic compounds of analytical grade were predominantly from BDH. Bio-Gel was obtained from Bio-Rad Laboratories, Bromley, Kent, U.K.

Results

Catabolism of proline by strain X278

It was previously reported that the four strains X210, X680, X278 and X277 could be grown to high cell densities when minimal medium was supplemented with proline, threonine and leucine (each 50 $\mu\text{g}/\text{ml}$) (Gamper & Moses, 1974). Repetition of this work showed that, although strain X210 did indeed give a final cell yield of 2.5×10^9 – 3.3×10^9 cells/ml ($A_{650} = 1.5$ – 2.0) on that medium, the three proline auxotrophs could attain only one-third of this value. Extra glycerol or $(\text{NH}_4)_2\text{SO}_4$ (the C and N sources respectively) failed to prevent the premature cessation of growth. The addition of all three amino acids, or of just proline and threonine together (to a final concentration of 200 μg of each/ml), was required to give a final cell yield of $A_{650} = 1.2$. This applied to all three Pro⁻ strains. An increase in the concentration of leucine had no effect, and neither did that of proline or threonine when individually increased.

Several lines of evidence suggest that strain X278 is able to catabolize proline; by making it unavailable for protein synthesis such catabolism may lead to errors in the results of the bioradiological assay system. The accompanying requirement for high concentrations of threonine was not considered in great detail, as its concentration in the bioradiological assay system was maintained at the non-limiting value of 200 $\mu\text{g}/\text{ml}$. The oxidation of proline to glutamate by *E. coli* has been discussed at some length (Frank & Ranhand, 1964). The enzymes concerned, proline oxidase and pyrroline-5-carboxylate dehydrogenase (EC 1.5.1.12), are induced by proline and subject to catabolite repression (Frank & Rybicki, 1961).

Growth on glucose decreased the doubling time, but, in a medium with threonine, leucine and proline (each 50 $\mu\text{g}/\text{ml}$), the early fall in the growth rate still occurred. However, increasing threonine to 200 $\mu\text{g}/\text{ml}$ and retaining proline at 50 $\mu\text{g}/\text{ml}$ gave a higher cell yield. Thus glucose had an effect when only threonine was non-limiting.

Proline was exhausted from the medium by the time the cell doubling-rate had changed (Fig. 1), glucose inhibiting the rate of this loss by 55%. Strain X278 also incorporated ^3H from [^3H]proline into cell protein at a rate that decreased if glucose or glutamate was present (Fig. 2), suggesting that some of the proline was being oxidized to glutamate by a catabolite-repressible system. The addition of glutamate did not alter the rate of loss of proline from the medium. Although the incorporation of ^3H

Fig. 1. Loss of proline from minimal medium during the growth of strain X278 (*proB*).

The culture contained, in medium 63, threonine and leucine (200 μg of each/ml) and proline (50 $\mu\text{g}/\text{ml}$). Either glycerol (●) or glucose (○) was present (0.32% and 0.2%, w/v, respectively). At zero time sufficient overnight culture was added to give $A_{650} = 0.1$. Thereafter, at 30 min intervals, the A_{650} of each flask was recorded and samples (1.0 ml) were withdrawn and stored at -20°C . Incubation was at 37°C with rotary shaking. The cells were removed from the thawed samples by centrifugation and samples (0.25 ml) assayed for proline content by the bioradiological method.

ceased abruptly on the exhaustion of proline, A_{650} continued to rise, albeit at a very much lower rate. It is possible that this represented intracellular polysaccharide synthesis (Frank & Rybicki, 1961).

Confirmation of proline catabolism was obtained by growing strain X278 on minimal medium with [^3H]proline. Analysis of the amino acid content of the cells revealed that [^3H]glutamate was present in the cellular protein. No other labelled products were detected; it is to be expected that ^3H would be lost from the carbon skeleton by passage through the tricarboxylic acid cycle.

The failure of glucose to increase the final cell yield of a culture of the proline auxotroph when threonine was at 50 $\mu\text{g}/\text{ml}$ indicates either a threonine-degradation system insensitive to catabolite repression or a defective active-transport

Fig. 2. Incorporation of [^3H]proline into the protein of strain X278 during growth on minimal medium

Cultures of strain X278 (*proB*) were grown on minimal medium 63 with threonine and leucine ($200\text{ }\mu\text{g}$ of each/ml) and [^3H]proline ($50\text{ }\mu\text{g}/\text{ml}$; $0.505\text{ }\mu\text{Ci}/\mu\text{mol}$). Also present were: glycerol (0.32% , w/v), $\bullet$; glycerol and glutamate (50 mM), $\circ$; glucose (0.2% , w/v), $\blacktriangle$; glucose and glutamate, $\square$. At 30 min intervals three 0.1 ml samples from each culture were taken for measurement of trichloroacetic acid-insoluble ^3H . The A_{650} values were determined from parallel cultures containing [^{12}C]proline.

system specific for this amino acid. It is noteworthy, however, that strain X210, also *thr*, and the parent strain X680, X278 and X277 did not require an increased concentration of threonine to give a high cell density. The significance of this finding has not been established.

Bioradiological assay of proline

The procedure described for proline assay determines the concentration of proline present in a sample at the start of the 1h assay incubation period; this was used to calibrate the assay of proline biosynthesis (where the amino acid was absent at zero time and then continuously produced).

In the calibration assays the rate of ^{14}C incorporation into trichloroacetic acid-insoluble material was independent of the initial proline concentration, although this determined the point at which incorporation abruptly ceases (Fig. 3a). By measuring the incorporation of ^{14}C for each proline concentration from the plateaux of Fig. 3(a), a calibration curve could be constructed (Fig. 3b). Numerous experiments revealed variation in the gradient of the line.

Fig. 3. Incorporation of [^{14}C]phenylalanine into trichloroacetic acid-insoluble material of strain X278 during the bioradiological assay of proline or pyrroline-5-carboxylate

(a) Kinetics of ^{14}C incorporation with proline present ($0.5\text{--}10.0\text{ }\mu\text{g}/\text{ml}$). (b) Calibration curve for the assay with proline ($\blacktriangle$) or pyrroline-5-carboxylate ($\bullet$). All values were corrected for ^{14}C incorporation into the cells in the absence of added proline or pyrroline-5-carboxylate.

It was therefore essential to construct a fresh calibration curve for each experiment.

Strain X278, being blocked in the synthesis of glutamic γ -semialdehyde from glutamate, can synthesize protein when supplied with either pyrroline-5-carboxylate, which it can reduce to proline, or proline itself. Comparing a calibration curve of the bioradiological assay of pyrroline-5-carboxylate with that produced with equimolar amounts of proline (Fig. 3b) shows more ^{14}C incorporation with pyrroline-5-carboxylate than with proline. Pyrroline-5-carb-

oxylate is catabolized at a lower rate than is proline (Frank & Ranhand, 1964), although the same subsequent pathway is involved. The reason for the difference is not yet clear, although it was suggested by those authors that proline oxidase and pyrroline-5-carboxylate dehydrogenase form a membrane complex. Thus it seems to us that, compared with endogenously generated pyrroline-5-carboxylate, external pyrroline-5-carboxylate may have reduced access to the pyrroline-5-carboxylate dehydrogenase.

The importance of pyrroline-5-carboxylate and proline giving very different calibration curves is apparent when one assays *E. coli* cell extracts for proline-biosynthetic activity. Should the assay cells be responding to pyrroline-5-carboxylate, and all assays were calibrated in terms of proline, then an overestimation of the rate of biosynthesis would result. With unfractionated cell extract from wild-type strains, which contain very active pyrroline-5-carboxylate reductase this would be of no consequence, as all the pyrroline-5-carboxylate would be converted into proline, but with extract partially purified by gel chromatography, or with other techniques that may be used in the future, there is the very real possibility of detecting pyrroline-5-carboxylate rather than proline and thus of introducing errors into the measurement of enzyme activities.

Proteolytic activity in the cell-free extract will presumably also liberate amino acids other than proline, and these will include phenylalanine. Thus the possibility of dilution of [^{14} C]phenylalanine by the unlabelled amino acid should also be considered. The observed linear relationship between the amount of extract supplied and the rate of ^{14} C incorporation into strain X278 might have resulted from a balance between increased incorporation due to proline produced versus decreased incorporation resulting from a lowering of the specific radioactivity of the phenylalanine.

Proline biosynthesis by cell-free extract from *E. coli*

Crude cell extracts contained extensive proteolytic activity, releasing proline in the absence of glutamate, ATP and NADPH. The rate of ^{14} C incorporation into the trichloroacetic acid-insoluble material of the strain X278 (a measure of the proline release) was proportional to the amount of extract supplied and was constant over an assay period of 1–2 h.

In the presence of glutamate, ATP and NADPH (the 'complete' assay system) the rate of ^{14} C incorporation increased considerably, giving biphasic kinetics (Fig. 4). There occurred first a period of rapid increase in the ^{14} C incorporation at a rate approximating to the fastest that the cells could attain when provided with unlimited proline in the

Fig. 4. Incorporation of [^{14} C]phenylalanine into strain X278 in the presence of a cell-free system synthesizing proline

Each reaction vessel contained, in a total volume of 1.2 ml: L-[U- ^{14} C]phenylalanine, 39 μ M (1.8 μ Ci/ μ mol); glutamate, 50 mM; ATP, 5 mM; NADPH, 0.4 mM; $MgCl_2$, 25 mM; proline-starved *E. coli* X278, 0.4 ml; cell-free extract of *E. coli* X210 (10 mg of protein/ml), 0.05–0.3 ml. All components other than the cell-free extract were dissolved in minimal medium 63 lacking $(NH_4)_2SO_4$ but containing glycerol (0.32%, w/v). Incubation and sampling were as described for the bioradiological assay of proline (see the Experimental section). Volume of cell-free extract/reaction vessel: 0.3 ml, $\bullet$; 0.25 ml, $\circ$; 0.2 ml, $\blacktriangle$; 0.15 ml, $\triangle$; 0.1 ml, $\blacksquare$; 0.05 ml, $\square$.

assay system (Phase I), succeeded by a linear slower phase (Phase II).

Effect of dilution of the extract on the specific activity of proline biosynthesis

To determine the specific activity of proline biosynthesis (μ mol of proline produced/min per mg of

protein, i.e. units/mg of protein) the ^{14}C recorded at each sampling time for both the 'complete' and the 'control' assay systems was corrected for the ^{14}C incorporation of the 'blank' vessel and then converted into quantities of proline by using the calibration curve. The values so obtained for the 'control' vessels were deducted from those of the equivalent 'complete' system to give the kinetics of proline biosynthesis (Fig. 5). The specific activities were calculated from the resulting Phase-II values.

This method demonstrated the effect of dilution described by Gamper & Moses (1974). The results from a number of experiments are shown in Fig. 6, illustrating the wide range of specific-activity values found with different batches of extract. Although this result is generally similar to that reported

previously (Gamper & Moses, 1974), the changes that we have made in the preparation of the cell-free extracts have increased the observed biosynthetic activities about 10-fold.

None of the curves of Fig. 6 passes through the origin. Since the fall in specific activity on dilution of the extract is explicable in terms of a readily dissociable protein complex, the integrity of which is required for the functioning of the proline-biosynthetic pathway, this would suggest that a minimum protein concentration is required to promote the aggregation of the enzymes. The dilution effect was seen whether Tris/HCl or phosphate buffer was used in the preparation of the extract, or for its dilution (although phosphate was always the buffer present in the medium 63 used as the main solvent in the assays); likewise, the presence or absence of bovine serum albumin to maintain a constant total protein concentration had no effect. The dilution effect was fully reversible after reconcentration by ultrafiltration.

Fig. 5. Kinetics of proline biosynthesis

Data from an experiment of the type shown in Fig. 4 are expressed in terms of proline synthesized. Cell-extract volumes (in reaction mixture volume of 1.2 ml): 0.3 ml, ●; 0.25 ml, ○; 0.2 ml, ■; 0.15 ml, □; 0.1 ml, ▲; 0.05 ml, △.

Fig. 6. Effect of dilution of the cell extract on the specific activity of proline biosynthesis

Data from four separate experiments of the type described in Fig. 5 are shown. In each case the specific activity is calculated from the Phase-II rate of [^{14}C]phenylalanine incorporation. All dilutions were made in the absence of albumin.

Effect of changes in the concentrations of the substrate and cofactors

Increasing the concentration of the glutamate gave kinetics of ^{14}C incorporation and of proline biosynthesis similar to those shown in Fig. 5, the system becoming saturated at 50mM-glutamate (Fig. 7a). The same pattern was seen with changes in the concentration of ATP (Fig. 7b), but with an inhibition of the reaction at concentrations greater than 10mM.

The requirements for glutamate, ATP and NADPH were absolute. Of nine amino acids tested for use as a substrate in the reaction (12 if one includes the threonine, leucine and phenylalanine present as essential nutrients in all the reaction vessels), only glutamate gave synthesis of proline. However, in the presence of ATP, NADPH and glutamate certain of the amino acids had an inhibitory effect, aspartate giving total inhibition, and glycine, alanine and serine giving at least 80% inhibition under the conditions pertaining in the assays (all amino acids at 50mM). None of the compounds tested affected the ability of X278 cells to incorporate ^{14}C phenylalanine in the presence of excess proline (1.0mg/ml).

Biphasic kinetics of proline biosynthesis

The biphasic kinetics reported above were observed with the 'complete' assay systems and with crude cell extracts from a proline prototroph of *E. coli*. Extracts from the proline auxotrophs X680 and X278 gave kinetics indistinguishable from those caused by proteolytic activity. Extracts of strain X277 were similar to those from strain X210, presumably because pyrroline-5-carboxylate produced by that extract served as a source of proline for the assay cells.

The shift from the kinetics of Phase I to those of Phase II was very rapid, as shown by sampling at 1min intervals, and both phases were linear. Pre-incubation of the extract with X278 cells for 30min at 37°C, but without the inclusion of ^{14}C phenylalanine or substrates, failed to eliminate the phase shift after the substrates and labelled phenylalanine were added later; neither did preincubation of substrates with the X278 cells before adding the label and the enzyme sample. Thus the Phase I/Phase II shift cannot be due to changes to the extract on its transfer from 4°C to 37°C and the different chemical environment of the assay system, or to changes to the cells on their contact with ATP

Fig. 7. Dependence of the specific activity of proline biosynthesis on the concentrations of glutamate (a) and ATP (b). The standard bioradiological assay procedure was followed, with all specific-activity values calculated from the resulting Phase-II values of ^{14}C incorporation. Each assay vessel contained 0.15 ml of crude extract from strain X210. The results of several independent experiments are shown in each diagram.

or NADPH. The initial rapid rate of ^{14}C incorporation could not have resulted from the presence of free proline in the extract, since this had just been desalted on Sephadex G-25 during its preparation. Desalting on Sephadex G-25 was important, since overnight dialysis was unable to remove free proline completely.

A consistent feature of the ^{14}C -incorporation pattern is that any change in the assay conditions, e.g. in the concentration of the crude extract or in the substrate or cofactor concentrations, affected only Phase-II kinetics; the greater the slope of Phase II the longer was the duration of Phase I. The other significant point is that the kinetic pattern observed is quite unlike that reported by Gamper & Moses (1974), who found that a single linear curve was produced, the gradient of which varied with the concentration of the extract in the reaction mixture.

Comparison of rates of proline biosynthesis in vivo and in vitro

It is possible to calculate the approximate specific activity for proline biosynthesis within the intact exponentially growing *E. coli*, on the basis of published data for the internal protein and proline contents of *E. coli* (Roberts *et al.*, 1955), cell size (Luria, 1960), the number of cells/ml of culture at $A_{650} = 1.0$ (Moses & Prevost, 1966) and the experimentally determined doubling time for strain X210 on glycerol, 74 min. This gives a specific activity of 3.43 nmol of proline/min per mg of total cell protein.

Several factors may be responsible for the discrepancy between the extrapolated and calculated rates of proline synthesis *in vivo*. The linear extrapolation may not apply to intracellular concentrations of protein perhaps because at much lower protein concentrations than those the proline-biosynthetic enzymes would be fully aggregated and incapable of greater catalytic activity. Furthermore, the cell-free system is probably not subject to normal intracellular regulatory factors, particularly as in our experiments the affinity of the starved auxotrophs for proline may maintain the ambient proline concentration below its normal intracellular value.

Complementation in vitro of extract from two Pro-strains of E. coli

It has been shown that the concentration of diluted cell extract by ultrafiltration restores the fall in specific biosynthetic activity caused by dilution, perhaps by allowing recombination of the separated enzymes into biosynthetically active aggregates. From this conclusion it was reasoned that combining extracts of strains X680 and X278, after prior dilution, would result in a mixture of the individual proteins

originally in the aggregates. Reconcentration by ultrafiltration should then give reassociation of the proteins to produce four types of aggregate. One of these would be expected to have both enzymes active, and hence the recombinant mixture should show proline biosynthesis with a specific activity 25% of that found with wild-type extract at an identical protein concentration.

Several attempts at dilution, mixing and reconcentration of the extracts from *proA* and *proB* strains failed to demonstrate complementation, suggesting either that one or both mutants had lesions affecting both of the proline genes concerned with the synthesis of pyrroline-5-carboxylate or that the concentration procedure was insufficient to give reassociation of the enzymes to a degree allowing detection of the small amounts of proline biosynthesis expected. The cells providing extracts for complementation experiments *in vitro*, being proline auxotrophs, were of course grown in the presence of proline. It could thus be argued that the synthesis of all proline-biosynthetic enzymes would have been repressed and thus no complementation was to be expected. However, unlike many biosynthetic systems, that for proline is unaffected by end-product repression (Baich, 1971).

Fractionation of crude cell extract by gel chromatography

The possibility of confirming the aggregation of the two proline-biosynthetic enzymes by means of their co-purification with gel chromatography was investigated. Chromatography of strain-X210 extract as described in the Experimental section yielded fractions with no detectable proline-biosynthetic activity. Concentration of the fractions by ultrafiltration before adding them to the assay vessels did, however, give ^{14}C incorporation greater than that corresponding to any proteinase activity (Table 1), again with biphasic kinetics. The upper and lower limits of the recorded activity gave a molecular-weight range of 2.9×10^5 for the active species (Fig. 8).

The addition to the assay system of cell extract (0.15 ml/assay vessel), without simultaneously supplying the substrates for proline biosynthesis, doubled the rate of ^{14}C incorporation in the presence of excess added proline. It therefore appears that crude cell extract increases the maximum rate at which strain X278 can incorporate [^{14}C]phenylalanine (and thus presumably assimilate proline), owing, perhaps, to nutrients from the extract raising the overall rate of proline synthesis and hence the rate of proline assimilation.

Removal of such factors by partial purification of crude cell extract might also affect the apparent kinetics of proline biosynthesis *in vitro*, particularly as the initial rate of biphasic ^{14}C incorporation

Table 1. *Specific activities of proline biosynthesis in concentrated fractions from a Bio-Gel A1.5M column*
 Fractions (2.1 ml), combined as indicated, were concentrated from 4.0 ml to 1.0 ml (Expt. 1), or from the volume resulting from the combining of the fractions to 1.0 ml (Expts. 2 and 3). Samples (0.3 ml) of the concentrates were assayed for proline-biosynthetic activity by the standard method. Specific activities are expressed as $10^3 \times \text{units/mg}$ of protein.

Expt.	Fraction no. ...	Specific activity														
		13	14	15	16	17	18	19	20	21	22	23	24	25	26	
1		0.0		0.0		0.0		0.0		0.64		0.64		0.0		
2		0.0					0.0				0.97					
3		0.0						0.0				2.8			4.0	

Fig. 8. *Calibration of the Bio-Gel A1.5M column*
 The elution volumes for a number of standard marker proteins of known molecular weights were determined. The vertical lines indicate the upper and lower limits of the proline-biosynthetic activity detected in the concentrated pooled fractions. 1, Blue Dextran; 2, thyroglobulin; 3, fibrinogen; 4, pyruvate kinase; 5, aldolase; 6, bovine serum albumin; 7, carbonic anhydrase; 8, cytochrome c.

equals the maximum possible rate under the experimental conditions used (Fig. 5).

Discussion

There are four potential sources of error when assaying for cell-free proline biosynthesis by using the bioradiological system.

(a) Variation in the extent to which a particular batch of strain X278 will catabolize proline. This can be corrected for by a fresh calibration curve for every experiment, but there is no certainty that the cells maintain a constant ratio of proline catabolized to that which is assimilated into cell protein under all

experimental conditions. Such factors as the cell extract, aeration of the reaction vessels, substrate concentrations (glutamate, ATP and NADPH) etc. may all have a significant effect on how proline is utilized.

(b) Uncertainty as to whether it is pyrroline-5-carboxylate or proline that is being taken up by the starved auxotrophs.

(c) The crude extract, by altering the capacity of strain X278 to assimilate proline, might affect the apparent kinetics of proline biosynthesis.

(d) The release of [^{14}C]phenylalanine by proteinases of the extract would dilute the [^{14}C]phenylalanine.

Clearly, although the bioradiological procedure possesses the twin advantages of very great sensitivity for proline (limited only by the specific radioactivity of the [^{14}C]phenylalanine used as the quantitative marker) and of removing biosynthetic proline *in situ* as fast as it is formed, it must be used in a quantitative sense with caution because of a number of influences that might affect its efficiency, and that are themselves difficult to evaluate.

The method used in the present work for the preparation of a crude cell-free extract with an active proline-biosynthetic system differed in several respects from that developed by Gamper & Moses (1974). The most significant of these were the shortening of the period of desalting from overnight dialysis to a rapid passage through the Sephadex G-25 column, and the use of frozen cells rather than the frozen cell extract as a source of enzymes. Together with a shortened centrifugation period, these all gave a more rapid preparation procedure which minimized any proteolytic action against the enzymes and was also more convenient to use.

The basic evidence supporting the existence of a proline-biosynthetic aggregate of enzymes is the decrease in the specific catalytic activity on dilution of the preparation. Evaluation of the specific activity, however, depends on the interpretation given to the biphasic kinetics of [^{14}C]phenylalanine incorporation

observed with the 'complete' assay system. There is no doubt that they are associated with proline biosynthesis, as they are found under all conditions where biosynthesis was expected, and only in such conditions.

Several explanations for the biphasic kinetics have been discussed above, particularly those that could be experimentally tested.

Other possibilities have been considered and discarded on theoretical grounds; for the time being we remain without a satisfactory explanation. The conclusions that we have reached about cell-free proline biosynthesis, based on our interpretation of the relevance of Phase-II rates, may thus have to be modified in the light of future investigations.

The assay procedure used throughout these studies, although convenient and sensitive, may have limitations for measuring enzyme activities *in vitro*. The proline auxotrophs, for example, might release metabolites during the period of the assay, affecting enzyme activities in the extract. One cannot be absolutely certain that the minimal medium, used as the universal solvent (apart from Tris/HCl buffer used in the preparation of the extract), does not influence the reaction. The presence of two independent biological entities in the same experimental system poses the difficulty of determining whether variations introduced into the system affect the aspect of primary interest (i.e. proline biosynthesis) or the response of the proline auxotrophs used to assay the proline produced. Thus proposed variations must be tested separately for effects on the proline assay system. It would, for instance, have been interesting to determine whether hydroxylamine was unable to inhibit glutamic γ -semialdehyde synthesis, since it is known to react with glutamyl phosphate (Levintow & Meister, 1956). The use of this reagent was, however, impossible because 5mm-hydroxylamine totally inhibited all ^{14}C incorporation in the proline auxotrophs.

The amount of information to be obtained by the use of crude cell extracts is necessarily limited. To obtain very high concentrations of the proline-biosynthetic enzymes to facilitate the isolation of the aggregate necessitates the development of techniques for their purification that must avoid an attendant increase in the rate of proteolysis, which would otherwise saturate the assay system with proline not derived from new synthesis. Nevertheless, one advantage of a crude-cell-free extract is that on disruption of the *E. coli* there is a chance that a proportion of the enzyme aggregates will remain intact and thus be detected. By first purifying the enzymes individually and then attempting to show complexing, one introduces the possibility of artificially creating an association that does not exist *in vivo*.

Preliminary attempts at fractionation have been attempted. The Bio-Gel A1.5M column gave fractions that, when concentrated, were able to convert glutamate into either glutamic γ -semialdehyde or proline. They were indisputably eluted well after the void volume and in a position corresponding to a mol.wt. of 300000–500000. The data do not distinguish between the fractions containing an intact aggregate when eluted or co-elution of individual enzymes. Elucidation of this problem is considered in the following paper (Hayzer & Moses, 1978).

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