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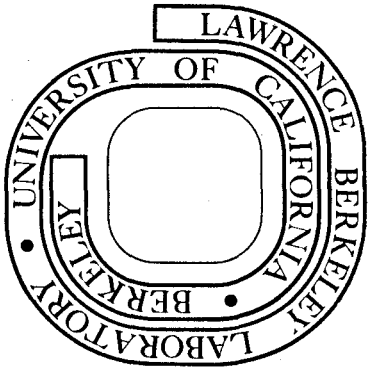


LBL-2065

## DISCLAIMER

# ENZYME ORGANIZATION IN THE PROLINE BIOSYNTHETIC PATHWAY OF ESCHERICHIA COLI

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SUMMARY

Employing a bioradiological trapping system, the conversion of glutamic acid to proline by a crude Escherichia coli extract was monitored. The activity was dependent upon the presence of both ATP- and NADPH-regenerating systems. The first two pathway enzymes appear to exist as a complex which stabilizes a high energy common intermediate postulated as γ-glutamyl phosphate. Attempted synthesis of this compound was unsuccessful due to its spontaneous cyclization to 2-pyrrolidone 5-carboxylate. Dissociation of the enzyme complex upon dilution of the extract is presumed responsible for an experimentally observed "dilution effect." Biosynthetic activity was largely unaffected by the presence of ammonia or imidazole. Stabilization of the enzyme-bound acyl phosphate precludes nucleophilic attack at the anhydride linkage. E. coli $pro_A^-$ and $pro_B^-$ auxotroph extracts failed to complement one another in the biosynthesis of proline. This failure is believed due to the lack of a dynamic equilibrium under the conditions of the experiment between the complex and its constituent enzymes.

An extract prepared from Proteus mirabilis was devoid of biosynthetic activity. It is presumed that the complex was destroyed upon preparation of the extract.

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*In vivo* studies with *E. coli* and baker's yeast showed no evidence for metabolic channeling in the final reaction of proline synthesis, the reduction of $\Delta^1$-pyrroline 5-carboxylate.

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## INTRODUCTION

In *Escherichia coli* the proline biosynthetic pathway converts glutamic acid to proline in a series of four steps. The first enzyme, γ-glutamyl kinase, catalyzes a proline-inhibitable, ATP-dependent activation of the γ-carboxyl group of glutamic acid[1]. In analogous reactions catalyzed by glutamine synthetase[2], γ-glutamyl cysteine synthetase[3], glutamate-tRNA$^{glutamine}$ amidotransferase[4], N-acetyl-γ-glutamyl kinase[5], β-aspartyl kinase[6], and various other enzymes[7], the cleavage of ATP to ADP is coupled with the formation of an intermediate acyl phosphate. By analogy, γ-glutamyl phosphate has been proposed as an intermediate in proline biosynthesis[8-11]. This compound is extremely labile[12] and has a marked tendency to cyclize to 2-pyrrolidone 5-carboxylate[8]. Where it exists as an intermediate in other biosynthetic pathways, it is found only as an enzyme-bound moiety[2-4]. Evidence to be presented in this paper supports the formation of enzyme-bound γ-glutamyl phosphate in proline biosynthesis.

γ-Glutamyl phosphate reductase, the second enzyme in the pathway, catalyzes the reduction with NADPH of γ-glutamyl phosphate to glutamic γ-semialdehyde. It has been partially purified, and its activity monitored in the reverse of the biosynthetic direction[13]. Since the substrate for this enzyme is postulated as being enzyme-bound, one might expect the first two pathway enzymes to exist as a complex or aggregate. Gene mapping studies

indicate that the respective alleles are contiguous with one another in Salmonella typhimurium and close to one another in E. coli[14,15]. While the two E. coli enzymes fail to copurify[13], it will be shown that dilution of the crude extract results in dissociation of a complex between the first two proline biosynthetic enzymes.

Glutamic γ-semialdehyde exists in rapid nonenzymatic equilibrium with $\Delta^1$-pyrroline 5-carboxylate, with the latter greatly predominating[16]. E. coli maintains nominal amounts of ornithine δ-transaminase, which acts as a link between the arginine and proline biosynthetic pathways[17]. This enzyme converts ornithine to glutamic γ-semialdehyde in the presence of α-ketoglutarate and in many gram-positive organisms serves as a secondary source of proline. However, in E. coli proline auxotrophs this secondary pathway is inconsequential.

The final enzyme in the sequence, $\Delta^1$-pyrroline 5-carboxylate reductase, reduces the double bond of the cyclic compound with NADPH to yield proline[18]. The enzyme of E. coli has not been characterized, but those from various other sources have been well studied[19,20]. Our studies indicate that in E. coli, $\Delta^1$-pyrroline 5-carboxylate reductase is not physically associated with the first two enzymes of the pathway.

The proline catabolic pathway, which converts proline to glutamic acid, is separate and independent from the biosynthetic enzymes. The pathway enzymes, proline oxidase and $\Delta^1$-pyrroline 5-carboxylate dehydrogenase, are induced by proline[21,22].

MATERIALS AND METHODS

Organisms and growth conditions

*E. coli* strains X210 (*thr*$^-$, *leu*$^-$), X277 (*thr*$^-$, *leu*$^-$, *pro*$_C^-$), X278 (*thr*$^-$, *leu*$^-$, *pro*$_B^-$) and X680 (*thr*$^-$, *leu*$^-$, *pro*$_A^-$) were gifts from Dr. R. Curtiss III. The *pro*$_A$ and *pro*$_B$ are believed to correspond to the mutually exclusive loss of γ-glutamyl kinase or γ-glutamyl phosphate reductase activity. Mutations at the *pro*$_C$ locus lead to a loss of $\Delta^1$-pyrroline 5-carboxylate reductase activity.

The cells were grown with aeration at 37° in minimal medium 63, supplemented with glucose (0.2%), thiamine (10 mg/l), and amino acids as required (50 mg/l of each)[23]. For experiments employing the bioradiological assay for cell-free synthesis of proline pathway products (see below), the medium was modified from minimal medium 63 by replacing $(NH_4)_2SO_4$ as a source of N with 20 mM glutamate. This medium, designated M64glyTL, also contained glycerol (0.2%), together with thiamine, threonine and leucine as above.

*Proteus mirabilis* WR1-PM1 (*nic*$^-$) was obtained from Dr. L. Baron, and was grown in the same medium together with nicotinamide (50 mg/l). Wild type *Saccharomyces cerevisiae* X2180-1B(α) was obtained from Prof. R. Mortimer and was grown as described previously[24].

Growth was measured turbidometrically with a Beckman DK-2 double-beam spectrophotometer, using $A_{650\ nm}$ for the bacteria, and $A_{540\ nm}$ for the yeast[25].

Measurement of the incorporation of $^{14}C$ from glucose into protein amino acids

The methodology was identical with that described previously[24], save for a different minimal medium for bacterial growth, as noted above. Briefly, cells were grown exponentially on [$^{14}C$] glucose as sole carbon source. At intervals, samples of the suspension were mixed with trichloroacetic acid, and the precipitated protein was washed with ethanol-water, ethanol and

ether, dried and subjected to acid hydrolysis. Individual amino acids in the hydrolysate were separated by paper chromatography, and their $^{14}C$ contents measured by conventional methods.

Materials

Ethyl acetamidomalonate was obtained from Nutritional Biochemicals, Cleveland, Ohio, acrolein and o-aminobenzaldehyde from J. T. Baker Chemical Co., Phillipsburg, N. J., N-carbobenzoxy-L-glutamic acid from Cyclo Chemical Co., Los Angeles, Calif., L-2-pyrrolidone 5-carboxylic acid and crude dibenzyl phosphite from Aldrich Chemical Co., Milwaukee, Wis., and hydrogen bromide from Matheson Gas Products, East Rutherford, N. J. Bis (trimethylsilyl) trifluoroacetamide, marketed as "Regisil," was a product of Regis Chemical Co., Chicago, Ill. N-Acetyl-L-glutamic acid was purchased from Sigma Chemical Co., St. Louis, Mo. L-[$^{14}C$] Phenylalanine and L-[$^{14}C$] proline were purchased from New England Nuclear Corp., Boston, Mass., and D-[$^{14}C$] glucose from ICN Corp., Irvine, Calif. All other biochemicals were obtained from Calbiochem, Los Angeles, Calif.

$\Delta^1$-Pyrroline 5-carboxylate

Solutions of this compound in aqueous 6 N HCl were prepared as described by Vogel and Davis[18]. Stock solutions kept at 0° retained biological activity for several months. Colorimetric determination of this compound with o-aminobenzaldehyde in ethanol[8] was simple and convenient, but the extinction coefficient of the colored complex increased with the age of the stock solution. The concentration of the stock solution was therefore standardized by a microbiological assay which compared overnight growth of *E. coli* X278 on $\Delta^1$-pyrroline 5-carboxylate with that on known concentrations of proline.

γ-Glutamyl hydroxamate

This compound was synthesized by the method of Roper and McIlwain[26] for use as a standard in the colorimetric determination of γ-glutamyl phosphate. The hydroxamate gave a millimolar extinction coefficient at 505 nm of 0.920

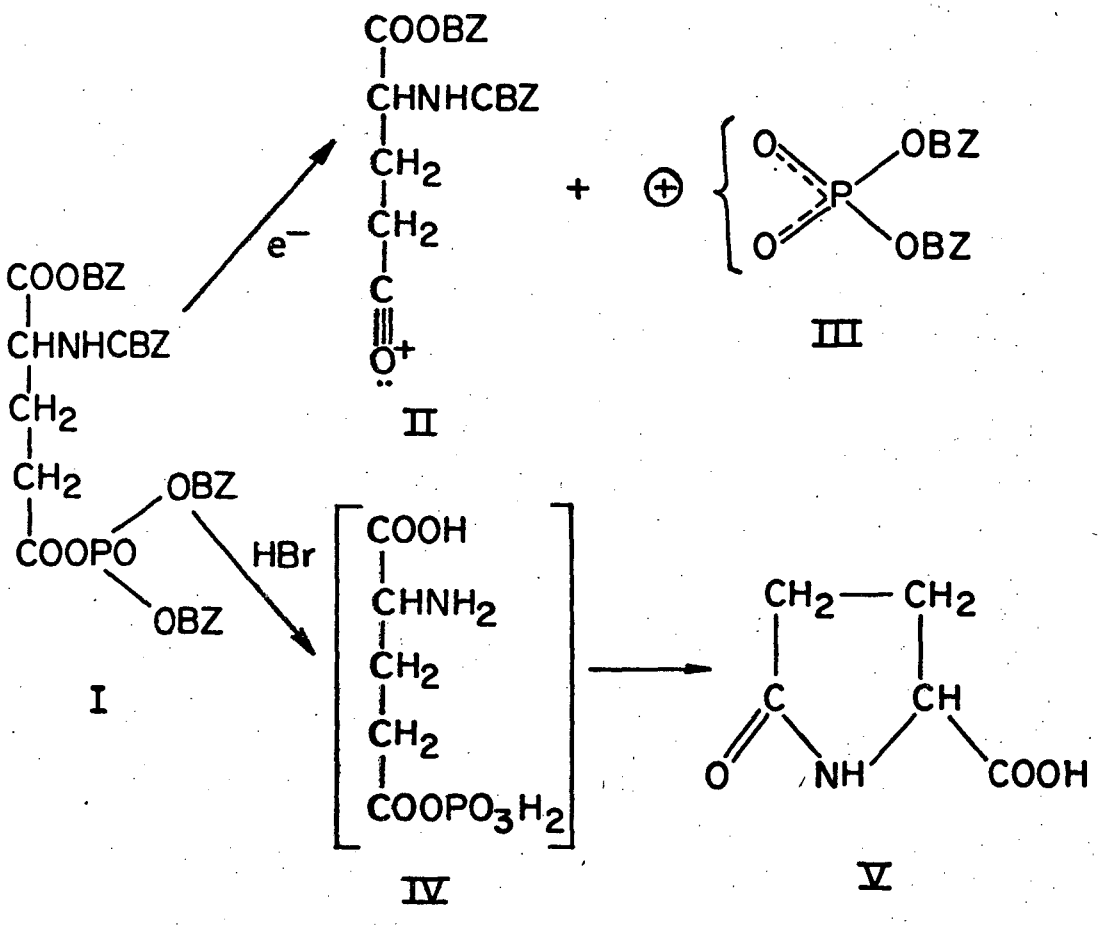
in the Hestrin method[27], 0.664 in the Lipmann and Tuttle method[28], and 1.120 in the modification of the latter by Iqbal and Ottaway[29].

γ-Glutamyl phosphate

N-Carbobenzoxy-L-glutamyl α-benzyl ester γ-dibenzyl phosphate (I) was synthesized from N-carbobenzoxy-L-glutamic acid as outlined by Katchalsky and Paecht[12]. NMR analysis of a 30% (w/v) solution of the protected phosphate in $CCl_4$ gave peaks at 1.7-2.4 δ, 4.8-5.1 δ, and 7.0-7.2 δ; the peaks had relative areas of 1.00 : 2.41 : 6.28. Mass spectrometric analysis failed to give a molecular ion, as the compound was cleaved at the anhydride linkage to give acylium ion (II) and dibenzyl phosphonium ion (III) as the only significant high molecular weight ions. Elemental analysis of $C_{34}H_{32}O_9NP$ requires C 64.6%, H 5.1%, N 2.2%, and P 4.9%; experimental values were C 64.42%, H 5.28%, N 2.12%, and P 4.99%.

The protective groups were removed by passage of HBr through a solution of the compound in dry $CCl_4$; prior to use, the HBr was passed through a tower of $P_2O_5$ to remove any traces of water. Upon reaction with the HBr, a heavy oil precipitated out on the sides of the flask. The $CCl_4$ solution was poured off and the oil washed with several portions of anhydrous ether. After the last traces of solvent had been removed by evaporation at room temperature under reduced pressure, the oil was immediately analyzed for acyl phosphate by a colorimetric method analogous to the one used by Katchalsky and Paecht[12]. A complete absence of any labile acyl phosphate was found, suggesting that the γ-glutamyl phosphate (IV) had decomposed upon synthesis. Katchalsky and Paecht[12], using the same procedure, obtained an oil which assayed as 92% γ-glutamyl phosphate.

The composition of the oil was determined by gas-liquid chromatography.




CBZ = carbobenzoxy

BZ = benzyl

XBL738-3854

Before analysis, the oil (0.74 g) was freed of entrained benzyl bromide by taking it up into 10 ml each of ether and water. After removal of the ether, the aqueous phase was washed with two additional 10 ml portions of ether. The oil was recovered from the water phase by evaporation at room temperature under reduced pressure. A portion of the oil (15 mg) was silylated by reflux with 500 µl of bis (trimethylsilyl) trifluoroacetamide in 0.5 ml of acetonitrile. After 1 h in a capped reaction vial at 90°, an aliquot of the mixture was chromatographed on a methyl siloxane (SE 30) column at a temperature of 170° and a flow rate of 100 ml/min. A comparison of the peaks obtained with peaks given by silylated standards, showed that the principal constituents of the oil were 2-pyrrolidone 5-carboxylic acid (V) and phosphoric acid. No glutamic acid was detected in the oil. We conclude that 2-pyrrolidone 5-carboxylic acid was produced by spontaneous ring closure of γ-glutamyl phosphate which, from the mode of synthesis, was presumed to be the intermediate product. Katchalsky and Paecht[12], in their reported synthesis of γ-glutamyl phosphate, made no mention of its marked tendency to cyclize.

Attempts to synthesize the acyl phosphate by a method similar to that used by Black and Wright[30] to prepare β-aspartyl phosphate also met with failure.

Preparation of cell-free extracts

The cells from an overnight culture were collected by centrifugation and washed once with 0.1 M potassium phosphate buffer, pH 7.0, containing 6 mM 2-mercaptoethanol. All subsequent operations were performed at 0°. The pellet was resuspended in similar buffer in the ratio of 10-20 ml for every 1 ℓ of culture, and the cells disrupted by passing the resultant slurry twice through a precooled French Press at 1,400 kg/cm$^2$. The suspension was then centrifuged

at 150,000 x $g_{avg.}$ for 90 min; the proline biosynthetic enzymes were found in the supernatant. Overnight dialysis of the supernatant against 50-100 volumes of phosphate buffer yielded the enzyme extract, and this was not purified further.

If the extract was to be used within 12 h it was held at 0°; for more prolonged storage it was subdivided into convenient aliquots, quick frozen in an acetone-dry ice bath, and kept at -20°. Stored in this way, extracts remained active for at least one month. When required for use, the samples were rapidly thawed out at 37°. The concentration of protein in the extract was determined by the method of Lowry et al.[31], using a dilute solution of bovine serum albumin fraction V powder as a standard. In those experiments where the extract was concentrated by ultrafiltration or where the resultant protein-free filtrate was required, collodion bags (pore size 0.5 nm) were used. Extracts to be used in conjunction with the proline-inhibitable γ-glutamyl kinase assay, or with some of the γ-glutamyl phosphate reductase assays, were prepared using 0.1 M tris-HCl buffer, pH 7.4, containing 6 mM 2-mercaptoethanol, instead of phosphate buffer.

Assay of γ-glutamyl kinase activity

Attempts to monitor proline-inhibitable γ-glutamyl kinase activity in the crude extract by a method similar to that of Baich[1], were unsuccessful. When the extract was incubated with 50 mM glutamate, 10 mM ATP, 25 mM $MgCl_2$, and 30 mM $NH_2OH$ in 0.1 M tris-HCl buffer, pH 7.4, the rate of formation of γ-glutamyl hydroxamate, as determined by colorimetric reaction with $Fe(NO_3)_3$[29], was unaffected by the presence or absence of 5 mM proline, indicating that only glutamine synthetase activity was being observed. Inclusion of an ATP-regenerating system (10 mM phosphoenolpyruvate and 20 E.U./ml

pyruvate kinase) in the assay, in order to prevent accumulation of inhibitory concentrations of ADP[11,32], did not help. Proline-inhibitable γ-glutamyl kinase in the crude extract was clearly much less active than glutamine synthetase in synthesizing γ-glutamyl hydroxamate. The colorimetric assay lacked sufficient sensitivity to detect the activity of the kinase in the presence of the glutamine synthetase system.

Assay of γ-glutamyl phosphate reductase activity

Baich[13] observed that the partially purified enzyme catalyzed the oxidation of $\Delta^1$-pyrroline 5-carboxylate to glutamate in the presence of $NADP^+$ and inorganic phosphate. The immediate product of oxidation was probably γ-glutamyl phosphate, which subsequently hydrolyzed to glutamate. The requirement for $NADP^+$ and inorganic phosphate distinguishes this reaction from the one catalyzed by $\Delta^1$-pyrroline 5-carboxylate dehydrogenase. In the reaction catalyzed by the catabolic enzyme, $NADP^+$ only partially replaced $NAD^+$ and inorganic phosphate was without effect[21].

If a crude extract was employed in the assay a dismutation reaction should occur. The last enzyme in the biosynthetic sequence, $\Delta^1$-pyrroline 5-carboxylate reductase, would be coupled to γ-glutamyl phosphate reductase, and $\Delta^1$-pyrroline 5-carboxylate would be converted to glutamate plus proline. When the crude extract was incubated with 1.0 mM $NADP^+$ and 2.0 mM DL-$\Delta^1$-pyrroline 5-carboxylate in 0.1 M potassium phosphate buffer, pH 7.0, the consumption of $\Delta^1$-pyrroline 5-carboxylate was followed colorimetrically[8]. Controls showed, however, that the activity was not dependent upon inorganic phosphate; $\Delta^1$-pyrroline 5-carboxylate dehydrogenase was catalyzing the reaction rather than γ-glutamyl phosphate reductase. It was concluded that the colorimetric assay lacked the sensitivity required to detect the $P_i$-dependent γ-glutamyl phosphate reductase activity in the reaction.

Assay of $\Delta^1$-pyrroline 5-carboxylate reductase activity

The final enzyme in the pathway possessed a high activity and was readily monitored by a simple colorimetric assay. Included in the incubation mixture were crude enzyme extract, 2.0 mM DL-$\Delta^1$-pyrroline 5-carboxylate, a NADPH-regenerating system (0.3 mM NADPH, 10 mM glucose 6-phosphate, and 2.8 E.U./ml glucose 6-phosphate dehydrogenase), and 0.5 mM 2-mercaptoethanol. The assay solvent was 0.1 M potassium phosphate buffer, pH 7.0 at 30°. Aliquots taken as a function of time were analyzed for $\Delta^1$-pyrroline 5-carboxylate[8]. Specific activity of the enzyme under these conditions was 68 E.U./mg total protein in the extract.

DL-$\Delta^1$-Pyrroline 5-carboxylate as a growth supplement for E. coli proline auxotrophs

E. coli X680 was grown overnight in medium supplemented with proline. The cells were filtered, washed and resuspended in media supplemented with various concentrations of L-proline or DL-$\Delta^1$-pyrroline 5-carboxylate. Four determinations for proline concentrations in the range $2.2 \times 10^{-5}$ - $3.3 \times 10^{-4}$ M gave a doubling time of $70 \pm 1.0$ min; for $\Delta^1$-pyrroline 5-carboxylate at four concentrations in the range $2 \times 10^{-4}$ - $1.5 \times 10^{-3}$ M the doubling time was $74 \pm 1.3$ min. Strain X277, mutant at the $pro_C$ locus, showed no growth with $\Delta^1$-pyrroline 5-carboxylate. We conclude that our preparation of this substance (see above) acted as a true intermediate in proline biosynthesis. For further experiments with this substance a standard concentration of 0.5 mM was employed.

Bioradiological assay of overall pathway activity

The conversion of glutamate to proline or $\Delta^1$-pyrroline 5-carboxylate by the crude enzyme extract was monitored by a bioradiological assay. An

overnight culture of E. coli X278, grown in medium supplemented with $\Delta^1$-pyrroline 5-carboxylate, was harvested and resuspended in a medium devoid of $\Delta^1$-pyrroline 5-carboxylate or proline. Incubation for 4 h at 37° with shaking resulted in a negligible increase in turbidity of the culture; the cells were at that stage presumed to be proline starved. The culture was collected by centrifugation and the cells were resuspended in M64glyTL medium to $A_{650\ nm} = 1.2 - 1.5$.

Unless otherwise noted, each assay contained, in a total volume of 6.0 ml, an ATP-regenerating system (5 mM ATP, 15 mM creatine phosphate, and 15 E.U./ml creatine phosphokinase), a NADPH-regenerating system (0.4 mM NADPH, 10 mM DL-isocitrate, and 0.6 E.U./ml isocitric dehydrogenase), 50 mM glutamate, 25 mM $MgCl_2$, 1-2 mM 2-mercaptoethanol, 1 ml enzyme preparation, 2 ml proline-starved X278, and $3.9 \times 10^{-5}$ M L-[$^{14}$C] phenylalanine, specific activity 0.36 $mC_i$/mmole. With the exception of the enzyme extract, which was made up in 0.1 M potassium phosphate buffer, pH 7.0, the assay components were dissolved in M64glyTL medium and adjusted to pH 7.0 before use. In the M64glyTL medium, glycerol was used in place of glucose as the carbon source, and glutamate in place of $(NH_4)_2SO_4$ as the nitrogen source, to prevent possible inhibition of the first biosynthetic step by ammonia or glucose 6-phosphate[11].

In this system, strain X278 readily concentrated proline, and to a lesser extent $\Delta^1$-pyrroline 5-carboxylate, and thereby precluded end product inhibition of $\gamma$-glutamyl kinase in the cell-free extract[1]. As X278 is a proline auxotroph, protein synthesis was dependent upon utilization of $\Delta^1$-pyrroline 5-carboxylate or proline produced by the enzymes of the

extract. The inclusion of the [$^{14}$C] phenylalanine allowed this utilization to be followed very sensitively, since incorporation of the phenylalanine into mutant cell protein was dependent upon incorporation of proline into that same protein. The production of $\Delta^1$-pyrroline 5-carboxylate or proline by the extract was monitored at regular intervals by transferring 0.5 ml of incubation mixture into 0.5 ml of 10% trichloroacetic acid. The resultant precipitated protein was collected on Millipore filters (pore size 0.45 μm), washed and prepared for counting as described by Moses and Prevost[25]. Eash assay included appropriate controls.

In a modification of the assay, biosynthesis was allowed to take place in the absence of the imino acid trapping system (i.e., [$^{14}$C] phenylalanine and X278 were omitted, and the solvent was 0.1 M potassium phosphate buffer, pH 7.0). In this modification biosynthesis was terminated by heating the reaction mixture to 100° for 5 min. After centrifugation to remove the precipitated protein, a portion of the supernatant was incubated with the components of the trapping system for radiomicrobiological assay of the imino acid produced.

## RESULTS

### Effect of DL-$\Delta^1$-pyrroline 5-carboxylate on [$^{14}$C] proline incorporation

Using strain X277, which requires proline for growth and cannot use $\Delta^1$-pyrroline 5-carboxylate, it was found that the latter compound was almost without effect on the incorporation of externally added [$^{14}$C] proline into acid-insoluble material (Table I).

### Competition experiments

Fig. 1 shows that 0.5 mM DL-$\Delta^1$-pyrroline 5-carboxylate in the medium

totally replaced glucose as a source of proline carbon in E. coli, confirming earlier results[34]. In baker's yeast, replacement was almost complete (Fig. 2). By comparison, there was no effect on the incorporation of glucose carbon into aspartic acid, glutamic acid, lysine, arginine, alanine, glycine or valine; kinetic data for valine, typical of all these amino acids, are included in Figs. 1 and 2.

Intracellular concentration of $\Delta^1$-pyrroline 5-carboxylate in E. coli

An exponentially growing culture of E. coli X210 was supplied with 0.5 mM DL-$\Delta^1$-pyrroline 5-carboxylate. At intervals up to 40 min thereafter, samples were withdrawn directly into trichloroacetic acid (final concentration 5% w/v). Parallel samples of the culture were simultaneously rapidly filtered through dry Millipore filters (0.45 μm pore size), and measured aliquots of the medium were added to trichloroacetic acid as for the whole suspension samples. Replicate measurements were made on each sample for content of $\Delta^1$-pyrroline 5-carboxylate. The results are presented in Table II. The turbidity ($A_{650\ nm}$) of the culture being known, the intracellular concentration of $\Delta^1$-pyrroline 5-carboxylate was calculated using the following assumptions: (1) at $A_{650\ nm} = 1.0$, the suspension contained 225 μg bacterial protein/ml[25]; (2) protein constitutes 11% of the bacterial wet wt.[34,35] and 56% of the dry wt.[34]; (3) the density of living bacteria is 1.1[35]; (4) the whole of the aqueous phase of the cell is accessible to the compound. The intracellular concentration was 1.47 times the extracellular concentration, viz., 0.74 mM. Thus, there was no marked accumulation of $\Delta^1$-pyrroline 5-carboxylate within the cells.

Standardization of the bioradiological assay

The assay was calibrated by incubating strain X278 for periods up to

5.5 h in growth medium containing graded amounts of L-proline or DL-$\Delta^1$-pyrroline 5-carboxylate. Aliquots were taken at regular intervals and their trichloroacetic acid-precipitable $^{14}C$ determined. Plots of $^{14}C$ incorporated versus time clearly demonstrated that protein synthesis in the mutant culture was dependent upon the presence of imino acid. When the latter was exhausted, protein synthesis stopped abruptly as shown by the absence of any further incorporation of $^{14}C$ into acid-insoluble material. The final maximum plateau level of $^{14}C$ incorporated was proportional to L-proline or DL-$\Delta^1$-pyrroline 5-carboxylate added to the reaction (Fig. 3). Proline was incorporated into the cell protein of X278 at a much greater rate than was $\Delta^1$-pyrroline 5-carboxylate. Utilization of 2.0 µg/ml of these imino acids required respectively 80 and 200 min incubation at 37°. The slower rate of $^{14}C$ incorporation observed with $\Delta^1$-pyrroline 5-carboxylate may reflect a decreased affinity of proline permease for this compound. Under conditions of the assay, the maximum observable rate of proline incorporation into mutant cell protein was 1.0 µg proline/ml/20 min at 37°.

Specificity of biosynthetic activity in extracts of E. coli X210

Extracts of strain X210 generate imino acid as monitored by the bioradiological assay (Fig. 4). The specificity of the reaction is shown in Table III. Biosynthetic activity by the extract required the addition of both ATP- and NADPH-regenerating systems and was dependent upon the glutamate concentration.

As the assay is very sensitive, it is conceivable that a major portion of the $\Delta^1$-pyrroline 5-carboxylate might have arisen from the conversion of N-acetylglutamate via the enzymes of the arginine biosynthetic

pathway and ornithine δ-transaminase. Reed and Lukens[17] monitored such a reaction, and concluded that E. coli extracts contain nominal amounts of ornithine δ-transaminase. If, in our experiments, appreciable amounts of glutamate were being acetylated and then converted to imino acid by this pathway, the activity should have been inhibited by L-arginine, since N-acetylglutamate synthetase is arginine-inhibitable[36]. However, inclusion of 10 mM arginine in the assay system was without effect (Table III), and there appears to be little doubt that the proline biosynthetic pathway was indeed being monitored.

In a companion assay, in which the imino acid trapping system was omitted from the incubation mixtures during enzymatic synthesis, various substrates were tested for activity (Table IV). Arginine, as before, was essentially without effect. The replacement of glutamate by N-acetylglutamate yielded no activity at all. Incubation of L-ornithine together with α-ketoglutarate as an assay for ornithine δ-transaminase activity yielded only 7% of the imino acid formed in the complete reaction plus trapping system, indicative of the very low activity of this enzyme in the crude extract.

In the assay described in Table III the extract showed a specific activity for $\Delta^1$-pyrroline 5-carboxylate synthesis of $1.2 \times 10^{-4}$ E.U./mg protein; the specific activity shown by the same extract in the $\Delta^1$-pyrroline 5-carboxylate reductase assay was 68 E.U./mg protein. It is therefore assumed that all $\Delta^1$-pyrroline 5-carboxylate produced in the bioradiological assay by an extract possessing the last enzyme will immediately be reduced to proline, and in such an assay it is the conversion of glutamate to proline that is monitored. Only when the extract lacks $\Delta^1$-pyrroline 5-carboxylate reductase can the formal conversion of glutamate to $\Delta^1$-pyrroline 5-carboxylate be monitored.

Baich[1] has demonstrated that γ-glutamyl kinase, the first enzyme in the proline biosynthetic pathway, is readily inhibited by very low concentrations of proline. This is confirmed by our own observation (Table IV) that the total yield of proline is markedly reduced in the absence of the trapping system during the period of imino acid biosynthesis.

Activity as a function of X210 extract concentration in the bioradiological assay

Fig. 5 shows the relationship between specific biosynthetic activity and the volume of X210 extract employed in the bioradiological assay. The initial portion of the curve is linear and extrapolates to the origin: when small quantities of extract were used, the specific activity was a linear function and the activity an exponential function of the extract concentration. Denaturation of one or more of the proline biosynthetic enzymes upon addition of the extract to the incubation mixture seemed unlikely. Bovine serum albumin was added to the reaction mixtures to provide identical total protein concentrations. Dilution of an unknown cofactor also seemed unlikely. The same specific activity relationships held when the volume of extract used in the bioradiological assay was balanced with an extract ultrafiltrate lacking protein.

A possible explanation of the effect of cell extract concentration on specific biosynthetic activity is that the first two proline biosynthetic enzymes exist together as a complex which dissociates upon dilution in accordance with the mass action law. Only the complex is presumed to be active. The probability of such a complex existing between the first two enzymes is very real. The product of the γ-glutamyl kinase reaction is most probably enzyme-bound γ-glutamyl phosphate. Thus, the second enzyme

in the sequence must come into close physical proximity to the kinase if the reaction is to ensue. Upon dissociation of the individual enzymes from the complex they are unable to catalyze the overall reaction glutamate to glutamic γ-semialdehyde (and hence to $\Delta^1$-pyrroline 5-carboxylate).

As the concentration of extract in the reaction mixture was increased, the previous relations no longer held. Total activity leveled off and specific activity decreased. This situation was not an accurate representation of the enzyme activity but reflected a saturation of the detection method with proline. From calibration experiments with the X278 trapping system, it is known that saturation of the latter occurred when proline was utilized at the rate of 29-35 ng/ml/min. This rate was approached when only 1.0 ml of the enzyme extract was included in an assay run. The maximum specific activity observed in this assay, $1.5 \times 10^{-4}$ E.U./mg protein, was roughly 5% of the in vivo rate, suggesting that if the capacity of the trapping system were greater, the linear portion of the specific activity curve would be extended considerably.

Reversibility of the complex dissociation

A portion of extract from E. coli X210 was diluted twofold with 0.1 M potassium phosphate buffer, pH 7.0. After thorough mixing, the diluted extract was reconcentrated to its original volume by ultrafiltration and allowed to stand 5 h at 0°. Relative $^{14}C$ incorporation associated with 1.0 ml aliquots of the original extract, the twofold diluted extract, and the reconcentrated extract in the bioradiological assay was respectively 100, 35, and 88 after subtraction of blanks lacking the ATP-regeneration system. The intermediate value obtained with the reconcentrated extract indicates that dissociation of the complex is not entirely reversible.

No investigation was made of the rate at which the individual enzyme subunits associate to form the active complex.

Proline biosynthesis in the presence of imidazole and ammonia

Acyl phosphates exhibit a high reactivity toward nucleophilic acyl substitution. Free γ-glutamyl phosphate would be expected to react rapidly with such nucleophiles as hydroxylamine, ammonia, or imidazole[37]. However, in the biosynthesis of proline this phosphate anhydride is believed to exist only as an enzyme-bound moiety. Its orientation within the active site of the complex enzyme, together with probable stabilization of the anhydride linkage, might preclude nucleophilic attack. If this is the case, biosynthesis should continue in the presence of such compounds as ammonia and imidazole.

Fig. 6 demonstrates the relative insensitivity of the proline biosynthetic pathway to the presence of the nucleophiles. Ammonia had no effect; imidazole was but slightly inhibitory. Prior to their use in the bioradiological assay, the cultures of X278 were incubated with 5 mM concentrations of the respective nucleophiles so as to minimize any nucleophile induced effect on the synthesis of protein by the cells during the actual assay. Preliminary growth studies had shown the growth rate of X278 to be slightly inhibited by imidazole: the doubling time ($A_{650\ nm}$ range 0.25–0.51) in the presence and absence of 5 mM imidazole was 5.1 h and 4.4 h respectively in M64glyTL medium supplemented with $\Delta^1$-pyrroline 5-carboxylate. Thus, the inhibition by imidazole in the bioradiological assay was probably to be attributed to effects on the trapping system rather than on imino acid biosynthesis by the extract.

The biosynthesis of proline in the presence of high nucleophile concentrations may be taken as evidence for stabilization of the hypothetical γ-glutamyl phosphate intermediate on the enzyme.

Failure of the acyl phosphate to react with ammonia is consistent with the behavior of other biosynthetic pathways in which γ-glutamyl phosphate is postulated as an intermediate[3,4]. Even with glutamine synthetase it appears that reaction of the enzyme-bound acyl phosphate with ammonia is enzyme catalyzed[38]. In the case of imidazole, the dubious effect of this compound on proline biosynthesis contrasts greatly with the efficacy in which it displaces the acyl phosphate from the partially purified γ-glutamyl kinase enzyme[1]. The biosynthetically active two-enzyme complex appears considerably more effective than the free kinase enzyme in stabilizing the acyl phosphate.

Attempted complementation of E. coli mutant extracts

The intimate association between the first two enzymes of the pathway prompted a study into the ability of various mutant extracts to complement one another. Three E. coli proline auxotrophs were available, each genetically distinct and presumed to be deficient in one of the three pathway enzymes. Each mutant strain was grown in cultures supplemented with 25 μg proline/ml. This proline concentration supported growth to $A_{650\ nm}$ = 1.0 approx. At this optical density, the cultures contained 225 μg bacterial protein/ml[25], of which 4.19%, or 9.4 μg/ml, was proline[33]. The remainder of the proline, 15.6 μg/ml, was presumably oxidized to glutamate by a proline induced catabolic system. Such a system in E. coli has been studied by Frank and Ranhand[21]. Extracts from the auxotroph cultures were prepared as described above.

The repressibility of the proline biosynthetic enzymes has been investigated by Baich[13,39]: only the first pathway enzyme was appreciably repressed by growth in the presence of proline. We have found that growth

of strain X210, fully competent in proline biosynthesis, in medium supplemented with 25 µg/ml of proline resulted in 24% repression of biosynthetic activity in extracts as monitored by the bioradiological assay. Fig. 7 shows the relationship of biosynthetic activity to supplemental proline concentration in the growth medium for strain X210. We presume that the extent of repression is similar for the proline auxotrophs derived from the parental strain, and that growth of the auxotrophs in the presence of 25 µg proline/ml would result in only partial repression of the first pathway enzyme.

As $A_{650\ nm}$ of the auxotroph cultures approached 1.0 the proline concentration was close to zero and the cells were effectively growing on limiting amounts of proline. This induced the synthesis of proteolytic enzymes[40], and as a result extracts from these cultures possessed high proteolytic activity. This activity interfered with the bioradiological assay for proline biosynthesis because the trapping system readily responded to the free proline and proline-containing oligopeptides resulting from proteolysis. Such activity was not subtracted out by an enzyme blank, and where proteolysis was appreciable such blanks could not be used. For complementation experiments with auxotroph extracts we therefore used as blanks the complete systems without the ATP-regeneration component (Table III). These blanks corrected for all nonspecific activity, including proteolysis.

Results of the complementation studies are given in Table V. Only three reactions showed activity. The $\underline{pro}_C^-$ extract, lacking the last enzyme, produced $\Delta^1$-pyrroline 5-carboxylate, while the wild-type extract and the mixed extracts from $\underline{pro}_B^-$ and $\underline{pro}_C^-$ strains produced proline. It is reasonable to assume that the mixed $\underline{pro}_B^-$ and $\underline{pro}_C^-$ extracts produced as

much proline as the $\underline{pro}_C^-$ extract produced $\Delta^1$-pyrroline 5-carboxylate. The lower activity of the $\underline{pro}_C^-$ extract, compared with mixed $\underline{pro}_B^-$ and $\underline{pro}_C^-$ extracts, probably resulted from the relative inefficiency with which the X278 trapping system utilized $\Delta^1$-pyrroline 5-carboxylate compared with proline. The relative value of 62% found for the activity of mixed $\underline{pro}_B^-$ and $\underline{pro}_C^-$ extracts may therefore be a true measure of the activity of the $\underline{pro}_C^-$ extract. Growth of the $\underline{pro}_C^-$ mutant in medium containing an initial proline concentration of 25 μg/ml resulted in 38% repression of activity. This compares closely with the value presented previously for the wild-type X210.

When corrected for blank values obtained in the absence of the ATP-regeneration system, the $\underline{pro}_A^-$ extract, $\underline{pro}_B^-$ extract, and a mixture of these two, all failed to show imino acid biosynthesis. The $\underline{pro}_A^-$ and $\underline{pro}_B^-$ mutants are unable to synthesize $\Delta^1$-pyrroline 5-carboxylate. Complementation of the two extracts to give biosynthetic activity would require the association of active γ-glutamyl kinase from one extract with active γ-glutamyl phosphate reductase from the other. Such complementation to give an active complex appears not to occur, and no biosynthetic activity was observed. It is conceivable that under the conditions of our assays the rate of subunit association was too slow to permit detection of activity. This failure to observe activity with mixed extracts from the $\underline{pro}_A^-$ and $\underline{pro}_B^-$ mutants supports the postulation that γ-glutamyl phosphate exists only as an enzyme-bound moiety. If a free intermediate were involved the mixed extracts would be expected to show some activity. This interpretation, of course, assumes that neither of the auxotrophs is a regulatory mutant and that repression in these strains is only partial as it is in the wild-type.

P. mirabilis extract activity in the bioradiological assay

Extracts of P. mirabilis were devoid of any proline biosynthetic activity. However, these extracts were active in the bioradiological assay. The activity was clearly proteolytic in nature since it was largely unaffected by omitting glutamate or the regeneration systems for ATP and NADPH from the bioradiological assay (Table VI). The absence of biosynthetic activity suggests that in P. mirabilis the two-enzyme complex which converts glutamate to $\Delta^1$-pyrroline 5-carboxylate is even more labile than it is in E. coli. The origin of the high proteolytic activity in the P. mirabilis extracts has not been investigated.

The specific activity of $^{14}C$ incorporation in the bioradiological assay was relatively constant over the range of P. mirabilis extract volumes employed. A strict proportionality between activity and extract volume is expected if proline was being formed via proteolysis. The slight increase in specific activity as the extract volume was increased (Fig. 8) was minimal when compared with the analogous effect for E. coli (Fig. 5). Fig. 8 illustrates the sensitivity of the bioradiological assay in monitoring proteolysis in the absence of proline biosynthesis.

## DISCUSSION

The difficulty reported by others[8,17] in obtaining a cell-free extract from E. coli capable of converting glutamic acid to $\Delta^1$-pyrroline 5-carboxylate or proline was probably due to the lability of the complex formed by the first two pathway enzymes, together with its very low specific biosynthetic activity. The high energy acyl phosphate intermediate, which necessitates the existence of the complex, is itself stabilized on the enzyme, thereby minimizing its tendency to undergo nucleophilic acyl

substitution and intramolecular cyclization. Studies on the reaction catalyzed by sheep brain glutamine synthetase, using the glutamic acid analogs β-glutamate[41] and cis-cycloglutamate[42] have provided direct evidence for the existence of enzyme-bound γ-glutamyl phosphate as an intermediate in this reaction. Similar studies conducted with the purified proline biosynthetic complex or its subunits would provide further more precise information on the nature of the γ-carboxyl activated glutamic acid intermediate which occurs in proline biosynthesis.

The lability of free γ-glutamyl phosphate would seem to preclude its use as a substrate in the study of proline biosynthesis. Our observations, as well as those of Strecker[8], indicate that the principal cause of this instability is the very rapid cyclization of γ-glutamyl phosphate to 2-pyrrolidone 5-carboxylate. Other investigators[2,4] have found that when enzyme-bound γ-glutamyl phosphate was released from the enzyme (glutamine synthetase or glutamate-tRNA$^{glutamine}$ amidotransferase) by denaturation, it was converted to 2-pyrrolidone 5-carboxylate. The cyclization reaction, however, was not observed by Katchalsky and Paecht[12] in their synthesis of γ-glutamyl phosphate.

The failure to observe biosynthetic activity with the P. mirabilis extract was attributed to the decomposition of the complex upon preparation of the extract. This contrasts greatly with the relative stability of the E. coli complex. As other organisms come to be examined for proline biosynthetic activity, the variability in complex stability may be found to widen. Specifically, complexes may exist stable enough for the two enzymatic activities to copurify as a single protein. Such a complex may

perhaps exist in Salmonella typhimurium, in which the genetic determinants are contiguous[14].

Yoshinaga et al.[10] have reported the conversion of glutamate to proline by a crude extract of Brevibacterium flavum. Activity formally required glutamate, ATP and $Mg^{2+}$. It was totally inhibited by ammonia and hydroxylamine. The extremely high activity, and the inhibition by ammonia, differentiate this system from that in E. coli. Indeed, the extract was prepared from a proline-excreting mutant, and the reaction mechanism may not be typical.

For the final reaction in proline biosynthesis, the reduction of $\Delta^1$-pyrroline 5-carboxylate, our evidence from in vivo studies in E. coli and in yeast, and from in vitro experiments with E. coli, indicates no organizational integration with the first two reactions of the pathway. This should be compared with the histidine pathway, which demonstrated channeling of intermediates in baker's yeast but not in S. typhimurium[24], and with glycolysis which does seem to involve some degree of metabolite channeling in E. coli[43,44]. It appears, therefore, that in the proline biosynthetic pathway channeling, and the transfer of an intermediate from one catalytic site to another bound to an enzyme surface, is not indicative of an overall organized macrostructure, but may rather be a solution to the biochemical problem of utilizing a potentially valuable intermediate which is nevertheless extremely unstable in aqueous solution.

It is of interest to note the position in arginine biosynthesis. Like the proline pathway, arginine biosynthesis starts from glutamic acid via the same two reactions leading to glutamic $\gamma$-semialdehyde, which is then transaminated to ornithine. In E. coli, P. mirabilis and

other bacteria, the intermediates between glutamate and ornithine are N-acetylated. Since N-acetyl γ-glutamyl phosphate does not cyclize in aqueous solution, N-acetylation has a twofold effect: (1) permitting the phosphorylation of the γ-carboxyl of glutamic acid, prior to its reduction to the aldehyde; and (2) preventing the utilization of arginine intermediates for proline biosynthesis because N-acetylglutamic γ-semialdehyde cannot cyclize to $\Delta^1$-pyrroline 5-carboxylate[45-49]. In the fungi, a group of organisms in which physical aggregation of enzymes cooperating in metabolic sequences is known[50,51], the intermediates are not N-acetylated, but are channeled[52-54]. Thus, γ-glutamyl phosphate is again stabilized, and glutamic γ-semialdehyde in the arginine pathway cannot cyclize to $\Delta^1$-pyrroline 5-carboxylate in the proline pathway. The separation of these two sets of potentially identical intermediates, either by channeling or by N-acetylation, may have the further effect of permitting the cell to exercise independent control over the two pathways. It would be interesting to know the organizational relationships between the enzymes of the arginine and proline pathways in a variety of bacteria and fungi.

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TABLE I

EFFECT OF $\Delta^1$-PYRROLINE 5-CARBOXYLATE ON THE INCORPORATION OF PROLINE INTO PROTEIN

A culture of strain X277, growing exponentially in medium 63 supplemented with glucose, threonine, leucine and proline, was divided into two portions, one of which received 1.5 mM DL-$\Delta^1$-pyrroline 5-carboxylate (P5C). After incubation at 37° for 20 min, [$^{14}$C] proline was added to each culture and samples removed at intervals for determination of trichloroacetic acid-insoluble $^{14}$C.

| Time after addition of [$^{14}$C] proline (min) | $A_{650}$ nm | | | Dis/min/ml culture | | |
|---|---|---|---|---|---|---|
| | -P5C | +P5C | Ratio $\frac{-P5C}{+P5C}$ | -P5C | +P5C | Ratio $\frac{-P5C}{+P5C}$ |
| 0 | 0.098 | 0.097 | 1.010 | 0 | 0 | - |
| 10 | 0.108 | 0.105 | 1.029 | 2360 | 2770 | 0.852 |
| 20 | 0.119 | 0.116 | 1.026 | 6060 | 5110 | 1.186 |
| 30 | 0.131 | 0.128 | 1.023 | 9910 | 8820 | 1.124 |
| 45 | 0.152 | 0.149 | 1.020 | 18000 | 16100 | 1.118 |
| 60 | 0.177 | 0.173 | 1.023 | 26700 | 24600 | 1.085 |
| 80 | 0.215 | 0.211 | 1.019 | 42200 | 40700 | 1.037 |
| 100 | 0.262 | 0.257 | 1.019 | 69600 | 64600 | 1.077 |
| 120 | 0.319 | 0.313 | 1.019 | 84700 | 82600 | 1.025 |
| Means ± S.D. | | | 1.021±0.005 | | | 1.063±0.099 |

TABLE II

UPTAKE OF DL-$\Delta^1$-PYRROLINE 5-CARBOXYLATE INTO *E. COLI*

Cells of strain X210, growing exponentially at 37° in medium 63 supplemented with glucose, threonine and leucine, received 0.5 mM DL-$\Delta^1$-pyrroline 5-carboxylate (P5C). At intervals, samples of the suspension were mixed with trichloroacetic acid (5% w/v final concn.). Simultaneously, other samples were filtered through dry Millipore filter discs (0.45 μm pore size) and aliquots of the filtrate mixed with trichloroacetic acid to give a final concn. of 5% (w/v). Replicate measurements were made of P5C concn. in each sample, and the intracellular concn. calculated as shown below.

| Time after adding P5C (min) | P5C concn. (mM) in total suspension | P5C concn. (mM) in filtrate | Ratio: total suspension / filtrate |
|---|---|---|---|
| 0 | 0.492 | 0.492 | 1.0000 |
| 2 | 0.494 | 0.496 | 0.9960 |
| 5 | 0.492 | 0.491 | 1.0020 |
| 8 | 0.492 | 0.491 | 1.0020 |
| 15 | 0.489 | 0.490 | 0.9979 |
| 25 | 0.487 | 0.487 | 1.0000 |
| 40 | 0.481 | 0.479 | 1.0041 |
| | | Mean ± S.E.M. | 1.000286± 0.00103 |

$A_{650}$ nm of cell suspension = 0.825 = 186 μg protein/ml[25].

Protein = 11% of cell wet wt.[34,35]; therefore cell wet wt. in suspension = 1.69 mg/ml.

TABLE II (Cont.)

Cell density = 1.1[36]; therefore cell vol. = 1.54 µl/ml suspension and medium vol. = 0.99846 ml/ml suspension.

Cells are 80% water[34]; therefore cellular water = 0.001232 ml/ml suspension.

Let the medium (i.e., filtrate) contain 1 unit P5C/ml; total suspension contains 1.000286 units P5C/ml (by experiment), therefore cells contain 1.000286 - 0.99846 = 0.001986 units P5C in 0.001232 ml cellular water.

Thus, intracellular concn. of P5C = 0.001986/0.001232 = 1.47 x extracellular concn.

TABLE III

SPECIFICITY OF E. COLI X210 EXTRACT IN THE BIORADIOLOGICAL ASSAY

The reaction mixtures contained 1.0 ml of cell-free extract (4.32 mg protein/ml) together with other components as described in Materials and Methods, in a total volume of 6.0 ml; the mixtures were shaken in air at 37° for 30 min. Samples (0.5 ml) were removed from each reaction mixture for determination of trichloroacetic acid-precipitable $^{14}C$.

| Components present in reaction | Trichloroacetic acid-precipitable $^{14}C$ (cpm/ml reaction mixture) |
|---|---|
| Complete, with 46 mM glutamate | 530 |
| plus arginine (10 mM) | 524 |
| minus extract of strain X210 | 0 |
| minus ATP-regenerating system | 0 |
| minus NADPH-regenerating system | 42 |
| Complete, with 16 mM glutamate | 186 |

TABLE IV

ACTIVITY OF VARIOUS SUBSTRATES IN THE BIORADIOLOGICAL ASSAY

The first reaction mixture contained the imino acid trapping system during the course of imino acid biosynthesis by the cell-free extract, as described in Materials and Methods.

In the other reactions the trapping system was absent during imino acid biosynthesis. The solvent was 0.1 M potassium phosphate buffer, pH 7.0, containing 6 mM 2-mercaptoethanol. Each reaction mixture was otherwise complete save that the ATP-regeneration system was omitted from No. 5; the total volume was 3.6 ml in each case. At the start of the assay, 0.6 ml of E. coli X210 extract (5.83 mg protein/ml) was added to each vessel. After 45 min of incubation at 37° the reactions were terminated by heating to 100° for 5 min; samples (2.5 ml) from each reaction were analyzed for proline by the radiomicrobiological assay (final volume 5.0 ml). Substrate blank values have been deducted.

| Substrate | Trichloroacetic acid-precipitable $^{14}C$ (cpm/ml in bioradiological assay) |
|---|---|
| 1. 50 mM glutamate (with trapping system) | 980 |
| 2. 50 mM glutamate | 176 |
| 3. 50 mM glutamate + 10 mM arginine | 170 |
| 4. 50 mM N-acetylglutamate | 0 |
| 5. 20 mM ornithine + 20 mM α-ketoglutarate minus ATP-regeneration system | 70 |

TABLE V

COMPLEMENTATION BETWEEN EXTRACTS OF *E. COLI* PROLINE AUXOTROPHS

The biosynthetic activity of each extract was determined with the bioradiological assay: the results reported below have been corrected by subtracting incorporated $^{14}C$ found in the absence of the ATP-regenerating system. The results presented in this Table are in each case the averaged values from several determinations. Protein contents of the extracts were: wild-type, 5.44 mg/ml; $pro_A^-$ mutant, 5.04 mg/ml; $pro_B^-$ mutant, 6.00 mg/ml; and $pro_C^-$ mutant, 4.88 mg/ml. Assay volume, 6.0 ml.

| Extract | Relative $^{14}C$ incorporation |
|---|---|
| 0.75 ml wild-type extract (strain X210) | 100 |
| 0.75 ml $pro_A^-$ extract (strain X680) | 0 |
| 0.75 ml $pro_B^-$ extract (strain X278) | 0 |
| 0.75 ml $pro_C^-$ extract (strain X277) | 12 |
| 0.75 ml $pro_A^-$ extract plus 0.75 ml $pro_B^-$ extract | 0 |
| 0.75 ml $pro_B^-$ extract plus 0.75 ml $pro_C^-$ extract | 62 |

TABLE VI

SPECIFICITY OF *P. MIRABILIS* EXTRACT IN THE BIORADIOLOGICAL ASSAY

The reaction mixtures contained 1.0 ml of cell-free extract (5.68 mg protein/ml), together with other components as described in Materials and Methods, in a total volume of 6.0 ml; the mixtures were shaken in air at 37° for 56 min. Samples (0.5 ml) were removed from each reaction mixture for determination of trichloroacetic acid-precipitable $^{14}C$. Blank values, obtained by the omission of the *P. mirabilis* extract, have been deducted.

| Components present in reaction | Trichloroacetic acid-precipitable $^{14}C$ (cpm/ml reaction mixture) |
|---|---|
| Complete, with 50 mM glutamate | 432 |
| *plus* arginine (10 mM) | 419 |
| *minus* extract of *P. mirabilis* | 0 |
| *minus* ATP-regenerating system | 436 |
| *minus* NADPH-regenerating system | 367 |
| Complete, with 15 mM glutamate | 432 |

FIGURE CAPTIONS

Fig. 1. Labeling patterns of proline and valine in E. coli X210. Cells were grown exponentially in medium 63 supplemented with threonine, leucine, and 0.125% glucose. As indicated by arrow A, [$^{14}$C] glucose (10 $\mu C_i$/ml) was added and the culture divided into three aliquots. One of these received 0.5 mM DL-$\Delta^1$-pyrroline 5-carboxylate (P5C) immediately; a second culture received P5C at arrow B, while the third received no P5C and served as a control. Samples were removed at intervals for measurement of $^{14}$C in protein amino acids as described in the text. ●——●, $^{14}$C in proline in the absence of P5C; x——x, $^{14}$C in proline, P5C being added at arrow A; ▲——▲, $^{14}$C in proline, P5C being added at arrow B; o——o, $^{14}$C in valine; Δ——Δ, $^{14}$C in valine, P5C being added at arrow B.

Fig. 2. Labeling patterns of proline and valine in S. cerevisiae X2180-1B(α). Cells were grown exponentially in Difco Bacto Yeast Nitrogen Base Without Amino Acids, supplemented with 0.1% glucose. As indicated by arrow A, [$^{14}$C] glucose (10 $\mu C_i$/ml) was added and the culture divided into two aliquots. One of these received 1 mM DL-$\Delta^1$-pyrroline 5-carboxylate (P5C) as indicated by arrow B; the other received no P5C and served as a control. Samples were removed at intervals for measurement of $^{14}$C in protein amino acids as described in the text. ●——●, $^{14}$C in proline in the absence of P5C; ▲——▲, $^{14}$C in proline, P5C being added at arrow B; o——o, $^{14}$C in valine; Δ——Δ, $^{14}$C in valine in the presence of P5C.

FIGURE CAPTIONS (Cont.)

Fig. 3. Relationship between trichloroacetic acid-precipitable $^{14}C$ and amount of imino acid supplied in the bioradiological assay. Incubation mixtures consisted of proline-starved cells of strain X278, L-[$^{14}C$] phenylalanine, and graded quantities of L-proline or DL-$\Delta^1$-pyrroline 5-carboxylate dissolved in 6.0 ml of M64glyTL medium. For further details see Materials and Methods. •——•, L-proline; o——o, DL-$\Delta^1$-pyrroline 5-carboxylate.

Fig. 4. Bioradiological assay of proline biosynthesis by cell-free extract of strain X210. The reaction mixture contained 1.75 ml cell-free extract (12 mg protein/ml) together with other components as described in Materials and Methods, in a total volume of 6.0 ml; the mixture was shaken in air at 37°. At intervals, samples (0.5 ml) were removed for determination of trichloroacetic acid-precipitable $^{14}C$. Blank values, obtained by omission of the cell-free extract, have been deducted.

Fig. 5. Specific activity versus extract volume in the bioradiological assay. The procedure described in Materials and Methods was followed. The volume of the X210 extract (12 mg protein/ml) added to each reaction vessel is plotted against the specific biosynthetic activity. Bovine serum albumin was added as necessary to each reaction mixture to give a final concentration of 4.0 mg total protein/ml. The total assay volume was 6.0 ml in each case. Aliquots (0.3 ml) were taken as a function of time for determination of trichloroacetic acid-precipitable $^{14}C$. Enzyme blank values have been deducted.

FIGURE CAPTIONS (Cont.)

Fig. 6. Effect of ammonia and imidazole on proline biosynthesis as monitored by the bioradiological assay. Prior to the assay, the culture of X278 was subdivided into two portions and incubated with 5 mM ammonia or 5 mM imidazole for 4 h at 37°, after which the two cultures were harvested and resuspended in fresh M64glyTL medium as described in Materials and Methods. A graded concentration of each nucleophile was tested for possible inhibition of proline biosynthesis. Each reaction mixture (total volume, 4.5 ml) contained 0.75 ml X210 extract (5.83 mg protein/ml). Aliquots (0.5 ml) were analyzed for trichloroacetic acid-precipitable $^{14}C$ after 10 min incubation with ammonia, or after 40 min incubation with imidazole. ●——●, imidazole; o——o, ammonia.

Fig. 7. Proline repression of γ-glutamyl kinase. Strain X210 was grown to $A_{650\ nm}$ = 1.0 approx. in the presence of the indicated quantities of L-proline. Extracts were prepared from the three cultures and the biosynthetic activity of each was monitored by the bioradiological assay. Each assay run had an initial volume of 4.5 ml. The concentration of enzyme extract was uniform in all reactions (0.75 mg protein/ml). The trichloroacetic acid-precipitable $^{14}C$ was obtained from 0.5 ml aliquots taken after 36 min incubation.

Fig. 8. Specific activity versus P. mirabilis extract volume in the bioradiological assay. The procedure described in Materials and Methods was followed. The volume of P. mirabilis extract (7.68 mg protein/ml) is plotted against the specific activity of proline production. Bovine serum albumin

FIGURE CAPTIONS (Cont.)

was added as necessary to each reaction mixture to give a final concentration of 2.56 mg total protein/ml. The total assay volume in each case was 6.0 ml. Aliquots (0.5 ml) were taken as a function of time for determination of trichloroacetic acid-precipitable $^{14}C$. Enzyme blank values have been deducted.

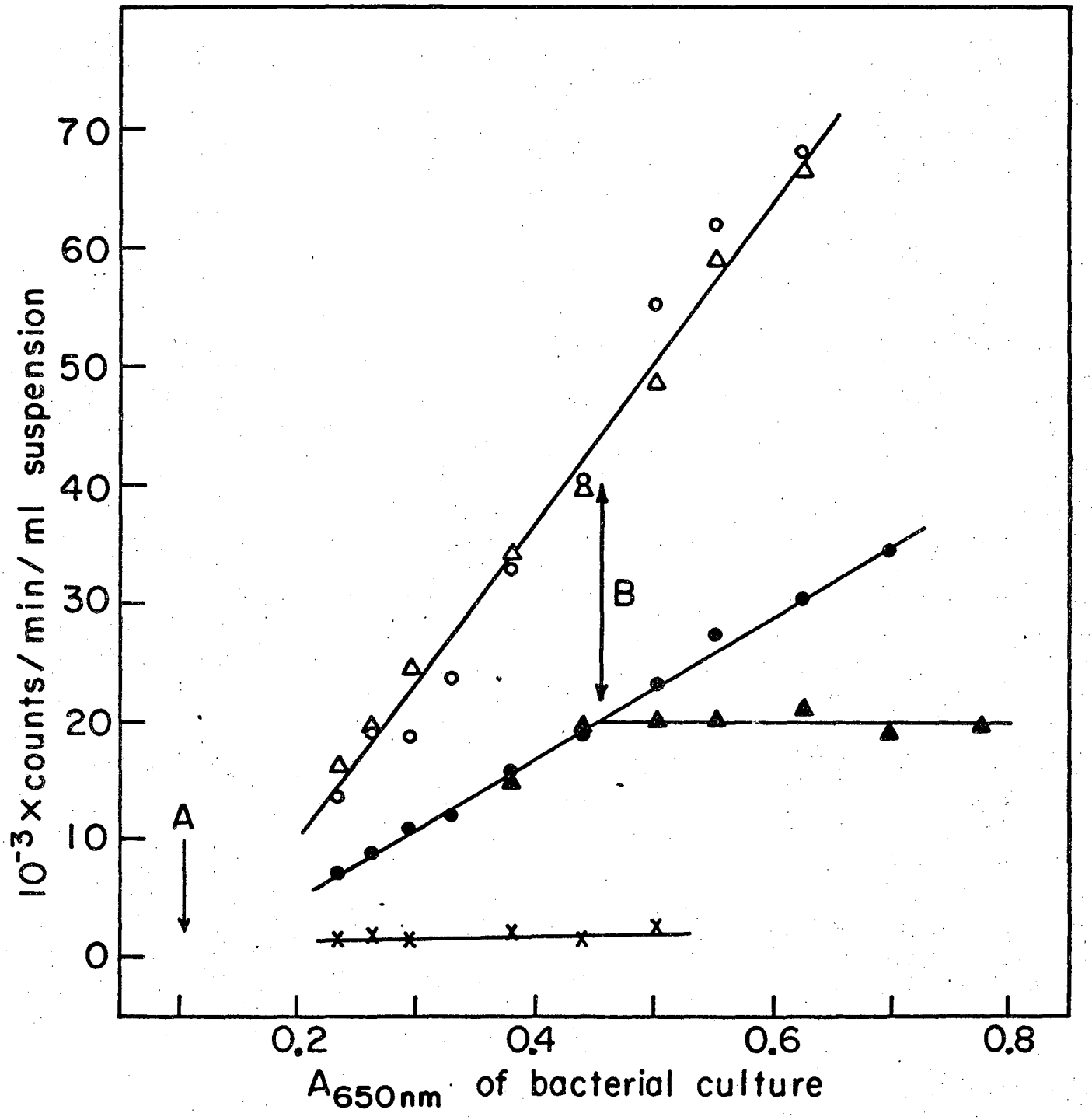


Fig. 1.

XBL738-3853

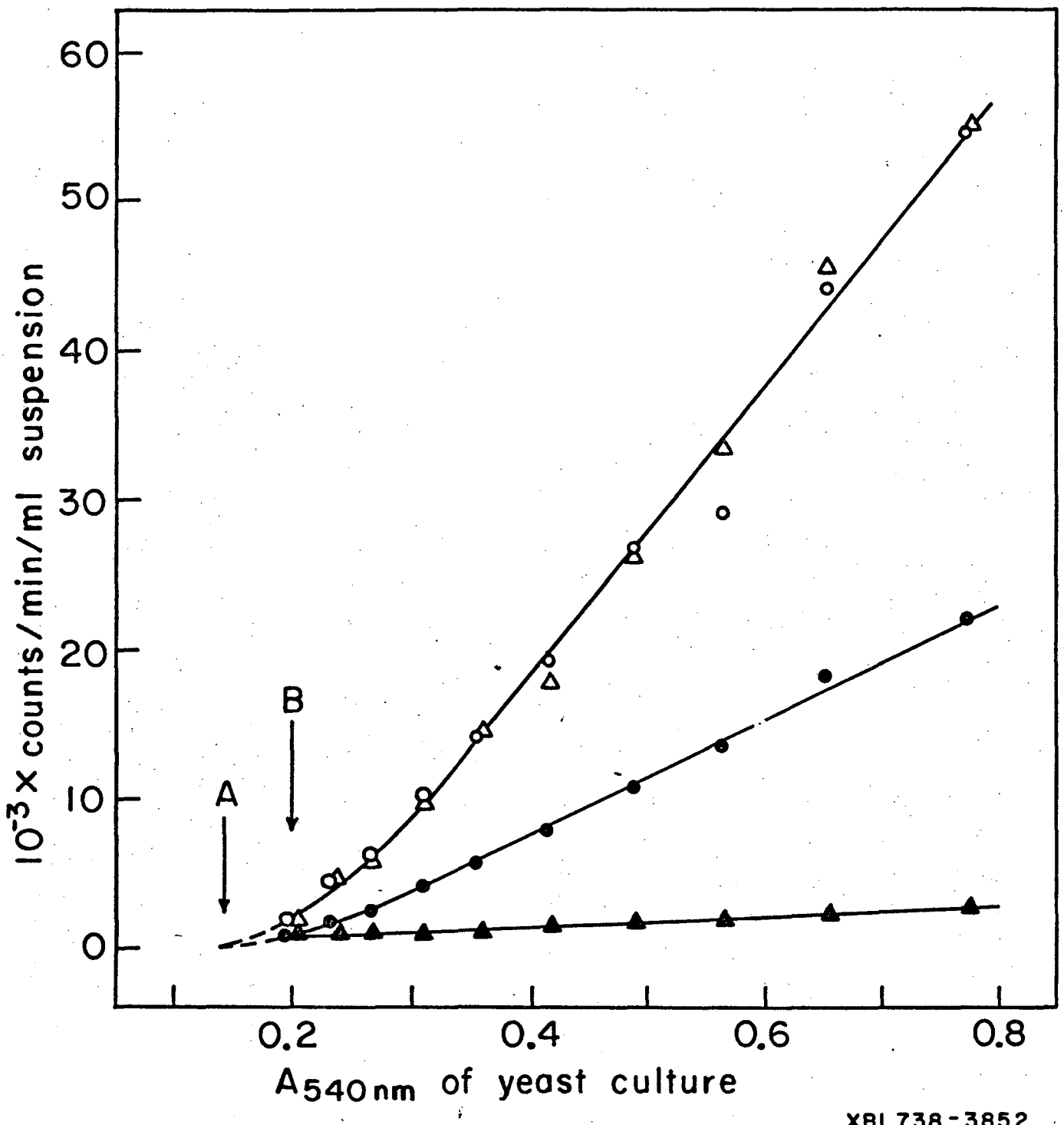
60
50
40
30
20
10
0
$10^{-3}$ x counts/min/ml suspension
A
B
0.2
0.4
0.6
0.8
$A_{540\,nm}$ of yeast culture
XBL738-3852

Fig. 2.

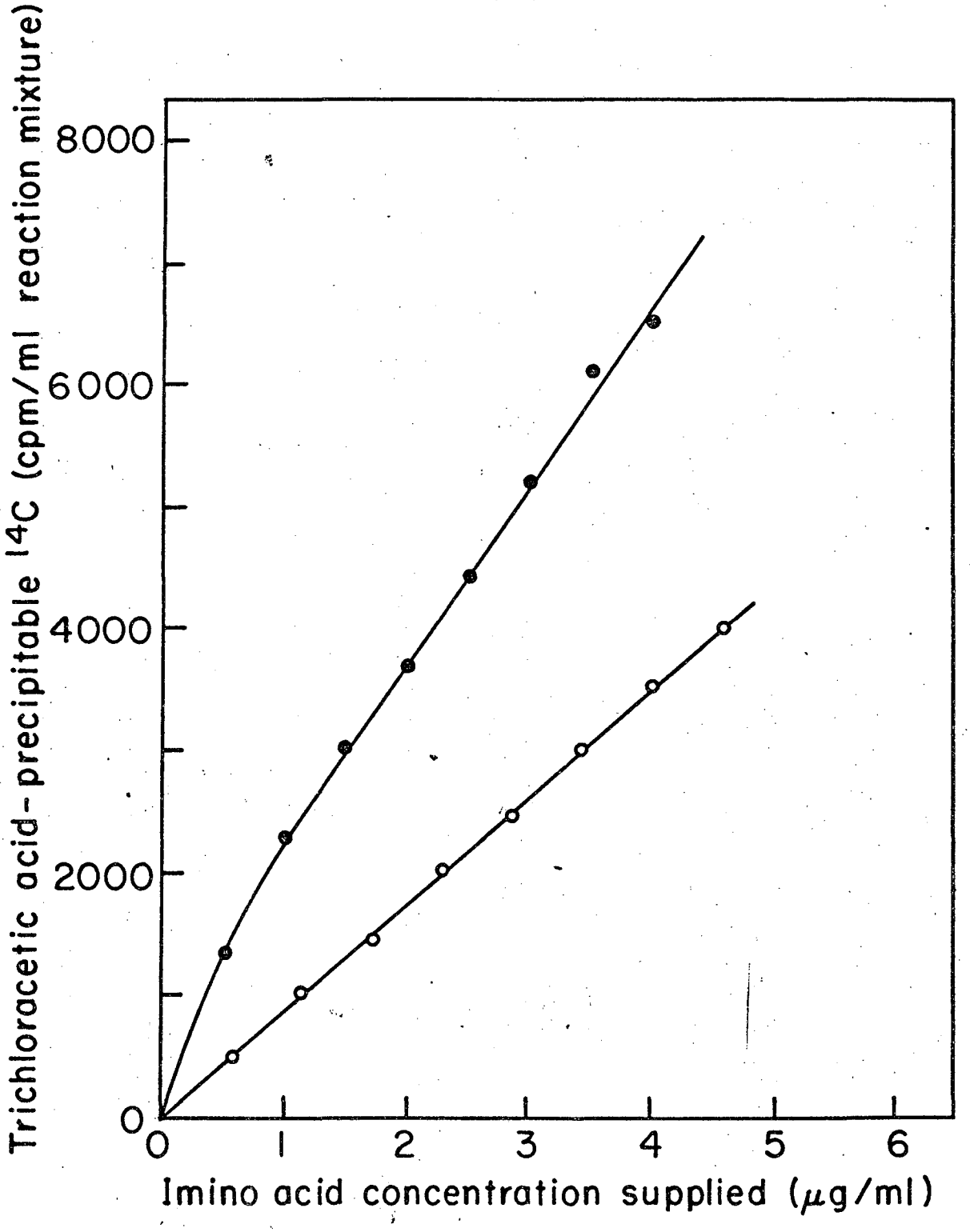


Fig. 3.

XBL738-3851

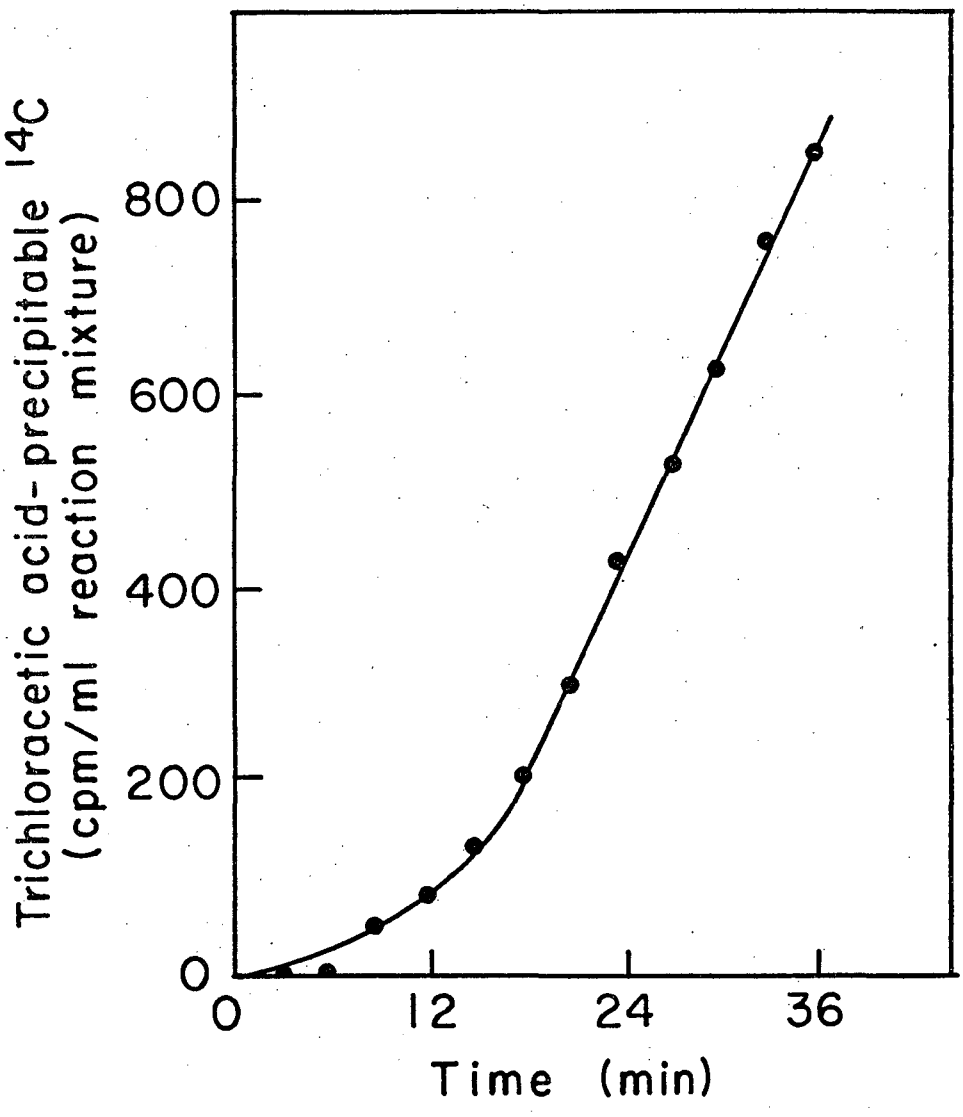


Fig. 4. XBL738-3850

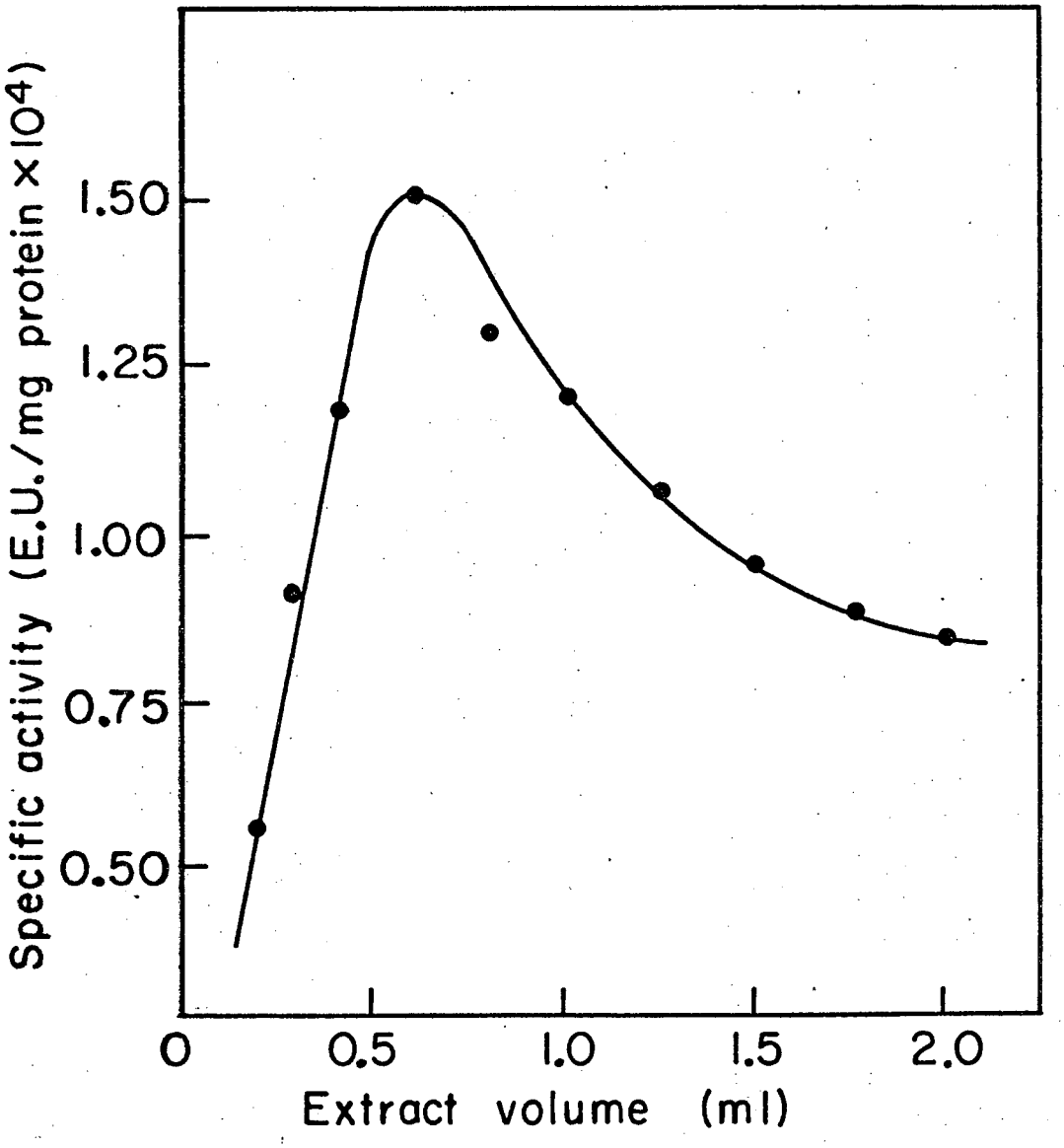


Fig. 5. XBL738-3849

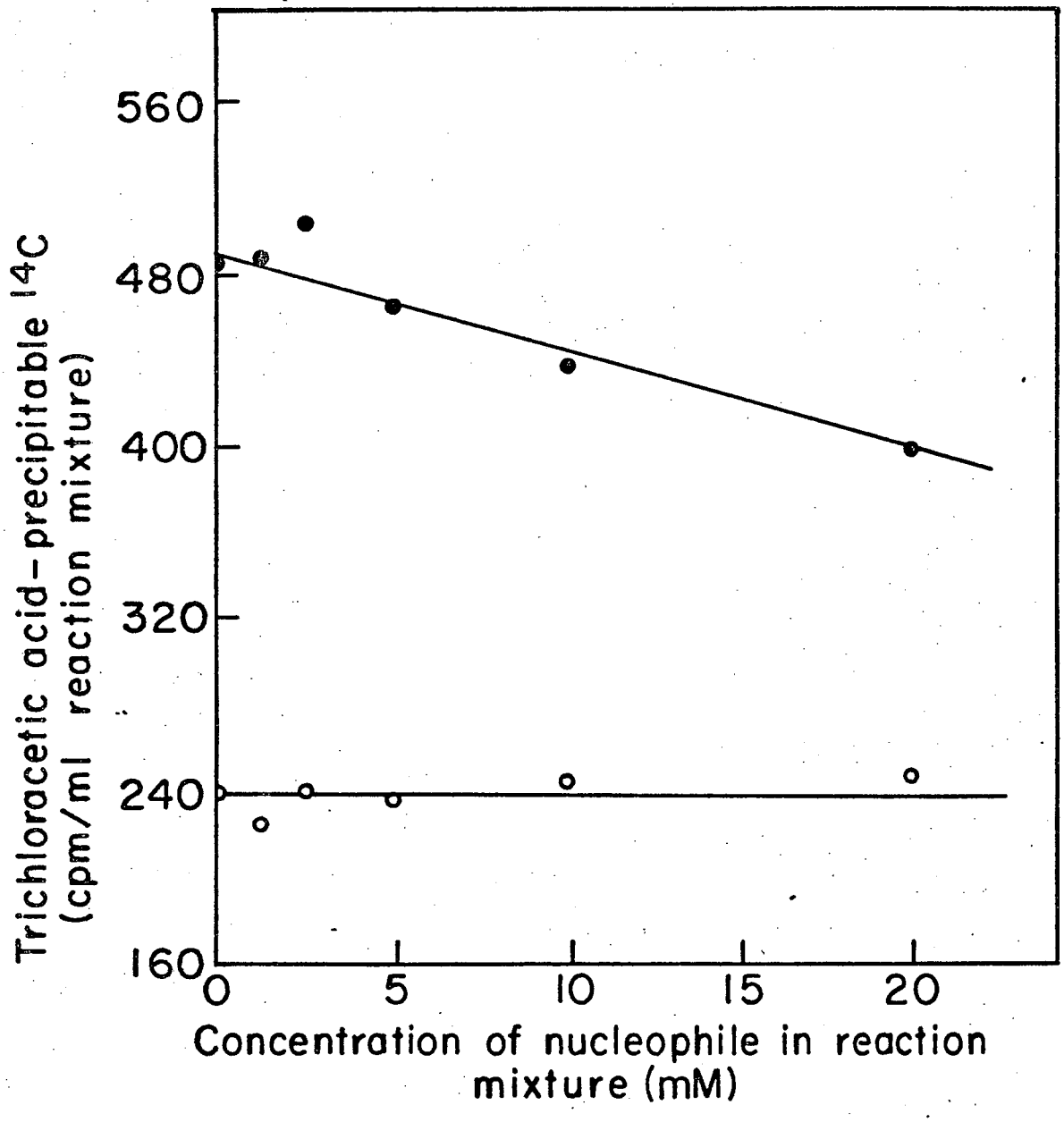


Fig. 6. XBL738-3848

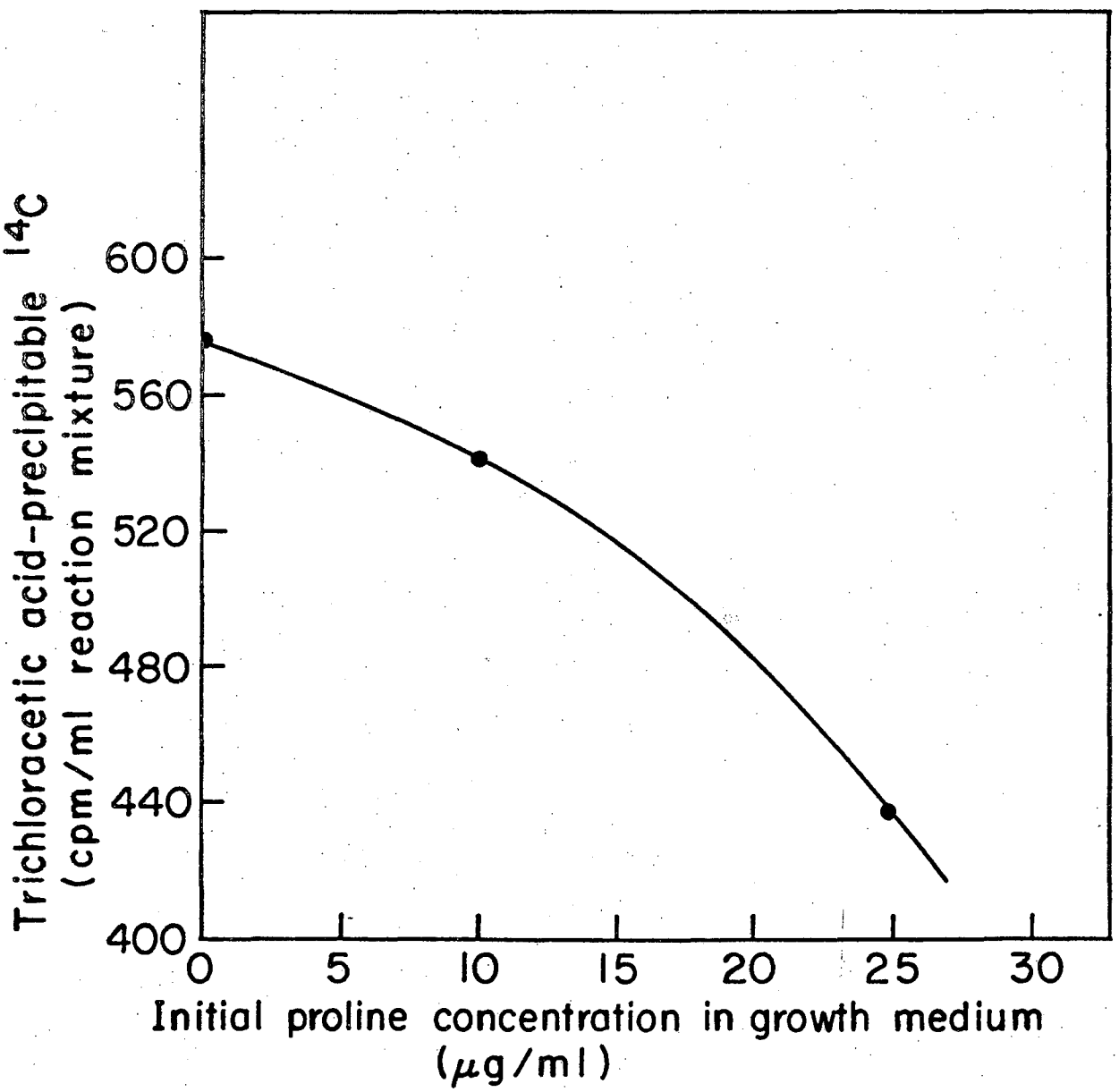
Trichloracetic acid-precipitable $^{14}C$
(cpm/ml reaction mixture)
600
560
520
480
440
400
0
5
10
15
20
25
30
Initial proline concentration in growth medium
(μg/ml)
XBL738-3847

Fig. 7.

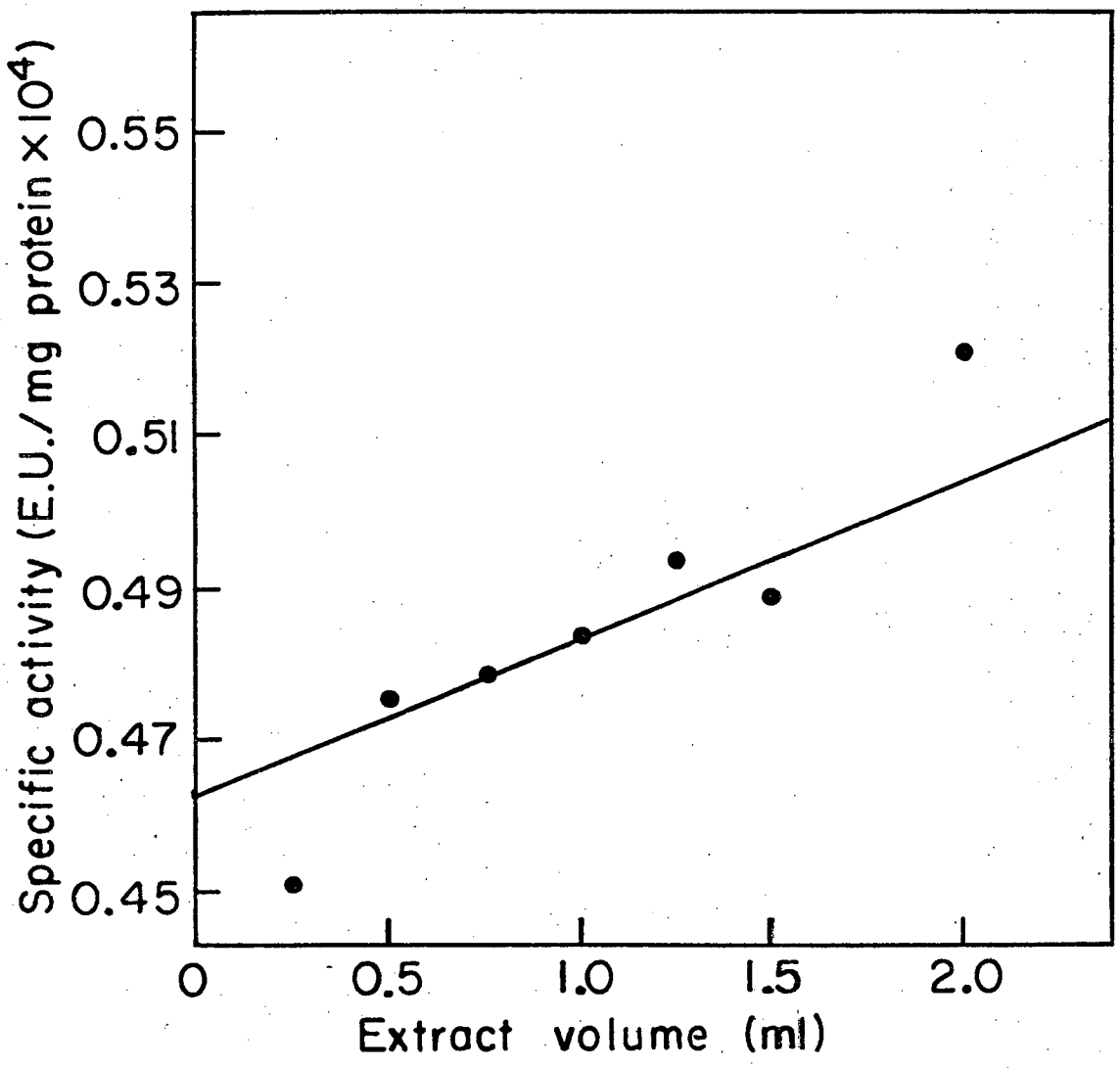
Specific activity (E.U./mg protein ×10⁴)
0.55
0.53
0.51
0.49
0.47
0.45
0
0.5
1.0
1.5
2.0
Extract volume (ml)
XBL738-3846

Fig. 8

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