Stabilization, Purification, and Characterization of Glutamate Synthase from *Clostridium pasteurianum*

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ABSTRACT: *Clostridium pasteurianum* possesses a high level of glutamate synthase (EC 1.4.1.14) activity and cell yield when grown on 4 mM ammonium chloride and molasses as the sole nitrogen and carbon sources, respectively. The enzyme activity is stabilized by addition of α-ketoglutarate, EDTA, and 2-mercaptoethanol. Ammonium sulfate precipitation and single-step combined gel and ion-exchange chromatography followed by fractional dialysis yield a homogeneous protein with 40% recovery of the glutamate synthase activity. The native enzyme ($M_r \simeq 590\,000$) gives five different subunits (as dimers) upon SDS gel electrophoresis. The enzyme has been characterized for pH and temperature optimum, substrate specificity, K_m^{app} values, energy of activation, half-life, and thermal stabilization. Metal ions and citric acid cycle metabolites do not affect the enzyme activity. Glutamate synthase shows fluorescence maximum at 370 nm when excited at 280 nm. The fluorescence is quenched upon the addition of NADH. Spectroscopic examination of the enzyme gave absorption maximum at 280 and none at 380 and 440 nm, indicating the absence of iron and flavin. The absence of iron and flavin was also confirmed by atomic absorption, chemical analysis, and fluoroscopy, respectively. The *C. pasteurianum* enzyme differs from that of other aerobic bacterial sources.

For a fairly long time it was thought that the principal mechanism of ammonia assimilation in a cell was via the action of glutamate dehydrogenase (GDH) coupled with transamination because both the enzymes were found virtually in all organisms

$$\alpha\text{-ketoglutarate} + \text{NADH/NADPH} + NH_4^+ \xrightleftharpoons{\text{GDH}} \text{glutamate} + NAD^+/NADP^+$$

$$\text{glutamate} + RC(O)COOH \xrightleftharpoons{\text{transaminase}} RCH(NH_2)COOH + \alpha\text{-ketoglutarate}$$

$$RC(O)COOH + \text{NADH/NADPH} + NH_4^+ \rightleftharpoons RCH(NH_2)COOH + NAD^+/NADP^+$$

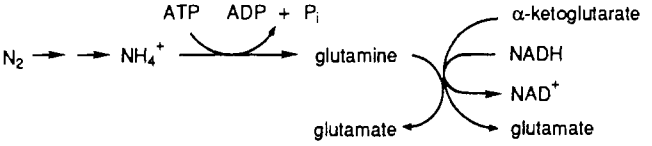
and there had been other pieces of strong evidence in favor of such a view, e.g., (i) the ability ofglutamate dehydrogenase to catalyze glutamate synthesis in vitro with a reaction equilibrium favoring such a synthesis and (ii) kinetic labeling data using *Candida utilis* (Folkes, 1959; Sims & Folkes, 1964; Folkes & Sims, 1974) and *Chlorella* (Bassham & Kirk, 1964).

However, all efforts to demonstrate this and other enzyme systems then known to incorporate NH_4^+ into organic molecules in the nitrogen-fixing anaerobic bacterium *Clostridium pasteurianum* were unsuccessful (Dainty & Peel, 1970), while experiments with this bacterium (Zelitch et al., 1951), using labeled dinitrogen gas or $^{15}NH_4^+$, had earlier shown the highest ^{15}N label in glutamate and glutamine.

The dilemma of ammonia assimilation in *C. pasteurianum* was solved when the following reaction was shown to occur in this bacterium (Nagatani et al., 1971; Dainty, 1972):

$$\text{L-glutamine} + \alpha\text{-ketoglutarate} + \text{NADH} \rightarrow 2 \text{ glutamate} + NAD^+$$

A similar reaction with NADPH as cofactor had earlier been reported in *Aerobacter aerogenes* (Tempest et al., 1970) and in several other microorganisms (Brown et al., 1970; Elmerich & Aubert, 1971). It was tentatively named glutamine amide-2-oxoglutarate aminotransferase (oxidoreductase, NADPH) (GOGAT). Since *C. pasteurianum* also contains glutamine synthetase activity (Hubbard & Stadtman, 1967), it was concluded that the two reactions combine together in the following manner to account for the dinitrogen assimilation in nitrogen-fixing bacteria:



Though a major role has been assigned to GOGAT for ammonia assimilation in *C. pasteurianum*, studies on this enzyme and its role have been confined to crude extracts only (Dainty, 1972; Kleiner, 1979). This may be due to the problems and challenges that anaerobes pose in their growth and processing. The enzyme systems in such bacteria are usually unstable and require precautions and relatively higher skill in their handling. In the present paper we report the stabilization, efficient purification procedure, and detailed characterization of glutamate synthase for the first time from an anaerobic, active nitrogen-fixing microbe, *C. pasteurianum*.

It may be pointed out that purification of this enzyme from aerobic bacteria such as *Escherichia coli* (Miller & Stadtman, 1972), *A. aerogenes* (Trotta et al., 1974), *Bacillus megaterium* (Hemmila & Mantsala, 1978), and *Gluconobacter suboxydans* (Tachiki et al., 1983) has been reported.

Experimental Procedures

Materials. Albizziin, L-asparagine, azaserine, biotin, bovine serum albumin, *cis*-aconitic acid, Coomassie brilliant blue R 250, 1-cyclohexyl-3-(2-morpholinoethyl)carbodiimide metho-*p*-toluenesulfonate, cysteine, dansyl chloride, dansyl L-amino acids, L-glutamine, iodoacetic acid, isocitric acid, α-ketoglutaric acid, 2-mercaptoethanol, methionine sulfoximine, 2-(*N*-morpholino)ethanesulfonic acid, β-nicotinamide adenine dinucleotide reduced form (NADH), β-nicotinamide adenine dinucleotide$_1$ phosphate reduced form (NADPH), phenyl-

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methanesulfonyl fluoride (PMSF),[1] pepstatin A, benzamidine, sodium dithionite, succinic acid, and tris(hydroxymethyl)-aminomethane *p*-aminodimethylaniline were purchased from Sigma Chemical Co. (St. Louis, MO). DEAE-Sephadex A-50, Sepharose 4B, and thyroglobulin (bovine thyroid) were procured from Pharmacia Fine Chemicals (Uppasala, Sweden). Agar agar, bromophenol blue, ethylenediaminetetraacetic acid (EDTA), Rose Bengal dye, and silica gel G were obtained from BDH (Poole, England). Acrylamide, L-glutamic acid, ammonium persulfate, *N,N'*-methylenebis(acrylamide), ninhydrin, *o*-phenanthroline, pyridine, and urea were bought from Merck (Darmstadt, Germany); *N,N,N',N'*-tetramethylethylenediamine (TEMED) was purchased from Koch Light Labs (England). Blue dextran and nitro blue tetrazolium (NBT) were acquired from Biochemicals Unit, Vallabhai Patel Chest Institute (India). Calibration proteins bovien serum albumin, aldolase, catalase, and ferritin were bought from Boehringer-Mannheim (Germany). Sodium dodecyl sulfate and the low molecular weight protein standards bovine serum albumin, ovalbumin, carbonic anhydrase, soybean trypsin inhibitor, and lysozyme were bought from Bio-Rad Laboratories (Richmond, CA). Phenylglyoxal hydrate and 2,3-butanedione were acquired from Fluka (Buchs, Switzerland), and tetranitromethane was from K&K Laboratories (Jamaica, NY).

All other chemicals used were of analytical grade available locally.

Organism and Cultivation. *Clostridium pasteurianum* (ATCC 6013) was obtained from American Type Culture Collection Center, Washington, DC, and grown on 4 mM NH_4Cl as a source of nitrogen according to the method of Dua and Burris (1963) except that molasses (equivalent sucrose content) was used as a source of carbon. The cells in the late log phase were harvested in a Sharples centrifuge, washed twice with cold 50 mM phosphate buffer (pH 7.2), and stored at −22 °C until used. The purity of *C. pasteurianum* cells in the final broth was occasionally checked by the method of Wilson and Miles (1975).

Molasses (50% sucrose) was diluted with an equal volume of distilled water, and pH of the solution was adjusted to 4.0 with 2 N H_2SO_4. It was then heated to 80 °C in a water bath for 10 min. The recipitate formed was centrifuged off, and the supernatant whose pH was adjusted to 7.0 with 2 N NaOH was then used for cell growth.

Buffers. Buffer A consisted of 200 mM potassium phosphate buffer (pH 7.2). Buffer B consisted of buffer A containing α-ketoglutarate (10 mM), 2-mercaptoethanol (5 mM), and EDTA (1 mM). Buffer C consisted of 10 mM potassium phosphate buffer (pH 7.2) containing α-ketoglutarate (10 mM), 2-mercaptoethanol (5 mM), and EDTA (1 mM).

Preparation of Cell-Free Extract. The cells (1 g wet weight/10 mL) were suspended in buffer B and disrupted for 2 min with a MSA ultrasonic disintegrator (20 kHz) at 0–4 °C. Cell debris was removed by centrifuging at 20000*g* for 30 min at 4 °C.

Enzyme Assay. Glutamate synthase activity was assayed by the method of Dainty (1972). The oxidation of NADH was monitored at 30 °C by following the decrease in absorbance at 366 nm with a Pye Unicam SP 500 spectrophotometer. The reaction mixture consisted of buffer A, enzyme (protein, 0.2 mg in 0.1 mL), α-ketoglutarate (5 mM), L-glutamine (5 mM), and NADH (0.25 mM) in a total volume of 3.0 mL. The control had no L-glutamine. The enzyme solution was dialyzed overnight against buffer A before assay. Enzyme shows linearity up to 80 μg of protein for 14 min. Therefore, the incubation of enzyme for 5 min was chosen for enzyme assay.

Enzyme activity is expressed as micromoles of NADH oxidized per hour.

Protein Determination. Protein was determined by the method of Lowry et al. (1951) using crystalline bovine serum albumin as standard. In all chromatographic procedures, protein concentration was determined spectrophotometrically by the method of Warburg and Christian (1941) employing a Pye Unicam SP 500 spectrophotometer.

Purification of Glutamate Synthase. All manipulations were carried out at 0–4 °C.

Ammonium Sulfate Precipitation. The cell-free extract (protein, 7.5 mg/mL) was brought to 30% saturation by adding powdered ammonium sulfate with constant stirring. The pH of the solution was maintained between 7.0 and 7.2 by the addition of 1 N KOH. The solution was allowed to stand for 40 min and then centrifuged at 20000*g* for 30 min. The pellet was discarded and ammonium sulfate again added to bring it to 50% saturation. After 40 min, the solution was centrifuged and the supernatant was discarded. The pellet was dissolved in buffer B to give 15.0 mg/mL protein.

Single-Step Combined Sepharose 4B/DEAE-Sephadex A-50 Column Chromatography. The 30–50% ammonium sulfate fraction (protein, 225 mg in 15 mL of buffer B) was directly loaded onto the Sepharose 4B column (2.2 × 90 cm) previously equilibrated with buffer B. When the glutamate synthase activity started appearing in the eluent (fraction 84 onward) (Figure 1A), the column was directly mounted on a DEAE-Sephadex A-50 column (2.2 × 35 cm) previously equilibrated with the same buffer, so that the eluent from the Sepharose 4B column was directly loaded on the ion-exchange column without disturbing the top of the gel bed. A flow rate of 15 mL/h was maintained in both the columns. The Sepharose 4B column was disconnected as soon as 51 mL of the eluent from the DEAE-Sephadex A-50 column (17 fractions of 3 mL each) had been collected. The DEAE-Sephadex A-50 column was now eluted with buffer B until no protein appeared in the eluent. Each fraction was monitored for protein content at 280 nm and assayed (0.2 mL) for glutamate synthase activity. The elution pattern is shown in Figure 1B. Glutamate synthase activity appeared in fractions 12–32.

Purification by Dialysis. Protein fractions obtained from the DEAE-Sephadex A-50 column chromatography, having significant activity, were pooled and concentrated in an Amicon ultrafiltration unit (membrane PM 30) under the positive pressure of nitrogen gas at 4 °C to 20 mL. The concentrated solution (protein, 20 mg) was dialyzed for 12 h against 1 L of buffer C. During dialysis, partial precipitation of the protein occurred. So the solution was centrifuged at 20000*g* for 30 min at 0 °C, transferred to a tube, and stored at 0 °C. The precipitate was first washed two or three times with 5 mL of buffer C and then dissolved in 2 mL of buffer B. Protein concentration and glutamate synthase activity were determined in the redissolved protein solution and the supernatant.

Polyacrylamide Disc Gel Electrophoresis. Homogeneity of the purified enzyme preparations was checked by disc gel electrophoresis according to the modified method of Davis (1964) by using a Shandon SAE 2717 gel electrophoresis apparatus using 7% and 5% gels at pH 8.3 and 7.5 in Tris–glycine buffer.

[1] Abbreviations: PMSF, phenylmethanesulfonyl fluoride; NBT, nitro blue tetrazolium; SDS, sodium dodecyl sulfate; DON, 6-diazo-5-oxo-L-norvaline; TCA, trichloroacetic acid; EDTA, ethylenediaminetetraacetic acid; Tris, tris(hydroxymethyl)aminomethane.

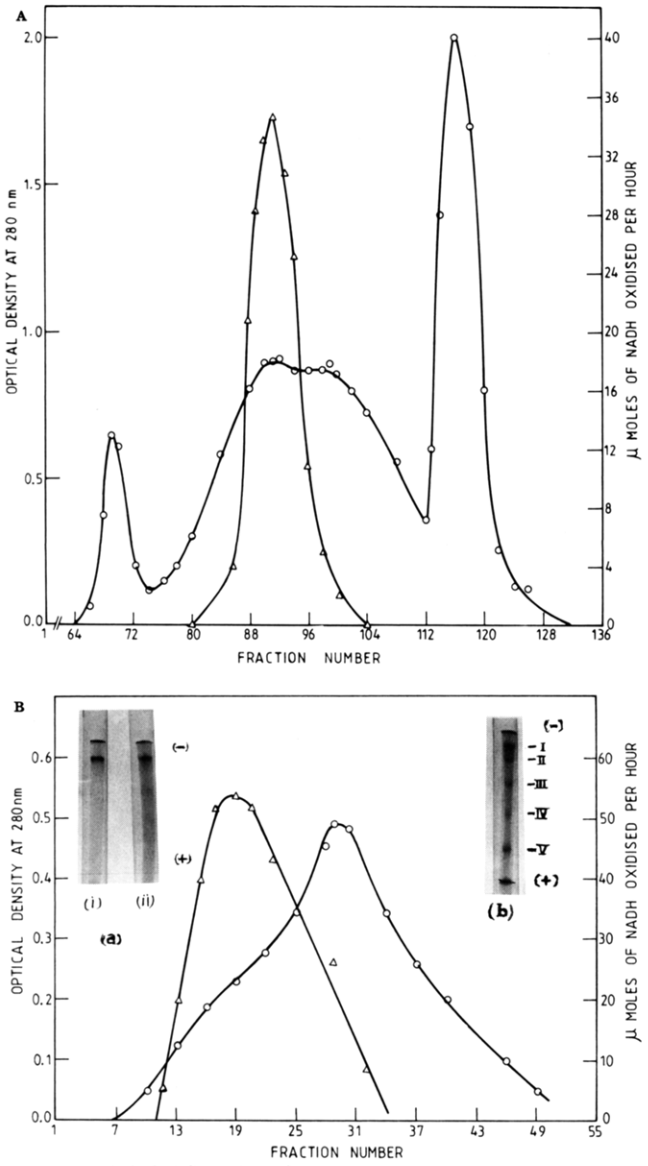

FIGURE 1: (A) Gel filtration of glutamate synthase on Sepharose 4B. The ammonium sulfate precipitated protein (0.3–0.5 saturation) redissolved in buffer B was applied onto a Sepharose 4B column (2.2 × 90 cm) previously equilibrated with the same buffer. Fractions (3 mL) were collected at a flow rate of 15 mL/h, and their absorbance was measured at 280 nm (O). Aliquots were withdrawn from each fraction and assayed for glutamate synthase activity (Δ). (B) Combined Sepharose 4B/DEAE-Sephadex A-50 chromatography of glutamate synthase. When the glutmate synthase activity started appearing in the eluent (fraction 84 onward) of the Sepharose 4B column, the column was mounted on a preequilibrated DEAE-Sephadex A-50 column (2.2 × 35 cm) maintaining a flow rate of 15 mL/h in both columns. The Sepharose 4B column was disconnected when 51 mL of the eluent from the DEAE-Sephadex A-50 column had been collected (that is, fractions 84–100 of the Sepharose 4B column). The DEAE-Sephadex A-50 column was then washed with buffer B. Each fraction (3 mL) collected was monitored for protein content at 280 nm (O) and glutamate synthase activity (Δ). (Inset a) Polyacrylamide disc gel electrophoresis of glutamate synthase under nondenaturing conditions: (i) Coomassie brilliant blue R 250 stained gel; (ii) NBT dye stained gel. (Inset b) SDS–polyacrylamide gel electrophoresis of glutamate synthase.

Activity Staining by Nitro Blue Tetrazolium Dye. Gels, after electrophoresis, were immersed in a tube containing 2-fold concentrated assay mixture required for glutamate synthase activity. After incubation for 30 min, the gels were washed with distilled water and dipped in NBT dye solution (Tarmy & Kaplan, 1968) until a colorless band against a dark blue background appeared at a place corresponding to the band obtained after staining with the Coomassie brilliant blue R.

Also, the NBT dye stained band could be obtained by preincubating the gel in 0.5 mM NADH solution in buffer A for 30 min, washing the gel twice in distilled water, and incubating it in NBT dye solution. In this case, a deep blue band was observed in contrast to a colorless one in activity stained gels. This was photographed for the record.

Ouchterlony Double-Immunodiffusion Test. Purified glutamate synthase (protein, 0.8–1.0 mg) was mixed with an equal volume of Freund's incomplete adjuvant and was injected into three areas of the back of a rabbit. The process was repeated after 2 weeks. Further intradermal injections of the enzyme (protein, 0.8–1.0 mg) in 200 mM potassium phosphate buffer, pH 7.4, 0.85% NaCl) were made at 2-week intervals, and blood was collected from an ear vein 7 days after each injection. Blood was allowed to clot and serum was collected by centrifuging at 10000*g* for 10 min and stored at 0 °C. Control serum was prepared from the blood of the same rabbit before immunization. The Ouchterlony double-immunodiffusion test (Ouchterlony, 1949) was done in 2% agar plate containing phosphate-buffered saline. It took 24–48 h for the precipitation lines to fully develop.

Identification of Enzymatically Formed Product. The reaction product was identified by running the reaction on a preparative scale. The reaction mixture contained NADH (0.25 mg), α-ketoglutarate (5 mM), and L-glutamine (5 mM) and enzyme (protein: 0.5 mg) in a total volume of 3 mL. The solution was incubated at 30 °C for 30 min. The reaction was stopped by transferring the reaction tubes to a boiling water bath for 10 min, cooling, and centrifuging at 20000*g* for 30 min. Control was also run without glutamine. The supernatant was concentrated in vacuo and dissolved in 0.5 mL of ethyl acetate, and the reaction product was identified by TLC using 1-butanol/acetic acid/water (80:20:20 v/v) as eluent.

Determination of the NH_2-Terminal Amino Acid Residue. NH_2-terminal amino acid analysis was performed as described by Gray (1976). The dansylation reaction of purified glutamate synthase (1 mL; protein, 0.2 mg/mL) was performed in the presence of 0.48 g of urea, 0.42 g of sodium carbonate, and 1.0 mL of dansyl chloride (20 mg/mL in redistilled acetone) at 37 °C for 12 h with continuous shaking.

Molecular Weight Estimation. The molecular weight of the purified glutamate synthase (protein, 2 mg/mL) was estimated by gel filtration on a Sepharose 4B column according to the method of Locascio et al. (1969). Marker proteins were (protein, 2 mg/mL) thyroglobulin (669000), ferritin (440000), catalase (240000), aldolase (158000), and bovine serum albumin (68000).

The subunit molecular weight of glutamate synthase was estimated by sodium dodecyl sulfate–polyacrylamide disc gel electrophoresis in Tris buffer by the method of Weber and Osborn (1975). Marker proteins used were lysozyme (14400), soybean trypsin inhibitor (21500), carbonic anhydrase (31000), ovalbumin (45000), and bovine serum albumin (66200).

Determination of Iron Content. (*a*) *Atomic Absorption Spectroscopy.* The sample for atomic absorption was prepared by the modified method of Peterson (1953) as follows: The enzyme solution (protein, 3 mg) was dialyzed first against 500 mL of 0.05 M phosphate–0.01 M EDTA buffer (pH 7.0) with three changes and then against 1 L of double-distilled deionized water for 24 h. The dialyzed protein was heated in sequence with (i) a mixture containing 0.1 mL each of concentrated HNO_3 and concentrated H_2SO_4, (ii) 0.1 mL of concentrated HNO_3, and (iii) 0.05 mL of 70% $HClO_4$ for 3–4 min and

Table I: Effect of Nitrogen Sources on Growth of *C. pasteurianum*[a] and Level of Glutamate Synthase Activity

nitrogen source	cell yield[b] (g/L)	sp act.[b] of glutamate synthase
N_2	1.75 ± 0.25	1.8 ± 0.2
NH_4Cl		
4 mM	2.3 ± 0.2	5.5 ± 1.0
40 mM	2.8 ± 0.2	4.5 ± 0.5
100 mM	2.5–3.5	3.8 ± 0.5
L-glutamate (5 and 20 mM)	2.3 ± 0.2	0.4 ± 0.1

[a] Similar differences in cell yield and specific activities were obtained when 2% sucrose or molasses (equivalent of sucrose) was used as the carbon source. [b] These results are average of four experiments.

evaporated to dryness at 80 °C in each case. Finally the residue was dissolved in 5 mL of deionized water and centrifuged at 10000*g* for 10 min. The clear supernatant was analyzed on Unicam SP 1900 atomic absorption spectrophotometer. A blank with all reagents except the enzyme solution was run simultaneously under identical conditions. All acids used were spectroscopically pure, and solutions were made in double-distilled deionized water.

(*b*) *Colorimetric Estimation.* The presence of iron was also determined colorimetrically by the method of Harvey et al. (1955) by precipitating protein by (i) boiling or (ii) treating the enzyme solution (2.0 mg of protein) with 5% trichloroacetic acid, centrifuging it after 10 min, reacting the supernatant with 0.2 mL of 1,10-phenanthroline (50% w/v ethanol), and reading the absorption at 512 nm. Control was run by taking the buffer solution.

Sulfide Content. Sulfide content was assayed by the methylene blue method of Fogo and Popowsky (1949) by adding alkaline zinc acetate solution to 2.2 mg of protein followed by *p*-aminodimethylaniline, 6 N HCl, and ferric chloride in that order and centrifuging it. The supernatant was read at 670 nm with a Unicam SP 500 spectrophotometer. Control sample without enzyme was run in parallel.

Flavin Assay. The enzyme solution (protein, 1.5 mg) was boiled for 5 min or tryptic digested at pH 8.4 and centrifuged. The supernatant was spotted on a silica gel thin-layer plate along with FMN and FAD reference standards and developed in either 3% ammonium acetate, pH 7.0, or 3% NH_4Cl, pH 4.0. Spots were located after drying under UV light. The rest of the supernatant was scanned and checked for its absorbance at 445 nm. The presence of flavin was also checked fluorometrically by the method of Burch et al. (1948). The enzyme solution (protein, 2.5 mg) was boiled for 5 min and centrifuged at 15000*g* for 10 min and its pH adjusted to 1 with 6 N HCl. The solution was again boiled for 15 min and cooled and its pH adjusted with 5 N NaOH to 6.8. These operations were done in the dark. The fluorescence of the solution was now read with a Hitachi Perkin Elmer MPF2A fluorescence spectrophotometer.

RESULTS

Bacterial Growth Experiments. The effect of varying ammonium salt concentrations on glutamate synthase levels was determined by growing the cells on media containing either 4, 40, or 100 mM NH_4Cl, except where N_2 or glutamate replaced NH_4^+ as the nitrogen source. The bacteria were harvested in the late log phase of growth, sonicated in buffer B at 4 °C, and assayed for glutamate synthase activity. As shown in Table I, the enzyme activity was highest in cells grown on limiting amount of ammonia and decreased with increasing ammonium chloride concentration. Cells grown on N_2 and glutamate had lower glutamate synthase activity. The lower level of glutamate synthase in cells grown on glutamate shows that the concentration of this amino acid in the media regulates the enzyme synthesis and suggests a regulatory role for this enzyme in biosynthetic pathways involving glutamate. In the case of *A. aerogenes*, though growth on limiting ammonium salts gave inconsistent results (Locascio et al., 1969), similar observations were made in case of growth on glutamate (Meers et al., 1970). Substituting molasses, a waste byproduct of the sugar industry, for sucrose/glucose as the sole source of carbon gave a higher cell yield (150%) and a higher level of glutamate synthase activity (200–300%). The improvement in cell yield and glutamate synthase activity could be attributed to the presence of certain unidentified ingredients in the molasses required for the optimal growth of the bacterium. Molasses is thus not only a cheaper but also a better growth medium.

Stability of Cell-Free Extract under Different Storage Conditions. Glutamate synthase in crude cell-free extract stored at 4 °C was found to be quite unstable; 80% of the activity was lost within 6 h, which made its purification difficult. Addition of proteolytic inhibitors such as PMSF (20 μg/mL), pepstatin A (2 μg/mL), and benzamidine (2 μg/mL) during sonication did not improve its stability. Hence, efforts were made to stabilize the enzyme by the addition of various stabilizing agents (5 mM) such as α-ketoglutarate, L-glutamine, NADH, glutathione (reduced) $Na_2S_2O_4$, EDTA, 2-mercaptoethanol, dithiothreitol, and glycerol (25% v/v) to 1-mL aliquots of enzyme solution (protein, 2 mg in 1 mL), stored at 4 °C. Aliquots (0.1 mL) were withdrawn after 16 h and assayed for glutamate synthase activity. Though glutamine and sodium dithionite stabilized the enzyme activity by retaining 82% against 8% activity in the control after storage for 16 h (results not shown), these could not be utilized because of the poor solubility of the first reagent at low temperatures and inhibitory effects of the second reagent. α-Ketoglutarate, EDTA, and 2-mercaptoethanol also stabilized the enzyme activity considerably. Therefore, they were tried in different combinations to see their collective effect on the stability. α-Ketoglutarate (10 mM) in the presence of EDTA (1 mM) and 2-mercaptoethanol (5 mM) improved the stability of the protein, and 35% of glutamate synthase activity was retained even after 24 h. Therefore, buffers used in purification routinely contained α-ketoglutarate (10 mM), EDTA (1 mM), and 2-mercaptoethanol (5 mM).

Purification of Glutamate Synthase. The enzyme was purified 58-fold with 40% recovery. The results of the purification are summarized in Table II. In an earlier procedure, which involved ammonium sulfate fractionation, overnight

Table II: Summary of Purification of Glutamate Synthase from *C. pasteurianum*

purification step	total volume (mL)	total act. units	total protein (mg)	sp act.	recovery (%)	purification (*x*-fold)
crude extract	100	2964.0	750.0	3.95	100	1.00
ammonium sulfate fractionation (0.3–0.5 saturation)	15	2273.6	225.6	10.1	76.7	2.61
Sepharose 4B/DEAE-Sephadex A-50 chromatography	20	1736.4	20.8	83.5	58.6	21.1
precipitation by dialysis against low ionic strength buffer	2	1185.9	5.1	230.70	40.0	58.4

dialysis against buffer B, chromatography on Sepharose 4B followed by pooling and concentration of the fractions against sucrose, dialysis, and then chromatography on a DEAE-Sephadex A-50 column, we obtained a 25-fold purification and 12% recovery (data not shown). The increased recovery achieved in the present procedure could be due to substantial time saving when the ammonium sulfate fractionated enzyme was directly loaded onto a Sepharose 4B column without prior dialysis of the protein solution. This procedure also eliminates steps involving concentration and dialysis of the pooled Sepharose 4B fractions, which further reduces purification time from 96 to just 24 h. It also prevented the remixing of the fractions that had been separated on the Sepharose 4B column. Dialysis of concentrated initial fractions containing high enzymatic activity, against buffer C, precipitated homogeneous glutamate synthase with 40% recovery, thus making this procedure a very simple one.

Most of the successful procedures for purification of this enzyme from other sources required six to eight steps and gave comparatively poor recovery. Glutamate synthase has been purified from *G. suboxydans* (Tachiki et al., 1983) with 3% recovery, from *E. coli* (Miller & Stadtman, 1972) with 16% recovery, and from *B. megaterium* (Hammila & Mantsala, 1978) with 30% recovery.

Tests for Homogeneity. The protein was subjected to various tests to verify the homogeneity of the preparation. The precipitate obtained on dialysis was resuspended in 25 mM phosphate buffer (pH 7.2) containing α-ketoglutarate (10 mM), 2-mercaptoethanol (5 mM), and EDTA (1 mM) and subjected to rechromatography on a DEAE-Sepharose CL-6B column (25 × 12 cm). The enzyme was eluted by using a linear gradient of 500 mL of 0.05–0.2 M of the same potassium phosphate buffer. The enzyme eluted as a single peak with no further increase in its specific activity. A similar result was obtained upon repeated chromatography on a Sepharose 4B column. Disc gel electrophoresis under nondenaturing conditions after each chromatography yielded a single band at pH 8.3 on 5% gel (Figure 1B, inset a) and at pH 7.5 on 5% and 7% gels (results not shown). This band was further checked by activity staining by NBT dye. Ouchterlony double-immunodiffusion test of glutamate synthase against its antibody also gave a single white precipitin line confirming the homogeneity of the enzyme preparation.

Molecular Weight. The molecular weight of the purified glutamate synthase was estimated by Sepharose 4B gel chromatography ($M_r \simeq 590\,000 \pm 4000$ as given under Experimental Procedures). The apparent molecular weight of glutamate synthase varies considerably in different species. The enzymes from *E. coli* (Miller & Stadtman, 1972), *Saccharomyces cerevisiae* (Masters & Meister, 1982) and *B. megaterium* (Hammila & Mantsala, 1978) have molecular weights around 800 000, 265 000, and 180 000, respectively. Thus, *C. pasteurianum* glutamate synthase is a large protein.

Purified glutamate synthase when denatured in the presence of SDS and subjected to electrophoresis exhibited five protein bands (Figure 1B, inset b). The molecular weights of the subunits were found to be 91 000, 86 000, 68 000, 31 000, and 17 500, respectively. Each band was eluted from the gel, incubated at room temperature for 30 min, and again subjected to SDS gel electrophoresis under similar conditions. In each case a single band was obtained, and its mobility remained unchanged, thus indicating that none of the slower moving band has arisen by polymerization of the faster moving band. Similar results were obtained when the buffer used during purification contained protease inhibitors. Also, protein obtained after sonication of cells when precipatated immediately with ammonium sulfate (0.3–0.5 saturation) in the presence or absence of protease inhibitors incubated at 35 °C for 0, 15, 30, and 60 min gave 17 bands on staining after SDS gel electrophoresis. The position and relative intensity of the five bands corresponding to those of purified glutamate synthase subunits remained unchanged, thus eliminating the possibility of either band arising from proteolytic fragment of the enzyme. Addition of the molecular weight of each band comes to 293 500, which indicates that against a molecular weight of 590 000 each band represents two similar subunits. There are thus 10 subunits in *C. pasteurianum* glutamate synthase. The enzymes from *E. coli* (Miller & Stadtman, 1972) and *S. cerevisiae* (Masters & Meister, 1982) were found to have two nonidentical subunits, each subunit containing four identical polypeptide chains. The molecular weights of the subunits in the former were 135 000 and 53 000 and 94 000 and 72 000 in the latter, while *Spinacia oleracea* (Tamura et al., 1980) glutamate synthase had one subunit.

NH_2-Terminal Amino Acid Analysis. Amino-terminal analysis was performed on the purified glutamate synthase (see Experimental Procedures). The amino terminals, valine, aspartic acid, and glutamic acid were identified in the enzyme. Though SDS disc gel electrophoresis of glutamate synthase yielded five nonidentical subunits, only three dansyl amino acid spots were observed on the chromatogram. This could arise due to two possibilities: (1) either the other two NH_2-terminal amino acid residues are masked and are not available for dansylation or (2) the other NH_2-terminal amino acid residues are identical with two of the three identified dansyl amino acids. Hence, further work will be needed to establish which of the two possibilities is correct. The NH_2-terminal analysis gives supportive evidence for multiplicity of glutamate synthase subunits. Only serine could be identified as NH_2-terminal in *E. coli* glutamate synthase (Miller, 1974), which was attributed to the low molecular weight subunit of the enzyme. None could be assigned to the high molecular weight subunit. They concluded that the NH_2-terminal of the high molecular weight subunit was so blocked as not to be available to react with dansyl chloride.

Ultraviolet Absorption Spectrum. The purified glutamate synthase shows maximum absorption at 280 nm with a trough near 250 nm (results not shown). The enzyme from *E. coli* (Miller & Stadtman, 1972), *A. aerogenes* (Trotta et al., 1974), and *S. cerevisiae* (Masters & Meister, 1982) absorbs maximally at 278–280, 375–380, and 440–450 nm, indicating the presence of an iron-containing flavoprotein. Glutamate synthase from rice leaves (Suziki & Gadal, 1982), *S. oleracea* (Tamura et al., 1980), and *Rhizobium lupini* bacteroids (Shugaev et al., 1980), on the other hand, absorbs only at 280 nm. *C. pasteurianum* glutamate synthase thus resembles the plant enzyme and has neither flavin nor non-heme iron, as in the case with aerobic bacterial enzyme. Absence of iron was also confirmed by atomic absorption and chemical methods and that of flavin by fluoroscopic and spectroscopic examination of the supernatant obtained either on TCA treatment or boiling of the enzyme.

Fluorescence Spectrum. The glutamate synthase shows fluorescence emission maximum at 370 nm when excited at 280 nm, which shifted to 465 nm on the addition of NADH (Figure 2). The quenching of enzyme fluorescence at 370 nm was proportional to the added NADH and reached a saturation value at 15–20 μM (Figure 2, inset). Addition of α-ketoglutarate/L-glutamine or both to the enzyme under similar conditions led to no significant change in fluorescence

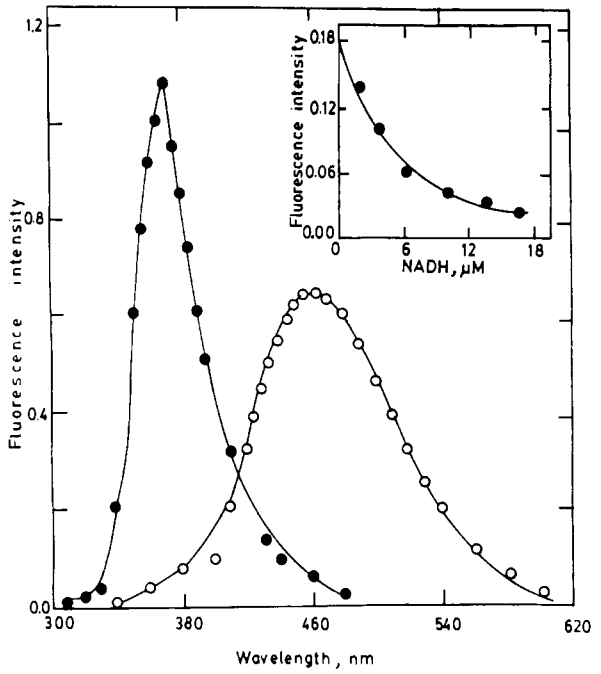

FIGURE 2: Fluorescence emission spectrum of glutamate synthase in the presence and absence of NADH. Fluorescence emission spectra were recorded of purified glutamate synthase (protein, 0.15 mg) (●) and of the enzyme in the presence of 0.025 mM NADH (○). Excitation wavelength was set at 280 nm. Reference cuvette contained buffer alone. (Inset) Effect of NADH concentration on glutamate synthase fluorescence.

intensity at 370 nm, nor did these additions influence the fluorescence at 465 nm obtained on the addition of NADH. These results, along with the earlier observation that NBT dye reacted with glutamate synthase preincubated with NADH, probably indicate that NADH binds first to the enzyme as in the case of lupin nodule (Boland, 1979) glutamate synthase.

Half-Life and Thermal Stability of Glutamate Synthase. Glutamate synthase (protein, 1.0 mg in 5 mL) was incubated at 50 °C; 0.5-mL aliquots were withdrawn at regular time intervals, cooled immediately in an ice bath, and assayed for residual glutamate synthase activity. The enzyme has a half-life of 10 min.

When the enzyme solution was incubated at different temperatures for 10 min, cooled in an ice bath, and assayed for enzyme activity, the enzyme started losing activity at 35 °C. Thus, half-life as well as thermal stability studies indicated that the enzyme is quite unstable. But the enzyme, as discussed later, showed optimum activity at 50 °C, which was rather unusual. This suggested that the enzyme was being stabilized by either substrate, cofactor, or product individually or in combination. Hence, the enzyme solution was preincubated with one of the substrates, cofactors, or products individually and in combination, heated to 50 °C for 10 min, cooled in an ice bath, and assayed for glutamate synthase activity. α-Ketoglutarate protected the enzyme only marginally, but the presence of both α-ketoglutarate and L-glutamine stabilized glutamate synthase activity against thermal denaturation significantly. Preincubation of the enzyme with NADH or the product, L-glutamate, made the enzyme more heat sensitive.

Optimum pH and Temperature. Glutamate synthase has a broad pH profile with optimal activity at pH 7.2. Glutamate synthase from *E. coli* (Miller & Stadtman, 1972), *S. cerevisiae* (Masters & Meister, 1982), *B. megaterium* (Hemmila & Mantsala, 1978), and corn leaf (Matoh et al., 1979) has optimal activity at pH 7.6, 7.1–7.7, 7.3, and 6.9, respectively. Thus, the pH optimum of *C. pasteurianum* enzyme falls within the same range as for the enzyme from other sources and resembles *S. cerevisiae* glutamate synthase in having a broad pH optimum. The enzyme shows optimum activity at 50 °C.

Energy of Activation. The energy of activation of glutamate synthase as determined from Arrhenius plot is 43.9 kJ mol^{-1}. Glutamate synthase from a thermophilic bacillus (Schmidt & Jervis, 1982) shows two energies of activation, one below 58 °C (82.8 kJ mol^{-1}) and the other above 58 °C (27.8 kJ mol^{-1}).

Apparent Michaelis–Menten Constants (K_m^{app}). For determining the apparent Michaelis–Menten constants of glutamate synthase for NADH, α-ketoglutarate, and L-glutamine, the concentrations of two substrates/cofactors were kept at saturation while the concentration of the third was varied and the enzyme activity was determined. Lineweaver–Burk plots ($1/v$ vs $1/s$) were drawn in each case. A straight line was obtained in all three cases. Values for K_m^{app} for NADH, α-ketoglutarate, and glutamine are 0.11, 0.17, and 0.16 mM, respectively. The enzyme from *E. coli* (Miller & Stadtman, 1972) had K_m values of 7.3 and 250 μM and that from *S. cerevisiae* (Masters & Meister, 1982) had 0.14 and 1 mM for α-ketoglutarate and glutamine, respectively. The enzyme from these sources had a K_m value of 7.7 and 2.6 μM for NADPH and NADH, respectively.

Identification of the Product Formed by Glutamate Synthase Catalyzed Reaction. The enzymatic reaction was run on a preparative scale as given under Experimental Procedures. L-Glutamic acid was found to be the sole reaction product by thin-layer chromatography.

Stoichiometry of Reaction. The stoichiometry of NADH oxidized to α-ketoglutarate utilized was determined by incubating the enzyme (protein, 0.2 mg in 0.1 mL) in the presence of saturating concentrations of NADH (250 μM) and glutamine (5 mM) but limiting concentrations of α-ketoglutarate (50 and 100 μM) and allowing the reaction to proceed to completion. The ratio of NADH oxidized to α-ketoglutarate consumed came to be nearly 1:1. When the experiment was repeated in the presence of a limiting concentration of glutamine and saturating concentrations of NADH and α-ketoglutarate, again a ratio of NADH oxidized to glutamine consumed came to be 1:1 (results not shown). Thus, for each mole of NADH oxidized, 1 mol of α-ketoglutarate and 1 mol of glutamine are consumed. Similar stoichiometry has been reported in the case of glutamate synthase from *A. aerogenes* (Trotta et al., 1974), *S. cerevisiae* (Masters & Meister, 1982), and lupin nodules (Boland, 1979).

Substrate/Cofactor Specificity of Glutamate Synthase. Substrate specificity of the enzyme was determined by assaying the enzyme in the presence of its natural substrates/cofactors and then in the presence of an analogue of one of them and keeping the other two the same (results not shown). Glutamate synthase needs NADH as reductant for its activity. Efforts to substitute NADPH, FADH, or FMNH for NADH in the reaction mixture failed to give any activity. Similarly, neither asparagine, NH_4Cl, nor other analogues of L-glutamine such as albizziin, DON, azaserine, and methionine sulfoximine exhibited any activity in the absence of L-glutamine. The four analogues of glutamine actually inhibited the glutamate synthase activity as reported in the *A. aerogenes* glutamate synthase (Trotta et al., 1974). α-Ketoglutarate could not be replaced by either oxaloacetate or pyruvate. Thus, glutamate synthase has a very narrow specificity for its substrates and cofactors. Glutamate synthase from *S. cerevisiae* (Masters & Meister, 1982) is also very specific for α-ketoglutarate,

L-glutamine, and NADH, but those from *A. aerogenes* (Trotta et al., 1974) and *B. megaterium* (Hemmila & Mantsala, 1978) can utilize ammonia in place of L-glutamine, while that from *Pisum sativum* (Matoh et al., 1980) can utilize methylviologen as electron donor in place of NADH. *Methanosarcina barkeri* (Kenealy et al., 1982) glutamate synthase can utilize hydrogen-reduced deazaflavin factor 420 or flavin mononucleotide but not NAD^+, $NADP^+$, or ferredoxin as electron donors.

Effect of Metal Ions on Glutamate Synthase Activity. Glutamate synthase was preincubated with different metal ions and then assayed for glutamate synthase activity. All the added metal ions inhibited the enzyme activity except Mn^{2+} and Ca^{2+}. Cu^{2+} and Fe^{2+} were most inhibitory, while Mg^{2+}, Co^{2+}, and Zn^{2+} inhibited only marginally. None of the metal ions enhanced the glutamate synthase activity, which shows that glutamate synthase requires no metal ion for its activity. Similar results have also been reported from *P. sativum* (Matoh et al., 1980) glutamate synthase.

Effect of Amino Acids. Glutamate synthase was assayed separately in the presence of 5 and 20 mM concentrations of glycine, L-glutamate, L-serine, L-histidine, L-asparagine, L-valine, L-methionine, L-aspartic acid, L-tryptophan, and L-arginine. Of the various amino acids tested, only L-glutamate inhibited the enzyme activity by 20% at 4 mM. This may be due to product inhibition. At 25 mM, only L-serine, L-histidine, L-methionine, and L-tryptophan produced 20–30% inhibition, which could be nonspecific as a result of overloading of the enzyme by high concentrations of these amino acids. In similar studies with *E. coli* (Miller & Stadtman, 1972) glutamate synthase, partial inhibition of enzyme activity was observed.

Effect of Tricarboxylic Acid Cycle Metabolites. Glutamate synathase was assayed separately in the presence of 5 and 25 mM concentrations of fumarate, succinate, isocitrate, maleate, *cis*-aconitate, and pyruvate. None of the added tricarboxylic acid cycle metabolites caused significant inhibition/activation of the enzyme activity. Pyruvate inhibited the enzyme only marginally, it being on analogue of α-ketoglutarate as in the case of *E. coli* (Miller & Stadtman, 1972) glutamate synthase.

Discussion

Experiments with labeled dinitrogen had shown glutamate and glutamine as early acceptors of fixed nitrogen in *C. pasteurianum*. So these amino acids are not only early acceptors of fixed nitrogen but also, in turn, primary donors of nitrogen for the synthesis of other nitrogenous compounds. Hence, purification of enzymes synthesizing glutamate and glutamine in nitrogen-fixing microbes and their regulatory mechanism are of great interest. While purification of glutamate synthase to homogeneity from aerobes (Miller & Stadtman, 1972; Trotta et al., 1974; Hemmila & Mantsala, 1978) had been reported, no such reports are available for anaerobes. This is due to instability of enzymes of anaerobic origin (Kleiner, 1979; Dua & Burris, 1963). Two observations helped us to purify this enzyme from *C. pasteurianum*. First, use of molasses and a starving level of NH_4Cl as the only source of carbon and nitrogen, respectively, gave 2–3-fold higher level of the enzyme. Systematic studies showed that enzyme is stabilized in the presence of $Na_2S_2O_4$, glutamine, mercaptoethanol, α-ketoglutarate, and EDTA. Due to the strong inhibitory effect of $Na_2S_2O_4$ and the low solubility of glutamine, α-ketoglutarate, EDTA, and mercaptoethanol were routinely used in buffers. They had a synergystic effect on enzyme stability. The investigation of the influence of ionic strength and nature of the buffer led to selection of 200 mM potassium phosphate buffer (pH 7.2). To keep the operational time to a minimum, quick precipitation of glutamate synthase was followed by its direct loading onto a combined gel filtration and ion-exchange column. Dialysis was avoided. This procedure gave 58-fold purification with 40% recovery.

Comparison of the properties of *C. pasteurianum* enzyme with those of aerobic microorganisms shows that this protein like *E. coli* and *B. megatarium* enzyme is a large protein. K_m for glutamine also falls in the range of these enzymes, but K_m of this enzyme for α-ketoglutarate is higher than that of *B. megaterium* and *E. coli* enzyme but lower than that of *A. aerogenes* enzyme. The NADH requirement for *C. pasteurianum* enzyme is absolute, and it shows no activity with NADPH, a reductant used by aerobic microbial enzymes. Its K_m for NADH is comparatively higher and is in conformity with its anaerobic character. Like *E. coli* enzyme, glutamine cannot be substituted by any other nitrogen donor. On the other hand, both *B. megaterium* and *A. aerogenes* enzyme show low levels of glutamate synthase activity when glutamine is substituted by ammonium salt, and these enzymes also have glutaminase activity which is absent in the case of *C. pasteurianum* enzyme. pH optima for aerobic and anaerobic enzymes are relatively close. The main difference in their properties lies in absorption spectrum. This enzyme shows no absorption at 370–380 or 440–450 nm and hence contains no flavin or iron. The absence of flavin in the supernatant of boiled or TCA-treated enzymes was confirmed by spectrophotometry, fluorometry, and thin-layer chromatography. Absence of iron was also checked by atomic absorption. The presence of iron, zinc, molybdenum, calcium, or magnesium in enzyme protein was at the same level as in the control. Chemical analysis of the purified glutamate synthase by the 1,10-phenanthroline method also gave negative results. The methylene blue method of Burch et al. (1948) indicated the absence of sulfur. Hence, *C. pasteurianum* enzyme, like the plant enzyme, is a non-flavin–Fe-S enzyme and thus differs from aerobic microbial enzymes.

E. coli and *B. megaterium* enzymes are tetramers of two different subunits, while *C. pasteurianum* enzyme is a dimer of five different subunits. The protein precipitated by ammonium sulfate (0.3–0.5 saturation) in the presence and absence of protease inhibitors like PMSF, pepstatin A, and benzamidine, incubated for 0, 15, or 30 min at 35 °C, and subjected to SDS electrophoresis showed no change in the relative intensity of stained bands corresponding to those produced by the purified glutamate synthase, thus eliminating the possibility of these subunits arising from proteolytic fragmentation.

Unlike *E. coli* enzyme, *C. pasteurianum* glutamate synthase activity is only slightly inhibited by glycine, serine, histidine, asparagine, methionine, or tryptophan and citric acid cycle metabolites. It seems, therefore, that the regulatory mechanism of glutamate synthesis in *C. pasteurianum* is different from that occurring in *E. coli*.

References

Bassham, J. A., & Kirk, M. (1964) *Biochim. Biophys. Acta 90*, 553–562.

Boland, M. J. (1979) *Eur. J. Biochem. 99*, 531–539.

Brenchley, J. E., Prival, M. J., & Magasanik, B. (1973) *J. Biol. Chem. 248*, 6122–6128.

Brown, C. M., Meers, J. L., & Tempest, D. W. (1970) *J. Gen. Microbiol. 61*, vii–viii.

Burch, M. B., Bessey, O. H., & Lowry, O. H. (1948) *J. Biol. Chem. 175*, 457–470.

Dainty, R. H. (1972) *Biochem. J. 126*, 1055–1056.

Dainty, R. H., & Peel, J. L. (1970) *Biochem. J. 117*, 573–584.
Davis, B. J. (1964) *Ann. N.Y. Acad. Sci. 121*, 404–427.
Dua, R. D., & Burris, R. H. (1963) *Proc. Natl. Acad. Sci. U.S.A. 50*, 169–175.
Elmerich, C., & Aubert, J. P. (1971) *Biochem. Biophys. Res. Commun. 42*, 371–376.
Fogo, J. K., & Popowsky, M. (1949) *Anal. Chem. 21*, 732–734.
Folkes, B. F. (1959) *Symp. Soc. Exp. Biol. 13*, 126–147.
Folkes, B. F., & Sims, A. P. (1974) *J. Gen. Microbiol. 82*, 77–95.
Gray, W. R. (1976) *Methods Enzymol. 11*, 139.
Harvey, E. A., Jr., Smart, J. A., & Anis, E. S. (1955) *Anal. Chem. 27*, 26–29.
Hemmila, I. A., & Mantsala, P. I. (1978) *Biochem. J. 173*, 45–52.
Hubbard, J. S., & Stadtman, E. R. (1967) *J. Bacteriol. 93*, 1045–1055.
Kenealy, W. R., Thompson, T. E., Schubert, K. R., & Zeikus, J. G. (1982) *J. Bacteriol. 150*, 1357–1365.
Kleiner, D. (1979) *Arch. Microbiol. 120*, 263–270.
Locascio, G. A., Tigler, H. A., & Batlle, A. M. (1969) *J. Chromatogr. 40*, 453–457.
Lowry, O. H., Rosebrough, N. J., Farr, A. L., & Randall, R. J. (1951) *J. Biol. Chem. 193*, 265–275.
Masters, D., Jr., & Meister, A. (1982) *J. Biol. Chem. 257*, 8711–8715.
Matoh, T., Suzuki, F., & Ida, S. (1979) *Plant Cell Physiol. 20*, 1329–1340.
Matoh, T., Ida, S., & Takahashi, E. (1980) *Plant Cell Physiol. 21*, 1461–1474.
Meers, J. L., Tempest, D. W., & Brown, C. M. (1970) *J. Gen. Microbiol. 64*, 187–194.
Miller, R. E. (1974) *Biochim. Biophys. Acta 364*, 243–249.
Miller, R. E., & Stadtman, E. R. (1972) *J. Biol. Chem. 247*, 7407–7419.
Nagatani, H., Shimizu, M., & Valentine, R. C. (1971) *Arch. Mikrobiol. 79*, 164–175.
Ouchterlony, O. (1949) *Acta Pathol. Microbiol. Scand. 26*, 507–515.
Peterson, R. E. (1953) *Anal. Chem. 25*, 1337–1339.
Schmidt, C. N. G., & Jervis, L. (1982) *J. Gen. Microbiol. 128*, 1713–1718.
Shugaev, N. I., Shaposhnikov, G. L., Aseeva, K. B., Evstigneeva, Z. G., & Kretovich, V. C. (1980) *Dokl. Akad. Nauk. SSSR 252*, 1513–1516.
Sims, A. P., & Folkes, B. F. (1964) *Proc. R. Soc. London B159*, 479–502.
Suziki, A., & Gadal, P. (1982) *Plant Physiol. 69*, 848–852.
Tachiki, T., Sung, H. C., Akisara, S. W., & Tochikura, T. (1983) *J. Ferment. Technol. 61*, 179–184.
Tamura, G., Oto, M., Hirasawa, M., & Aketagawa, J. (1980) *Plant Sci. Lett. 19*, 209–215.
Tarmy, E. M., & Kaplan, N. O. (1968) *J. Biol. Chem. 243*, 2579–2586.
Tempest, D. W., Meers, J. L., & Brown, C. M. (1970) *Biochem. J. 117*, 405–407.
Trotta, P. P., Platzer, K. E. B., Haschemeyer, R. H., & Meister, A. (1974) *Proc. Natl. Acad. Sci. U.S.A. 71*, 4607–4611.
Warburg, O., & Christian, W. (1941) *Biochem. Z. 310*, 384–421.
Weber, K., & Osborn, M. (1975) *Proteins 1*, 179.
Wilson, G. S., & Miles, A. (1975) in *Topley and Wilson's Principles of Bacteriology, Virology and Immunity*, Vol. 1, pp 1129–1130, Arnold, London.
Zelitch, I., Rosenblum, E. D., Burris, R. H., & Wilson, P. W. (1951) *J. Bacteriol. 62*, 747–752.