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RIBULOSE-1, 5-BISPHOSPHATE CARBOXYLASE FROM COMFREY: ISOLATION AND CHARACTERIZATION*

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Abstract—Ribulose-1,5-bisphosphate carboxylase/oxygenase has been purified to electrophoretic homogeneity from comfrey, *Symphytum* spp. Sodium dodecyl sulfate polyacrylamide and polyacrylamide gel electrophoresis studies on the purified product showed no extraneous proteins. Comparisons of the electrophoretic mobilities of the subunits to those of standard proteins indicated a large subunit MW of 50 000 and a small subunit of 12 700, which for an octameric structure of each subunit indicates a native MW of 502 000. Specific activities of the comfrey enzyme ranged from 1.2 to nearly 2 μmol $^{14}CO_2$ fixed/min·mg of protein over several preparations and were maintained for months when stored from the sucrose gradient at $-70°$. The specific activities depended critically on the amounts of enzyme used in the assay even under saturating conditions of substrates and cofactors. The effective pH dependence for carboxylase catalysis peaked near 7.4, which apparently is the lowest effective optimum yet reported for this enzyme from any source. However, on a constant carbon dioxide basis the pH dependence profile was reversed with a maximum near pH 8.6 which was 0.4 units higher than the value for the spinach enzyme. The K_ms for carbon dioxide and ribulose-1,5-bisphosphate at pH 7.5 were 130 μM and 30 μM, respectively, which are comparable to the accepted values for the carboxylase from spinach at pH 7 2

INTRODUCTION

Ribulose-1,5-bisphosphate carboxylase/oxygenase (EC 4 1 1 39) catalyses the carboxylation and cleavage of ribulose-1,5-bisphosphate to form two molecules of 3-phospho-D-glycerate [1–5] In the presence of oxygen the Calvin cycle enzyme also catalyses the oxygenation and subsequent cleavage of RuBP to form one molecule of 3-phospho-D-glycerate and one molecule of phosphoglycolate [6], which probably represents the first step in photorespiration This key enzyme has now been isolated from a variety of organisms ranging from the traditional sources, spinach and tobacco, to several chemosynthetic bacteria [7]. We have purified and partially characterized RuBPCase from comfrey, a long-lived root propagated borage plant whose leaves rank among the highest known in nitrogen content [8] and which in the U.S.A is grown mostly for its herbal properties The long lifespan and abundant leafy material from only a single plant assured a genetically consistent source and a convenient year-round supply of material when greenhouse grown.

RESULTS

Purification

Initially it was found that under the conditions described a 15–30 sec homogenization time was adequate to release the RuBPCase from comfrey leaves Protein was precipitated with 40% ammonium sulfate and the redissolved 40% pellet applied directly to a linear 0 2–0.8 M sucrose gradient [9] and centrifugation to equilibrium produced a single peak on the A_{280} profile with two broad minor bands The same profile was obtained from all preparations and also if the redissolved 40% pellet was first dialysed against buffer B. Specific activities in that case, however, were considerably lower, indicating degradation during dialysis. Only the fractions with the highest specific activity were pooled for storage It could be stored in the sucrose density gradient medium at $-70°$ retaining over 85% of its original activity after 5 months The A_{280}/A_{260} ratio for the pooled fractions was 1 99. The A_{280}/A_{260} ratio for the minor bands was much lower indicating considerable contamination of those frctions with nonprotein material The enzyme was thawed and warmed for 10 min at 40° before application to the DEAE column which gave further slight purification. Table 1 summarizes the results of the purification scheme which shows roughly a 25-fold increase in specific activity from crude extract to purified enzyme.

SDS-PAGE (5–10 μg protein per well) was run at each stage of the isolation scheme. The crude extract showed

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Abbreviations Tris, tris-hydroxylamino methane, MOPS, morpholinopropane sulfonic acid, PMSF, phenylmethylsulfonyl fluoride, PVP, polyvinyl-polypyrrolidone, SDS, sodium dodecyl sulfate, PAGE, polyacrylamide gel electrophoresis, DTT, dithiothreitol, RuBPCase, ribulose-1,5-bisphosphate carboxylase/oxygenase, DEAE, diethylaminoethane, TCA, trichloroacetic acid, RuBP, ribulose-1,5-bisphosphate (tetra sodium salt)

Table 1 Purification of RuBPCase from comfrey leaves

	Protein (mg)	μmol $^{14}CO_2$ fixed/min mg protein	Degree of purification
Crude extract	2455*	0 05	1
40% ammonium sulfate precipitate	107	0 54	11
Pooled sucrose gradient	60	0 97	20
DEAE-cellulose	26	1 3	26

*From 100 g fr wt of deribbed leaves

major bands at MWs of 50 000 and 13 000 which corresponded to the large and small subunits, respectively, of the purified enzyme and six fainter bands at MWs corresponding to 90 000, 77 000, 68 000, 46 400, 39 700 and 36 900 Of these, only the 39 700 band was retained along with the RuBP Case subunits in the 40% precipitate, but a new band (60 000) became visible The pooled RuBP Case fractions from the sucrose gradient also showed the 60 000 band but the 39 700 band was no longer detectable With very heavily loaded disc gels additional bands became visible between those for the large and small RuBPCase subunits All of the extraneous bands were removed, however, in the DEAE cellulose step indicating a homogeneous preparation The fainter band near the origin has been seen with the stored enzyme on other occasions and may have represented higher aggregates of RuBCase Other preparations run on 5 5% PAGE disc gels gave the same results

Characterization of RuBPCase from comfrey

The MWs of the large and small subunits of the comfrey enzyme were estimated to be 50 000 and 12 700, respectively, based on a linear regression analysis of log MW vs distance migrated for bovine serum albumin, alcohol dehydrogenase, chymotrypsinogen and lysozyme. Assuming an L_8S_8 structure, where L refers to the large subunit and S to the small, which has been found for all higher plant RuBPCases, the results predict a native MW of slightly over 500 000 for the comfrey enzyme

The assay conditions were optimized in regard to concentrations of substrates and cofactors, amounts of enzyme, pH and preincubation conditions. A preincubation time of 45 min at 25 ± 1° in buffer C was found to give maximal counts because of the slow attainment of isotopic equilibrium between labeled and unlabeled HCO_3^- This was verified by the addition of 175 units of carbonic anhydrase to the preincubation mixture which reduced the time required to achieve maximum counts to less than 1 min Carbonic anhydrase was not used routinely in the assays, however, and the 45 min preincubation was retained under normal conditions.

In regard to substrate and cofactor concentrations it was established that 20 mM sodium bicarbonate and 10 mM Mg^{2+} gave full activity in the presence of a large excess (0.7 mM) of RuBP (Fig 1) The slight decrease above 20 mM was reproducible and resulted from weak inhibition by HCO_3^- [Bonsall, R F, Bolden, T D and Mueller, D D, unpublished results] The effects of sodium bicarbonate and Mg^{2+} were interdependent For example, from other experiments at 1 mM sodium bicarbonate and 1 mM Mg^{2+} the specific activity was found to be only

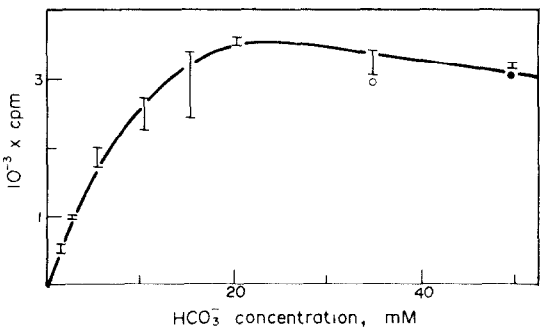

Fig 1 Specific activity vs HCO_3^- concentration at saturating levels of RuBP and Mg^{2+} The DEAE purified enzyme (20 μg) was activated in a sealed vial for 45 min in 490 μl 50 mM Tris–HCl, 10 mM Mg^{2+}, 2 mM DTT and 1 mM EDTA containing the indicated concentrations of HCO_3^- The 90 sec assay was initiated by adding 10 μl RuBP The error bars represent the extremes of duplicate determinations (○) 17 5 mM, and (●) 25 mM Mg^{2+} The average cpm at 20 mM HCO_3^- corresponded to a specific activity of 1 33 μmol $^{14}CO_2$ fixed/min mg protein

0 12 μmol $^{14}CO_2$ fixed/min·mg protein but rose to 0.66 units upon increasing the Mg^{2+} concentration to 5 mM which was over 40% of the 1 6 units observed under saturating conditions Saturating Mg^{2+} levels were achieved, however, at 10 mM as shown in Fig 1 where raising the Mg^{2+} concentration to 17 5 mM at 35 mM sodium bicarbonate and to 25 mM at 50 mM sodium bicarbonate failed to increase the specific activity The slight decrease in activity probably was real and has been noted previously at high Mg^{2+} concentrations [10]

To optimize the relative amounts of enzyme in the assay under otherwise saturating conditions the specific activity was measured using various amounts of protein at a fixed saturating concentration of RuBP (Fig 2) At low concentrations of enzyme from the pooled sucrose gradient fractions (high ratios of substrate to active sites) the specific activity was substantially reduced As the enzyme concentration was increased a maximum in activity was observed near 5 μg of protein in the standard assay At higher concentrations still there was a gradual but steady decline in activity as the substrate levels became less saturating Based on this evidence, 10–20 μg RuBPCase was used in all assays containing 0 7 mM RuBP to keep the conditions in the plateau region The same results were obtained with DEAE cellulose purified enzyme (data not shown).

The effective pH optimum for carboxylase activity

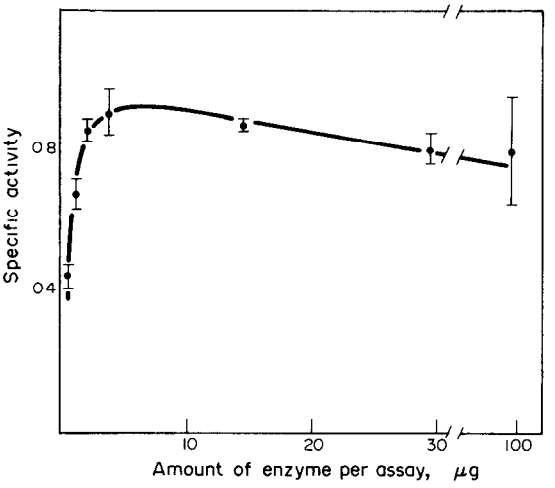

Fig 2 Dependence of the carboxylase activity on amount of enzyme at saturating levels of Mg^{2+}, HCO_3^- and RuBP Varying amounts of enzyme from the pooled sucrose gradient fractions were activated in sealed vials in 50 mM Tris–HCl, 20 mM $NaHCO_3$ containing *ca* 0·3 μCi NaH $^{14}CO_3$, 10 mM $MgCl_2$, 2 mM DTT and 1 mM EDTA, pH 7·8 at 25 ± 1° (540 μl) The reaction was initiated with 10 μl 39 mM RuBP and quenched after 60 sec

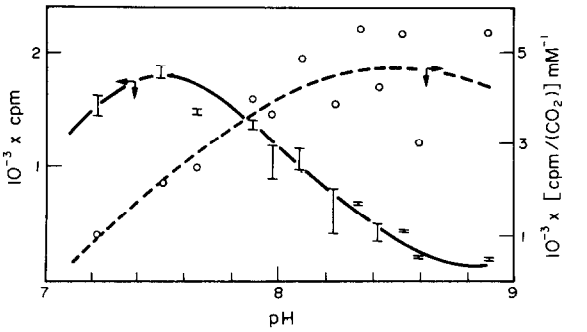

Fig 3 Dependence of the carboxylase activity on pH DEAE cellulose purified enzyme was activated for 45 min in 10 mM Tris–HCl, 20 mM $NaHCO_3$ containing *ca* 1 μCi NaH $^{14}CO_3$, 10 mM $MgCl_2$, 2 mM DTT and 0.1 mM EDTA, pH 8·0, in a sealed vial The reaction was initiated by adding 20 μg of the activated enzyme solution (13 μl) to 487 μl of 25 mM Tris–HCl and 25 mM MOPS–HCl, which contained identical concentrations of the other constituents except for the addition of 0·7 mM RuBP and adjustment of pH The reactions were quenched after 90 sec (——) (Right hand ordinate) represents the effective pH dependence and the error bars represent the extremes of duplicate determinations (– – – –) (Left hand ordinate) represents the estimated pH dependence of the same data replotted on a constant 1 mM CO_2 basis where the points (○) are averages of the duplicate determinations

under saturating conditions was 7.4 (Fig. 3) which was lower than the 8.0 reported for the tobacco enzyme [11] at the same Mg^{2+} concentration. This same profile was obtained with other enzyme preparations at 1 mM sodium bicarbonate [Esser, M D and Mueller, D D, unpublished results] and also showed a steady decline in activity from 7·4 to 6.5 Carbon dioxide, however, is a substrate of RuBPCase and its concentration changes with pH when sealed from the atmosphere. Therefore, the data were replotted on a constant 1 mM carbon dioxide basis (Fig. 3) using $7·4 \times 10^{-7}$ M as the constant for the overall equilibrium between carbon dioxide and HCO_3^- at 25° [12]. This method resulted in a reversal of the pH dependence profile and indicated a true pH optimum of at least 8·6, for catalysis by the comfrey enzyme, which was somewhat higher than the 8.2 obtained at a constant carbon dioxide concentration for the spinach enzyme by Andrews *et al* [13] at 25 mM Mg^{2+}.

K_m values for RuBP and carbon dioxide were determined from initial velocities (V_i) of the reactions with fully activated enzyme. For the reactions with varying low amounts of RuBP at 20 mM sodium bicarbonate the rate was linear for at least 50 sec and four–five data points were used to find V_i. In the corresponding studies with varying low concentrations of carbon dioxide and saturating levels of RuBP the rate remained linear only for *ca* 25 sec, possibly due to inactivation of the preincubated enzyme in the assay mixture and only the first three data points were used to calculate each V_i The bicarbonate concentrations were converted to carbon dioxide concentrations using $K = 7.4 \times 10^{-7}$ as before. The Lineweaver–Burk plots for RuBP yielded a K_m of 30 μM and a V_{max} of 1·3 μmol $^{14}CO_2$ fixed/min mg protein whereas the plot for carbon dioxide gave a K_m of 130 μM and a V_{max} of 2.6 units. The differences in V_{max} were disappointing but the data were obtained on different preparations and may reflect real variations.

One of the unique aspects of the comfrey enzyme besides its pH optimum was its inactivation under the combined effects of moderate salt concentrations and low temperatures. When the enzyme in buffer B was made 0.25 M in sodium chloride and exposed to 4° the enzyme was quickly inactivated. The inactivation depended on sodium chloride levels and temperature. In the normal buffer B, which is 52·5 mM in Cl^-, the enzyme could be frozen for extended periods without substantial loss of activity. Similarly at 0.25 M added sodium chloride the enzyme remained active at room temperature for several days. The inactive product produced in 0.25 M sodium chloride at 4° could not be reactivated by removal of the salt and/or warming to 40–50°K for various times up to 2 hr. Concentrations of Cl^- above 0·25 M, however, did affect the activity of the enzyme at 25° dropping it from over 1 unit in 0.25 M to essentially zero in 0.8 M added salt.

DISCUSSION

The procedures selected to isolate RuBPCase from comfrey leaves are similar to those which have evolved over the years in several other laboratories [9, 14, 15]. Among the major modifications were the use of dithionite as a reducing agent through the ammonium sulfate precipitation step and keeping the crude extract virtually continuously in contact with insoluble PVP during the early manipulations These modifications kept the solutions green, until they became colorless on the sucrose gradient, and resulted in higher overall yields and greater specific activity. Another change from existing procedures was the elimination of an ammonium sulfate cut during the purification No advantage was apparent with a cut and its elimination saved *ca* 3 hr of preparation time which in itself was no doubt advantageous in maintaining

the integrity of the enzyme The product produced by this procedure was electrophoretically pure, had good activity and stored excellently

The specific activities of the comfrey RuBPCase in 20 mM sodium bicarbonate and 10 mM Mg^{2+} ranged from 1 2 to nearly 2 μmol $^{14}CO_2$ fixed/min mg protein over several preparations, which was quite comparable to that of spinach RuBPCase, even though it was activated and assayed at pH 7 8 for a number of the studies reported here Presumably somewhat higher activities would have been obtained in these cases if it had been assayed at pH 7 4, the effective optimum for catalysis by the comfrey enzyme The activity observed depended strongly on the amount of enzyme used in the assay When less than 5 μg was used the specific activity decreased sharply A decrease such as this could result from dissociation of the subunits at low concentrations RuBPCases from higher organisms (L_8S_8 structure) usually are dissociable only under denaturing conditions, making this possibility less attractive Alternatively, it has been shown [16–18] that RuBP binds the unactivated form of RuBPCase more tightly thereby effectively inactivating the enzyme. Again this seems unlikely because the enzyme was fully activated immediately prior to addition of RuBP A more likely explanation appears to be contamination of RuBP with isomerization products, such as xyulose-1,5-bisphosphate, which is a potent inhibitor of RuBPCase activity [19] This would have had to occur, however, despite storage of the dissolved RuBP in small aliquots frozen under liquid nitrogen, never reusing a thawed solution and not using any material 1 month after dissolution

The comfrey enzyme had a number of properties in common with those of spinach and other higher plants MWs of the large and small subunits, Mg^{2+} requirements, activation by carbon dioxide and K_m for RuBP and carbon dioxide The value of 30 μM for K_m (RuBP) at pH 7 4 agreed well with those reported for the spinach enzyme at pH 7 8 [20] Similarly, the K_m (CO_2) of 130 μM at pH 7.5 was close to the value of 147 μM observed for spinach RuBPCase at pH 7 2 [21]. K_m (CO_2) for spinach, however, depended on pH since it dropped to 35 μM at pH 8 0 and to 15 μM at pH 8.8 [21] Assuming the comfrey enzyme would show a similar dependence on pH, it becomes possible to compare the pH dependence of activity to that of K_m (CO_2) The activity for comfrey RuBPCase increased *ca* five-fold between pH 7 2 and 8 0 (Fig 3) Over the same range K_m (CO_2) decreased by a factor of 4 2 for the spinach enzyme Clearly, the changes are comparable and suggest that most of the pH dependence of carboxylase activity arises from changes in binding of carbon dioxide to the active site In turn this suggests that the group(s) in the active site responsible for the carboxylation of RuBP probably does not ionize further in this pH range. On the other hand, the disparity between the pH 7 2 and 8 8 changes [10-fold for K_m (CO_2) and still *ca* five-fold for activity] implies that an ionization(s) unfavourable for catalysis may occur as the pH is raised above 8 0 Even above pH 8 the enzyme should have remained saturated with carbon dioxide because the 10-fold decrease in carbon dioxide in equilibrium with HCO_3^- is matched by a corresponding decrease in K_m (CO_2) between pH 7.2 and 8 8 These conclusions do require that there were no significant pH dependent changes in the state of activation of the enzyme during the 90 sec assay It was for that reason that the enzyme was activated at pH 8 0, the midpoint of the pH range investigated

Although the comfrey enzyme shares several properties with the spinach enzyme, there are differences Besides apparently storing better, the RuBPCase from comfrey seems to have the lowest effective pH optimum for catalysis (7 4) yet reported The true pH optimum observed in a constant carbon dioxide basis, however, was higher than that reported for the spinach enzyme by at least 0 4 units Since the pH rise in the stroma immediately following illumination is thought to play an important role in light activation of RuBPCase [22–25], the slightly higher pH optimum suggests that comfrey chloroplasts may function at a correspondingly higher pH or that its carboxylase operates in a somewhat less efficient state

The sensitivity to salt and cold also appears unique to the comfrey enzyme, at least in the manner of its manifestations Other RuBPCases are known to be sensitive to cold, especially over prolonged periods of storage [26–28] and the activity of the tobacco enzyme apparently decreases in the presence of moderate concentrations of Cl^- In these cases, however, the inactivation or inhibition was reversible either by lowering the salt concentration or warming the solutions after extended cold storage Neither of these procedures produced any detectable recovery of activity with the enzyme from comfrey This property is currently under further investigation

EXPERIMENTAL

Comfrey (*Symphytum* spp) crowns were obtained from North Central Comfrey Products, Glidden, Wisconsin, and apparently were of the Quaker variety as judged by their lavender blossoms [8] The plants were grown in 30 gallon trash barrels filled with soil and watered weekly with a standard plant nutrient soln Some crowns also were garden-grown without any special attention Prior to homogenization the freshly harvested leaves were packed on ice for transport, H_2O washed, deribbed and cut into small pieces *Ca* 50 g of this material was added to 600 ml of a 50 mM Tris–HCl, 50 mM $NaHCO_3$, 10 mM $MgCl_2$, 5 mM $Na_2S_2O_3$, 1 0 mM EDTA, 0 1 mM PMSF, pH 8 0 soln (buffer A) containing 48 g insoluble PVP in a 1 l fluted flask and homogenized for 15–30 sec at full power, on a Virtis-45 instrument equipped with five knife blades between Teflon spacers Immediately the homogenate was vacuum filtered through four layers of miracloth onto another 35 g quantity of PVP The above procedure was repeated for an additional 50 g portion of leaves The combined filtrates were stirred for 30 min, centrifuged at 10 000 *g* for 5 min and the supernatant recovered The extract was made 40% satd with $(NH_4)_2SO_4$ (244 g/l), stirred for 15 min and allowed to stand for 30 min The resulting suspension was centrifuged at 13 000 *g* for 30 min, the pellets redissolved in 20 ml buffer A and recentrifuged for 30 min at 13 000 *g* *Ca* 3 ml of the supernatant was layered on top of each of six Polyallomer tubes containing 34 ml of a linear 0 2–0 8 M sucrose gradient prepared in buffer B, which had 2 mM DTT replacing the dithionite of buffer A, and spun for 20 hr at 27 000 rpm in a Spinco SW-27 rotor 1 ml fractions were collected from the bottom and the RuBPCase located by A_{280} and enzymatic activity The peak tubes were pooled and stored under N_2 in sealable cryotubes (Vanguard, International, Neptune, NJ) at −70 Prior to most studies the sucrose solns were further purified on a 3 × 1 5 cm DEAE cellulose column equilibrated with buffer B, washed free of sucrose with the same buffer and eluted with buffer B made 0 25 M in NaCl In some preparations 0 1 mM PMSF was

maintained throughout all procedures No differences in specific activity or electrophoretic mobility of the enzyme from these preparations were noted All isolations were done at 4°

Protein determinations on the purified enzyme soln were based on A_{280} measurements using $E^{1\%}_{1cm} = 16$ which is typical of higher plant enzymes [29] With less purified samples a modified Bradford Coomassie brilliant blue G-250 procedure [30] was used Before analysis of the crude preparations by this method the protein was ppted overnight at 4° with 10% TCA, washed × 3 with cold Me_2CO and dissolved in 0 1 M NaOH

Activity measurements were done at room temp in 7 ml scintillation vials using essentially the $NaH\ ^{14}CO_3$ method reported by Paulson and Lane [14] but utilizing prior activation with CO_2 and Mg^{2+} as described by Lorimer *et al* [31] Typically, 20 μg RuBPCase was incubated for 45 min in 50 mM Tris–HCl, 19 mM $NaHCO_3$ containing 0 2–1 μCi $NaH\ ^{14}CO_3$, 10 mM $MgCl_2$, 2 mM DTT, 1 0 mM EDTA, pH 7 8 (buffer C) in a total vol of 0 54 ml The reaction was initiated with 10 μl 39 mM RuBP (Sigma Chemical Co) dissolved in the same buffer, quenched after 60 sec with 0 2 ml HOAc and taken to dryness overnight at 85° in an aluminum block with holes drilled to a depth of 4 0 cm which was placed on a hot plate After resolution with 0 5 ml deionized H_2O, 6 5 ml of a standard H_2O dissolving scintillation cocktail similar to that of Patterson and Greene [32] were added and the samples counted on a Beckman liquid scintillation counter Residual ^{14}C counts in the samples were compared to unheated and heated controls prepared in the same manner but without RuBP

Disc PAGE expts generally were run at 3 mA/tube on 7% gels in 13 cm long tubes held in a H_2O cooled assembly using a Bio-Rad Model 500 power supply and a 5 mM Tris–HCl, 38 4 mM glycine, pH 8 3 electrode buffer SDS-PAGE expts used 10% gels, 8 mA/tube and a 72 mM Na_2HPO_4, 28 mM NaH_2PO_4, 3 5 mM SDS buffer Slab SDS-PAGE expts were performed at 4° on 20 × 20 × 0 15 cm, 15% polyacrylamide running gels at 2 5 mA using a 9 9 mM Tris–HCl, 76 6 mM glycine, 3 0 mM SDS buffer, pH 8 2 Prior to electrophoresis the samples were reduced with β-mercaptoethanol in SDS (1% BME, 1% SDS) by heating the soln in a boiling water bath for *ca* 5 min Before this treatment, however, the 40% supernatant samples were ppted with 10% TCA overnight at 4° and washed with cold deionized H_2O The gels were stained with Coomassie brilliant blue R-250 and destained with dilute HOAc according to standard procedures

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REFERENCES

1 Wildman, S W and Bonner, J (1947) *Arch Biochem* **14**, 381
2 Quayle, J R, Fuller, R C, Benson, A A and Calvin, M (1954) *J Am Chem Soc* **76**, 3610
3 Weissback, A, Smyrniotis, P Z and Horecker, B L (1954) *J Am Chem Soc* **76**, 3611
4 Racker, E (1955) *Nature (London)* **175**, 249
5 Jacoby, W S, Brommond, D O and Ochoa, S (1956) *J Biol Chem* **218**, 811
6 Bowes, G, Ogren, W L and Hageman, R H (1971) *Biochem Biophys Res. Commun* **50**, 532
7 McFadden, B A (1980) *Acc Chem Res* **13**, 394
8 Hart, R H, Thompson, A J, III, Elgin, J H, Jr and McMurtrey, J E, III (1981) *Agron J* **73**, 737
9 Goldthwaite, J J and Bogorad, L (1971) *Analyt Biochem* **41**, 57
10 Sugiyama, T, Nakayama, N and Akasawa, J (1968) *Biochem Biophys Res Commun* **30**, 118
11 Chollet, R and Anderson, L L (1976) *Arch Biochem Biophys* **176**, 344
12 Gibbons, B H and Edsall, J T (1963) *J Biol Chem* **238**, 3502
13 Andrews, T J, Badger, M R and Lorimer, G H (1975) *Arch Biochem Biophys* **171**, 93
14 Paulson, J M and Lane, M D (1966) *Biochemistry* **5**, 2350
15 Lawlis, V B, Gordon, G L R and McFadden, B A (1979) *J Bacteriol* **139**, 287
16 Wishnick, M, Lane, M D and Scrutton, M C (1970) *J Biol Chem* **245**, 4939
17 Laing, W A and Christeller, J T (1976) *Biochem J* **159**, 563
18 Paech, C and Tolbert, N E (1978) *J Biol Chem* **253**, 7864
19 McCurry, S D and Tolbert, N E (1977) *J Biol Chem* **252**, 8343
20 Jensen, R G and Bahr, J J (1977) *Annu Rev Plant Physiol* **28**, 379
21 Bowes, G, Ogren, W L and Hageman, R H (1975) *Plant Physiol* **56**, 630
22 Packer, L, Murakami, S and Mehard, C W (1970) *Annu Rev Plant Physiol* **21**, 271
23 Walker, D A (1973) *New Phytol* **72**, 209
24 Heldt, H W, Werdan, K, Milovancev, M and Geller, G (1973) *Biochim Biophys Acta* **314**, 224
25 Werden, K, Heldt, H W and Milovancev, M (1975) *Biochim Biophys. Acta* **396**, 276
26 Kawashima, N, Singh, S and Wildman, S G (1971) *Biochem Biophys Res Commun* **42**, 664
27 Ryan, F J and Tolbert, N E (1975) *J Biol Chem* **250**, 4229
28. Hall, N P, McCurry, S D and Tolbert, N E (1981) *Plant Physiol* **67**, 1220
29 Tomimatsu, Y (1980) *Biochim Biophys. Acta* **622**, 85
30 Stoops, J D (1978) M S Thesis, Kansas State University, Manhattan, U S A
31 Lorimer, G H, Badger, M. R and Andrews, T J (1976) *Biochemistry* **15**, 529
32 Patterson, M D and Greene, R C (1965) *Analyt Chem* **37**, 854