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THE ISOLATION, PURIFICATION, AND CHARACTERIZATION OF RIBULOSE-1,5-BISPHOSPHATE CARBOXYLASE/OXYGENASE FROM COMFREY

by

SCOTT ANTHONY SIMPSON

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Department of Biochemistry

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Approved by:

Delbert D. Muellu
Major Professor

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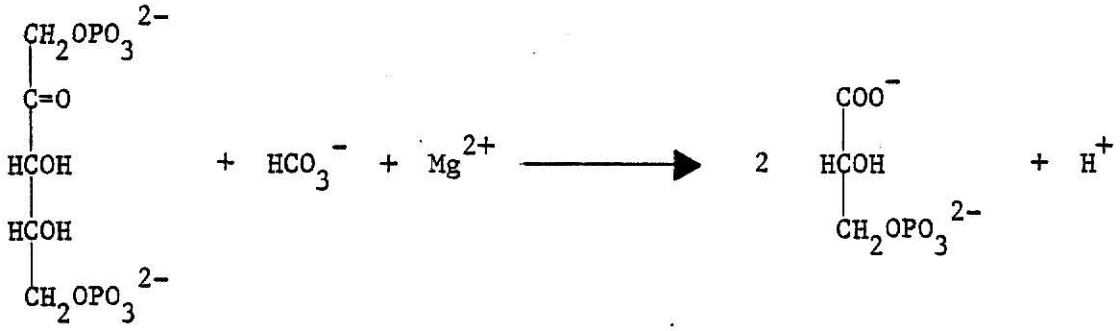
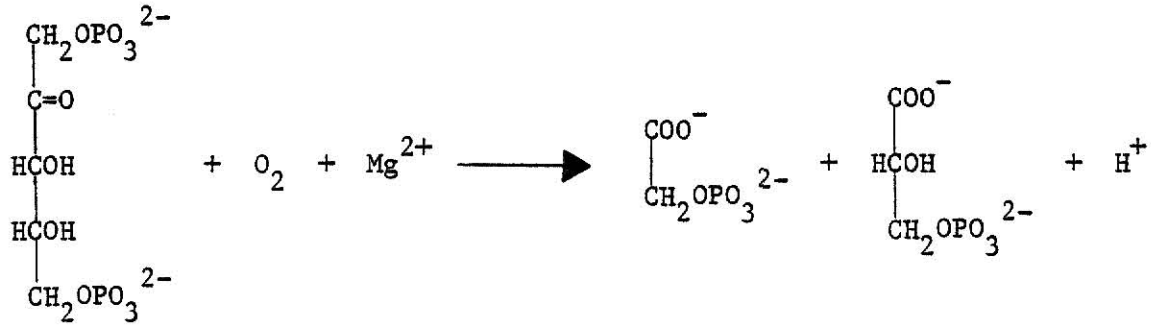
INTRODUCTION

Ribulose-1,5-bisphosphate carboxylase/oxygenase (EC 4.1.1.39) catalyzes the carboxylation and cleavage of ribulose-1,5-bisphosphate to yield two molecules of 3-phosphoglycerate as illustrated (Quayle et al, 1954; Weisbach et al, 1954; Racker, 1955; Jacoby et al, 1956).



This enzyme also possesses an oxygenase activity, catalyzing the oxygenation of ribulose-1,5-bisphosphate, producing one molecule each of phosphoglycolate and 3-phosphoglycerate (Bowes et al, 1971; Andrews et al, 1973; Ryan and Tolbert, 1975).



Ribulose-1,5-bisphosphate carboxylase/oxygenase (RuBPCase) is a

highly regulated enzyme as evidenced by the effects of a number of Calvin cycle metabolites on its activity, most notably CO_2 and Mg^{2+}. Other compounds that have been found to stimulate the carboxylase reaction are fructose-6-phosphate, ADP, ATP, UDP-glucose, and glucose-6-phosphate, while fructose-1,6-bisphosphate has been found to inhibit the reaction, (Buchanan and Schurmann, 1972, 1973). Chu and Bassham (1973, 1975), on the other hand, reported that fructose-6-phosphate was an inhibitor. The early literature was repleat with numerous discrepancies such as this and many were resolved when it was found that the order of addition of reagents was frequently crucial and when it was discovered that CO_2 and Mg^{2+} were essential activators of the enzyme.

There have been many isolation procedures reported for the enzyme from a variety of sources. Most of these were based on the purification scheme developed for the spinach enzyme by Paulsen and Lane (1966). After the initial homogenization and centrifugation, their procedure used a 35-50% ammonium sulfate cut of the supernatant followed by a 39-45% precipitation to further refine the product. The redissolved pellet was passed through a DEAE-Sephadex G-25 column, the fractions containing activity were then applied to a DEAE-Cellulose column and eluted stepwise with 0.005, 0.1, and 0.2 M phosphate buffers. The pooled fractions containing RuBPCase activity were precipitated by bringing it to 55% saturation with ammonium sulfate. Following centrifugation, the redissolved pellet was applied again to the DEAE-Sephadex G-25 column, followed by batchwise elution using 0.005 and 0.025 M phosphate buffers on a hydroxylapatite column.

Buchanan and Schurmann (1972) used essentially the same procedure as Paulsen and Lane (1966) for their spinach enzyme preparations, except

they used a 10-40% linear sucrose density gradient, which had been first used by Goldthwaite and Bogorad (1971), instead of the final DEAE-Cellulose and hydroxylapatite column chromatography steps. A sucrose gradient step was also used by Tabita and McFadden (1974) and by Tabita and Stevens (1975) to purify RuBPCase from three microbial sources and a blue-green algae, respectively.

To isolate the enzyme from the green algae Chlamydomonas reinhardi, Givan and Criddle (1971) used a rather exhaustive procedure. After ultrasonic disintegration, the broken cells were centrifuged at 40,000 x g, followed by a 140,000 x g spin for 1 hr on the resulting supernatant. A 35-55% ammonium sulfate cut was obtained on the supernatant and the redissolved pellet was applied to a 5-70% discontinuous sucrose gradient. Fractions containing activity were applied to a DEAE-Cellulose column. The resulting pooled fractions were brought to 60% saturation with ammonium sulfate, and after centrifugation, the redissolved pellet was again applied to a 5-70% discontinuous sucrose gradient.

Schloss and Hartman (1977) used yet another procedure to isolate the enzyme from the bacterium Rhodospirrilum rubrum. After obtaining a 35-60 % ammonium sulfate cut on the cell homogenate, the redissolved pellet was applied to a DEAE-Cellulose column and eluted with a 0.05-0.15 M linear potassium phosphate gradient. Fractions containing activity were pooled and brought to 90% saturation with ammonium sulfate, followed by application of the redissolved pellet to an A-50 DEAE-Sephadex column and elution with a 0.0-0.3 M linear NaCl gradient. The pooled fractions were concentrated using pressure dialysis, followed by further purification on a hydroxylapatite column.

All of the above procedures have been reported to produce essentially

homogeneous enzymes with specific activities in the range of 1.2 to 3.24 μmoles $^{14}CO_2$ fixed/min/mg protein.

With these various techniques in mind, my research project was to design an isolation scheme which would give a homogeneous enzyme from a new source, comfrey, and to perform some initial characterization studies, such as pH optimum, substrate Km, and molecular weights on the preparation. The reason for choosing comfrey for this project was that it is a long-lived plant which will grow year-round in a green house, and thereby provide a convenient source of RuBPCase. Furthermore, since this root-propagated plant grows prolifically, one crown will supply ample material for most studies on the enzyme, which also assures a genetically consistent source of RuBPCase.

EXPERIMENTAL

Homogenization

Ribulose-1,5-bisphosphate carboxylase/oxygenase (RuBPCase) was extracted from comfrey leaves using a Tris (50 mM), sodium bicarbonate (50 mM), magnesium chloride (10 mM), EDTA (0.1 mM), sodium hydrosulfite (5 mM), PMSF (0.1 mM), pH 8.0, buffer system (TBMESP). Approximately 50 g of freshly harvested comfrey leaves were washed with distilled water, deribbed, and added to a fluted homogenization flask containing 48 g of polyvinylpolypyrrolidone (PVP) and 600 mL of cold TBMESP buffer. This mixture was homogenized on ice with a Virtis 45 homogenizer for 15-30 sec. The resulting suspension was filtered through four layers of Miracloth into a flask kept on ice which contained 37 g of PVP wetted with 230 mL of cold TBMESP buffer. The above procedure was repeated for another 50 g of comfrey leaves. The two filtrates were combined and stirred for 30 min in the cold room. This suspension was centrifuged at 10,000 x g for 10 min at 4°. The resulting supernatant was referred to as "crude extract".

Ammonium Sulfate Fractionation

The following procedures were performed at 4° except where noted. The "crude extract" was brought to 40% saturation with 244.33 g of solid ammonium sulfate/liter of "crude extract". After the last ammonium sulfate was added, the solution was stirred for an additional 15 min and allowed to stand for 30 min. It was then centrifuged at 13,000 x g for 30 min. The pellets were dissolved in a total of 20 mL of TBMESP

buffer by gently agitating the suspension and allowing them to stand at room temperature for 15 min. This mixture was centrifuged at 10,000 x g for 10 min to remove the insoluble material. The resulting supernatant was referred to as "redissolved 40% ammonium sulfate pellet". In earlier preparations attempts were made to obtain more complete dissolution of the "redissolved 40% ammonium sulfate pellet" by warming the suspension briefly to 40-45°. However, no increased solubilization was detectable with that procedure.

Linear Sucrose Density Gradient

A 0.2 M and a 0.8 M sucrose (Fisher Scientific Co.) solution were prepared in TBMESP buffer and filtered by gravity through No. 597 Schleicher and Schuell filter paper to remove the insoluble material. A 16 mL portion of the 0.8 M sucrose solution was placed in the front mixing chamber of a 50 mL universal conical bore gradient maker (Buchler Instruments Div.) and a 16 mL portion of the 0.2 M sucrose solution was poured into the back chamber. A vibrating stirrer (Buchler Instruments Div.) was placed in the front mixing chamber to within 1 cm of the bottom and turned on to a medium speed. The interconnecting stopcock was opened after the outlet had been connected to a 40 mL (25.4 x 88.9 mm) polyallomer centrifuge tube (VWR Scientific Inc.) which had been placed in the swinging bucket for the SW-27 rotor. After the last of the resulting mixture had been collected, the bucket was placed in the refrigerator for at least one hour prior to addition of the enzyme. Approximately 3.33 mL of the "redissolved 40% ammonium sulfate pellet" were applied to each of the six cooled 0.2-0.8 M linear sucrose density gradients (opposite buckets were carefully balanced) and then centrifuged in a SW-27 swinging bucket rotor on a Spinco L3-50 ultracentrifuge at

27,000 rpm for 20 hr. One-ml fractions were collected from the bottom of the gradient tubes after piercing with an 18-gauge needle. Based on A_{280} and enzymatic assay determinations, the peak tubes were pooled and stored as 1 mL aliquots in 1.5 mL cyrotubes (Vanguard International Inc.) at -70°.

DEAE-Sephadex Column Chromatography

The gradient preparation was further purfied before use with DEAE-Sephadex column chromatography, which was carried out at room temperature. The pooled sucrose fractions, warmed at 40° for 10 min, were applied to a 1 x 3 cm DEAE-Sephadex G-25 column which had been equilibrated with TBME buffer with 2 mM dithiothreitol (TBMET) after washing with 5 bed volumes of 1 M NaCl and exhaustive washing with deionized H_2O. To remove the sucrose the column was washed with two bed volumes of the equilibration buffer and the enzyme was eluted with 0.25 M NaCl in TBMET buffer. One-mL fractions were collected. Based on A_{280} and enzymatic assay determinations, peak tubes were pooled and used immediately for the particular experiment.

RuBP Carboxylase Assay

Activity measurements were carried out at room temperature in 7 mL scintillation vials. To 400 µL of TMES or TMET buffers, pH 8.0, 20 µg of the enzyme in 100 µL of TBMESP or TBMET buffers, pH 8.0, were added and allowed to incubate at room temperature for 45 min. To this mixture 43.5 µL of 0.124 M $NaHCO_3$ and 0.0015 M $NaH^{14}CO_3$ (3.56 mCi/mmole) in TME buffer, pH 8.0, were added and allowed to equilibrate for at least 10 sec. The reaction was started by adding 10 µL of the substrate, D-ribulose-1,5-bisphosphate (RuBP), 0.03875 M, in TME buffer at pH 8.0, (stored at -20° in 45 µL fractions under N_2 in 1.5 mL cyrotubes (Vanguard International Inc.)) and allowed to react for 60 sec. The reaction was quenched with

0.2 mL of glacial acetic acid and then slowly taken to dryness on a hot plate, followed by additions of 0.5 mL of deionized water and 6.5 mL of liquid scintillation cocktail (8 g PPO, 0.2 g POPOP, 1 liter Triton X-100, and 2 liters of toluene). Radioactivity measurements were carried out on a Beckman Liquid Scintillation Spectrometer using a ^{14}C isoset and a gain setting of 2.5. Reproducibility of the assay is given in Table I. Specific activity was calculated as illustrated.

$$\frac{B1 - B2}{10.438} = \frac{\text{CPM (Experimental)} - B2}{X}$$

$$\frac{X}{\text{min} \cdot \text{mg protein}} = \text{Specific Activity}$$

where: B1 = total possible counts, i.e., the cold blank (no RuBP)

B2 = background counts, i.e., the heated blank (no RuBP)

X = μmoles $^{14}CO_2$ fixed

10.438 = total number of μmoles HCO_3^- present in assay

Polyacrylamide Disc-Gel Electrophoresis

Non-SDS polyacrylamide disc-gels were made according to the procedure described by Clark and Switzer (1977) which was the original disc-gel electrophoresis system of Ornstein (1964) and Davis (1964). Each polyacrylamide gel consisted of a 7% acrylamide running gel and a 2.5% acrylamide stacking gel. Electrophoresis was carried out in 0.005 M Tris, 0.0384 M glycine, pH 8.3, at 3 milliamps per tube until the bromophenol blue tracking dye moved to within 1 cm of the end of the tube. The gels were then stained for 25 min in 0.25% Coomassie blue in

TABLE I

Reproducibility of Ribulose-1,5-Bisphosphate Carboxylase Enzymatic Assay

B1	B2	DEAE-Sephadex	Specific Activity
47	131252	1382	1.2043
54	129715	1454	1.2694
52	134640	1456	1.2713
Ave.= 51	135652		Ave.= 1.25
	130315		σ = 0.04
	127471		
	Ave.= 131471		

methanol : acetic acid : deionized water = 5 : 1 : 5, and destained in 7% acetic acid and 7% methanol in deionized water by replacing the destaining solution three or four times over a period of 24 hours. The electrophoretic unit consisted of a Bio Rad-Model 150 A gel electrophoresis cell and a Bio-Rad Model 500 power supply.

SDS polyacrylamide disc-gels were made according to the procedure described by Clark and Switzer (1977), which is a modified version of Weber and Osborn (1969). All gels were 10% acrylamide. Electrophoresis was carried out in 0.072 M Na_2HPO_4, 0.0283 M NaH_2PO_4, and 0.0035 M SDS, at 8 milliamps per tube until the bromophenol blue tracking dye moved to within 1 cm of the end of the tube. The gels were stained in 0.025% Coomassie blue in 25% isopropanol and 10% acetic acid in deionized water for 24-36 hours and destained in 25% isopropanol and 10% acetic acid in deionized water. The electrophoretic unit used was the same one used for the non-SDS gels.

Study of the Km for RuBP

To 500 µg of the enzyme in 500 µL of TBMET buffer, 2 mL of TMET buffer were added. This incubation mixture was warmed in a 40° water bath for 10 min and then allowed to stand at room temperature for 45 min. The additional TBMET buffer was added (Table II) to maintain the same concentrations of all materials in the final reaction mixture, followed by 217.5 µL of 0.124 M $NaHCO_3$ and 0.0015 M $NaH^{14}CO_3$ in TME buffer, pH 8.0. The indicated µmoles of substrate (0.03875 M RuBP) were added (Table II) and at the various times shown in the same table after the addition of the substrate, 500 µL of this final reaction mixture were withdrawn and pipetted into a 7 mL scintillation vial which contained 0.2 mL of glacial acetic acid. It was then slowly

TABLE II

nmoles RUBP	µL TBMET additional	Reaction Times (sec)	$\frac{1}{V}$	$\frac{1}{[S]}$
8.27	130.31	15,30,45,60,75	0.0556	64,741
16.55	0	15,30,45,60,75	0.0281	32,371
33.09	255.46	15,30,45,60,75	0.0151	16,185
66.19	250.30	10,20,30,40,50	0.0088	8,093
132.38	239.97	10,20,30,40,50	0.0061	4,046

The lowest three substrate levels used a 1:100 dilution of the substrate (0.0388 M) so that these small amounts of substrate could be pipetted more accurately.

taken to dryness on a hot plate, followed by the additions of 0.5 mL deionized water and 6.5 mL liquid scintillation cocktail. Radioactive determinations were performed as described earlier.

pH Studies

The pH dependence of activation by CO_2 was obtained by enzymatic assay determinations in which the pH of the TMET incubation buffer was varied at constant ionic strength. The pH of the incubation buffer was adjusted from 6.0 to 7.2 in 0.3 unit steps, and with the addition of NaCl as shown in Table III, all buffers had the same ionic strength. Table IV shows how the ionic strengths of each species were calculated. Upon addition of $NaH^{14}CO_3$ and RuBP, the pH of the final reaction mixture was 7.55 ± 0.05. The assays were then performed as explained previously.

The dependence of activity on pH was determined from pH 7.5 to 9.0 by incubating and assaying the enzyme at higher pH values. The pH of the incubation buffer was adjusted from 7.5 to 9.0 in 0.3 unit steps and all buffers were maintained at the same ionic strength by the addition of NaCl (Table III). The assays were then performed as described previously. The pH values reported are those for dummy solutions prepared in exactly the same manner except for the presence of 0.0015 M $NaH^{14}CO_3$.

Two-Dimensional Polyacrylamide Gel Electrophoresis

Two-dimensional polyacrylamide gel electrophoresis of the purified enzyme was performed by Mrs. Dana Tyrell according to the procedure described by O'Farrell and O'Farrell (1977) with a few modifications. The isoelectric focusing gels were 17 mm long and the amount of protein used was 15-50 µg. The gels were electrophoresed for a total of 1700 volt hours. Since the enzyme was not radioactively labeled, the gels

TABLE III

NaCl Additions to Maintain Ionic Strength of Incubation Buffer

pH	Buffer Ionic Strength	g NaCl Added to 25 mL TMET Buffer to Give a Total Ionic Strength of 0.1353
6.0	0.1195	0.0231
6.3	0.1266	0.0127
6.6	0.1324	0.0043
6.9	0.1352	0.0039
7.2	0.1353	0
7.5	0.1323	0.0044
7.8	0.1264	0.0130
8.1	0.1185	0.0245
8.4	0.1104	0.0364
8.7	0.1039	0.0459
9.0	0.0995	0.0524

TABLE IV

Ionic Strength Calculations

BICARBONATE

$$\frac{[H_2CO_3]}{1 + \frac{K_1}{[H^+]}} (1)^2 + \frac{[H_2CO_3]\ K_1}{[H^+] + K_1} (2)^2$$

EDTA

$$\frac{[EDTA]}{1 + \frac{K_3}{[H^+]}} (2)^2 + \frac{[EDTA]\ K_3}{[H^+]\ K_3} (3)^2$$

RuBP

$$\frac{[RuBP]}{1 + \frac{K_2}{[H^+]} \quad \frac{K_2{}^2}{[H^+]}} (2)^2 + \frac{[RuBP]}{\frac{[H^+]}{K_2} + 1 + \frac{K_2}{[H^+]}} (3)^2 + \frac{[RuBP]}{\frac{[H^+]^2}{} + \frac{[H^+]}{K_2} + 1}$$

TRIS

$$pH = pK_a + \log \frac{[TRIS^+]}{[TRIS^\bullet]}$$

$Mg^{2+} = 0.04$

$Cl^- = 0.02$ from $MgCl_2$

$[Cl^-] = [TRIS^+]$ from TRIS·HCl

were stained for 10 min in 10% TCA, 50% methanol, and 0.25% Coomassie Brilliant Blue in deionized water and destained with 25% ethanol and 10% acetic acid in deionized water.

The second dimension gel was a 20 x 20 cm slab composed of 12% acrylamide rather than an acrylamide gradient and was run for approximately 17 hours at 5-7 milliamps per slab until the tracking dye moved to the end of the slab. Again, since the enzyme was not radioactively labeled, the slab was stained in 50% methanol, 12% acetic acid, and 0.2% Coomassie Brilliant Blue in deionized water for four hours, followed by destaining in 10% ethanol and 5% acetic acid in deionized water.

Protein Determinations

Protein concentration was determined in the "crude extract" by bringing it to 10% in TCA and allowing it to set overnight at 4°. Following centrifugation, the pellet was repeatedly washed with acetone until it became colorless, indicating the removal of most of the chlorophyll. The pellet was then dissolved in a minimum amount of 0.2 N NaOH and protein concentration was obtained using a Coomassie Brilliant Blue stain (Stoops and Mueller). The "redissolved 40% ammonium sulfate pellet" protein concentration was also obtained using the Coomassie Brilliant Blue stain. However, this was performed directly on the redissolved pellet since the residual ammonium sulfate did not appear to interfere with the assay.

Protein concentrations of the sucrose fractions and eluants from the DEAE-Sephadex column were obtained using a $\varepsilon^{1\%}_{1cm} = 16$ for the spinach enzyme, since Tomimatsu (1980) reported $\varepsilon^{1\%}_{1cm}$ ranging from 14.1 to 18.2 for all higher plant enzymes thus far investigated.

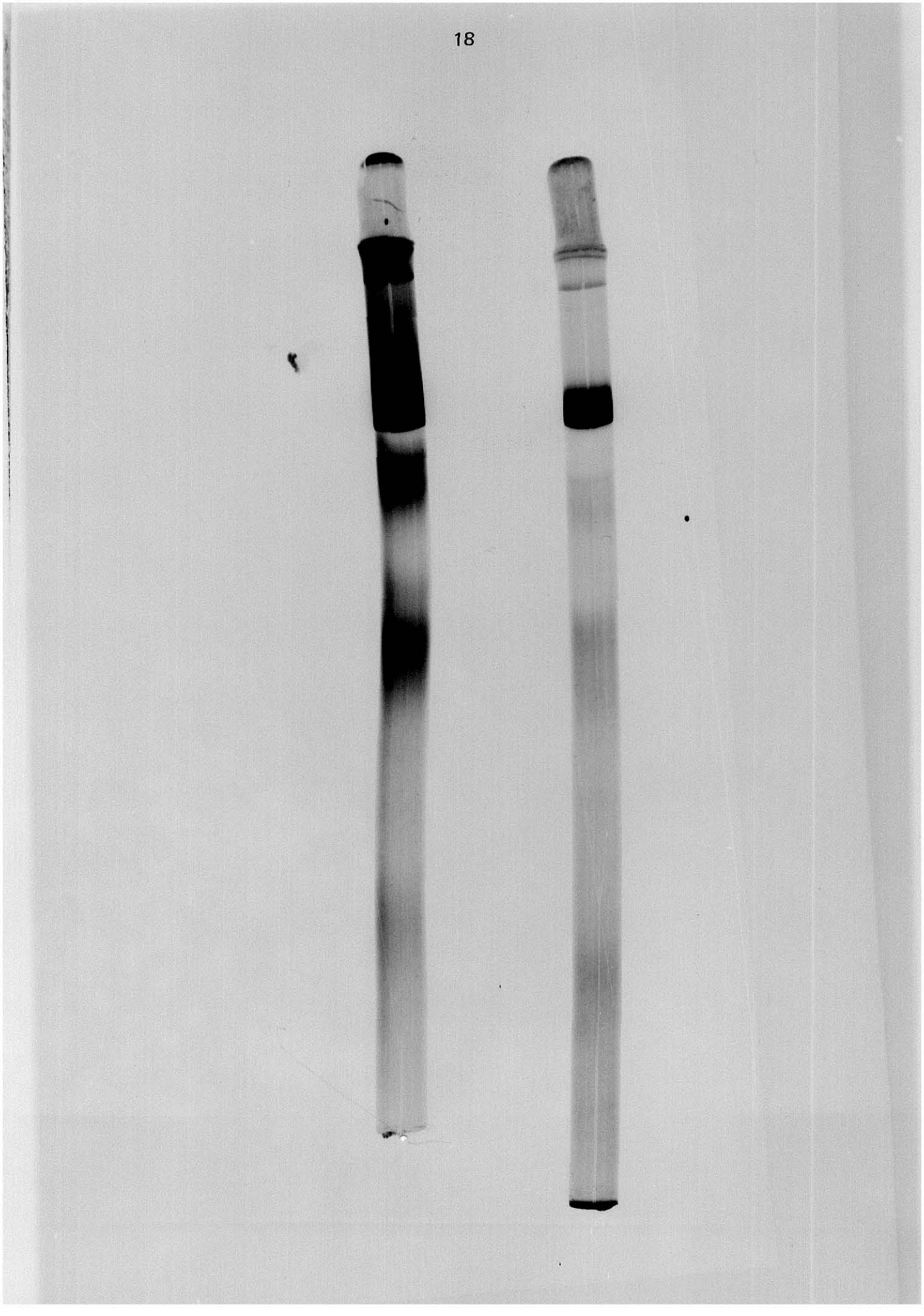
RESULTS

Initial Isolation and Purification Experiments

Initial experiments were conducted to determine the homogenization time required to obtain maximum protein. Using 20, 40, and 60 sec homogenizations on a Virtis 45 instrument, protein concentrations of 1.97, 0.62, and 1.3 mg/mL were obtained. Even though there was more scatter in the concentrations than is desirable, it did appear that there is no distinct advantage in homogenizing for over 20 sec. By visually noticing when the leaves are thoroughly chopped up, it was found that a homogenization time of 15-30 sec was adequate. A Virtis 45 homogenizer with a 5-blade system was chosen over a Waring blender because it was found that it did a better job of homogenizing the leaves in less time.

To determine at what percentage ammonium sulfate would precipitate RuBPCase, aliquots of the "crude extract" were brought to 10%, 20%, 30%, and 40% saturation with solid ammonium sulfate. Specific activities of the respective dialyzed supernatants were 0.24, 0.35, 0.39, and 0.09 μmoles $^{14}CO_2$ fixed/min/mg protein, revealing that RuBPCase was brought down largely in the 40% ammonium sulfate pellet. Furthermore, non-SDS polyacrylamide disc-gels revealed large amounts of RuBPCase in the 40% pellet, but very little in the 40% supernatant (Fig. 1). In addition, it was found that an ammonium sulfate cut was not needed, since the linear sucrose density gradient showed a great preponderance of RuBPCase (Fig. 2). Doing an ammonium sulfate precipitation to concentrate the

Figure 1. Non-SDS polyacrylamide disc-gel electrophoresis of the 40% supernatant and redissolved 40% pellet from ammonium sulfate precipitation. All gels were electrophoresed in 0.005 M Tris, 0.0384 M glycine, pH 8.3, at 3 milliamps per tube and consisted of a 7% acrylamide running gel and a 2.5% stacking gel. The left gel is the 40% supernatant; the right gel is the redissolved 40% pellet. Both gels were loaded with 60 µg of protein.



protein prior to application to the sucrose gradient rather than an ammonium sulfate cut saved 3-4 hours in the preparation. Non-SDS polyacrylamide gels also showed that very little protein was removed in the 10%, 20%, and 30% precipitations.

The "redissolved 40% ammonium sulfate pellet" contains an unknown amount of ammonium sulfate, and since sulfate is an inhibitor of the enzyme, the specific activity was no doubt higher than that reported (Table V). Paulsen and Lane (1966) reported a sulfate Ki of 8.1×10^{-3} M for spinach RuBPCase. Attempts to dialyze the "redissolved 40% ammonium sulfate pellet" against TBMESP buffer in the cold met with varying success, perhaps due to residual protease activity. Consequently, desalting efforts were discontinued as a routine procedure. Quick passage of the pellet through a small gel permeation column may be the best approach to solve this problem and has been used in other experiments.

DEAE-Sephadex G-25 column chromatography was used to further purify the enzyme. It was found that 0.25 M NaCl in TBMET buffer would elute the RuBPCase from the column. Typically, one-half the protein applied appeared in the eluting peak.

Overall, the purification described above can be carried out in about 36 hours with 20 hours of this time on the centrifuge for the linear sucrose density gradient.

Purification

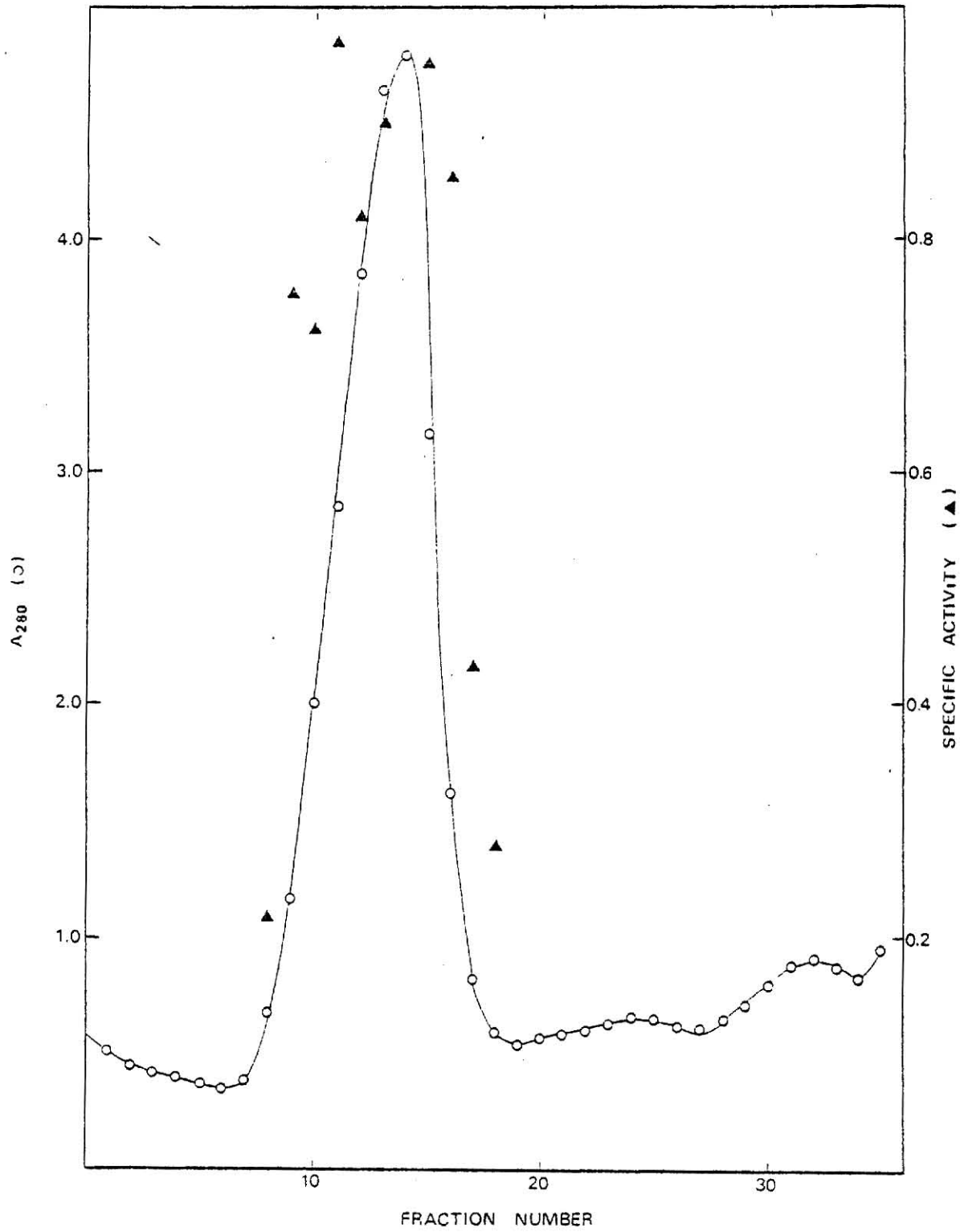
The degree of purification at each step in the scheme and overall yield of RuBPCase are given in Table V. Starting with 100 g of fresh leaf weight, a yield of 26 mg of enzyme was obtained with a 26-fold purification.

TABLE V

Purification of Ribulose-1,5-Bisphosphate Carboxylase

	Mg Protein	µmoles $^{14}CO_2$ fixed/ min/mg protein	Purification
Crude Extract	2455	0.05	1
Dissolved 40% Pellet from Ammonium Sulfate precipitation	107	0.54	10.9
Pooled Sucrose Gradient	59.7	0.97	19.5
0.25 M NaCl Eluant from DEAE-Sephadex Column Chromatography	26	1.29	26

Figure 2. Sucrose gradient elution profile of RuBPCase. One-mL fractions were collected from the bottom of the sucrose gradient tube. The carboxylase assay was performed as described in the experimental section. Tubes 9-15 were pooled and stored in 1 mL aliquots at -70°.


4.0
3.0
2.0
1.0
A_{280} (○)
0.8
0.6
0.4
0.2
SPECIFIC ACTIVITY (▲)
10
20
30
FRACTION NUMBER

The sucrose gradient profile (Fig. 2) obtained from the "redissolved 40% ammonium sulfate pellet" revealed one major and two minor peaks based on A_{280}, and from enzymatic assay determinations, the large peak was found to contain RuBPCase activity. There was scatter of the activities over the peak beyond that predicted from the reproducibility of the assay (Table I) due to the variable brown coloration, and hence quenching, which resulted from drying the assay samples in the presence of sucrose. Attempts to correct for quenching by spiking with $NaH^{14}CO_3$ met with little success, therefore, the data are presented without quench corrections. Tubes 9-15 were pooled and stored in the sucrose at -70° for future studies. Even though tubes 16 and 17 had relatively high specific activities, they were not pooled because of the possible presence of proteases which are usually much smaller molecules than RuBPCase and would be expected to migrate less in the sucrose gradient. This of course lowered the overall yield by about 25%. At this stage the pooled sucrose had a specific activity (SA) of 0.97 μmoles $^{14}CO_2$ fixed/min/mg protein and a A_{280}/A_{260} ratio of 1.99. Table VI reveals the A_{280}/A_{260} ratios of various sucrose fractions and their protein concentrations.

Prior to most studies on the enzyme it was further purified on a DEAE-Sephadex G-25 column and the enzyme was eluted with TBMET buffer containing 0.25 M NaCl. After this step the SA increased to 1.29 μmoles $^{14}CO_2$ fixed/min/mg protein. Other experiments have shown up to a two-fold increase in SA after the DEAE-Sephadex column step.

Homogeneity

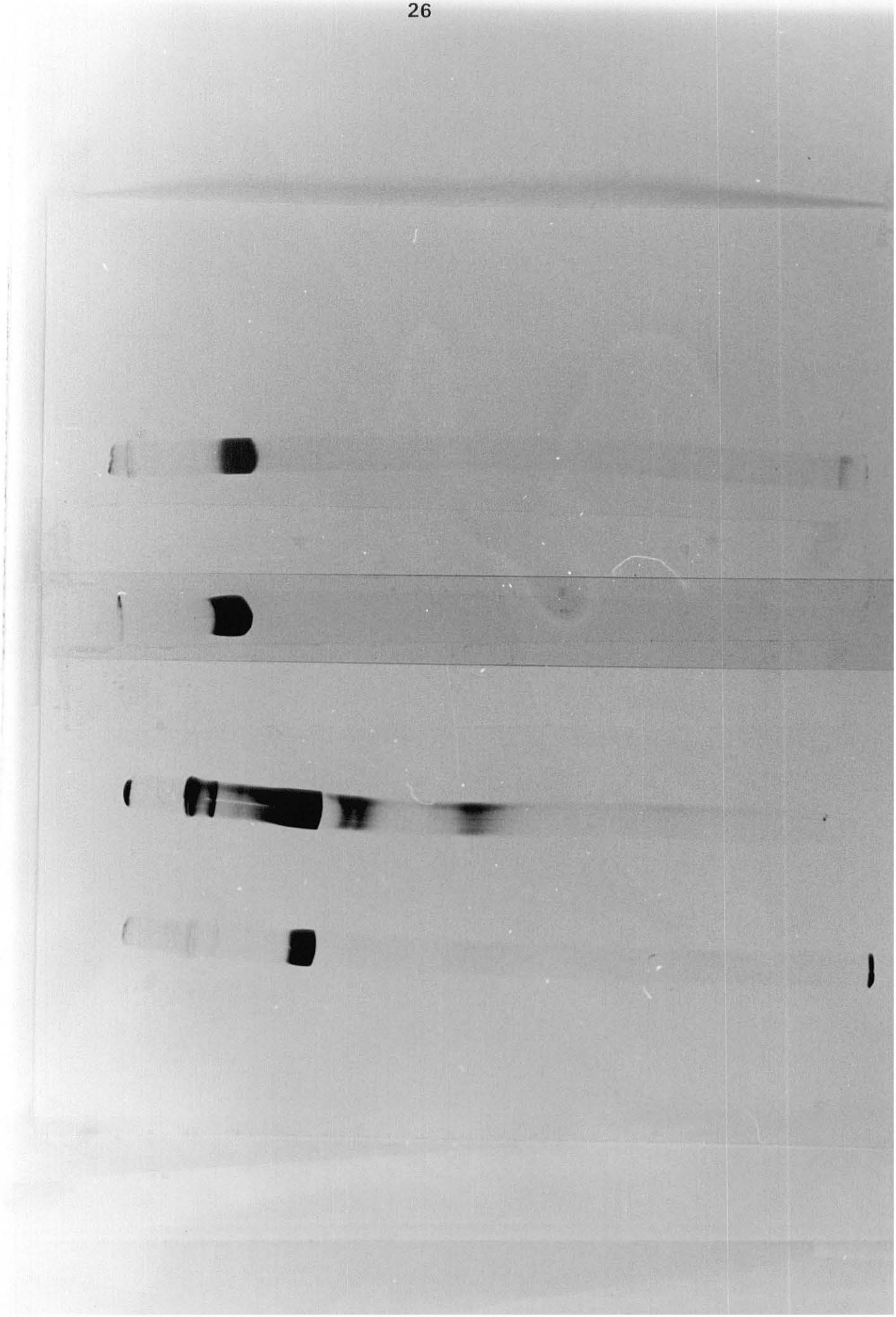
Homogeneity of the RuBPCase was determined at each step in the purification scheme using polyacrylamide disc-gel electrophoresis procedures. Non-SDS polyacrylamide disc-gels revealed a decreasing

TABLE VI

A_{280}/A_{260} Ratios of Various Sucrose Gradient Fractions

Fraction Number	A_{280}/A_{260}	RuBPCase Conc. (mg/mL)
4	0.7465	
9	1.650	0.52
10	1.905	1.08
11	1.832	1.43
13	1.940	2.42
16	1.924	1.11
25	1.323	

Figure 3. Non-SDS polyacrylamide disc-gels at various steps in the purification scheme. Electrophoresis was performed as described in the legend to Fig. 1. From left to right: crude extract (15 µg), redissolved 40% pellet from ammonium sulfate precipitation (60 µg), pooled sucrose gradient (60 µg), and 0.25 M NaCl eluant from DEAE-Sephadex column chromatography (60 µg).



number of proteins proceeding through the scheme (Fig. 3). To visualize these contaminants, however, it was necessary to use very heavily loaded gels. After DEAE-Sephadex column chromatography, there were only two bands, representing RuBPCase and a higher molecular weight protein at the origin. The higher molecular weight band may well be polymerized RuBPCase since the spinach enzyme is known to produce aggregates upon storage at -20° (Andrews et al, 1973), as does the tobacco enzyme (Gray et al, 1980). The R_f value corresponded to 0.15, which is almost identical to that of spinach RuBPCase (Sigma Chemical Co.), that being 0.14. Since the molecular weight of the spinach enzyme is 550,000 daltons, the native comfrey enzyme must be of very similar size.

Molecular Weights of the Large and Small Subunits

Heavily loaded SDS polyacrylamide disc-gels of the pooled sucrose gradient revealed two dark bands, representing the large and small subunits, plus several minor bands (Fig. 4). At normal loading (2-10 µg/gel), only the two types of subunits were visible. Overall, the enzyme appeared to be reasonably pure at this step. The molecular weights of the large and small subunits determined by SDS polyacrylamide disc-gel electrophoresis were 50,000 and 12,200 daltons, respectively (Fig. 5).

Time Course of the RuBP Carboxylase Assay and the Effect of Enzyme/Substrate Molar Ratios on Specific Activity

The time course of activity under the carboxylase assay conditions described previously and the effects of enzyme/substrate molar ratios on specific activity were studied during the initial characterization of the enzyme. The data from the time course study (Fig. 5) were linear through 80 sec. Therefore, all routine enzymatic assays were allowed to react for 60 sec. For maximum activity, it appears from Fig. 6 that a enzyme/

Figure 4. SDS polyacrylamide disc-gel electrophoresis of the pooled sucrose gradient. Electrophoresis was performed in 0.072 M Na_2HPO_4, 0.028 M NaH_2PO_4, and 0.0035 M SDS at 8 milliamps per tube. The gel represents 40 µg of enzyme.

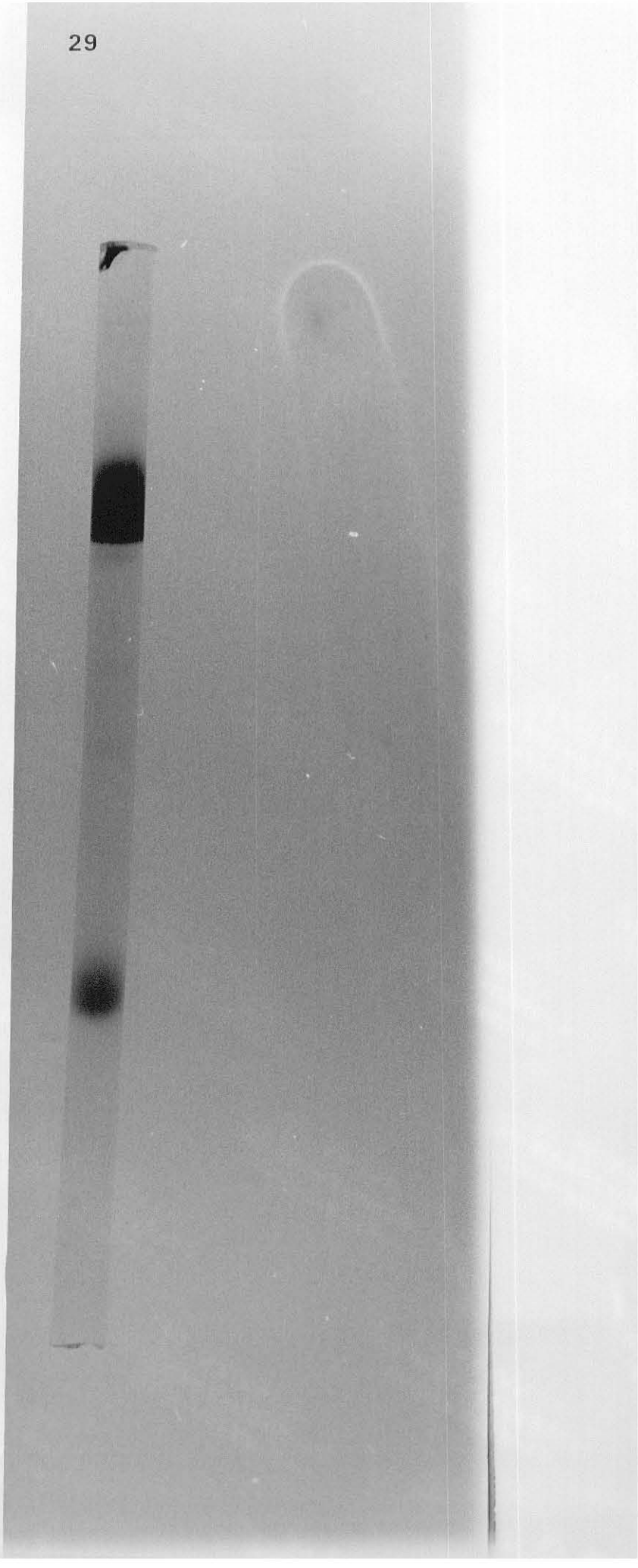


Figure 5. Molecular weight determination of the large and small subunits by SDS polyacrylamide disc-gel electrophoresis. Electrophoresis was performed as described in legend to Fig. 4. The amount of each protein applied was 2 µg. From left to right:

Gels 1 and 2: standard proteins, from top to bottom: BSA, alcohol dehydrogenase, chymotrypsinogen, and lysozyme.

Gels 3 and 4: RuBPCase

Gels 5 and 6: Standard proteins and RuBPCase

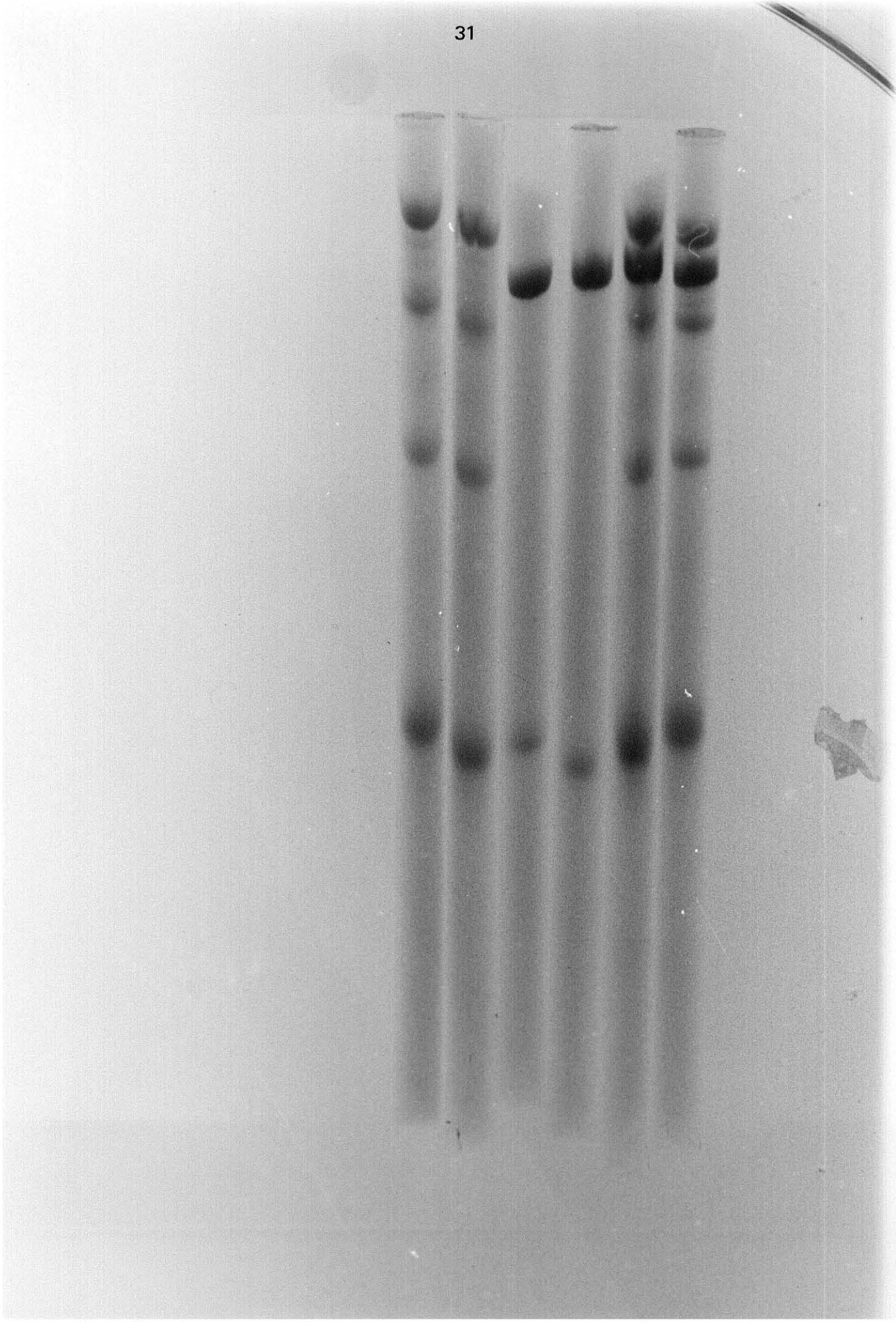


Figure 6. Time course of the RuBP carboxylase assay. The enzymatic assay was the standard assay described in the experimental section, except the time of the reaction was varied from 10-90 sec.

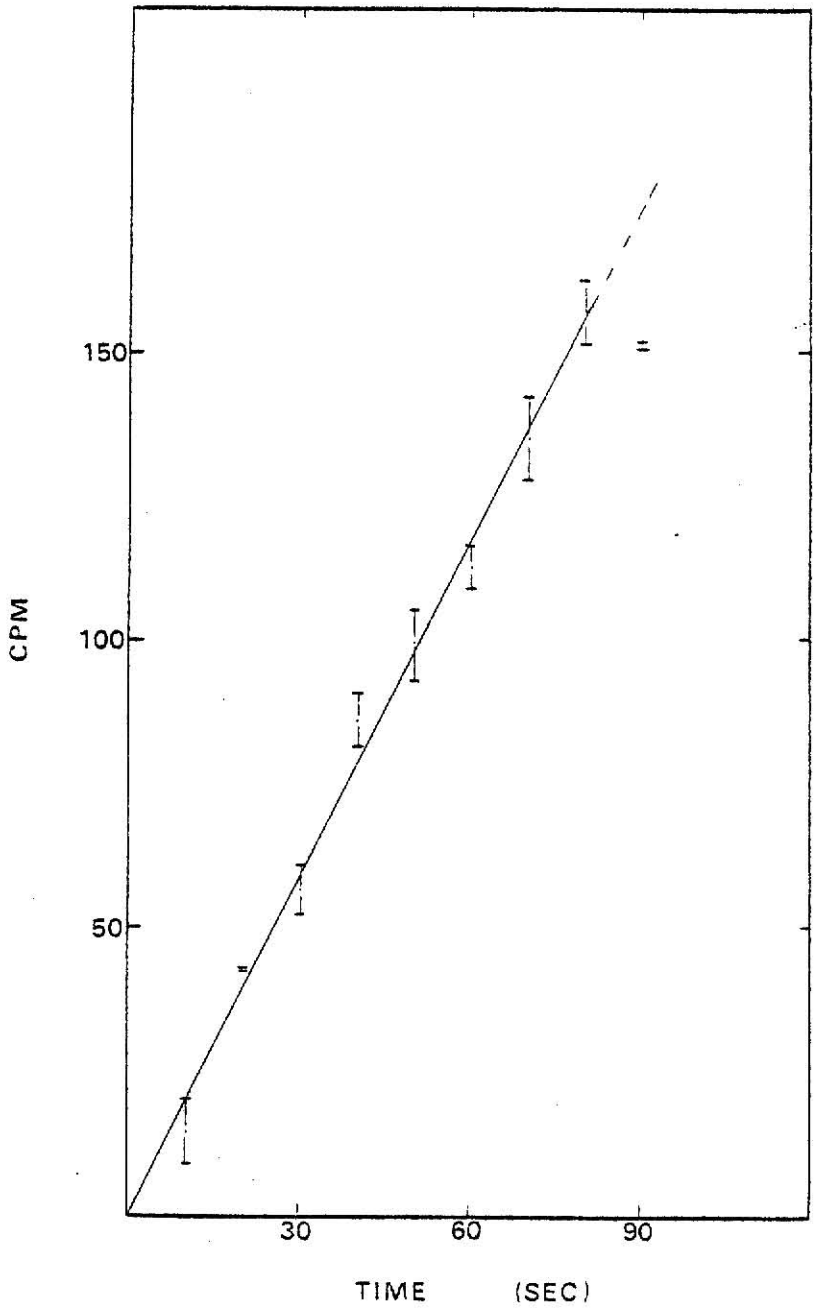
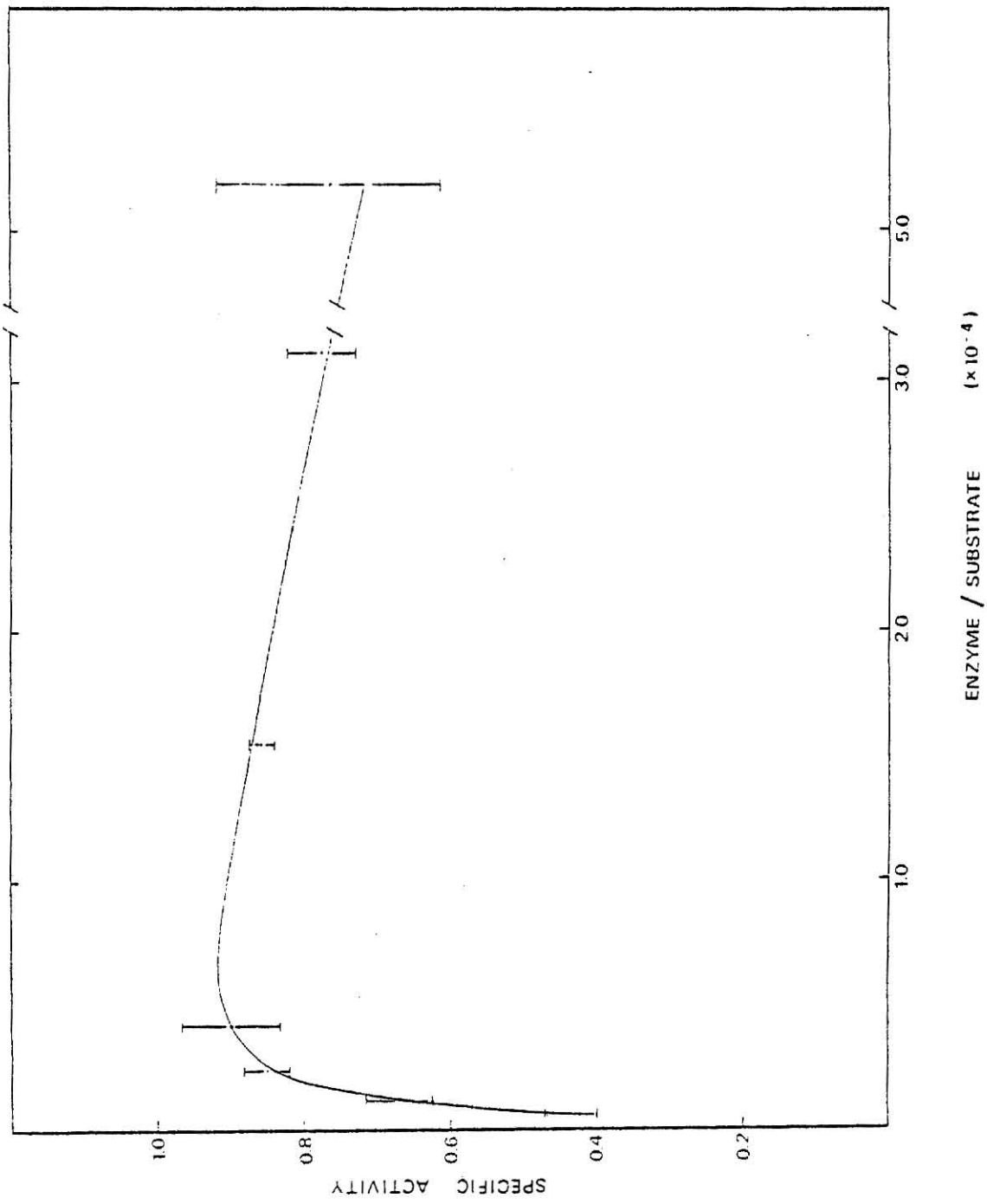

150
100
50
CPM
30
60
90
TIME (SEC)

Figure 7. Effect of varying the enzyme/substrate molar ratio on specific activity. The enzymatic assay was the standard assay described in the experimental section, except the amount of enzyme varied from 0.5 to 60 µg.


SPECIFIC ACTIVITY
1.0
0.8
0.6
0.4
0.2
1.0
2.0
3.0
5.0
ENZYME / SUBSTRATE (×10⁻⁴)

substrate molar ratio of 6×10^{-5} should be maintained, which corresponds to 20 µg of protein (MW = 500,000 daltons) when 0.388 µmoles of the substrate, RuBP, were used. Table VII shows the amount of protein used for the particular enzyme/substrate molar ratio.

Km for RuBP

The kM for RuBP was determined from initial slopes of activity vs time data collected for enzyme/substrate molar ratios varying from 4.69×10^{-2} to 1.47×10^{-3} and using the Lineweaver-Burk plot as shown in Fig. 7. Table II reveals pertinent information concerning variables for each substrate level. A Km of 4.1×10^{-4} M is within the range of values reported for the spinach enzyme (Kawashima and Wildman, 1970; Jensen and Bahr, 1977)

pH Studies

In higher plants, algae, and the bacterial RuBPCases, CO_2 is both an activator as well as a substrate. Accordingly, the pH dependence of both steps have been investigated. For the dependence of activation on pH, the incubation buffer was adjusted from 6.0 to 7.2 in 0.3 unit steps and the assays performed in the standard mixture which had been adjusted to give a final pH of 7.5 to 7.6 upon addition of $NaH^{14}CO_3$ and RuBP (all buffers had the same ionic strength). To 400 µL of TMET buffer, 20 µg of the pooled enzyme from the sucrose gradient were added and incubated for 45 min which corresponded to 10 mM Mg^{2+} and 10 mM $NaHCO_3$. This long time was chosen to assure complete activation at each of the selected pH values. The vials were tightly sealed throughout the experiment. Fig. 8 shows the change in activity with pH over the region specified which indicated that activation was probably maximal by about pH 7.5.

TABLE VII

Specific Activities for Various Enzyme/Substrate Molar Ratios

µg Protein per Assay	Specific Activity	Enzyme/Substrate Molar Ratio
1.175	0.44	6.09×10^{-6}
2.35	0.67	1.22×10^{-5}
4.70	0.85	2.44×10^{-5}
10.00	0.90	5.18×10^{-5}
30.00	0.86	1.55×10^{-4}
60.00	0.77	3.11×10^{-4}
100.00	0.76	5.18×10^{-4}

Figure 8. Lineweaver-Burk plot for the Km of RuBP. V is the slope of CPM vs time data collected from various enzyme/substrate ratios.

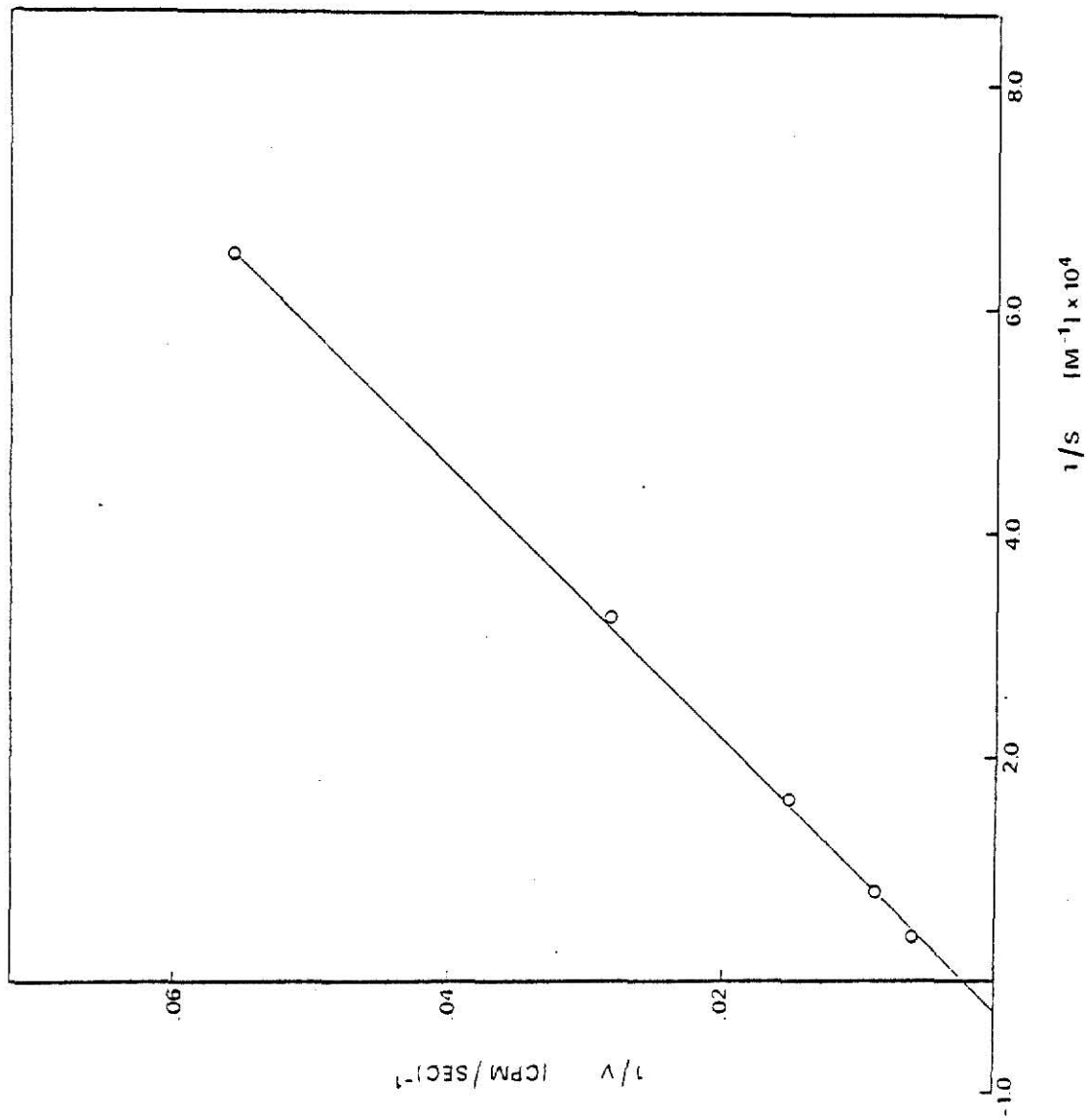
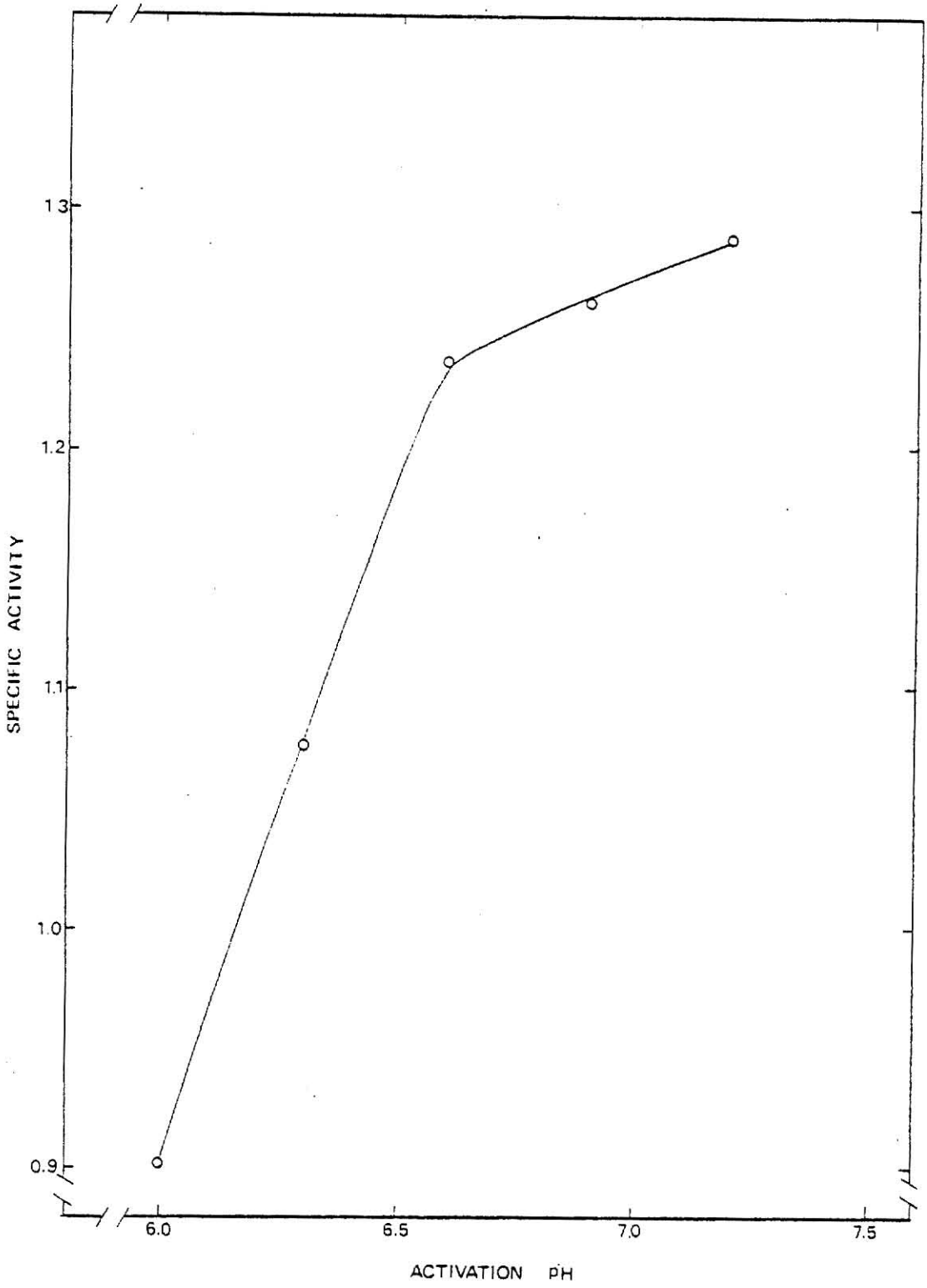

1/V (CPM/SEC)⁻¹
.06
.04
.02
-1.0
2.0
4.0
6.0
8.0
1/S [M⁻¹] x 10⁴

Figure 9. Effect of activation pH on specific activity. The enzymatic assay was the standard assay described in the experimental section, except the pH of the incubation buffer was varied at constant ionic strength.


SPECIFIC ACTIVITY
1.3
1.2
1.1
1.0
0.9
6.0
6.5
7.0
7.5
ACTIVATION pH

Alternatively, the dependence of activity on pH was determined from pH 7.5 to 9.0 by incubating and assaying the enzyme at higher pH values (again, all buffers had the same ionic strength). This method was anticipated to yield maximal activation over the pH interval since activation appears to occur at lysine residues which presumably remain reactive as the pH is increased. Fig. 9 reveals that maximal activity occurred near pH 7.5, which was lower than that reported for other higher plant enzymes by 0.3 to 0.7 pH units (Badger, 1980; Lorimer, Badger, and Andrews, 1976).

Two-Dimensional Polyacrylamide Gel Electrophoresis

Isoelectric focusing in the first direction to non-equilibrium of the DEAE-Sephadex purified enzyme in 9 M urea revealed six bands, with pI's of 5.76, 5.80, 5.90, 6.00, 6.59, and 7.03 (Fig. 10). It appeared that the four most acidic bands were the large subunits, but as shown by their stain intensities, they were not present in equal concentrations. The two most basic bands were most likely the small subunits, but on this experiment alone it could not be ruled out that some of the bands arose from extraneous proteins. Evidence that these multiple bands arose solely from RuBPCase was obtained when a gel with 0.4% SDS was run in the second dimension. The four bands which corresponded to the four different large subunits ran the same distance in the second dimension, indicating very similar molecular weights (Fig. 11). The same was true for the two different small subunits, in that they traveled the same distance in the second dimension, signifying similar molecular weights. It is important to point out that the enzyme used for the isoelectric focusing gel (Fig. 10) was isolated in the absence of PMSF, a serine protease inhibitor, while the gel run in the second

Figure 10. Effect of pH of the final reaction mixture on specific activity. The enzyme was incubated and assayed at various pH values which were maintained at constant ionic strength.

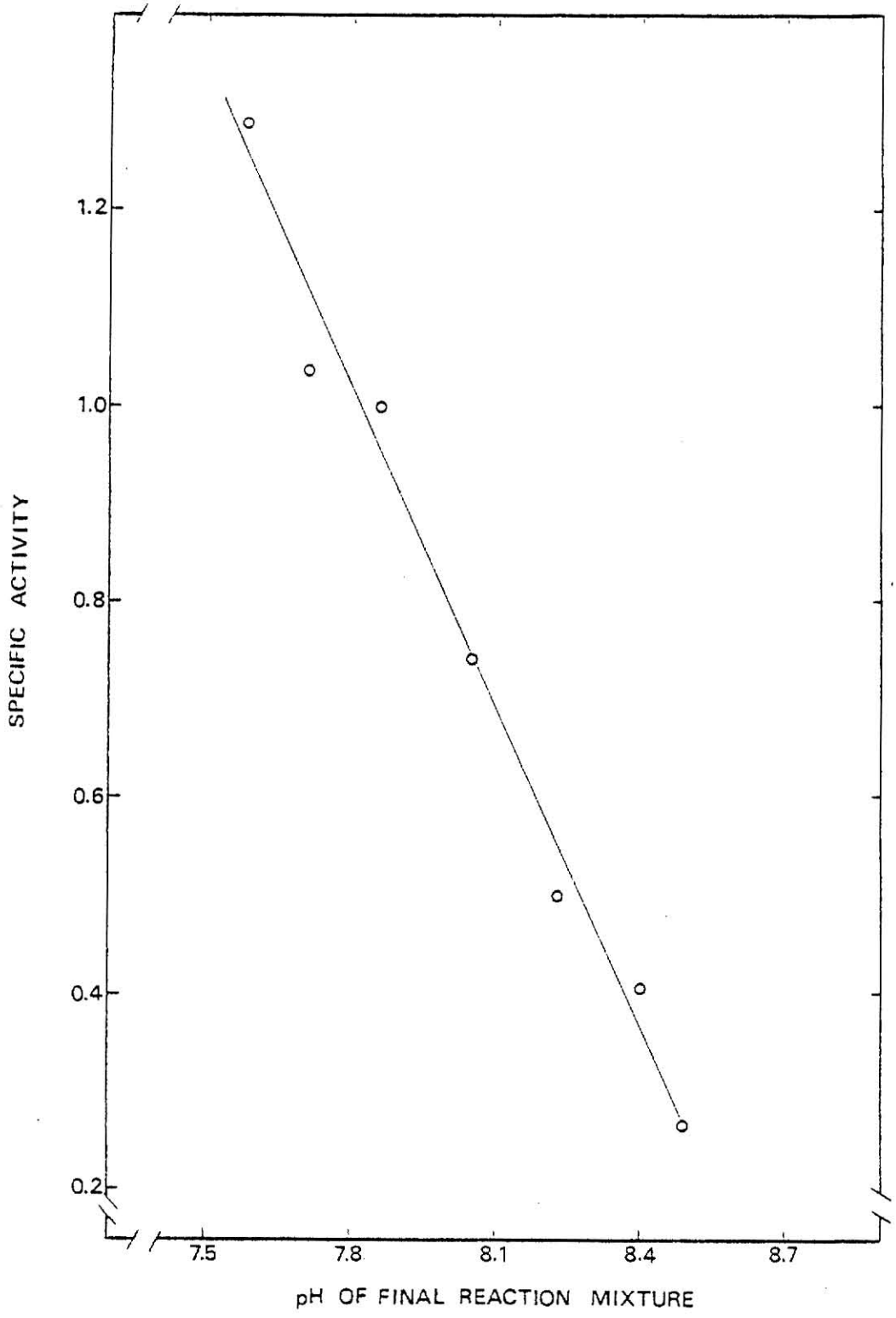

SPECIFIC ACTIVITY
1.2
1.0
0.8
0.6
0.4
0.2
7.5
7.8
8.1
8.4
8.7
pH OF FINAL REACTION MIXTURE

Figure 11. Isoelectric focusing gel of purified RuBPCase.

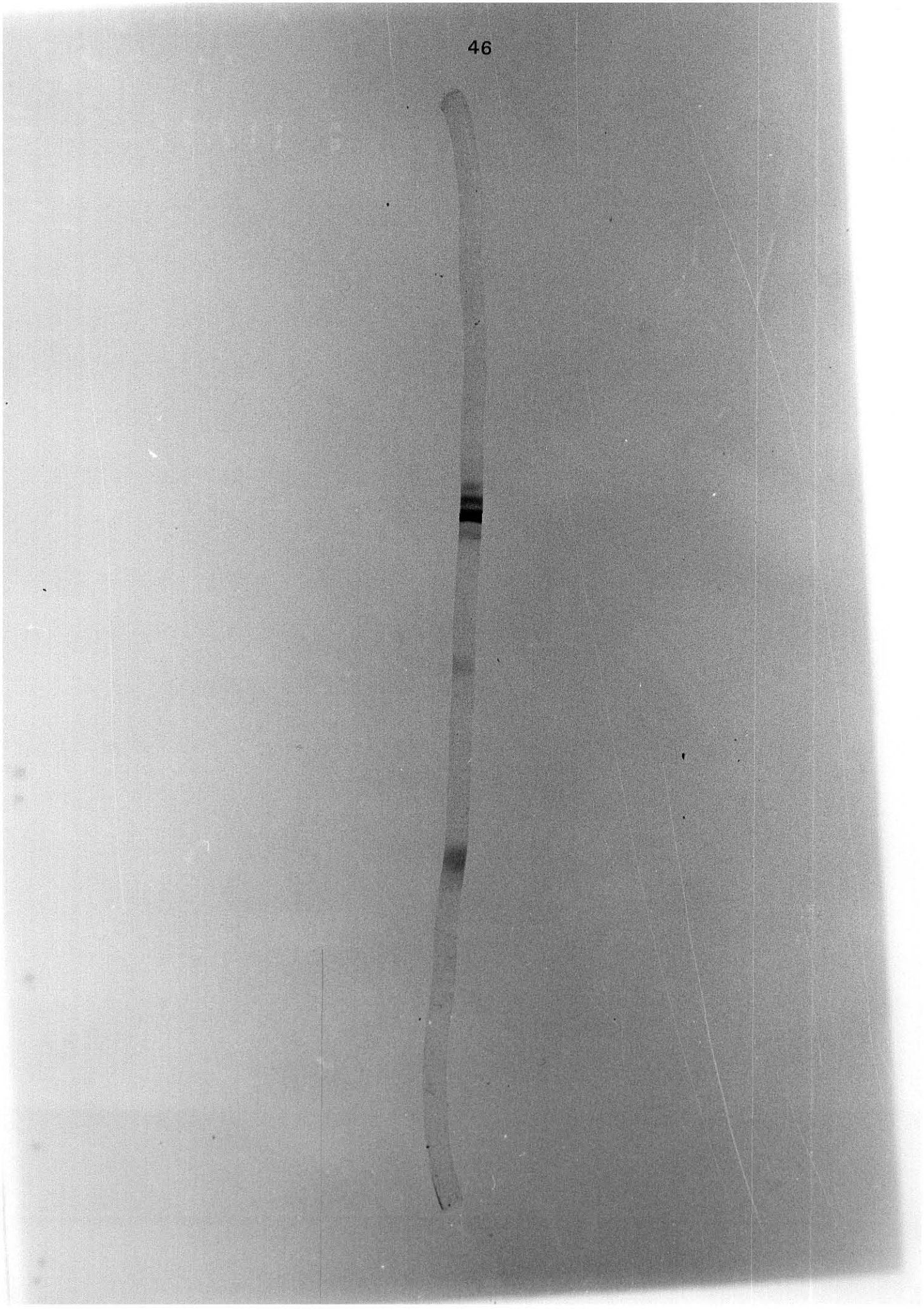
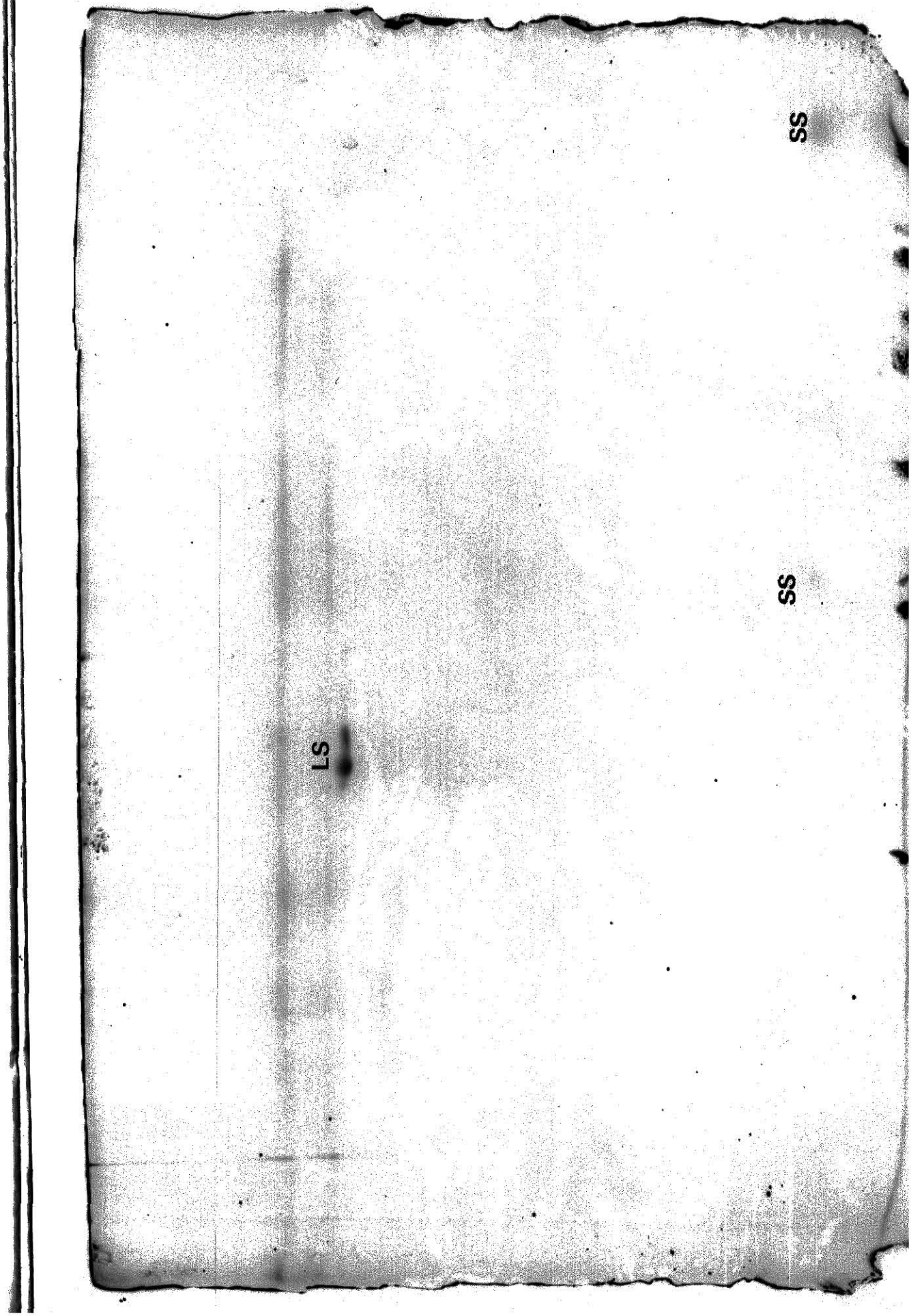


Figure 12. Second dimension polyacrylamide gel electrophoresis of purified RuBPCase.


SS
SS
LS

dimension used enzyme that had been isolated in the presence of PMSF. No differences in the number or intensities of the isoelectric focusing bands were detectable.

Effect of NaCl on Activity

The effect of NaCl on activity was determined to see if the enzyme was inhibited by 0.25 M NaCl when eluted off the DEAE-Sephadex column. The incubation buffer was made 0.0, 0.2, 0.4, 0.6, and 0.9 M in NaCl at pH 8.0 and the carboxylase assay was then performed as described previously using enzyme from the pooled sucrose. As Fig. 12 reveals, specific activity appeared to drop off cooperatively with increasing NaCl concentration or ionic strength. The ionic strengths reported in Fig. 12 are those for the final reaction conditions. This dramatic effect probably does not mitigate our results with the enzyme assays performed with enzyme solutions off the DEAE-Sephadex column. For these experiments, 50 μL or less was used in order to have 20 μg of protein for the reaction. However, when 50 μL of the enzyme in 0.25 M NaCl was added to the incubation buffer (400 μL of TMET and 50 μL of TBMET buffers), it was diluted by a factor of 10, thus bringing the NaCl concentration down to 0.025 M. When the ionic strength of the 0.025 M NaCl was calculated, the specific activity corresponding to this ionic strength was approximately one per cent less as compared to the incubation buffer which had no NaCl.

Effect of 3-PGA on Activity

To determine if there is product inhibition, 3-PGA was added to the incubation mixture at concentrations of 0.1, 1.0, and 10.0 times the concentration of active sites on the enzyme. Table VIII reveals that at a concentration of 10 times the concentration of active sites,

Figure 13. Effect of NaCl on specific activity. The enzymatic assay was the standard assay described in the experimental section, except for the presence of NaCl in concentrations ranging from 0.0 to 0.9 M in the incubation buffer.

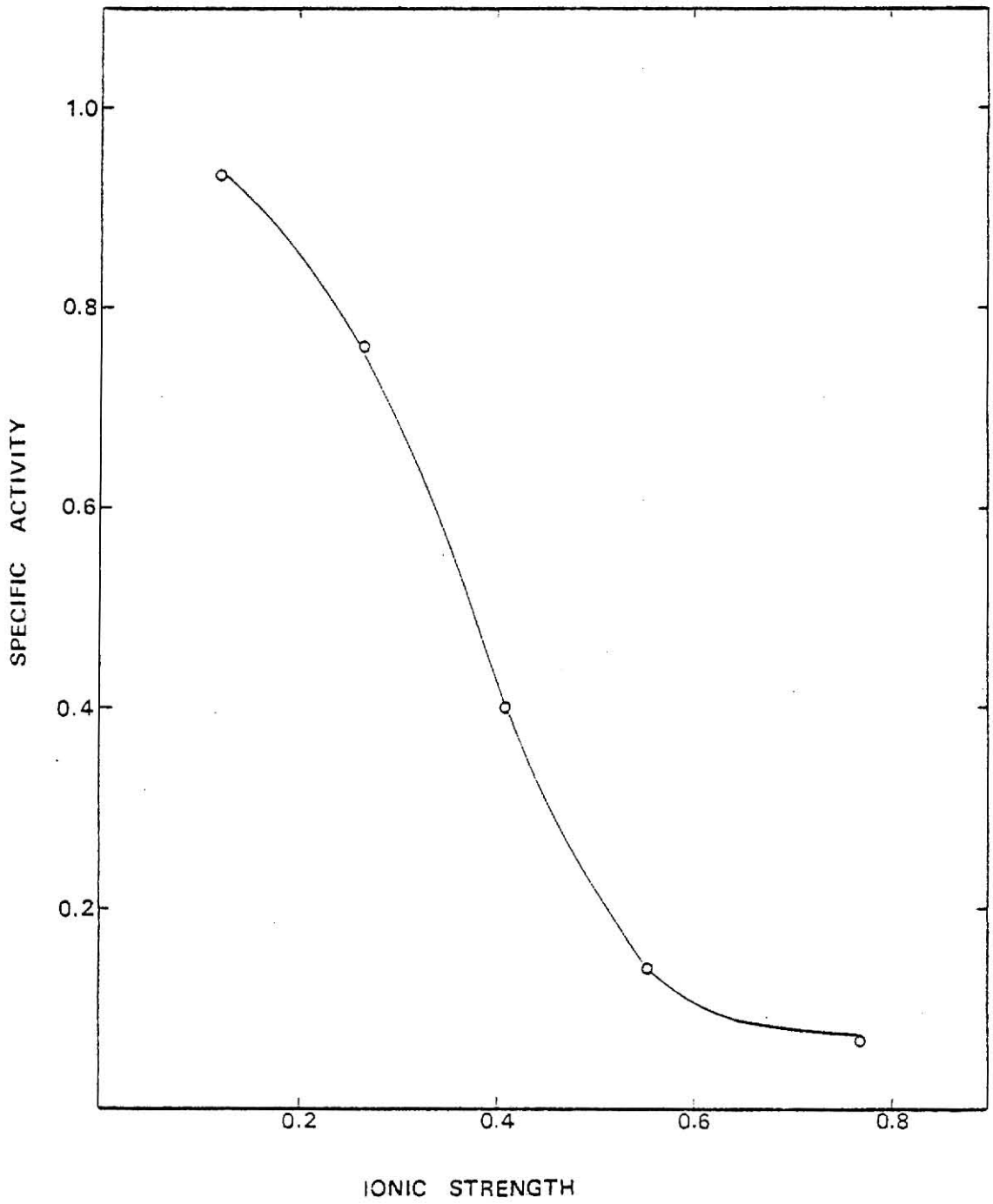

1.0
0.8
0.6
0.4
0.2
SPECIFIC ACTIVITY
0.2
0.4
0.6
0.8
IONIC STRENGTH

TABLE VIII

Inhibition of RUBPCase Activity by 3-PGA

$\frac{[PGA]}{[Active\ Site]}$ Molar Ratio	Specific Activity	% Inhibition
Control	1.92	
0.1 PGA	1.86	3%
1.0 PGA	1.69	12%
10.0 PGA	1.28	33%

3-PGA inhibited the carboxylase reaction by 33%. It should be pointed out that in one minute at an enzyme/substrate molar ratio of 6×10^{-5} in the standard carboxylase assay, the amount of product formed is 95 times the concentration of the active sites on the enzyme. However, less inhibition is seen (Fig. 6) than predicted by the above experiments. When 3-PGA was incubated with the enzyme, binding occurred. After 45 min of incubation, a 10,000-fold excess of RuBP over active sites was added, followed by the addition of $NaH^{14}CO_3$ 1 minute later (this is just the opposite order in a standard carboxylase assay). The reaction was stopped 1 min after the addition of the $NaH^{14}CO_3$. Therefore, RuBP had two minutes to chase the inhibitor from the enzyme's active sites. Since a noticeable inhibition is observed, but which was less than that predicted from the data of Table VIII, this would indicate that once the inhibitor binds, its release was quite slow. Conversely, in a standard carboxylase assay, the product formed had to chase the RuBP from the enzyme's active sites. The release appeared slow in this direction as well, since there is only a small amount of inhibition even at high enzyme/substrate molar ratios as shown in Fig. 6, where relatively large amounts of 3-PGA are formed. An alternate explanation could be that 3-PGA acted as an allosteric inhibitor which interfered with activation during incubation and deactivated the enzyme during the assay.

DISCUSSION

Initial Isolation and Purification Experiments

Initial purification attempts involved using Sepharose-6B column chromatography (Lorimer, Badger, and Andrews, 1976) at room temperature to purify crude spinach RuBPCase (Sigma Chemical Co.). This met with little success, as the activity of the pooled fraction was much lower compared to the activity obtained when a 0.2-0.8 M linear sucrose density gradient was substituted for this column step. Therefore, during purification of the comfrey enzyme, the Sepharose-6B column chromatography step after the ammonium sulfate precipitation was eliminated and a linear sucrose density gradient was used in its place.

The first several preparations using comfrey as the source involved dialyzing the "redissolved 40% ammonium sulfate pellet" at 4° against the incubation buffer prior to loading on the sucrose gradient to prevent destabilization of the gradient by high salt concentrations. Fearing that proteases might damage the enzyme during this 12 hour dialysis step, one preparation was performed in which the "redissolved 40% ammonium sulfate pellet" was applied directly to the sucrose gradient without dialyzing. It was found this procedure had no effect on the A_{280} profile from the sucrose gradient and that the activities were slightly improved. Therefore, this dialysis step was not performed in future preparations.

Another dialysis step was removed from the preparation when it was found that the pooled sucrose fraction could be applied directly to the

DEAE-Sephadex column. This was initiated when it was found that upon dialysis of the pooled sucrose fraction, a little activity was lost, most likely again due to protease activity. Therefore, this preparation involves no dialysis steps, whereas most other procedures from various investigators involved several dialysis steps. This not only helped preserve the enzyme before purification was complete, but reduced the time required from about three days to 36 hours.

After the sucrose gradient step, the enzyme was applied to a DEAE-Sephadex column and eluted with 0.3 M NaCl in TBMET buffer. The initial column run was performed at 4° and it was found that A_{280} readings were obtained up to the point where 0.3 M NaCl was coming through the column, at which point A_{280} readings could not be obtained, even upon dilution of the fractions with water. Carboxylase assays were performed on those fractions which would not give an A_{280} reading, but showed no activity even when activated for 45 min. We knew that the RuBPCase was eluted off the column with 0.3 M NaCl, because previous experiments at room temperature and with a smaller column gave A_{280} readings plus excellent activities. Knowing that the capacity of the column had not been overloaded, it was concluded that a combination of high salt concentration and cold in some way had inactivated the enzyme. A previous study on the tobacco enzyme (Kawashima, Singh, and Wildman, 1971) revealed that it also was cold inactivated but could be reactivated by incubating in a 50° water bath for 20 min. This method was tried, but no activity was obtained. It was therefore decided to store the enzyme as the pooled sucrose fraction at -70° and to run the enzyme through a 1 x 3 cm DEAE-Sephadex G-25 column at room temperature when needed for the various studies. Also the NaCl concentration was lowered to 0.25 M,

as it was determined that this was the lowest concentration that would elute the enzyme.

Although it was found that the levels of ammonium sulfate present in the redissolved 40% pellet did not affect the sucrose gradient, it does spread the protein bands on non-SDS polyacrylamide disc-gels. To alleviate this problem, the 40% supernatant and the 40% redissolved pellet from the ammonium sulfate precipitation were passed through a 0.5 x 7.0 cm P-30 column to reduce the ammonium sulfate concentrations to levels where no adverse effects were detectable. The presence of sucrose had no effect on either SDS or non-SDS polyacrylamide disc-gels.

Specific Activity and A_{280}/A_{260} Ratio of Purified RuBPCase

The purification scheme developed for RuBPCase from comfrey led to an essentially homogeneous enzyme preparation as judged by SDS and non-SDS polyacrylamide disc-gel electrophoresis. A specific activity of nearly 1.3 μmoles $^{14}CO_2$ fixed/min/mg protein was obtained which was comparable to the activities of purified spinach (Paulsen and Lane, 1966), tobacco (Chollet and Anderson, 1976), and bacterial (Givan and Criddle, 1972) enzymes. The A_{280}/A_{260} ratio of the purified enzyme was 1.99, which was a higher ratio than any reported for the spinach enzyme (Ryan and Tolbert, 1974; Paulsen and Lane, 1966).

Molecular Weight of the Native Enzyme and the Large and Small Subunits

The molecular weight estimate obtained by comparison to the spinach enzyme using non-SDS polyacrylamide disc-gels showed a similar native molecular weight to that of spinach and to the molecular weights of other higher plant enzymes, which range from 559,000 to 600,000 daltons (Kawashima and Wildman, 1970). SDS polyacrylamide disc-gels revealed molecular weights of 50,000 and 12,200 daltons for the large and small

subunits, respectively, which was compatible with an L_8S_8 structure. Than an L_8S_8 structure could exist for RuBPCase seems to have been verified by X-ray diffraction studies (Baker et al, 1975; Baker et al, 1977) and electron micrograph (Bovien and Mayer, 1978) investigations. All higher plants that have been investigated have this L_8S_8 structure along with the green algae Chlamydomonas reinhardi and Chorella ellipsoidae (Kawashima and Wildman, 1970) and also Chromatium D (Takabe and Akazawa, 1975). In more primitive blue green algae (Tabita et al, 1975), RuBPCase exists as a L_8 structure apparently devoid of the small subunit. Similarly, the photosynthetic bacterium Rhodospirillum rubrum (Tabita and McFadden, 1974) exists a dimer of the large subunits only.

Time Course of the RuBP Carboxylase Assay and the Effect of Enzyme/Substrate Molar Ratios on Specific Activity

One of the initial experiments performed was a time course study of the RuBP carboxylase assay, and it was found that the data were linear through 80 sec. Whitman, Martin, and Tabita (1977) performed a time course experiment on the enzyme from Rhodospirillum rubrum through 75 sec with similar assay conditions and also found a linear time course. Consequently, all routine assays were carried out for 60 sec. It was also found that an enzyme/substrate molar ratio of 6×10^{-5} should be maintained in the carboxylase assays for maximum activity, which corresponds to 20 µg of protein in the standard mixture.

Km for RuBP

A Km of 4.1×10^{-4} M was determined for RuBP, which lies almost in the middle of the range of 1.0×10^{-4} to 7.0×10^{-4} M reported from other plant sources (Kawashima and Wildman, 1970). The assays for the Km of RuBP from comfrey were performed at pH 8.0, but as shown in Fig. 8,

the pH optimum was 7.5. Therefore, this may mean that the increase in activity noted between pH 8.0 and 7.5 was primarily due to a decrease in the Km for RuBP which might bring it to the lower end of the Km range for higher plant enzymes.

Comparison of pH Optimum to Other Sources

The effects of pH on the activation and catalysis of RuBPCase have been reported from various sources. Our studies revealed that activation was maximal by pH 7.5, which is slightly less than the values thus far reported for the enzyme from other sources. Buchanan and Schurmann (1973) found the pH optimum for activation was approximately 7.8 for incubations at both 1 mM and 20 mM Mg^{2+} for the spinach enzyme. Lorimer, Badger, and Andrews (1976) reported a pH optimum for activation of 8.5 in the presence of 20 mM Mg^{2+} from spinach. For the enzyme from tobacco leaves, a pH value of 8.0 was found for activation (Chollet and Anderson, 1976).

Two-Dimensional Polyacrylamide Gel Electrophoresis

The nature of the comfrey RuBPCase subunits were investigated by two-dimensional gel electrophoresis and the data suggested the large subunit was composed of four different types while the small subunit was composed of two types. Spinach enzyme revealed a similar pattern upon isoelectric focusing (Rjada, Johal, and Chollet, 1980), except there were two additional faint bands a short distance away on either side of the four distinct bands representing the large subunits. Whereas the pI's of the large subunits from comfrey ranged from 5.76 to 6.00, the spinach had pI's in the pH 4 range for the large subunits. However personal communications with one of these authors suggested that there may be an error in their pH measurements. Spinach also revealed two types of small subunits, but were reported to be more acidic than the

small subunits from comfrey.

Effect of NaCl on Specific Activity

It was found that increasing concentrations of NaCl decreased the specific activity of RuBPCase. Since Fig. 12 is sigmoidal, this could signify cooperative dissociation. However, it could merely mean that high Cl^- concentrations are inhibitory. To verify the first assumption, the experiment should be performed with other larger anions which are too big to fit into the active site to see if they have the same effect as NaCl. If they do, this would be an excellent method to dissociate the subunits.

Effect of 3-PGA on Specific Activity

Table VIII reveals that 3-PGA inhibited the carboxylase reaction when present in the incubation mixture. However, in a standard carboxylase assay, even though the concentration of the product formed is 95 times the concentration of the active sites on the enzyme after 60 sec, there was only a small amount of inhibition (Fig. 7). This could have been due to the fact that RuBP did not have to chase the 3-PGA from the active sites as it had to when the product was present in the incubation mixture. Consequently, since the RuBP present virtually saturated the active sites (a 10,000/1 molar ratio of RuBP/active sites), and since 3-PGA must bind less tightly than RuBP, there was only slight inhibition when 3-PGA was introduced after the RuBP. Again, considering the inhibition experiments where 3-PGA was added to the incubation mixture, this implies that the release of 3-PGA is quite slow. In that case, there were two minutes for the overwhelming excess of RuBP to chase the previously bound product, but yet a higher degree of inhibition was observed. Another explanation could be that 3-PGA may be an allosteric

inhibitor of RuBPCase and that RuBP can displace it from the proposed second site.

CONCLUSIONS

1. A purification scheme has been developed to isolate RuBPCase from comfrey which led to an essentially homogeneous enzyme preparation with a specific activity of nearly 1.3 μmoles $^{14}CO_2$ fixed/min/mg protein. The A_{280}/A_{260} ratio of the purified RuBPCase was 1.99.

2. Comfrey yielded 26 mg of purified enzyme per 100 g of fresh leaves, which made it a reasonably good source of the enzyme.

3. The molecular weight of the native enzyme was found to be 500,000 daltons from the molecular weights of the large and small subunits which were 50,000 and 12,200 daltons, respectively, and an apparent L_8S_8 structure.

4. Activity decreased at high enzyme/substrate ratios, probably due to product inhibition. Maximal activity was found to occur at an enzyme/substrate molar ratio of 6×10^{-5}.

5. The Km for the substrate RuBP was determined to be 4.1×10^{-4} M, which lies in the range of values reported for the spinach enzyme.

6. The pH dependence of the activation by CO_2 and Mg^{2+} appeared to be maximal by pH 7.5. Specific activity also appeared to have a pH optimum near 7.5.

7. Preliminary two-dimensional gel electrophoresis data indicated four types of large subunits and two types of small subunits.

8. There is a possibility that high levels of NaCl could be a means of dissociating the subunits. In any event, however, the combination of cold and high salt are to be avoided for routine work

on the comfrey enzyme.

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THE ISOLATION, PURIFICATION, AND CHARACTERIZATION OF RIBULOSE-1,5-BISPHOSPHATE CARBOXYLASE/OXYGENASE FROM COMFREY

by

SCOTT ANTHONY SIMPSON

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Ribulose-1,5-bisphosphate carboxylase/oxygenase (RuBPCase, EC 4.1.1.39) catalyzes the addition of CO_2 to, and cleavage of, ribulose-1,5-bisphosphate to form two molecules of 3-phosphoglycerate. The same protein, functioning as an oxygenase, catalyzes the oxygenation of ribulose-1,5-bisphosphate to yield one molecule each of phosphoglycolate and 3-phosphoglycerate.

RuBPCase has been purified from comfrey leaves, using ammonium sulfate precipitation, linear sucrose gradient centrifugation, and DEAE-Sephadex column chromatography. RuBPCase was located in the sucrose gradient by A_{280}, and from enzymatic assay determinations, those fractions with the highest specific activity were pooled and stored at -70°. This preparation was further purified before use by DEAE-Sephadex column chromatography. These procedures resulted in an essentially homogeneous enzyme as judged by polyacrylamide disc-gel electrphoresis, which had a specific activity of nearly 1.3 μmoles CO_2 fixed/min/mg protein at pH 8.0.

The molecular weight of the native RuBPCase was very similar to that of the spinach enzyme (550,000 daltons) based on polyacrylamide gel electrophoresis studies. SDS-polyacrylamide disc-gel electrophoresis of the comfrey enzyme gave molecular weights of 50,000 and 12,200 daltons, respectively, for the large and small subunits, which implies an L_8S_8 structure for the native enzyme as has been found in other higher plant sources. Isoelectric focusing data revealed multiple bands, indicating four types of large and two types of small subunits. The pI's for the large subunits ranged from 5.76 to 6.0, while one of the small subunits had a pI of 6.59 and the other 7.03.

All RuBPCases thus far investigated have revealed activation by CO_2 and Mg^{2+} in the pH range of 7.8 to 8.5. Activation by CO_2 for the comfrey enzyme became optimal by pH 7.5 in a Tris buffer containing 10 mM Mg^{2+}, and maximum activity also appeared to occur at pH 7.5. In addition,

activity was affected by the molar ratio of enzyme/substrate. Maximal activity was found at a ratio of 6×10^{-5} when assayed at pH 8.0. The decrease above that ratio appears to be due to product inhibition, since incubation of 3-PGA with the enzyme revealed decreased activity. Increasing concentrations of NaCl at pH 8.0 dramatically decreased the specific activity of RuBPCase, and a plot of ionic strength vs specific activity was sigmoidal, possibly indicating cooperative dissociation. The Km for the substrate, D-ribulose-1,5-bisphosphate, was 4.1×10^{-4} M at pH 8.0.