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EVIDENCE FOR A CONFORMATIONALLY SENSITIVE ANION BINDING SITE ON RIBULOSE -1,5-BISPHOSPHATE CARBOXYLASE/OXYGENASE ISOLATED FROM COMFREY

by

ROBERT F. BONSALL

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Major Professor

TABLE OF CONTENTS

TABLE OF CONTENTS, Continued page

LIST OF TABLES

LIST OF FIGURES

INTRODUCTION

Ribulose -1,5-bisphosphate carboxylase/oxygenase (EC 4.1.1.39) is ragarded as the "key catalyst in the primary synthetic process that fuels all living systems" (Ellis 1979). In addition it is thought to be the most abundant protein in nature since it constitutes up to 65% of the total soluble protein in leaf extracts. The enzyme consists of eight small (S) subunits (molecular weights of 12,000 - 14,000 daltons) and eight large (L) subunits (molecular weights of 50,000-55,000 daltons). It is apparently a branch point enzyme which catalyzes the CO_2 fixation reaction of the Calvin Cycle and the first reaction process in photorespiration which are illustrated below.

Carboxylase Reaction of the Calvin Cycle

CO_2 + D-ribulose -1,5-bisphosphate (RuBP) + $H_2O \xrightarrow{Mg^{++}}$ 2 (3-phospho-glyceric acid, 3-PGA)

Oxygenase Reaction of Photorespiration

O_2 + RuBP $\xrightarrow{Mg^{++}}$ 3-PGA + 2-phosphoglycolate

Ribulose -1,5-bisphosphate carboxylase/oxygenase (RuBPcase) is a highly regulated enzyme. It has been found that PGA formation from RuBP occurs only when the enzyme has been exposed to Mg^{++} and CO_2. The Km values reported for RuBP with spinach RuBPcase ranges from 1×10^{-4} mM to 7×10^{-4} mM which suggests a strong affinity for substrate (Weissbach et al., 1956; Paulsen and Lane, 1966; Kieras and Haselkorn, 1968; Sugiyama et al., 1968a; Bassham et al., 1968). Furthermore, RuBPcase appears to have a low affinity for bicarbonate since the Km values for HCO_3^- range from 1.1×10^{-2} mM

to 5.6×10^{-3} mM (Weissbach et al, 1956; Racker, 1957; Paulsen and Lane, 1966; Kieras and Hasselkorn, 1968; Sugiyama et al., 1968b; Bassham et al. 1968). However, CO_2 is the real substrate, not HCO_3^- (Cooper et al., 1969) and Km (CO_2) is much smaller after converting Km (HCO_3^-) to Km (CO_2). This results because at pH optimum (7.8 for the spinach enzyme) and $CO_2 + H_2O \rightleftarrows H_2CO_3 \rightleftarrows HCO_3^- + H^+$ equilibria lie far to the right. Indeed, at pH 7.8 less than 2% of the total bicarbonate concentration is CO_2. The currently accepted value of Km (CO_2) for the purified spinach enzyme is 20 μM (Badger and Anderson, 1974).

During investigations leading to the nature of the RuBP binding site it was discovered that both phosphate and sulfate anions had inhibitory effects on RuBPcase. Further investigations on the types of inhibition patterns demonstrated by these two anions with RuBP lead to discrepencies. Trown (1965) presented data which indicated that phosphate and sulfate were noncompetitive inhibitors while Paulsen and Lane (1966) suggested that the two anions were competitive with respect to RuBP. Further investigation of Paulsen's and Lane's data indicated a spread in the extrapolated Km values for RuBP (of 0.9×10^4 - 1.4×10^{-4}), where depending on how the extrapolated lines were drawn, conclusions on the type of inhibition could be questionable as to being either competitive or noncompetitive with respect to RuBP (Kawashima and Wildman, 1970).

In regard to the chemical composition of RuBPcase 90 SH groups/mole of wheat or spinach RuBPcase have been detected (Sugiyama et al., 1967; Sugiyama et al., 1968c) which translates to slightly over 11 SH groups per LS pair. In spinach beet RuBPcase the molar ratio of cysteine with respect to the total amino acids suggested that a protein of 5.15×10^5 daltons would have 84 SH groups (Ridley, 1967).

This lead to the question of whether these SH groups were associated with the catalytic site of RuBPcase.

By spectrophotometric analysis it was discovered that 5,5' dithiobis (2-nitrobenzoic acid), DTNB, reacted very quickly with 4 SH groups whereas the reaction of DTNB to an additional 6-7 SH groups was much slower and the enzyme became completely inactivated (Trown, 1964). In addition, only two of the 4 SH groups involved in the fast reaction were found to react with DTNB after the enzyme was first exposed to RuBP. As a result it was concluded that RuBP altered the reactivity of two sulfhydryl groups, suggesting that these sulfhydryl groups were in the active site and may have a catalytic role.

Further investigation by Akazawa's laboratory (Sugiyama, et al., 1968a) discovered that p-chloromercuribenzoic acid (PCMB), which is a strong inhibitor of 3-PGA formation, reacted with ten SH groups per mole of enzyme causing no loss in catalytic activity. Complete inhibition was observed only after it reacted with 30 SH groups. Using iodoacetoamide (IAA), another inhibitor of 3-PGA formation, they discovered that 30-40 SH groups/mole of RuBPcase could be alkylated but that loss of activity occurred after only 8-10 SH groups had reacted (Sugiyama, et al., 1968a). It was estimated that rapid reaction occurred with 7-8 SH groups compared to a much slower reaction of IAA to the remaining 30 or so SH groups. When the enzyme was exposed to RUBP before IAA the RuBP was found to mask 5 fast reacting SH groups which was larger than that reported by Trown and Rabin using TBNB. In addition, the presence of CO_2 and Mg^{++} seemed to abolish RuBP protection against akylation and raised the fast reacting SH groups

from 7-8 to 13-14 in the presence of these effectors. Tryptic digestion of RuBPcase subjected to akylation in the presence or absence of RuBP gave random distribution of alkylated cysteine sulfhydryl groups throughout the peptides and also with those which were protected by RuBP. It should also be mentioned that RuBP when bound to RuBPcase retards degradation of the protein by proteolytic enzymes, urea and sodium dodecyl sulfate, SDS, (Sugiyama, et al., 1968d). Therefore, Akazawa's group concluded that specific sulfhydryl groups were not involved in the binding of RuBP but instead functioned to lock the enzyme in a specific configuration. When exposed to Mg^{++} and CO_2 this configuration was altered.

It has been shown that carbamyl phosphate and hexose mono- and diphosphates will protect spinach RuBPcase sulfhydryl groups from IAA akylation (Argyroudi - Akoyunoglou et al., 1967). In addition these substances are not competitive with RuBP for the catalytic site and do not inhibit the enzymatic reaction (Argyroudi - Akoyunoglou et al., 1967; Sugiyama et al., 1968a). It should be noted that carbamyl phosphate was discovered to mask two of the four or five sulfhydryls found to be protected by RuBP against IAA akylation without inhibiting enzymatic activity. This is not in agreement with Trown's conclusions that two sulfhydryl groups are intergrated with the catalytic site for RuBP. All of the above chemical modification data suggest that conformational changes can be easily induced in spinach RuBPcase.

3-PGA, the end product of the carboxylase reaction, has been discovered to be a competitive inhibitor for the binding of HCO_3^- and noncompetitive with respect to the RuBP binding site (Paulsen and Lane, 1966). It has been found that the presence

of Mg^{++} or HCO_3^- will slightly protect the enzyme from proteolytic degradation as well as urea and SDS denaturation. If all three are present the protection against denaturation is greater but there is not any protection against IAA akylation (Rabin et al., 1964). As a result it can be concluded that the sulfhydryl groups do not directly participate in Mg^{++} and HCO_3^- binding.

It has been reported (Akoyunoglou et al., 1967; Argyroudi - Akoyunoglou 1967) that HCO_3^- can bind directly to spinach RuBPcase thus forming a stable enzyme CO_2 - complex. It also has been found that just before RuBP carboxylation a Mg^{++} and CO_2 complex forms with the enzyme (Pon et al., 1963). If RuBPcase is incubated in the presence of substrate (RuBP) followed by exposure to Mg^{++} and HCO_3^- then there is no effect on the catalytic activity of the enzyme. If RuBP pretreatment is extended, the enzyme becomes inactivated. Pretreatment with Mg^{++} alone at 0^o produced some activation. However, pretreatment with both Mg^{++} and HCO_3^- was found to give substantial activation (Pon et al., 1963). It is known that CO_2 and Mg^{++} are absolutely required to activate the enzyme (Lorimer et al., 1976) and that activation occurs at a lysine residue separate from the active site (Lorimer and Miziorko, 1980).

The binding of RuBP to the enzyme has been discovered to give rise to a difference spectra that consists of positive peaks at 268 and 288 nm together with a negative peak at 298 nm (Rabin et al., 1964; Racusen et al., 1964). The difference spectra did not change upon HCO_3^- addition but disappeared upon the addition of Mg^{++} (where the negative peak disappeared more quickly than the two positive peaks). This disappearance of the negative peak during Mg^{++} addition

was concluded to be related to the cleavage and disappearance of RuBP and CO_2 (where the carboxylation step was the rate limiting step). Furthermore the disappearance of both positive peaks were concluded to possibly signify conformational changes which might arise from the dissociation of Mg^{++} thus having the enzyme return to its native conformation to start the catalytic process over again.

RuBPcase from Nicotiana tabacum leaves was found to undergo a profound change in solubility when exposed to RuBP (Kwok et al., 1971). Sedimentation velocity experiments suggested that there was no gross change in quaternary structure (arrangement of two types of subunits with respect to one another) since the difference between the sedimentation coefficients of RuBPcase and RuBP-treated RuBPcase was found to be less than 1%.

Difference spectrophotometry was also used by Kwok (1974) to investigate conformational change of RuBPcase on RuBP binding. The difference spectra were recorded for various ratios of RuBP: RuBPcase (2:1; 4:1; 8:1; 12:1) where equivalent amounts of RuBPcase alone were used as a reference. A negative absorbance difference at 296 nm was observed and interpreted as changes in the environment of buried tryptophyl and tyrosyl residues which became oriented towards the outside. As a result the change in solubility due to exposure to RuBP was related to a change in the tertiary structure (folding within the subunits) rather than a change in the quaternary structure of the enzyme.

It was the purpose of this thesis to investigate the effects of bicarbonate inhibition with respect to comfrey RuBPcase and compare it to other anions such as nitrate and chloride to see if a separate binding site did exist. Nitrate could have in vivo effects with

RuBPcase activity because it is known that nitrate and nitrite are transported across the chloroplast membrane (Rudolf et al., 1980). Also differential spectroscopy at various chloride concentrations was to be used to determine the nature of this anion site, if present and to see if a conformational change occurred upon anion binding. To further investigate any possible conformational changes upon anion binding, CD analyses were to be obtained on the enzyme exposed to various amounts of bicarbonate. It should be mentioned that CD spectra were also obtained concerning urea denaturation with respect to changes in the secondary structure of RuBPcase. Furthermore, Km data for RuBP were calculated at zero (extrapolated) chloride concentrations and low nitrate levels (which were shown not to have inhibitory effects on the enzyme). In addition the homogeneity of RuBPcase at each step of the purification scheme was investigated by SDS polyacrylamide gel electrophoresis.

EXPERIMENTAL

Isolation and Purification of Ribulose -1,5-bisphosphate carboxylase/oxysenase (RuBPcase)

RuBPcase was isolated from freshly cut garden-or greenhouse-grown comfrey leaves by the procedure of Simpson (1980). Briefly summarized the procedure consisted of homogenization on ice in the presence of insoluble polyvinylpolypyrrolidone (PVP) in a 50 mM Tris, 50 mM $NaHCO_3$, 10 mM $MgCl_2$, 1 mM EDTA, 5 mM $Na_2S_2O_6$, .1 mM phenylmethylsulfonylflouride at pH=7.5 (TBMESP) buffer system. This was followed by filtration through miracloth, stirring for 30 minutes with additional PVP, and centrifugation at 10,000 x g for 5 minutes at 4^oC in order to obtain the crude extract. The crude extract was brought to 40% saturation with ammonium sulfate followed by centrifugation at 13,000 x g for 20 minutes (40% pellets). The 40% pellets were dissolved in a minimum of TBMESP buffer and allowed to stand at room temperature for 15 minutes followed by centrifugation at 10,000 x g for 10 minutes. The redissolved 40% pellets were applied to .2-.8M linear sucrose density gradients and centrifuged in a SW-27 swinging bucket rotor on a Spinco L3-50 ultracentrifuge at 27,000 rpm for 20 hours. The sucrose gradients were collected in 1 ml fractions and the peak tubes located by A_{280} measurements. The peak tubes with the highest specific activity were pooled and the enzyme was stored in 1.5 ml cryotubes under nitrogen atmosphere at -70^oC. Activity measurements for RuBPcase were those of Paulsen and Lane (1966) as modified by Simpson and Mueller (in preparation). Protein determinations were made using the $E^{1\%}_{cm} = 17$ determined at 280 nm by Bolden and Mueller (unpublished results).

Ion Exchange Column Chromatography

All sucrose gradient preparations were further purified before use by ion exchange chromatography on G-25 DEAE Sephadex as described by Simpson (1980). The equilibration buffer which was used for the column depended on the type of buffer system needed for the particular experiment to be performed, but in all cases RuBPcase was eluted by making the buffer 0.2M in NaCl.

SDS Polyacrylamide Slab - Gel Electrophoresis

SDS slab gels were run on the crude extract 40% supernatant, 40% pellet, sucrose gradient and ion exchange preparations of the purified enzyme. The standards consisted of a mixture of BSA, lysozyme and chymotrypsinogen A obtained individually from the Sigma Chemical Company. The equilibration buffer used for the DEAE column was 50mM Tris, 1mM $NaHCO_3$, 10mMgCl_2, .1mM EDTA at pH 7.5 (TB'ME). The polyacrylamide gel consisted of a 15% acrylamide and 2% N,N'-methylene bis acrylamide running gel with a 30% acrylamide and 0.8% N,N'-methylene bisacrylamide stocking gel. The polymerizing buffers were Tris chloride at pH - 8.9 and Tris chloride at pH - 6.7 for running and stocking gels, respectively. The protein solution was heated in an SDS, β-mercaptoethanol buffer (5 µl/50 µl of protein) for 5 minutes at 100°C. The electrophoresis was carried out at 2.5 milliamps with a Bio Rad Model 500 power supply in a cold room for 5½ hours. The electrophoresis buffer consisted of 9.9 mM Tris, 76.6 mM glycine and 10% SDS. The gel was stained overnight in 0.25% Coomassie blue, 25% ethanol and 10% glacial acetic acid while slowly stirring. This was followed by destaining for 10 hours in 25% ethanol and 10% glacial acetic acid where the destaining solution was changed three times. The

electrophoresis unit consisted of a slab gel electrophoresis cell which was constructed by the Machine Shop at Kansas State University.

Ki Determination for Chloride Inhibition of RuBPcase

The buffer used for the chloride Ki determination was 50 mM Tris, 20 mM $NaH^{14}CO_3$, 10 mM $MgCl_2$ and .1 mM EDTA in 2 mM dithioerythritol (DTE) at pH = 7.5. A pooled reaction mixture was prepared for each set of five reaction times which contained appropriate quantities of buffer, enzyme, inhibitor and RuBP for eight assays (five times, one heated blank, one unheated blank and one "extra"). Each assay consisted of a 540 µl aliquot withdrawn and quenched between 10 and 50 seconds after initiation of the reaction with RuBP. Therefore, each pool contained 8 x 540 = 4320 µl prepared in the following manner. To a predetermined volume of buffer, a volume of enzyme in TB'ME plus 0.2M NaCl was added to give a total of 160 µg of protein (typically between 80 and 160 µl). In turn, a volume of 2 M NaCl in the reaction buffer described above was added to give chloride ion concentrations between 68 and 392 mM. Consequently, the volume of buffer was 4320 µl minus the sum of the volumes of enzyme, 2 M NaCl and RuBP (to be added later). The volumes of buffer and stock NaCl solutions are listed for each pool in Table I, Appendix A.

This pooled reaction mixture was incubated in a sealed vial for 45 minutes to activate the enzyme and to achieve isotopic equilibrium. After incubation, the appropriate amount of RuBP in TME buffer, pH 7.5, was injected to give the nmoles of RuBP per 540 µl listed in Table I, Appendix A, which are corrected for the percent purity listed by the manufacturer. Immediately after addition of the RuBP solution, the

mixture was swirlled rapidly and 540 µl aliquots were quenched at 10 second intervals by injection into 0.2 ml of glacial acetic acid in a 7 ml scintillation vial. The exact quenching times are given in Table II, Appendix A. All volumes were measured with P-20, P-200 and P-1000 Pipetman adjustable micropipets and all experiments were done at room temperature in a fume hood, except that the stock RuBP solutions were held on ice until use. The above procedures were repeated for the next highest inhibitor level using the same RuBP concentration until all five inhibitor concentrations had been measured. The entire procedure was repeated at the next highest RuBP concentration until all the 5 x 5 x 5, substrate x chloride x time experiments were completed. Finally, the quenched mixtures were evaporated to dryness overnight at 85^{o}-90^{o}C in a specially designed aluminum block set on a hot plate in an exhaust hood. This was followed by the addition of 0.5 ml of deionized water and 6.5 ml of liquid Scintillation cocktail (8g PPO, .2g POPOP, 1 liter Triton X-100 and 2 liters of toluene). All samples were counted on a Beckman Liquid Scintillation spectrometer using a ^{14}C isoset with a gain setting of 2.5. The cpm obtained represented the amount of $^{14}CO_2$ incorporated into acid stable products (3-PGA) under each set of experimental conditions. In combination with the cpm obtained for the heated (background) and unheated (full counts) it is possible to convert these to specific activities.

Ki Determination for Nitrate Inhibition of RuBPcase

The procedure used for the determination of the Ki for nitrate inhibition with RuBPcase was the same as that described for chloride inhibition except 10 mM Tris was substituted for the 50 mM Tris to

reduce the background chloride level. The amounts of buffer, 4M $NaNO_3$ stock solution, and substrate which were added to the pooled reaction mixtures together with the various quenching times are listed in Table III[a&b], Appendix A. Also a standard (containing 1.0176 ml of buffer, 18 µl of radioactive bicarbonate and 40 µg of enzyme per pool) was run before each successive substrate concentration in order to test for changes in enzymatic activity during the 6-8 hours required to complete the experiment.

Effect of Bicarbonate on Chloride Inhibition

Enzymatic activities at various levels of chloride ion were investigated as a function of bicarbonate concentration. RuBPcase activity was assayed at 49 µM RuBP using 20 µg of enzyme and varying concentrations of bicarbonate and chloride ions in a total of 540 µl per assay. A constant amount of $NaH^{14}CO_3$ was added regardless of the total concentration of bicarbonate investigated. Four concentrations of bicarbonate were selected (1, 10, 20 and 40 mM) and the activities compared at chloride ion levels ranging from 31.6 (no added chloride) to 463 mM. The assays were quenched 50 seconds after the addition of substrate. Heated and unheated control samples containing all ingredients except RuBP were run in parallel with the samples to allow quantitation of the data.

Km Determination for RuBP

The procedure was essentially that described for the Ki determination of nitrate inhibition with RuBPcase. The amounts of buffer, 1 M $NaNO_3$ stock solution, nmoles of substrate and quenching times are listed in Table IV[a,b&c](Appendix A). In addition, a Km value for RuBP was determined using reaction velocities extrapolated to zero chloride concentrations.

pH Dependence of Bicarbonate Inhibition on RuBPcase

The buffers which were used consisted of 10 mM Tris, 10 mM $MgCl_2$, 2 mM DTE and .1 mM EDTA at the various pH values and cold bicarbonate concentrations which are listed in Table V (Appendix A). To pooled samples containing the listed amounts of buffer and hot bicarbonate, 20 µg of enzyme per assay were added followed by incubation for 45 minutes. It should be noted that the ratio of radioactive bicarbonate to cold bicarbonate was made the same for all solutions. After the 45 minutes incubation period the solution was made 0.7 mM in RuBP (saturating) and reacted for 60 seconds followed by quenching of 540 µl of final pool reaction mixture with 200 µL of glacial acetic acid. The quenched mixtures were then dryed and counted as described above.

Circular Dichrosim (CD) Measurements

All CD spectra were measured on a Cary 60 spectropolarimeter using a Model 6001 CD attachment and a .5 cm cell. The ellipticities were given in units of deg - cm^2/d - mol. These calculations were obtained by using a mean molecular weight of 100 for the amino acid residues. The concentration of RuBPcase used was estimated at 0.64 mg/ml by A_{280} measurements of the protein solution after urea denaturation using an $E^{1\%}_{1cm}$ = 17 at 280 nm. After transporting the purified enzyme solutions to the laboratory of Dr. John Cann, University of Colorado Medical, Denver, Colorado, for CD analysis some of the protein precipitated in the cell and the exact concentrations at which the spectra were obtained are not known. It should be noted that the actual concentration obtained at the end of the experiments was probably less than .64 mg/ml indicated above since the addition of urea redissolved the sample and since denaturation usually increases protein extinction coefficients.

RESULTS

SDS Polyacrylamide Slab - Gel Electrophoresis

SDS polyacrylamide slab gels were run in series on the crude extract 40% supernatant, 40% pellet, sucrose gradient and ion exchange preparations in order to determine the homogeneity of RuBPcase at each step of the purification scheme (Figure 1 [a] . The crude extract (track 1) showed the large and small subunits of RuBPcase and six additional bands as indicated by the arrowheads. Three of the bands appeared above that of the large subunit (90.0, 76.9 and 68.2 K daltons) and three below (46.4, 39.7 and 36.9 K daltons). In track 2 the TCA precipitated supernatant from the 40% ammonium sulfate precipitation also showed six extra bands. At least four of these bands (74.2, 65.2, 44.4 and 34.4 K daltons) appeared to correspond to those in track 1 and possibly a fifth (83.7 K dalton), but this is less certain even considering the curvature of the gel. One new band is apparent at 57.0 K and the band at 35.7 K in the crude extract is missing. The 40% pellet shown in track 3 displayed one extra protein which banded at 38.8 K daltons and probably corresponded to the 39.7 K band seen in track 1 but missing in track 2. The pooled fractions from the sucrose density gradient are shown in track 5. Again only one non-RuBPcase band was seen which migrated as a 60.8 K protein which did not seem to correspond to either the 65.2 or 57.0 K protein band seen in the 40% supernatant. No efforts have been made to identify the extraneous proteins at this time. The pooled RuBPcase fractions from DEAE cellulose chromatography (track 6) showed no extraneous bands and indicated a homogenous preparation.

In addition the molecular weights for both large and small

subunits of RuBPcase were determined from a series of standards (track 4) consisting of BSA (MW = 66,700) chymotrypinogen A (MW = 25,000) and lysozyme (MW = 14,300). The molecular weight values were obtained from a plot of Rf vs log MW for each standard (Figure 1^{b}). From this plot the molecular weight values were determined to be 50,000 daltons and 12,700 daltons for the large and small subunits, respectively.

Km Determination for RuBP

The activity vs time data obtained together with their least squares analyses are presented in Table $VI^{(a-h)}$ (Appendix B). Some additional low concentration nitrate data were included since no effects on initial velocity could be detected up to 20 mM inhibitor. The nitrate concentrations which were utilized in the Km determinations were 0 mM, 1.25 mM, 2.5 mM, 3.75 mM, 6.25 mM, 10 mM and 20 mM. A Km was also determined using the extrapolated values (Y-intercepts) at zero chloride concentrations Table $VII^{(a,b\&c)}$ (Appendix B) and from the zero nitrate data of the nitrate Ki measurements. Initial velocities were determined from linear regression analyses of the cpm vs time data for each set of experiments. Overall the correlation coefficients (CC) indicated that the velocities were quite linear with time.

Sample Lineweaver - Burk plots using the initial velocities as obtained above are shown for the data at zero mM nitrate and zero mM chloride in Figure $2^{(a\&b)}$ and all the Km values are listed in Table $1^{(a,b\&c)}$ The Km values for RuBP ranged from a high of 133 µM (obtained from the initial velocities at zero chloride) down to a low of 21 µM (in the presence of 2.5 mM nitrate). The average of all listed Km values was 56 µM, while the average of those in zero or low nitrate (32 mM chloride from buffer salts) alone averaged 46 µM.

Figure 1[(a)]. SDS Polyacrylamide Gel Electrophoresis (track 1: crude extract, track 2: 40% supernatant, track 3: 40% pellet, track 4: from top to bottom, BSA, chymotrypsinogen A, lysozyme, track 5: pooled sucrose gradient fractions; track 6: pooled DEAE column chromatography fractions).

The gels were electrophoresed at 4°C in 9.9 mM Tris, 76.6 mM glycine and 10% SDS at 2.5 milliamps. The gel consisted of 15% acrylamide and and 2% N,N'-methylene bis acrylamide running gel with a 30% acrylamide and .8% N,N'-methylene bis acrylamide stacking gel. Each track was loaded with 4-6 µg of protein obtained as described in the experimental section except track 4 which was loaded with 6 µg of each standard protein. The arrowheads indicate protein bands which were visible on the original gel but which did not reproduce well on the photograph. L refers to large subunit and S to small.

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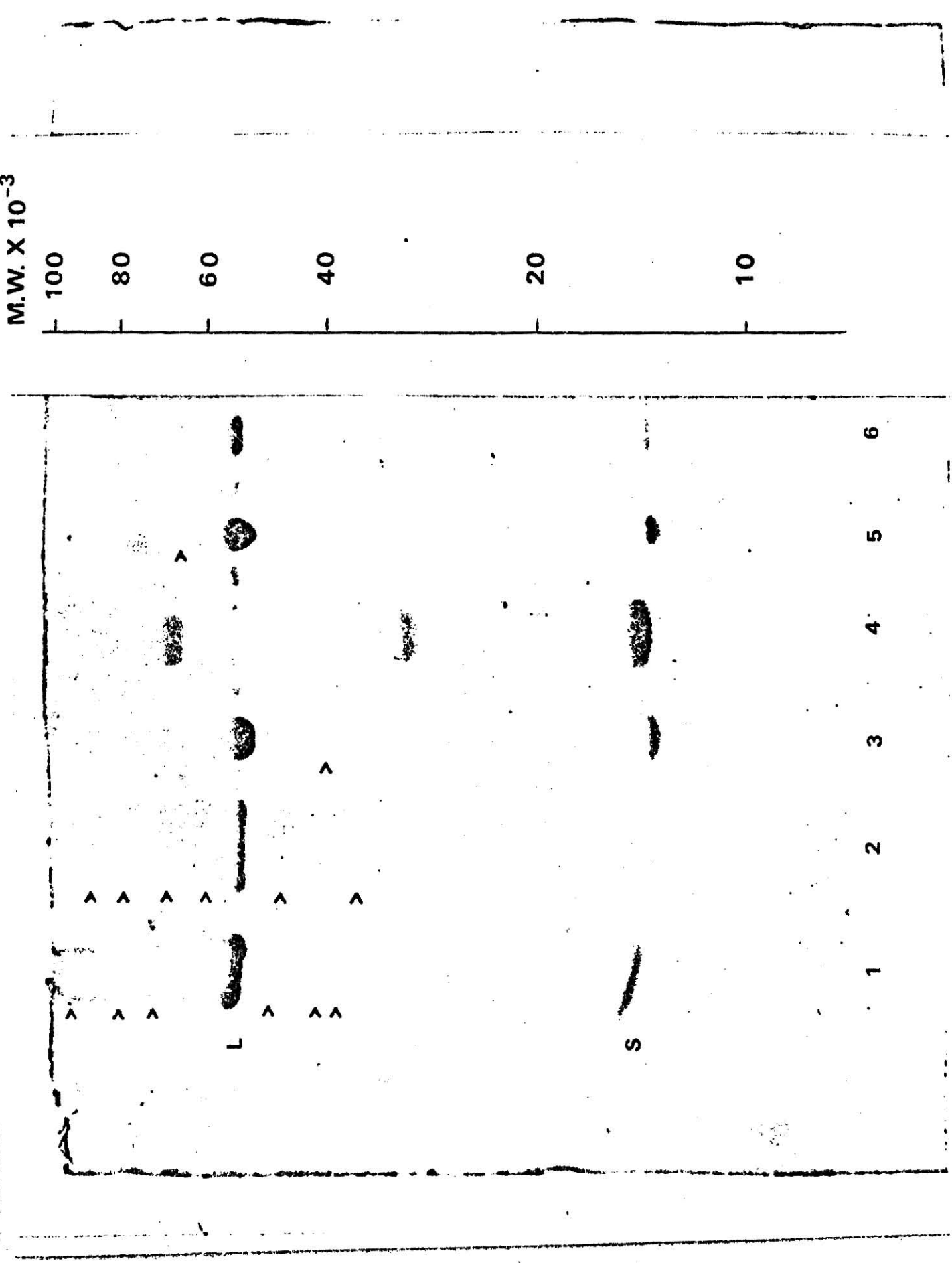
M.W. X 10^{-3}
100
80
60
40
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10
L
S
1
2
3
4
5
6

Figure $1^{(b)}$. Log MW vs Rf for Molecular Weight Determinations of the Large and Small Subunits for RuBPcase

An average Rf value of .19 was obtained for the large subunit (utilizing tracks 1-3 and 5 and 6 of Figure $1^{(a)}$) where the average Rf value for the small subunit was .60 (which was determined by using tracks 2, 3, 5, and 6 of Figure $1^{(a)}$). From these Rf values and by the use of Figure $1^{(b)}$ log Mw values of 4.7 and 4.1 were obtained for the large and small subunits respectively.

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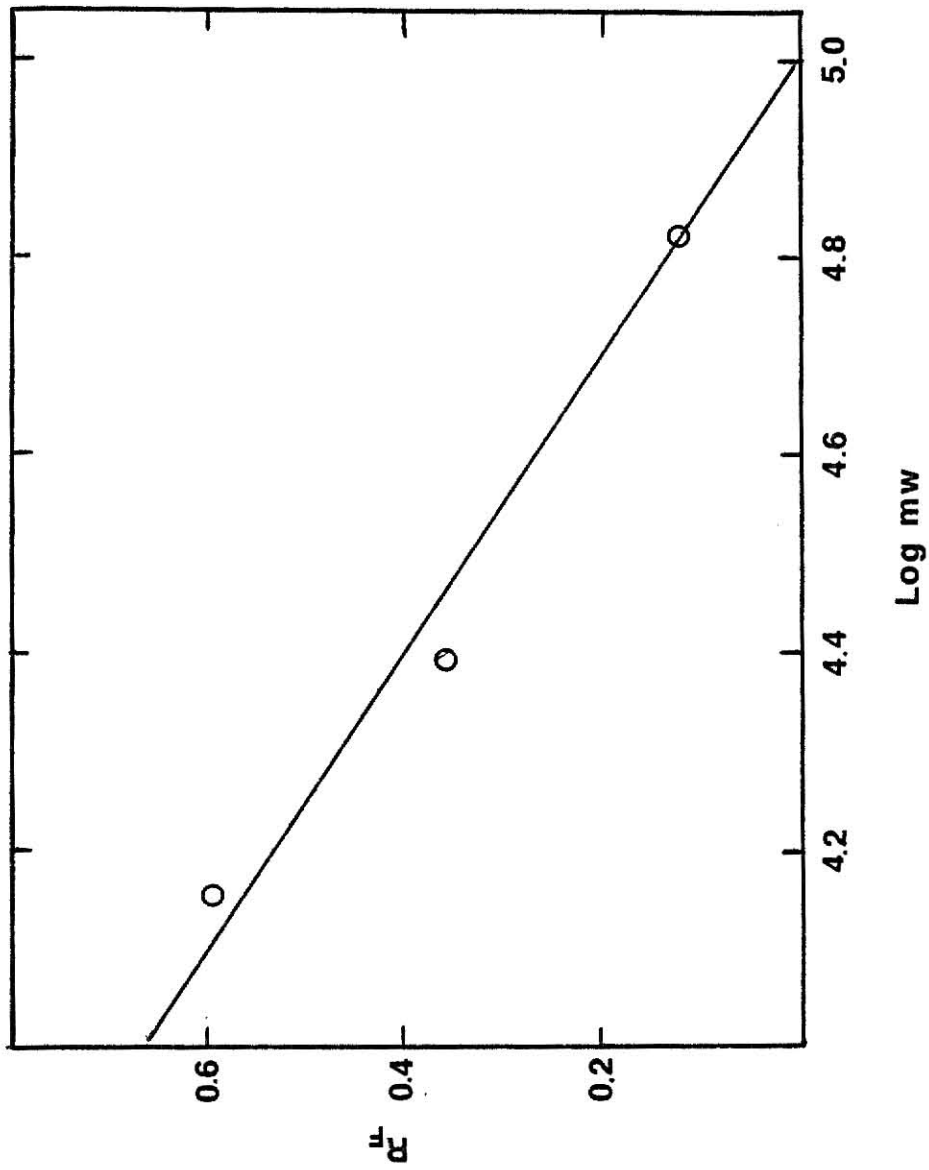

0.6
0.4
0.2
R_f
4.2
4.4
4.6
4.8
5.0
Log mw

Figure $2^{(a)}$. Sample Lineweaver - Burk Plot for Km Determination of RuBP

The 1/S axis intercepts were -.0075 and -.0333 for chloride (O) and nitrate (Δ) inhibition lines respectively. These corresponded to Km values of 133 μM (chloride) and 30 μM (nitrate). The lines are from linear regression of the data shown in Table $VI^{(i)}$ and $VII^{(c)}$ of Appendix B.

Note: For the nitrate line there was an extra point ($1/Vi = 0.31\ (cpm/sec)^{-1}$; $1/S = 0.34(\mu M)^{-1}$) which was not shown to allow a larger scale for the remaining data.

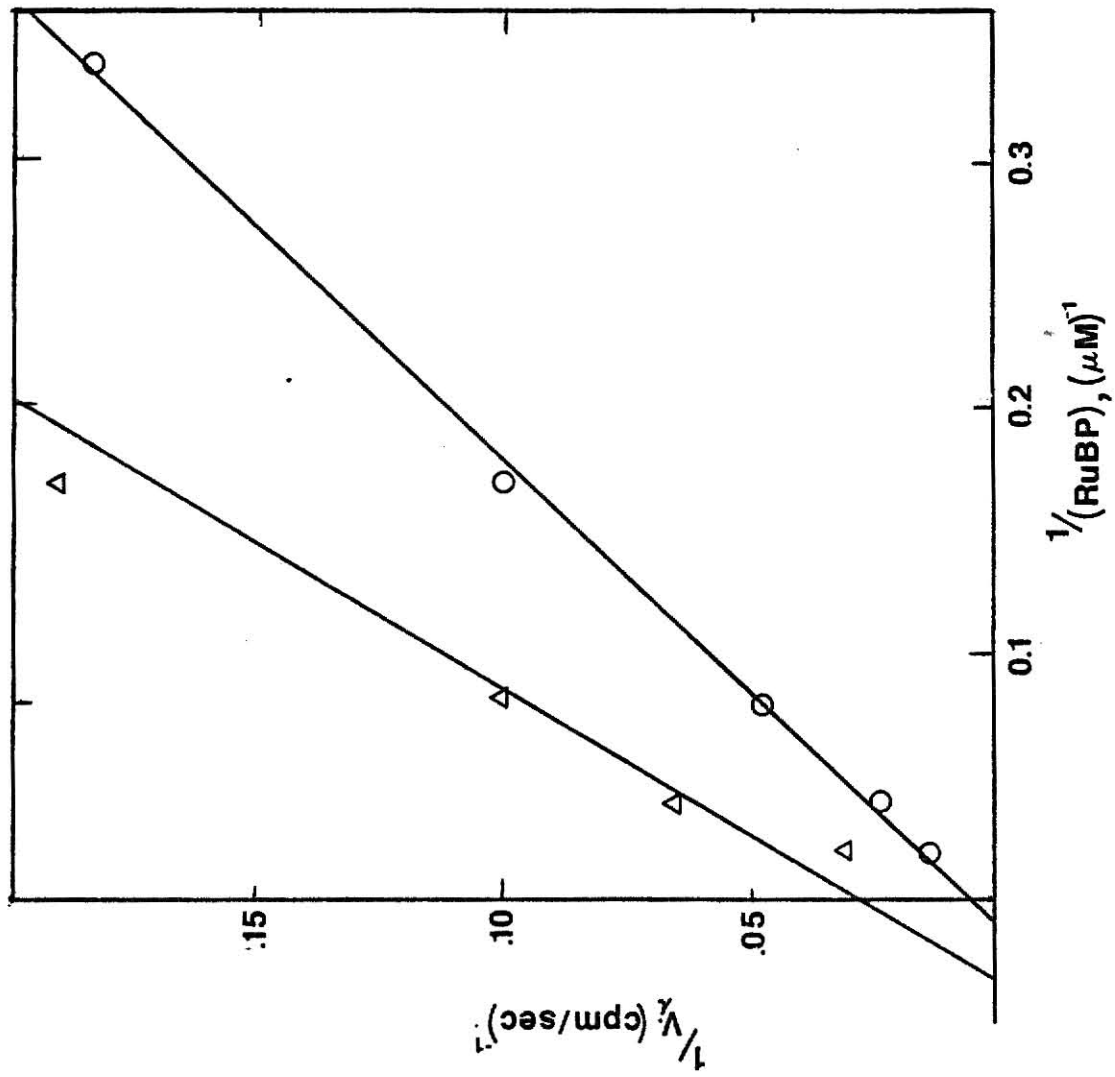

.15
.10
.05
1/V_i (cpm/sec)^-1
0.1
0.2
0.3
1/(RuBP), (μM)^-1

Figure $2^{(b)}$. Blown Up Sample Lineweaver - Burk Plot for Km Determination of RuBP

Shown in Figure $2^{(b)}$: ○, from zero chloride data; △, from zero nitrate data.

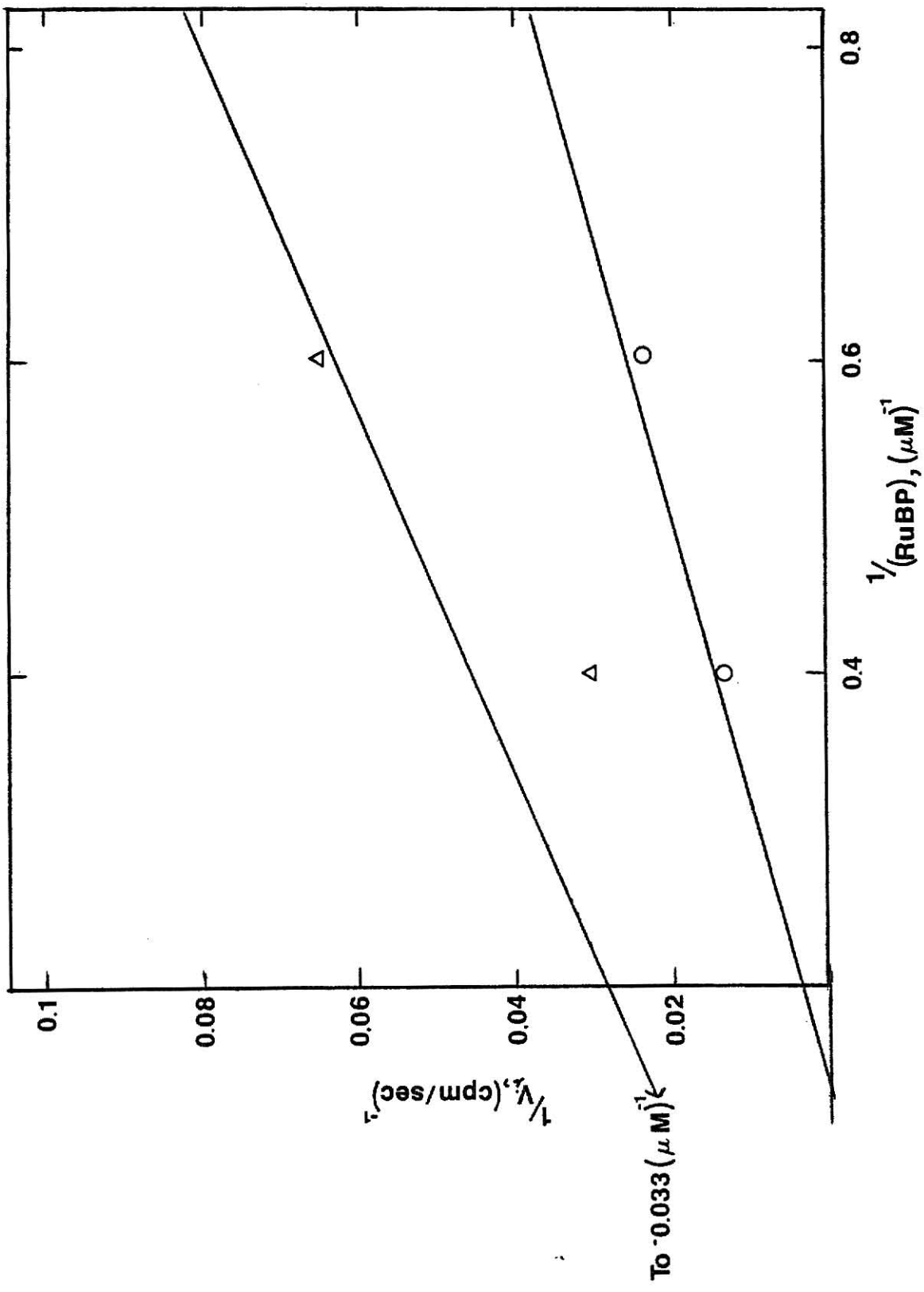

0.1
0.08
0.06
0.04
0.02
0.4
0.6
0.8
1/Vi, (cpm/sec)-1
1/(RuBP), (μM)-1
To -0.033 (μM)-1

TABLE I

Km of RuBP

(a)

NO_3^- (mM)	zero	1.25	2.50	3.75	6.25
Km (μM)	30	120	21	36	37

(b)

NO_3^- (mM)	zero	10	20	zero
Km (μM)	40	46	43	40

(c)

Cl- (mm)	zero (extrapolsted)
Km(μM)	133

The Effect of Bicarbonate Inhibition on RuBPcase as a Function of pH

The effect of bicarbonate inhibition on RuBPcase was studied by measuring the enzymatic activity of bicarbonate concentrations at fixed (saturating) levels of RuBP (Table II). Other data from our laboratory has shown 20 mM to be saturating with respect to 20 µg of RuBPcase. The higher bicarbonate data reveal that as the concentration is increased above 30 mM the activity of RuBPcase decreases for two of the three pH values investigated and at all pH values above 40 mM.

Determination of Inhibition of RuBPcase Activity by Anions

Anion inhibition of RuBPcase activity was determined from initial velocities measured in a substrate vs inhibitor vs time (5 x 5 x 5) set of experiments. The individual sets of raw data (cpm vs time) were analyzed by a two parameter linear regression fit. A three-parameter power curve fit was also tried but did not yield significantly improved initial velocities based on standard deviations. The data are listed in Table VIII$^{(a-l)}$ in Appendix B. Computed initial velocities for each substrate and anion concentration were displayed as Lineweaver-Burk plots for analysis (Segel 1975). The same data were analyzed by a computer program based on Cleland's procedure (Cleland 1975). The results for this analysis are summarized in Table IX$^{(a\&b)}$ (Appendix B).

It should be noted that the enzymatic activity was checked periodically (at the beginning of each successive substrate concentration) in order to ascertain the changes in enzymatic activity over the course of the entire experiment, about 8 hrs. (Table III).

TABLE II

The Effect of Bicarbonate Inhibition on RuBPcase as a Function of pH.

pH / HCO_3^- Conc.	pH = 7.5	pH = 7.8	pH = 8.1
20 mM	1516.20 cpm	1196.95 cpm	1271.7 cpm
30 mM	1150.80 cpm	1369.80 cpm	1081.05 cpm
40 mM	1366.35 cpm	1104.65 cpm	1091.40 cpm
50 mM	1180.15 cpm	1035.30 cpm	918.75 cpm

TABLE III

Enzyme Activity at the Start of Each Successive Substrate Concentration Set

Substrate*	Activity
1st	1558 cpm
2nd	1526 cpm
3rd	1257 cpm
4th	1270 cpm
5th	1030 cpm

* The sequence of experiments started with the lowest substrate concentration and ran through all the nitrate concentrations (1st set). This procedure was repeated for the next highest substrate concentration until all five substrate sets were completed. This required about 8 hrs. Therefore, about 1.6 hrs elapsed between substrate sets.

Inhibition by Chloride

In Figure $3^{(a\&b)}$ the lowest line corresponds to the initial velocities at zero chloride concentrations which were obtained by constructing a plot of Vi vs chloride concentration followed by extrapolating the linear regression line to zero chloride concentration (Figure $3^{(c)}$). The data for Vi vs chloride concentrations together with their least squares analyses are listed in Table X (Appendix B). The reciprocal plot for each fixed chloride concentration (Figure $3^{(a\&b)}$), utilizing the two-parameter fit, clearly demonstrates that as the inhibitor concentration (chloride) increases so does the slope of each inhibition curve together with the 1/Vi axis intercept, $1/V_{max}^{app}$. A pattern of generally decreasing x-intercepts ($-1/K_m^{app}$) and increasing 1/Vi intercepts with increasing chloride (excluding the highest chloride concentration) was suggestive of mixed competitive, noncompetitive inhibition (Segel 1975). Computer analysis by Cleland's method for competitive and noncompetitive inhibition patterns gave variance values of 16.70 and 17.25 respectively. Therefore, by comparison it was concluded initially that chloride inhibition of RuBPcase might be mixed.

The replot (slope of reciprocal plot vs chloride concentration) shown in Figure $3^{(d)}$ was found to be virtually linear up to 279 mM chloride but to curve upward sharply at 392 mM. The calculated Ki value (based on the linear portion of data) was 285 mM which can be compared to the 139 mM and 157 mM values for nomcompetitive and competitive inhibition patterns, respectively, obtained by Cleland's method. It should be noted that neither the linear portion nor the upward curvature (concave) nature of the replot data are compatible to

That expected for straightforward mixed inhibition. To the contrary, the concave nature of the replot curve is traditionally taken as an indication of multiple binding sites. The abrupt upward curvature above 300 mM, however, suggests that other factors, such as conformational changes, may be accompanying chloride inhibition.

Figure 3[(a)]. Plot of $1/V_i$, $(cpm/sec)^{-1}$ vs $1/RuBP$, $(\mu M)^{-1}$ for Chloride Inhibition

The experiments were performed as described in the text. The lines were determined from linear regression analyses of the data: ● , zero (extrapolated) chloride, □ 68; △ 92; ○ 167; ■ 279 and ▲ 392 mM chloride.

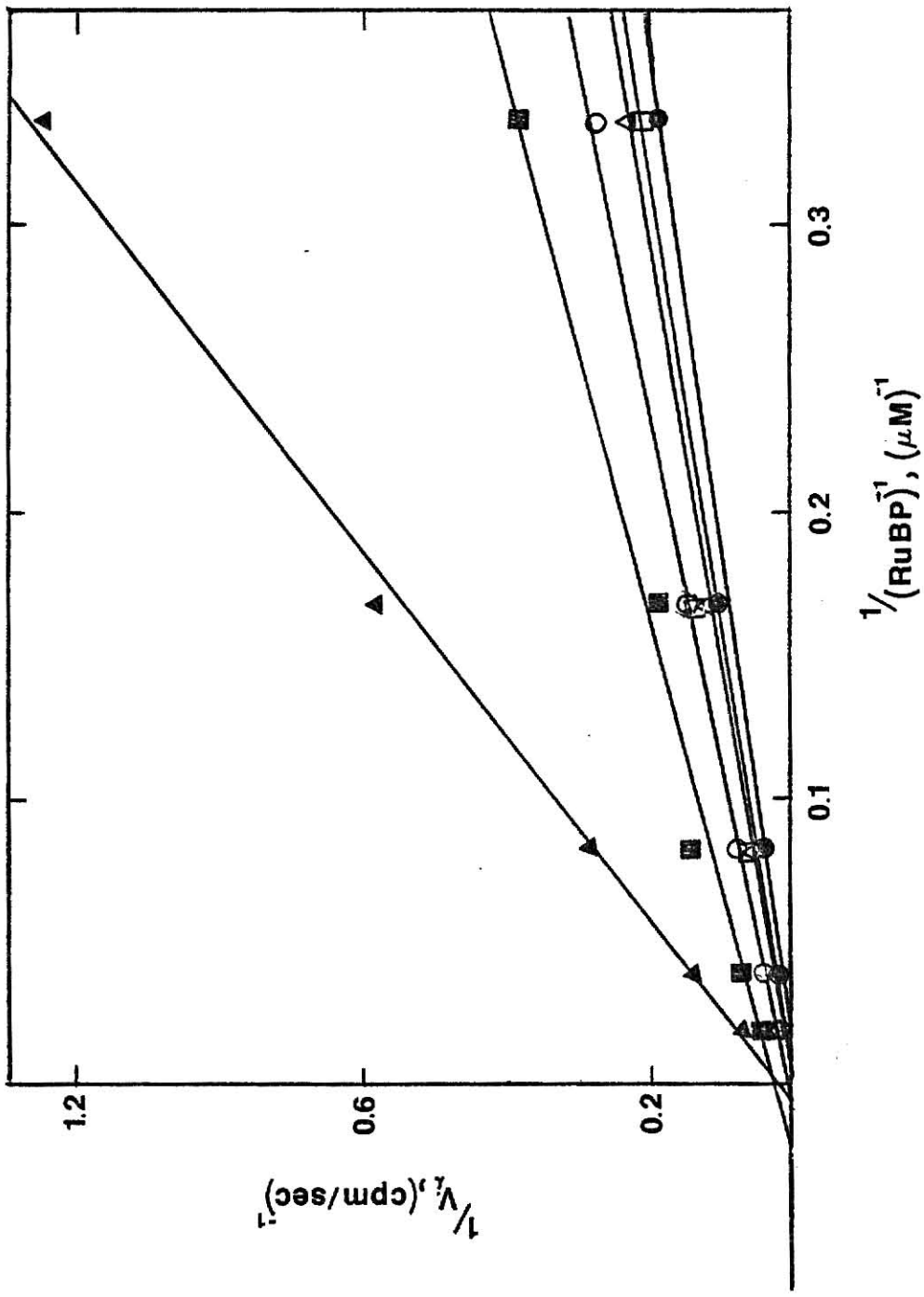

1.2
0.6
0.2
1/V_i, (cpm/sec)^-1
0.1
0.2
0.3
1/(RuBP), (μM)^-1

Figure $3^{(b)}$. Blown Up Plot of 1/Vi, $(cpm/sec)^{-1}$ vs 1/RuBP, $(\mu M)^{-1}$ vs 1/RuBP, $(\mu M)^{-1}$ for Chloride Inhibition shown in Figure $3^{(a)}$.

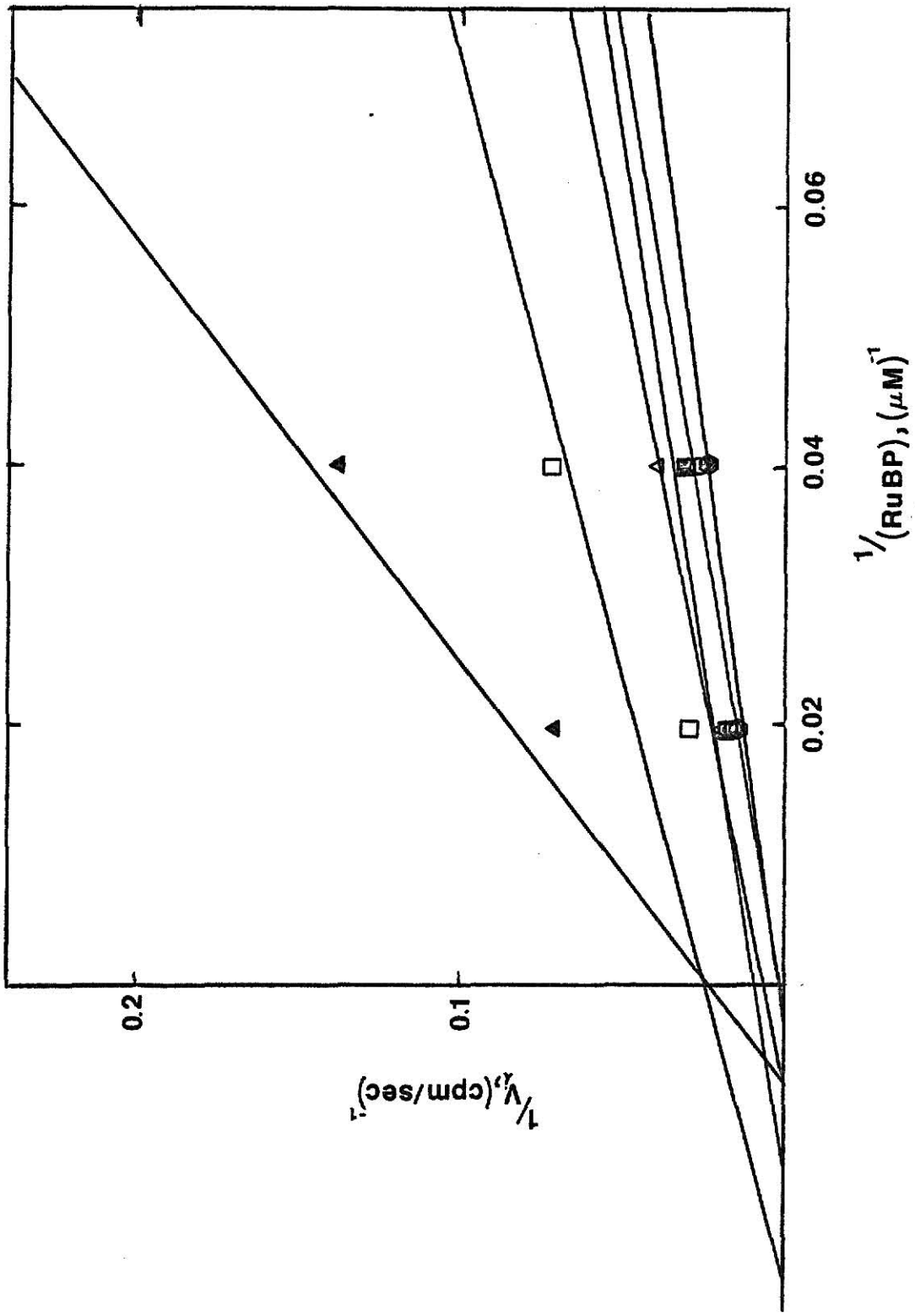

0.2
0.1
1/V, (cpm/sec)-1
0.02
0.04
0.06
1/(RuBP), (μM)-1

Figure 3[(c)]. Vi, (cpm/sec) vs Chloride (mM).

The values obtained for Vi, (cpm/sec) at zero chloride from extrapolation of the linear regression lines (Table VII[(a)]) were 5.462, 9.615, 20.81, 41.87 and 72.82 for substrate concentrations of 2.966 µM (□), 5.936 µM (●), 11.86 µM (△), 24.73 µM (■), and 49.45 µM (○), respectively, which corresponded to chloride ion concentrations of 67.66 mM, 92.00 mM, 167 mM, 279 mM and 392 mM.

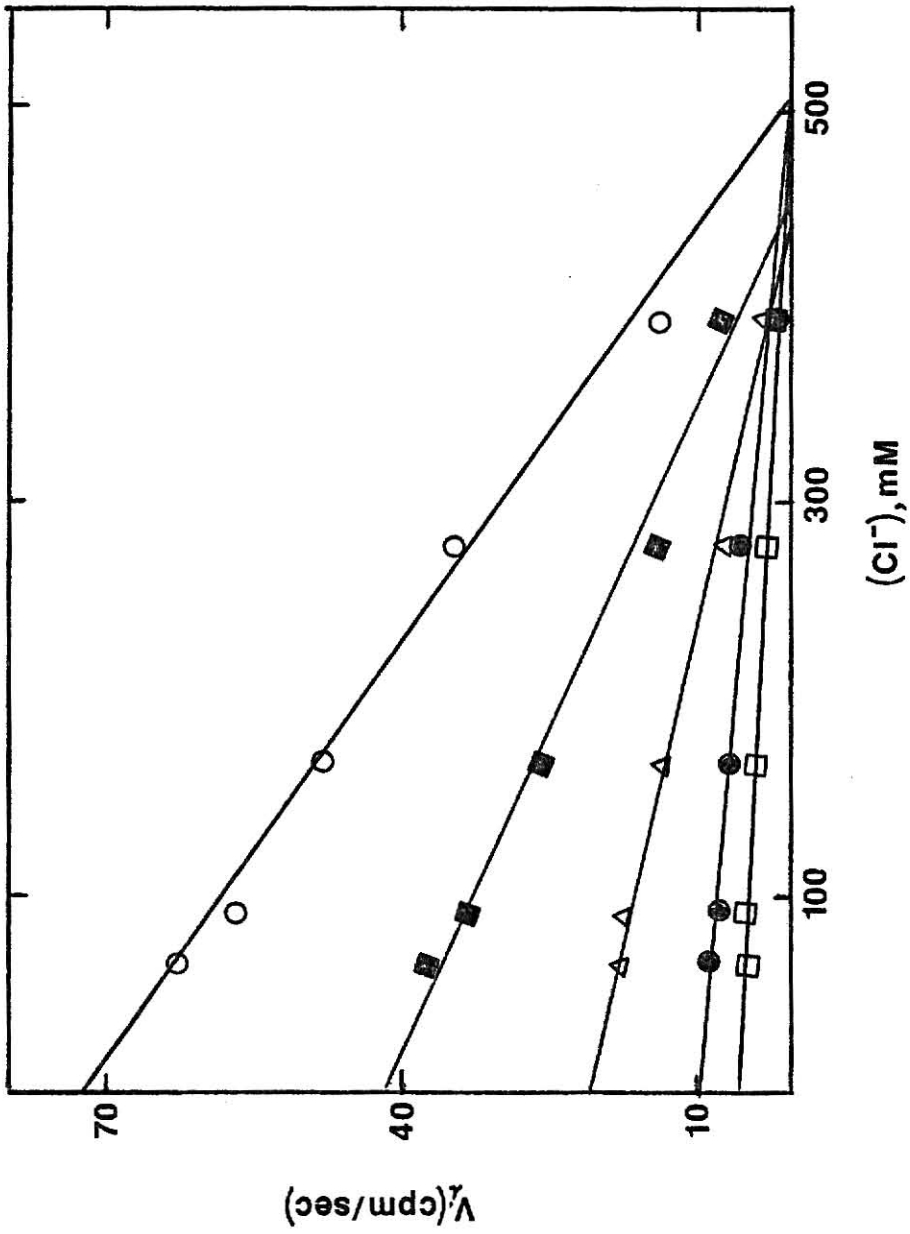
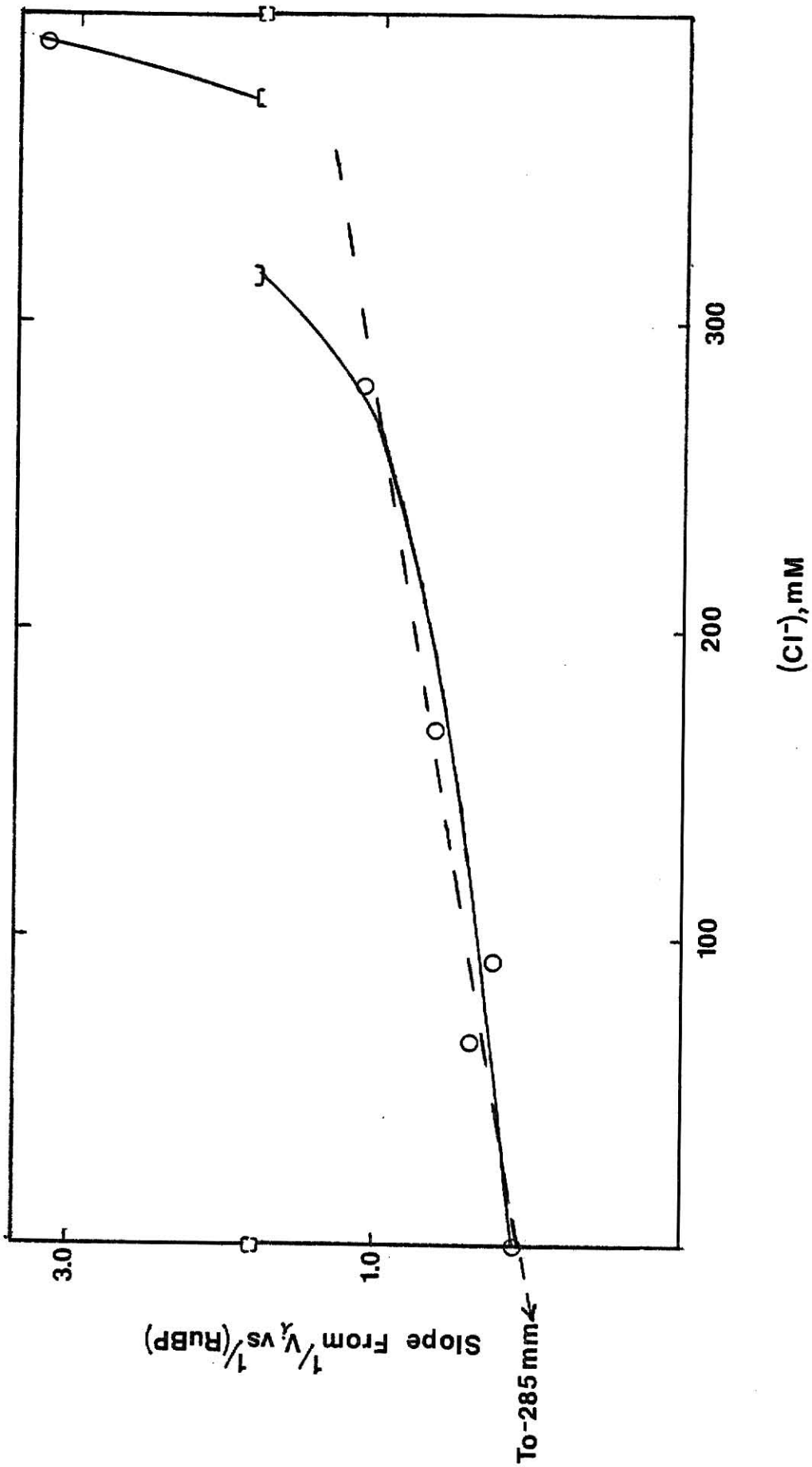

70
40
10
V_i(cpm/sec)
100
300
500
(Cl^-), mM

Figure $3^{(d)}$. Replot for Chloride Inhibition

Slope from 1/Vi vs 1/RuBP (Figure $3^{(a)}$) are plotted against chloride concentration. The line was calculated from linear regression analysis of the points through 279 mM chloride ion.


3.0
1.0
Slope From $^{1}/_{V_i}$ vs $^{1}/_{(RuBP)}$
To -285 mm
100
200
300
(Cl^-), mM

(Segel 1975).

Inhibition by Nitrate

The Lineweaver - Burk plot for nitrate inhibition (Figure 4(a&b)) which utilized the two-parameter fit of cpm vs time appeared to be similar to that observed for chloride with the exception that the same concentration of nitrate gave greater inhibition. It should be noted that at 400 mM nitrate concentrations there were no measurable velocities (Table VIII(j) Appendix B). The variance values obtained by Cleland's method were 1.80 and 1.56 for competitive and noncompetitive inhibition patterns respectively. Thus, the inhibition pattern for nitrate also appeared to be mixed.

The replot for nitrate inhibition (Figure 4(c)) also was found to be essentially linear up to 100 mM nitrate and then curve rapidly upwards to 200 mM nitrate. The Ki which was determined from the replot was 47 mM where those by Cleland's method for competitive and noncompetitive inhibitors were 21 mM and 46 mM, respectively. Again, the concave curvature of the replot data is not consistent with normal mixed inhibition patterns and the abruptness of the curvature seems to suggest factors other than multiple sites changing the activity.

Effects of Bicarbonate on Chloride Inhibition

The effects of increasing bicarbonate ion concentrations on the degree of inhibition by chloride ion are given in Table IV. In each case the percent of control was calculated from the cpm obtained for a given concentration of bicarbonate in 31.6 mM chloride (no added NaCl) taken as 100%. The data showed that increasing (HCO_3^-) at a fixed concentration of chloride ion decreased the inhibition by chloride (increased % of control). This indicated that HCO_3^- reversed

the inhibition provided by chloride possibly by competing directly for the same anion binding site.

Figure 4(a). Plot of $1/V_i$ $(cpm/sec)^{-1}$ vs $1/RuBP$, $(\mu M)^{-1}$ for Nitrate Inhibition

The experiments were performed as described in the text. The lines were determined from linear regression analyses of the data: ● , zero, □ 50, △ 100, ○ 200 mM nitrate.

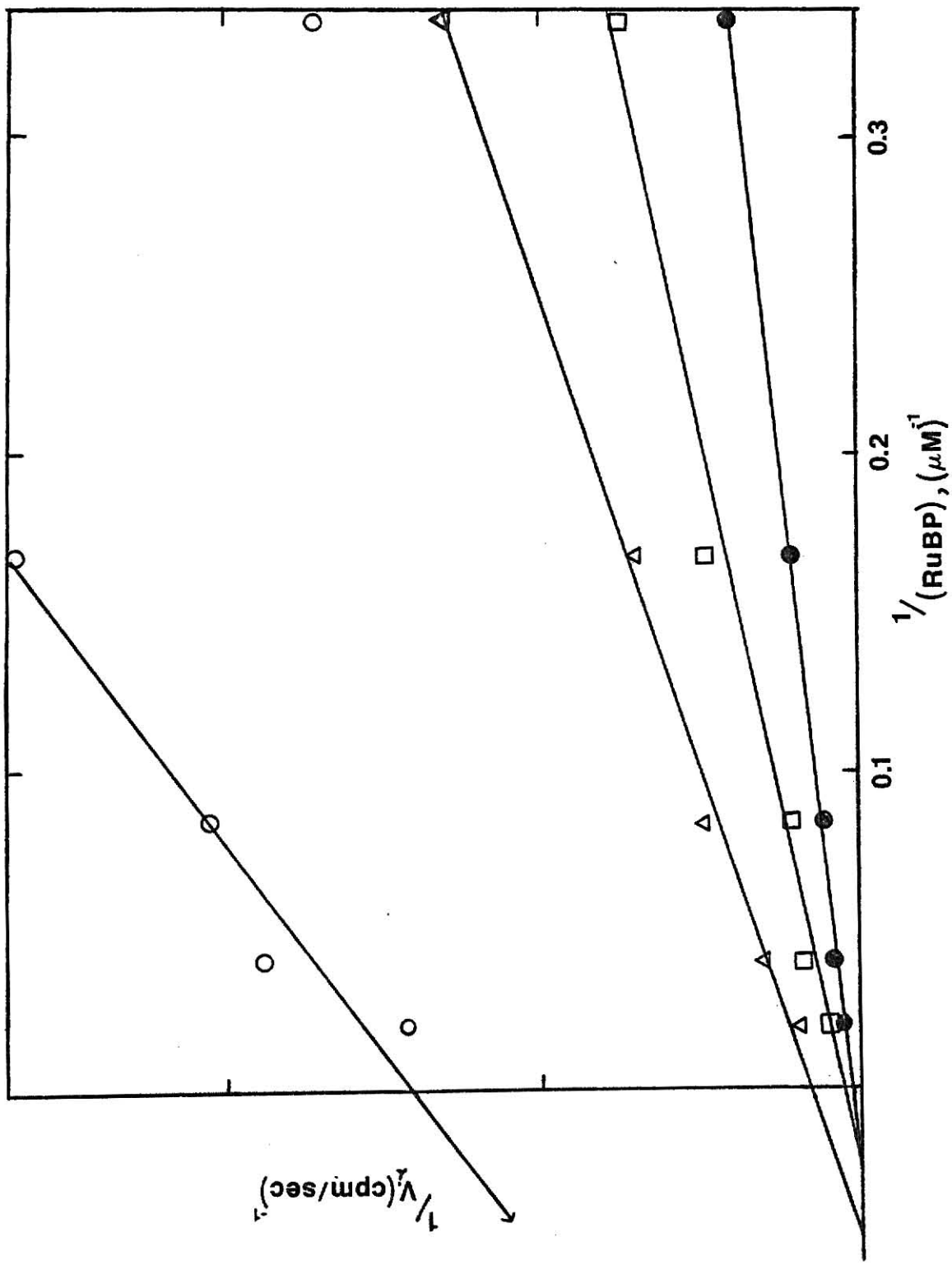

0.1
0.2
0.3
1/(RuBP), (μM)⁻¹
1/V_i (cpm/sec)⁻¹

Figure $4^{(b)}$. Blown Up Plot of $1/Vi(cpm/sec)^{-1}$ vs $1/RuBP\ (\mu M)^{-1}$ for Nitrate Inhibition

Shown in Figure $4^{(b)}$ ● from zero chloride, Δ from zero nitrate, (31.6 mM Cl^-) ■ from 50 mM nitrate and O from 100 mM nitrate.

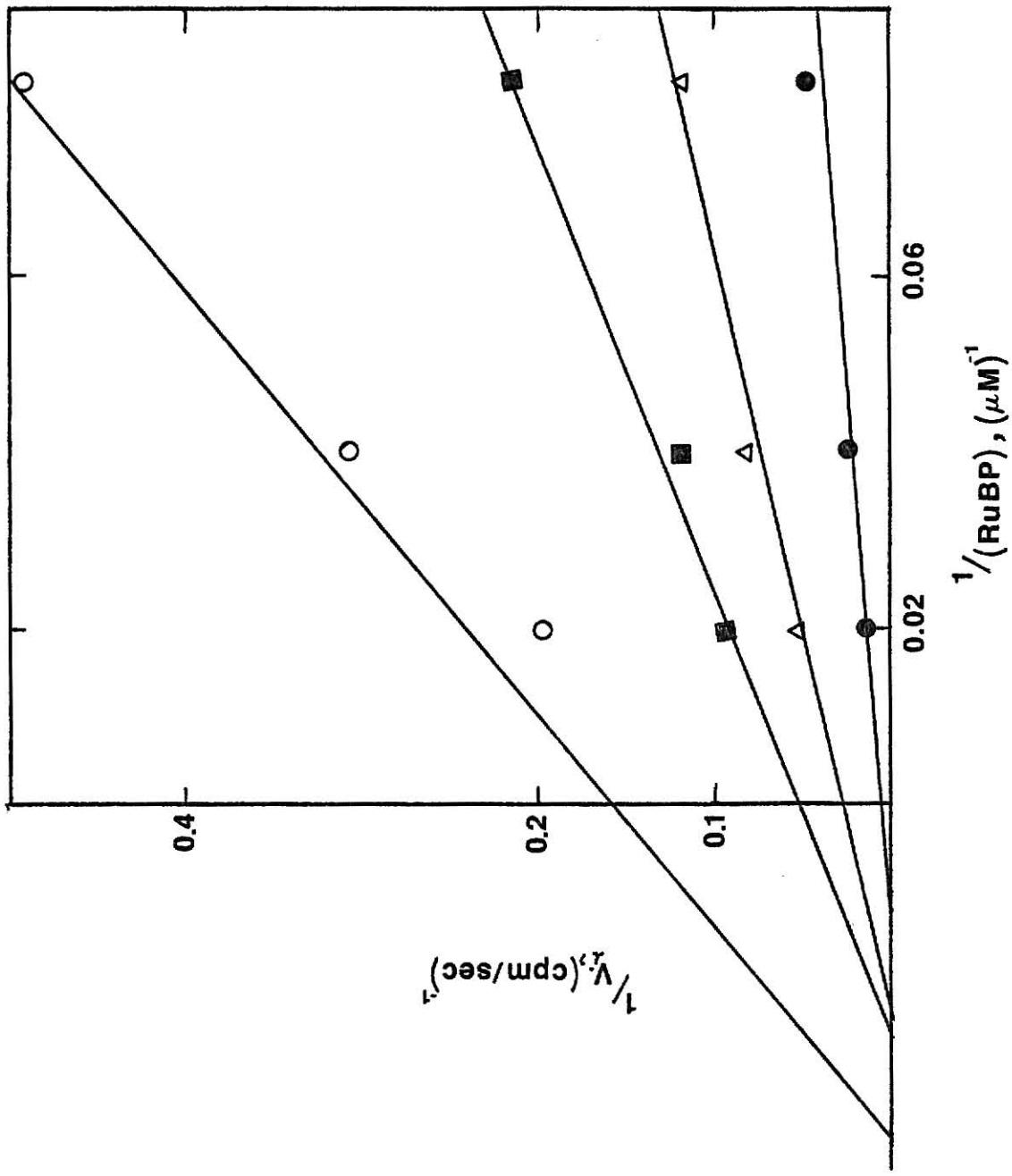

0.4
0.2
0.1
0.02
0.06
1/V_x,(cpm/sec)^-1
1/(RuBP), (μM)^-1

Figure $4^{(c)}$. Replot for Nitrate Inhibition

Slope from 1/Vi vs 1/RuBP (Figure $4^{(a)}$) plotted against nitrate concentration. The calculated Ki was based on the linear portion up to 100 mM nitrate.

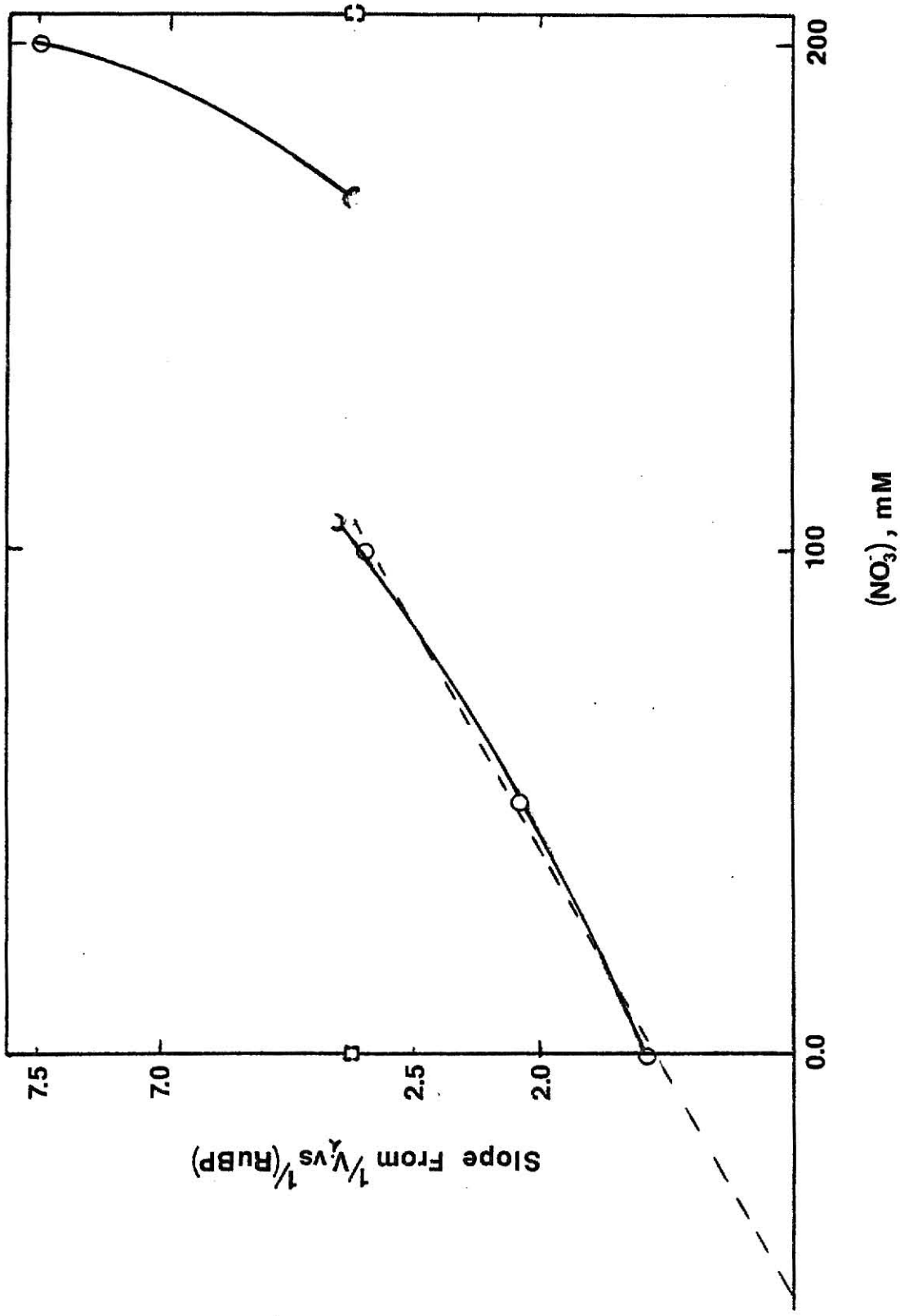

7.5
7.0
2.5
2.0
0.0
100
200
Slope From $^{1}/_{V_A}$ vs $^{1}/_{(RuBP)}$
(NO_3^-), mM

TABLE IV

Percentage Inhibition of RuBPcase Activity as a Function of Chloride and Bicarbonate Concentrations at Saturating Levels of RuBP.

	Chloride mM		
Bicarbonate mM	110	160	210
1	38	56	n.d.
10	28	37	53
20	n.d.	n.d.	19
40	n.d.	20	n.d.

n.d. = not determined

Preliminary CD Spectra of RuBPcase

The Chen reference spectra (Chen 1972) and poly-L-lysine reference spectra for pure α-helix, β-sheet and random coil in the far UV region are shown in Figures $5^{(a\&b)}$, respectively. The comparisons of the experimental far UV CD spectrum of RuBPcase with the best fit calculated (computer program of Dr. John R. Cann) from the Chen and poly-L-lysine far UV CD reference spectra are given in Figure $5^{(c\&d)}$. In the region 206-218 nm (Figure $5^{(c)}$) the experimental CD curve for RuBPcase has a more negative ellipticity than that of the theoretical Chen CD spectral fit, while in the region 218-250 nm the experimental CD curve has a more positive ellipticity. The experimental CD spectrum of RuBPcase (Figure $5^{(d)}$) seems to match better with that of the theoretically determined poly-L-lysine curve except in the 212 nm - 219 nm and 230 nm - 250 nm regions. The calculated % α-helix, β-sheet and random coil for RuBPcase employing both methods of analysis are listed in Table V.

Figure $5^{(e)}$ demonstrates the near UV CD spectra of RuBPcase which was due to the aromatic amino acid residues present in the enzyme. From this spectrum as many as nine distinct CD bands were observed between 256 nm - 268 nm and 278 nm - 294 nm.

The far UV CD spectra of RuBPcase was also measured in the absence and presence of $NaHCO_3$ (10 mM and 50 mM) in order to determine if conformational changes occurred in the enzyme with increasing bicarbonate concentrations (Figure $5^{(f)}$). It appeared that as the concentration of bicarbonate was increased conformational changes may have occurred which reduced the amount of α-helix as evidenced by the decreased ellipticities in the 206 - 234 nm region.

The far UV CD spectra of RuBPcase in the absence and presence of urea (2M, 5M, 6M) was also measured (Figure $5^{(g)}$). From Figure $5^{(g)}$ it was observed that as the urea concentration was increased the ellipticities became more positive in the 214 - 246 nm regions. This figure demonstrates the destruction of secondary structure that occurs in RuBPcase upon urea denaturation. At 6 M urea there was virtually no secondary structure remaining.

TABLE V

% α-Helix, β-Sheet and Random Coil as Determined by Chen Reference and Poly-L-Lysine Reference Spectra

Chen Theoretically Calculated		Poly-L-Lysine Theoretically Calculated	
Alpha	40%	Alpha	17%
Beta	38%	Beta	57%
Random coil	22%	Random coil	26%

Figure 5[(a)]. Chen Reference (1) Spectra for Pure α-Helix, β-Sheet and Random Coil.

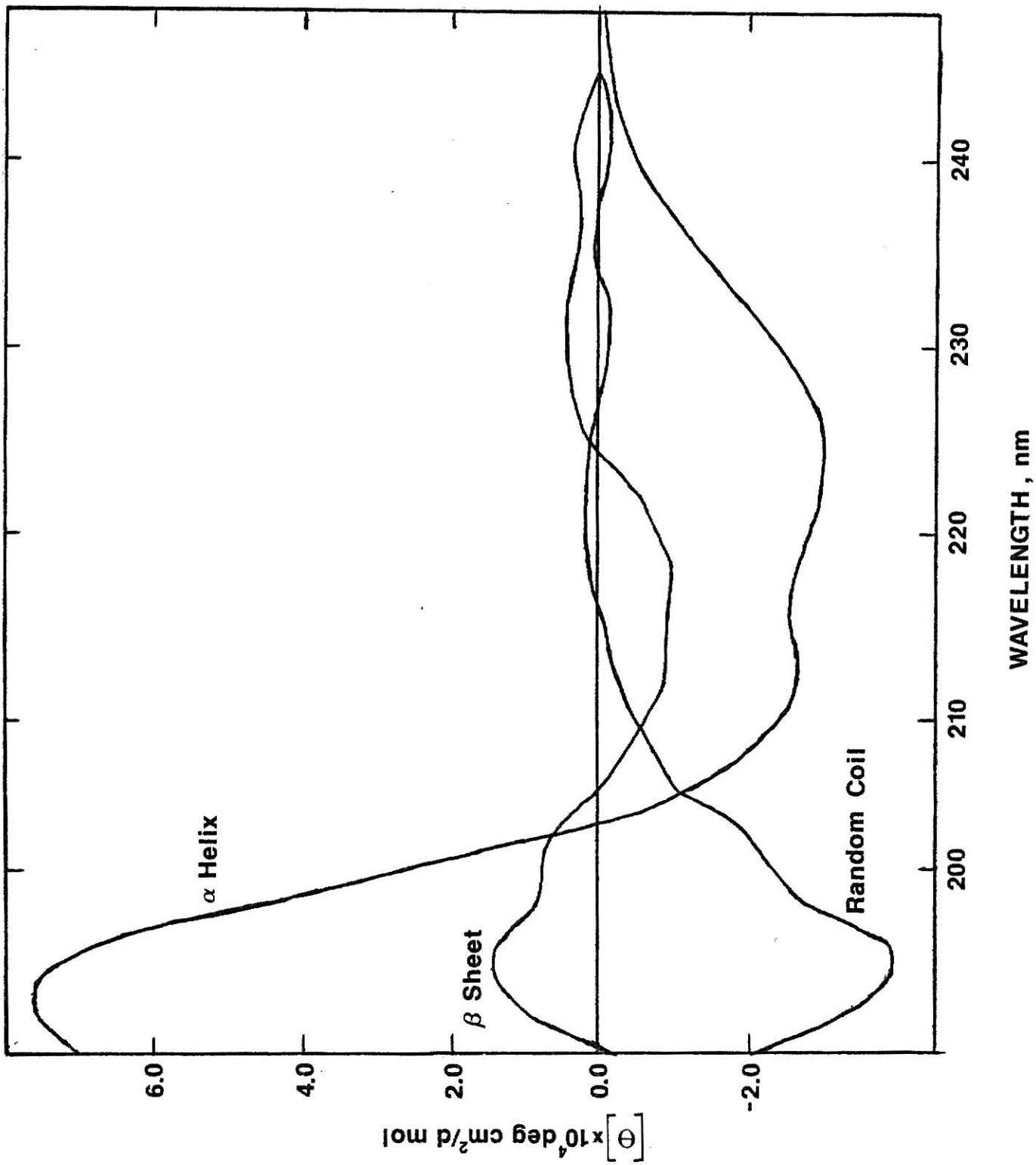

α Helix
β Sheet
Random Coil
6.0
4.0
2.0
0.0
-2.0
[Θ]x10⁴deg cm²/d mol
200
210
220
230
240
WAVELENGTH, nm

Figure 5(b). Poly-L-Lysine Reference (1) Spectra for Pure α-Helix, β-Sheet and Random Coil.

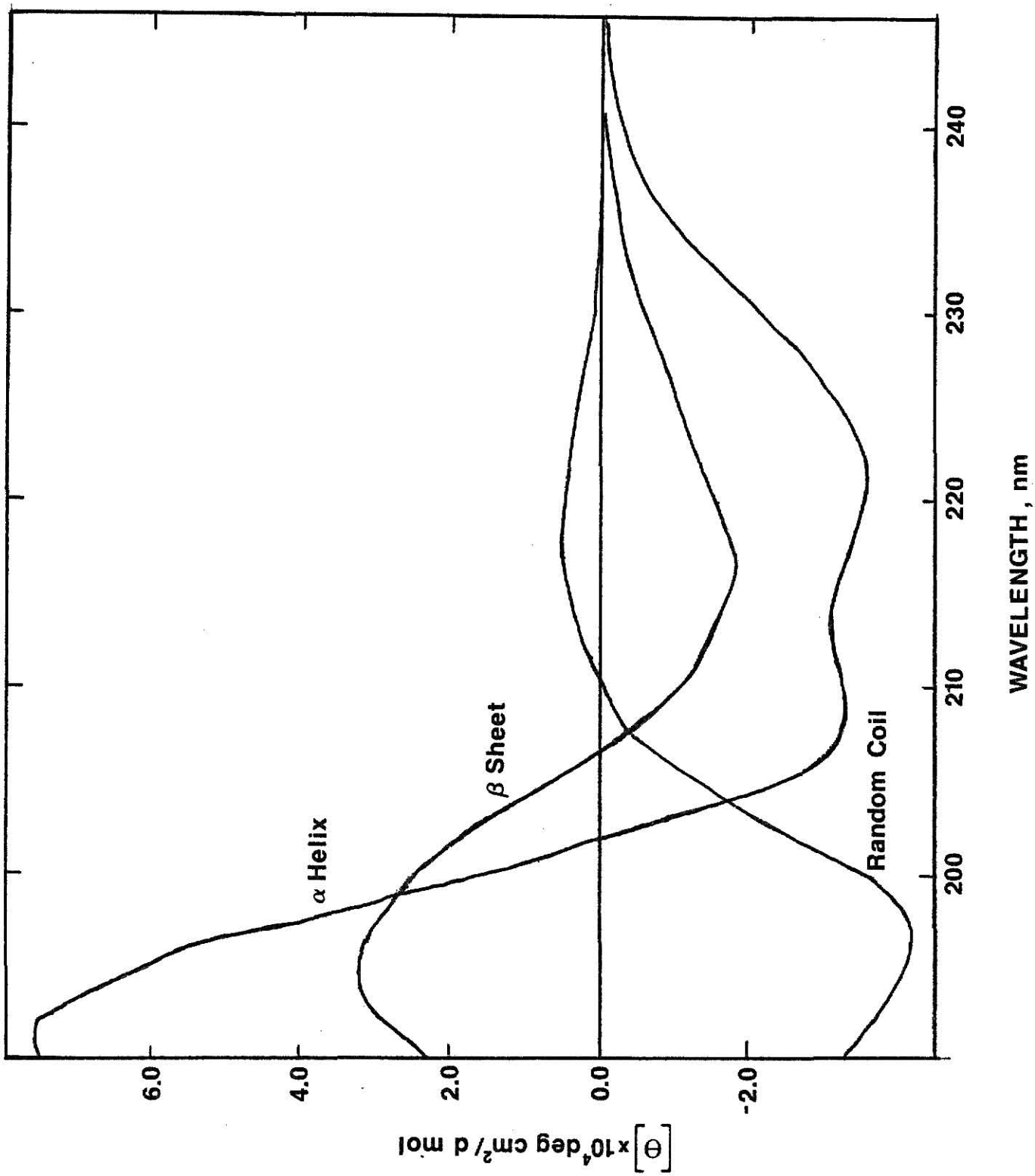

α Helix
β Sheet
Random Coil
6.0
4.0
2.0
0.0
-2.0
200
210
220
230
240
WAVELENGTH, nm
[Θ] x10⁴deg cm²/ d mol

Figure 5(c). Theoretical Chen far UV CD Spectral Fit and Experimental Far UV CD Curve for RuBPcase

a....Theoretical curve

b....Experimental curve

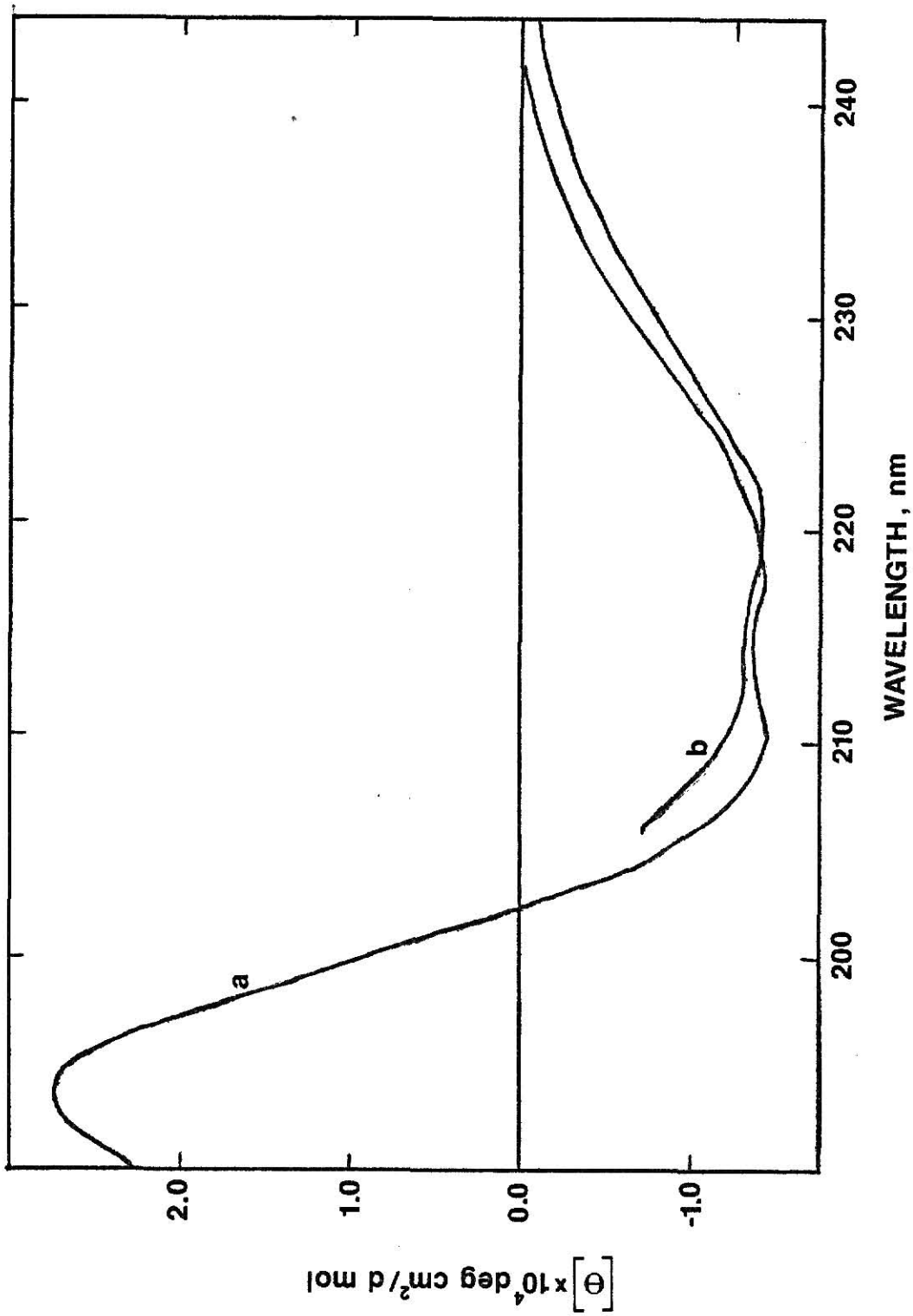

[Θ] x 10⁴ deg cm²/d mol
2.0
1.0
0.0
-1.0
200
210
220
230
240
WAVELENGTH, nm
a
b

Figure 5(d). Theoretical Poly-L-Lysine Far UV CD Spectral Fit and Experimental for UV CD Curve for RuBPcase

a....Theoretical curve

b....Experimental curve

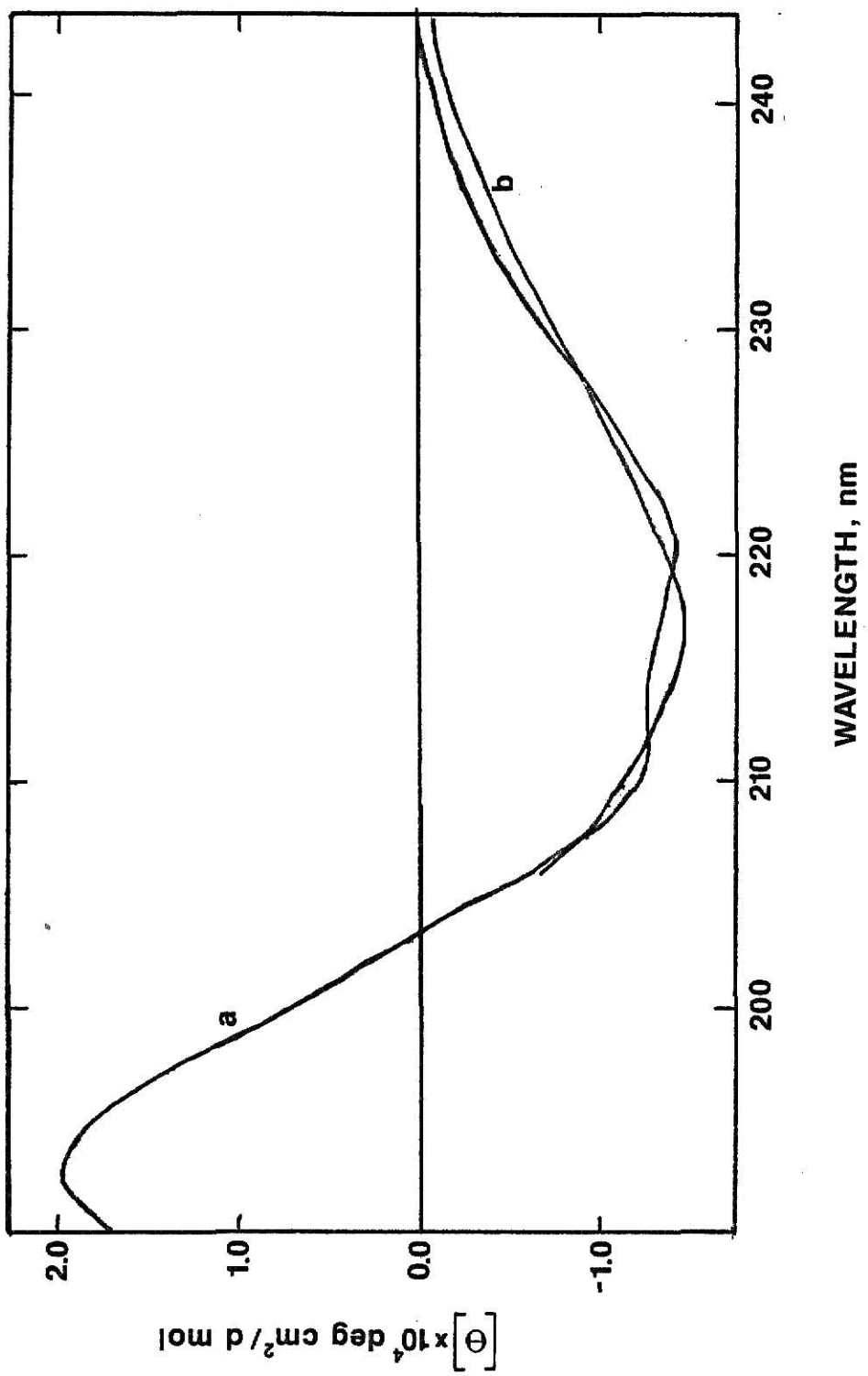

2.0
1.0
0.0
-1.0
200
210
220
230
240
a
b
WAVELENGTH, nm
[Θ]x10⁴ deg cm²/d mol

Figure 5(e). The Near UV CD Spectra (nm) of RuBPcase

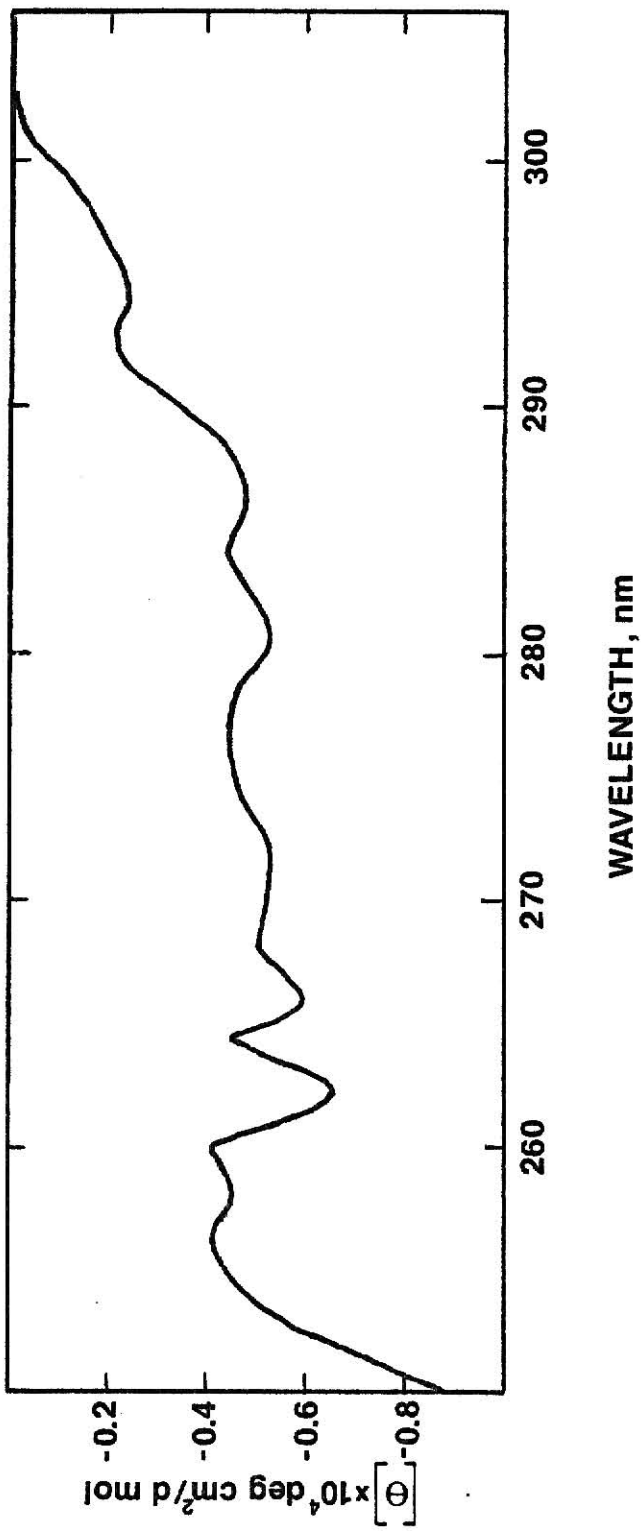

-0.2
-0.4
-0.6
-0.8
[Θ] x10⁴ deg cm²/d mol
260
270
280
290
300
WAVELENGTH, nm

Figure 5(f). The Far UV CD Spectra of RuBPcase in the Presence and Absence of Bicarbonate

Bicarbonate concentrations are shown as:(a) zero mM, (b) 10 mM and (c) 50 mM.

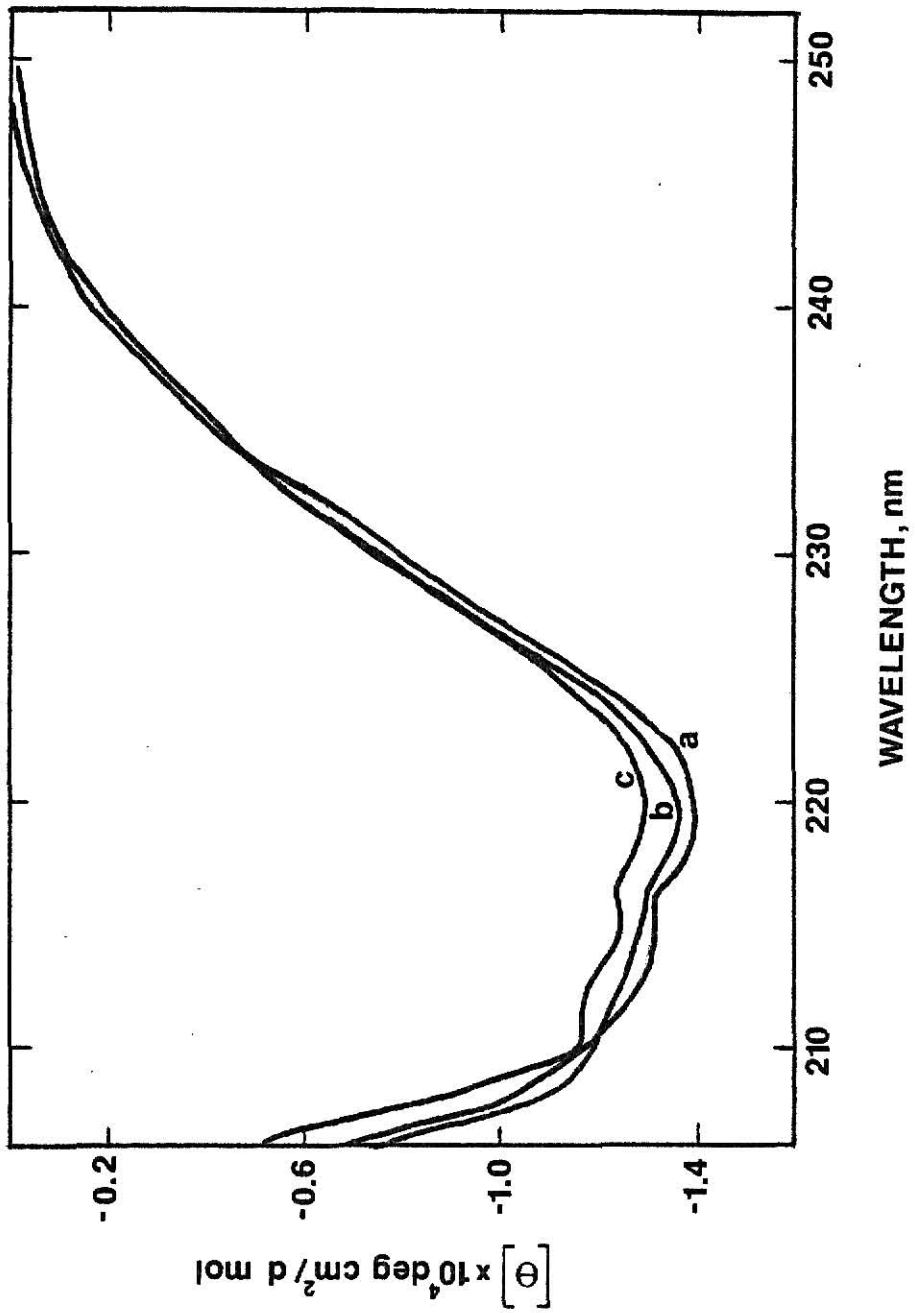
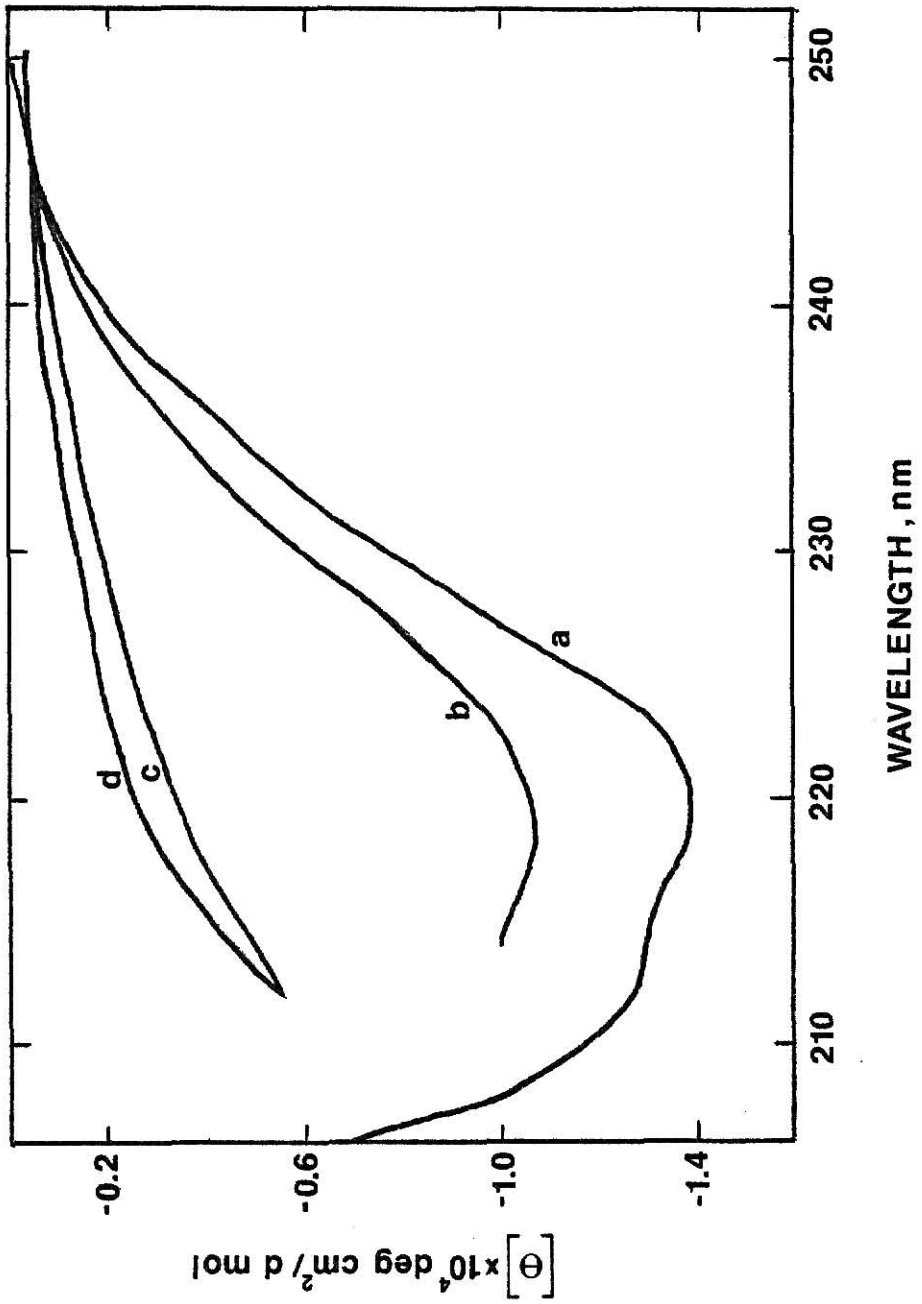

-0.2
-0.6
-1.0
-1.4
[Θ] x 10⁴deg cm²/d mol
210
220
230
240
250
WAVELENGTH, nm
a
b
c

Figure 5(g). The Far UV CD Spectra of RuBPcase in the Presence and Absence of Urea

Urea concentrations are shown as:(a) zero, (b) 2 M, (c) 5 M, (d) 6M.


-0.2
-0.6
-1.0
-1.4
210
220
230
240
250
a
b
c
d
WAVELENGTH, nm
[Θ] x10⁴ deg cm²/ d mol

CONCLUSIONS

SDS polyacrylamide electrophoresis data on the crude extract of RuBPcase from comfrey revealed six discernible protein bands in addition to the large and small subunits of RuBPcase. Five of these extraneous bands remained in the 40% ammonium sulfate supernatant and only one co-precipitated with the 40% pellet. Since the one extra band in the pellet was readily separated from RuBPcase in the sucrose density gradient step, it was not necessary to make an ammonium sulfate cut prior to application of the redissolved pellet to the gradient. The comfrey enzyme appears to be unique among the higher plant enzymes in this regard. Density gradient centrifugation alone, however, did not appear to yield a truly homogeneous preparation since another band was apparent in the pooled fractions. Its molecular weight (60.8 K daltons) does not match very well any of those seen at the earlier stages of purification. Its molecular weight is, however, close to that of one large and one small subunit together and could possibly represent some residual quarternary structure stabilized against dissociation with SDS by sucrose. If this were the case, the enzyme would be homogeneous after density gradient centrifugation. Very heavily loaded gels, however, show a band running a little ahead of the large subunit which is removed by DEAE in cellulose chromatography. In any event the DEAE step was used routinely and does yield a homogeneous product as judged by SDS polyacrylamide gel techniques.

The molecular weights of both large and small subunits of RuBPcase were determined by SDS PAGE techniques. The molecular weights were calculated to be 50,000 daltons and 12,700 daltons for

large and small subunits respectively. These values were found to be in excellent agreement with the 50,000 and 12,000 dalton values obtained by Simpson (1980) on a different preparation and using disc gel techniques.

The Km values for RuBP from all determinations ranged from 21 μM to 133 μM with an average value of 56 μM, whereas the average of the Km values for RuBP from the low nitrate data alone averaged 46 μM. The value obtained by Simpson (1980) was listed as 250 μM determined on the enzyme from the pooled sucrose fractions. These differences in Km values could be due to the different states of purity of the enzyme.

Inhibition patterns for RuBPcase with chloride and nitrate determined from Lineweaver-Burk plots were suggestive of mixed competitive and noncompetitive inhibition. Computer analysis by Cleland's method for competitive and noncompetitive inhibition patterns gave very similar variance values for chloride and for nitrate inhibition, respectively. The replots for both chloride and nitrate were found to be essentially linear at low concentrations and to curve upward strongly at high concentrations thus forming concave curves instead of the convex curves which should be obtained from normal mixed inhibition patterns. Traditionally upward curvature is taken as an indication of multiple binding sites. The Ki value obtained for chloride and nitrate from the linear portion of the replot curve were calculated to be 285 mM and 47 mM, respectively. The Ki values obtained from Cleland's method for noncompetitive and competitive inhibition patterns for chloride were 139 mM and 157 mM, while those for nitrate were 21 mM and 46 mM, respectively. Therefore,

the inhibition patterns for both nitrate and chloride were found to be similar but nitrate was the more potent inhibitor. It should be noted that ionic strength experiments reported by Simpson (1980) revealed that specific activity dropped sigmoidally with increasing NaCl concentration. It is possible that these replot curves which were obtained for chloride and nitrate could also be suggesting a sigmoidal effect. As a result it was concluded that since chloride and nitrate demonstrated similar types of i-hibition patterns, which did not seem to follow straightforward kinetics, that some type of conformational change might be occurring within the enzyme which caused a decrease in its activity.

The data for bicarbonate inhibition as a function of pH clearly showed that there was a decrease in activity as bicarbonate concentrations were increased above saturation levels. It is believed that since bicarbonate and nitrate are both trigonal planar monovalent anions, bicarbonate inhibition could possible occur at the same site as that of nitrate and in turn chloride. The fact that chloride and bicarbonate ions are competitive was shown by the ability of bicarbonate to overcome the inhibition of chloride, although it was in itself a more potent inhibitor. As a result it was concluded that chloride, nitrate and bicarbonate were binding to an anion site which altered enzymatic activity substantially.

To determine if this loss of activity was due to a conformational change in the enzyme upon anion binding Bolden (unpublished) performed divverential UV spectroscopy on RuBPcase in 267, 367, and 667 mM NaCl (Figure 6). Bolden discovered that there was clearly a change in ΔA at the 280 nm region as salt concentration was increased.

This suggests the binding of chloride occurs at a specific anion site involving one or more tyrosine residues. Another study utilized CD spectra of RuBPcase in the presence and absence of bicarbonate anion. Comparisons of the experimental far UV CD spectra of RuBPcase with the reference spectra Chen and from poly-L-lysine demonstrate that the poly-L-lysine computations gave a better fit (Figure $5^{(c\&d)}$). The far UV CD spectra of RuBPcase in the absence and presence of bicarbonate (Figure 5^{f}) shows that as the bicarbonate concentration was increased the ellipticities became less negative which implies a loss of secondary structure. As a result the CD spectra support the hypothesis of a conformational change upon binding of an anion to a specific site. At least part of the ΔA_{280} observed upon anion binding may also reflect this conformational change.

The near UV CD spectra of RuBPcase (Figure 5^{e}) gave distinct CD bands between 256 nm - 268 nm and 278 nm - 294 nm. It might be valuable to repeat the spectrum together with varying bicarbonate concentrations in order to observe specific conformational changes involving tertiary structure of the enzyme with respect to aromatic amino acid residues.

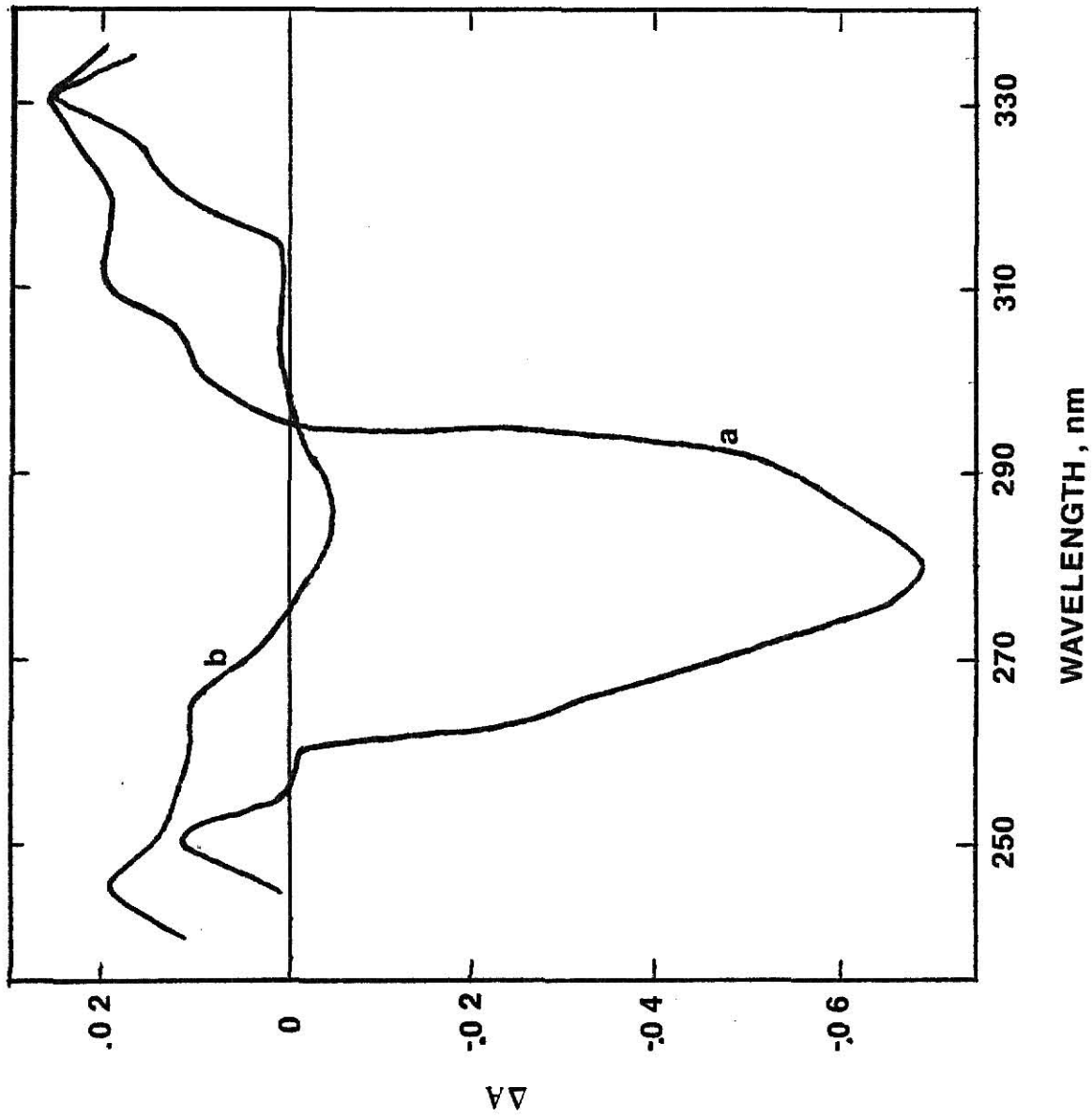
The far UV CD spectra of urea denaturation with respect to RuBPcase (Figure 5^{f}) showed that as urea concentrations were increased the secondary structure of RuBPcase decreased. This was demonstrated by more positive ellipticity values with increasing urea concentration. It also should be noted that all CD work was just preliminary and should be repeated on at least one other preparation.

Figure 6. Differential UV Spectroscopy on RuBPcase in the Presence of NaCl.

A baseline containing 267 mM NaCl was used to determine ΔA at 367 mM and 667 mM NaCl.

a....667 mM NaCl

b....367 mM NaCl


.0 2
0
-0.2
-0.4
-0.6
ΔA
250
270
290
310
330
WAVELENGTH , nm
a
b

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APPENDIX A

TABLE 1

Determination of Chloride Inhibition of RuBPcase

(Amounts of substrate, buffer and chloride)

Substrate (nmoles)/540 µl	Amounts of Buffer (µl)				
	No added Cl^- (67.56 mM Cl^-)*	92 mM Cl^- (52.55 µl of stock)	167 mM Cl^- (214.55 µl of stock)	279 mM Cl^- (457.55 µl of stock)	392 mM Cl^- (700.55µl of stock)
1.838	4,137.5	4,084.9	3,922.9	3,679.9	3,436.9
3.678	4,117.9	4,065.4	3,903.3	3,660.3	3,417.3
7.350	4,075.2	4,022.6	3,860.6	3,617.6	3,374.6
15.32	4,143.5	4,090.9	3,928.9	3,685.9	3,442.9
30.64	4,126.4	4,073.9	3,911.9	3,668.9	3,425.9

* 67.56 mM chloride which was already present was due to Tris-chloride, $MgCl_2$ buffer and further purification from DEAE cellulose using .2 m NaCl.

TABLE II

Quenching Times for Chloride Inhibition Study

Sample	Time Seconds				
A (67.56mM)	17	27	37	47	57
B	9	19	29	39	49
C	10	20	30	40	50
D	10	20	30	40	50
E	11	21	31	41	51
F (92 mM)	13	23	33	43	53
G	10	16	30	40	50
H	11	21	31	41	51
I	8	18	28	38	48
J	11	21	31	41	51
K (167 mM)	15	25	35	45	55
L	12	22	32	42	52
M	10	20	30	40	50
N	10	20	30	40	50
O	10	20	30	40	50
P (279 mM)	12	22	32	42	52
Q	13	23	33	43	53
R	11	21	31	41	51
S	10	20	30	40	50
T	10	20	30	40	50
U (392 mM)	10	20	30	40	50
V	11	21	31	41	51
W	10	20	30	40	50
X	10	20	30	40	50
Y	10	20	30	40	50

TABLE III[(a)]

Determination of Nitrate Inhibition with RuBPcase

(Amounts of buffer, salt and substrate added)

Substrate (nmoles)/540µl	Amounts of Buffer (µl)				
	No added $NaNO_3^-$ (31.59 mM Cl^-)*	50 mM $NaNO_3^-$ (47.25 µl of stock)	100 mM $NaNO_3^-$ (94.50 µl of stock)	200 mM $NaNO_3^-$ (189 µl of stock)	400 mM $NaNO_3^-$ (378 µl of stock)
1.838	3,647.6	4,155.5	3,553.1	3,458.6	3,269.6
3.678	3,628.9	4,135.9	3,534.4	3,439.9	3,250.9
7.350	3,591.6	4,093.2	3,497.1	3,402.6	3,213.6
15.32	3,651.3	4,161.5	3,556.8	3,462.3	3,273.3
30.64	3,636.4	4,144.4	3,541.9	3,447.4	3,258.4

*The amount of Cl^- present due to Tris chloride, $MgCl_2$ buffer and resulting from further purification by DEAE cellulose (where .2 m NaCl was used to wash off the protein) was 31.59 mM.

TABLE III[b]

Quenching times for Nitrate Inhibition Study

Sample	Time (Seconds)				
A (0 mM NO_3^-)	6	16	26	36	46
B	5	15	25	35	45
C	5	15	25	35	45
D	6	16	26	36	46
E	5	15	25	35	45
F (50 mM NO_3^-)	6	16	26	36	46
G	5	15	25	35	45
H	5	15	25	35	45
I	5	15	25	35	45
J	5	15	25	35	45
K (100 mM NO_3^-)	6	16	26	36	46
L	5	15	25	35	45
M	5	15	25	35	45
N	5	15	25	35	45
O	5	15	25	35	45
P (200mM NO_3^-)	5	15	25	35	45
Q	5	15	25	35	45
R	5	15	25	35	45
S	5	15	25	35	45
T	5	15	25	35	45
U (400mM NO_3^-)	5	15	25	35	45
V	6	16	26	36	46
W	5	15	25	35	45
X	5	15	25	35	45
Y	5	15	25	35	45

TABLE IV[a]

Km Determination for RuBP (Amounts of buffer, salt and substrate added)

Substrate (nmoles) per 540 µl	Amounts of Buffer (µl)							
	No added $NaNO_3^-$	1.25mM $NaNO_3^-$ (5.4µl stock)	2.5mM $NaNO_3^-$ (10.8µl stock)	3.75mM $NaNO_3^-$ (16.2µl stock)	6.25mM $NaNO_3^-$ (27 µl stock)	No added $NaNO_3^-$	10 mM $NaNO_3^-$ (37.8µl stock)	20mM $NaNO_3^-$ 75.6µl stock
1.838	4,160.9	4,155.5	4,150.1	4,144.7	4,133.9	3,623.9	3,586.1	3,548.3
3.678	4,141.3	4,135.9	4,130.5	4,125.1	4,114.3	3,605.1	3,567.3	3,529.5
7.350	4,098.6	4,093.2	4,087.8	4,082.4	4,071.6	3,567.8	3,529.8	3,482.2
15.32	4,166.9	4,161.5	4,156.1	4,150.7	4,139.9	3,576.3	3,538.5	3,500.7
30.64	4,149.8	4,144.4	4,139.0	4,133.6	4,122.8	3,510.1	3,472.3	3,434.5

TABLE IV(b)

Quenching Times for Ribulose-1,5-Bisphosphate Km Study

Sample	Time Seconds				
A (0 mM NO_3^-)	11	21	31	41	51
B	11	21	31	41	51
C	8	18	28	38	48
D	7	17	27	37	47
E	7	17	27	37	47
F (1.25 mM NO_3^-)	10	20	30	40	50
G	11	21	31	41	51
H	8	18	28	38	48
I	8	18	28	38	48
J	7	17	27	37	47
K (2.50mM NO_3^-)	9	19	29	39	49
L	10	20	30	40	50
M	7	17	27	37	47
N	7	17	27	37	47
O	7	17	27	37	47
P (3.75mM NO_3^-)	9	19	29	39	49
Q	10	20	30	40	50
R	7	17	27	37	47
S	7	17	27	37	47
T	8	18	28	38	48
U (6.25mM NO_3^-)	8	18	28	38	48
V	10	20	30	40	50
W	6	16	26	36	46
X	7	17	27	37	47
Y	7	17	27	37	47

TABLE IV(c)

Quenching Times for Ribulose-1,5-Bisphosphate Km Study

Sample	Time Seconds				
A (0 mM NO_3^-)	7	17	27	37	47
B	7	17	27	37	47
C	6	16	26	36	46
D	6	16	26	36	46
E	5	15	25	35	45
F (10 mM NO_3^-)	5	15	25	35	45
G	6	16	26	36	46
H	5	15	25	35	45
I	5	15	25	35	45
J	5	15	25	35	45
K (20 mM NO_3^-)	5	15	25	35	45
L	6	16	26	36	46
M	4	14	24	34	44
N	5	15	25	35	45
O	5	15	25	35	45

TABLE V

pH Dependence of Bicarbonate Inhibition on RuBPcase

Total Bicarbonate Concentration (mM)	Hot Bicarbonate Concentration (mM)	Cold Bicarbonate Concentration (mM)	Buffer (Added to pool) (10 mM Tris 10 mM $MgCl_2$) .1 mM EDTA xmM CBC & HBC) µl
20	2 mM	18	1,013.6
30	3	27	1,005.5
40	4	36	996.8
50	5	45	988.1

*Note: All solutions were made at pH = 7.5, pH = 7.8 or pH = 8.1

APPENDIX B

TABLE VI

Data for Km of RuBP (cpm vs time)

(a) zero nitrate

Time (Sec)	2.97 μM (RuBP)	Time (Sec)	5.94 μM (RuBP)	Time (Sec)	11.9 μM (RuBP)	Time (Sec)	24.7 μM (RuBP)	Time (Sec)	49.5 μM (RuBP)
11	406.5 cpm	11	505.8 cpm	8	580 cpm	7	270.6 cpm	7	324.9 cpm
21	455.1 cpm	21	604.4 cpm	18	678.4 cpm	17	420.2 cpm	17	614.7 cpm
31	471.7 cpm	31	654.8 cpm	28	785 cpm	27	569.7 cpm	27	954.3 cpm
41	528.4 cpm	41	680.2 cpm	38	902.1 cpm	37	735.7 cpm	37	1275.4 cpm
51	531.3 cpm	51	729.9 cpm	48	941.3 cpm	47	880.3 cpm	47	1616.6 cpm
Slope	3.229		5.240		9.463		15.35		32.44
Y Int	378.50		427.6		512.4		160.9		81.27
X Int	-117.2		-90.19		-54.15		-10.48		-2.505
CC	.9711		.9719		.9907		.9998		.9996

TABLE VI Continued

(b) 1.25 mM Nitrate

Time (Sec)	2.97 µM (RuBP)	Time (Sec)	5.94 µM (RuBP)	Time (Sec)	11.9 µM (RuBP)	Time (Sec)	24.7 µM (RuBP)	Time (Sec)	49.5 µM (RuBP)
10	427.3 cpm	11	526.4 cpm	8	573.1 cpm	8	294.4 cpm	7	325.1 cpm
20	461.4 cpm	21	606.1 cpm	18	652.7 cpm	18	400.3 cpm	17	573.2 cpm
30	492.2 cpm	31	658.3 cpm	28	793.9 cpm	28	589.0 cpm	27	905.7 cpm
40	511.0 cpm	41	704.0 cpm	38	899.8 cpm	38	752.5 cpm	37	1230.6 cpm
50	532.7 cpm	51	749.2 cpm	48	979.2 cpm	48	914.2 cpm	47	1497.9 cpm
Slope	2.604		5.431		10.59		15.92		30.03
X Int	406.8		480.5		483.1		144.4		95.69
Y Int	-156.2		-88.47		-45.61		-9.070		-3.187
CC	.9921		.9918		.9955		.9971		.9988

TABLE VI Continued

(c) 2.5 mM Nitrate

Time (Sec)	2.97 µM (RuBP)	Time (Sec)	5.94 µM (RuBP)	Time (Sec)	11.9 µM (RuBP)	Time (Sec)	24.7 µM (RuBP)	Time (Sec)	49.5 µM (RuBP)
9	453.4 cpm	10	503.8 cpm	7	552.1 cpm	7	279.0 cpm	7	302.2 cpm
19	445.0 cpm	20	585.6 cpm	17	641.4 cpm	17	444.8 cpm	17	596.3 cpm
29	483.0 cpm	30	647.9 cpm	27	751.3 cpm	27	627.6 cpm	27	919.9 cpm
39	499.3 cpm	40	685.9 cpm	37	901.9 cpm	37	825.7 cpm	37	1230.3 cpm
49	523.7 cpm	50	743.9 cpm	47	954.7 cpm	47	947.4	47	1509.8 cpm
Slope	3.949		5.805		10.66		17.18		29.93
X Int	346.4		459.3		472.5		161.1		109.1
Y Int	-87.71		-79.116		-44.34		-9.380		-3.646
CC	.9382		.9918		.9920		.9977		.9995

TABLE VI Continued

3.75 mM Nitrate

(d)

Time (Sec)	2.97 µM (RuBP)	Time (Sec)	5.94 µM (RuBP)	Time (Sec)	11.9 µM (RuBP)	Time (Sec)	24.7 µM (RuBP)	Time (Sec)	49.5 µM (RuBP)
9	417.1 cpm	10	523.6 cpm	7	549.3 cpm	7	303.8 cpm	8	339.2 cpm
19	460.0 cpm	20	576.4 cpm	17	478.8 cpm	18	623.8 cpm	18	623.2 cpm
29	489.0 cpm	30	633.8 cpm	27	761.4 cpm	27	737.0 cpm	28	913.3 cpm
39	538.4 cpm	40	693.7 cpm	37	874.7 cpm	37	953.6 cpm	38	122.6 cpm
49	545.1 cpm	50	744.4 cpm	47	930.9 cpm	47	1066.6 cpm	48	1495.5 cpm
Slope	3.344		5.585		9.945		20.00		29.12
Y Int	392.9		466.9		483.4		167.9		103.4
X Int	-117.5		-83.60		-48.61		-8.391		-3.551
CC	.9821		.9997		.9944		.9932		.9999

TABLE VI Continued

(e) 6.25 mM Nitrate

Time (Sec)	2.97 µM (RuBP)	Time (Sec)	5.94 µM (RuBP)	Time (Sec)	11.9 µM (RuBP)	Time (Sec)	24.7 µM (RuBP)	Time (Sec)	49.5 µM (RuBP)
8	418.2 cpm	10	525.6 cpm	6	522.6 cpm	7	328.6 cpm	7	244.3 cpm
18	445.5 cpm	20	591.8 cpm	16	654.1 cpm	17	583.3 cpm	17	330.8 cpm
28	475.8 cpm	30	641.3 cpm	26	746.7 cpm	27	791.8 cpm	27	438.4 cpm
38	515.0 cpm	40	669.7 cpm	36	878.8 cpm	37	1028.5 cpm	37	537.5 cpm
48	505.3 cpm	50	698.4 cpm	46	955.0 cpm	47	1196.1 cpm	47	649.7 cpm
Slope	2.437		4.235		10.90		21.80		3.131
Y Int	403.7		498.3		468.2		197.0		367.4
X Int	-165.7		-117.7		-42.97		-9.036		-117.4
CC	.9508		.9811		.9966		.9979		.9948

TABLE VI Continued

(f) Zero Nitrate

Time (Sec)	2.97 µM (RuBP)	Time (Sec)	5.94 µM (RuBP)	Time (Sec)	11.9 µM (RuBP)	Time (Sec)	24.7 µM (RuBP)	Time (Sec)	49.5 µM (RuBP)
7	384.2 cpm	7	386.5 cpm	10	398.5 cpm	6	438.0 cpm	5	434.4 cpm
17	428.0 cpm	17	446.6 cpm	20	521.5 cpm	16	616.7 cpm	15	669.2 cpm
27	453.5 cpm	27	473.0 cpm	30	650.9 cpm	26	759.7 cpm	25	993.9 cpm
37	478.9 cpm	37	530.2 cpm	40	749.6 cpm	36	863.7 cpm	35	1277.1 cpm
47	515.3 cpm	47	587.0 cpm	50	837.5 cpm	46	1144.2 cpm	45	1668.8 cpm
Slope	3.131		4.846		10.98		16.59		30.77
Y Int	367.4		353.8		345.3		333.0		239.5
X Int	-117.4		-73.01		-31.44		-20.07		7.785
CC	.9948		.9940		.9962		.9873		.9966

TABLE VI Continued

10 mM Nitrate

(g)

Time (Sec)	2.97 µM (RuBP)	Time (Sec)	5.94 µM (RuBP)	Time (Sec)	11.9 µM (RuBP)	Time (Sec)	24.7 µM (RuBP)	Time (Sec)	49.5 µM (RuBP)
5	369.9 cpm	6	391.4 cpm	5	358.6 cpm	5	432.1 cpm	5	450.2 cpm
15	392.8 cpm	16	455.7 cpm	15	364.2 cpm	15	579.4 cpm	15	370.6 cpm
25	447.2 cpm	26	505.5 cpm	25	374.4 cpm	25	786.9 cpm	25	962.1 cpm
35	489.2 cpm	36	581.3 cpm	35	417.3 cpm	35	1024.4 cpm	35	1291.4 cpm
45	492.7 cpm	46	629.5 cpm	45	419.2 cpm	45	1258.4 cpm	45	1603.0 cpm
Slope	3.420		5.998		1.743		20.98		30.26
Y Int	352.9		356.5		343.2		211.8		218.9
X Int	-103.2		-59.44		-196.9		-13.91		-7.232
CC	.9721		.9979		.9398		.9962		.9905

TABLE VI Continued

(h) 20 mM Nitrate

Time (Sec)	2.97 µM (RuBP)	Time (Sec)	5.94 µM (RuBP)	Time (Sec)	11.9 µM (RuBP)	Time (Sec)	24.7 µM (RuBP)	Time (Sec)	49.5 µM (RuBP)
5	356.6 cpm	6	371.5 cpm	4	365.7 cpm	5	378.1 cpm	5	448.2 cpm
15	366.6 cpm	16	465.0 cpm	14	358.1 cpm	15	442.3 cpm	15	683.0 cpm
25	371.5 cpm	26	468.7 cpm	24	389.3 cpm	25	725.2 cpm	25	985.3 cpm
35	376.8 cpm	36	571.2 cpm	34	400.4 cpm	35	964.8 cpm	35	1252.3 cpm
45	377.1 cpm	46	552.2 cpm	44	401.1 cpm	45	1458.4 cpm	45	1626.6 cpm
Slope	.5120		4.676		1.131		26.83		29.26
Y Int	356.9		364.1		355.8		123.0		267.6
X Int	-697.1		-77.88		-314.6		-4.584		-9.144
CC	.9514		.9261		.8971		.9657		.9967

TABLE VI Continued

Km Data for Nitrate

(i)

(1/S vs 1/Vi)

Substrate (μM)	1/S	1/Vi $(^{cpm}/_{sec})^{-1}$							
		0 mM NO_3^-	1.25 mM NO_3^-	2.5 mM NO_3^-	3.75 mM NO_3^-	6.25 mM NO_3^-	0mM NO_3^-	10mM NO_3^-	20 mM NO_3^-
2.966	.3371	.3097	.3840	.2532	.2990	.4103	.3194	.2924	.3031
5.936	.1685	.1908	.1841	.1723	.1791	.2361	.2064	.1667	.2139
11.863	.0843	.1057	.0944	.0939	.1006	.0918	.0911	(.5737)	(.7634)
24.726	.0404	.0652	.0628	.0582	.0500	.0459	.0603	.0477	.0373
49.452	.0202	.0308	.0333	.0334	.0343	.0983	.0325	.0330	.0342
	Slope	.8620	1.0962	.6889	.8402	.6125	.9151	.8267	.8935
	Y Int	.0283	.0091	.0326	.0233	.0301	.0229	.0174	.0206
	X Int	-.0328	-.0083	-.0473	-.0277	-.0267	-.0250	-.0217	-.0231
	CC	.9937	.9983	.9863	.9959	.9753	.9897	.9986	.9757

Note: ( ) = not used in calculations

TABLE VII

Vi (cpm/sec) Extrapolated to Zero Chloride Concentration

(a)

Cl^- (mM) \ Substrate (μM)	2.966	5.936	11.86	24.73	49.45
65.56	4.354	8.461	17.68	37.14	62.78
92	4.775	7.374	17.0	33.16	56.30
167	3.621	6.652	13.39	25.84	52.98
279	2.613	5.315	7.014	13.95	34.84
392	.9537	1.716	3.508	7.233	13.99
Slope	-.0110	-0.186	-.0457	0.924	-1.438
Y Int	5.462	9.615	20.81	41.87	72.82
X Int	494.6	515.9	455.3	453.0	506.2
CC	-.9816	-.9726	-.9946	-.9928	-.9874

TABLE VII Continued

(b) Initial Vi at Zero Chloride

Substrate (nm)	1.838	3.678	7.350	15.32	30.64
Vi	5.462	9.615	20.81	41.87	72.82

(c) 1/S vs 1/Vi for Chloride

Substrate (μM)	1/S	1/Vi (cpm/Sec)$^{-1}$
		Zero mM Cl^-
2.966	.3371	.1831
5.936	.1685	.1040
11.863	.08430	.04054
24.726	.04044	.02398
49.452	.02022	.01373
	Slope	.5469
	Y Int	.0019
	X Int	-.0035
	CC	.9959

TABLE VIII

Determination of Anion Inhibition of RuBPcase: Chloride Inhibition (cpm vs time)

No added chloride (67.56 mM)

(a)

Time (Sec)	2.97 μM (RuBP)	Time (Sec)	5.94 μM (RuBP)	Time (Sec)	11.9 μM (RuBP)	Time (Sec)	24.7 μM (RuBP)	Time (Sec)	49.5 μM (RuBP)
17	507.4 cpm	9	587.4 cpm	10	673.1 cpm	10	775.8 cpm	11	948.1 cpm
27	581.2 cpm	19	681.6 cpm	20	906.0 cpm	20	1190.4 cpm	21	1577.7 cpm
37	625.0 cpm	29	792.0 cpm	20	1109.6 cpm	30	1629.5 cpm	31	2287.9 cpm
47	657.6 cpm	39	888.9 cpm	40	1271.7 cpm	40	1956.2 cpm	41	2847.2 cpm
57	686.9 cpm	49	906.8 cpm	50	1374.1 cpm	50	2250.0 cpm	51	3452.6 cpm
Y Int	450.5		8.461		17.68		37.14		62.78
Slope	4.354		526.0		536.4		446.1		276.4
ST DV	16.23		31.06		46.71		57.59		43.12

TABLE VIII Continued

(b) 92 mM Chloride

Time (Sec)	2.97 µM (RuBP)	Time (Sec)	5.94 µM (RuBP)	Time (Sec)	11.9 µM (RuBP)	Time (Sec)	24.7 µM (RuBP)	Time (Sec)	49.5 µM (RuBP)
13	486.8 cpm	10	528.2 cpm	11	676.1 cpm	8	680.1 cpm	11	948.4 cpm
23	561.3 cpm	16	585.3 cpm	21	892.4 cpm	18	1045cpm	21	1520 cpm
33	632.5 cpm	30	694.4 cpm	31	1086.7 cpm	28	1423 cpm	31	2164 cpm
43	645.2 cpm	40	773.5 cpm	41	1222.8 cpm	38	1742 cpm	41	2741 cpm
53	683.6 cpm	50	818.1 cpm	51	1361.0 cpm	48	1989 cpm	51	3153 cpm
Y Int	444.3		7.374		17.00		33.16		56.30
Slope	4.775		464.6		520.7		447.3		359.7
ST DV	22.96		14.25		34.03		48.13		73.60

TABLE VIII Continued

167 mM chloride

(c)

Time (Sec)	2.97 µM (RuBP)	Time (Sec)	5.94 µM (RuBP)	Time (Sec)	11.9 µM (RuBP)	Time (Sec)	24.7 µM (RuBP)	Time (Sec)	49.5 µM (RuBP)
15	483.1 cpm	11	544.3 cpm	10	604.3 cpm	10	695.1 cpm	10	887.1 cpm
25	536.2 cpm	21	620.4 cpm	20	749.4 cpm	20	955.8 cpm	20	1342 cpm
35	580.2 cpm	31	703.0 cpm	30	908.5 cpm	30	1234 cpm	30	1948 cpm
45	601.5 cpm	41	750.2 cpm	40	998.6 cpm	40	1503 cpm	40	2447 cpm
55	631.5	51	812.0 cpm	50	1149 cpm	50	1714 cpm	50	2984 cpm
Y Int	439.8		473.1		480.34		445.02		332.0
Slope	3.621		6.652		13.39		25.84		52.98
ST DV	11.54		11.83		19.16		21.96		35.97

TABLE VIII Continued

279 mM Chloride

(d)

Time (Sec)	2.97 µM (RuBP)	Time (Sec)	5.94 µM (RuBP)	Time (Sec)	11.9 µM (RuBP)	Time (Sec)	24.7 µM (RuBP)	Time (Sec)	49.5 µM (RuBP)
12	444.2 cpm	13	488.0 cpm	11	514.2 cpm	10	570.7 cpm	10	807.0 cpm
22	470.9 cpm	23	542.9 cpm	21	578.0 cpm	20	712.9 cpm	20	1154 cpm
32	511.0 cpm	33	587.2 cpm	31	662.0 cpm	30	896.9 cpm	30	1545 cpm
42	528.4 cpm	43	648.6 cpm	41	627.2 cpm	40	986.4 cpm	40	1779 cpm
52	546.1 cpm	53	700.9 cpm	51	790.3 cpm	50	1132 cpm	50	2237 cpm
Y Int	416.5		418.1		436.9		441.1		459.1
Slope	2.613		5.315		7.014		13.95		34.84
ST DV	7.861		4.157		6.408		24.17		52.97

TABLE VIII Continued

392 mM Chloride

(e)

Time (Sec)	2.97 µM (RuBP)	Time (Sec)	5.94 µM (RuBP)	Time (Sec)	11.9 µM (RuBP)	Time (Sec)	24.7 µM (RuBP)	Time (Sec)	49.5 µM (RuBP)
10	460.8 cpm	11	451.3 cpm	10	451.8 cpm	10	490.1 cpm	10	609.0 cpm
20	440.8 cpm	21	460.8 cpm	20	508.2 cpm	20	562.9 cpm	20	739.2 cpm
30	451.4 cpm	31	492.4 cpm	30	521.8 cpm	30	654.5 cpm	30	872.3 cpm
40	454.7 cpm	41	502.8 cpm	40	563.0 cpm	40	712.6 cpm	40	1022 cpm
50	478.3 cpm	51	516.1 cpm	50	599.8 cpm	50	776.9 cpm	50	1167 cpm
60	504.4 cpm	51	516.1 cpm	50	599.8 cpm	50	776.9 cpm	50	1167 cpm
Y Int	431.7		431.5		423.7		422.4		462.3
Slope	.9537		1.716		3.508		7.233		13.99
ST DV	16.06		6.191		10.11		10.31		7.676

TABLE VIII Continued

Nitrate Inhibition (cpm vs time)

Zero mM Nitrate

(f)

Time (Sec)	2.97 µM (RuBP)	Time (Sec)	5.94 µM (RuBP)	Time (Sec)	11.9 µM (RuBP)	Time (Sec)	24.7 µM (RuBP)	Time (Sec)	49.5 µM (RuBP)
6	234.2 cpm	5	237.1 cpm	5	240.4 cpm	6	266.5 cpm	5	285.0 cpm
16	256.5 cpm	15	276.5 cpm	15	321.8 cpm	16	394.1 cpm	15	429.0 cpm
26	242.6 cpm	25	347.0 cpm	25	381.4 cpm	26	473.6 cpm	25	614.0 cpm
36	310.5 cpm	35	377.4 cpm	35	488.7 cpm	36	577.7 cpm	35	841.5 cpm
46	328.6 cpm	45	418.7 cpm	45	588.7 cpm	46	794.7 cpm	45	1030 cpm
Y Int	211.4		215.3		188.3		178.8		164.25
Slope	2.428		4.641		8.635		12.40		19.03
ST DV	20.76		10.77		15.89		43.00		25.66

TABLE VIII Continued

(g) 50 mM Nitrate

Time (Sec)	2.97 µM (RuBP)	Time (Sec)	5.94 µM (RuBP)	Time (Sec)	11.9 µM (RuBP)	Time (Sec)	24.7 µM (RuBP)	Time (Sec)	49.5 µM (RuBP)
6	229.9 cpm	5	223.0 cpm	5	242.2 cpm	5	257.5 cpm	5	259.6 cpm
16	238.1 cpm	15	248.9 cpm	15	279.6 cpm	15	359.7 cpm	15	360.4 cpm
26	255.4 cpm	25	282.9 cpm	25	343.7 cpm	25	437.8 cpm	25	451.4 cpm
36	266.5 cpm	35	287.1 cpm	35	379.6 cpm	35	411.1 cpm	35	579.5 cpm
46	282.7 cpm	45	336.0 cpm	45	425.6 cpm	45	607.4 cpm	45	697.8 cpm
Y Int	219.68		2.642		4.668		8.512		10.59
Slope	1.340		209.53		217.44		221.9		201.4
ST DV	2.488		10.54		7.310		8.992		10.07

TABLE VIII Continued

100 mM Nitrate

(h)

Time (Sec)	2.97 µM (RuBP)	Time (Sec)	5.94 µM (RuBP)	Time (Sec)	11.9 µM (RuBP)	Time (Sec)	24.7 µM (RuBP)	Time (Sec)	49.5 µM (RuBP)
6	212.5 cpm	5	209.9 cpm	5	219.3 cpm	5	229.4 cpm	5	244.9 cpm
16	222.5 cpm	15	214.6 cpm	15	239.5 cpm	15	262.8 cpm	13	256.7 cpm
26	243.5 cpm	25	239.0 cpm	25	263.2 cpm	25	280.7 cpm	25	297.3 cpm
36	235.5 cpm	35	242.6 cpm	35	278.9 cpm	35	316.2 cpm	35	370.1 cpm
46	244.2 cpm	45	265.6 cpm	55	301.2 cpm	45	366.0 cpm	45	443.6 cpm
Y Int	211.8		1.414		2.032		3.266		5.108
Slope	.7640		198.8		209.6		209.4		194.8
ST DV	7.735		5.899		1.977		9.818		24.67

TABLE VIII Continued

200 mM Nitrate

(i)

Time (Sec)	2.97 µM (RuBP)	Time (Sec)	5.94 µM (RuBP)	Time (Sec)	11.9 µM (RuBP)	Time (Sec)	24.7 µM (RuBP)	Time (Sec)	49.5 µM (RuBP)
5	214.5 cpm	5	204.0 cpm	5	224.7 cpm	5	209.6 cpm	5	(222.9 cpm)
15	219.5 cpm	15	211.2 cpm	15	212.8 cpm	15	233.4 cpm	15	225.2 cpm
25	220.0 cpm	25	211.7 cpm	25	233.5 cpm	25	230.2 cpm	25	227.1 cpm
35	233.7 cpm	35	216.3 cpm	35	234.0 cpm	35	(213.3 cpm)	35	235.8 cpm
45	(227.8 cpm)	45	(212.2 cpm)	45	(220.0 cpm)	45	(229.2 cpm)	45	245.6 cpm
Y Int	210.3		203.3		216.5		219.8		212.4
Slope	.5810		.3740		.4860		.5300		.6990
ST DV	4.159		1.940		9.430		6.940		2.936

TABLE VIII Continued

400 mM Nitrate

(j)

Time (Sec)	2.97 µM (RuBP)	Time (Sec)	5.94 µM (RuBP)	Time (Sec)	11.9 µM (RuBP)	Time (Sec)	24.7 µM (RuBP)	Time (Sec)	49.5 µM (RuBP)
5	231.0 cpm	6	277.8 cpm	5	239.0 cpm	5	223.9 cpm	5	240.9 cpm
15	233.7 cpm	16	275.5 cpm	15	219.0 cpm	15	230.7 cpm	15	227.9 cpm
25	227.3 cpm	26	280.8 cpm	25	230.0 cpm	25	222.7 cpm	25	227.1 cpm
35	227.9 cpm	36	269.3 cpm	35	229.3 cpm	35	235.7 cpm	35	241.8 cpm
45	221.8 cpm	46	277.2 cpm	45	223.3 cpm	45	223.1 cpm	45	235.4 cpm
Y Int	234.4		-0.740		-2110		.0340		.0290
Slope	-.2420		278.0		233.4		226.4		233.9
ST DV	26.64		4.737		7.857		6.615		8.010

TABLE VIII Continued

1/Vi vs 1/(RuBP) for Chloride

(k)

Substrate (μM)	$1/S_{(\mu M)^{-1}}$	$1/Vi\ (cpm/sec)^{-1}$					
		Extradated 0 mM Cl^-	65.6 mM Cl^-	92 mM Cl^-	167 mM Cl^-	279 mM Cl^-	392 mM Cl^-
2.966	.3371	.1831	.2297	.2094	.2762	.3827	1.049
5.936	.1685	.1040	.1182	.1356	.1503	.1881	.5828
11.86	.0843	.0405	.0566	.0588	.0747	.1426	.2851
24.73	.0404	.0239	.0269	.0302	.0387	.0717	.1383
49.45	.0202	.0137	.0159	.0178	.0189	.0287	.0715
	Slope	.5469	.6813	.6191	.8112	1.057	3.099
	Y Int	.0019	.0008	.0098	.0062	.0252	.0221
	X Int	-.0035	-.0000	-.0158	-.0077	-.0241	-.0071
	CC	.9959	.9998	.9885	.9991	.9909	.9984

TABLE VIII Continued

1/Vi vs 1/(RuBP) for Nitrate

(1)

Substrate (μM)	1/S $(\mu M)^{-1}$	1/Vi $(cpm/sec)^{-1}$			
		No NO_3^-	50 mM NO_3^-	100 mM NO_3^-	200 mM NO_3^-
2.966	.3371	.4119	.7463	1.309	(1.721)
5.936	.1685	.2155	.4852	.7072	2.674
11.86	.0843	.1158	.2142	.4921	2.058
24.73	.0404	.0806	.1175	.3062	1.887
49.45	.0202	.0526	.0944	.1958	1.431
	Slope	1.1306	2.140	3.405	7.587
	Y Int	.0282	.0531	.1590	1.418
	X Int	-.0249	-.0248	-.0467	-.1869
	CC	.9993	.9891	.9975	.9702

TABLE IX

Cleland Computer Fit for Competitive Noncompetitive and Uncompetitive Inhibition Patterns

Chloride Inhibition

(a)

Inhibition	Variance	Ki (mM)
Competitive	16.70	157.3
Noncompetitive	17.25	139.1
Uncompetitive	755.8	---

Nitrate Inhibition

(b)

Inhibition	Variance	Ki (mM)
Competitive	1.796	20.70
Noncompetitive	1.560	45.90
Uncompetitive	3.413	---

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EVIDENCE FOR A CONFORMATIONALLY SENSITIVE ANION BINDING SITE ON RIBULOSE -1,5-BISPHOSPHATE CARBOXYLASE/OXYGENASE ISOLATED FROM COMPHREY

by

ROBERT F. BONSALL

B.A., Hartwick College, 1978

AN ABSTRACT OF A THESIS

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Graduate Biochemistry Group

KANSAS STATE UNIVERSITY

Manhattan, Kansas

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Ribulose -1,5-bisphosphate carboxylase/oxygenase (RuBPcase) was isolated from freshly cut garden or greenhouse grown comphrey leaves. The isolation procedure was essentially that developed by Simpson (1980) and involved homogenization on ice in TBMESP buffer (50 mM Tris, 50 mM $NaHCO_3^-$, 10 mM $MgCl_2$, .1 mM EDTA, 5 mM $Na_2S_2O_6$, .1 mM PMSF at pH = 7.5), filtration, stirring for 30 minutes in poly-vinylpyrrolidone and centrifugation at 10,000 x g for 5 minutes to yield the crude extract. The crude extract was ammonium sulfate precipitated followed by centrifugation at 13,000 xg for 20 minutes (40% pellets). The 40% pellets were redissolved in a minimum TBMESP buffer and then applied to a linear sucrose gradient. Sucrose gradients were collected in 1 ml fractions where peak tubes were located by A_{280} measurements. The isolated enzyme was stored in 1.5 ml cryotubes under nitrogen atmosphere at -70^{o}C. Before use all sucrose gradient preparations were further purified by ion exchange chromatography on G-25 DEAE Sephadex.

SDS polyacrylamide slab-gel electrophoresis was performed in series on the crude extract, 40% supermatant, 40% pellet, sucrose gradient and ion exchange preparations of the purified enzyme. The data demonstrated that the crude extract contained six protein bands together with the large and small subunits of RuBPcase. It was found that five of these bands remained in the 40% ammonium sulfate supernatant with one that co-precipitated with the 40% pellet. The sucrose density gradient preparation wasn't completely homogeneous since another band (60.8 K dalton) was apparent in the pooled fractions. This band did not seem to match very well with any of the bands observed at the earlier stages of the purification. It

was believed to represent some residual quaternary structure which was stabalized against dissociation with SDS by sucrose. The DEAE preparation was found to yield a homogeneous product. In addition the molecular weights of both large and small subunits of RuBPcase were determined by SDS PAGE and calculated to be 49,992 and 12,657 daltons respectively.

The Km for RuBP was determined at zero (extrapolated) chloride concentrations and low nitrate levels (which were shown not to have inhibitory effects on the enzyme). The values from all determinations ranged from 21 μM to 133 μM with an average value of 56 μM. The average Km values from the nitrate data alone averaged 46 μM.

Inhibition patterns for RuBPcase with chloride and nitrate determined from both Lineweaver - Burk plots and by Cleland's method suggested mixed competitive and noncompetitive inhibition. The replots for both chloride and nitrate were linear at low concentrations and curved upward at high concentrations thus forming concave curves instead of convex curves for normal mixed inhibition. It was concluded that since chloride and nitrate demonstrated similar types if inhibition patterns, which didn't seem to follow normal straight forward kenetics, that some type of conformational change might be occurring within the enzyme which caused a decrease in its activity.

Data was also obtained for bicarbonate inhibition as a function of pH which clearly demonstrated a decrease in RuBPcase activity as bicarbonate concentrations were increased above saturating levels. Furthermore, it was discovered that chloride and bicarbonate ions were at least partially competitive since increasing the bicarbonate

concentration overcame the inhibition of chloride. Since bicarbonate and nitrate are both trigonal planar monovalent anions of similar size bicarbonate inhibition probably occurred at the same anion site as that of nitrate and chloride which in turn altered the enzymatic activity substantially.

In order to demonstrate a specific binding site and to probe the nature of this site differential UV spectroscopy at various NaCl concentrations was performed by T. D. Bolden of our laboratory. The difference spectra showed a large change in Δ A in the 280 nm region as salt concentration was increased. This suggested that the binding of chloride was occurring at a specific anion site which involved one or more tyrosine residues.

To investigate any possible conformational change upon anion binding, far UV CD spectra were acquired on RuBPcase in the absence and presence of various bicarbonate concentrations. These spectra showed that as the bicarbonate concentration was increased the ellipticities became less negative which implied a loss of secondary structure. Therefore, the CD spectra supported the hypothesis of a conformational change induced by binding of an anion to a specific site. In addition, near UV CD spectra for RuBPcase was found to give several distinct bands between 256 - 268 nm and 278 - 294 nm. The far UV CD spectra of urea denaturation of RuBPcase revealed that as urea concentrations were increased the secondary structure of RuBPcase decreased and virtually disappeared at 5 - 6 M urea.