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PHYSICAL, CHEMICAL, AND BIOCHEMICAL STUDIES WITH ISOLATED CHLOROPLASTS AND PURIFIED ENZYMES

A. M. El-Badry
(Ph. D. Thesis)

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Physical, Chemical, and Biochemical Studies with Isolated Chloroplasts and Purified Enzymes

by

A. M. El-Badry

Abstract

Fructose-1,6-diphosphatase (E.C. 3.1.3.11) has been isolated, purified, and crystallized, from previously isolated spinach chloroplasts. The effects of various anions, cations, and sulfhydryl reagent were tested, and activation by Mg^{++}, glycine, HCO_3^-, and sulfhydryl reagent is described. The purified enzyme is very specific for fructose-1,6-diphosphate and does not attack sedoheptulose-1,7-diphosphate. The S_{20} value of the enzyme was 7.7, from which the molecular weight of the enzyme was estimated as 140,000.

Quenching studies of the intrinsic fluorescence due to the tryptophan residues in fructose-1,6-diphosphatase showed that the conformation of the enzyme is affected by the substrate fructose-1,6-diphosphate and possibly by the product fructose-6-phosphate. The fluorescent probe 1-anilino-8-naphthalene sulfonate (ANS) binds to some hydrophobic region on the enzyme. However, its extrinsic fluorescence is not sensitive to the conformational change of the enzyme induced by added ligands. Depolarization fluorescence studies revealed conformational change due to the binding of the

substrate, the allosteric affector and the divalent cation Mg^{++}.

γ-irradiation of fructose-1,6-diphosphatase was found to damage the catalytic activity of the enzyme. The substrate fructose-1,6-diphosphate protected the site from damage. Mg^{++} and fructose-1,6-diphosphate in the presence of magnesium ion protected the site to a lesser extent. The allosteric function of the enzyme was also damaged by γ-irradiation. Mg $ATP^{\equiv}$ protected the allosteric site from γ-irradiation damage. Mg^{++} or anionic ATP resulted in partial protection of the site.

Ribulose-1,5-diphosphate carboxylase has been isolated from previously isolated spinach chloroplasts by a procedure which facilitates simultaneous isolation of several photosynthetic enzymes. The purification and storage procedure gives a stable enzyme. The enzyme activity is enhanced 40 to 180% by nearly physiological levels of fructose-1,6-diphosphate when Mg^{++} is in the range of 1 to 10 mM.

An alkaline, magnesium ion dependent inorganic pyrophosphatase has been isolated from previously isolated spinach chloroplasts. The activity of the enzyme was increased 100-fold, with a 42% yield, upon purification from the total soluble chloroplast enzymes. The pH optimum for the enzyme shifts from 9.0 at 5 mM Mg^{++} to 7.0 at 40 mM Mg^{++}. The substrate for the reaction appears to be Mg pyrophosphate, and anionic pyrophosphate is an effective inhibitor. There seems to be also an activating effect of Mg^{++} ion on the enzyme at pH 7. No other cation substitutes for Mg^{++} ion

in activating the hydrolysis of pyrophosphate. Among anions tested, only F^- caused severe inhibition. The enzyme is inactive towards fructose-1,6-diphosphate, thiamine pyrophosphate, ATP, and ADP. The possibility that this enzyme is subject to metabolic regulation is discussed in relation to an indicated role of pyrophosphate in the regulation of photosynthetic carbon reduction.

Nitroxide free radicals were found to inhibit CO_2 fixation by isolated chloroplasts. The inhibitory potency of these radicals depended on the polarity of the substituent on the ring. Compounds containing free carboxyl groups were poor inhibitors. A hydroxyl or an amide group on the ring was optimal for inhibition of CO_2 fixation. The size of the ring seemed not to influence the potency of the compounds as inhibitors of CO_2 fixation. The presence of a double bond in the ring reduced the effectiveness of the compound as an inhibitor. All of the nitroxide free radicals which were effective inhibitors of CO_2 fixation were shown also to be good Hill oxidants. As in the case of CO_2 fixation, the ability of the radical to act as a Hill oxidant is a function of its ability to penetrate the lipoprotein membrane of the chloroplasts.

The radicals exerted their action by acting as electron acceptors in the electron transport chain. The effect of CO_2 fixation is hence a consequence of the action of the radical on the electron transport chain. The radical is reduced by the chloroplast either in the chloroplast or outside the chloroplasts by a reducing substance which leaks

out the chloroplast upon illumination. Analysis of reduction of the radical by measuring the height of the EPR signal shows that reduction outside the chloroplasts and deactivation of the compound due to binding to a macromolecule or a particle are two mechanisms to explain the decay and leveling off of the EPR signal.

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PART I

STUDIES WITH FRUCTOSE-1,6-DIPHOSPHATASE

Chapter I

FRUCTOSE-1,6-DIPHOSPHATASE FROM SPINACH CHLOROPLASTS: PURIFICATION, CRYSTALLIZATION AND SOME PROPERTIES

A. INTRODUCTION

The enzyme fructose-1,6-diphosphatase (D-fructose-1,6-diphosphate 1-phosphohydrolase EC 3.1.3.11) catalyzes the hydrolysis of fructose-1,6-diphosphate.

$$\text{Fructose-1,6-diphosphate} \xrightarrow{Mg^{++}} \text{Fructose-6-phosphate} + \text{inorganic phosphate}$$

Fructose-1,6-diphosphatase (FDPase) is an important enzyme in the regulation of gluconeogenesis in mammalian and microbial systems[1-4]. The inhibition of mammalian and bacterial FDPase by adenosine 5' phosphate (AMP) suggests allosteric regulation[5-8]. Mendicino *et al.* have shown that FDPase is inhibited by a specific mitochondrial enzyme system[9]. Nakashima *et al.*[10,11] have altered the catalytic properties of rabbit liver and rabbit kidney fructose diphosphatase by treating these enzymes with coenzyme A or acyl carrier protein from *E. coli*. This treatment increased the activity at the neutral pH several fold and shifted the pH optima from pH 8.8 to pH 7.5 in the presence of Mg^{++}.

Hydrolysis of fructose-1,6-diphosphate (Fru-1,6-P_2)* to fructose-6-phosphate is also an important reaction during the fixation of carbon dioxide in photosynethetic systems. It has been suggested that activity of FDPase in photosynthetic *Chlorella* cells is subject to regulation[12].

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*Abbreviations: FDPase, fructose-1,6-diphosphatase; Fru-1,6-P_2, fructose-1,6-diphosphate; EDTA, ethylene diamine tetraacetic acid.

That is, although FDPase is active during the photosynthetic fixation of carbon dioxide, its activity appears to decrease markedly when Chlorella cells are transferred to non-photosynthetic conditions by turning off the light. This suggests light activation of FDPase enzyme: the activation appears to decay after about 2 min of darkness.

Morris[13] found that the activity of partially purified FDPase from spinach chloroplasts is inhibited by magnesium pyrophosphate ($MgP_2O_7^{=}$), $MgATP^{=}$ and $MgADP^{-}$ at concentrations of 1 to 3 mM. The degree of inhibition by all these inhibitors decreases with increasing concentrations of Mg^{++} and substrate (Fru-1,6-P_2). $MgP_2O_7^{=}$ and $MgADP^{-}$ are approximately equally effective inhibitors; both are less effective than $MgATP^{=}$.

FDPase is a widely distributed enzyme in living systems. It has been isolated from many mammalian sources including rabbit liver[14], rabbit muscle[15-17], rabbit kidney[18], swine kidney[19,20] and rat tissues[21]. The enzyme has also been isolated from a variety of microbial systems including Dictyostelium discoideum[22], Candida utilis[23,24], Polysphondylium pallidum[25], Pseudomonas saccharophila[26], E. coli[27], and Acinetobacter sp.[28].

FDPase has also been isolated from some plant sources. These include castor bean endosperm and leaf[29,30], navy bean[31] and wheat embryos[32]. Racker and Schroeder isolated an aklaline FDPase which they claimed could have little role in the reductive pentose phosphate cycle due to its apparent localization outside the chloroplast[33]. Smillie[34] has further investigated the localization of FDPase and found that most of the enzymic activity is localized in the chloroplast. This finding was confirmed by Latzko and Gibbs[35]. In this paper we report a method for the purification and crystallization of fructose-1,6-diphosphatase from spinach chloroplasts. Some of the properties and characteristics of the

enzyme are also reported.

B. MATERIALS AND METHODS

1- Chemicals. Fructose-1,6-diphosphate, fructose-6-phosphate, ribulose-1,5-diphosphate, glucose-1-phosphate, glucose-6-phosphate, ribulose-5-phosphate, AMP, ADP, ATP, NADP, sorbitol, 2-N morpholino ethane sulfonic acid, deoxyribonuclease, ribonuclease, phosphoglucose isomerase and glucose-6-phosphate dehydrogenase were obtained either from Cal Biochem or from Sigma Chemical Co. Tetrasodium pyrophosphate was from Allied Chemicals. All inorganic salts were analytical reagents. DEAE-cellulose (Cellex-D) and phosphocellulose (Cellex-P) were obtained from Biorad Lab.

Both DEAE-cellulose and phosphocellulose were further purified by suspending in water and decanting the fines. For every 40 to 80 g of the absorbent, one liter of 1 N NaOH was added, followed after 15 min by 1100 ml of 1 N HCl. The acidic suspension is filtered on a Buchner funnel and rinsed with a little water. The residue was again suspended in 1 liter of 1 N NaOH and after 15 min is filtered again and washed until the effluent is neutral with distilled water.

The free DEAE-cellulose base was then equilibrated with a solution of 0.05 M tris-HCl, pH 7.4, containing 0.001 M EDTA. The buffer was changed several times until pH 7.4 was achieved. The adsorbent was stored (at 4°) in a 30-50% suspension in tris-HCl, 0.05 M, pH 7.4.

The sodium salt of phosphocellulose was converted to the acid form for storage by equilibrating it with 2 N HCl for 3 h. The resin was then washed on a Buchner funnel until the effluent is nearly neutral. A 30-50% suspension of the acid form of phosphocellulose was titrated with 1 N NaOH to pH 8. After 30 min of stirring, the pH was readjusted and

the material was allowed to settle; then it was resuspended in 1 liter of 0.05 M tris-HCl, pH 8.0. The pH was checked and adjusted for a week before use.

2- Assay of enzymic activity. The enzyme D-fructose-1,6-diphosphatase was assayed by the determination of NADPH produced, when the FDPase reaction is coupled with the enzymatic reactions of phosphoglucose isomerase (E.C.5.3.1.9) and glucose-6-phosphate dehydrogenase (E.C.1.1.1.49). The latter reaction requires $NADP^+$, and NADPH produced by this reaction is directly proportional to the quantity of fructose-6-phosphate produced by the FDPase reaction. The measurement of change in optical absorption at 340 nm is used to estimate the quantity of NADPH formed. The reaction mixture (1.0 ml) contained tris-HCl buffer, 0.05 M, pH 8.7; $MgCl_2$, 5 mM; ethylene diamine tetraacetic acid, dipotassium salt (EDTA), 2 mM; fructose-1,6-diphosphate, 1 mM; $NADP^+$ (sodium salt), 0.2 mM; phosphoglucose isomerase, 5 μg; glucose-6-phosphate dehydrogenase, 1.5 μg; and FDPase in the range of 0.01 to 0.02 unit. The temperature was 22°. One unit of enzyme activity is defined as the amount of enzyme activity catalyzing the hydrolysis of 1 μmole of fructose-1,6-diphosphate to fructose-6-phosphate and inorganic phosphate per minute and specific activity is expressed as units per mg of protein.

For studying the substrate specificity of FDPase, the hydrolysis of the sugar phosphates was assayed by the direct determination of inorganic phosphate released in the enzymic reaction by the procedure of Fiske and SubbaRow[36,37]. The incubation mixture (1ml) contained tris-HCl buffer, 0.05 M, pH 8.7; $MgCl_2$, 5 mM; EDTA, 2 mM, the sugar phosphate tested, 1 mM, and enzyme in an amount sufficient to release 0.05 to 0.1 mM of inorganic phosphate in 10 min at 22°. The reaction was stopped by the addition of 5 N H_2SO_4 for the phosphate determination. The

unit of enzyme activity was expressed in terms of micromoles of inorganic phosphate formed under these conditions.

3- Determination of protein. Protein concentration was determined spectrophotometrically by measuring the absorption at 280 nm or by using the Lowry method of protein determination[38]. Bovine serum albumin was used as the standard in each of the two methods.

4- Disc gel electrophoresis. The method of Davis [39] was used to analyze for the homogeneity of the enzyme at the different stages of the purification procedure. Cross-linked polyacrylamide gel at pH 8.4 was used. Samples of 10-20 µl containing 50 to 100 µg of protein were used. All reagents used in the procedure were obtained from Canalco.

5- Determination of molecular weight. The sedimentation velocity method described by Chervenka[40] was used for the determination of S_{20} value of the enzyme. The run was performed using an An-D rotor at 59,780 rpm.

6- Isolation of spinach ferredoxin. Spinach ferredoxin was isolated and purified by a procedure which involved acetone fractionation, according to San Pietro and Lang[41], followed by chromatography on DEAE-cellulose based on the method of Lovenberg *et al.*[42]. Its quantitative determination was based on the spectrum of the purified material.

7- Methods of reducing ferredoxin.

(a) Reduction by illuminated chloroplasts. Ferredoxin can be reduced in the presence of chloroplast particles and light[42]. Chloroplasts were isolated by the method of Jensen and Bassham[43]. A 200 µl of the chloroplast slurry (containing 0.4 mg chlorophyll) in 0.05 *M* tris-HCl, pH 7.4, was added under nitrogen to a 1 ml tris-HCl, pH 7.4, solution containing

3 mg of purified ferredoxin. The tube was irradiated under intense light for 5 min. The reduction of ferredoxin was measured spectrophotometrically at 420 nm by the use of Cary Model 14 spectrophotometer. Ferredoxin photoreduction by illuminated chloroplasts was stable, and ferridoxin could be largely reoxidized by NADP even after a considerable period in the dark.

(b) Reduction by hydrogen. Tagawa and Arnon[44] showed that molecular hydrogen can reduce ferredoxin in the dark if the enzyme hydrogenase from *C. pasteurionum* is present. The bacterial hydrogenase was isolated by the following method[45]. Thirty grams of a frozen cell paste of *C. pasteurianum* was thawed overnight and suspended in water with the aid of a magnetic stirrer to a final volume of about 200 ml. Small amounts of deoxyribonuclease and ribonuclease were added to obtain a free-flowing suspension. Fifty-ml aliquots of this suspension were subjected to sonication for 10 min by the Biosonik. Cell debris was removed by a 15-min centrifugation at 36,000 x g. The supernatant was then heated for 10 min at 60° under hydrogen. The precipitate was centrifuged off, and the supernatant was dialyzed against 0.05 *M* tris buffer at pH 7.4. The crude enzyme was dialyzed before use to minimize the level of inorganic phosphate present in the enzyme preparation. Ferredoxin was reduced by bubbling hydrogen through tubes containing the ferridoxin solution and the bacterial hydrogenase. Spectrophotometric evidence was the criterion for ferredoxin reduction.

(c) Chemical reduction. To 1 ml solution of 0.05 M tris-HCl, pH 7.4, containing 3 mg/ml of ferredoxin was added 30 µl of 0.05 M sodium dithionite under nitrogen. Ferredoxin reduced by this method was reversibly oxidized to a large extent with oxygen. Bleaching of ferredoxin was

also reversible upon exposure to oxygen. The reduced ferredoxin solution was dialyzed against 0.05 M tris-HCl buffer, pH 7.4, before use. Determination of the reduction was carried out spectrophotometrically.

C. ISOLATION OF THE ENZYME D-FRUCTOSE-1,6-DIPHOSPHATASE

Fructose-1,6-diphosphatase was purified by the following method:

1- Source of the enzyme. Field-grown, relatively young, spinach leaves were harvested and immediately stored over crushed ice in polyethylene bags in large ice chests. The leaves were deribbed and washed with ice-cold water and were dried between two sponges. The deribbed leaves were then chopped and divided into 50-g batches.

2- Isolation of chloroplasts. Each 50-g batch was homogenized for 5 to 8 sec in a Waring blendor with 200 ml of solution A[43] (containing Sorbitol, 0.33 M; $NaNO_3$, 0.002 M; EDTA (dipotassium salt), 0.002 M; sodium isoascorbate, 0.02 M; $MnCl_2$, 0.001 M; KH_2PO_4, 0.0005 M; 2-N-morpholinoethane sulfonic acid, 0.05 M, adjusted with NaOH to pH 6.1 and NaCl, 0.02 M). The blendorate was then forced through six layers of cheesecloth to strain out fibrous material.

The homogenate was centrifuged at 2000 x g for 3 min. The supernatant was decanted and each pellet was suspended in 10 ml of "basic buffer" (0.05 M tris-HCl buffer (pH 7.4) - 0.002 M dithiothreitol - 0.0002 M EDTA - 0.001 M $MgCl_2$).

3- Sonication. The chloroplast suspension in basic buffer was sonicated for 30 sec in batches of 50 ml, using the Biosonik (Model BPI, Bronwill Scientific Co., Rochester, N. Y.) at 0°.

The sonicated suspension was centrifuged at 36,000 x g for 2 h and the suspension was saved as the crude enzyme preparation(I).

4- Acetone fractionation. Acetone was added to the crude enzyme

fraction to a concentration of 30%. The acetone had been precooled in the freezer at -14° and was added to the crude enzyme solution slowly while stirring at 4°. The enzyme in 30% acetone was allowed to stand in the cold room (4°) for 30 min, and the mixture was centrifuged at 13,200 x g for 4 min. The supernatant was collected and the acetone concentration in the supernatant was brought to 75%. The enzyme in 75% acetone was allowed to stand in the freezer at -14° for 1-2 h. A copious precipitate formed and settled toward the bottom of the container. The upper layer of 75% acetone solution was decanted. The lower layer containing the precipitated enzyme and some other proteins was then centrifuged for 1 min at 5000 x g and the pellets were collected.

The greyish-white precipitate was suspended in the smallest possible volume of basic buffer and was dialyzed against cold water (4°) for 4 h. Then it was dialyzed against basic buffer twice for 8 h each time.

The dialyzed mixture was centrifuged in the Sorvall, at 36,000 x g for 10 min, and the supernatant (II) was saved.

5- DEAE-cellulose column. A DEAE-cellulose column was prepared and pre-equilibrated with 0.05 M tris-HCl (pH 7.4). The supernatant (II) was applied to the column and the column was washed with 0.05 M tris-HCl buffer (pH 7.4). FDPase did not stick to the DEAE-cellulose under these conditions and most of the activity passed down the column in the 0.05 M tris-HCl (pH 7.4) eluting buffer. This step is a very useful purification step since a considerable number of proteins were bound to the DEAE-cellulose and were therefore removed from the FDPase solution (III).

6- Ammonium sulfate fractionation. The protein eluted from the DEAE-cellulose column (III) was subjected to ammonium sulfate

fractionation. Enzyme grade ammonium sulfate, previously crushed into a powder by a mortar and pestle, was added slowly to the enzyme solution while stirring. The pH of the mixture was maintained at about pH 7 using ammonium hydroxide solution and pH paper as indicator. The precipitate formed at 45% ammonium sulfate saturation was centrifuged at 13,200 x g for 10 min and was discarded. Ammonium sulfate powder was added to the supernatant to a concentration of 60% saturation and the precipitate was collected by centrifugation at 13,200 x g for 10 min and was dissolved in basic buffer (IV).

7- Phosphocellulose column. Fraction (IV) was dialyzed against 0.05 M tris-HCl buffer (pH 8.0) for 6 h. It was then applied to a cellulose-phosphate column that has been pre-equilibrated with 0.05 M tris-HCl buffer (pH 8.0). After loading the column with the enzyme, the column was washed with 0.05 M tris-HCl buffer, pH 8.0, until no protein was coming out in the washing buffer. The column was then eluted with 10^{-3} M fructose-1,6-diphosphate in 0.05 M tris-HCl, pH 8.0. The fractions containing FDPase were pooled and were dialyzed against a saturated solution of ammonium sulfate preadjusted with ammonium hydroxide to pH 8.0 (using pHydrion paper). The protein precipitated in the dialysis tubing and the suspension mixed with it was removed from the dialysis tubes and the precipitate was collected by centrifugation at 36,000 x g for 20 min and was dissolved in "basic buffer" (V).

8- Chromatography on Sephadex G-100 column. In most preparations fraction (V) protein was homogeneous and no further purification was needed. However, in one of our preparations a trace of a contaminating protein was observed by polyacrylamide disc gel electrophoresis. In this case,fraction V was chromatographed on a sephadex G-100 column

which was preequilibrated with tris-HCl buffer, pH 8.0, and was eluted with the same buffer. The fractions containing FDPase were pooled and were concentrated in the same manner as described for the protein eluted from the phospho-cellulose column (VI). The specific activity of the homogeneous protein at this stage was 21.1 units per mg protein (Table I).

9- Crystallization of FDPase. The concentration of protein in 1 ml of fraction (VI) was adjusted to 7 mg/ml in a buffer containing tris-HCl, 0.1 M, pH 8.0, and magnesium chloride, 10 mM. The solution was then titrated with a cold (4°) saturated solution of ammonium sulfate until a slight turbidity was observed. The solution was allowed to warm to room temperature where the turbidity disappeared. It was then centrifuged at 36,000 x g for 10 min and the clear supernatant was cooled to cold room temperature gradually by placing the tube containing the solution in a beaker containing water at room temperature and placing this assembly in the cold room (4°C). The tube was then transferred to an ice bucket full of crushed ice and was kept there on ice for a few days. The crystalline enzyme was obtained (VII). The enzyme in its crystalline state was not stable for over a month in crystallization suspension. However, redissolving the enzyme in basic buffer and storing the solution at 0°C kept the enzyme activity stable for over six months.

D. HOMOGENEITY AND MOLECULAR WEIGHT OF FDPASE

Fractions (V) and (VI) were examined for their protein homogeneity using disk gel electrophoresis. Both fractions contained only one major band. In one run the phosphocellulose eluate (fraction V) contained another minor band which was eliminated from gel filtration on a Sephadex G-100 column.

TABLE I

PURIFICATION OF SPINACH CHLOROPLAST FRUCTOSE-1,6-DIPHOSPHATASE

	Fraction	Protein Concentration (mg/ml)	Specific activity (units/mg protein)	Yield (%)
I	Sonicate supernatant	10	0.02	100
II	Acetone fraction	21	0.68	60
III	DEAE column	7.5	3.3	49
IV	45-60% ammonium sulfate fraction	20	8.2	73
V	Phospho-cellulose column	22	20.3	58
VI	Sephadex G-100 column	21	21.1	52

Plate 1. Crystals of Fructose-1,6-diphosphatase as seen by the phase contrast microscope.

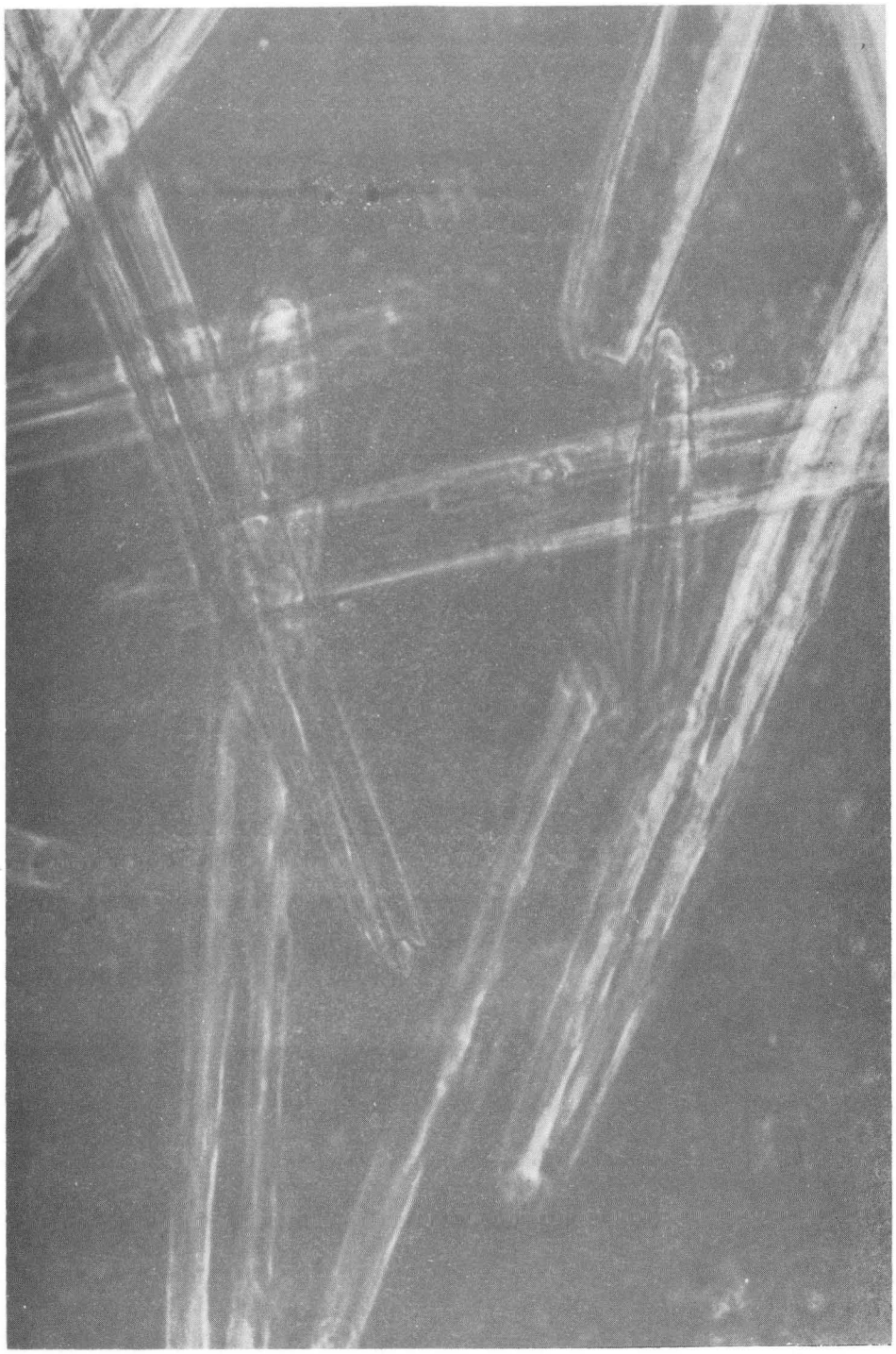

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Plate I-1

Sedimentation rates data gave an S_{20} value of 7.7. Assuming a spherical protein with a partial specific volume of 0.725 cm, the molecular weight of the protein was estimated to be 140,000. This value makes spinach chloroplast FDPase not much different from the mammalian FDPase[14] in molecular weight. The enzyme is somewhat larger than the Candida utilis FDPase which is reported to have a value of 100,000.[24]

E. EFFECT OF MAGNESIUM ION CONCENTRATION ON THE pH OPTIMUM OF FDPASE

In the study of effect of Mg^{++} concentration on the pH optimum of chloroplast FDPase, Mg^{++} concentrations of 5 to 40 mM were used and the pH of the enzymic reaction was varied from pH 6.0 to pH 9.5. At lower concentration of magnesium (1 mM and 5 mM) the pH optimum is around 8.5 (Fig. 1). As the Mg^{++} concentration in the assay mixture increases the pH optimum shifts towards the neutral pH and at 40 mM Mg^{++} ion concentration the pH optimum is at pH 7. Our results are similar to those of Preiss et al.[46] obtained with partially purified FDPase from spinach chloroplasts and are also similar to the results obtained with purified rabbit liver FDPase[47].

The possible regulatory significance of this shift is apparent. However, the observation with the rabbit liver enzyme that dinitrophenylation[48,49] causes similar shifts in the pH optima of the enzyme suggests that the action of Mg^{++} is to block some negative charges near the active site which renders the active site more accessible to the negatively charged substrate (fructose-1,6-diphosphate or its magnesium salt). At the neutral pH the enzyme conformation is such that the negative charges are concentrated near the active site; thus more Mg^{++} concentration is needed to neutralize these charges in order to make the active site more accessible to the negatively charged substrate. As

Fig. 1. Effect of Mg^{++} concentration on the pH optimum of FDPase.

O--O 1 mM Mg^{++}

□--□ 5 mM Mg^{++}

●--● 10 mM Mg^{++}

△--△ 20 mM Mg^{++}

■--■ 40 mM Mg^{++}

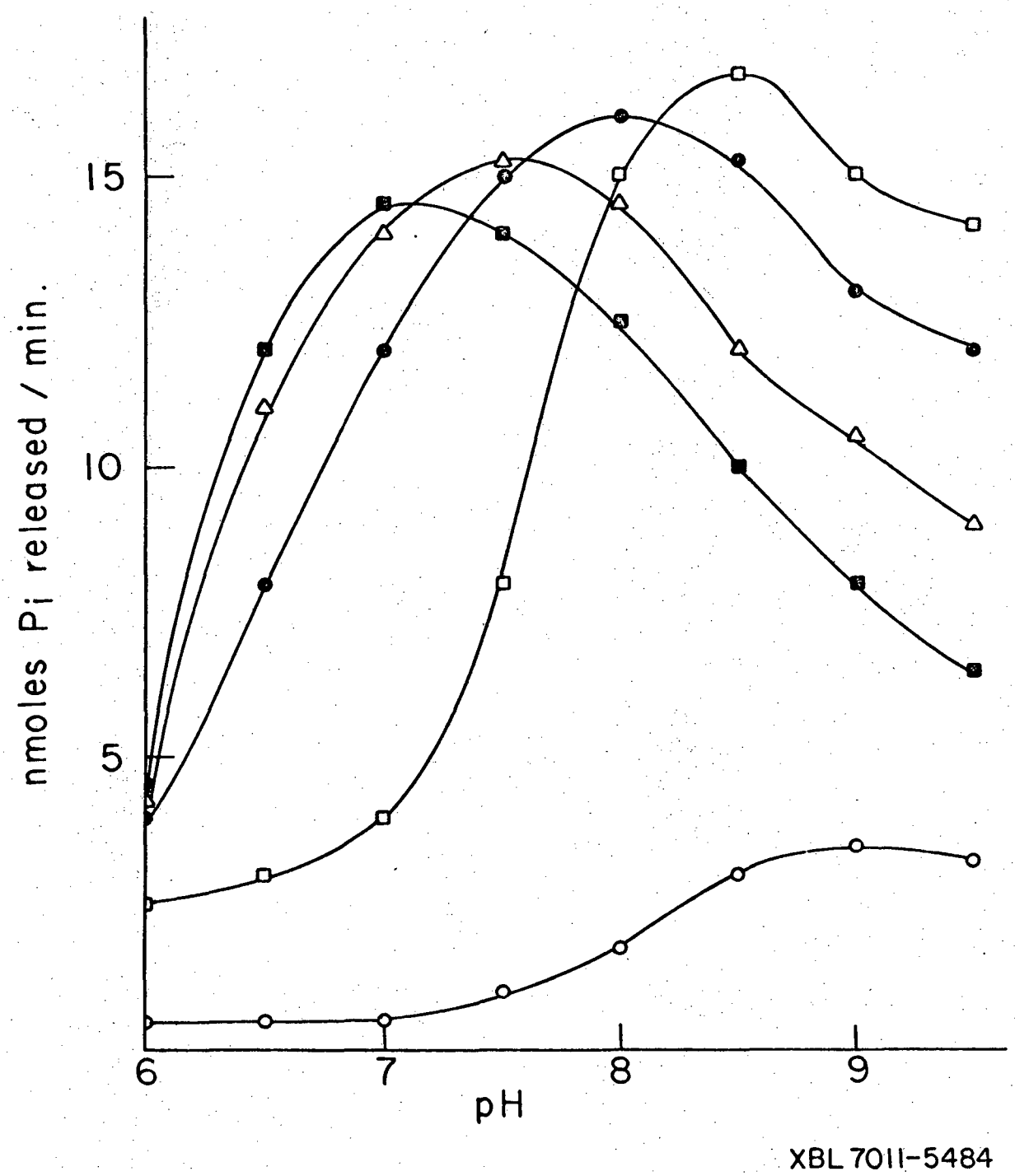

Figure I-1

the pH shifts to the alkaline pH the enzyme conformation changes and consequently the negative charges become remote from the site; thus the Mg^{++} concentration needed for maximum activity is much lower than that required at the neutral pH.

Another explanation for the shift of pH optima as a function of Mg^{++} concentration is based on the chelate association constant for the Mg^{++} and the ionic Fru-1,6-P_2. McGilvery[50] calculated that a solution, 1 mM in each of Fru-1,6-P_2 and $MgCl_2$ with sodium salts to bring the ionic strength to 0.077 (at 25° at pH 7), is expected to contain 19% as Mg Fru-1,6-P_2^{-2}, and 3% Mg H Fru-1,6-P_2^{-}. If the pH was raised so as to completely ionize Fru-1,6-P_2, the concentration of the chelate would still only represent 26% of the total Fru-1,6-P_2. It is apparent from these calculations that the activity of FDPase cannot be explained in terms of action of the Mg^{++} on the substrate, as it is the case in some other enzymes such as chloroplast inorganic pyrophosphatase[51]. The great shift in enzymic activity depending on the pH and Mg^{++} concentration excludes or minimizes this assumption. In addition, we were not able to obtain significant inhibition of chloroplast FDPase by increasing the concentration of the anionic Fru-1,6-P_2. Fig. 2 shows that Mg^{++} has a direct effect on FDPase. The sigmoidal shape of the curve of the dependence of activity of FDPase on Mg^{++} concentration shows that at both high and low pH (pH 7.0 and 8.7) the response of enzymic activity to Mg^{++} concentration is sigmoidal at low Mg^{++} concentrations. Such behavior may be interpreted as allosteric effect of Mg^{++} on FDPase.[52]

F. EFFECT OF CATIONS ON FDPASE ACTIVITY

Several other cations were tested for their possible activation of the enzyme. Mg^{++} was by and large the most activating of all

Fig. 2. Dependence of FDPase activity on Mg^{++} ion concentration.

O--O pH 8.7

Δ--Δ pH 7.0

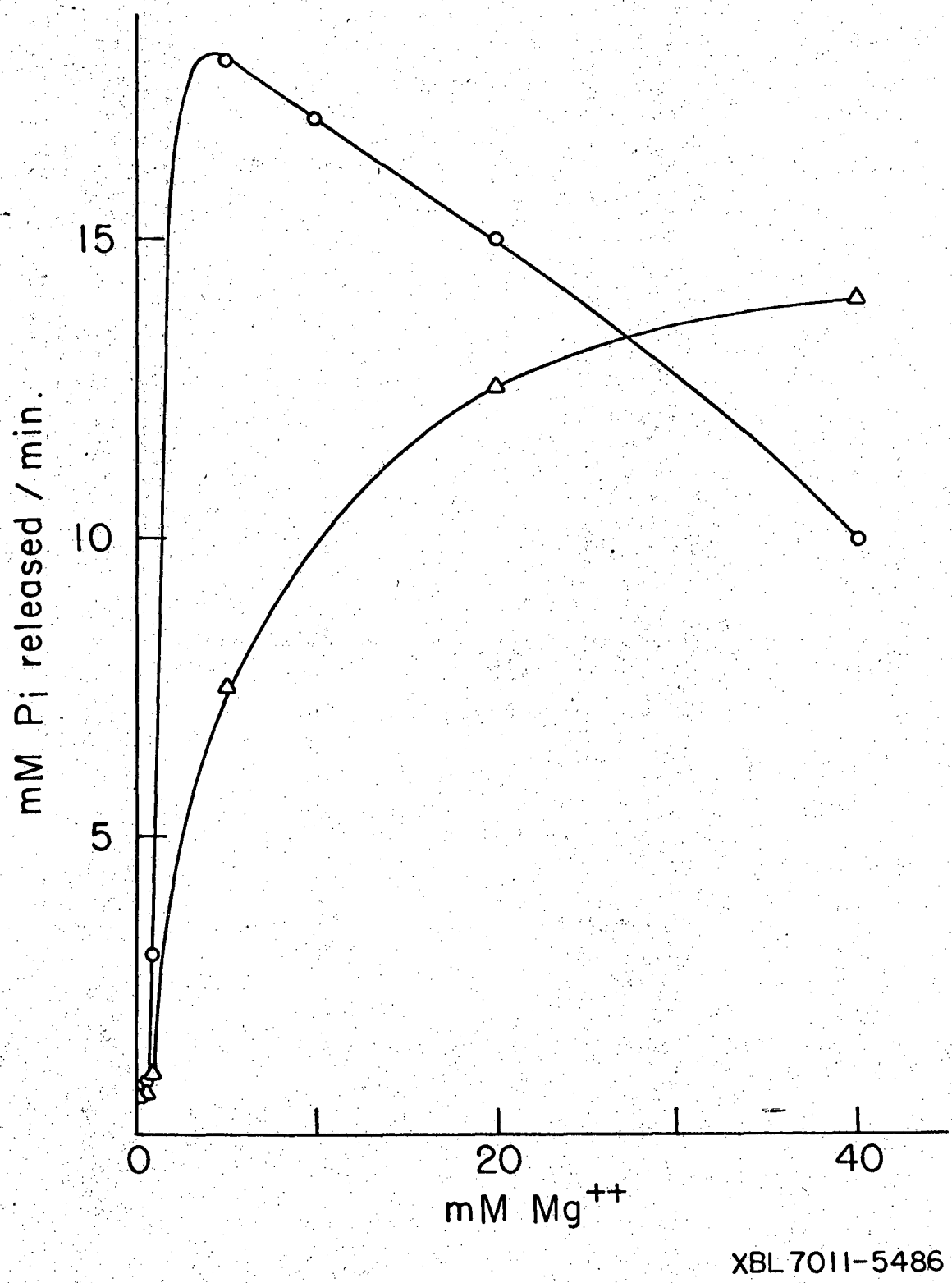

Figure I-2

cations tested. Mn^{++} partially replaced Mg^{++} as the divalent cation required for the catalytic hydrolysis of FDP by the enzyme. All other cations tested exhibited no activating effect on FDPase (Table II). No activity for the hydrolysis of sedoheptulose-1,7-diphosphate, fructose-1-phosphate, or ribulose-1,5-diphosphate was induced by the presence of Zn^{++}, Mn^{++} or Ca^{++}. The lack of activity of chloroplast FDPase towards the hydrolysis of SDP in the presence of Mg^{++} had suggested the possible activation by other divalent cation in the assay mixture. No study of the antagonism or synergism between any of the metal ions and Mg^{++} was carried out.

G. EFFECT OF ANIONS ON THE ACTIVITY OF FRUCTOSE-1,6-DIPHOSPHATASE

The effect of anions on the activity of FDPase was carried out in assay mixtures 5 mM in magesium ion. The sodium salt of the anions desired were used throughout this experiment. The concentrations of the anions were varied from 5 to 40 mM at pH 8.7. None of the anions tested (F^-, Cl^-, Br^-, I^-, SO_4^{--} and HPO_4^{--}) had any stimulating or inhibitory effect on the enzyme. The lack of inhibition by the F^- ion of FDPase may provide another piece of evidence for the idea that the action of Mg^{++} is on the enzyme and not on the substrate. The solubility product of MgF_2 is very low. This low solubility product of MgF_2 has resulted in the inhibition of enzymes which require the magnesium salt of the ligand acting as substrate. An example of such enzymes is chloroplast inorganic pyrophosphatase which requires the magnesium salt of pyrophosphate as the substrate. Pyrophosphatase was inhibited by the fluoride ion due to the unavailability of Mg^{++} in the reaction mixture. This was not the case with FDPase. Thus there may be

TABLE II

EFFECT OF CATIONS ON FRUCTOSE-1,6-DIPHOSPHATASE ACTIVITY

Cation	nmoles of P_i released per ml* per min			
	5	10	20	40 mM cation
Mg^{++}	18	17	16.3	15.5
Mn^{++}	8.6	8.5	7	5
Zn^{++}	2.5	2.0	1.0	0.05
Fe^{3+}	0.5	0.5	0.2	0.05
Co^{++}	1.5	1.4	0.5	0.2
Ni^{++}	1.0	0.8	0.3	0.05
Au^{++}	0.4	0.2	0.1	0.05
Cd^{++}	1.0	0.5	0.5	0.08
Ca^{++}	1.5	1.0	1.0	1.5

* Containing 1 μg of fraction VI protein

a strong binding between magnesium and the enzyme and a direct effect of Mg^{++} on FDPase, changing its conformation into one which is active towards the hydrolysis of Fru-1,6-P_2.

H. ACTIVATION OF FDPASE BY GLYCINE

Glycine was found to activate FDPase towards the hydrolysis of FDP as shown in Fig. 3. The activity of the enzyme is increased by about 50%. The activation by glycine is not magnesium dependent. The glycine activation of the isolated enzyme raises the possibility of the regulation of FDPase by glycine in vivo. The diversion of carbon from the carbon reduction cycle to the synthesis of amino acids and proteins may be regulated at the FDPase point.[53] We have no simple explanation of a mechanism of this activation. However, it appears that a balance in favor of increased amount of soluble glycine results in the activation of FDPase and an increase in the flow of F6P to the synthesis of amino acids. The participation of glycine as a regulatory substance was further substantiated when it was found that glycine activates and enhances the carbon dioxide fixation by isolated chloroplasts in a parallel way to its activation to FDPase.[54]

I. ACTIVATION OF FDPASE BY BICARBONATE

Bicarbonate which is the substrate for the enzyme ribulose-1,5-diphosphate carboxylase (E.C. 4.1.1.39) (which catalyzes the fixation of CO_2 using the five-carbon sugar diphosphate RuDP and resulting in the production of PGA), the first enzymic reaction in the carbon reduction cycle was found to be an activator of spinach chloroplast FDPase. This finding might suggest that carbon dioxide not only acts as the substrate for RuDPase but also as the activator for some enzymes involved in the

Fig. 3. Activation of FDPase by glycine, bicarbonate, and dithiothreotol, as a function of concentration of the actovator.

□--□ glycine

■--■ bicarbonate

○--○ dithiothreotol

●--● control

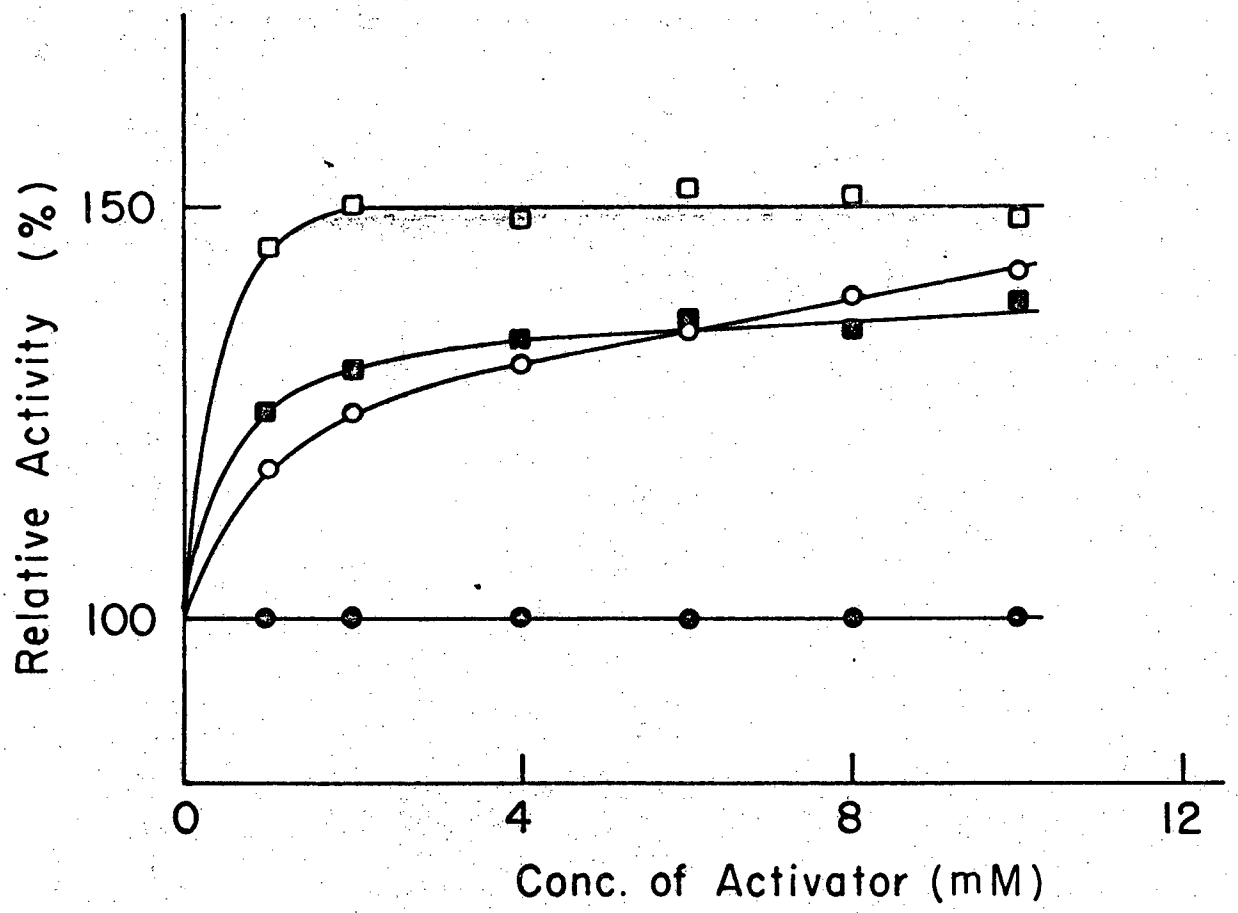

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Figure I-3

cycle. This regulatory mechanism may prove to be an important one and needs to be studied further.

J. ACTIVATION OF FDPASE BY FERREDOXIN AND SULFHYDRYL REAGENTS

Buchanan et al.[55,56] claimed that reduced ferredoxin activates the enzyme FDPase. Our results do not confirm this finding. Reduced ferredoxin, dithiothreotol and glutathion were found to act as protective agents against the oxidation of the enzyme's sulfhydryl groups. We have no evidence of direct activation of the enzyme by ferredoxin.

The use of illuminated chloroplasts resulted in the reduction of ferredoxin, but this system was not suitable for the assay of FDPase activity. The chloroplast suspension contributed a high background level of inorganic phosphate, and also contained a considerable level of FDPase activity. This interfered with the determination of inorganic phosphate by the Fiske-SubbaRow method.

The use of hydrogen reduction catalyzed by bacterial hydrogenase has the drawback of interference due to high level of bacterial FDPase activity. Because of lack of kinetic and other data on the interaction of FDPase's from the two sources, spinach chloroplasts and C. pasterianum, it was not possible to obtain conclusive results.

Because of these difficulties we turned to chemical reduction. Reduction by dithionite proved to be an efficient method for reducing ferredoxin. Unfortunately, the presence of hydrosulfite ion interfered with the activity of FDPase. Therefore a method was devised to achieve reduction of ferredoxin and eliminate the excess hydrosulfite ion and its oxidation products from the medium. Ferredoxin was mixed with sodium dithionite under nitrogen in a closed system. The resulting reduced ferredoxin was then transferred to a dialysis tubing and dialysis was

carried out under nitrogen with change of buffer (0.05 M tris, pH 7.4) every 3 h, for a total of four changes. The dialysis was done at 0°C. Reduced ferredoxin was introduced into the assay mixture just prior to the introduction of FDPase. The reaction was allowed to proceed for 10 min, then was stopped by adding 0.1 ml of 50% trichloroacetic acid. The precipitate was centrifuged out, and the inorganic phosphate produced in the enzymatic reaction was determined by the Fiske-SubbaRow method.[36,37]

Fig. 4 shows that Mg^{++} concentration did not affect the relative amount of activation achieved in the presence of 100 μg of ferredoxin in a 1-ml assay mixture. We had expected that reduced ferredoxin may replace Mg^{++} in blocking the negative charges near the active site; thus, at lower Mg^{++} concentrations a greater amount of activation would be expected. Our results, however, do not confirm this assumption and we obtained relatively the same degree of activation (about 10%) at all levels of Mg^{++} tested. Dithiotheitol followed a similar pattern of activation with the relative degree of activation at lower magnesium concentration (5-10 mM) about double that obtained at 40 mM Mg^{++}.

The activation of FDPase by ferredoxin and DTT as a function of pH (Fig. 5) shows that the mechanism of activation of FDPase by sulfhydryl reagents is different from that obtained by ferredoxin at lower pH values (pH 6.5-7.5) the activation of the enzyme by sulfhydryl reagents was more than 100% of the activity in the absence of the -SH reagent while at higher pH the activation was much smaller. Ferredoxin, on the other hand, gave almost a constant small amount of activation at pH 6 to 9.5).

This finding induced us to study the effect of mixing ferredoxin and dithiotheitol and Table III shows that the effect is additive, which

Fig. 4. Activation of FDPase by glycine, bicarbonate, dithiothreotol, and ferredoxin as a function of magnesium ion concentration.

△--△ 10^{-3} M glycine

□--□ 10^{-3} M bicarbonate

■--■ 10^{-3} M dithiothreotol

○--○ 100 μg ferredoxin

●--● control

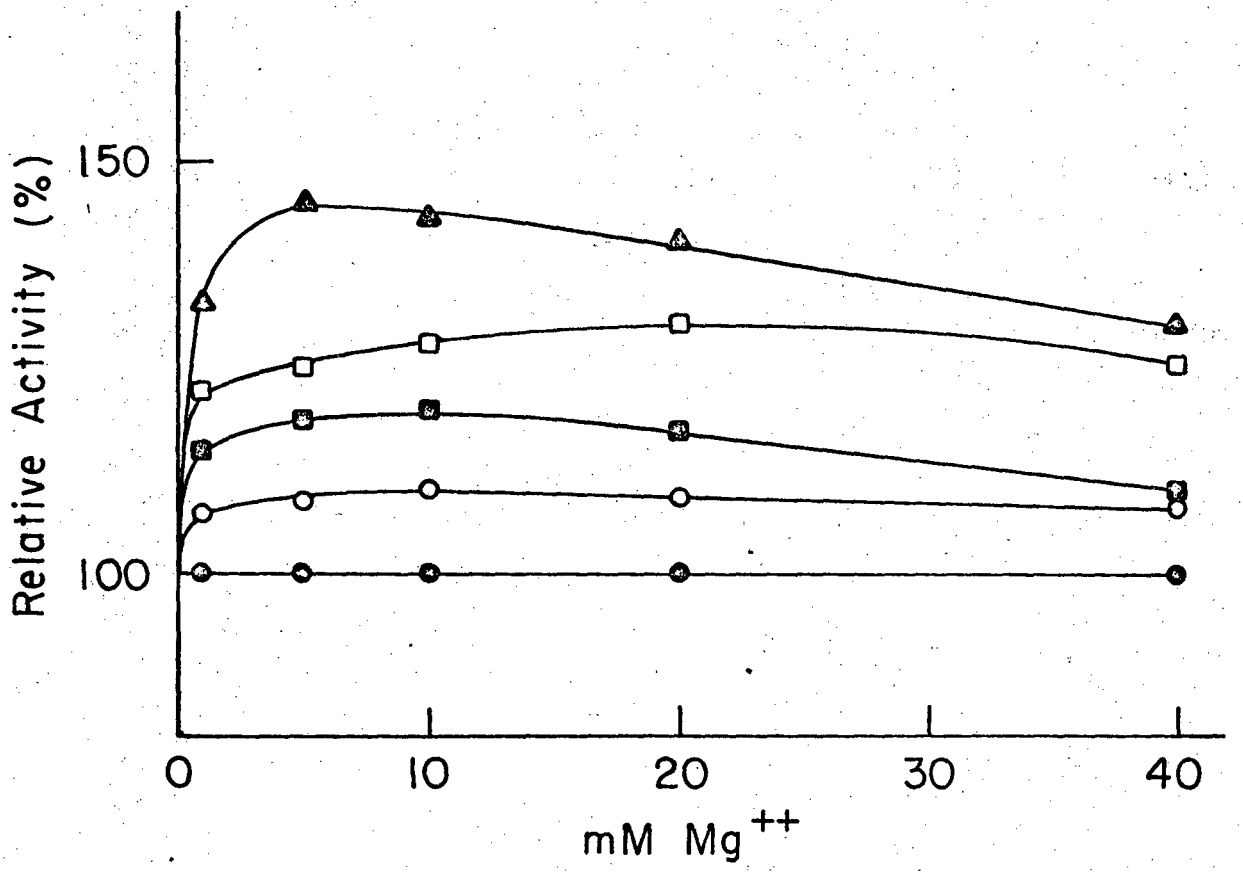

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Figure I-4

Fig. 5. Effect of pH on the activation of FDPase by DTT and ferredoxin.

O--O control (1 μg enzyme, no ferredoxin or DTT)

□--□ 1 μg enzyme + 100 μg ferredoxin

△--△ 1 μg enzyme + 10^{-3} M DTT

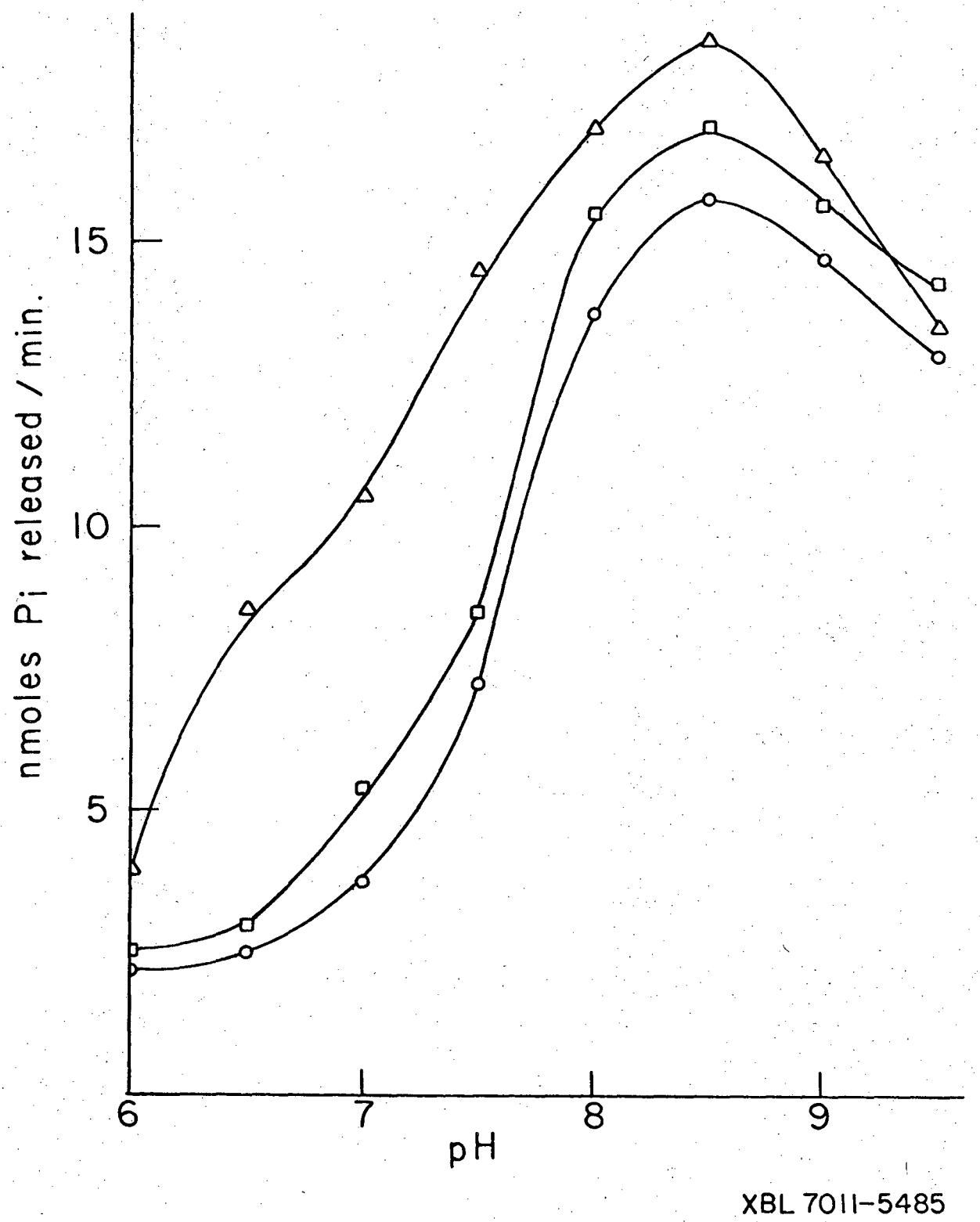

Figure I-5

TABLE III

THE ACTIVATION OF FDPASE BY CHEMICALLY REDUCED FERREDOXIN AND DTT

	Inorganic phosphate liberated/ml* per min (nmoles)
Complete	24.6
- Ferredoxin	23
- DTT	21
- Ferredoxin and DTT	18.5
- FDPase	0.05
- FDP	0.04

* Containing 1 μg of fraction VI protein

reinforces our previous assumption that the mechanism of action of ferredoxin and sulfhydryl reagents on FDPase are different.

K. ACTIVATION OF FDPASE BY AMMONIUM SULFATE

In the purification process activation of spinach chloroplast FDPase was observed upon treatment with ammonium sulfate. This activation was unobtainable if ammonium sulfate was used for fractionation before the use of acetone fractionation. This may suggest the presence of an acetone extractable inhibitor of the enzyme which may mask the activation of the enzyme. The nature of this inhibitor is under study. It may be that acetone results in dissociation of an enzyme inhibitor complex freeing a site for ammonium sulfate to act on the enzyme as an activator. Concentrates of the 75% acetone supernatant obtained during the purification of the FDPase was inhibitory to the purified enzyme.

L. SPECIFICITY OF FDPASE

Spinach chloroplast fructose-1,6-diphosphatase is highly specific for fructose-1,6-diphosphate. The enzyme failed to attack sedoheptulose-1,7-diphosphate, ribulose-1,5-diphosphate, fructose-6-phosphate, fructose-1-phosphate, glucose-1-phosphate, glucose-6-phosphate, ribulose-5-phosphate, pyrophosphate, AMP, ADP or ATP. Changing the cation in the assay mixture from Mg^{++} to Zn^{++}, Mn^{++} or Ca^{++} and/or changing the pH to neutral pH (pH 7) did not induce any enzymic activity towards sedoheptulose-1,7-diphosphate, ribulose-1,5-diphosphate or fructose-1-phosphate.

M. DISCUSSION

The properties of the chloroplast FDPase, including its pH optima shift with Mg^{++} and its activation by glycine and by carbonate ion, are

additional evidence that the chloroplast enzyme, like FDPase from many other tissues, plays an important regulatory role in carbon metabolism in chloroplasts. This role was indicated earlier[12] by kinetic studies of Chlorella pyrenoidosa photosynthesizing in the presence of $^{14}CO_2$ and ^{32}P-labeled phosphate, in which rapid changes in levels of fructose-1,6-diphosphate and fructose-6-phosphate accompanied the light to dark and dark-light transition and the addition of several chemicals which produced general regulator effects.

In view of the similar evidence from in vivo studies that the conversion of sedoheptulose-1,7-diphosphate to sedoheptulose-7-phosphate is also regulated[12], it is interesting that the purified FDPase did not convert sedoheptulose-1,7-diphosphate to its monophosphate. Present evidence does not permit us to decide whether two separate enzymes are present in the chloroplasts for the hydrolysis of these two sugar diphosphates or the isolated enzyme has lost its capacity to convert sedoheptulose diphosphate, either through lability or because of lack of conditions necessary for activation of this function.

Kinetic studies of the properties of the enzyme isolated by the procedures in this report provide additional strong evidence for the allosteric properties of this enzyme[53]. We have found no evidence for a large activation of the enzyme by reduced ferredoxin at any pH of Mg^{++} ion concentration studied. However, Buchanan et al.[55,56] who reported such activation by reduced ferredoxin, also reported a requirement for a protein factor of low molecular weight. Since we have not isolated such a factor, our experimental results are not necessarily in disagreement with those reports, in which no specific activity of the enzyme was given.

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Chapter II

FLUORESCENCE STUDIES WITH CHLOROPLAST FRUCTOSE-1,6-DIPHOSPHATASE

A. INTRODUCTION

Enzymic action is generally associated with conformational change of the protein catalyzing the specific reaction. This change could be affected either by the substrate or by the allosteric affector[1,2].

Fluorescence techniques are very useful in revealing facets of conformational changes and dynamics. They are helpful in measuring distances between groups on proteins, determining the extent of flexibility of a protein, and measuring the rate of very rapid conformation transitions. In addition, the degree of polarity of sites on the protein can be measured using probes such as 1-anilino-8-naphthalene sulfonate (ANS) in a highly polar environment[3].

In this report we have investigated the conformational change of fructose-1,6-diphosphatase, isolated from spinach chloroplasts, induced by the substrate fructose-1,6-diphosphate, the product fructose-6-phosphate, and the divalent metal ion required for the enzymic catalysis, Mg^{++}. In addition, conformational changes caused by the allosteric affectors, ADP, ATP, and inorganic pyrophosphate, were also studied.

In these studies we have used the techniques of quenching fluorescence and depolarization fluorescence. Two types of fluorescence were investigated: The intrinsic fluorescence of the enzyme FDPase resulting from the presence of tryptophan and tyrosine residues in the protein, and the extrinsic fluorescence induced in the presence of ANS.

B. MATERIALS AND METHODS

1- Chemicals. Fructose-1,6-diphosphate, fructose-6-phosphate, ATP, ADP, AMP and cyclic AMP were highest purity products obtained from Cal Biochem. Tetrasodium pyrophosphate was obtained from Allied Chemicals. Trizma base, reagent grade, was obtained from Sigma Chemical Company. Magnesium chloride, $MgCl_2 \cdot 6H_2O$ was obtained from J. T. Baker Chemical Co. N Acetyl-L-tyrosine ethyl ester, Grade 1, and N Acetyl-L-tryptophan ethyl ester, Grade 1, were obtained from Cyclo Chemical Co.

2- Preparation of enzyme. The enzyme was prepared from spinach chloroplast as described by El-Badry[4] and the homogeneous protein had a specific activity of about 20 units/mg protein.

Protein concentration was determined spectrophotometrically by measuring the absorption at 280 nm or by using the Lowry method of protein determination[5]. Bovine serum albumin was used as the standard in each of the two methods.

The enzyme was assayed by coupling the FDPase reaction with that of glucose phosphate isomerase and glucose-6-phosphate dehydrogenase, and the NADPH produced in the reaction was measured spectrophotometrically at 340 nm[4].

3- Instrumentation

Two spectrophotofluorimeters were used in this investigation: (1) A modified Aminco-Bowman spectrophotofluorimeter with an improved detection system and (2) a Hitachi MPF-2A fluorimeter.

The Aminco-Bowman fluorimeter had the standard optical system with Xenon lamp including the power supply. In the cell compartment a tuning fork (from American Time Company) was mounted; it was oscillating at

200 cycle/sec. A driving unit for the fork produced simultaneously a reference signal which was used for the phase sensitive measuring system. An RCA 6199 end-on tube was mounted in a Princeton Applied Research (PAR) photomultiplier housing. A photometric preamplifier (PAR) RIM221 was in the same housing near the photomultiplier. The load resistor of the photomultiplier could be selected externally. The signal from the preamplifier went into a PAR JB-4 lock-in amplifier. The output was attached to a Hewlett-Packard X-Y recorder with time base. The versatility of this instrument with the possibility of expanding the scale, shifting the base line, choosing different time constant and optimizing the signal-to-noise ratio in different stages of the detecting system made it suitable for fluorescence quenching experiments and for kinetic measurements. In addition, the response curve of the RCA 6199 with an S-11 characteristic showed that this photomultiplier itself works as a cut-off filter for suppressing scattered light from the excitation of the protein fluorescence. The spectra reported in this paper are uncorrected for monochromator transmission and for photomultiplier response.

We used the well designed optical system of the Hitachi MPF-2A fluorimeter with two Beckman polarizers to determine the polarization spectra. With an S-20 response photomultiplier, the correction curve in the region of the intrinsic protein fluorescence was very flat; therefore, nearly "true" fluorescence spectra were recorded, and used to determine the peak position of the fluorescence.

FDP and F6P had a slight absorption in the exciting wavelength region; due to this absorption the fluorescence was corrected according to the formula of Förster[6], which was approximated in our case to the following equation:

$$\text{Fluorescence (corrected)} = \text{Fluorescence (measured)} \left(1 + \frac{OD}{2}\right)$$

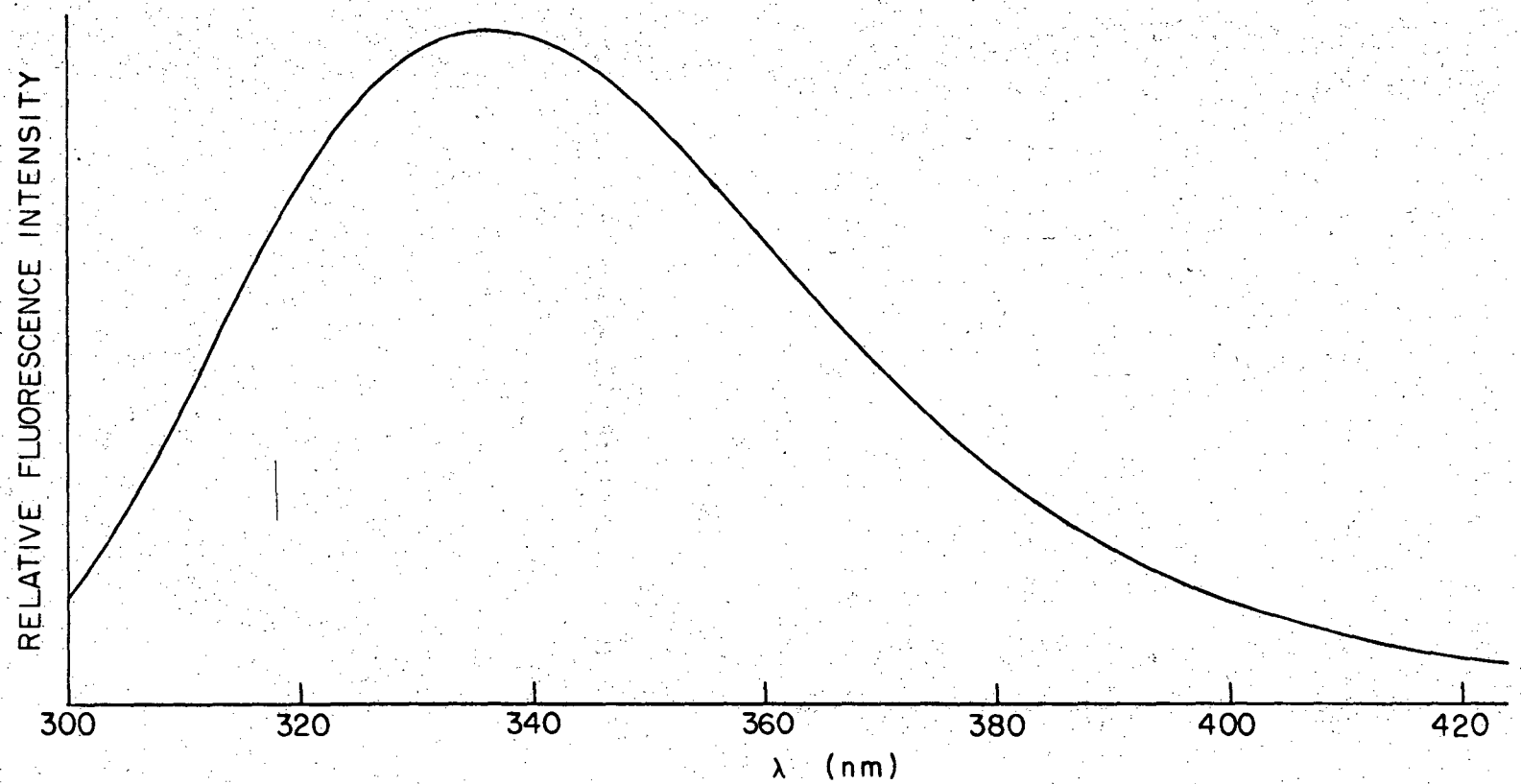
where OD is the optical density of FDP or F6P at the exciting wavelength.

To account for the small dilution effect by adding substrate and inhibitors to the enzyme solution, we measured first the decrease of the fluorescence by adding the same amount of buffer solution.

C. INTRINSIC FLUORESCENCE

The intrinsic fluorescence spectrum of the enzyme measured with MPF-2A (Fig. 1) shows that the peak fluorescence occurs at 337 nm and is independent of the excitation wavelength. This demonstrates that most of the fluorescence is due to tryptophan emission, which occurs normally between 332 if the residue is in non-polar environment to 342 in polar environment, and that about half of the tryptophan residues see a polar and the others have a non-polar environment. This suggests that some residues are exposed to the aqueous environment. That none or little fluorescence can be seen from tyrosine residues arises from the fact that very efficient quenching mechanisms exist for tyrosine. Some of these quenching mechanisms originate in the interaction with the microenvironment of the protein[7]. The efficiency of energy transfer from tyrosine to tryptophan has been shown, after some controversial investigations, to be as high as 0.56[8]. Tryptophan fluorescence in proteins can increase or decrease by binding with other molecules depending on the local environmental change of the emitting species. There are many different quenching mechanisms for tryptophan fluorescence, and it seems that not one of them is dominant in proteins. For instance, proton donation from -COOH and NH_3^+ groups, etc., electron ejection in the excited state to electro-negative groups in the neighborhood of the indole ring, changes in the intersystem crossing, intermolecular electron transfer, hydrogen bonding and so on (for literature survey see 7 and 9). This complicated

Fig. 1. Intrinsic fluorescence spectrum of chloroplast fructose-1, 6-diphosphatase measured with Hitachi (Perkin-Elmer) MPF-2A fluorescence spectrophotometer.



XBL 7012-4936

Figure II-1

situation makes it difficult to make specific statements upon detecting some changes in the fluorescence intensity of enzymes by binding of substrates and inhibitors. If there are tryptophan residues in the active center, then this can be quenched directly by binding of the substrates. Figs. 2 and 3 show the activation and fluorescence spectra of FDPase with and without FDP and F6P and we see that in both cases decrease of fluorescence intensity occurs. To decide if this quenching was the result of direct tryptophan-FDP, F6P interaction we investigated the fluorescence of the substance N-acetyl tryptophan amide, and we found that both FDP and F6P are weak sensitizers of the tryptophan ester amide fluorescence. This suggests that the decrease of fluorescence intensities is the result of conformational change of the enzyme.

Figs. 4 and 5 show the quenching of the enzyme fluorescence as a function of FDP and F6P concentrations respectively. These curves can be used to give a kind of averaged binding constant. Because the number of active sites on this FDPase is not known, we assume as approximation that the different active sties are independent of each other and that binding to each site produces the same amount of quenching. We then have

$$\frac{[B]\ [S]}{[BS]} = K$$

where [BS] represents the concentration of binding sites bounded with substrate S and [B] is the concentration of free sites. In addition, we have

$$[B_{total}] = [BS] + [B]$$

If all the binding sites are occupied we get maximal quenching,

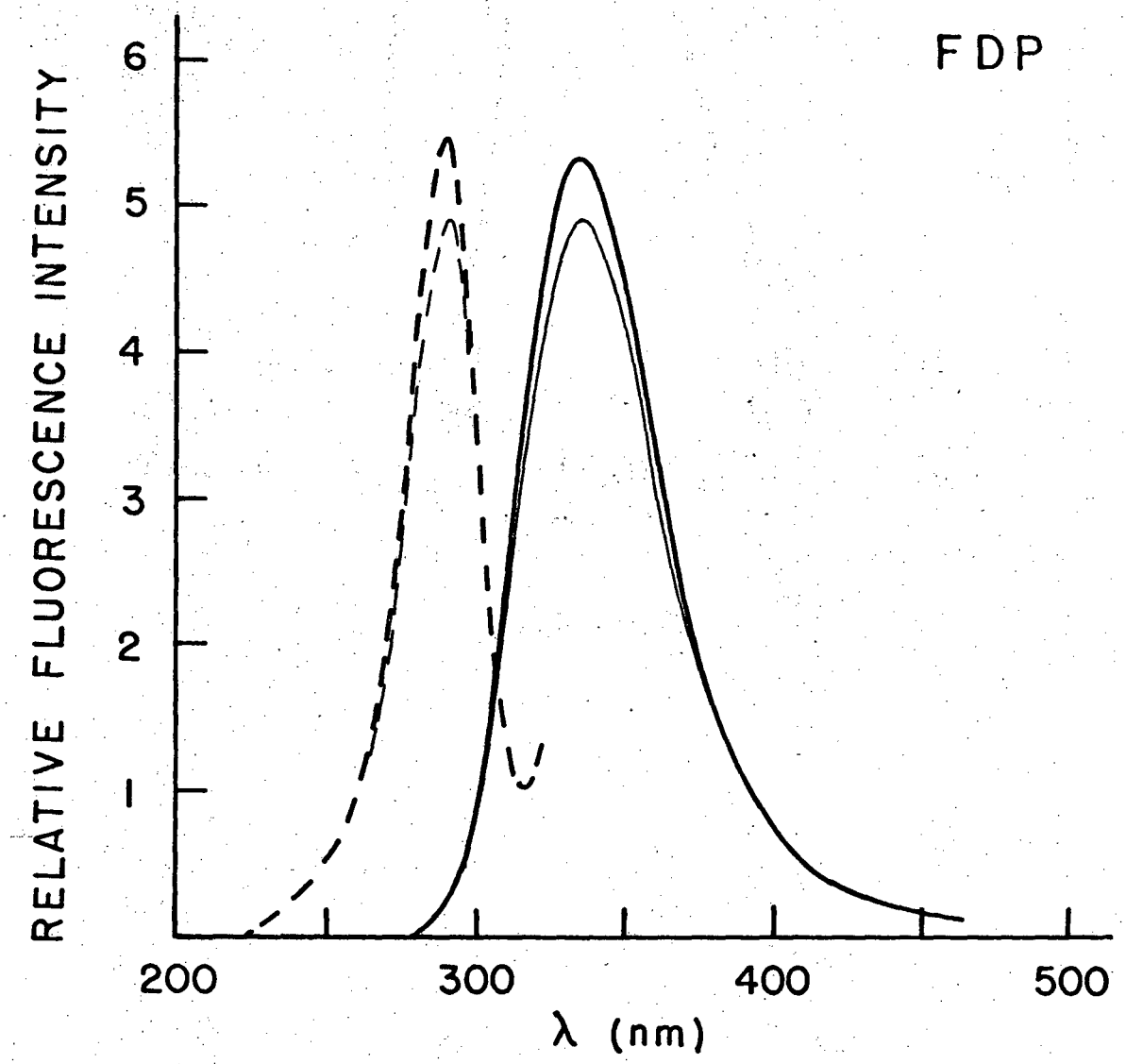
Fig. 2. Quenching of fluorescence and activation spectra of FDPase by FDP.

▬▬▬ Fluorescence spectrum of FDPase for activation at 290 nm

——— Fluorescence spectrum of FDPase + 5 mM FDP for activation at 290 nm

▬ ▬ ▬ Activation spectrum of FDPase for fluorescence at 340 nm

- - - Activation spectrum of FDPase + 5 mM FDP for fluorescence at 340 nm



XBL708-5327

Figure II-2

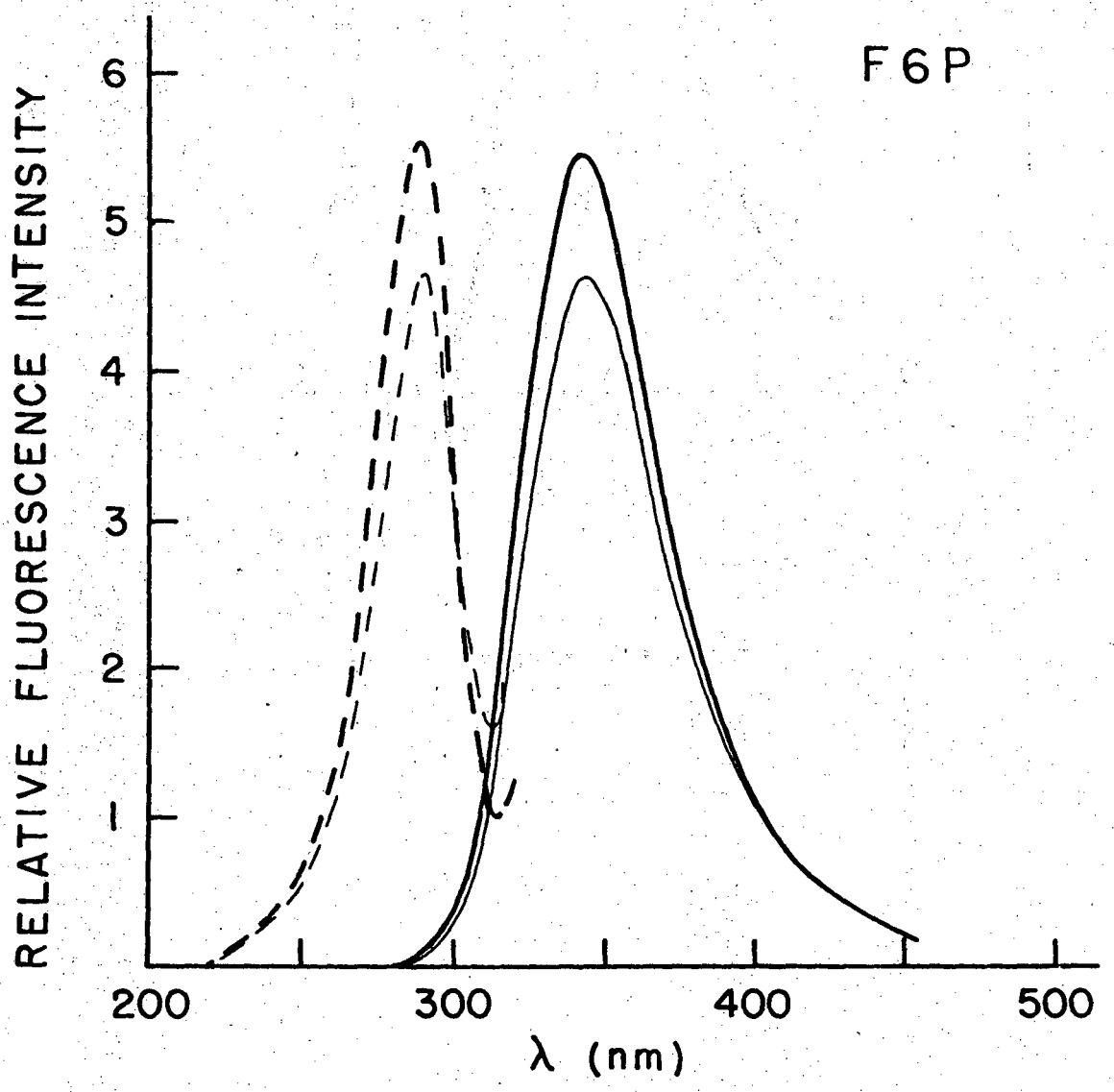
Fig. 3. Quenching of fluorescence and activation spectra of FDPase by F6P

▬▬▬ Fluorescence spectrum of FDPase for activation at 290 nm

——— Fluorescence spectrum of FDPase + 5 mM F6P for activation at 290 nm

▬ ▬ ▬ Activation spectrum of FDPase for fluorescence at 340 nm

- - - Activation spectrum of FDPase + 5 mM F6P for fluorescence at 340 nm



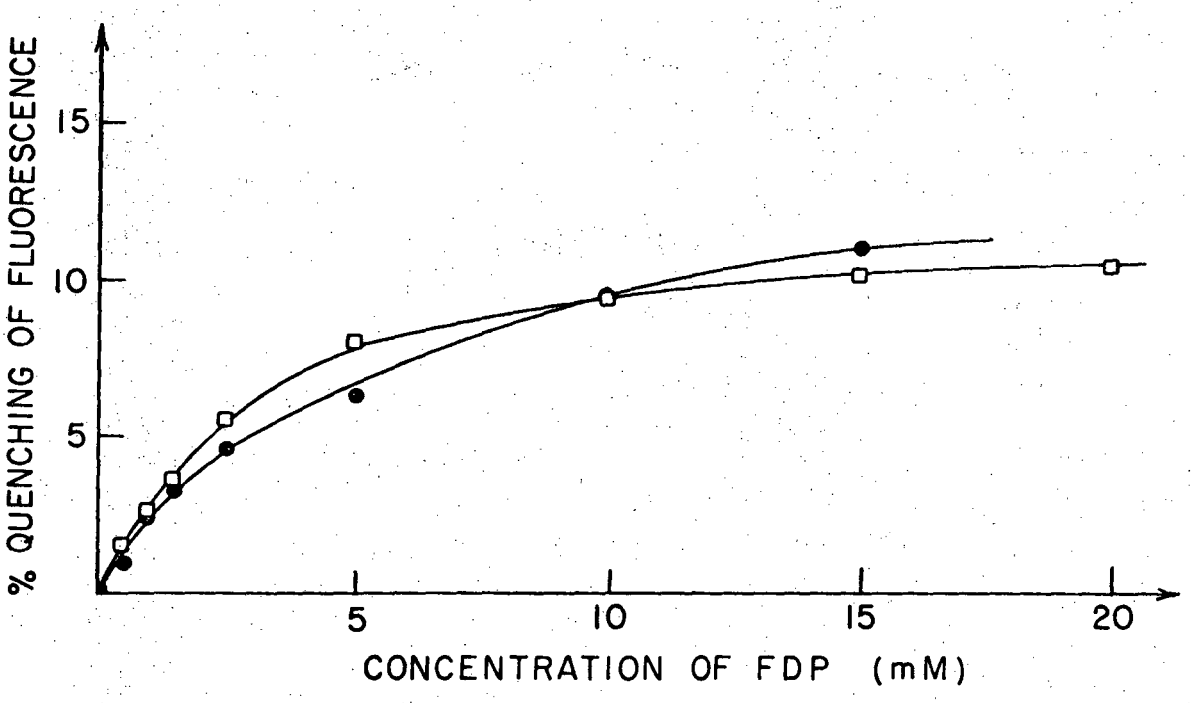
XBL708-5324

Figure II-3

Fig. 4. Quenching of fluorescence as a function of FDP concentration.

□--□ Fluorescence of FDPase solution in 0.05 M tris-HCl, pH 8.7 at 22°, as a function of FDP concentration

O--O Fluorescence of FDPase solution in 0.05 M tris-HCl, pH 8.7, in the presence of 5 mM $MgCl_2$



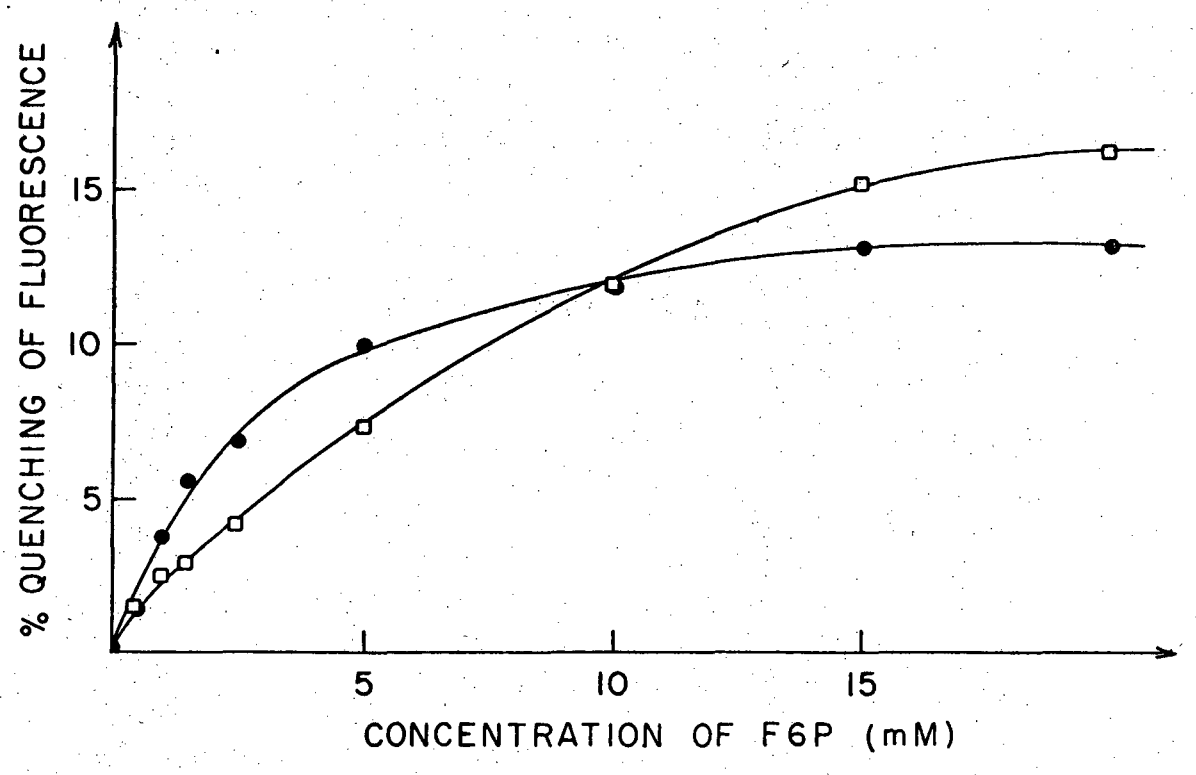
XBL 704-5155

Figure II-4

Fig. 5. Quenching of fluorescence as a function of F6P concentration.

□--□ Fluorescence of FDPase solution in 0.05 M tris-HCl at 22°, as a function of F6P concentration

O--O Fluorescence of FDPase solution in 0.05 M tris-HCl, pH 8.7, in the presence of 5 mM $MgCl_2$



XBL 704-5156

Figure II-5

f_{max}, otherwise we have $f = f_{max} \frac{[BS]}{[B_{total}]}$ and with the help of the above equations

$$f = \frac{[S]\ f_{max}}{K + [S]}$$

Thus we see that for $[S] = K$, $f = \frac{f_{max}}{2}$.

And this relationship can be used for determining K. For FDP half of the maximal quenching in presence of Mg^{++} is achieved for $[S] = 3.8 \times 10^{-3}$ M = K.

For F6P we get $K = 2.2 \times 10^{-3}$ M.

These binding constants are very low and suggest that these molecules are held to the enzyme with weak forces like dipole-dipole, dipole-charge, or hydrogen bonding interactions. These low binding constants could explain the slow kinetics exhibited by FDPase.

The difference in quenching of FDPase by FDP and F6P makes it possible to follow the kinetics of conversion of FDP to F6P by fluorescence intensity measured as a function of time. Fig. 6 demonstrates that the fluorescence kinetics correspond to the kinetics determined by following the reduction of NADP as described in the Methods section.

The temperature dependence of the fluorescence of FDPase is presented in Fig. 7. In presence of magensium ion the curve is very smooth, suggesting that no structural change occurs in the investigated temperature range. It seems that there are minor local structural changes in the case of FDPase in the absence of magensium ions at 22°C, suggesting that there are binding sites for Mg^{++} on the enzyme that produce local stability.

The pH dependence is shown in Fig. 8. Probably a strong structural

Fig. 6. Kinetics of the enzymatic hydrolysis of FDP as measured by change in fluorescence intensity as a function of time. The enzymic solution contained 5 mM $MgCl_2$ in tris-HCl buffer, pH 8.7; the reaction was measured at 22°.

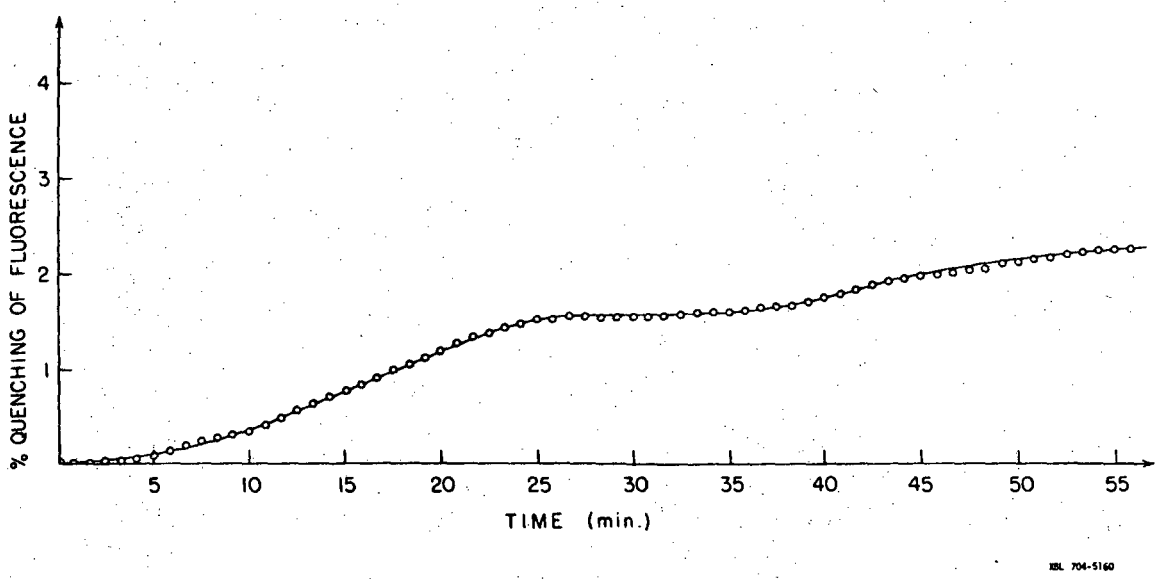
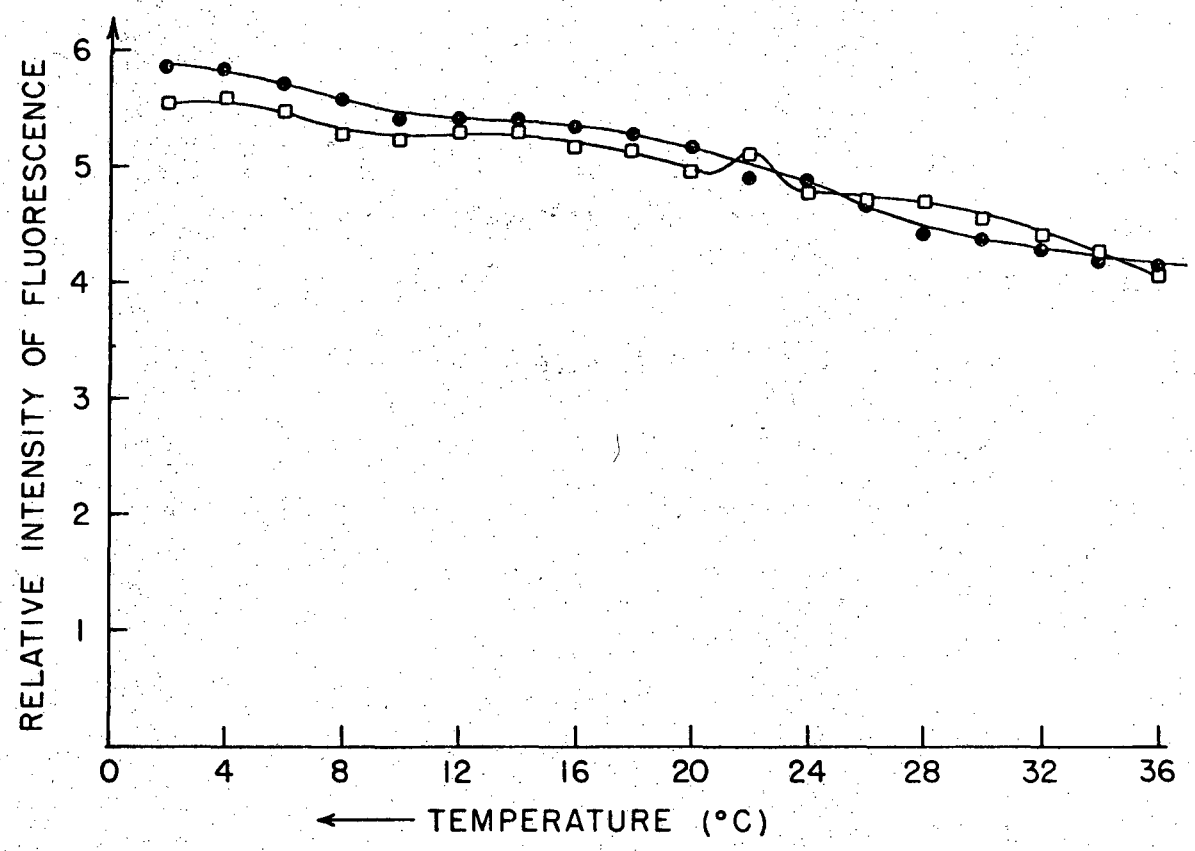

% QUENCHING OF FLUORESCENCE
1
2
3
4
5
10
15
20
25
30
35
40
45
50
55
TIME (min.)
XBL 704-5160

Figure II-6

Fig. 7. Temperature dependence of FDPase intrinsic fluorescence.

□--□ Enzyme solution in 5 mM tris-HCl, pH 8.7

O--O Enzyme solution in 5 mM tris-HCl, pH 8.7, in the presence of 5 mM Mg^{++}



XBL 704-5157

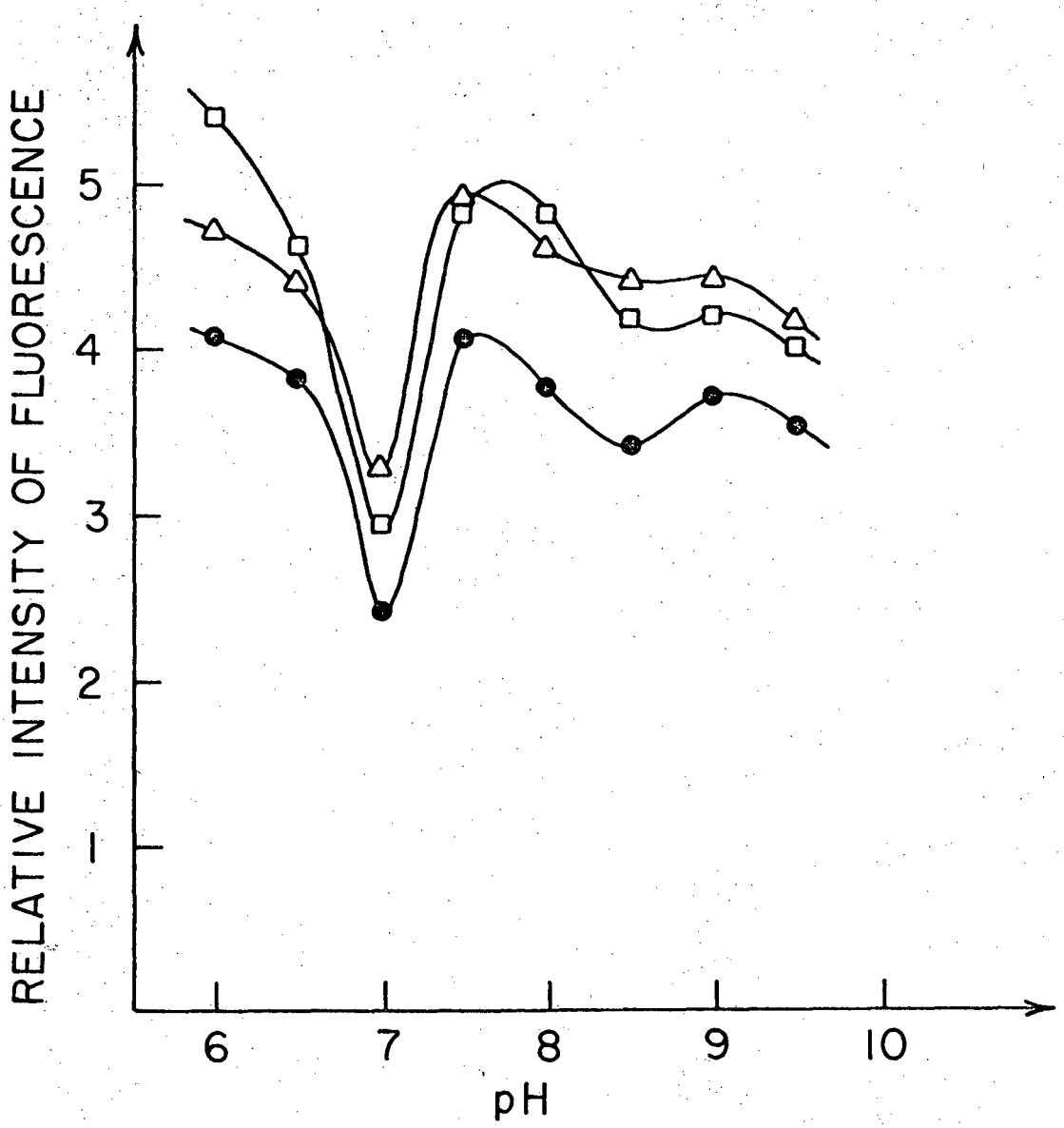
Figure II-7

Fig. 8. pH dependence of FDPase intrinsic fluorescence.

□--□--□ Enzyme solution in tris-HCl, pH 8.7, at 22°

O--O--O Enzyme solution in tris-HCl in the presence of 5 mM $MgCl_2$ at 22°

△--△--△ Enzyme solution in tris-HCl in the presence of 40 mM $MgCl_2$ at 22°



XBL 704-5158

Figure II-8

transition produces the minimum in the fluorescence versus pH curve at pH 7. FDPase exhibits very low activity at pH 7 and magnesium ion concentration (5 mM) is pH 8.5. This optimum shifts to the neutral pH as the Mg^{++} concentration in the medium increases to 40 mM[4]. The structural change at pH 7 could be responsible for the low activity seen at pH 7.

Although the pK of imidazole ring in histidine is 6.0, the pK of imidazole is 6.9; one might expect the pK of imidazole ring in the side chain of a protein to have a pK of close to 7; thus the ionization of the imidazole may be the cause for the strong quenching observed, around pH 7, of the fluorescence intensity of the enzyme FDPase.

In Figs. 9 and 10 we present the activation and fluorescence spectra of the enzyme with ADP and ATP present. The narrowing of the activation spectrum is a result of the strong overlap of ADP and ATP absorption with the absorption of FDPase. This overlap in absorption makes it very difficult to draw any quantitative conclusions about the effect of ADP and ATP on the fluorescence of the enzyme. To obtain information about the interaction of these molecules with FDPase, we measured the fluorescence polarization where only ratios of intensities are important and no correction of the absorption overlap needs to be done.

D. EXTRINSIC FLUORESCENCE

Another method of investigating the interaction of ATP, ADP and AMP with the enzyme was made by labeling the enzyme with a fluorescence dye like ANS where no absorption overlap exists. In Fig. 11 we demonstrate that ANS was bound by the enzyme in a non-polar region. ANS in aqueous and polar environment has a very low quantum yield, but in non-polar, hydrophobic environment this yield is high. The activation and

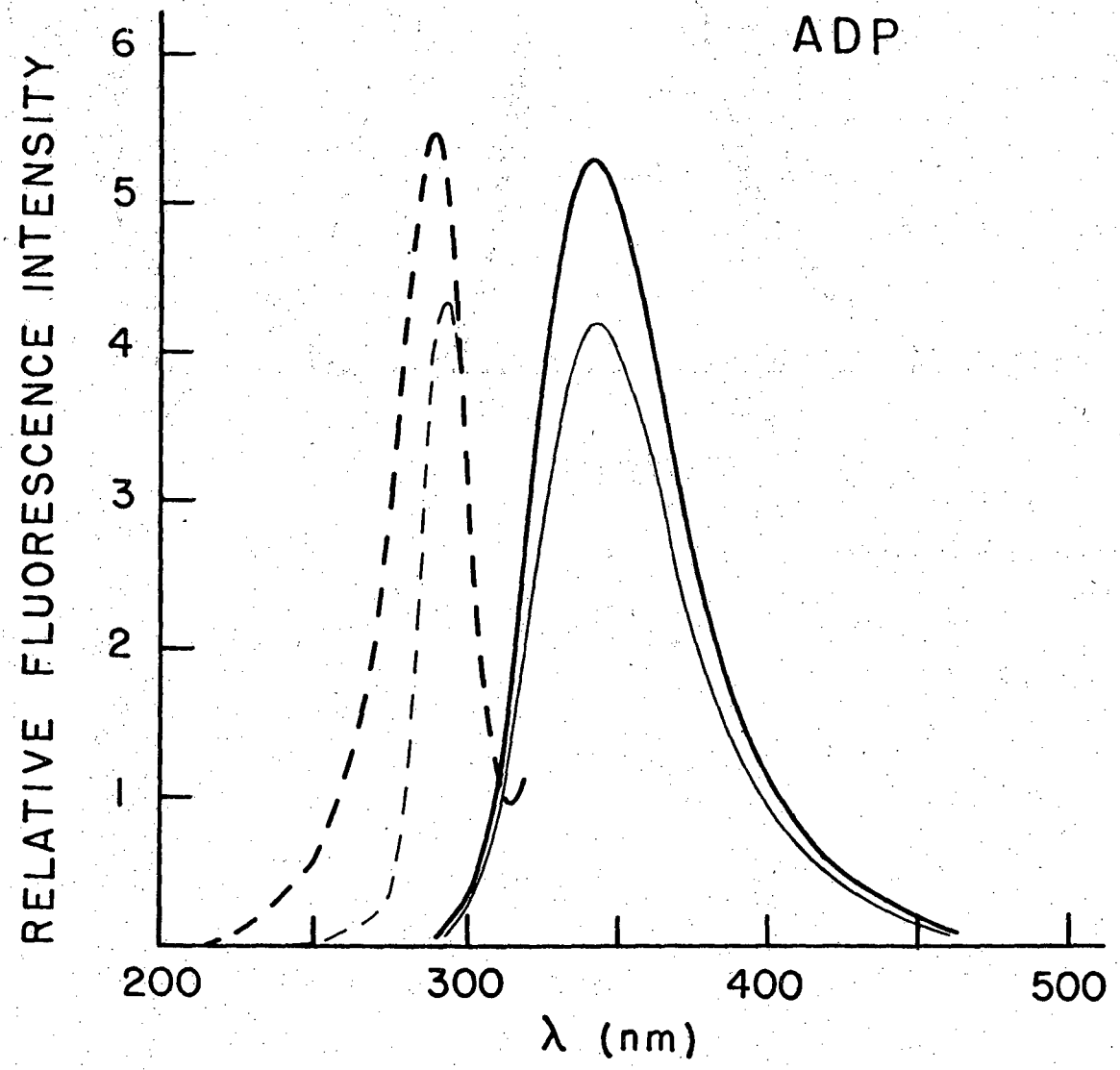
Fig. 9. Quenching of fluorescence and activation spectra of FDPase by ADP.

▬▬▬ Fluorescence spectrum for activation at 290 nm

——— Fluorescence spectrum in the presence of 5 mM ADP for activation at 290 nm

▬▬ ▬▬ Activation spectrum for fluorescence at 340 nm

-- -- Activation spectrum in the presence of 5 mM ADP for fluorescence at 340 nm



XBL708-5326

Figure II-9

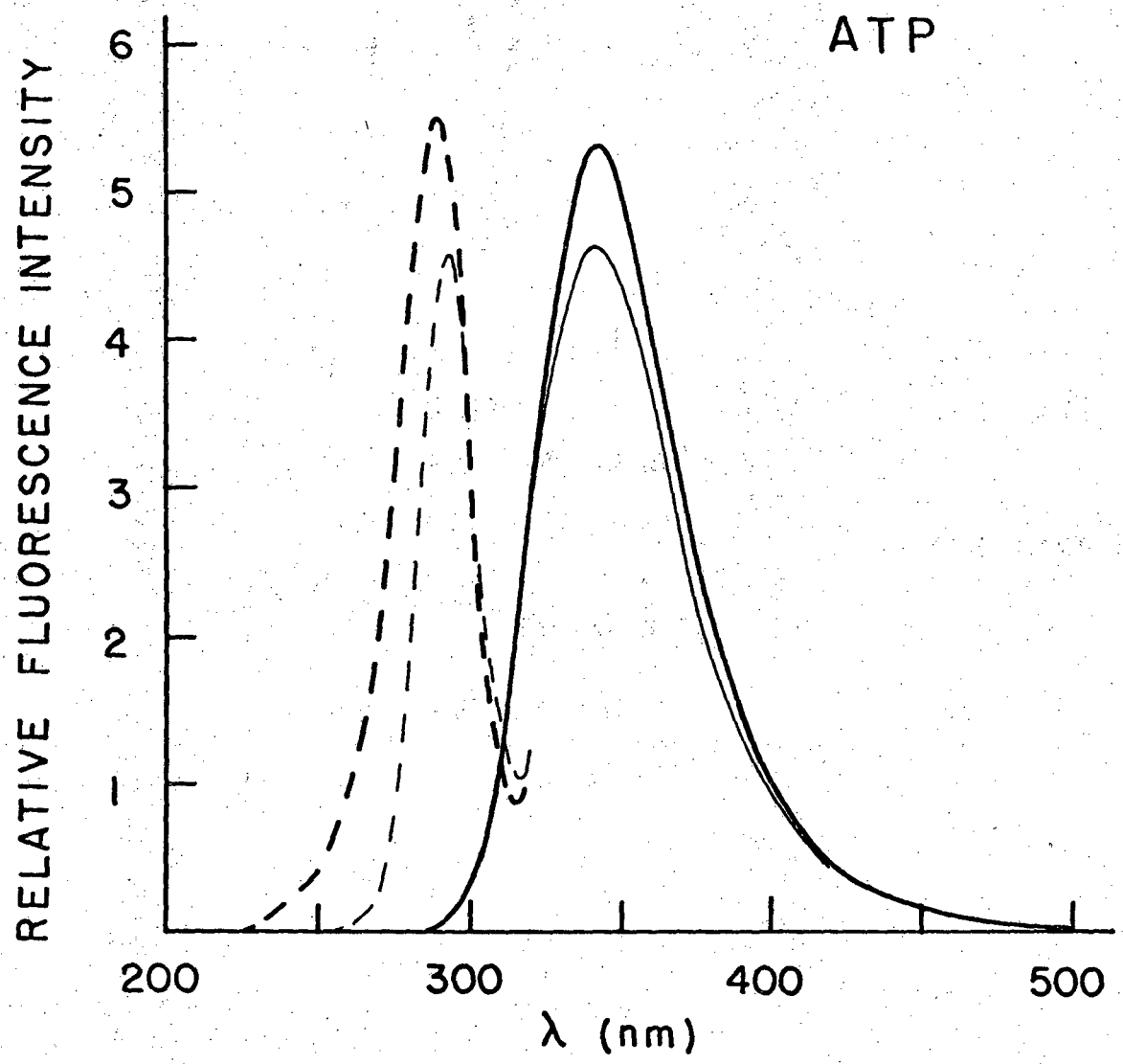
Fig. 10. Quenching of fluorescence and activation spectra of FDPase by ATP.

▬▬▬ Fluorescence spectrum for activation at 290 nm

——— Fluorescence spectrum in the presence of 5 mM ATP for activation at 290 nm

▬ ▬ Activation spectrum for fluorescence at 340 nm

-- -- Activation spectrum in the presence of 5 mM ATP for fluorescence at 340 nm



XBL708-5328

Figure II-10

Fig. 11. Extrinsic fluorescence of the enzyme induced by ANS.

—— · —— Fluorescence spectrum of ANS for activation at 403 nm

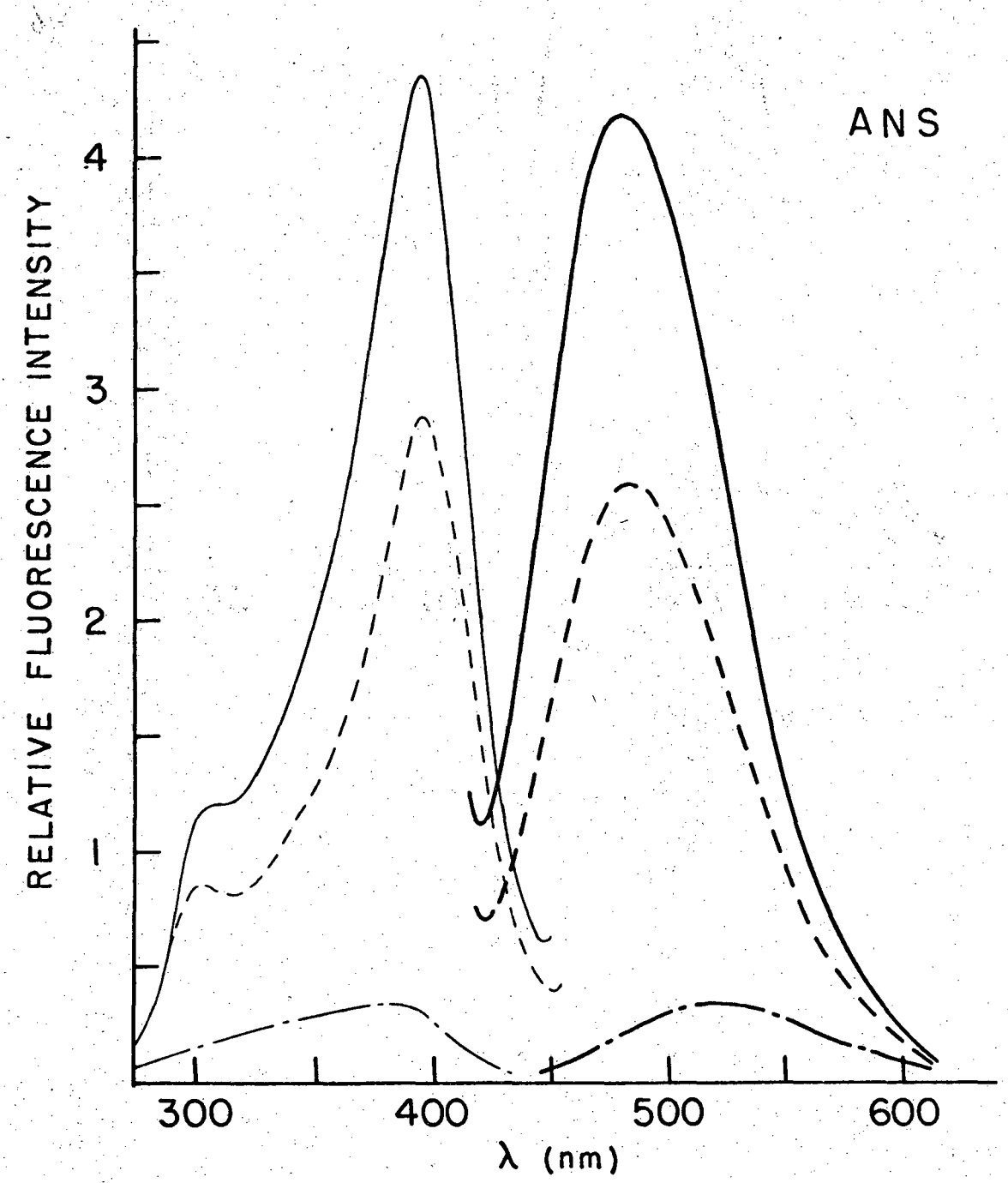
—— · —— Activation spectrum of ANS for fluorescence at 510 nm

-- -- Fluorescence spectrum of ANS for activation at 403 nm in the presence of 1.2×10^{-5} M FDPase

-- -- Activation spectrum of the enzyme for fluorescence at 510 nm in the presence of 1.2×10^{-5} M FDPase

—— Fluorescence spectrum of ANS for activation at 403 nm in the presence of 2.4×10^{-5} M FDPase

—— Activation spectrum of ANS for fluorescence at 510 nm in the presence of 2.4×10^{-5} M FDPase



XBL708-5329

Figure II-11

fluorescence spectra of ANS in the buffer alone shows small intensities, but by the addition of the enzyme, the fluorescence increases and the peaks are shifted. There is a saturation in fluorescence intensity when all ANS binding sites are occupied (Fig. 12).

We were unable to show quenching of the FDPase-ANS fluorescence by adding FDP, F6P, ADP and ATP. This suggests that the conformational change due to binding of any of these ligands does not affect the hydrophobic enviornment of the ANS in the protein. However, we found some sensitization of the protein-ANS complex fluorescence by Mg^{++} in agreement with the results obtained in the study of the temperature dependence of FDPase fluorescence in the presence of Mg^{++}. We conclude that there is magnesium protein interaction.

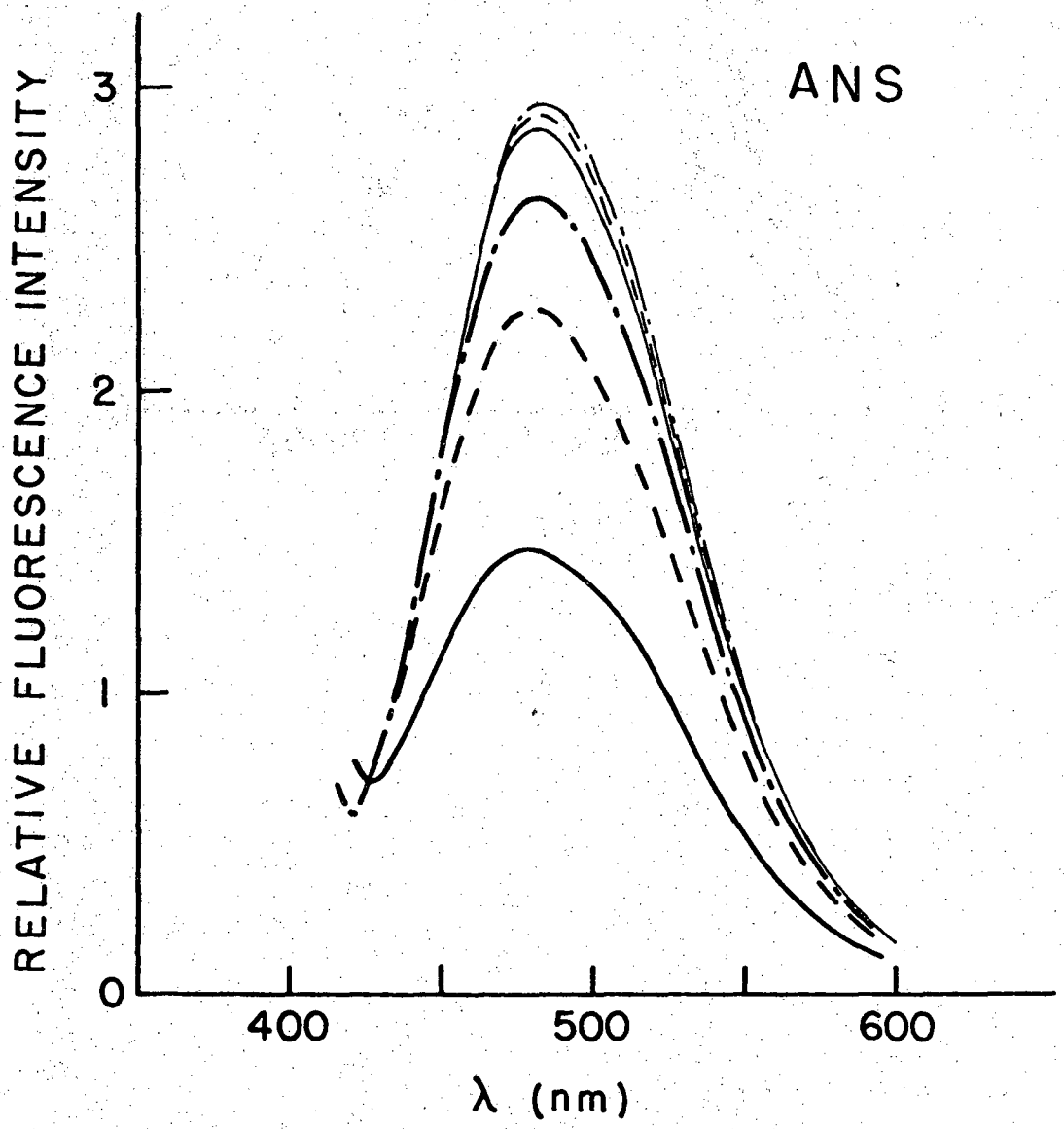
E. DEPOLARIZATION FLUORESCENCE

The interpretation of the fluorescence polarization spectra is very difficult. The problem starts with the fact that the electronic structure of the transition around 280 nm in tryptophan is not well understood. The polarization spectrum of tryptophan alone[10] shows two maxima at 270 nm and at 305 nm; at 290 nm there is a minimum. Weber explains this behavior by assuming a special geometrical relationship of the energy surfaces of two excited singlet states, so that the 270 and 305 nm peaks arise from one singlet state and the 290 nm minimum from the other. The very unusual observation that the polarization factor varies with the fluorescence wavelength suggests that both singlet states contribute to the fluorescence[7].

The intrinsic factors influencing the polarization are the geometrical relationship between absorbing and emitting transition moments, modified

Fig. 12. Fluorescence spectrum for activation at 403 nm of 1.2×10^{-5} M FDPase in the presence of:

———	0.05 mM ANS
- - - -	0.1 mM ANS
— • —	0.15 mM ANS
———	0.2 mM ANS
-- --	0.25 mM ANS
— • —	0.3 mM ANS



XBL708-5325

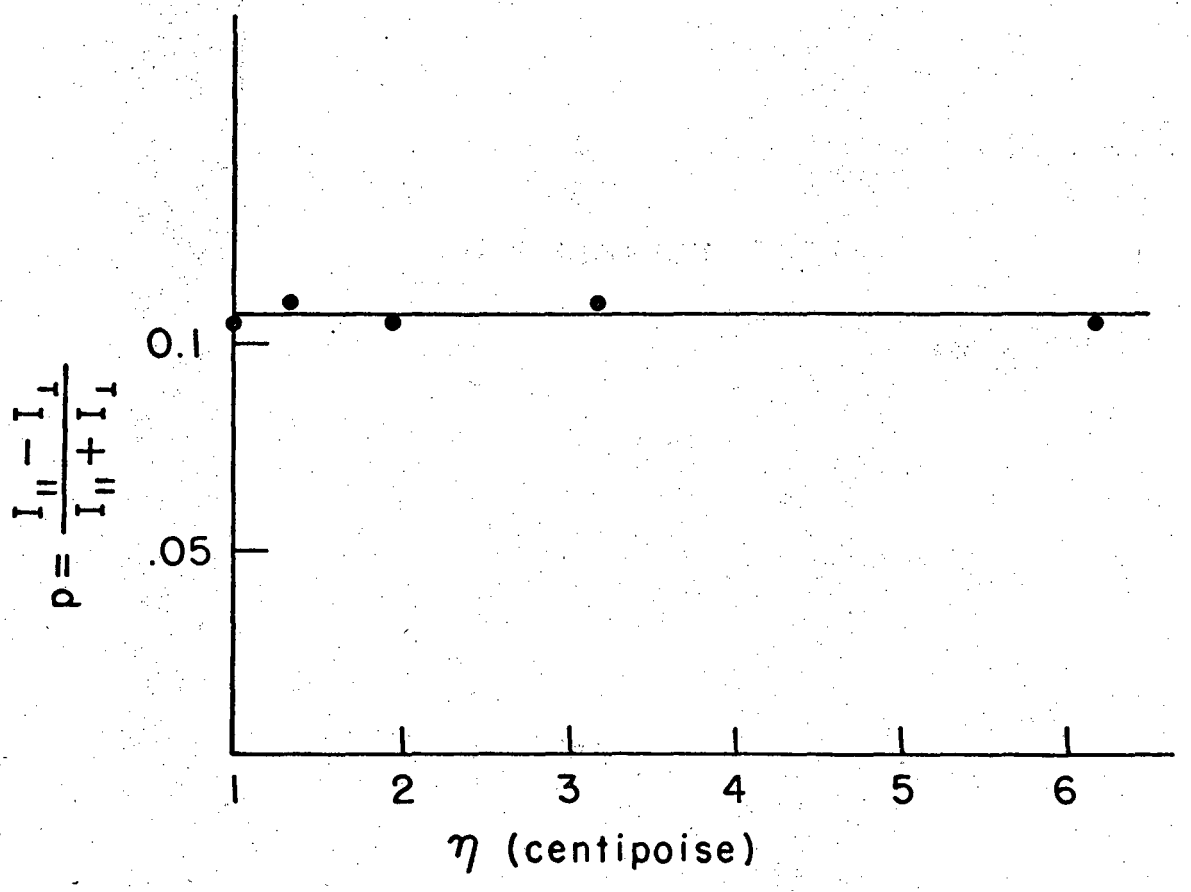
Figure II-12

by its environment. The extrinsic factors contributing to the depolarization are rotation of the transition dipoles between absorption and emission and energy transfer mechanisms. It was shown by Weber that tryptophan excited in the long wavelength edge of the absorption band is not able to participate in energy transfer[10]. A change in the polarization for 295 nm represents change in the environment or local rotation of the fluorescent tryptophan residues. FDPase as a whole is too big and rotates too slowly for contributing to depolarization (Fig. 13). Energy transfer between tryptophan residues in proteins was never demonstrated, so a change in the polarization below 295 nm represents change in the environment of the tryptophan residues or change in energy transfer of tyrosine to tryptophan. Figs. 14 to 20 show the polarization spectra of FDPase with FDP, F6P, ATP, ADP, PP_i, AMP and cyclic AMP.

In all the polarization spectra the region 290-300 nm stays unchanged by binding of different molecules except FDP (Fig. 14). FDP has the strongest effect in the 300-310 nm region. The interaction of the fluorescent tryptophan residues with neighboring groups changes drastically by conformational change; that is also shown by the different p values around 270 nm. The geometrical deformation probably turns the angle of the energy donating tyrosine residues with the energy accepting tryptophan molecules in a favorable direction for energy transfer.

F6P has a small effect on the polarization spectra (Fig. 15). The inhibitors ATP, ADP and PP_i show big deviations in the p values at short wavelengths, suggesting changes in the energy transfer from tyrosine to tryptophan. AMP and cyclic AMP are not inhibitors of spinach chloroplasts FDPase and as can be seen from Figs. 19 and 20 they do not affect the polarization at shorter wavelenghts; there are only deviations around

Fig. 13. The polarization, P, of FDPase as a function of the viscosity of the surrounding medium.



XBL 708-5323

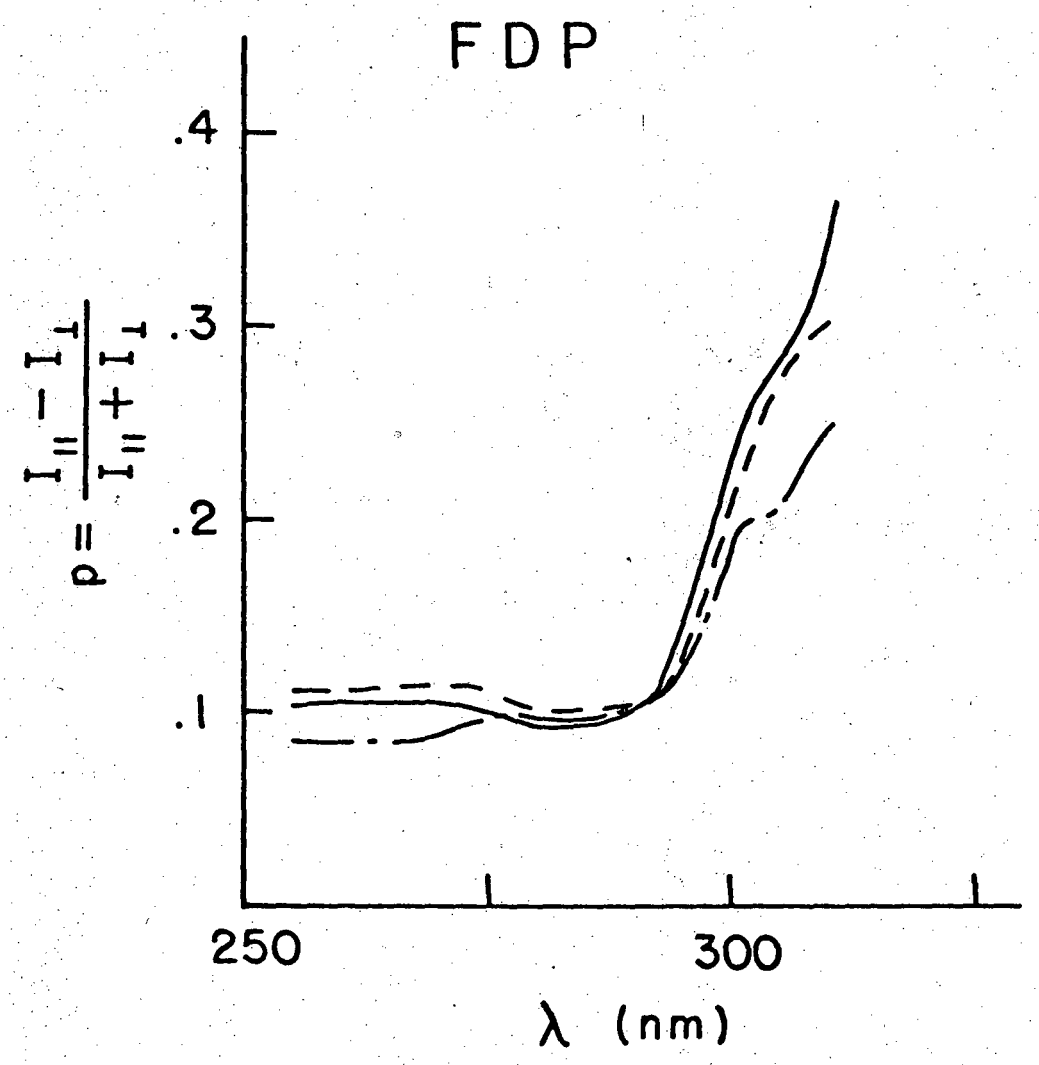
Figure II-13

Fig. 14. Effect of FDP on the fluorescence polarization spectrum of FDPase in tris-HCl, pH 8.7, at 22°.

——— Enzyme (1.2×10^{-5} M)

-- -- Enzyme in the presence of 5 mM FDP

— · — Enzyme in the presence of 5 mM FDP + 5 mM Mg



XBL 708-5322

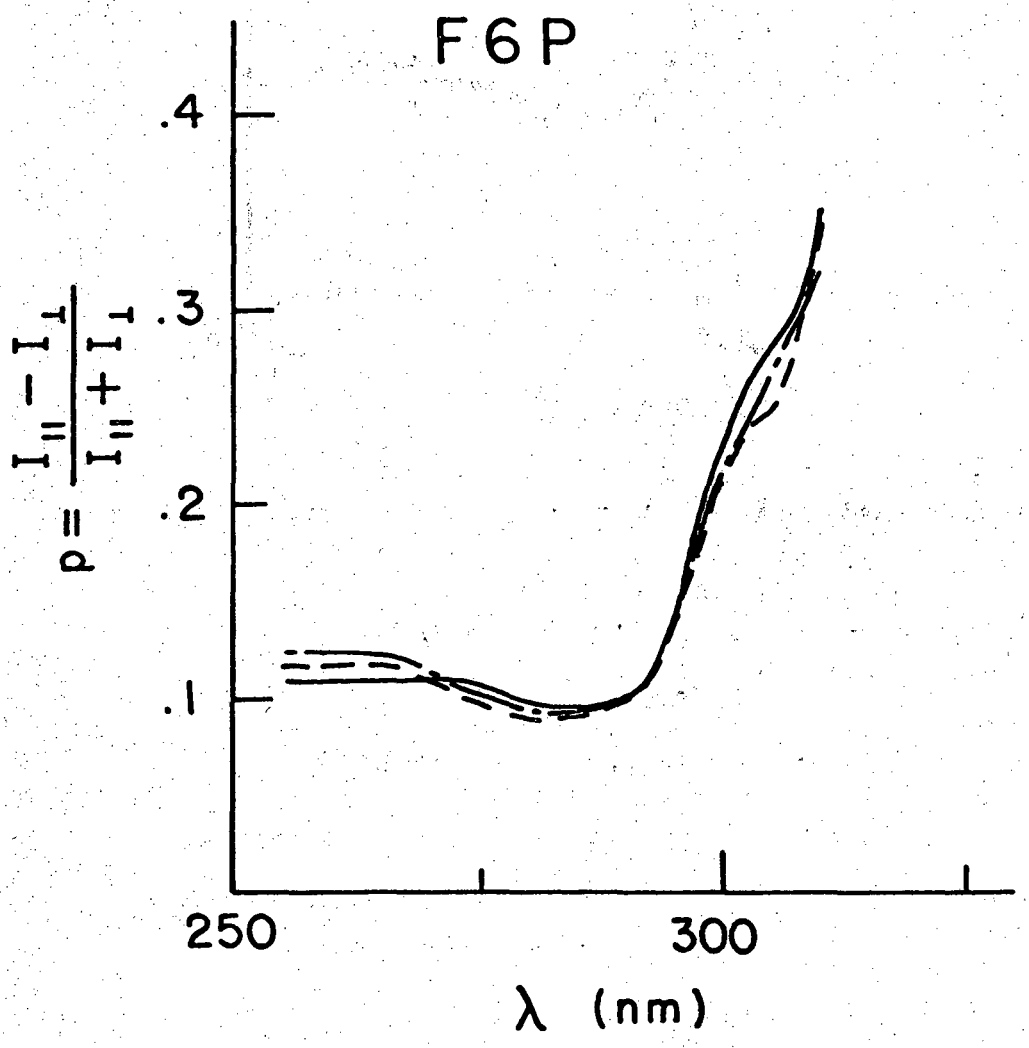
Figure II-14

Fig. 15. Effect of F6P on fluorescence polarization spectrum of FDPase in tris-HCl, pH 8.7, at 22°.

——— Enzyme (1.2×10^{-5} M)

-- -- Enzyme in the presence of 5 mM F6P

— · — Enzyme in the presence of 5 mM F6P and 5 mM Mg



XBL 708-5321

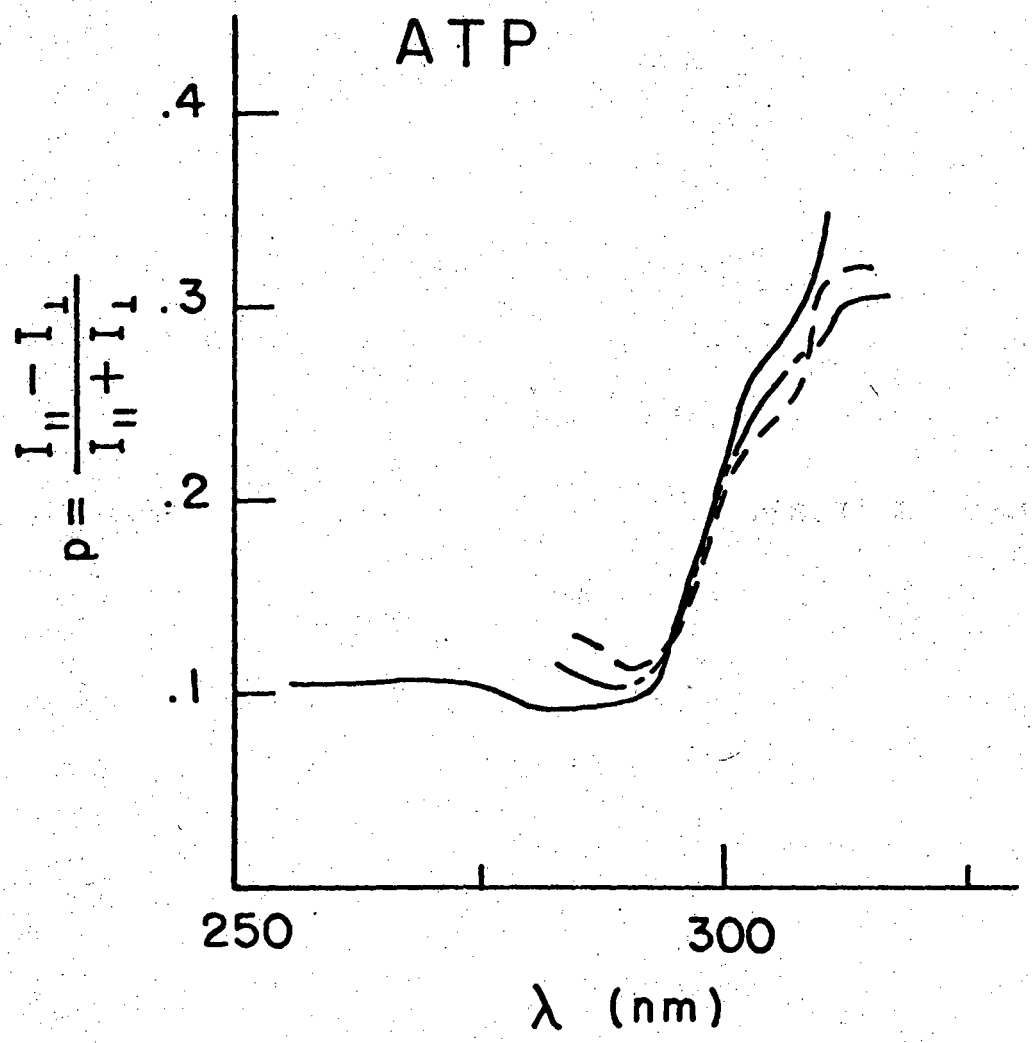
Figure II-15

Fig. 16. Effect of ATP on fluorescence polarization of FDPase in tris-HCl, pH 8.7, at 22°.

——— Enzyme (1.2×10^{-5} M)

-- -- Enzyme in the presence of 5 mM ATP

— · — Enzyme in the presence of 5 mM ATP and 5 mM Mg^{++}



XBL 708-5319

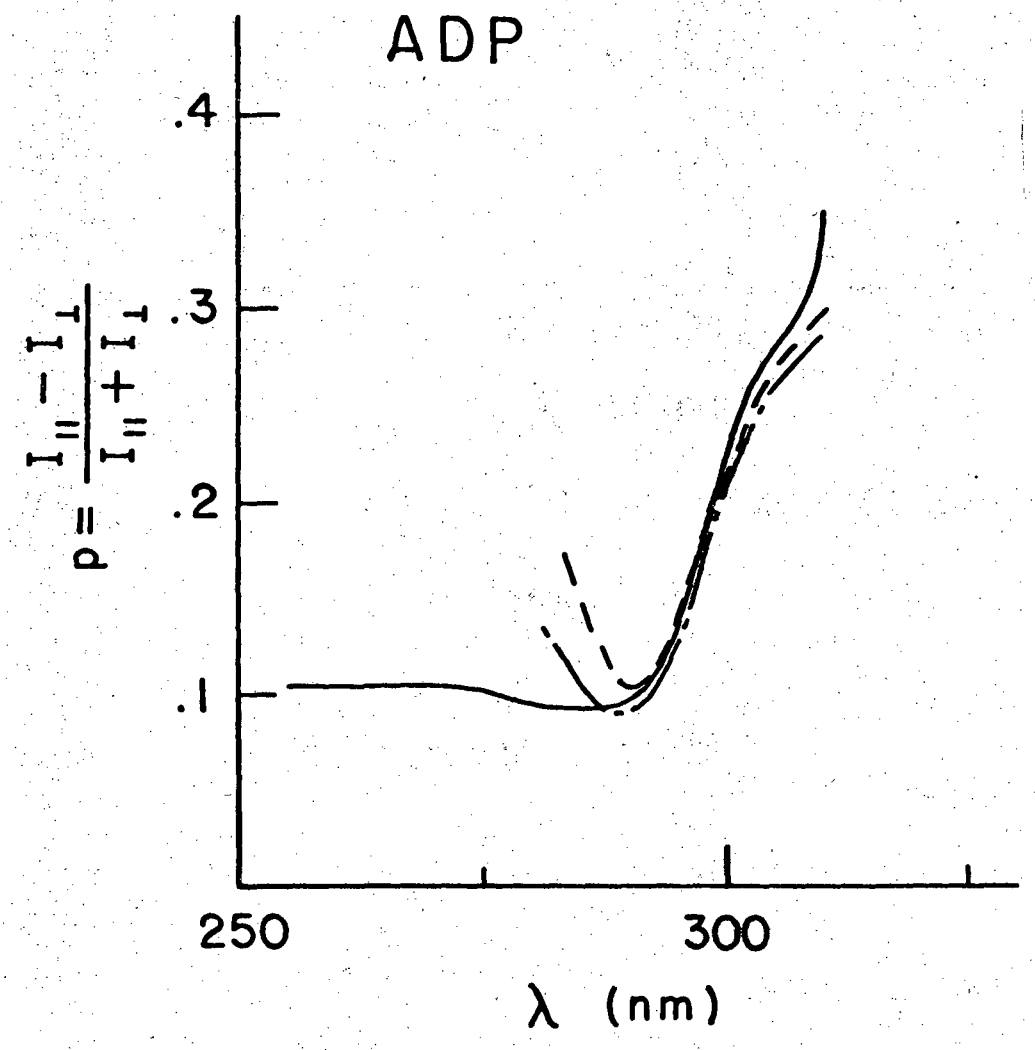
Figure II-16

Fig. 17. Effect of ADP on fluorescence polarization of FDPase in tris-HCl, pH 8.7, at 22°.

——— Enzyme (1.2×10^{-5} M)

-- -- Enzyme in the presence of 5 mM ADP

— · — Enzyme in the presence of 5 mM ADP and 5 mM Mg^{++}



XBL 708-5317

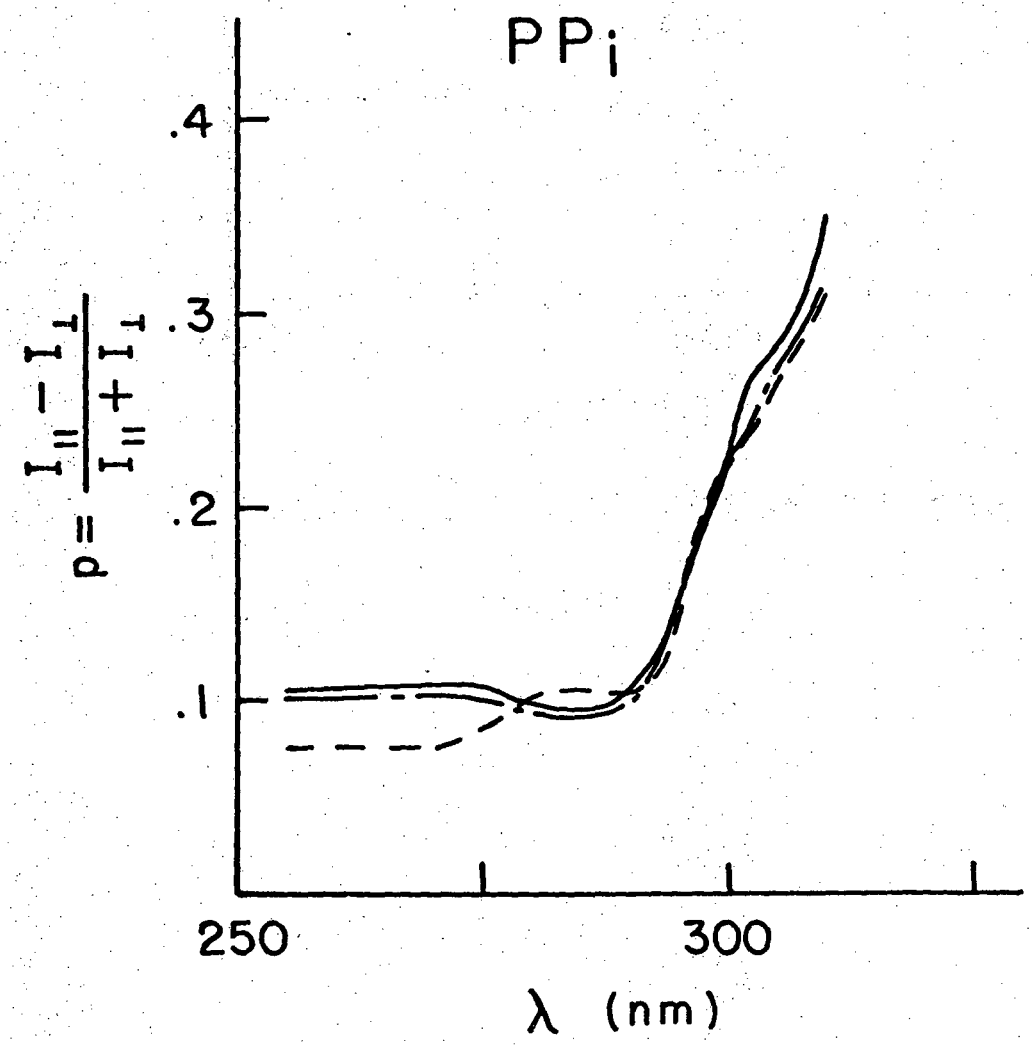
Figure II-17

Fig. 18. Effect of PP_i on fluorescence polarization of FDPase in tris-HCl, pH 8.7, at 22°.

——— Enzyme (1.2×19^{-5} M)

-- -- Enzyme in the presence of 5 mM PP_i

— • — Enzyme in the presence of 5 mM PP_i and 5 mM Mg^{++}



XBL 708-5316

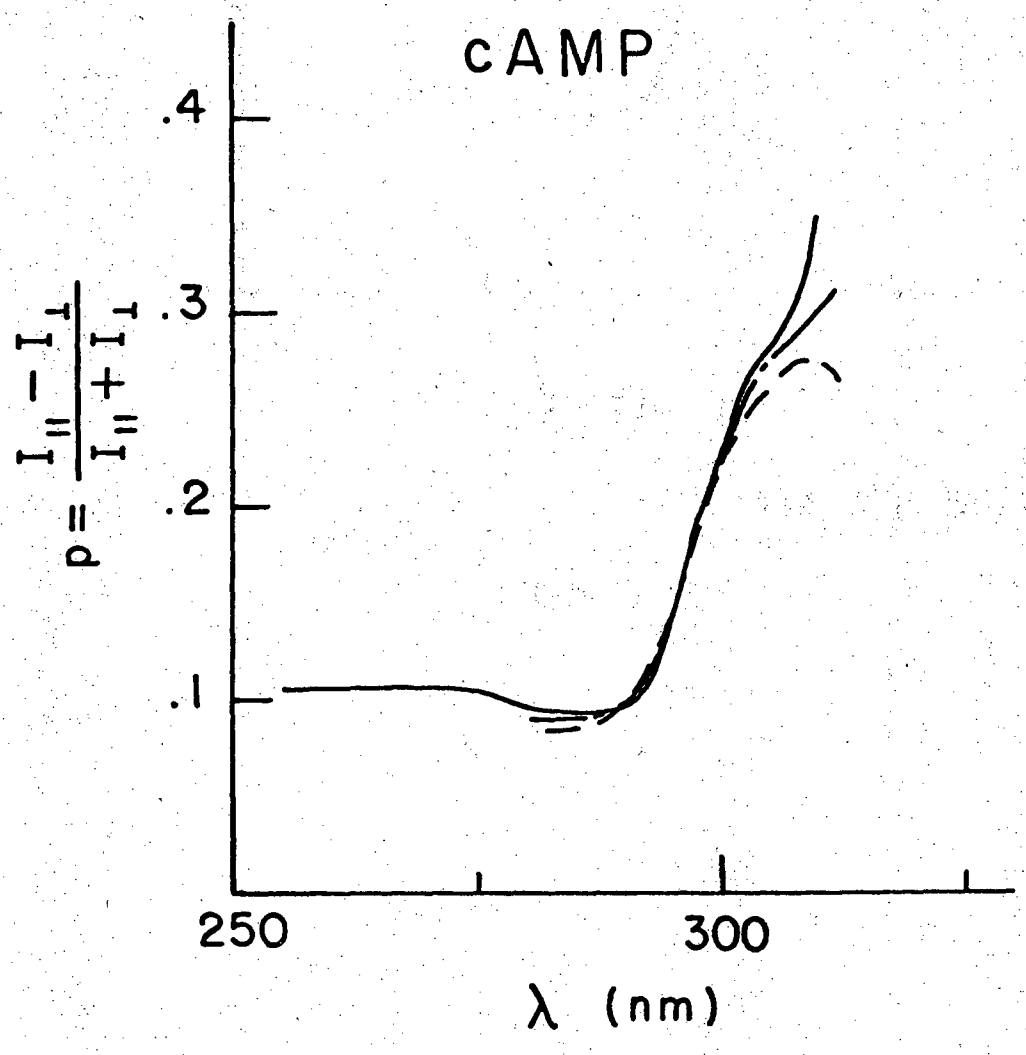
Figure II-18

Fig. 19. Effect of cyclic AMP on fluorescence polarization of FDPase in tris-HCl, pH 8.7, at 22°.

——— Enzyme (1.2×10^{-5} M)

-- -- Enzyme in the presence of 5 mM cAMP

— · — Enzyme in the presence of 5 mM cAMP and 5 mM Mg^{++}



XBL 708-5320

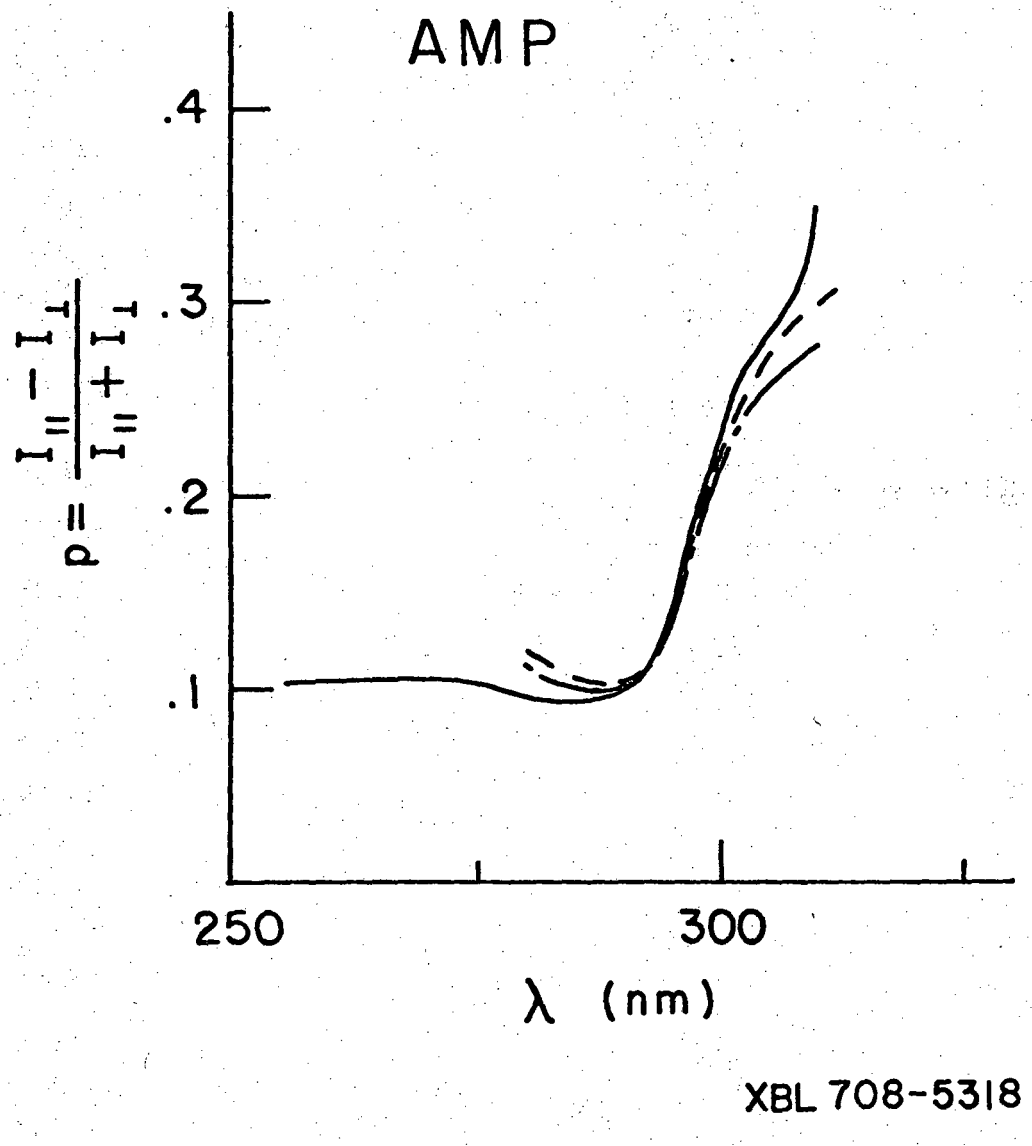
Figure II-19

Fig. 20. Effect of AMP on fluorescence polarization of FDPase in tris-HCl, pH 8.7, at 22°.

——— Enzyme (1.2×10^{-5} M)

-- -- Enzyme in the presence of 5 mM AMP

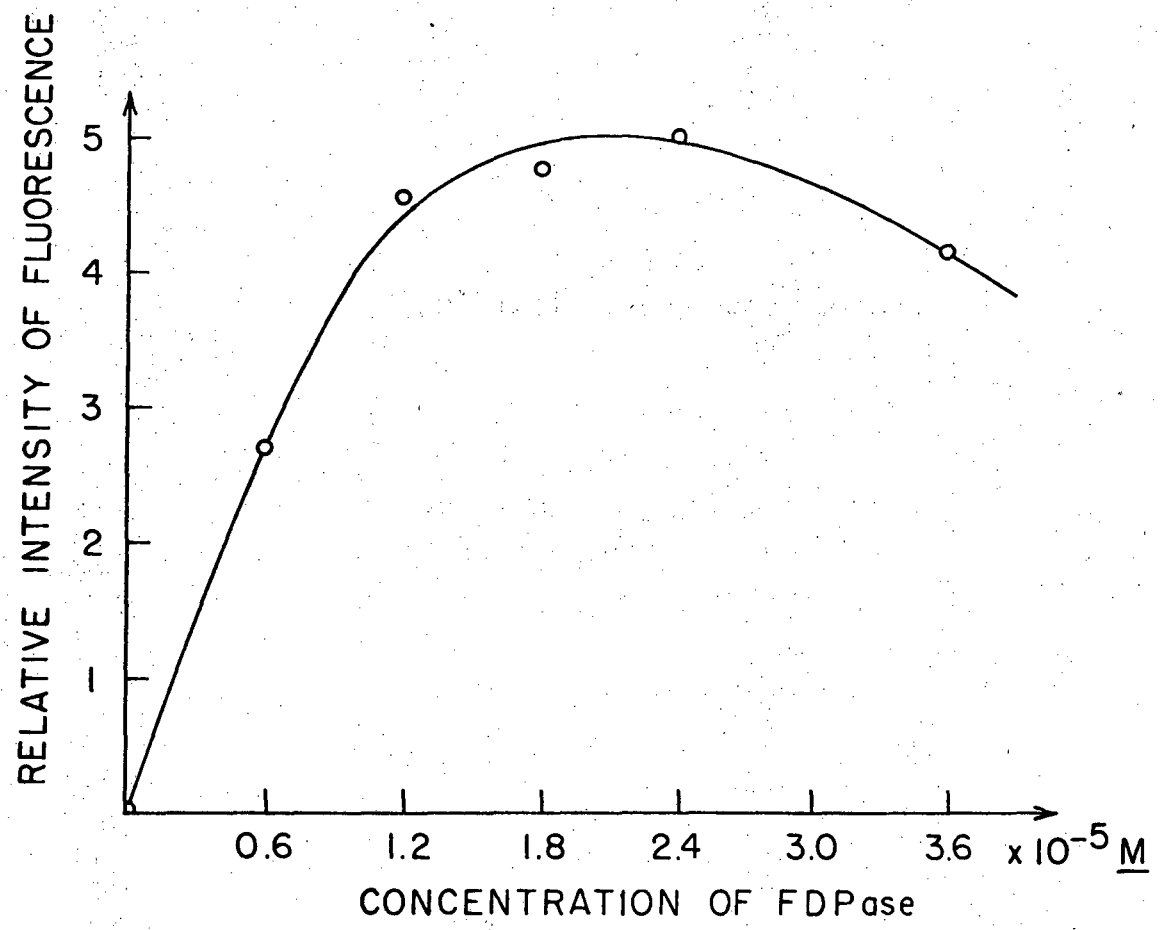
— : — Enzyme in the presence of 5 mM AMP and 5 mM Mg^{++}



XBL 708-5318

Figure II-20

Fig. 21. Dependence of fluorescence on FDPase concentration.



XBL 704-5159

Figure II-21

310 nm. It seems that cyclic AMP and AMP are bound to the enzyme producing environmental change of the tryptophan residues near the allosteric site, but they do not produce a conformational change of the enzyme as a whole.

F. MIXED QUENCHING EXPERIMENTS

To study the sites of fluorescence quenching by different ligands on the enzyme FDPase, an experiment was designed where the quenching by one ligand was studied in the presence of another at a concentration which results in the maximum "quenching saturating concentration." The results can be concluded as follows:

(1) The fluorescence of an FDPase solution which has reached its quenching saturation by F6P was further quenched by FDP. This indicates that the site of quenching of the substrate is not the same as that of the product. Presumably the product leaves the site soon after its formation; and it exhibits its quenching of the enzymic fluorescence at a site different from that of the substrate.

(2) This assumption is further substantiated by observing that the fluorescence of an enzyme which has reached its quenching saturation by FDP was further quenched by F6P.

(3) A more interesting observation is that the fluorescence of an enzyme solution containing a high concentration (20 mM) of the allosteric affector, pyrophosphate, was quenched in an identical manner to an enzymic solution untreated with pyrophosphate. This is a direct and simple method to determine the type of inhibition exhibited by a ligand on the enzyme. It is clear in our case that PP_i binds and exerts its inhibitory effect on FDPase at a different site from the catalytic site, that is, PP_i is an allosteric affector of spinach chloroplast FDPase.

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Chapter III

γ-RAY EFFECT ON THE CATALYTIC AND ALLOSTERIC SITES AND ON THE CONFORMATION OF CHLOROPLAST FRUCTOSE-1,6-DIPHOSPHATASE

A. INTRODUCTION

Enzymes are biological catalysts which not only mediate biochemical reactions but also control and regulate the levels and concentrations of different metabolites in the living cell. Radiation effect on enzymes therefore may be achieved by altering their catalytic activity, their response to their allosteric affectors, or both. This effect could be due to direct effect on the catalytic or allosteric site or due to change in the general conformation of the protein. Pihl and coworkers[1-3] studied X-ray modification of aspartate transcarbamylase, phosphorylase b and rabbit liver FDPase and their results indicate that the allosteric functions of these enzymes were much more sensitive to radiation damage than were their catalytic activities. This may suggest that radiation damage has greater effect on the regulatory sites than on their catalytic activity [4].

Spinach chloroplast fructose-1,6-diphosphatase (E.C. 3.1.3.11) catalyzes the hydrolysis of Fru-1,6-P_2 produced in the carbon reduction cycle of photosynthesis to Fru-6-P and inorganic phosphate. Bassham and coworkers[5] suggested that FDPase in photosynthetic Chlorella cells is subject to light activation. In this report we investigated the direct effect of γ-irradiation on the catalytic and allosteric functions of spinach chloroplast fructose-1,6-diphosphatase.

B. MATERIALS AND METHODS

1- Chemicals. Fructose-1,6-diphosphate, fructose-6-phosphate, AMP, ADP, ATP, phosphoglucose isomerase and glucose-6-phosphate dehydrogenase were obtained from Cal Biochem. Tetrasodium pyrophosphate was from Allied Chemicals.

2- Preparation of enzyme. The enzyme was prepared from spinach chloroplast as described by El-Badry[6] and the homogeneous protein had a specific activity of about 20 units/mg of protein.

Protein concentration was determined spectrophotometrically by measuring the absorption at 280 nm or by using the Lowry method of protein determination[7]. Bovine serum albumin was used as the standard in each of the two methods. The enzyme was assayed by coupling the FDPase reaction with that of phosphoglucose isomerase and glucose-6-phosphate dehydrogenase and the NADPH produced in the reaction was measured spectrophotometrically at 340 nm[6].

3- Irradiation of the enzyme. Enzyme solutions were irradiated in Pyrex glass tubes, 5 mm in diameter, in the presence of air at 0°. The tubes were placed vertically in a double wall stainless steel sample cup surrounded by crushed ice. The samples were then irradiated in a custom made cobalt-60 source which features radial arrangement of 16 ^{60}Co elements (slugs containing the isotope in elemental form) in a horizontal plane. The circular arrays can be varied in diameter to change the intensity within certain limits. In these experiments the circle diameter was 6 inches. The source has an automatic moving stage on which the sample cup travels sideways and up into the center of the field. The stage provides automatic rotation of the sample cup about

the vertical center axis. The 2000 curie, ^{60}Co, γ-ray source emission was at a rate of 2.0 megarads (±15%) per h.

C. RESULTS

1- Effect of irradiation on the catalytic function of chloroplast FDPase. As seen in Fig. 1, γ-ray irradiation affected the catalytic activity of the enzyme FDPase. We have not observed any activation of the catalytic activity at lower doses of irradiation as was observed with the rabbit liver enzyme[3]. The effect was only damaging to the catalytic activity of the enzyme with an ED_{50} = 250 kilorads.

To determine the nature of the radiation damage to the enzyme, that is, whether the effect is on the general conformation of the enzyme or directly on the active site, an experiment was designed in which the enzyme was irradiated in the presence of the substrate fructose-1,6-diphosphate (2 mM), the divalent ion required by the enzyme, magnesium ion (5 mM), or in the presence of both.

Fig. 1 shows that Fru-1,6-P_2 was most effective in protecting the catalytic activity of the enzyme. Fru-1,6-P_2 and magnesium together followed in effectiveness. However, magnesium alone had the least effect on protecting the active site of the enzyme. Fru-1,6-P_2 binds to the site tightly and is not hydrolyzed in the absence of the divalent ion magnesium. FDP therefore provides the greatest amount of protection from radiation damage to the site. Magnesium ion holds the enzyme in a conformation which is less sensitive to radiation damage. In the presence of both magnesium and FDP protection of the active site from γ-radiation damage is achieved partially due to the effect of Mg^{++} directly on the conformation of the enzyme and partially due to the protection of FDP

Fig. 1. Effect of irradiation on the catalytic activity of FDPase.

□--□ FDPase irradiated in the presence of FDP

△--△ FDPase irradiated in the presence of FDP and magnesium

O--O FDPase irradiated in the presence of magnesium

O--O Enzyme irradiated in the absence of ligands

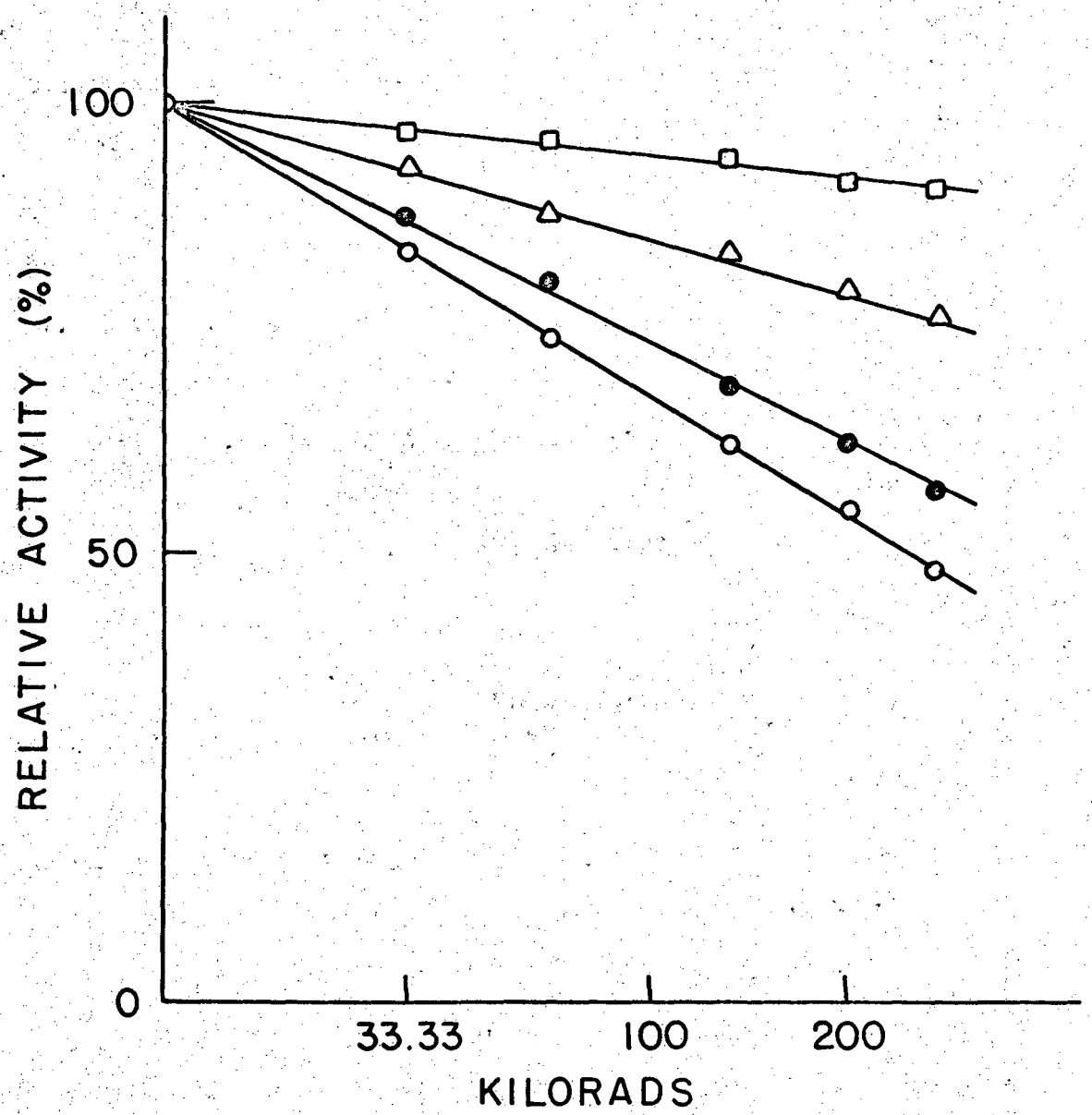

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Figure III-1

to the active site by binding to some functional groups rendering them less sensitive to radiation damage. Fru-1,6-P_2 and magnesium offer less protection to the active site than FDP alone, probably because Fru-1,6-P_2 alone in the absence of magnesium binds to the enzyme without being hydrolyzed; however, in the presence of magnesium the substrate is hydrolyzed and the product leaves the site rendering it accessible to radiation damage.

It seems to us that the decrease in catalytic activity of chloroplast FDPase is due to both the direct damaging effect of the radiation on the active site of the enzyme, possibly due to the destruction of some functional groups in the active site, and to the effect of the radiation on the conformation of the enzyme. The direct effect on the active site as seen from Fig. 1 has more damaging effect to the catalytic activity of FDPase. The effect on the conformation may be due to the destruction of the magnesium binding sites, preventing the magnesium from binding to the enzyme, and thus preventing the formation of a catalytically active conformation.

2- Effect of irradiation on the allosteric function of the enzyme. Spinach chloroplast FDPase is inhibited by the allosteric effectors, ATP, ADP, and pyrophosphate. To study the effect of γ-radiation on the allosteric site, inhibition by ATP was chosen as the parameter for determining the effect of γ-rays on the allosteric site of the enzyme. The allosteric function is defined as the degree of inhibition by ATP, expressed in percent of inhibition of the unirradiated enzyme. Fig. 2 shows that γ-irradiation has damaged the allosteric site and that the damage to the active site was essentially prevented in the presence of

Fig. 2. Effect of γ-ray irradiation on the allosteric function of FDPase.

O--O Enzyme irradiated in the absence of ATP and Mg^{++} and tested in the absence of ATP

O--O Enzyme irradiated in the presence of ATP and Mg^{++} and tested for ATP inhibition

□--□ Enzyme irradiated in the presence of ATP only and tested for ATP inhibition

△--△ Enzyme irradiated in the absence of ATP and Mg^{++} and tested for ATP inhibition

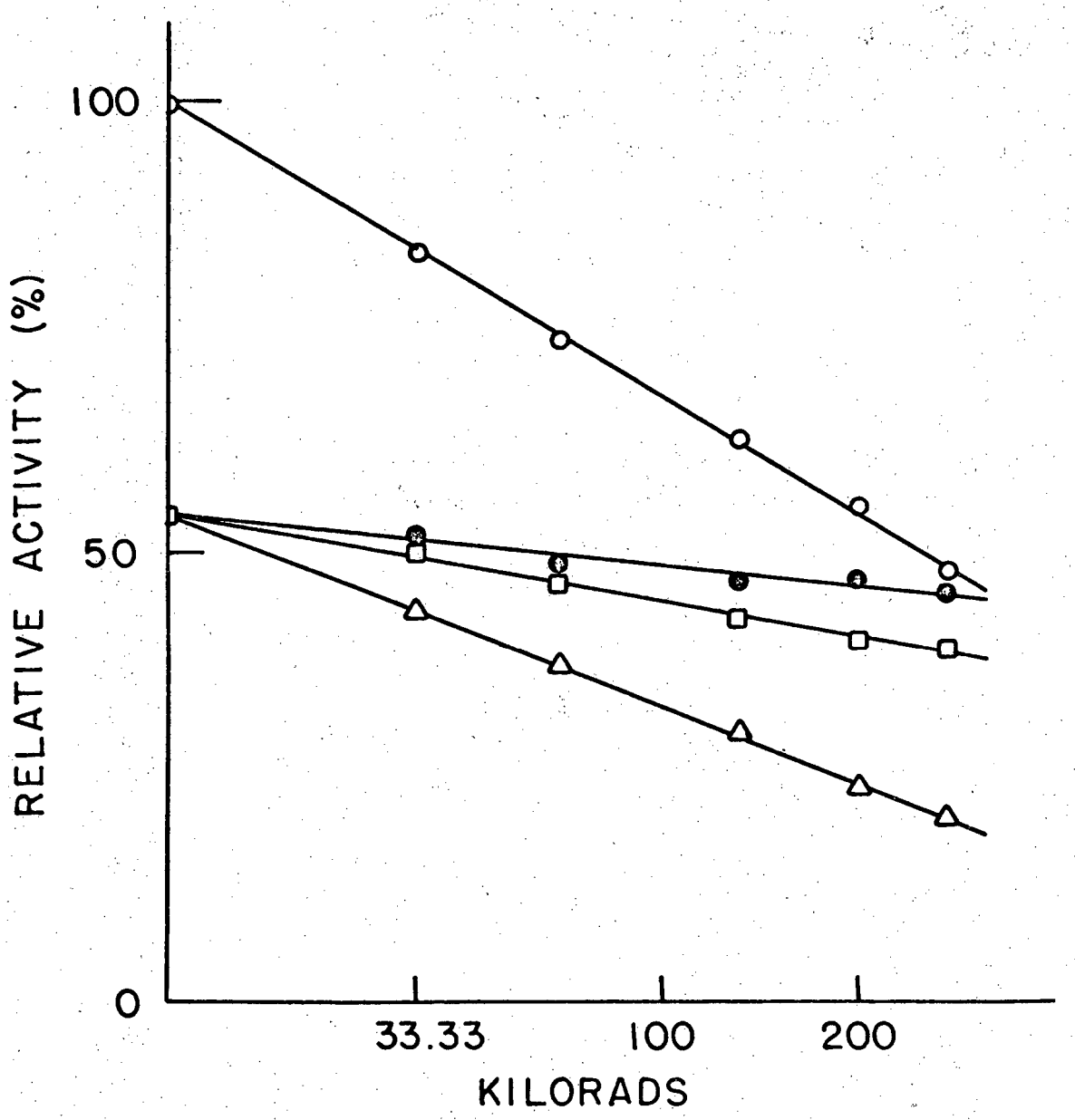

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Figure III-2

the allosteric affector ATP and the divalent cation magnesium. The effect of magnesium here is probably a dual effect, partially due to holding the enzyme in a conformation which is resistant to radiation damage and partially due to its effect on ATP; that is, the allosteric affector Mg $ATP^{=}$ is more effective than anionic ATP in protecting the allosteric site from γ-irradiation damage.

D. DISCUSSION

Radiation effect on chloroplast FDPase was seen to be due to three types of effects.

(1) The effect on the catalytic site of the enzyme is probably due to the destruction of some functional groups in the active site. These groups could be involved in the binding of the substrate to the enzyme or in the hydrolysis of the phosphate group at the C-1 position of the substrate. This direct effect on the chemical nature of the active site resulted in the greatest damage to the catalytic activity of the enzyme.

(2) The second effect of the γ-irradiation on the enzyme we interpret as an effect on the conformation of the enzyme. This effect could be due to the breakage of some disulfide bonds or other bonds responsible for the general geometry and conformation of the enzyme. More likely though, this effect is due at least partially to the destruction of the magnesium binding sites on the enzyme. The destruction of these sites makes the enzyme unable to change to a conformation which is favorable to the catalytic hydrolysis of Fru-1,6-P_2 in the presence of magnesium. This effect could be thought of as an effect on the catalytic function of the enzyme due to unfavorable conformation. It can also be explained as an effect on the site of binding of the allosteric activator, the

divalent cation magnesium.

(3) The third type of effect is an effect on the allosteric site of the enzyme. This effect is mostly due to the destruction of the binding sites to which the allosteric inhibitor ATP binds to the allosteric site.

The effect on the allosteric site does not seem to be as great as the effect on the catalytic site, as seen from comparing the slopes of the dosage response curves in Fig. 2. However, this is probably due to the sensitivity of the catalytic site to the effect on the conformation of the enzyme caused by radiation damage, while the allosteric site is not affected as much by the deformation in the enzyme conformation.

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PART II

STUDIES WITH RIBULOSE-1,5-DIPHOSPHATE CARBOXYLASE

Chapter IV

RIBULOSE-1,5-DIPHOSPHATE CARBOXYLASE FROM SPINACH CHLOROPLASTS: AN IMPROVED PURIFICATION METHOD AND EFFECTS OF FRUCTOSE-1,6-DIPHOSPHATE

A. INTRODUCTION

The enzyme ribulose-1,5-diphosphate carboxylase, RuDPCase* [3-phospho-D-glycerate carboxylase (dimerizing), EC 4.1.1.39] catalyzes the fixation of carbon dioxide, in the carbon reduction cycle, with ribulose-1,5-diphosphate to form two identical molecules of 3-phospho-D-glycerate.

Cooper and Filmer[1] have shown that the active species utilized by RuDPCase is CO_2 and not HCO_3^-. Deuterium incorporation experiments carried out by Mullhofer and Rose[2] showed that deuterium and CO_2 became affixed to carbon atom that was originally the C-2 position of ribulose diphosphate. This finding indicates that the cleavage occurs at the C-2 - C-3 bond of the ribulose diphosphate during the carboxylation reaction.

The reaction is essentially irreversible[3] and the enzyme is highly specific for RuDP, which cannot be replaced by ribulose-5-phosphate, ribose-1,5-diphosphate, ribulose-1-phosphate or xylose-1,5-diphosphate[4,5]. Paulsen and Lane[6] have shown that orthophosphate and sulfate inhibit the carboxylation reaction competitively with respect to ribulose diphosphate. They also reported that 3-phosphoglycerate inhibits competitively with respect to HCO_3^- and non-competitively with respect to ribulose-1,5-diphosphate, while ribulose-1,5-diphosphate inhibits at concentrations greater than 7×10^{-4} M. Rutner and Lane[7] showed that the enzyme is composed of two distinct kinds of non-covalently linked polypeptide chains

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* Abbreviations: RuDPCase, ribulose-1,5-diphosphate carboxylase.

which differ in molecular weight and amino acid composition.

Bassham and coworkers[8] have found that the pH optimum of RuDPCase shifts to physiological pH with the increase of Mg^{++} concentration in the assay mixture. This may provide a mechanism of regulation of the enzyme *in vivo*.

Bassham and coworkers using *Chlorella pyrenoidosa* cells[9] and isolated chloroplasts[10] and employing steady-state photosynthetic conditions showed that upon going from light to dark, the rate of CO_2 fixation decreased almost immediately. This decrease has occurred in the presence of sufficient amount of ATP, RuDP and HCO_3^-. When the *Chlorella* cells and the spinach chloroplasts were illuminated an increase in the rate was in effect within a very short time. Bassham has explained the loss of CO_2 fixation upon transition to the dark and its increase upon illumination as light activation of RuDPCase.

Wildman and Criddle[11] claimed activation of RuDPCase in crude extracts of *Rhodospirillum rubrum*, some marine algae, and leaves of higher plants upon exposure to light at 325 nm. They have extracted a light activating factor (LAF) from chloroplasts of tomato leaves using cold absolute methanol. Purified tomato enzyme did not respond to light; however, activation of the purified enzyme was achieved in the presence of LAF[12]. Such indiciations of activation may explain the discrepancies between the low activities of RuDPCase found in crude extracts and the high CO_2 fixation rate observed in isolated chloroplasts and intact leaves.

The enzyme RuDPCase has been isolated from many sources: *Hydrogenomonas eutropha* and *Hydrogenomonas facilis*[13], *Rhodospirillum rubrum*[14] and *Rhodopseudomonas spheroides*[15], *Chlorella ellipsoidea*[16], rice leaves[17]

and tomato leaves[12]. Several methods of isolation have also been reported for spinach leaf RuDPCase[4,5,6,18]. However, the enzyme isolated by these methods is reported to be unstable in the purified form.

In this paper we report a method for the isolation of RuDPCase of considerable stability. The enzyme isolated from spinach chloroplasts by our method is stable for over six months. In addition, our procedure provides a means for isolating large quantities of the enzyme which was not feasible before[6] and which is needed for kinetic studies. RuDPCase isolated by our method has also higher specific activity than previously reported[4,6]. Also, we report the stimulation by fructose-1,6-diphosphate at a concentration close to the probable physiological level.

B. MATERIALS AND METHODS

1- Chemicals. Fructose-1,6-diphosphate and ribulose-1,5-diphosphate were purchased from Sigma. Commercial RuDP was in the dibarium form and was converted to the sodium salt by treatment with Bio-Rex 70, sodium form (obtained from Biorad Labs). Sorbitol and 2-N-morpholinoethane sulfonic acid were from Cal Biochem. All inorganic salts were analytical reagents. DEAE-cellulose (Cellex-D) was from Biorad Labs. It was further purified by suspending in water and decanting the fines. For every 40 to 80 grams of the adsorbent, 1 liter of 1 N NaOH was added, followed after 15 min by 1100 ml of 1 N HCl. The acidic suspension was filtered on a Buchner funnel and rinsed with a little water. The residue was again suspended in 1 liter of NaOH and after 15 min is filtered again and washed, until the effluent was neutral, with distilled water. The free DEAE-cellulose was then equilibrated with 0.05 M solution of 0.05 M tris-HCl, pH 7.4, containing 0.001 M EDTA. The buffer was changed

several times until pH 7.4 was achieved. The adsorbent was stored at 4°C in a 30-50% suspension in tris-HCl, 0.05 M, pH 7.4. Sephadex G-100 was obtained from Pharmacia-fine Chemicals. Glyceraldehyde-3-phosphate dehydrogenase (E.C.1.2.1.12), 3-phosphoglycerate kinase (E.C.2.7.2.3) and α -glycerophosphate dehydrogenase-triose phosphate isomerase (E.C.1.1.99.5) from Cal Biochem.

2- Assay of enzymic activity. The enzyme was assayed spectrophotometrically in a coupled enzymatic reaction. The reaction mixture (1 ml) contained: tris-HCl, pH 8.0, 0.1 M; NADH, 0.12 mM; glutathione, 5 mM; glyceraldehyde-3-phosphate dehydrogenase, 0.25 mg; 3-phosphoglycerate kinase, 5 μg; α-glycerophosphate dehydrogenase-triose phosphate isomerase, 25 μg; RuDP, 0.5 mM; ATP, 12 mM; $MgCl_2$, 10 mM; $KHCO_3$, 75 mM; and 10-20 μg of RuDPCase. In this method of assay for each micromole of RuDP cleaved 4 micromoles of NADH are oxidized; this can be followed spectrophotometrically at 340 nm.

Assay of enzymic activity using radioactive $KH^{14}CO_3$ was also used. The complete assay mixture (0.5 ml) contained tris-HCl, pH 8, 0.1 M; RuDP, 0.7 mM; $KH^{14}CO_3$, 0.5 mM (30 C/mole); $MgCl_2$, 10 mM; and 50 μg of RuDPCase. The assay mixture was incubated at 25° for 10 min and was stopped by the introduction of 0.05 ml of glacial acetic acid. The mixture was then spotted on (2 x 3 inches) pieces of Whatman No. 1 filter paper and was counted after drying in a stream of hot air, using a pair of large Geiger-Müller tubes with thin windows.

3- Determination of protein. Protein concentration was determined by measuring its absorption at 280 nm or by using the Lowry method of protein determination[19]. Bovine serum albumin was used as the standard in each of the two methods.

4- Disc gel electrophoresis. The method of David[20] was used to analyze for the homogeniety of the protein at the different stages of purification. Cross-linked polyacrylamide gel at pH 8.4 was used. Samples of 10-20 µl containing 50-100 µg of protein were used. All reagents used in the procedure were obtained from Canalco.

C. ISOLATION OF RIBULOSE-1,5-DIPHOSPHATE CARBOXYLASE

Spinach chloroplast RuDPCase was purified by the following method:

1- Source of the enzyme. Field grown, relatively young spinach leaves were harvested and immediately stored over crushed ice in polyethylene bags in large ice chests. The leaves were deribbed and washed with ice-cold water and were dried between two sponges. The deribbed leaves were then chopped and divided into 50 g batches.

2- Isolation of chloroplasts. Each 50-g batch was homogenized for 5 to 8 sec in a Waring Blendor with 200 ml of solution A[21] (containing Sorbitol, 0.33 M; $NaNO_3$, 0.002 M; EDTA (dipotassium salt), 0.002 M; sodium isoascorbate, 0.002 M; $MnCl_2$, 0.001 M; KH_2PO_4, 0.0005 M; 2-N-morpholinoethane sulfonic acid, 0.05, adjusted with NaOH to pH 6.1, and NaCl, 0.02 M). The blendorate was then forced through six layers of cheesecloth to strain out fibrous material.

The homogenate was centrifuged at 2000 x g for 3 min. The supernatant was decanted and each pellet was suspended in 10 ml of "basic buffer" [0.05 M tris-HCl buffer (pH 7.4) - 0.002 M dithiothreitol - 0.0002 M EDTA - 0.001 M $MgCl_2$].

3- Sonication. The chloroplast suspension in basic buffer was sonicated for 30 sec in batches of 50 ml, using the Biosonik (Model BPI, Bronwill Scientific Co., Rochester, N. Y.) at 0°.

The sonicated suspension was centrifuged at 36,000 x g for 2 h and the supernatant was saved as the crude enzyme preparation (I).

4- Acetone fractionation. Acetone was added to the crude enzyme fraction to a concentration of 30%. The acetone had been precooled in the freezer at -14° and was added to the crude enzyme solution slowly while stirring at 4°C. The enzyme in 30% acetone was allowed to stand in the cold room (4°) for 30 min and the mixture was centrifuged at 13200 x g for 4 min. The supernatant was brought to 75%. The enzyme in 75% acetone was allowed to stand in the freezer at -14° for 1-2 h. A copious precipitate formed and settled toward the bottom of the container. The upper layer of 75% acetone solution was decanted. The lower layer containing the precipitated enzyme and some other proteins was then centrifuged for 1 min at 5000 x g and the pellets were collected.

The greyish-white precipitate was suspended in the smallest possible volume of basic buffer and was dialyzed against cold water (4°) for 4 h. Then it was dialyzed against basic buffer twice for 8 h each time.

The dialyzed mixture was centrifuged in the Sorvall at 36,000 x g for 10 min and the supernatant (II) was saved.

5- DEAE-cellulose column. A DEAE-cellulose column was prepared and preequilibrated with 0.05 M tris-HCl (pH 7.4). The supernatant (II) was applied to the column and the column was washed with 0.05 M tris-HCl buffer (pH 7.4). RuDPCase did not stick to the DEAE-cellulose under these conditions and most of the activity passed down the column in the 0.05 M tris-HCl (pH 7.4) eluting buffer. A considerable degree of purification was achieved by this step since a great deal of protein other than RuDPCase was removed from the enzyme solution (III).

6- Ammonium sulfate fractionation. The protein eluted from the DEAE-cellulose column (III) was subjected to ammonium sulfate fractionation. Enzyme-grade ammonium sulfate, previously powdered by a mortar and pestle, was added slowly to the enzyme solution with stirring. The pH of the mixture was maintained at about pH 7 using ammonium hydroxide solution and pH paper as indicator. The precipitate formed at 30% ammonium sulfate saturation was centrifuged at 13,200 x g for 10 min and was discarded. Ammonium sulfate powder was added to the supernatant to a concentration of 40% saturation and the precipitate was collected by centrifugation at 13,200 x g for 10 min and was dissolved in a "storage buffer" containing tris-HCl, pH 8, 0.1 M; $KHCO_3$, pH 8, 0.1 M; and $MgCl_2$, 0.005 M (IV).

7- Sephadex G-100 column. A Sephadex G-100 column was prepared and was pre-equilibrated with the "storage buffer". Fraction (IV) protein was applied to the column and was eluted with the same buffer. Tubes containing RuDPCase activity were pooled and the protein was concentrated by dialyzing against a saturated solution of ammonium sulfate pre-adjusted with ammonium hydroxide to pH 8 (using pHydrion paper). The protein precipitated in the dialysis tubing and the suspension mixed with it was removed from the dialysis tubes and the precipitate was collected by centrifugation at 36,000 x g for 20 min and was dissolved in basic buffer and was dialyzed against storage buffer twice for 8 h each (V).

D. HOMOGENEITY OF RUDPCASE

Fractions (IV) and (V) were examined for protein homogeneity. Fraction (IV) contained one major band of protein and minor traces of two other bands. Fraction (V), however, was homogeneous and showed only one band of protein which is RuDPCase.

TABLE I

PURIFICATION OF SPINACH CHLOROPLAST RIBULOSE-1,5-DIPHOSPHATE CARBOXYLASE

Fraction	Protein concentration (mg/ml)	Specific activity (units/mg protein)	Yield (%)
I Sonicate supernatant	10	0.51	100
II Acetone fraction	21	1.30	89
III DEAE-cellulose column	7.5	2.20	60
IV 30-40% ammonium sulfate fraction	12	2.75	38
V Sephadex G-100 column	15	3.32	27

E. EFFECT OF FDP ON THE ACTIVITY OF RUDPCASE

Fructose-1,6-diphosphate (Fru-1,6-P_2) which is the substrate for the enzyme fructose-1,6-diphosphatase (EC3.1.3.11) in the photosynthetic carbon reduction cycle, was found to have an activating or inhibiting effect on the enzyme RuDPCase, depending on both the concentration of FDP and magnesium ion in the assay mixture. Fig. 1 shows that Fru-1,6-P_2 increased the RuDPCase activity by 180% at 1 mM magnesium and at an Fru-1,6-P_2 concentration of 0.4 mM. At higher concentrations of Fru-1,6-P_2 the activating effect on the enzyme disappears, and no inhibitory effect on the enzyme is seen if the magnesium ion concentration in the assay mixture is low (1 mM). As the magnesium ion concentration increases the activating effect of Fru-1,6-P_2 on the activity of RuDPCase decreases. The activation by 0.4 mM Fru-1,6-P_2 at 1 mM is more than twice that achieved at 5 mM Mg^{++} and approximately six times that achieved at 10 mM magnesium ion. At 40 mM magnesium Fru-1,6-P_2 becomes inhibitory at all levels of FDP concentrations.

The curve for RuDPCase activity vs. Fru-1,6-P_2 concentration is slightly sigmoidal in nature. The sigmoidal nature of the curve is more apparent at higher concentrations of magnesium, especially at 10 mM (Fig. 1). At 40 mM magnesium, FDP becomes inhibitory at all concentrations. These observations may indicate an allosteric effect of Fru-1,6-P_2 on RuDPCase[22].

F. DISCUSSION

The isolation procedure reported here can be easily adapted to the isolation of larger quantities, and is convenient for simultaneous isolation of some other chloroplast enzymes, such as FDPase[23] and pyrophosphatase[24]. The use of bicarbonate during the enzyme preparation and in the storage buffer leads to greater enzyme activity and stability.

Fig. 1. Effect of FDP on the activity of RuDPCase at different magnesium levels.

O--O 1 mM Mg^{++}

●--● 5 mM Mg^{++}

□--□ 10 mM Mg^{++}

△--△ 40 mM Mg^{++}

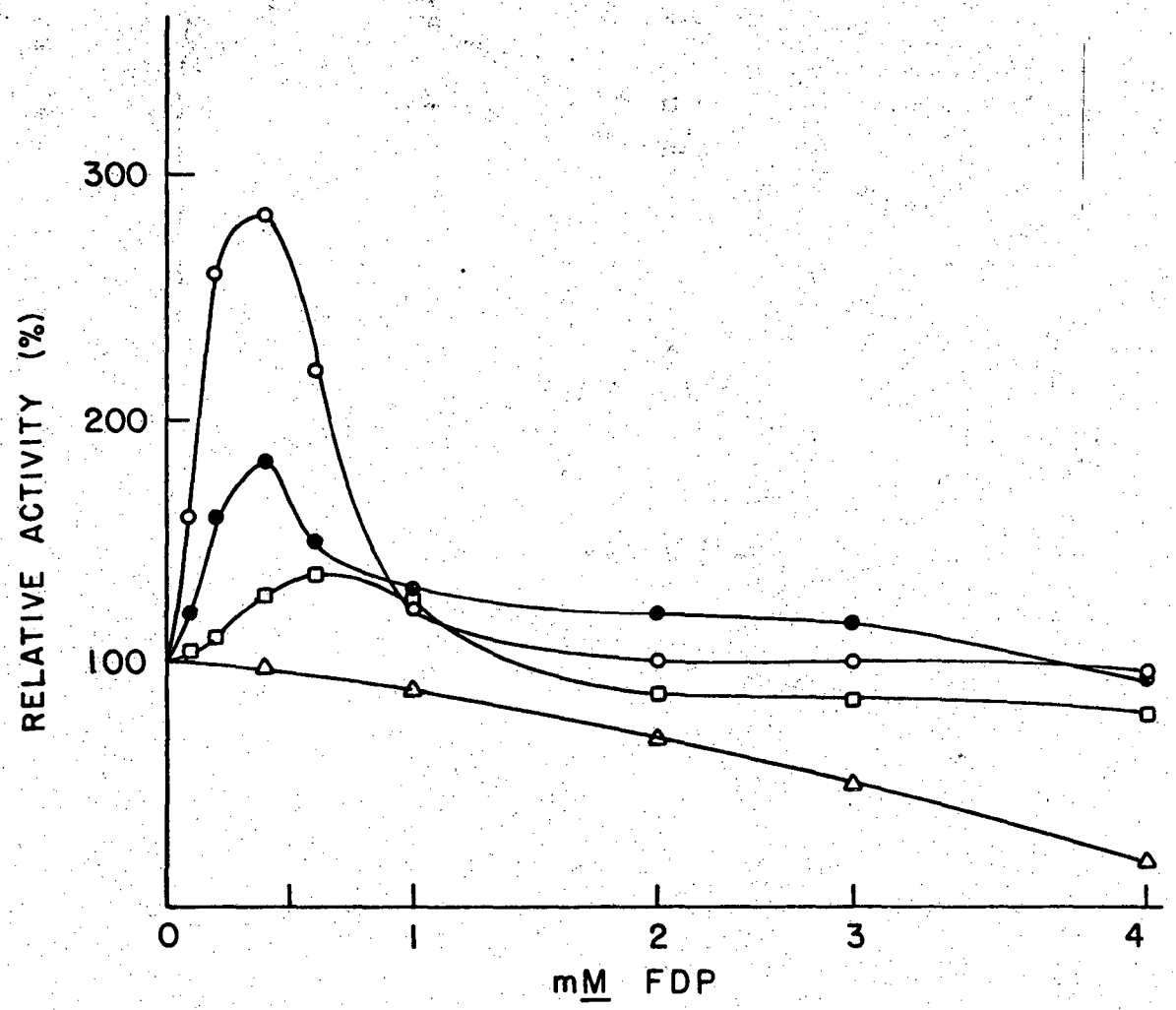

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Figure IV-1

That Fru-1,6-P_2 exhibits activation and inhibition of RuDPCase activity may indicate an interaction between two regulated steps of the carbon reduction cycle. Thus we see an effect of Fru-1,6-P_2 on the activity of RuDPCase, while there is an activating effect by bicarbonate on the activity of fructose-1,6-diphosphatase[23]. The reactions catalyzed by these two enzymes are considered to be control points in the carbon reduction cycle.[9,25-27] Interaction between the substrate of each of these enzymes with the activity of the other may prove to be an important aspect of regulation of photosynthetic carbon metabolism.

The level of Fru-1,6-P_2 in Chlorella pyrenoidosa photosynthesizing under steady-state conditions has been estimated at 0.1 mM[25]. There is evidence that the level of Mg^{++} ions in the osmotically-responding space of spinach chloroplasts may be as high as 16 mM[28]. Thus the activation of RuDPCase by 0.4 mM FDP at 1 mM or 5 mM Mg^{++}, or by 0.6 mM FDP at 10 mM Mg^{++}, is not far from physiological levels.

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PART III

STUDIES WITH INORGANIC PYROPHOSPHATASE

CHAPTER V

CHLOROPLAST INORGANIC PYROPHOSPHATASE, PURIFICATION AND PROPERTIES OF THE ENZYME

A. INTRODUCTION

Enzymes which hydrolyze inorganic pyrophosphate (PP_i) to orthophosphate have been found to be widely distributed in animal, plant, and bacterial systems[1-18]. However, the enzyme, obtained from previously isolated chloroplasts, is required for studies of the possible role of this reaction in photosynthesis. For example, if chloroplast pyrophosphatase activity is subject to metabolic regulation, this control might be effected by a different means in the photosynthetic system than in other metabolic systems. This is the case for fructose diphosphatase[19,20]. We report here the finding of alkaline pyrophosphatase activity in previously isolated spinach chloroplasts, methods of purification and characterization of the enzyme, and the dependence of its activity on Mg^{++} ion concentration and pH. Simmons and Butler[20] found that maize leaves contain alkaline inorganic pyrophosphatase activity, with a specific requirement for Mg^{++} and a pH optimum between 8 and 9.

B. MATERIALS AND METHODS

1. Chemicals. Tetrasodium pyrophosphate, Allied Chemical; AMP, ADP, ATP, TPP, p-nitrophenylphosphate, Cal Biochem, grade A; fructose-1,6-diphosphate, sodium salt,

Sigma Chemical Co. All inorganic salts were analytical reagents.

2. Enzyme assay. The enzymatic hydrolysis of inorganic pyrophosphate was determined in a 1 ml reaction mixture containing 2 mM tetrasodium pyrophosphate, 50 mM Tris buffer (pH 7-9), 5 to 40 mM $MgCl_2$, 5 mM EDTA, and the required amount of the enzyme for obtaining a detectable amount of orthophosphate. The reaction was stopped by the introduction of 0.1 ml of 50% trichloroacetic acid in aqueous solution and the protein was spun down. Aliquots of the supernatant were used for analyzing the released orthophosphate by the method of Fiske and Subbarow[21,22].

3. Protein determination. In the process of enzyme purification, in order to determine the specific activity of the enzyme, protein determinations were carried out using UV absorption at 280 nm for preliminary estimation. The Lowry[23] method of protein determination was used for more precise results. Bovine serum albumin was used as the standard in each of the two methods.

C. RESULTS

1. Isolation of the enzyme inorganic pyrophosphatase. Spinach chloroplast inorganic pyrophosphatase (PPase) was isolated using the following method:

a. Source of the enzyme

Field-grown spinach leaves were harvested and immediately stored over ice in polyethylene bags in large

ice chests. The leaves were deribbed and washed with ice-cold water and were dried between two sponges. The deribbed leaves were then chopped and divided into 50-gram batches.

b. Isolation of chloroplasts

Each 50-gram batch was homogenized for 8 sec in a Waring blendor with 200 ml of solution A[24] (containing 0.33 M sorbitol, 0.002 M $NaNO_3$, 0.002 M EDTA (dipotassium salt), 0.002 M Na isoascorbate, 0.001 M $MnCl_2$, 0.001 M $MgCl_2$, 0.0005 M KH_2PO_4, 0.05 M [2-N-Morpholinoethane sulfonic acid] adjusted with NaOH to pH 6.1, and 0.02 M NaCl. The blendorate was then forced through six layers of cloth to strain out fibrous material.

The homogenate was centrifuged at 2000 x g for 3 min. The supernatant was decanted and each pellet was suspended in 10 ml of "basic buffer" [0.05 M Tris buffer, pH 7.4, 0.002 M dithiolthreotol (DTT), 0.0002 M EDTA, and 0.001 M $MgCl_2$].

c. Sonication

The chloroplast suspension was sonicated for 30 sec in batches of 50 ml, using the Biosonik (Model BPI, Bronwill Scientific Co., Rochester, N.Y.) at 0°C.

The sonicated suspension was centrifuged at 36,000 x g for 2 hours and the suspension was saved as the crude enzyme preparation (I).

d. Acetone fractionation

Acetone was added to the enzyme to a concentration of 30%. The acetone had been precooled in the freezer at

-14°C and was added to the crude enzyme solution slowly while stirring at 4°C. The enzyme in 30% acetone was allowed to stand in the cold room (4°C) for 30 min and the mixture was centrifuged at 13,200 x g for 4 min. The supernatant was collected and the acetone concentration in the supernatant was brought to 75%. The enzyme in 75% acetone was allowed to stand in the freezer at -14°C for 1 to 2 hours. A copius precipitate formed and settled toward the bottom of the container. The upper layer of 75% acetone solution was decanted. The lower layer containing the precipitated enzyme and some other proteins was then centrifuged for 1 min at 5000 x g and the pellets were collected.

The greyish-white precipitate was suspended in the smallest possible volume of basic buffer and was dialyzed against cold water (4°C) for 4 hours. Then it was dialyzed against basic buffer twice, for 8 hours each time.

The dialyzed mixture was centrifuged in the Sorvall, at 36,000 x g for 10 min, and the supernatant (II) was saved.

e. First DEAE-cellulose column

A DEAE-cellulose column was prepared and pre-equilibrated with 0.05 M tris-HCl, pH 7. The supernatant was applied to the column and the column was washed with 0.05 M tris-HCl (pH 7.4) buffer until no more protein was found in the eluate. Then the column was eluted with a buffer containing 0.15 M tris-HCl and 0.28 M NaCl (pH 7.4) (III).

f. Second DEAE column

The protein eluted from the first column (III) was diluted to bring the salt concentration down to 0.05 M and

was applied to a DEAE column that had been equilibrated with 0.05 M tris-HCl, pH 7. A gradient of 0.05 M NaCl and 0.05 M tris buffer (pH 7.4) to 0.15 M tris-HCl and 0.5 M NaCl (pH 7.4) was used for elution, and fractions that contained pyrophosphatase activity were pooled (IV).

The specific activity of the purified enzyme (IV) was increased 100-fold (see Table I) as compared with the total soluble chloroplast protein (I).

2. Stabilization of pyrophosphatase activity. Among the sulfhydryl reagents used, DTT had the greatest stabilizing effect on the enzyme. Glutathione at a comparable molarity (0.002 M) also had a good stabilizing effect. The enzyme is very stable in tris-HCl buffer at a pH of 6 to 9.5. EDTA is needed at a concentration of 0.0002 M to stabilize pyrophosphatase activity. EDTA at the relatively high concentration of 0.002 M was tolerated by pyrophosphatase. The high concentration of EDTA was essential as a bacteriostatic agent in the storage solution. Among all bactericidal and bacteriostatic agents tested, EDTA at 0.002 M was the most satisfactory. Pyrophosphatase activity remained unchanged for two months in 0.05 M tris buffer, pH 6.2, 0.002 M EDTA, 0.002 M DTT at 0°C. However, pyrophosphatase activity was not decreased after heating for 20 min at 60°C.

3. Effect of substrate concentration on the activity of pyrophosphatase at different Mg^{++} ion concentrations and the corresponding pH optima. This experiment was designed to study the substrate effect at two dif-

TABLE I

Purification of Spinach Chloroplast Inorganic Pyrophosphatase

A unit of enzyme activity is that amount which will hydrolyze 1 μmole of PP_i per min in the described assay.

Fraction	Protein mg/ml	Specific Activity units/mg protein	Yield %
I Sonicate supernatant	10	12	100
II Acetone fraction	20	230	57
III First DEAE-cellulose column	10	520	49
IV Second DEAE-cellulose column	6	1230	42

ferent Mg^{++} ion concentrations. These were Mg^{++} concentrations of 5 mM at pH 9 and 40 mM at pH 7. The enzyme concentration was constant and the only variable in this experiment was the concentration of the substrate inorganic pyrophosphate. It was found that pyrophosphatase was inhibited by the substrate at higher concentrations (Fig. 1). At 5 mM Mg^{++} concentration and pH 9, the optimum pyrophosphate concentration for maximum pyrophosphatase activity was 5 mM. At pH 7 and higher Mg^{++} concentration, the optimum substrate concentration was also 5 mM; however, only slight inhibition of pyrophosphatase activity was observed at higher substrate concentrations. This observation could be taken to indicate that Mg^{++} exerts an effect on the substrate. Magnesium pyrophosphate may be the specific substrate for the enzyme and anionic pyrophosphate itself an inhibitor. Thus the higher magnesium concentration would result in the availability of a higher concentration of the specific substrate magnesium pyrophosphate and therefore would counteract the effect of the competitive inhibitor anionic inorganic pyrophosphate.

4. <u>Effect of Mg^{++} on the pH optimum of pyrophosphatase</u>. In studying the effect of Mg^{++} ion on the pH optimum for inorganic pyrophosphatase activity, Mg^{++} concentrations of 5 mM to 40 mM were used and the pH of the reaction mixture was varied from pH 7 to 9.5. Figure 2 shows the shift of two pH units from an optimum of pH 9 at a magnesium ion concentration of 5 mM to an optimum of pH 7 at 40 mM Mg^{++}. The effect of Mg^{++} ion on pH optimum could be due to one or both of the following factors:

Figure 1. Effect of pyrophosphate concentrations on pyrophosphatase activity at two levels of magnesium ion concentration, 5 mM Mg^{++} (o——o) and 40 mM Mg^{++} (Δ——Δ) concentrations. Assays were carried out in 1 ml of assay mixture containing 40 mμg of Fraction IV protein.

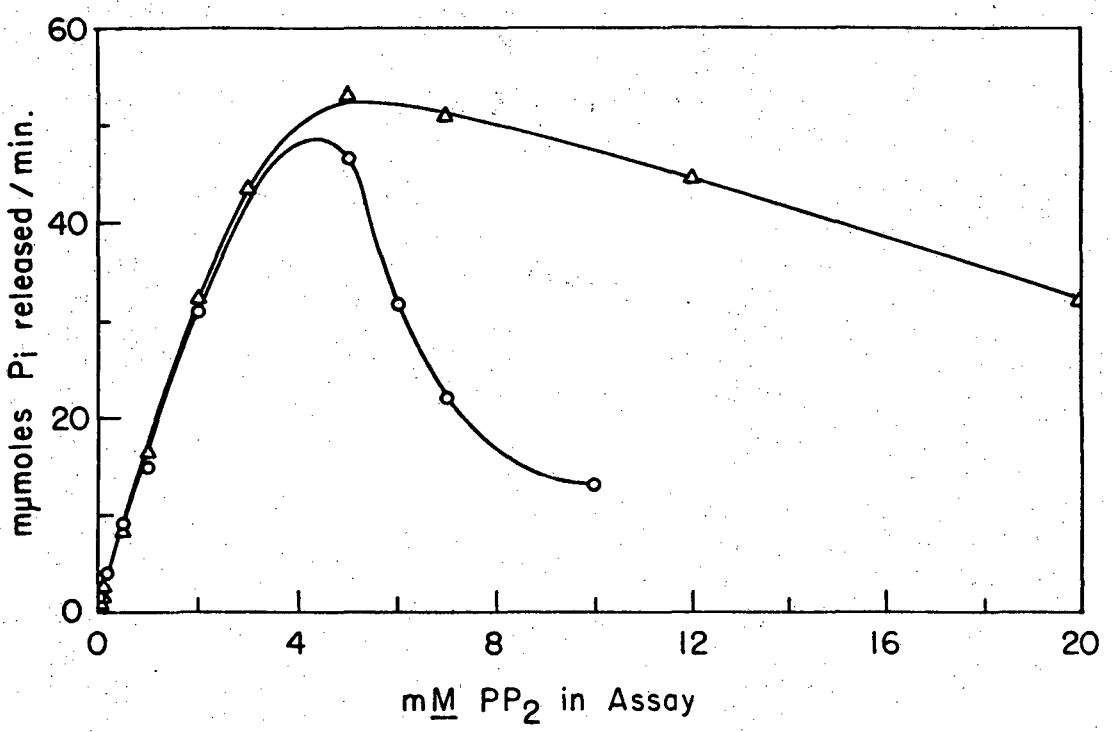

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Figure V-1

Figure 2. Effect of magnesium ion concentration on pH optimum. Assays were carried out in 1 ml assay mixture containing 40 mμg of Fraction IV protein. ○——○, 5 mM Mg^{++}; □——□, 10 mM Mg^{++}; ●——●, 20 mM Mg^{++}; and Δ——Δ, 40 mM Mg^{++}.

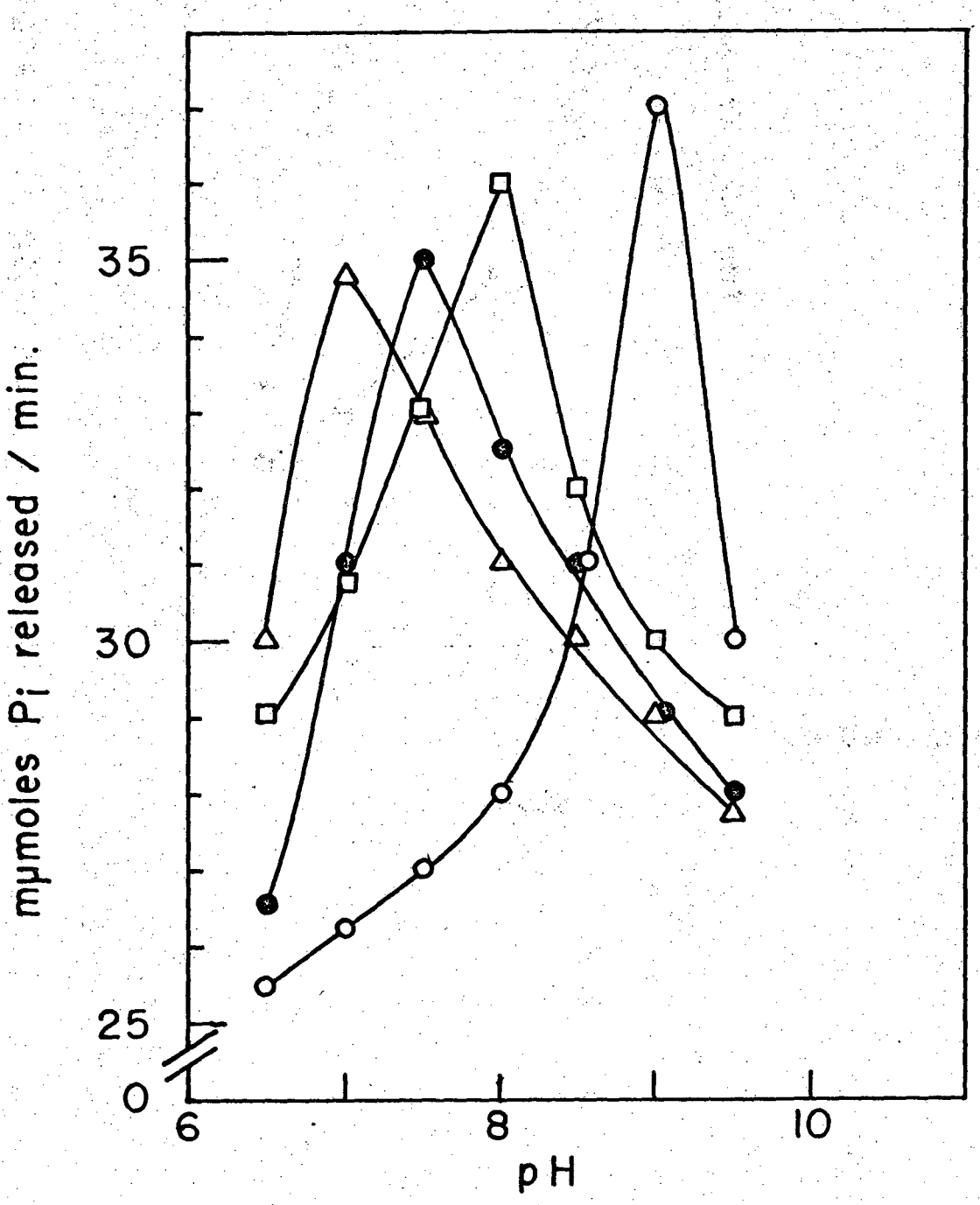

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Figure V-2

a. The effect of Mg^{++} is on the substrate. That is, the substrate for the enzyme is not free pyrophosphate anion, but magnesium pyrophosphate.

b. Magnesium ion exerts its effect directly on the enzyme. That is, Mg^{++} alters the conformation or the ionic properties of the active site of the enzyme pyrophosphatase. The direction of the shift in pH optimum seems to favor the assumption that Mg^{++} affects the enzyme as well as the substrate. One explanation could be that Mg^{++} helps cover some negative charges near the active site at pH 7 which interfere with the accessibility of the active site to the substrate. If, at pH 9, the conformation of the enzyme has changed so that the negative charges are removed from the vicinity of the active site, Mg^{++} would be needed only to make the specific substrate, magnesium pyrophosphate. Thus, these results suggest that Mg^{++} exerts its effect on both substrate and enzyme.

The effect of lower concentrations of magnesium ion on the pyrophosphatase activity was determined in an assay mixture containing 2 mM tetrasodium pyrophosphate, 50 mM Tris buffer at pH 9 in the absence of EDTA. However, 5 mM of EDTA was found to have no effect on the enzyme activity at all levels of magnesium ion concentration. The sigmoidal dependence of enzyme activity on low concentrations of Mg^{++} ion (Fig. 3) provides evidence of an allosteric effect of Mg^{++} ion on the activity of the enzyme[25]. Anionic pyrophosphate may have also contributed to the sigmoidal shape of the curve at low Mg^{++} ion concentrations.

Figure 3. Effect of lower concentrations of magnesium ion on the activity of pyrophosphatase. Assays were carried out in 1 ml mixtures containing 40 mμg of Fraction IV protein.

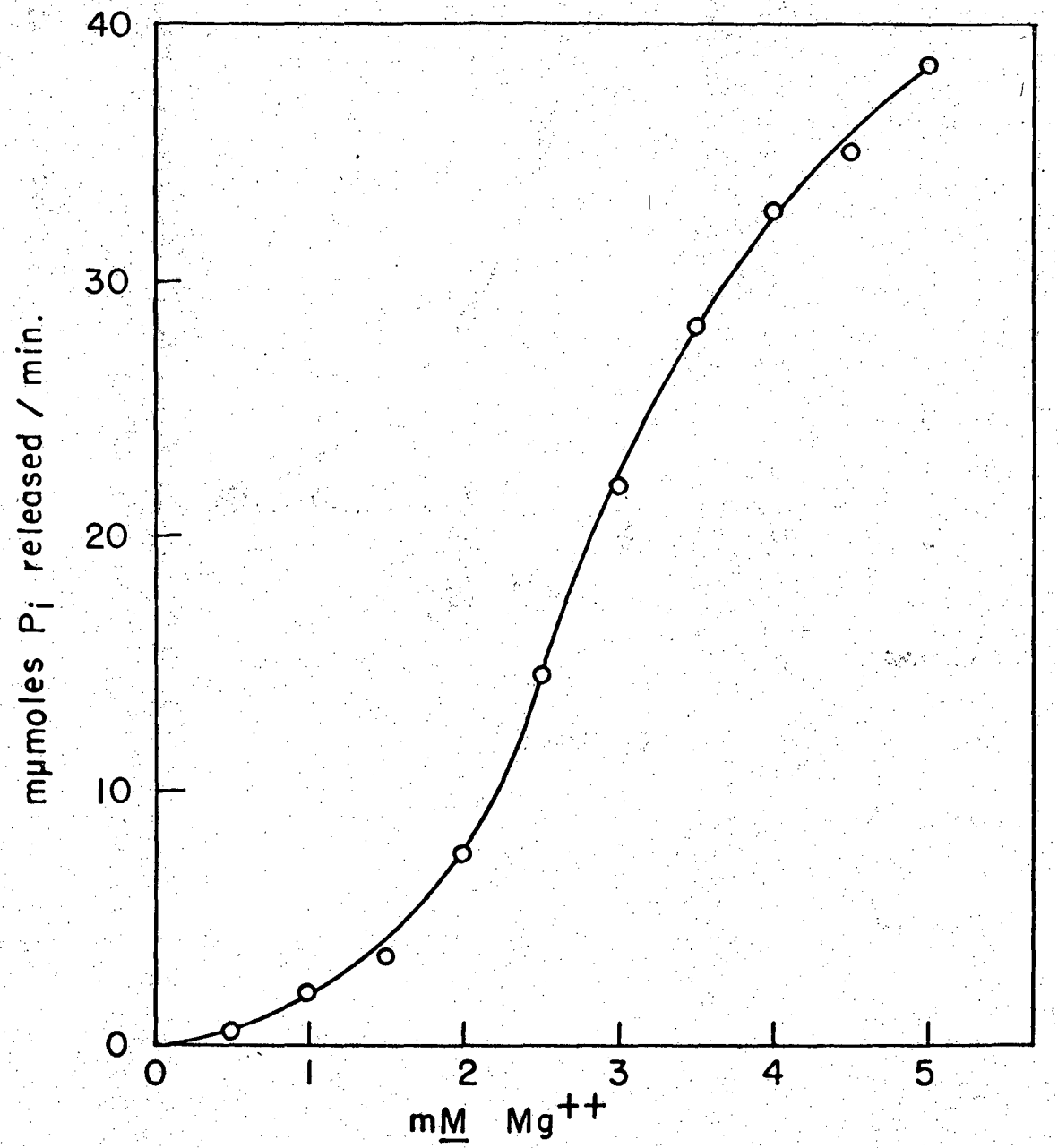

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Figure V-3

5. Effect of cations on pyrophosphatase activity. Among the 12 cations (Table II) tested, only Mg^{++} markedly stimulated the activity of the enzyme pyrophosphatase, Au^{++}, Fe^{+++}, Co^{++}, Ni^{++}, Cd^{++}, and Ca^{++} had no activating effect at low concentration and an inhibitory one at higher concentrations. No activities for hydrolysis of ATP, ADP, or TPP were induced by presence of Zn^{++} or Mn^{++} ions as was found for pyrophosphatase from yeast[26]. None of the cations tested could replace or match the activating effect of Mg^{++} on pyrophosphate.

6. Effect of anions on the activity of pyrophosphatase. In testing for the effect of anions on the activity of pyrophosphatase, Mg^{++} was always present at a concentration of 5 mM. The pH of the reaction medium (0.05 M tris-HCl) was 9 and pyrophosphate was present in sufficient amounts (2 mM) to yield detectable quantities of inorganic orthophosphate product. The anions F^-, Br^-, Cl^-, I^-, $SO_4^=$, and $HPO_4^=$ were tested individually at a concentration of 5 mM. Only F^- was found to have an inhibitory effect on the enzyme in the presence of Mg^{++} (Table III). The effect of F^- may be due to prevention of Mg^{++} ion activation of the pyrophosphatase enzyme due to the low solubility product of MgF_2. The fluoride inhibition was striking.

7. Specificity of pyrophosphatase. In order to determine the specificity of pyrophosphatase, different potential substrates were incubated with the enzyme at pH 9 and in the presence of 5 mM Mg^{++}. The reaction was run at room temperature for 20 min. Pyrophosphatase was found to be very

TABLE II

Effect of Cations on Pyrophosphatase Activity

Cation	mμmoles of P_i Released/ml*/min			
	5 mM cation	10 mM cation	20 mM cation	40 mM cation
Mg^{++}	34	32.5	30	26
Mn^{++}	2.1	1.5	1.5	1.5
Zn^{++}	2.75	2.0	1.0	1.0
Fe^{+++}	0.5	0.4	0.25	0.05
Co^{++}	1.6	1.1	1.5	1.6
Ni^{++}	1.25	1.0	1.25	1.0
Au^{++}	0.53	0.5	1.0	1.0
Cd^{++}	1.0	1.0	1.0	1.5
Ca^{++}	1.5	1.0	0.75	1.25

*Containing 40 mμg fraction IV protein.

TABLE III

Effect on Anions on Pyrophosphatase Activity

Anion	mμmoles of Pi released/ml*/min			
	5 mM anion	10 mM anion	20 mM anion	40 mM anion
F^-	3.85	3.5	1.25	0.5
Cl^-	31.5	29.5	30.0	27.0
Br^-	31.5	28.0	27.0	30.0
I^-	31.5	28.0	27.0	29.5
$SO_4^=$	26.5	25.0	27.25	23.0

*Containing 40 mμg fraction IV protein.

specific for inorganic pyrophosphate. When tested at pH 9 and 5 mM Mg^{++}, pyrophosphatase was inactive toward fructose-1,6-diphosphate, ATP, ADP, thiamine pyrophosphate, and para-nitrophenylphosphate.

8. Effect of inhibitors of CO_2 fixation on the activity of pyrophosphatase. In studying the effect of several inhibitors of CO_2 fixation on pyrophosphatase activity, optimum conditions for enzyme activity were employed (pH 9, Mg^{++} concentration of 5 mM, 4 mM pyrophosphate) and the reaction was run at room temperature. Vitamin K_5, caprylic acid, and the natural spinach inhibitor (factor B)[31] were tested. Vitamin K_5, caprylic acid, and the spinach juice factor showed no inhibitory effect on the pyrophosphatase activity. AMP, ADP, and ATP in the presence of Mg^{++} fail to exert any inhibitory effect on catalytic activity of the enzyme towards pyrophosphate in contrast to the enzyme from E. coli[12].

D. DISCUSSION

The finding of Mg^{++} dependent, inorganic pyrophosphatase in previously isolated spinach chloroplasts supports the proposal that the level of PP_i inside the chloroplasts is controlled within the chloroplasts. The dependence of the pH optimum on Mg^{++} level in the range of 5 mM to 40 mM shows some similarity to the behavior of fructose-1,6-diphosphatase[20] (E.C. 3.1.3.10) and of ribulose diphosphate carboxylase[27] (E.C. 4.1.1.39), both of which exhibit Mg^{++} dependent shifts in pH optima over about the same range of pH and Mg^{++}

concentration. The absolute change in activities with pH and Mg^{++} concentration are different for each of the three enzymes.

It has been suggested that the mechanism of light-dark regulation of diphosphatase and carboxylase enzymes of the carbon reduction cycle might depend on changes in Mg^{++} ion concentration and pH resulting from light-pumping of ions through the thylakoid membranes, leading to a higher pH and Mg^{++} ion concentration in the stroma region of the chloroplasts in the light[28]. Light-induced pumping of these ions in chloroplasts has been reported[29], but unfortunately information about the possible changes induced in the stroma region of intact chloroplasts is not available. If the suggested mechanism is corect, and if pyrophosphatase is regulated, then a similar mechanism might be responsible for pyrophosphatase regulation. Otherwise, the Mg^{++}-pH dependence might be an indication of other, more specific allosteric properties. In any event, any regulation of pyrophosphatase in chloroplasts would be effected by a different mechanism than that found in other systems, since the activity of the enzyme in chloroplasts is unaffected by ATP, ADP, or AMP.

Inorganic pyrophosphate added to a suspension of isolated spinach chloroplasts stimulates the photosynthetic reduction of CO_2[24,30]. The inhibitory effects on such fixation of factors isolated from spinach leaves depends on the ratio of added pyrophosphate to added factors[31]. The level of ^{32}P-labeled pyrophosphate in <u>Chlorella pyrenoidosa</u> which

have been photosynthesizing in the presence of ^{32}P-labeled inorganic phosphate changes suddenly during the transition from light to dark[32], and also in the light upon the addition of fatty acids which are known to cause changes in the activity of regulated enzymes of the carbon reduction cycle and inhibition of photophosphorylation[33]. Furthermore, the addition of octanoic acid causes a transient increase in pyrophosphate at the same time that the level of ATP declines and while the synthesis of carbohydrates and PP_i in the chloroplasts must be presumed to decrease.

The presence of PP_i in the green tissue at levels comparable to those of other metabolites suggests that the activity of inorganic pyrophosphatase may be limited, and thus possibly subject to regulation. The rise in PP_i level, along with the inactivation of two regulated enzymes, fructose diphosphatase and phosphoribulokinase, upon addition of octanoic acid also suggests pyrophosphatase regulation. Since PP_i itself appears to affect other biochemical activities, control of its level through the action of a regulated pyrophosphatase could be part of a feedback control, from carbohydrate synthesis to CO_2 fixation rate, or through the distribution of carbon fixed by the carbon reduction cycle to non-carbohydrate biosynthesis.

It seems likely that properties of pyrophosphatase enzyme in some tropical grasses (maize, sugar cane, etc.) which contain a special CO_2 fixing pathway[34] may be different from properties of the enzyme in spinach and the majority of

green plants which do not contain this special pathway. Such a difference would be due to the fact that PP_i is formed in one step of that special pathway, during the pyruvate inorganic phosphate dikinase reaction[35]. Nevertheless, the properties of the purified enzyme isolated from spinach chloroplasts are similar in some respects to properties described for pyrophosphatase activity in a homogenate of maize leaves[20].

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PART IV

ELECTRON PARAMAGNETIC RESONANCE STUDY OF THE INTERACTION OF NITROXIDE RADICALS WITH SPINACH CHLOROPLASTS

CHAPTER VI

INTERACTION OF NITROXIDE RADICALS WITH THE ELECTRON TRANSPORT CHAIN IN PHOTOSYNTHESIS: RELATION OF STRUCTURE TO ACTIVITY AND ELECTRON PARAMAGNETIC RESONANCE STUDY OF THE MECHANISM OF ACTION

A. Introduction

Nitroxide free radicals are useful labels in studying molecular structure and biological function. They possess great sensitivity to changes in the local environment and an ability to monitor very rapid molecular motion in addition to monopolizing the role of probing the environment with their electron paramagnetic signals[1-5]. Nitroxide radicals have been used to probe the conformational changes at specific sites of biological macromolecules, as their EPR spectra provide information about the rotational freedom at the sites in question[2,3]. Thus information is obtained about the local viscosity at a site and the changes of this viscosity with change in conformation. Nitroxide free radicals have been used to prove conformational changes in proteins and nucleic acids[1-8] and for studying the deformations of nerve membranes during excitation[9,10]. Furthermore, the radicals have the special property of being equally useful in optically transparent as well as optically opaque solutions[2].

In this chapter we have studied the interaction of nitroxide radicals with isolated chloroplasts and the effect of the radical structure on the potency of the nitroxide

radicals as Hill oxidants and as inhibitors of CO_2 fixation.

B. MATERIALS AND METHODS

1. Isolation of chloroplasts. Young spinach plants were obtained from a nearby farm, and the leaves were immediately chilled on ice and kept on ice until use. The spinach used for some experiments was grown in growth chambers under light conditions simulating an eight-hour day and sixteen-hour night at a temperature of 15°C.

Chloroplasts were isolated either by the method of Jensen and Bassham[11] or by a modification of this method using sucrose-tricine buffer. In the method of Jensen and Bassham, each of three solutions used in the isolation and assay of chloroplasts contained the following: 0.33 M Sorbitol, 0.002 M $NaNO_3$, 0.002 M EDTA (dipotassium salt), 0.002 M isoascorbate (added just before use to avoid its oxidation), 0.001 M $MnCl_2$, 0.001 M $MgCl_2$, and 0.0005 M KH_2PO_4.

In addition, solution A contained 0.02 M NaCl and 0.05 M MES, adjusted with NaOH to pH 6.1; solution B contained 0.02 M NaCl and 0.05 M HEPES, adjusted with NaOH to pH 6.7; solution C contained 0.005 M $Na_4P_2O_7 \cdot 10\ H_2O$ (added just before use) and 0.05 M HEPES, adjusted with NaOH to pH 7.6. All solutions were stored at 4°C.

Solution D, which is used in the alternate method to that of Jensen and Bassham, contained 0.5 M sucrose and 0.1 M tricine, pH 7.6.

The procedure for making chloroplasts can be summarized as follows: ten grams of spinach leaves were washed, the midribs were removed, and the blades were sliced with a scalpel on a glass plate. These slices and 30 ml of cold soln. A (4°) were placed in a semimicro Monel homogenizing vessel on a Waring blendor and blended for 5 sec at high speed. The slurry was filtered under mild pressure through six layers of cheesecloth (42 threads per inch), and the resulting juice was centrifuged for 50 sec at 2000 x g. The pellet was suspended in soln. B at 0°C, to give a suspension which contained about 2 mg chlorophyll/ml. This suspension was used within two hours, to assay CO_2 fixation, oxygen evolution, or radical decay. Soln. D was used in place of solns. A, B, and C, in an alternate method using the same procedures described above.

2. Assay of photosynthetic rate. Spinach chloroplasts were assayed for the rate of photosynthesis in the presence of various additions as described previously.[11,12] A typical experiment starts with the injection of 25 µl of chloroplasts suspended in soln. B to each of several 15-ml round-bottom flasks stoppered with serum caps and containing 450 µl of soln. C. The flasks are mounted on a shaking rack and illuminated from below in a 20°C bath as described before[11]. The chloroplasts in the swirling flasks are preilluminated for 5 min; ^{14}C-labeled bicarbonate is added and photosynthesis is allowed to continue for another 5 minutes. The reaction is terminated by the addition of 4.5 ml

of methanol to each flask.

In order to measure the total ^{14}C fixed by isolated chloroplasts, an aliquot of the chloroplast-methanol mixture is spotted on a piece of filter paper, treated with acetic acid, dried, and its radioactivity measured in a gas-flow counter. From the known counter efficiency and the specific activity of the ^{14}C used, the amount of CO_2 fixed in the 5-minute period is calculated.

The amount of chlorophyll present is determined by extracting an aliquot of the chloroplast suspension in soln. B with 80% acetone and measuring its absorption at 665 and 649 nm, as described previously[13].

The rate of photosynthesis (μmoles fixed per mg chlorophyll/hr) is calculated from the chlorophyll content and the CO_2 fixation data.

3. Measurement of photosynthetic product distribution. An aliquot of 500 μl of each chloroplast-methanol suspension was spotted on chlomatographic paper and subjected to two-dimensional elution in phenol/acetic acid/water and butanol/propionic acid/water solvent systems as described elsewhere[14].

The spots were detected by autoradiography and were counted semiautomatically by a gas-flow counting device.

4. Measurement of oxygen evolution. Oxygen evolution measurements were carried out with a Pickett-type, Teflon-covered stationary rate electrode[15]. This electrode is suitable for measuring rates of photosynthesis on aliquots of a pretreated suspension of chloroplasts. A silver/silver

chloride reference electrode was used in a buffered electrolyte solution containing 0.5 M KCl and 0.5 M $KHCO_3$. A platinum electrode was at -0.6 v with respect to the Ag/AgCl electrode. A Bell and Howell projector with a 300 W tungsten lamp was used as the light source. The light was passed through 8-inch-diameter plane-convex lenses, then through IR and UV filters (Corning 1-69 and 3-74 filters). Chloroplast samples containing 0.2 mg of chlorophyll/ml were suspended in soln. C. Soln. C was also used as the circulating medium (Fig. 1). The detection circuit was an adaptation from that of Myers and Graham[16].

5. EPR Measurements. EPR measurements were made using a Varian E-3 spectrometer. An aqueous sample cell was used; the EPR cavity containing the cell was illuminated by light from a projection lamp (115 V). To study decay of the signal, EPR spectra were taken repeatedly at two-minute intervals; alternatively, the magnetic field of the spectrometer was set on the first peak of the spectrum and the decay with time was recorded directly.

Figure 1. A diagram of the oxygen electrode assembly for measuring the rates of oxygen evolution on aliquots of pretreated chloroplast suspension.

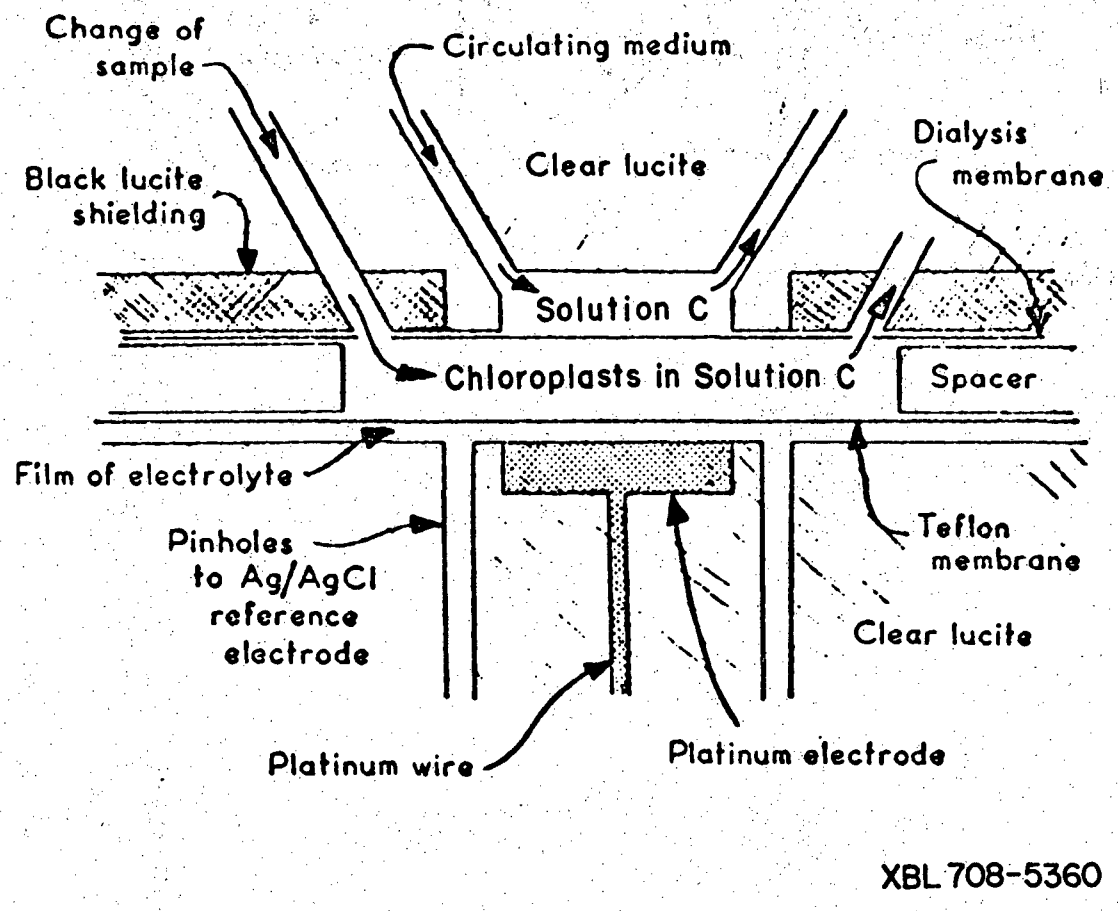
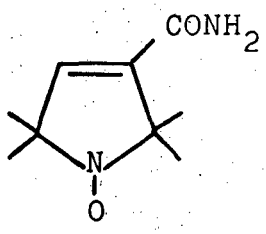

Change of sample
Circulating medium
Clear lucite
Dialysis membrane
Black lucite shielding
Solution C
Chloroplasts in Solution C
Spacer
Film of electrolyte
Pinholes to Ag/AgCl reference electrode
Teflon membrane
Clear lucite
Platinum wire
Platinum electrode
XBL 708-5360

Figure 6-1

6. <u>The radicals</u>.



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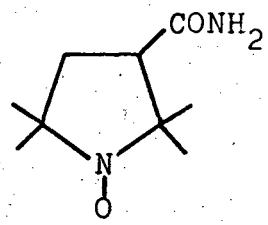

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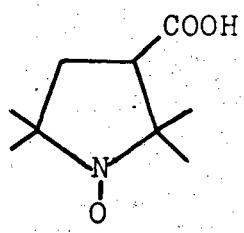

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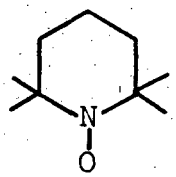

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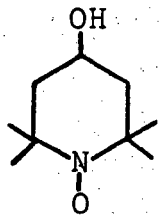

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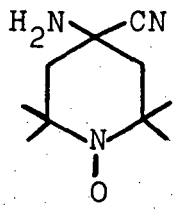

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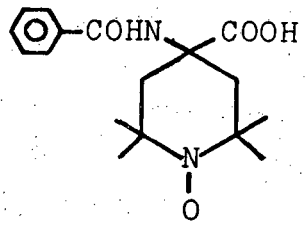

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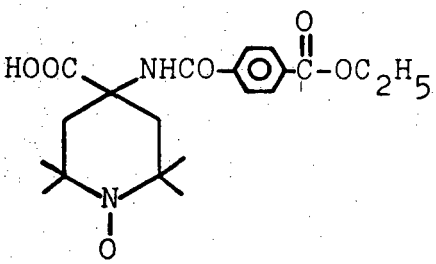

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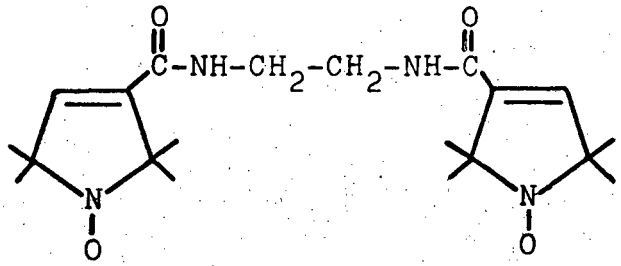

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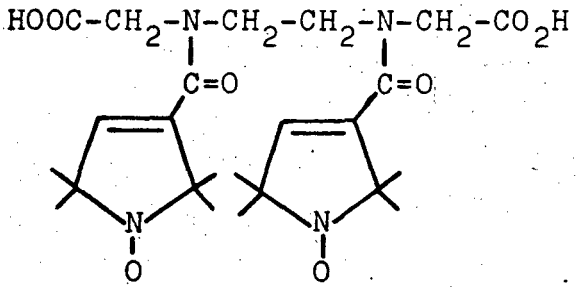
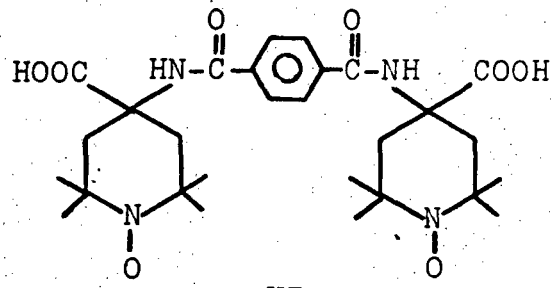


X



XI

2,2,5,5-Tetramethyl-3-Carbamidopyridine-1-oxyl (I), 2,2,5,5-Tetramethyl-3-Carbamidopyrrolidine-1-oxyl (II), 2,2,5,5-Tetramethyl-3-Carboxypyrrolidine-1-oxyl (III) were prepared as described by Rotzantzev and Krinitzkaya[17].

2,2,6,6-Tetramethylpiperidine-1-oxyl (IV) was synthesized by the method of E. G. Rozantzev and Neiman[18].

2,2,6,6-Tetramethyl-4-oxopiperidine-1-oxyl (V) was synthesized as reported by Rozantzev[19].

N,N'-Bis(1-oxyl-2,2,5,5-tetramethylpyrroline-3-carboxy)-1,2-diaminoethane (IX) and N,N'-Bis(1-oxyl,2,2,5,5-tetramethyl-pyrroline-3-carboxy)ethylenediamine-N,N'-diacetic acid were synthesized as described by Ferruti et al.[20].

The synthesis of compound VI, VII, VIII AND XI will be reported elsewhere.

7. Flashlamp system. Xenon flashes were produced from a Model L-391 flash lamp system manufactured by ILC Co. This system affords a choice of 15 flash durations, from 20 micronseconds to 7.5 milliseconds, as well as timing circuits and switches allowing the discharge of four different capacities through the same flash lamp at preselected intervals.

C. RESULTS AND DISCUSSION

1. Relation of nitroxide radical structure to inhibitory effect on the rate of CO_2 fixation. To study the effect of the radicals on CO_2 fixation rate, chloroplasts isolated in sorbitol buffer were used. Table I shows the effect of eleven radicals on the rate of CO_2 fixation by

TABLE I

Effect of Nitroxide Radicals on the Rate of $^{14}CO_2$-Fixation by Isolated Chloroplasts (5 min exposure)

Radicals	Rate (μmoles ^{14}C/mg Chl/hr)	Inhibition (%)
Control	143	-
I	108	25
II	7	95
III	93	35
IV	12	92
V	4	97
VI	21	85
VII	90	37
VIII	55	62
IX	115	20
X	128	11
XI	106	26

Concentration of radical = 10^{-6} M, Chl content = 0.05 mg/flask, total volume with each flask = 0.5 ml Soln. C.

chloroplasts. It is evident from these data that inhibitory power is inversely related to polarity of the compound. The chloroplast membrane, like other membranes, is composed of lipoproteins[21]. The membrane and the aqueous solution external to the chloroplasts may be thought of as a two-phase system in which hydrophilic solutes will tend to collect in the aqueous phase (except where active-transport phenomena are involved) but lipophilic (or hydrophobic) solutes will tend to partition into the membrane phase. Thus for a compound to penetrate the membrane it must have a partition coefficient favoring its solubilization by components of the membrane. It is no surprise, then, that compounds which possess a free carboxylic group (such as III, VII, VIII, X, and XI) are relatively poor inhibitors. Among these compounds containing a free carboxylic group, inhibition of the CO_2 fixation rate is clearly a function of the ratio of the size of the nonpolar moiety to the number of free carboxyl groups. Thus compound VIII, which has a larger lipid-soluble moiety, is a better CO_2-fixation inhibitor than compound VII, although the latter possesses similar structural features. A similar comparison can be made between biradicals X and XI. Both biradicals possess two carboxylic groups, but the hydrophobic moiety in radical XI is relatively larger than that in radical X, and correspondingly the inhibition of CO_2 fixation exhibited by radical XI is greater than that exhibited by radical X. Compound III, containing a free acid group on the saturated 5-membered ring, causes a 35% inhibition of

CO_2 fixation; amidation of the acid moiety (compound II) increases the inhibitory power of the compound, yielding 95% inhibition. Compounds such as IV, V and VI which possess structural features favorable to permeation through the lipoprotein membrane of the chloroplasts, are potent inhibitors of the CO_2 fixation rate. Among the nitroxide radicals tested for inhibition of CO_2 fixation, the size of the ring containing the nitroxide group did not appear to be a critical factor; five-membered-ring radicals with structural features favorable to permeation were just as effective as inhibitors as were six-membered-ring compounds possessing similar features. Compounds III and V are good examples. A close look at CO_2 fixation rates also reveals that the presence of an unsaturation in the ring greatly decreases the capacity of the radical to inhibit CO_2 fixation; this is apparent in comparing radicals I and II. This may also explain the poor inhibitory potency of compound IX, which possess no polar functional groups and thus should be able to permeate the membrane.

The data in Table II indicate that the presence of the ditertiary butyl nitroxide function is essential for inhibition of CO_2 fixation. The ditertiary butyl amine group is not inhibitory. Thus, either the molecules do not penetrate the chloroplast membrane or the nitroxide group is essential for the effect.

2. <u>Irreversibility of inhibition of CO_2 fixation by the nitroxide radicals</u>. In an experiment designed to determine whether the nitroxide group is a

TABLE II

Effect of Amines (and Corresponding Nitroxides) on $^{14}CO_2$ Fixation by Chloroplasts

(5 min exposure)

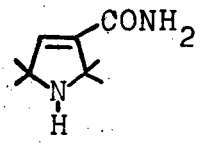
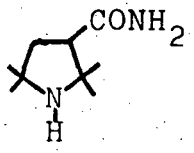
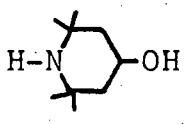
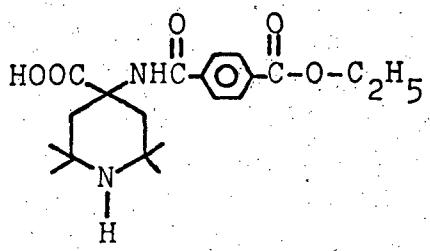
Compound	Rate (μmoles ^{14}C/mg Chl/hr)	Inhibition (%)
Control	107	-
I	76	29
II	63	41
V	3	97
VIII	37	65
	103	4
	104	3
	110	0

TABLE II (continued)

Compound	Rate (μmoles ^{14}C/mg Chl/hr)	Inhibition (%)
	101	6

Concentration of radical = 10^{-6} M, Chl content = 0.05 mg/flask, total volume each flask = 0.5 ml Soln. C.

photosynthestat (reversible inhibition) or a photosynthetocide (irreversible inhibition) chloroplasts were treated with radical III (a poor inhibitor of CO_2 fixation) or radical V (a potent inhibitor of CO_2 fixation) at a concentration of 10^{-6} M. The chloroplasts were then illuminated for ten minutes in the absence of CO_2, washed with solution C, and centrifuged for one minute at 2000 x g. These chloroplasts which were washed after treatment with the radical, along with control chloroplasts not treated with inhibitor but illuminated and washed in a similar manner, were then assayed for CO_2 fixation rate by a second incubation with $H^{14}CO_3^-$ in the light for five minutes. (A standard control, in which chloroplasts not treated with inhibitor were assayed for CO_2 fixation without preillumination or washing, was also run.) The results shown in Table III indicate that the inhibitory power of these compounds is irreversible (i.e., is not removed by washing) and provide additional evidence for the dependence of inhibitory activity on the permeability of the chloroplast membrane to the compounds tested. Compound III, which exhibits only a weak inhibitory activity toward unwashed chloroplasts (see e.g. Table I), is a relatively potent inhibitor of CO_2 fixation when the integrity of the chloroplast membrane is altered through illumination, washing, and additional centrifugation.

3. Relationship of radical structure to function as a Hill oxidant. To study the potency of the nitroxide radicals as Hill oxidants, three monoradicals and two biradicals were used (Table IV and Fig. 2). The

TABLE III

Effect of Washing on the Rate of $^{14}CO_2$ Fixation by Isolated Chloroplasts (5 min exposure) in the Presence and Absence of Radicals

Radical	Rate (μmoles ^{14}C/mg Chl/hr)	Inhibition
Control	133	-
Chloroplasts washed with Soln. C	73	46
III	26	64
V	2	97

Concentration of radical = 10^{-6} M, Chl content = 0.05 mg/flask, total volume each flask = 0.5 ml Soln. C.

Chloroplasts were illuminated for 5 min in the presence of the radical, then were spun down at 2000 x g for 50 sec, washed with Soln. C, and again centrifuged for 50 sec and used for determining the rate of $^{14}CO_2$ fixation.

TABLE IV

Effect of Nitroxide Radicals on Initial O_2 Evolution Rate (with Picket Electrode)

(15 min Contact with Radical in the Dark Before Test)

Compound	picomoles O_2 Evolved/30 sec (in 1 μℓ Electrode Chamber)	Increase in Rate of O_2 Evolution (μmoles/mg Chl·min)
Control	9.67	-
I	13.98	43.1
V	57.28	476.1
VIII	13.52	38.5
IX	13.28	36.1
X	13.39	37.2
$K_3Fe(CH)_6$	38.35	286.8
IX + $K_3Fe(CN)_6$	13.23	35.6
V + $K_3Fe(CN)_6$	43.17	335.0

Figure 2. Rates of oxygen evolution measured on aliquots of chloroplast suspensions pretreated with different nitroxide radicals. The measurements were carried out on chloroplast samples containing 0.2 mg of chlorophyll/ml. These were suspended in Soln. C which was also used as the circulation medium. Radicals were premixed with the chloroplasts in the dark and were then injected into the sample compartment of the O_2 detection assembly and were allowed to stand in the sample chamber in the dark for 15 min until a stable base line was obtained. The light was turned on for 30 sec and the rates were followed by recording with a strip chart recorder.

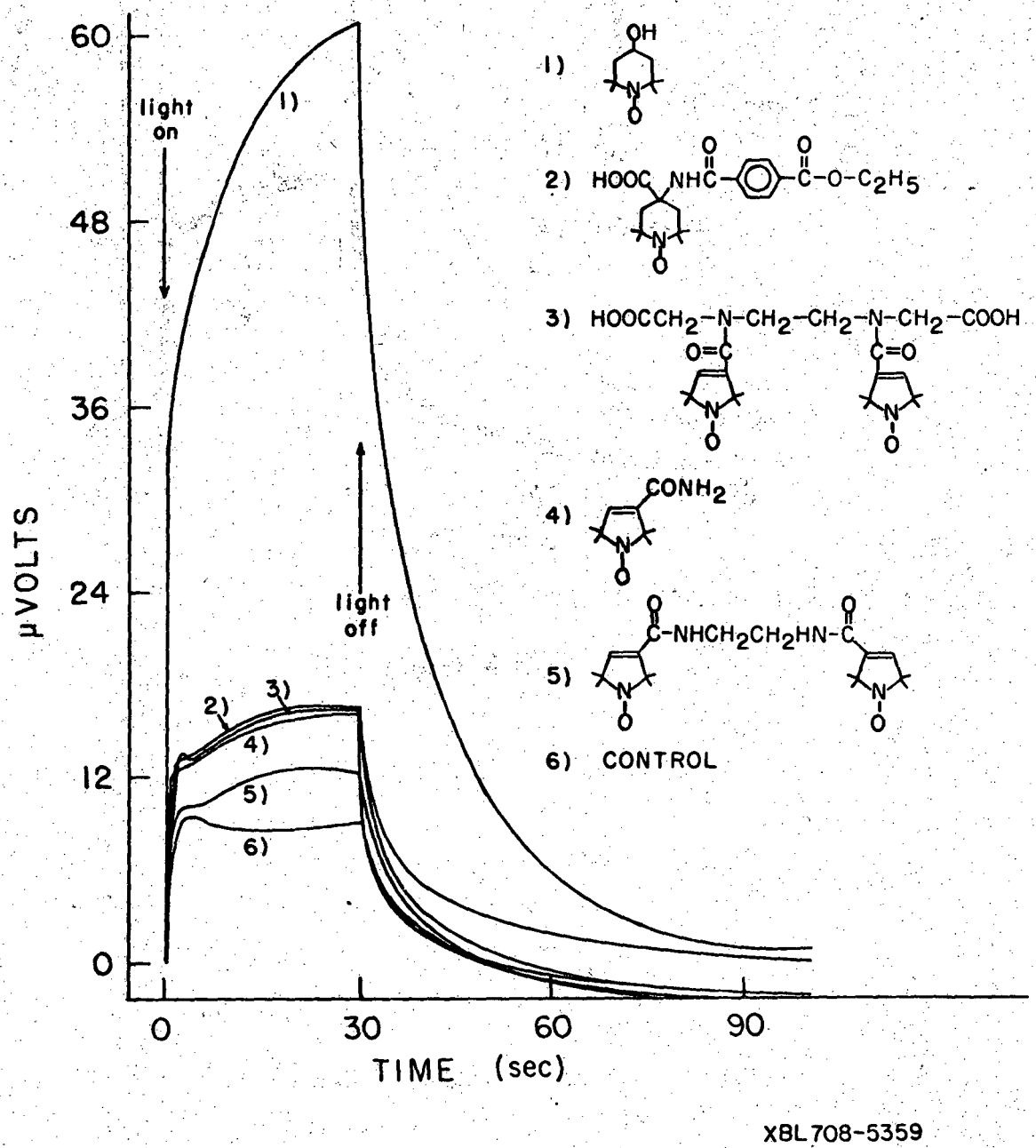

60
48
36
24
12
0
μVOLTS
light on
light off
1)
2)
3)
4)
5)
6)
0
30
60
90
TIME (sec)
1)
2) HOOC NHC C-O-C2H5
3) HOOCCH2-N-CH2-CH2-N-CH2-COOH
O=C C=O
4) CONH2
5) C-NHCH2CH2HN-C
6) CONTROL
XBL708-5359

Figure 6-2

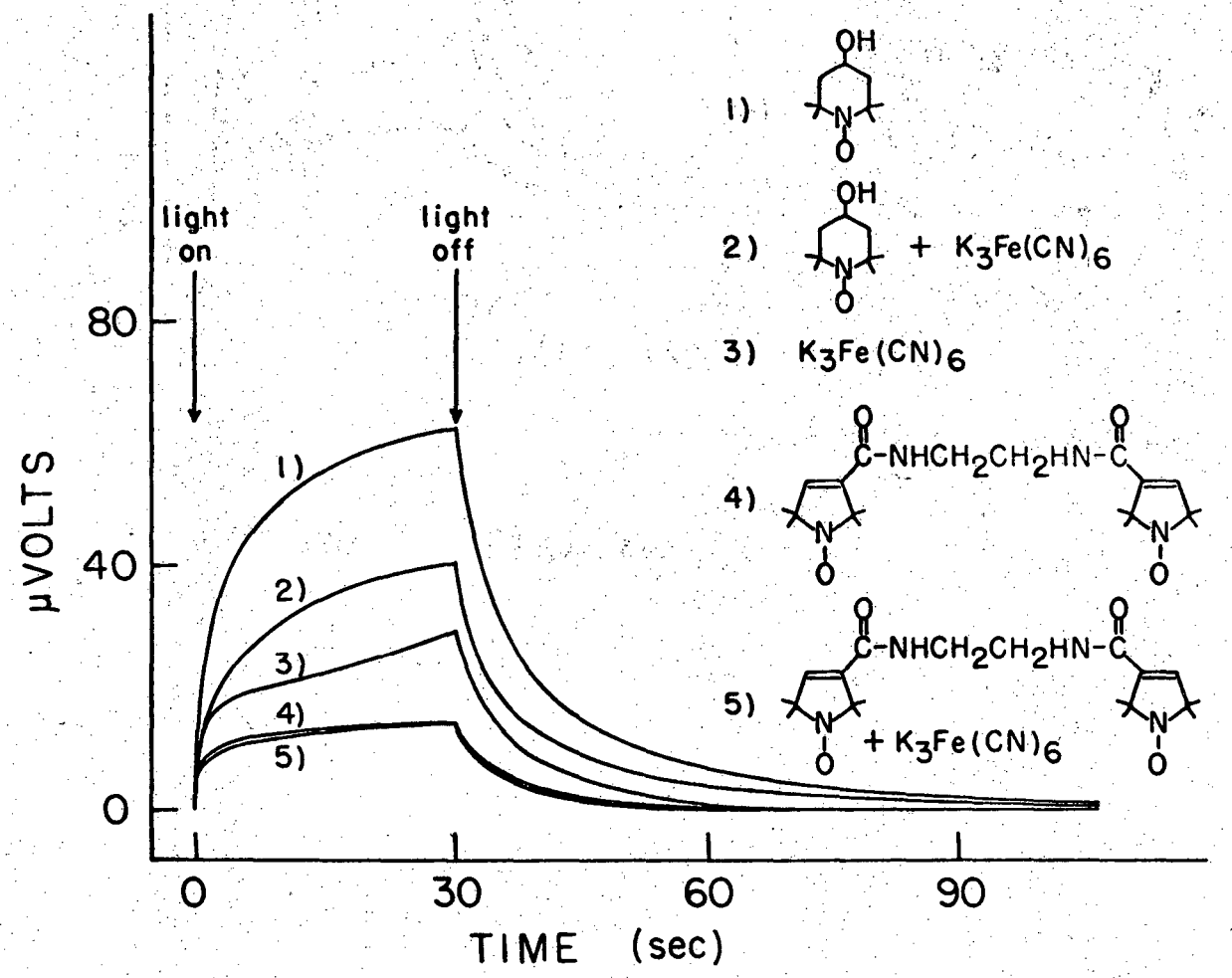
radicals were premixed with the chloroplasts in the dark and were then injected into the sample compartment of the O_2 detection assembly and were allowed to stand in the sample chamber in the dark for 15 min until a stable base line was obtained. Our data for oxygen evolution suggest that the structural requirements for a successful Hill oxidant are similar to those required in a potent inhibitor of CO_2 fixation. Permeability of the chloroplast membrane to the radical is the most important factor. This can be seen if we compare radical V which is better able to penetrate the membrane than radicals containing free carboxyl groups. The biradical X and the non-radical XII trigger much less oxygen evolution than does the monoradical V. It also appears that the existence of an unsaturation in the radical ring renders the radical less active as a Hill oxidant. The radical I, which contains no free carboxylic group, has as low a potency for Hill oxidation as the monoradical VIII which does possess a free carboxylic group. Both biradicals tested for oxygen evolution (see Table IV) were poor Hill oxidants, and they both contain unsaturation in the ring containing the radical. Our data also indicate that the amount of oxygen evolution triggered by the nitroxide radicals probably does not depend on the size of the ring.

4. Effect of ferricyanide on oxygen evolution triggered by the radical. Ferricyanide in quantities equimolar to those of the radicals tested was used as a control in oxygen evolution experiments, since it is one of the best Hill oxidants known. Ferricyanide proved to be more potent

as a Hill oxidant than were most of the radicals tested in these experiments (Fig. 3 and Table IV). However, the radical V was almost twice as effective in Hill oxidation as was $K_3Fe(CN)_6$. This again may indicate the importance of penetration into the chloroplast membrane. Ferricyanide is known to be a very good Hill oxidant[22], yet it triggers less oxygen evolution than does the radical V, presumably because the chloroplast membrane presents a permeability barrier to ferricyanide as contrasted to the facility of penetration of the membrane by the radical V.

When ferricyanide and the radical V are mixed together and tested for their combined effect on oxygen evolution, the level of Hill oxidation observed is greater than that for $K_3Fe(CN)_6$ alone but less than would be expected from the sum of the effects of each compound tested individually. However, when ferricyanide is mixed with the biradical IX the interaction is such that observed oxygen evolution is much lower than that seen for either the biradical or ferricyanide alone. These results might suggest that ferricyanide interacts with the radicals to form complexes of very low membrane solubility. The complex containing monoradical and ferricyanide ion may slowly dissociate to its components (thus freeing them to penetrate the membrane) due to depletion of the radical from the medium through uptake by the chloroplasts. The complex between ferricyanide and biradical, on the other hand, may have such a low dissociation constant that, once the complex is formed, virtually no dissociation

Figure 3. Effect of ferricyanide on the rate of oxygen evolution triggered by the radicals, ferricyanide and a mixture of both the radicals and ferricyanide. Conditions are the same as in Figure 2.



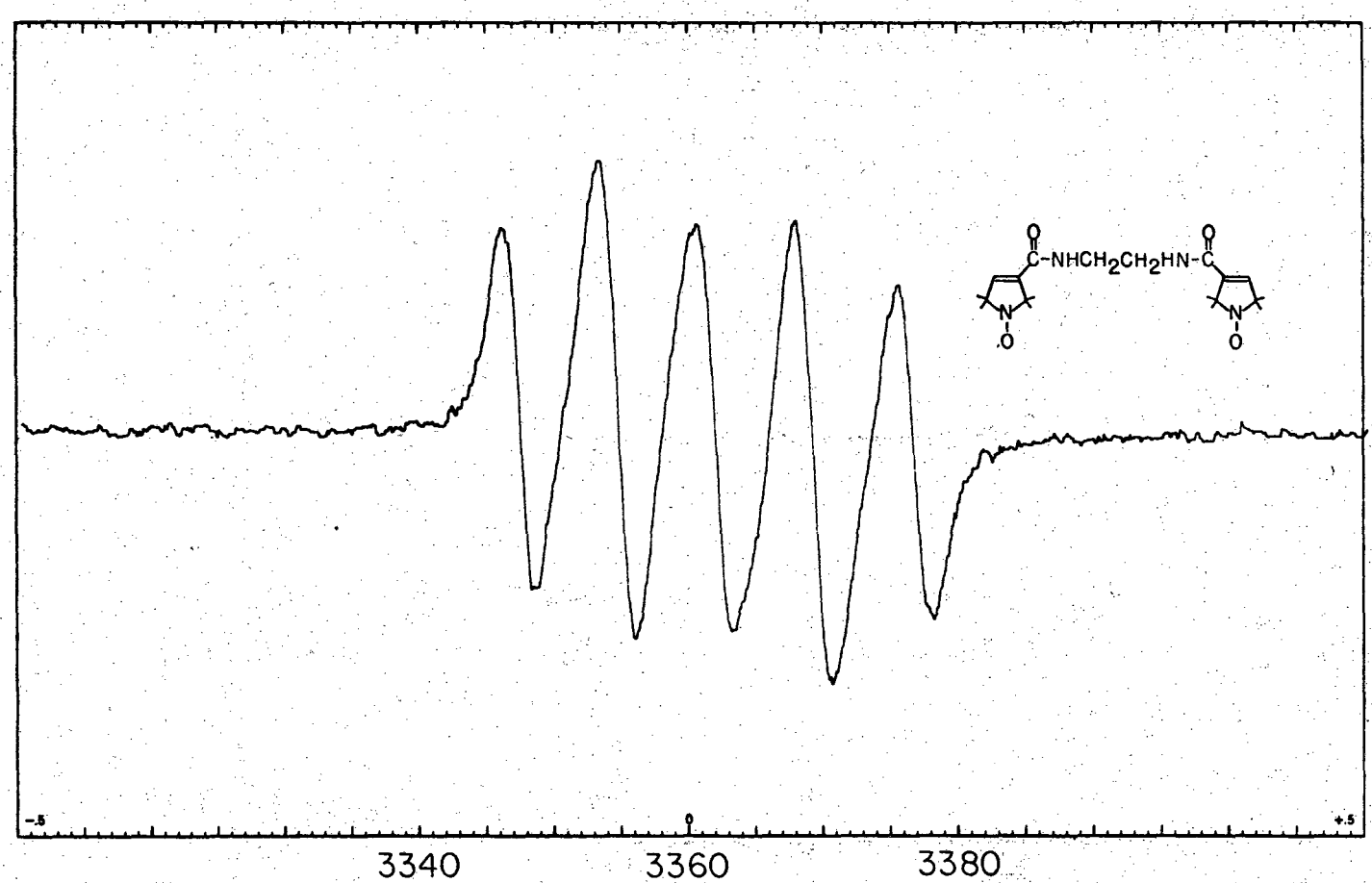
XBL708-5358

Figure 6-3

occurs. EPR study of the interaction between the biradical IX and ferricyanide shows that there is interaction between the biradical and ferricyanide. This interaction results in the change of relative peak height of the five line spectrum as seen in Fig. 4 and 5.

5. Interaction of the nitroxide radicals with chloroplasts. When chloroplasts were pretreated with nitroxide radicals in the dark and then placed in the EPR cavity (also in the dark) there was a very small, insignificant decay of the monoradical signal, and some essentially insignificant changes in the five-line signal of the biradical also occurred. If the chloroplasts were illuminated in the EPR cavity through the grid of the cavity, the decay of the monoradical signal was enhanced and all three peaks continued to decrease with time until they reached a plateau (Fig. 6-8). This plateau in the decay of the radical was achieved at different levels of decay, depending on the type and concentration of the radical, the age of the chloroplasts and on the suspending medium, as we will discuss below. Upon illuminating chloroplasts pretreated with biradical IX the odd-numbered peaks (1,3 and 5) increased in height; however, a decrease in the intensity of the even-numbered peaks (2 and 4) was observed (Fig. 10-12) implicating the reduction of one half of the biradical or binding of the radical to a membrane or a macromolecule resulting in slower exchange between the two halves of the biradical. The presence of ferricyanide did not seem to alter the mode of decay of the monoradical V (Fig. 9). However, the

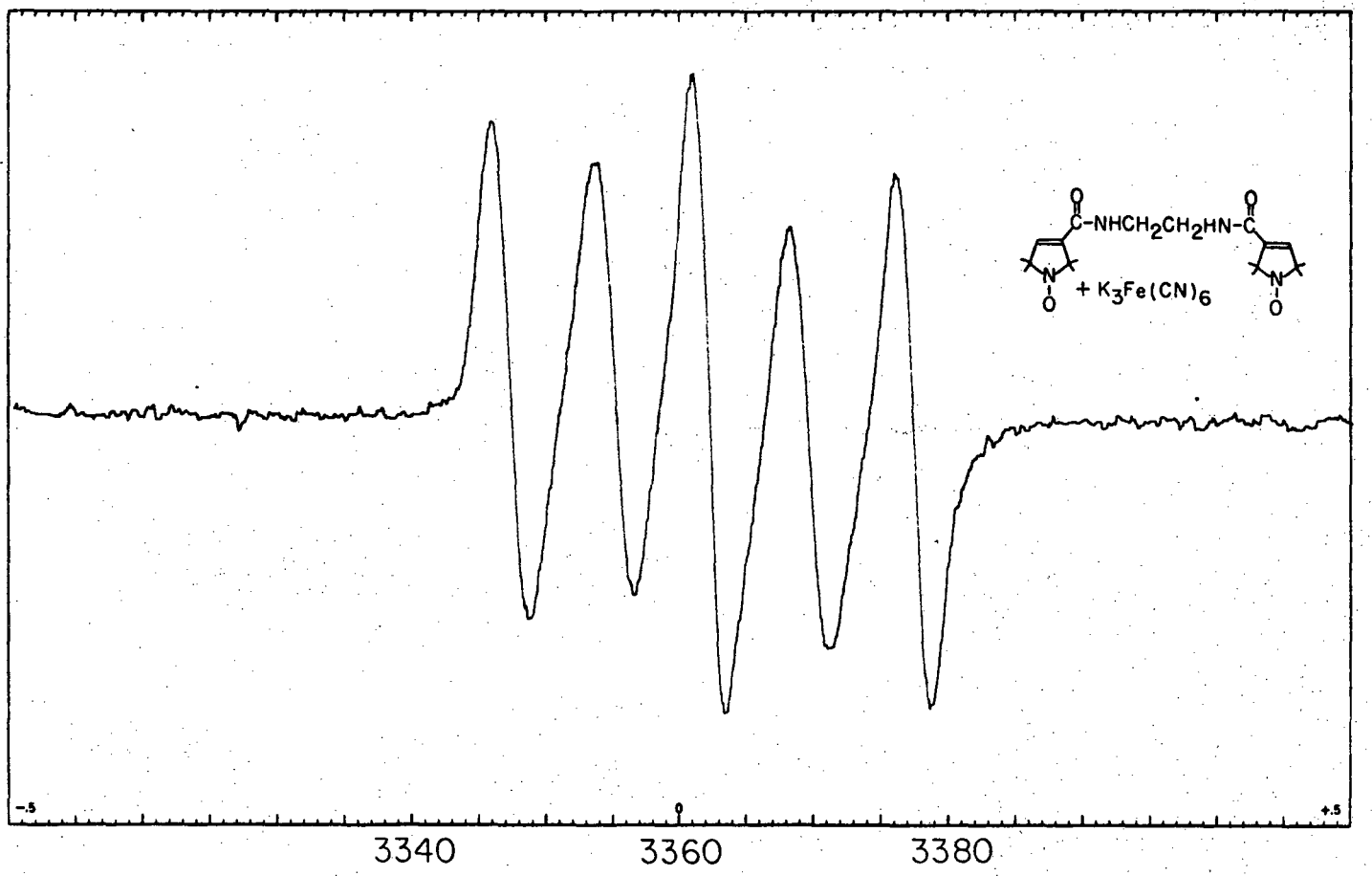
Figure 4. EPR spectrum of the biradical IX in solution C.



XBL 7012-4937

Figure 6-4

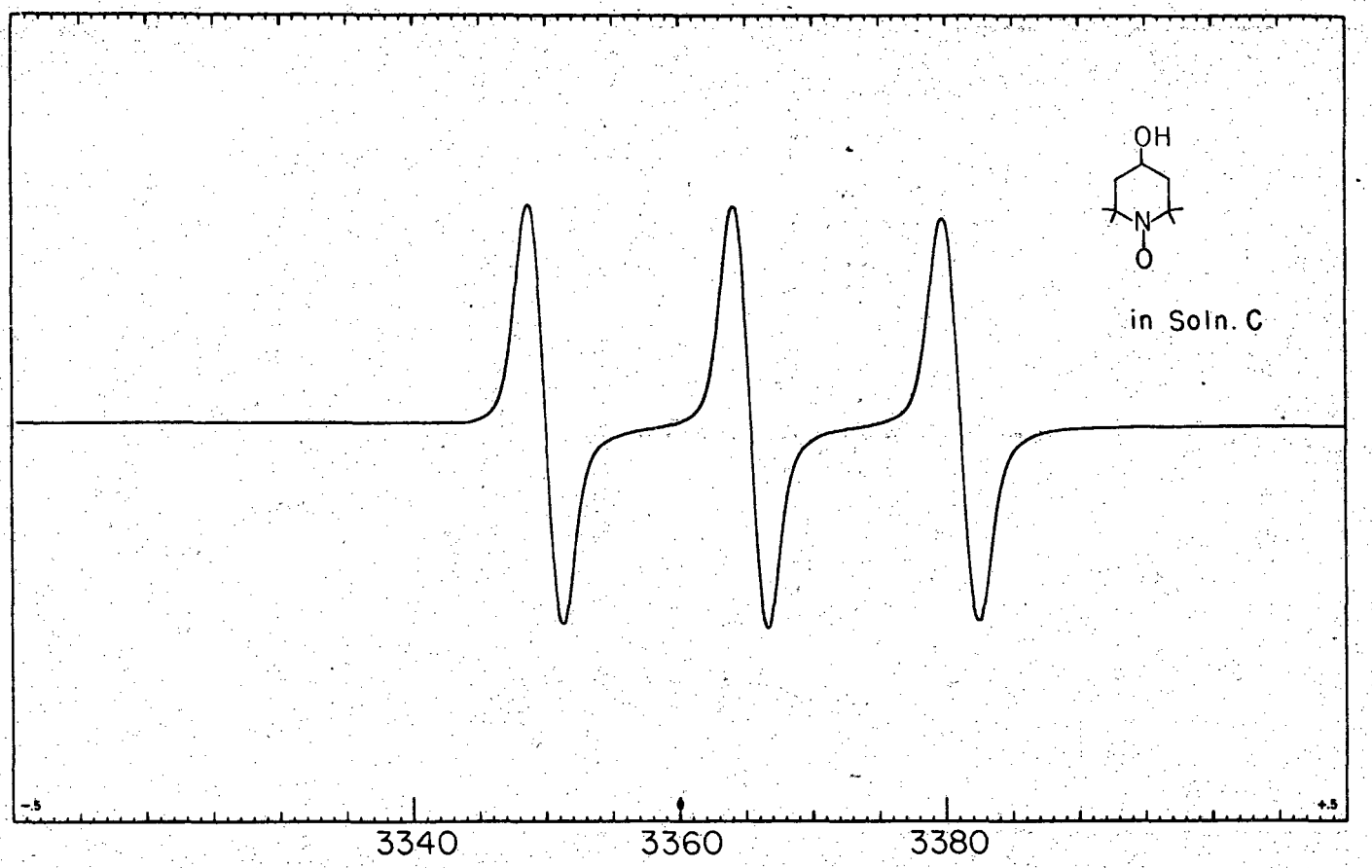
Figure 5. EPR spectrum of biradical IX in the presence of an equimolar quantity of potassium ferricyanide.



XBL 7012-4940

Figure 6-5

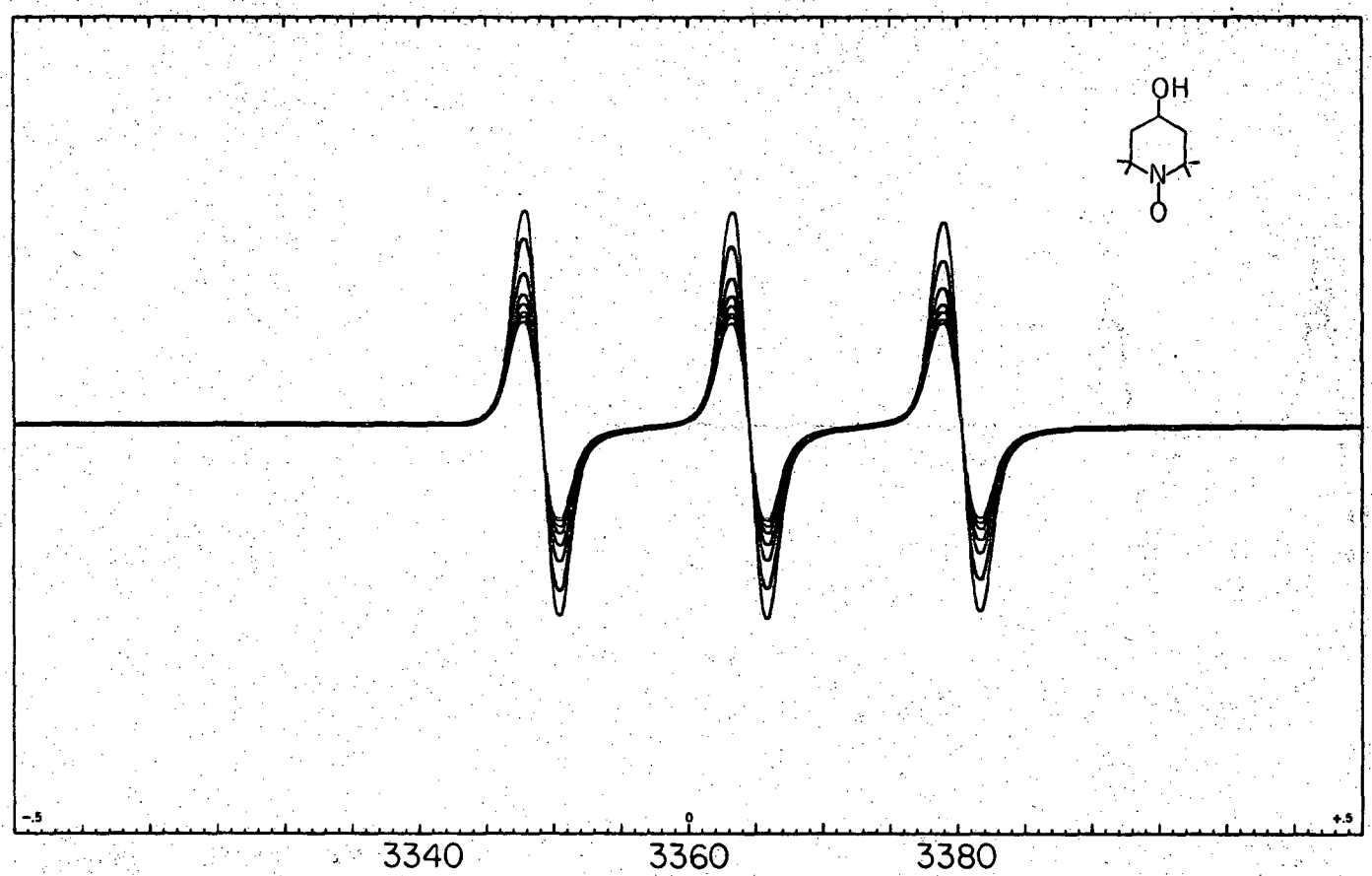
Figure 6. EPR spectrum of radical V in solution C.



XBL 708-5363

Figure 6-6

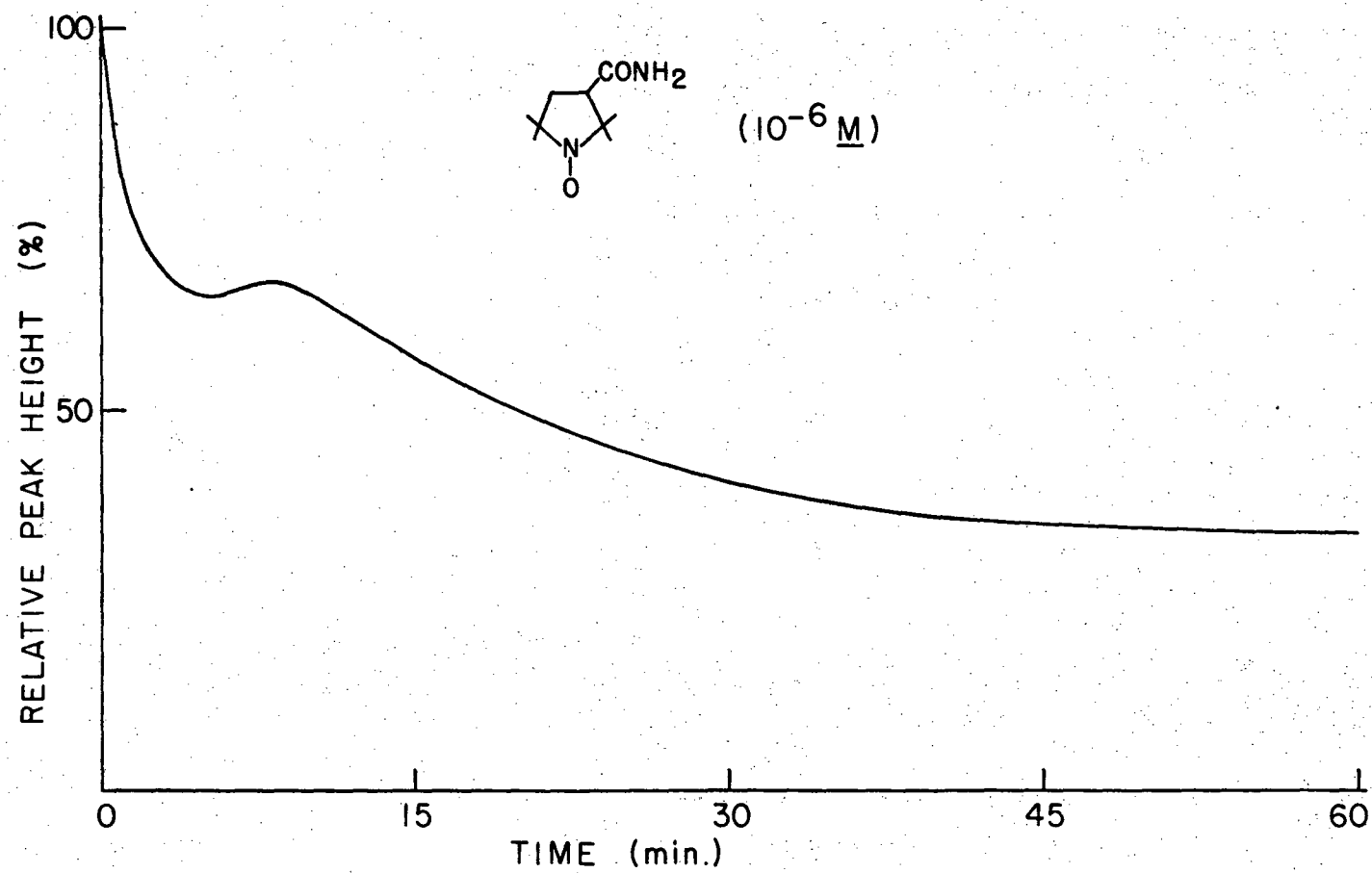
Figure 7. Decay of the EPR signal of radical V in the light in the presence of chloroplasts containing 0.2 mg chlorophyll/ml. Spectrum was taken every 2 minutes.



XBL 708-5364

Figure 6-7

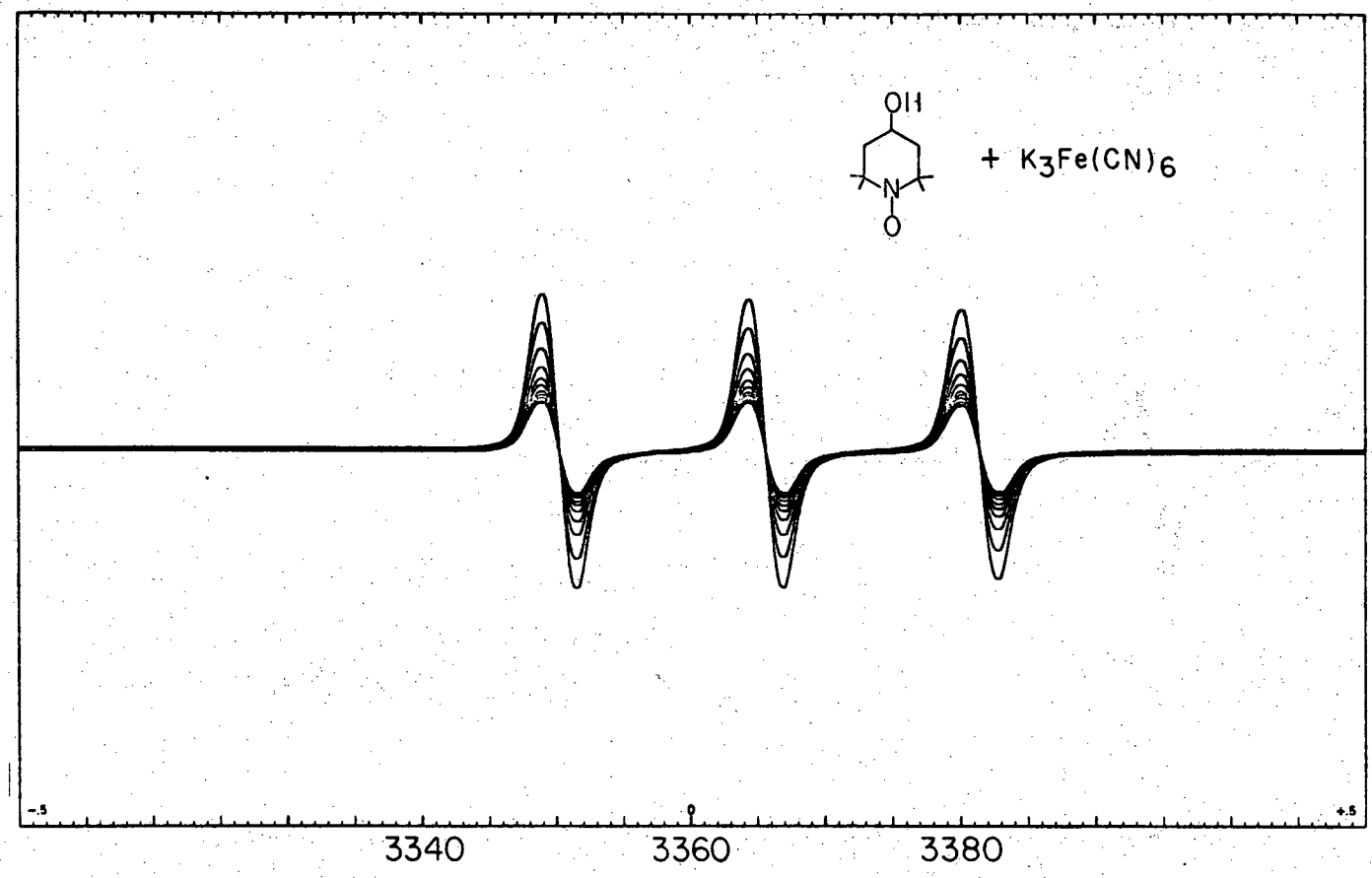
Figure 8. Decay of the EPR signal of radical II. The magnetic field of the EPR spectrometer was fixed on the first peak and the decay of the signal upon illumination in the presence of chloroplasts was followed as a function of time.



XBL 708-5343

Figure 6-8

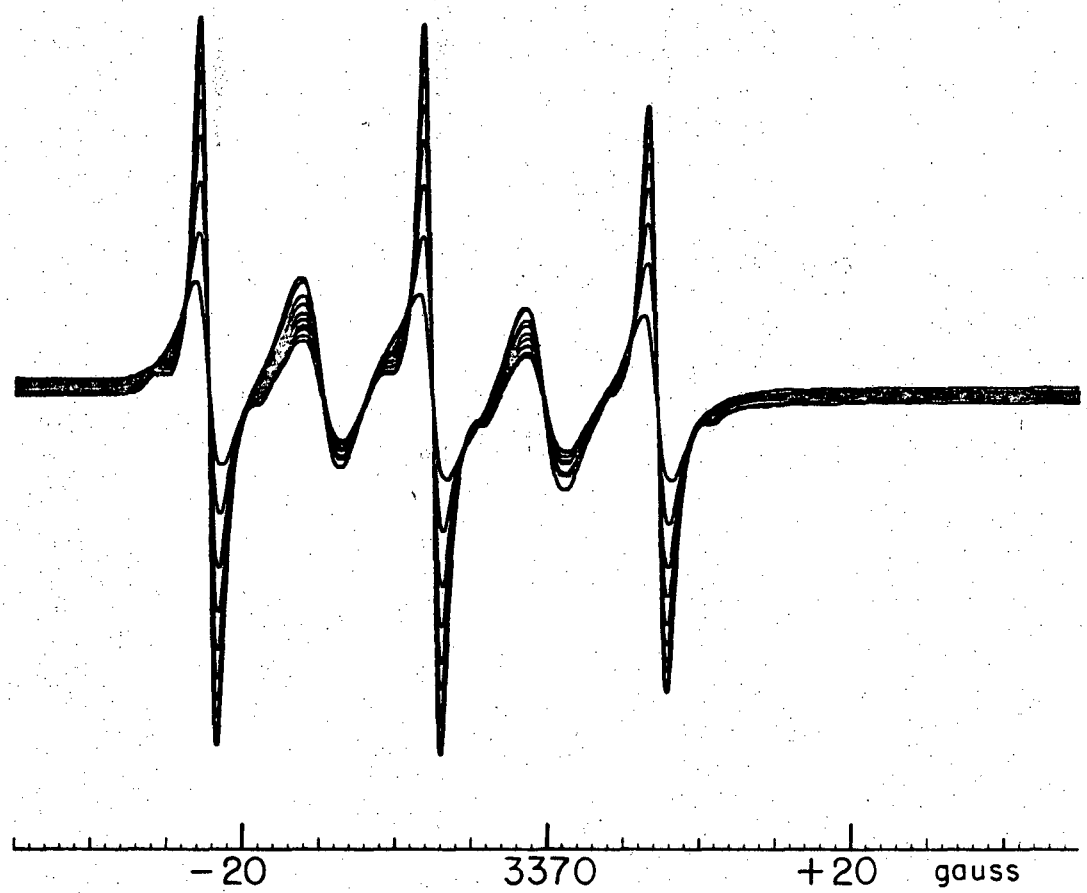
Figure 9. Decay of EPR signal of radical V in the presence of equimolar quantity of ferricyanide in the presence of chloroplasts. Spectrum was taken every 2 minutes.



XBL 708-5365

Figure 6-9

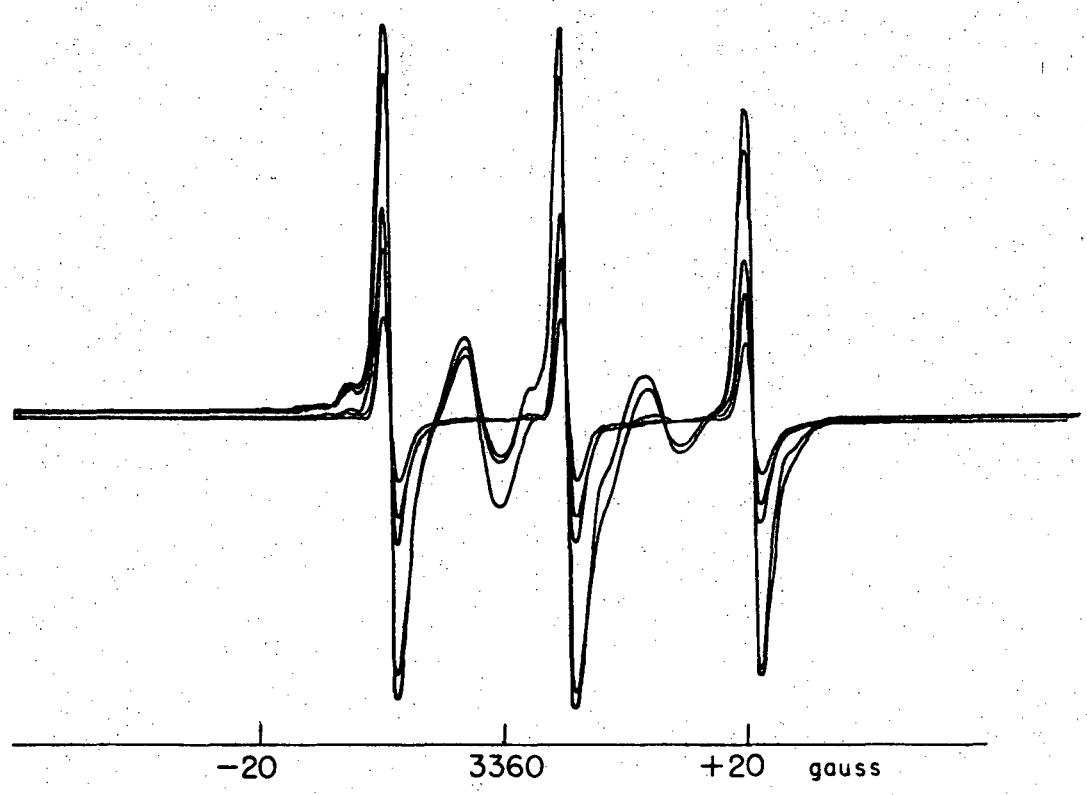
Figure 10. Decay of EPR signal of biradical IX upon illumination in the presence of chloroplasts ? spectrum taken every 2 minutes. The odd numbered peaks increase in height while the even numbered ones decrease.



XBL 6911-5358

Figure 6-10

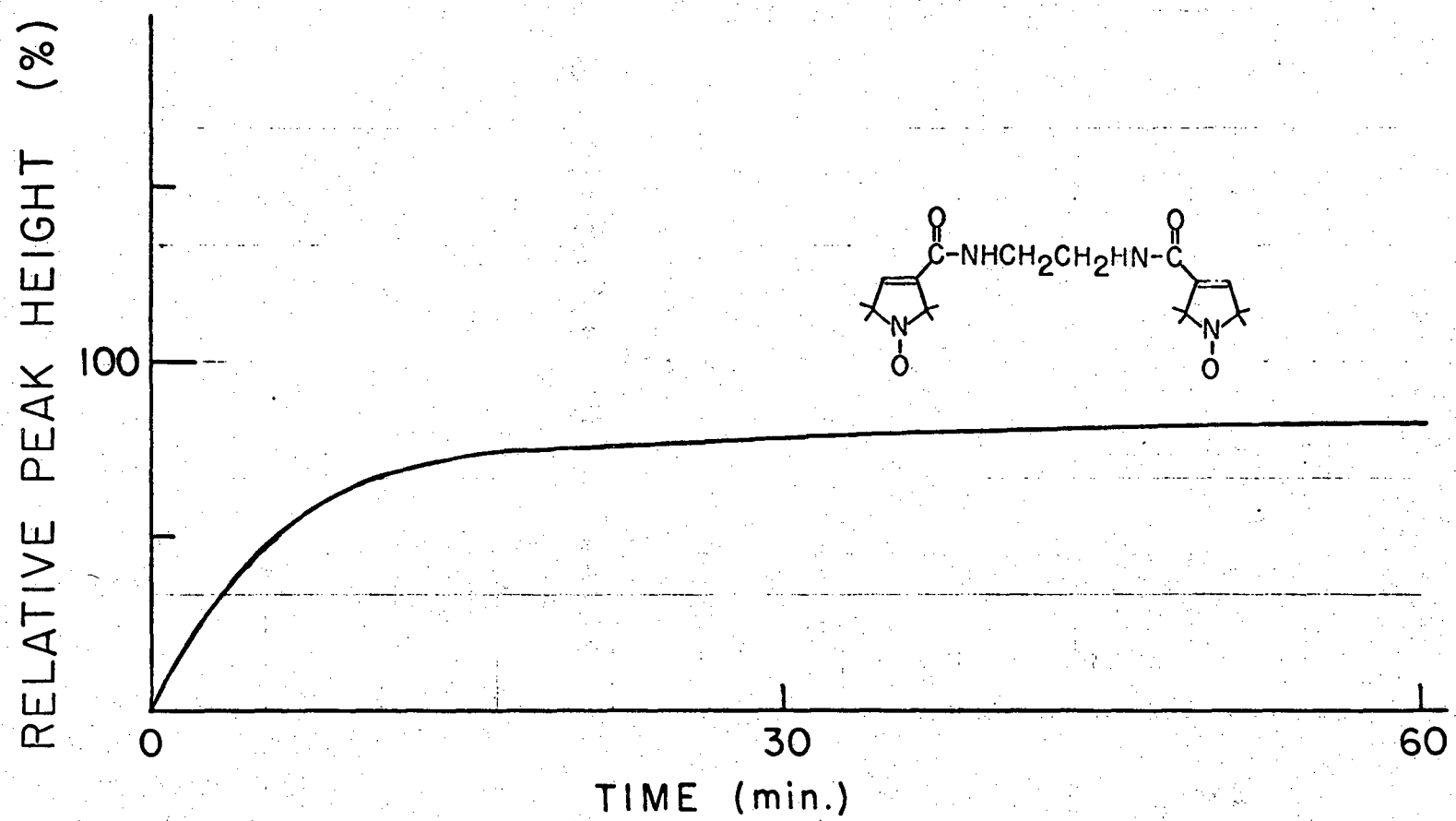
Figure 11. Change of the five line signal of biradical IX into a 3 line signal upon continued illumination in the presence of chloroplasts. All peaks start to decrease until a 3 line spectrum is obtained.



XBL6911-5352

Figure 6-11

Figure 12. Increase of intensity of the first peak of the five line spectrum of biradical IX upon illumination in the presence of chloroplast. The field was fixed on the first peak and the change with time was recorded.



XBL 7012-4942

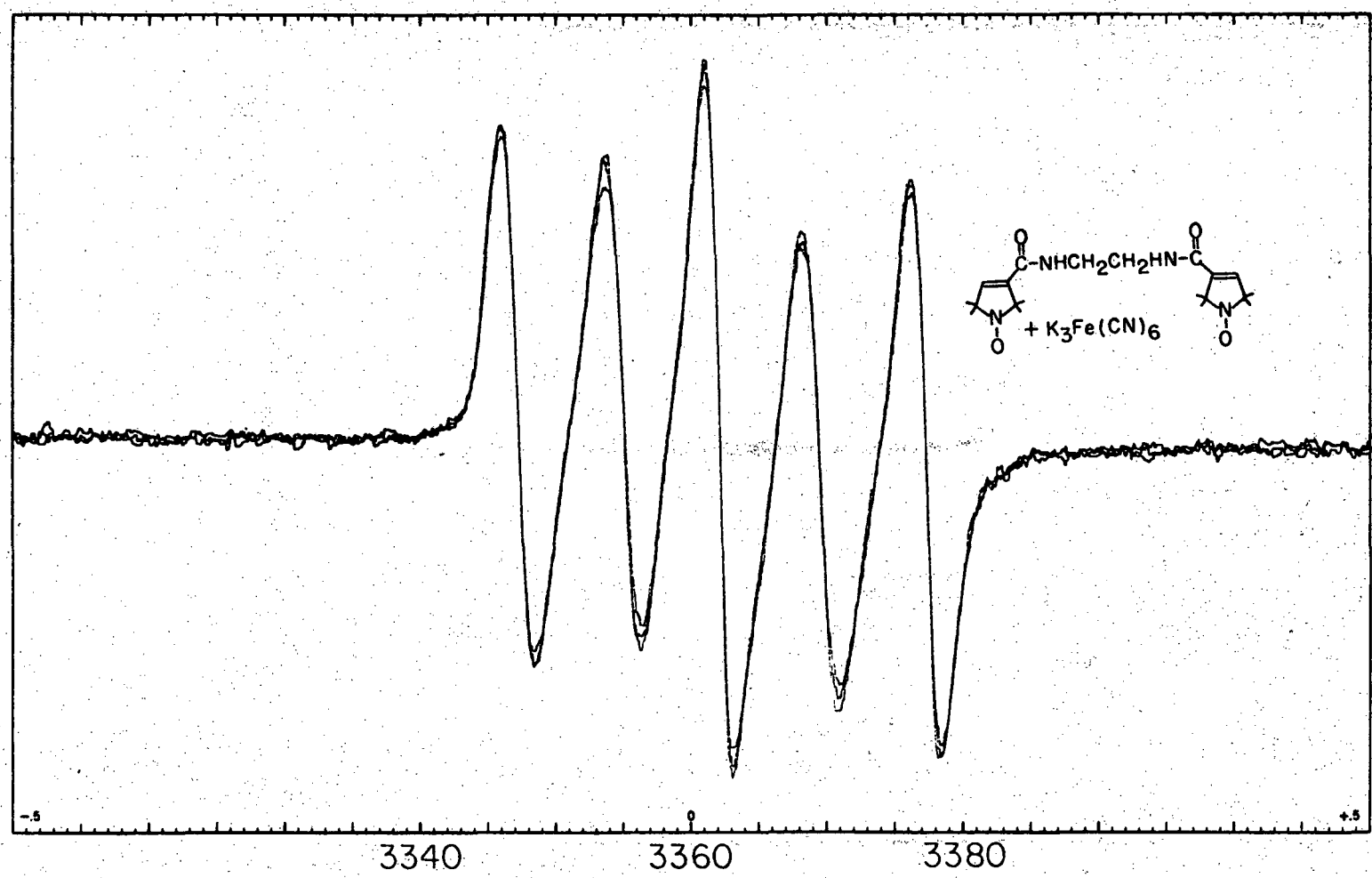
Figure 6-12

biradical IX changed its mode of decay slightly as seen in Fig. 13 and 14.

6. Effect of the integrity of the chloroplasts on decay of the radical spin signal. The first question to come to mind was whether the decay of the radical spin signal was associated with the intricate organization of the chloroplasts. To answer this question, an experiment was designed in which intact chloroplasts pretreated with the monoradical I or the biradical IX were compared with a similar preparation of chloroplasts which had been prediluted fivefold with water in order to disrupt the chloroplasts by osmotic shock. The results showed that the decay of the radical does not depend on the integrity of the chloroplast membrane.

7. Determination of the site of decay of the radical. In order to establish the site of reduction of the nitroxide radical and whether the radical is associated with the chloroplasts or dissolved in the suspending medium, pretreated chloroplast suspensions each containing one of the radicals were allowed to shake in round-bottom flasks for 10 min under conditions of intense illumination in a water bath at 20°C. The chloroplasts were then centrifuged to separate them from the supernatant and the EPR signal was taken for both the supernatant and pellet (after washing and resuspending the pellet in Soln. C). It was clear from this experiment that the bulk of the radical was in the suspending medium; however, weaker intensities of EPR signals were detected in the

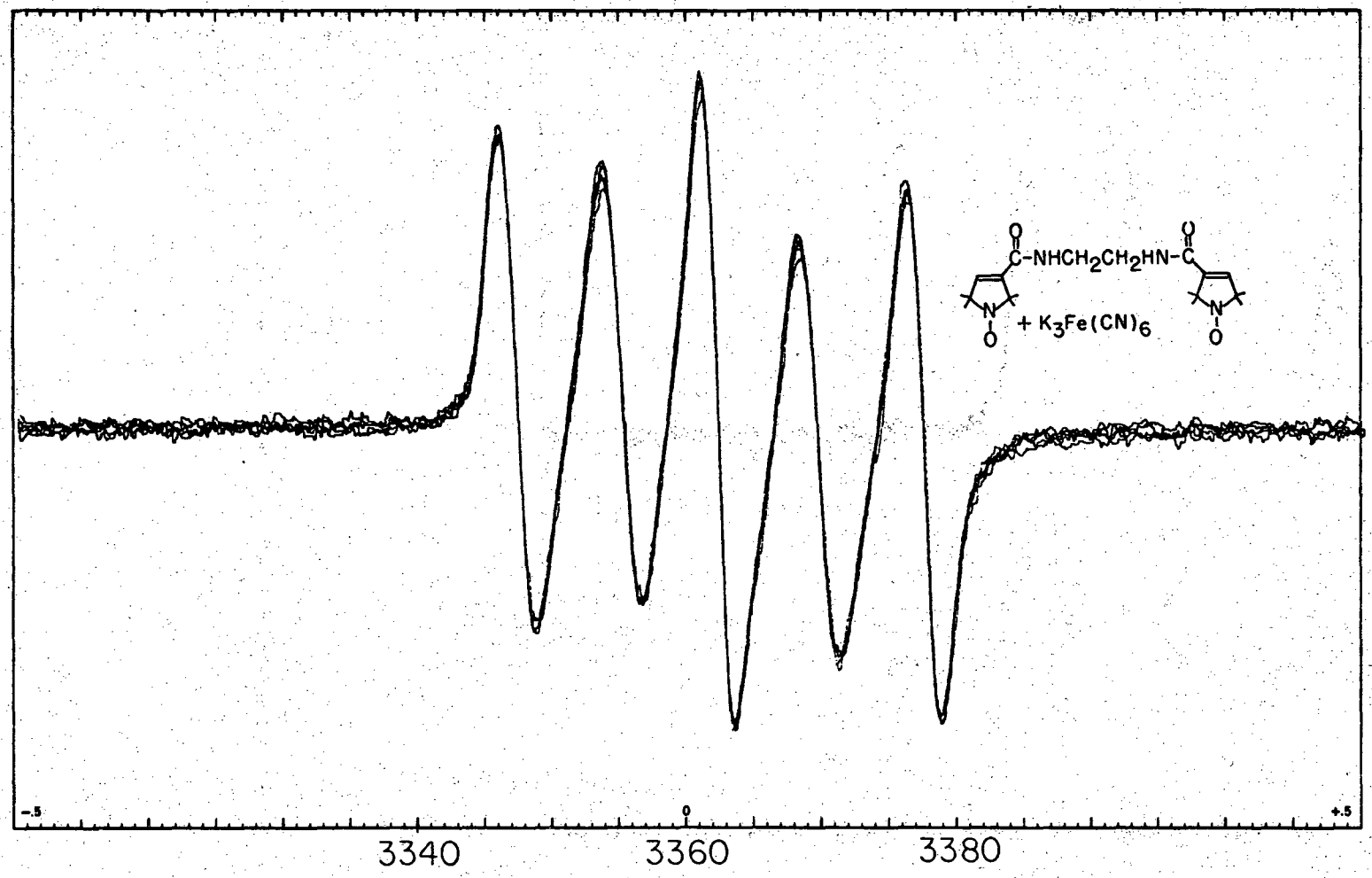
Figure 13. Change in signal of biradical IX in the presence of ferricyanide and chloroplasts. The first changes are increase of intensity of all peaks.



XBL 7012-4938

Figure 6-13

Figure 14. Change in signal of biradical IX in the presence of ferricyanide. Continued illumination of the radical under the same conditions as in Figure 13 results in the decay of signal similar to that of the biradical in the absence of ferricyanide.



XBL 7012-4939

Figure 6-14

chloroplasts. The weak signals associated with the chloroplasts were observed with radical II, IV and V and to a much lesser degree with all others. This lack of signal could be due to the decay of the small amounts of radical associated with the chloroplasts or due to the failure of significant quantities of these radicals to enter the chloroplasts because of unfavorable partitioning. One must also note that the volume of chloroplasts is about 1/50 of that of the suspending medium.

8. Effect of the age of chloroplasts on the decay of the radical signal. Chloroplast age was found to affect the rate of decay of the EPR signal of the radical. Freshly prepared chloroplasts were much more efficient in reducing the radical. The rate of reducing the radical, Fig. 15, shows that the rate of reducing the radical by freshly prepared chloroplasts is much faster than the rate of reduction caused by 12-hours old chloroplasts. The percentage of the signal reduced at the plateau was also higher in the case of the freshly prepared chloroplasts than in the case of the 12-hours old chloroplasts.

9. Effect of the chloroplast suspension medium on the decay of the radical signal. Three radicals were chosen for this study; these were radical I, V and VIII. Chloroplasts prepared by the Jensen and Bassham method, in sucrose tricine buffer, were treated with each of these radicals separately. The treated suspension was placed in the aqueous solution cell which was inserted into the EPR

Figure 15. Effect of chloroplast age on the decay of the signal of radical II, _____ freshly prepared chloroplasts, ___.___ 12 hrs old chloroplasts.

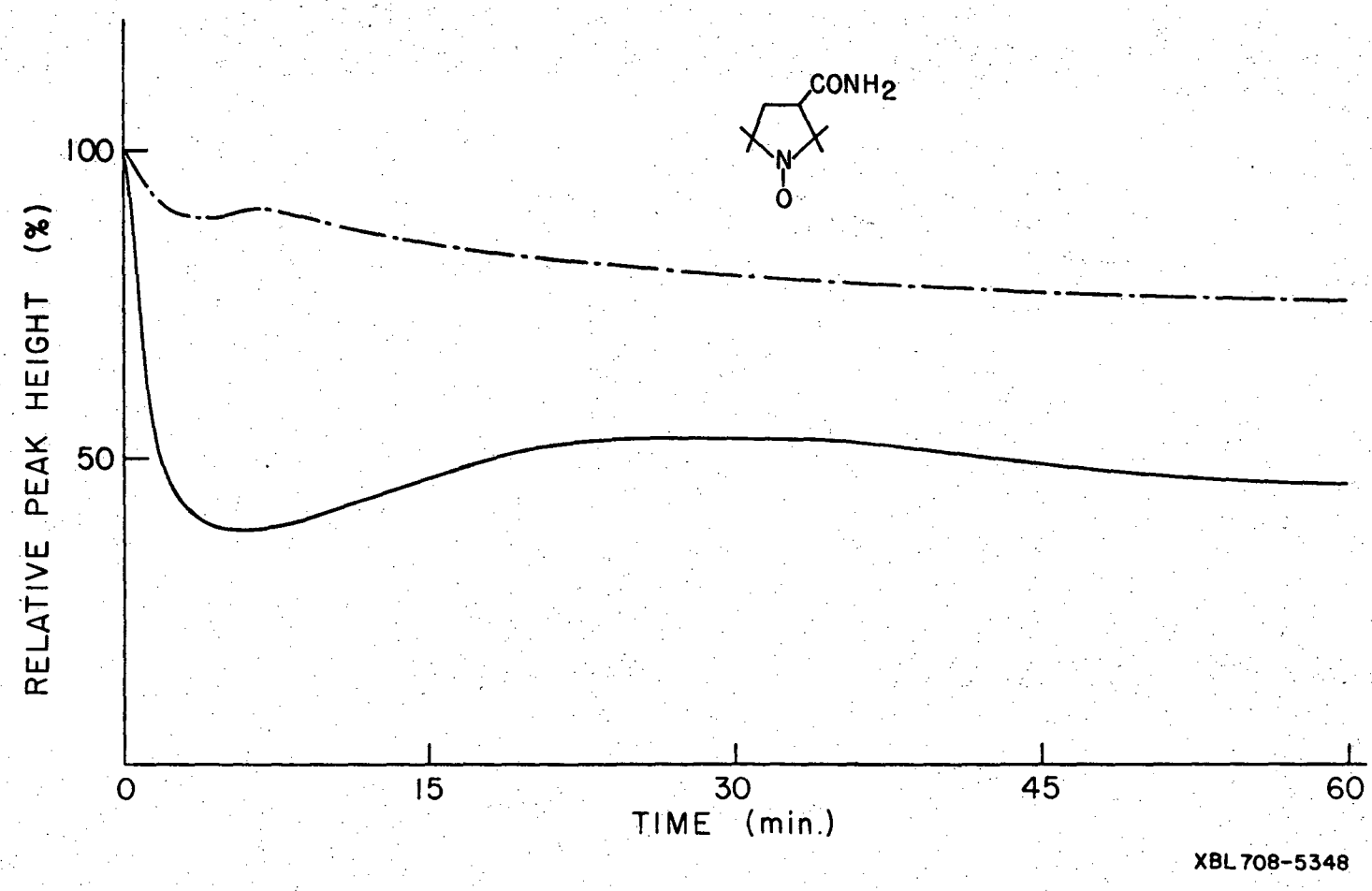

CONH2
N
O
100
50
RELATIVE PEAK HEIGHT (%)
0
15
30
45
60
TIME (min.)
XBL 708-5348

Figure 6-15

cavity in the dark. The magnetic field of the EPR spectrometer was fixed on the first peak and the decay of each radical on illumination of the cavity was observed. As we see in Figures 16-18, the suspending medium is an important factor in studying the interaction of the nitroxide radicals with chloroplasts. It seems as though the sucrose-tricine suspending medium slows down the decay of the radical, perhaps due to limitation of the rate of diffusion of a reducing intermediate which leaks out of the chloroplasts in the light to interact with the radicals chemically, physically, or both, rendering the spin signal less intense. This can be documented by the fact that the kinetics of decay of the monoradical V are relatively insensitive to the suspending medium, but the kinetics of decay of the two other radicals differ significantly depending on the suspending medium.

10. Effect of preillumination of the chloroplasts on the decay of the radical. When a suspension of chloroplasts in Sorbitol buffer (Soln. C) was preilluminated with ten Xenon flashes (6 joules, 2×10^{-6} sec at 2 sec intervals) and then the preilluminated chloroplasts were mixed with the biradical IX in the dark and the EPR signal was measured in the dark, the result was a complete destruction of the five-line signal of the biradical to a very weak three-line signal (Fig. 19). This adds further evidence to support our hypothesis that an intermediate diffuses out of the chloroplasts into the suspending medium in the presence of light to interact with the radical and that independently of the presence or absence of light such

Figure 16. Effect of suspending medium on the decay of radical VII, ——— in solution D, ----- in solution C.

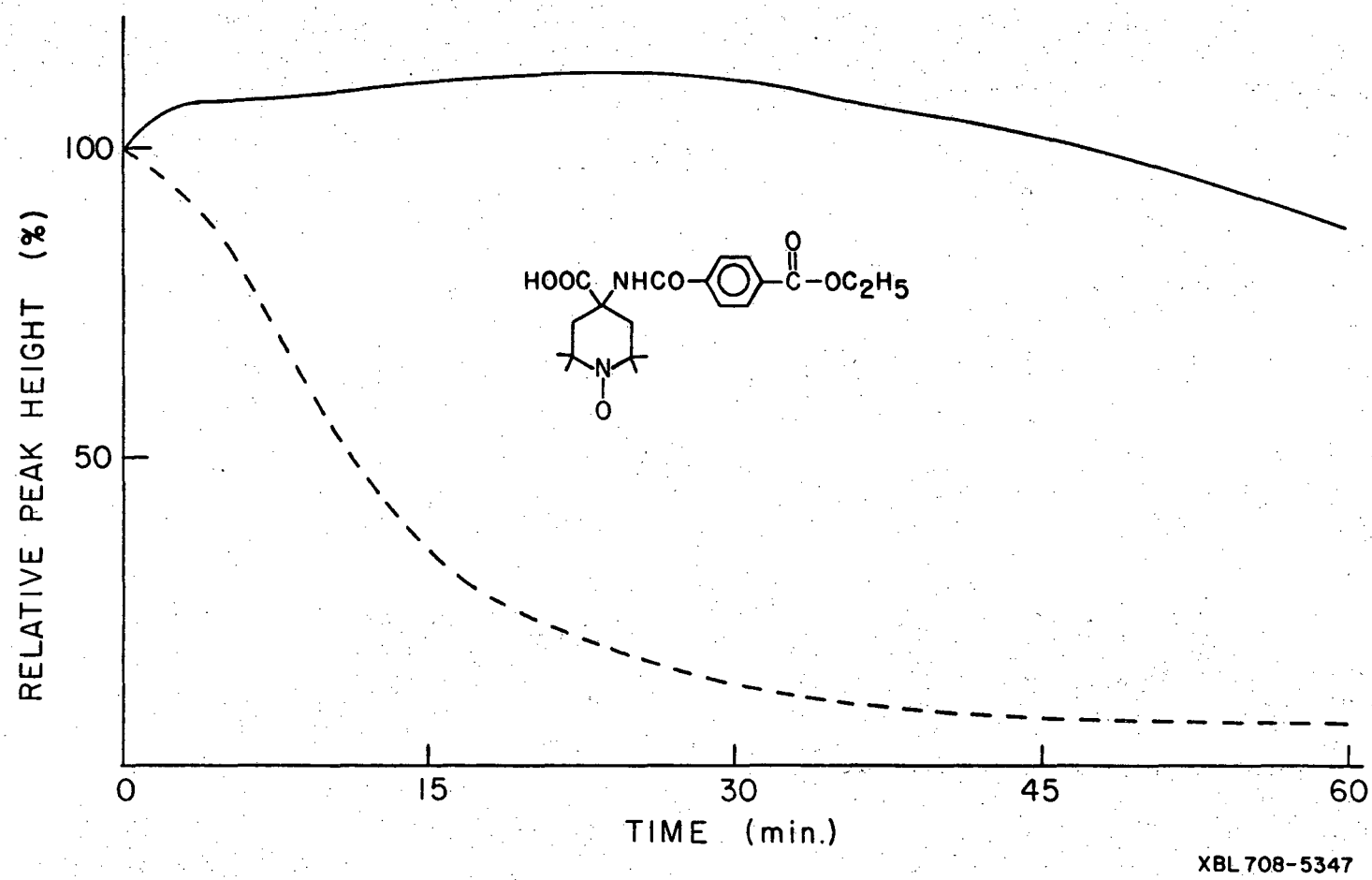

RELATIVE PEAK HEIGHT (%)
100
50
0
15
30
45
60
TIME (min.)
HOOC
NHCO
O
C
OC2H5
N
O
XBL 708-5347

Figure 6-16

Figure 17. Effect of suspending medium on the decay of radical V, ——— in solution D, ----- in solution C.

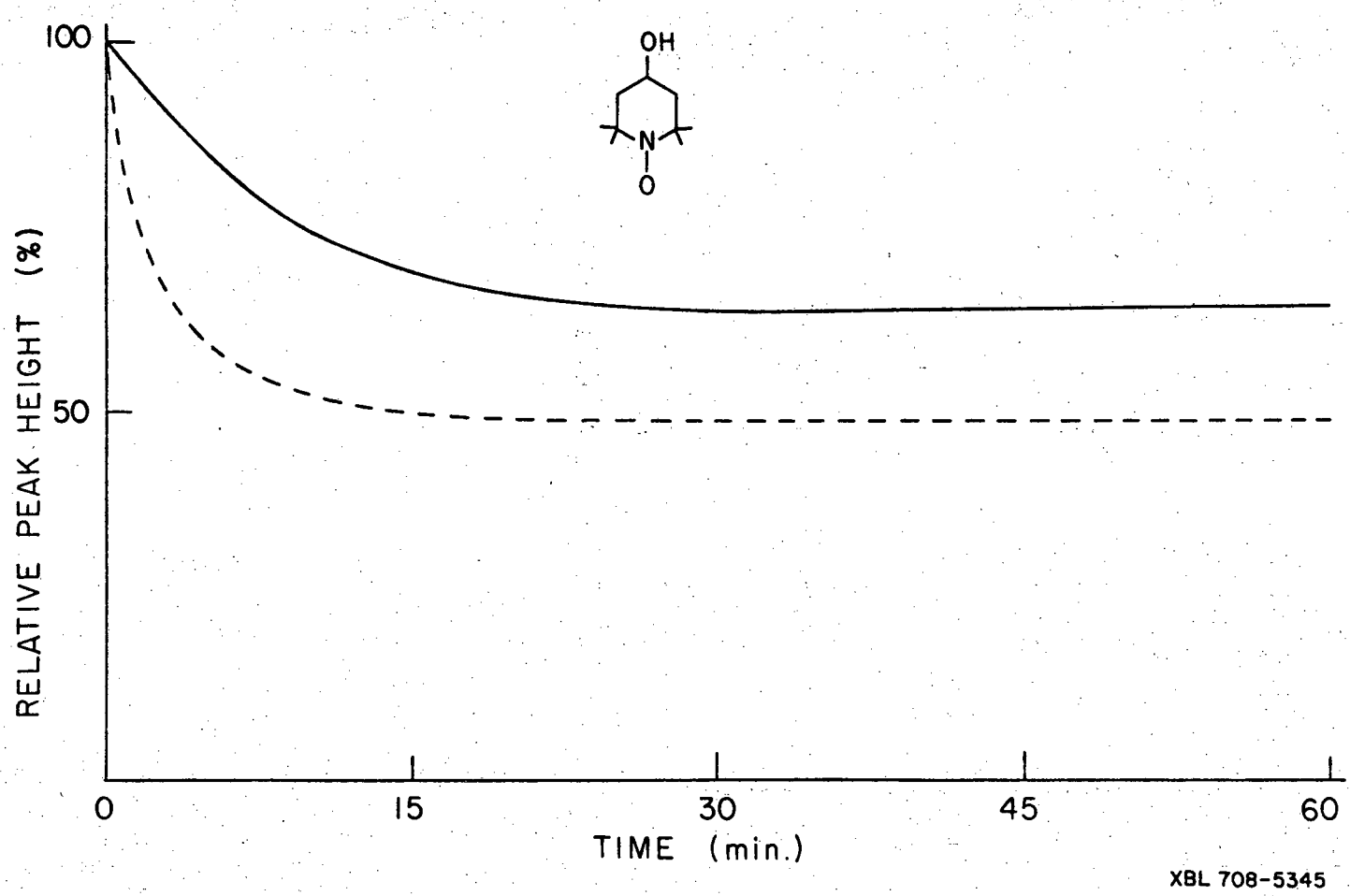

100
50
RELATIVE PEAK HEIGHT (%)
0
15
30
45
60
TIME (min.)
OH
N
O
XBL 708-5345

Figure 6-17

Figure 18. Effect of suspending medium on the decay of radical I, ——— in solution D, ----- in solution C.

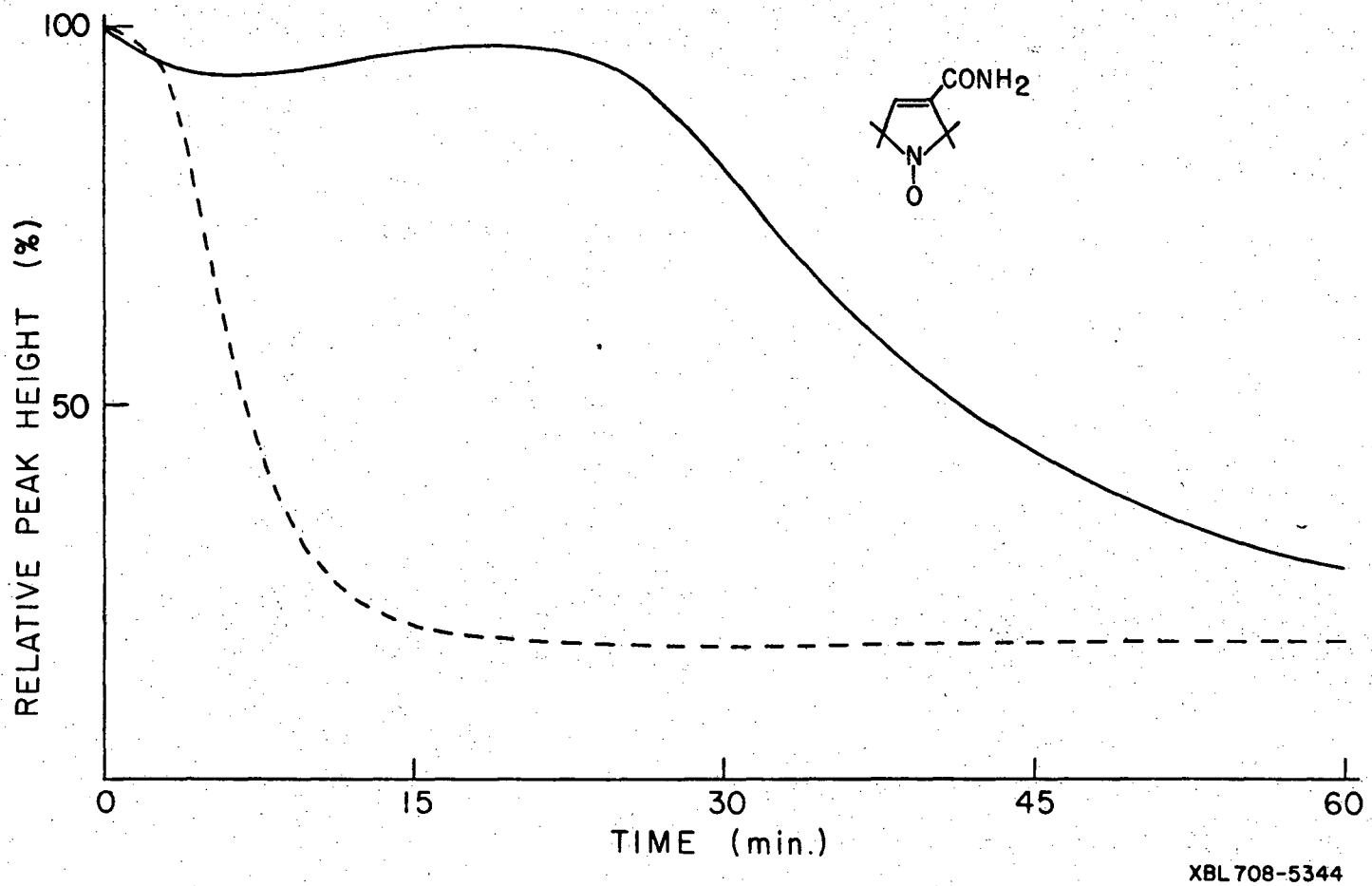

100
50
RELATIVE PEAK HEIGHT (%)
0
15
30
45
60
TIME (min.)
CONH2
N
O
XBL 708-5344

Figure 6-18

Figure 19. Effect of preillumination on the decay of biradical IX. Chloroplasts were preilluminated with ten xenon flashes (6 joules, 2×10^{-6} sec at 2 sec intervals). The biradical was introduced in the dark and the spectrum was taken in the dark.

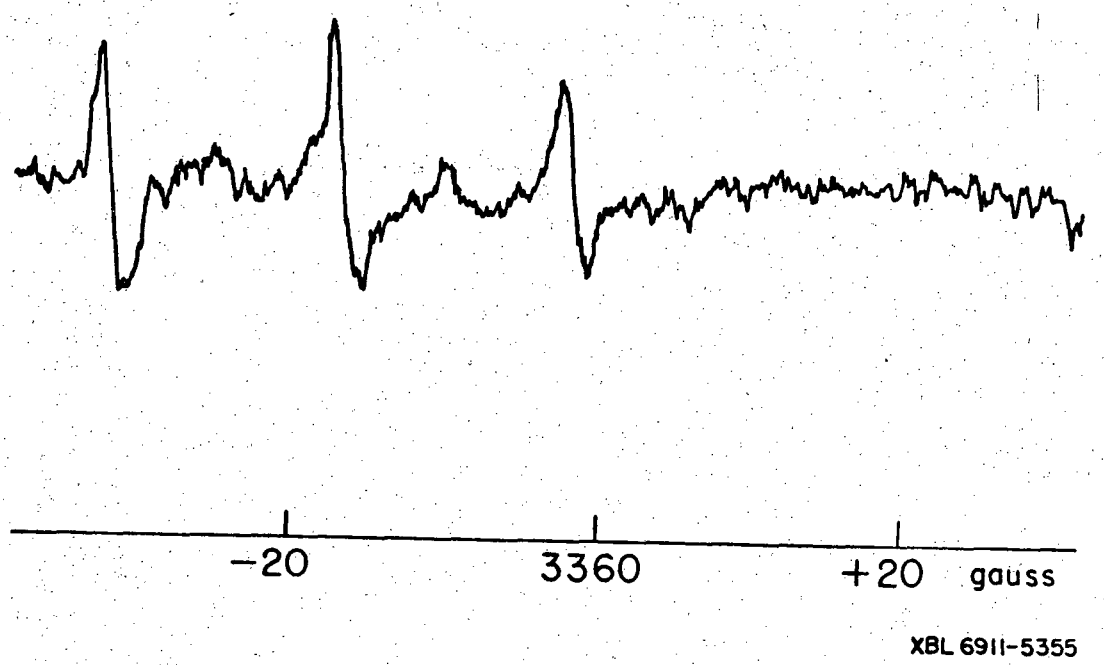


Figure 6-19

interaction results in the destruction of the signal.

11. Effect of the concentration of the radical on the decay of the signal. The radical V was used for these studies. Three concentrations, 1×10^{-5}, 1×10^{-6}, and 5×10^{-7} M, were employed. The radical was mixed with chloroplasts (containing 0.2 mg chlorophyll/ml soln. C) in the dark and then the chloroplast suspension was placed in the EPR cavity and was illuminated for a period of one hour. Figure 20 shows that the rate of decay of the radical decreases as concentration of the radical is increased.

In view of these results we conclude that we must be dealing with two competitive processes, one being the destruction of the radical and the other being the ability of the radical to reach its site of action. The use of lower initial concentrations of the radical results in its essentially complete destruction before any significant amount reaches the site of action. If the initial concentration is increased, a significant amount of the radical can reach the site of action before the radical is destroyed. Apparently the destruction of the radical takes place chiefly outside the chloroplast while the primary site of action (biochemical lesion) of the radical is inside the chloroplast. This idea is substantiated by allowing the signal from chloroplasts pretreated with the radical V (10^{-6} M) to reach a plateau and then adding another aliquot of the same radical or a second radical VIII (at concentration of 1×10^{-6} M). The height of the new spin peak appearing after the addition of the

Figure 20. Effect of the concentration of radical V on its rate of decay upon illumination in the presence of chloroplasts.

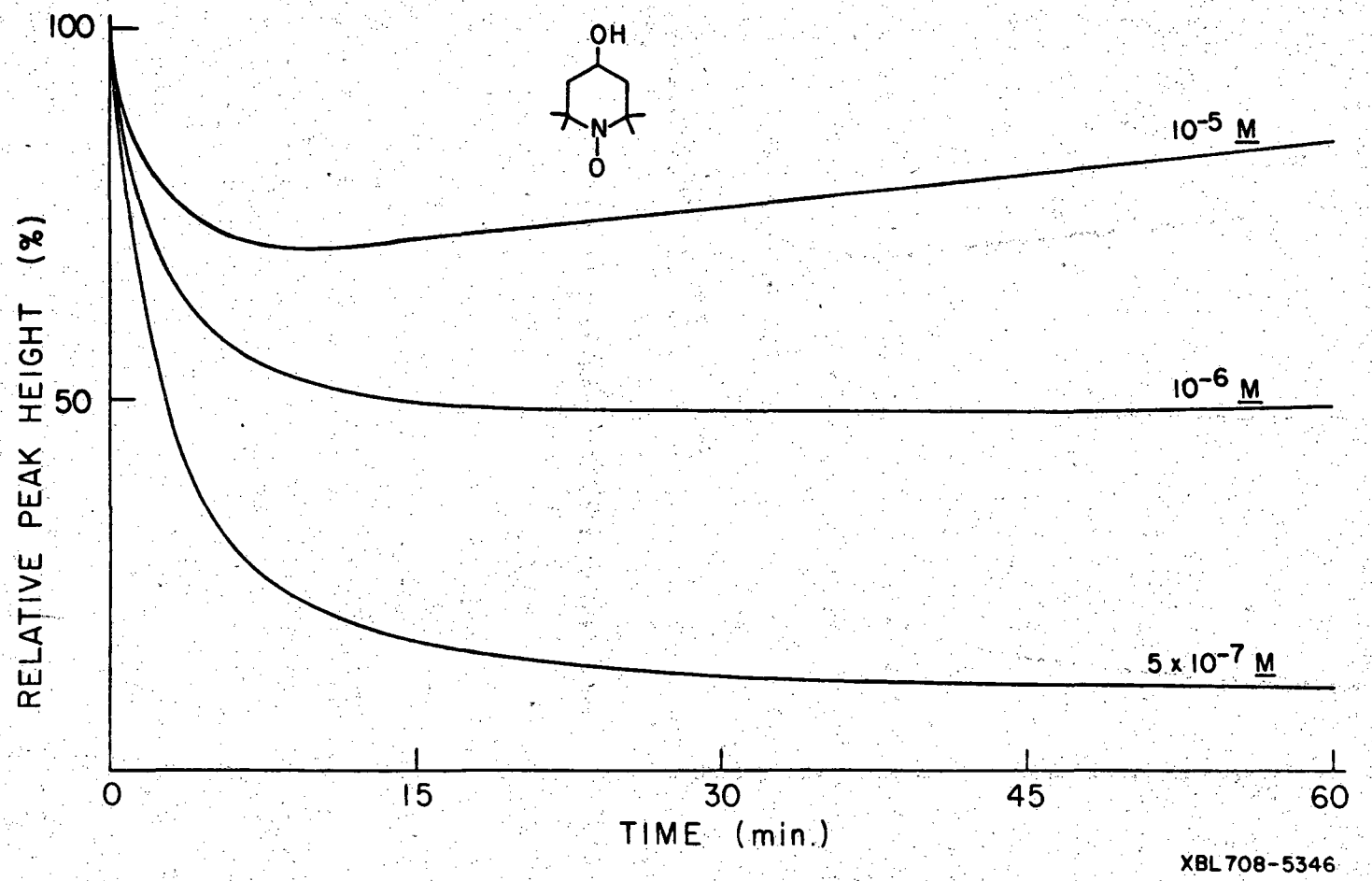
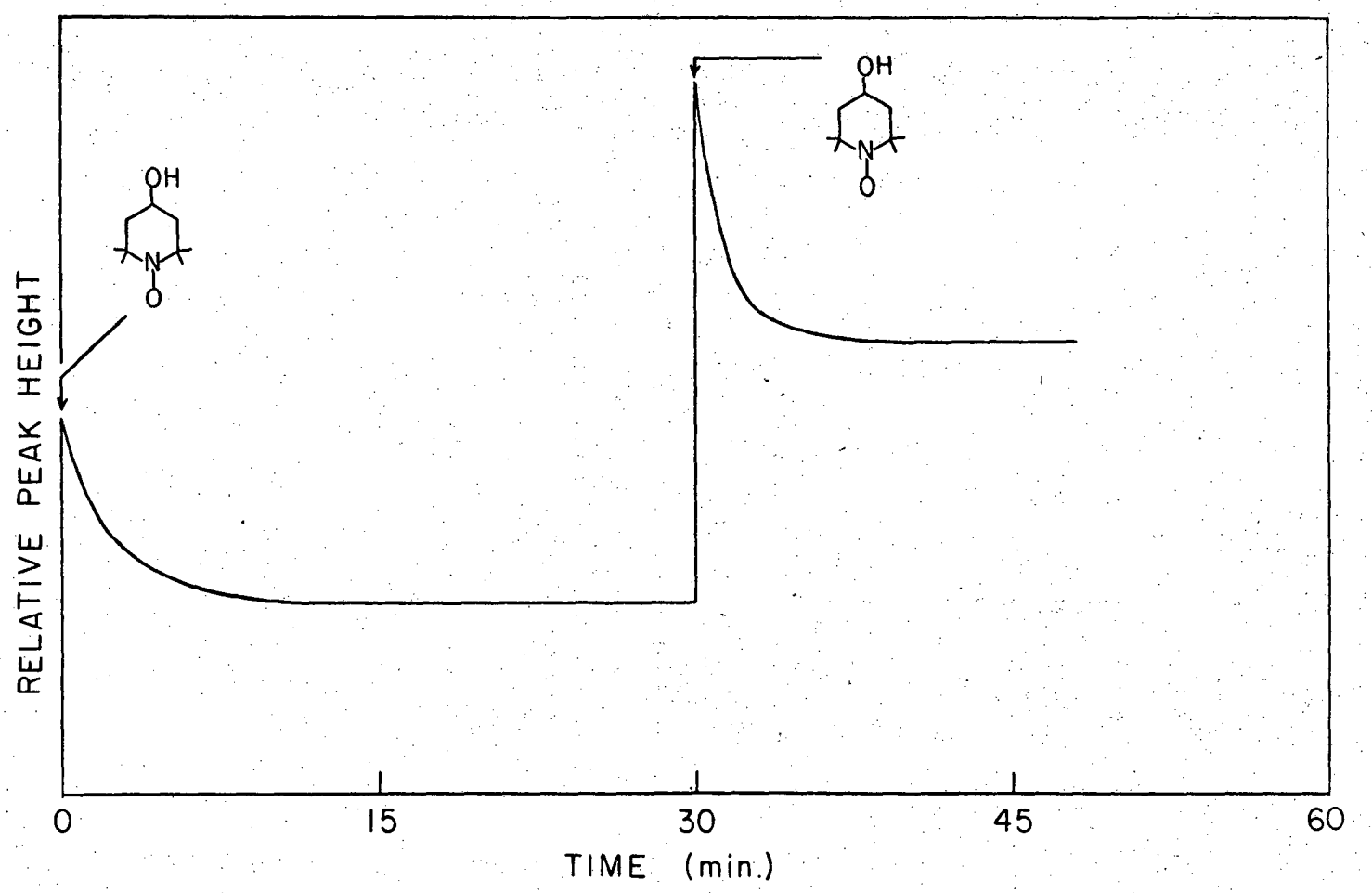

100
50
RELATIVE PEAK HEIGHT (%)
OH
N
O
10^{-5} M
10^{-6} M
5×10^{-7} M
0
15
30
45
60
TIME (min.)
XBL708-5346

Figure 6-20

second radical continued to decrease until it reached a plateau at a level which is higher than the original plateau reached by the radical V alone (Fig. 21, 22). This indicates that the destruction of radical V by illuminated chloroplasts has stopped, probably because the radical has bypassed the destructive site and reached a site which is compartmentalized in such a manner that no destruction of the radical is possible. (Alternatively, stabilization of the radical may be due to its binding to a larger molecule which protects it from destruction.) However, the potential ability of the chloroplasts to destroy the radical is still operative, as is apparent from the initial decay of the signal upon the introduction of a new radical to the system. The new plateau is higher, probably because a fraction of the secondarily introduced radical has reached a site at which it bypasses the destruction mechanism. For example, by binding to some macromolecule or partitioning into a site which is inaccessible to the reducing agent, the radical becomes protected from reduction.

12. Restoration of the EPR signal. Attempts to restore the EPR signal by pretreating chloroplasts with a radical and then turning the lights off after illumination for varying periods of time failed to restore the EPR signal significantly -- that is, only a very small amount of restoration was achieved. It is possible that the plateau occurring in EPR signal decay may be due to the fact that at the site of action the radical is undergoing an oxidation-

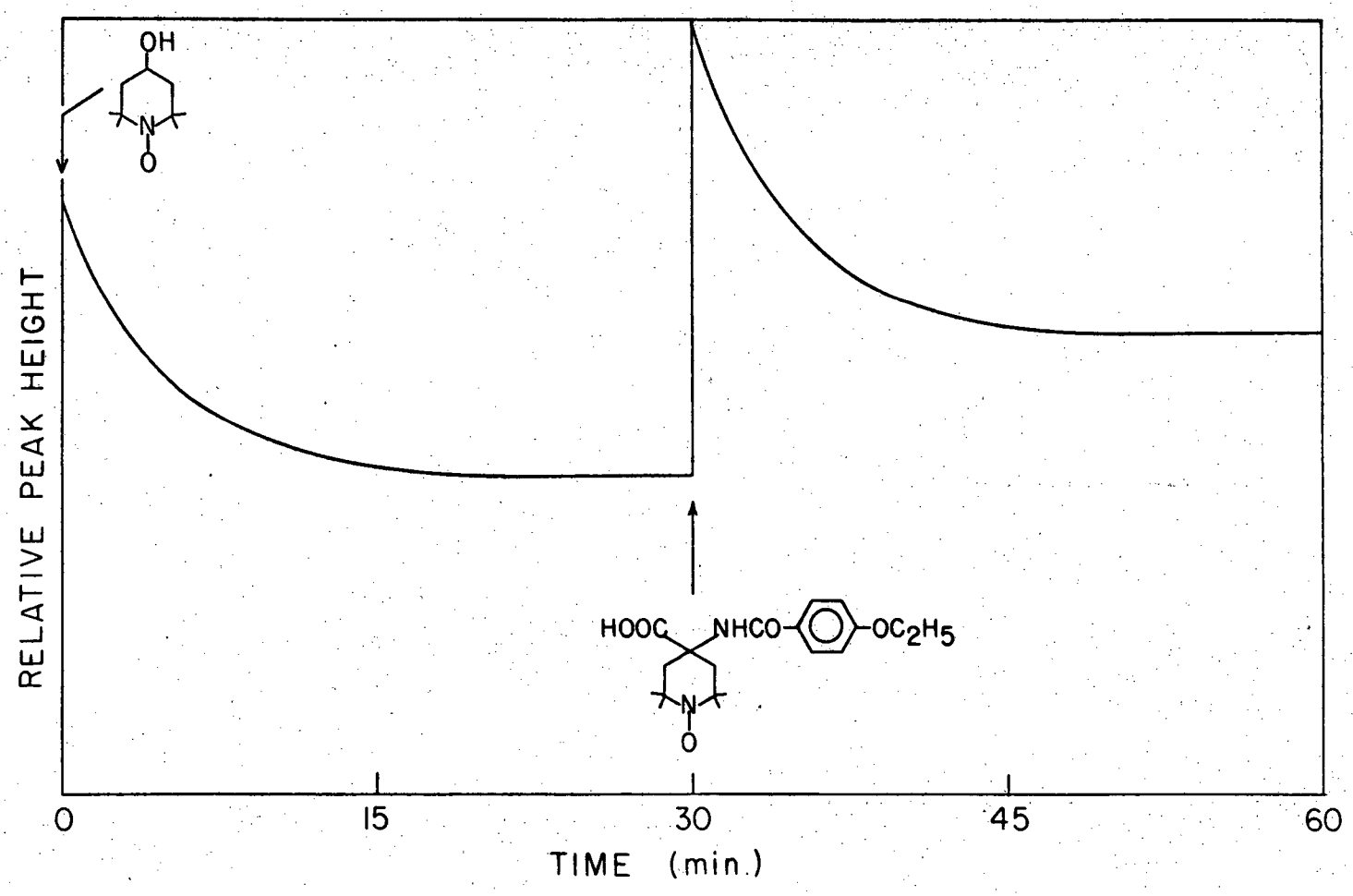
Figure 21. Effect of introduction of a second quantity of radical V on the decay of the signal after it has reached its plateau. (Both first and second additions were at 10^{-6} M.)



XBL 708-5367

Figure 6-21

Figure 22. Effect of introduction of a radical VIII (10^{-6} M) on the decay of the signal obtained by 10^{-6} M of radical V after a plateau has been achieved.



XBL 708-5366

Figure 6-22

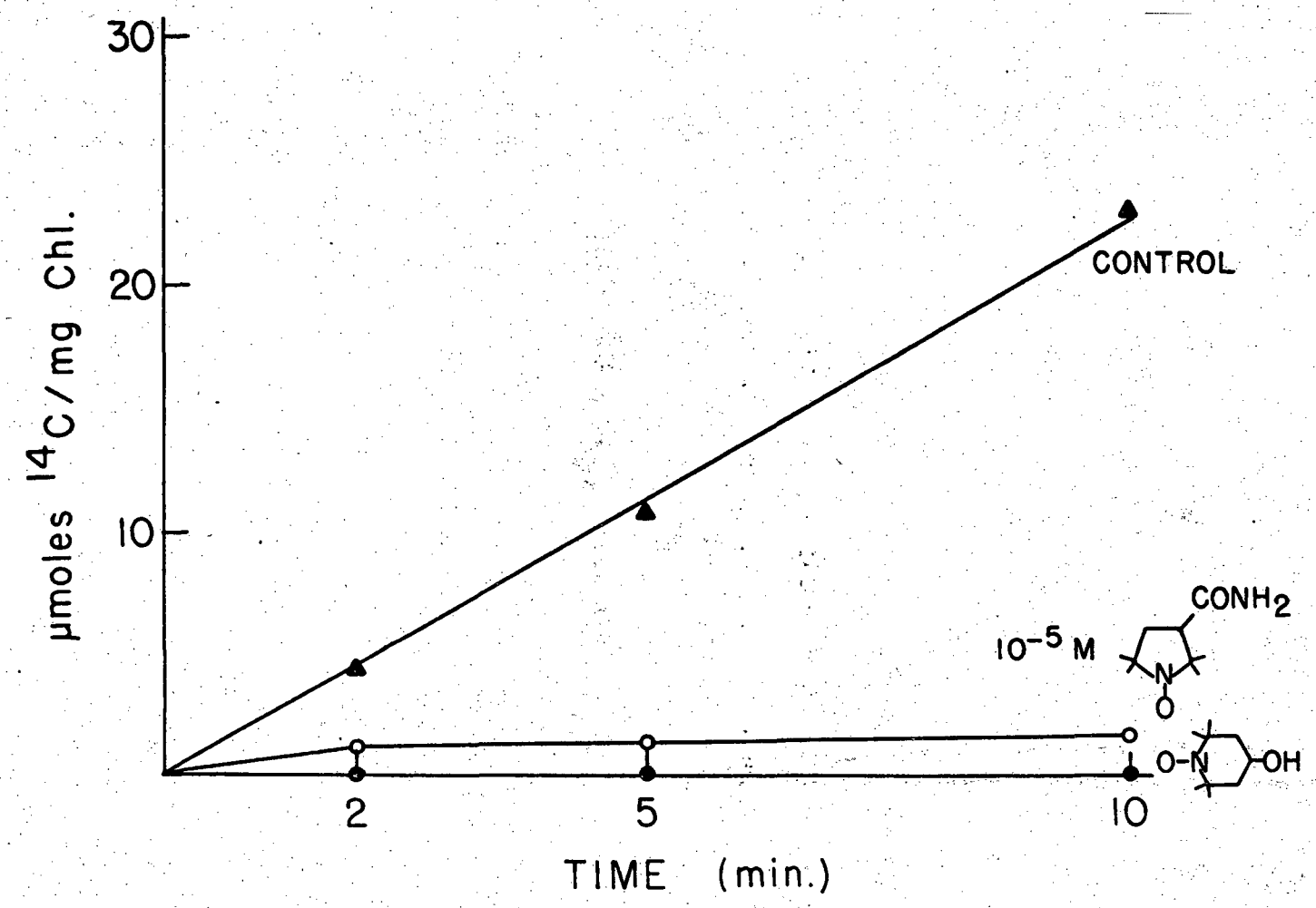
reduction reaction in which it accepts electrons from an intermediate in the electron transport chain and donates them to another intermediate in the chain. The occurrence of a plateau would in this case be due to the achievement of a steady-state situation where the rate of reduction of the radical is equal to the rate of its oxidation. Experiments to verify this idea were conducted by illuminating the chloroplasts for much longer times (2-4 hrs). This illumination resulted in partial to full restoration of the EPR signal after its initial decay. This may be explained by the failure of the reductive mechanisms of the chloroplasts to operate for the duration of illumination while the oxidation step continues to function. Addition of ferricyanide to the pretreated chloroplasts speeded the restoration of the signal, which indicates that the radical probably acts at a step before ferricyanide in the electron transport chain, or that ferricyanide speeds the rate of reoxidation of the reduced radical by accepting electrons from this reduced form of the radical directly or indirectly.

13. Effect of the radicals on the photosynthetic product distribution. Although all evidence points to the fact that the primary site of action (biochemical lesion) of the radicals involves the electron transport system of photosynthesis, we have attempted also to determine the effect of the radicals on the photosynthetic product distribution of the different intermedaites in the carbon reduction cycle. Two experiments were carried out. One involved determination

of the kinetics of ^{14}C incorporation into various intermediates of the cycle within 10 min of CO_2 fixation in the presence of the radical V. This experiment shows that the inhibition of CO_2 fixation as well as the inhibition of formation of different ^{14}C-labeled intermediates in the carbon reduction cycle occurred with no delay in time (Fig. 23-30). The second experiment was carried out using three radicals of different inhibitory potencies (Table V). The results show an indifferent (i.e., non-specific) type of inhibition which suggests that the carbon reduction cycle is not the biochemical lesion of the nitroxide radicals. Instead, the inhibition appears to be a consequent step to the primary effect on the electron transport system. The radical III, which is a poor inhibitor, showed a similar pattern of inhibition when its access to the site of action was facilitated by washing the chloroplasts with soln. C and pelleting them by centrifuging at 2000 x g for 1 minute (Table VI).

14. <u>Effect of dichlorophenyl dimethyl urea (DCMU) on the decay of the EPR signal of the radical</u>. Upon illumination the EPR signal of radical V was found to decay at a much lower rate in the presence of DCMU than in its absence. This may indicate that there are more than one site of reduction of the radical; and that DCMU blocks some of these sites resulting in a lower rate of reduction of the radical, thus allowing more radical to bypass the reduction mechanism as indicated by the fact that the plateau (Fig. 31) is reached at a higher intensity of the original signal.

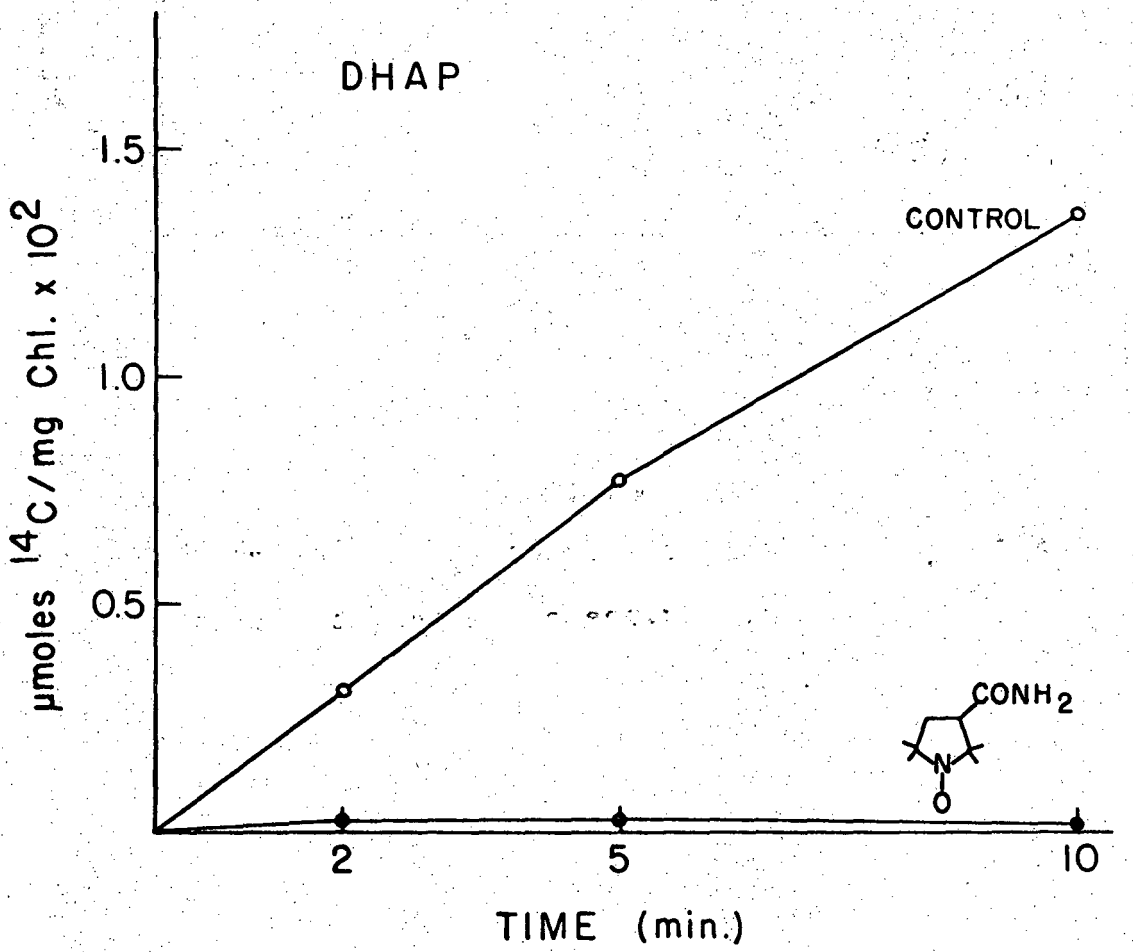
Figure 23. Effect of radicals II and V on the CO_2 fixation rate by isolated chloroplasts.



XBL 708-5357

Figure 6-23

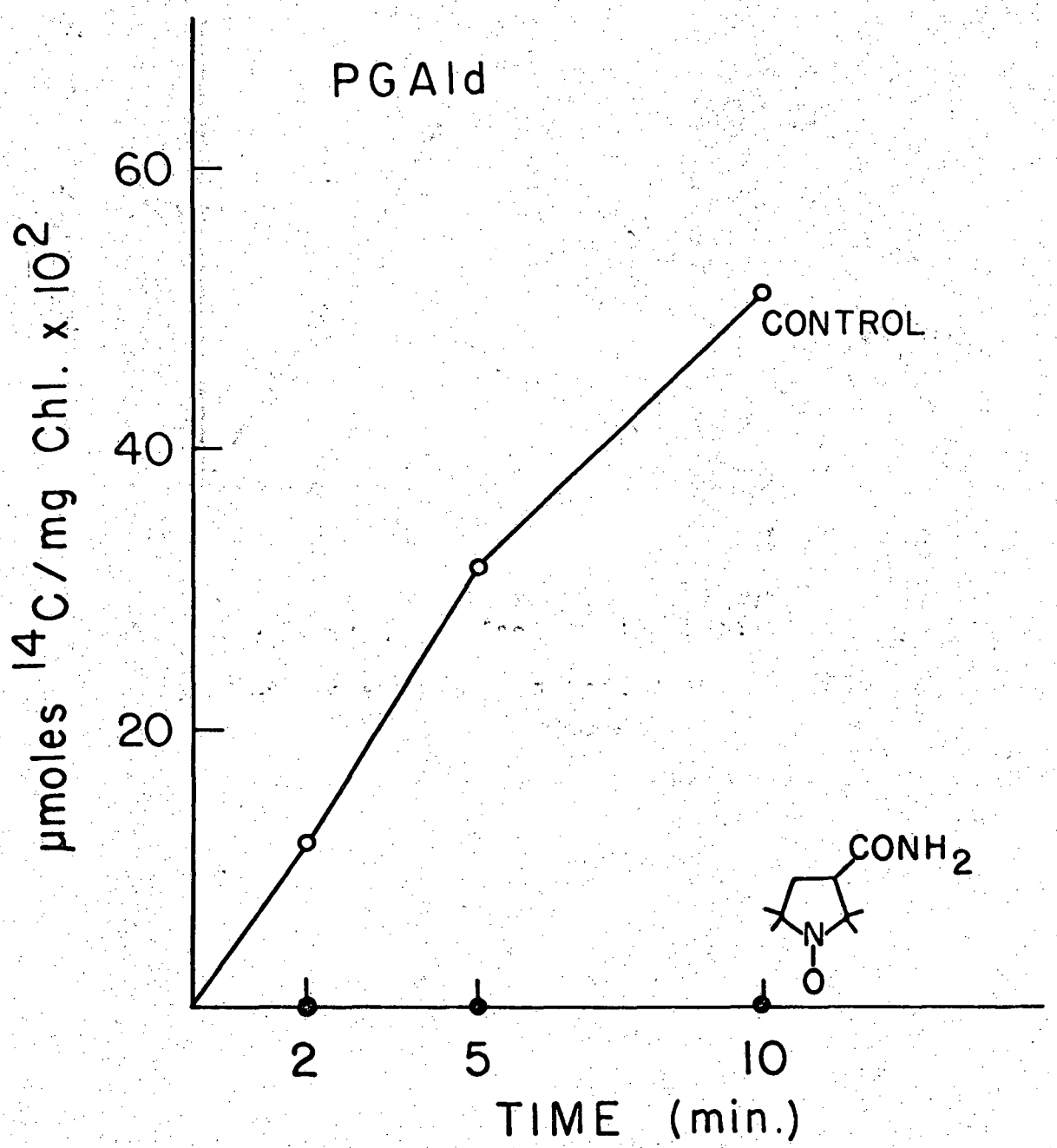
Figure 24. Effect of radical II on the rate of formation of dihydroxyacetone phosphate by isolated chloroplasts.



XBL 708-5355

Figure 6-24

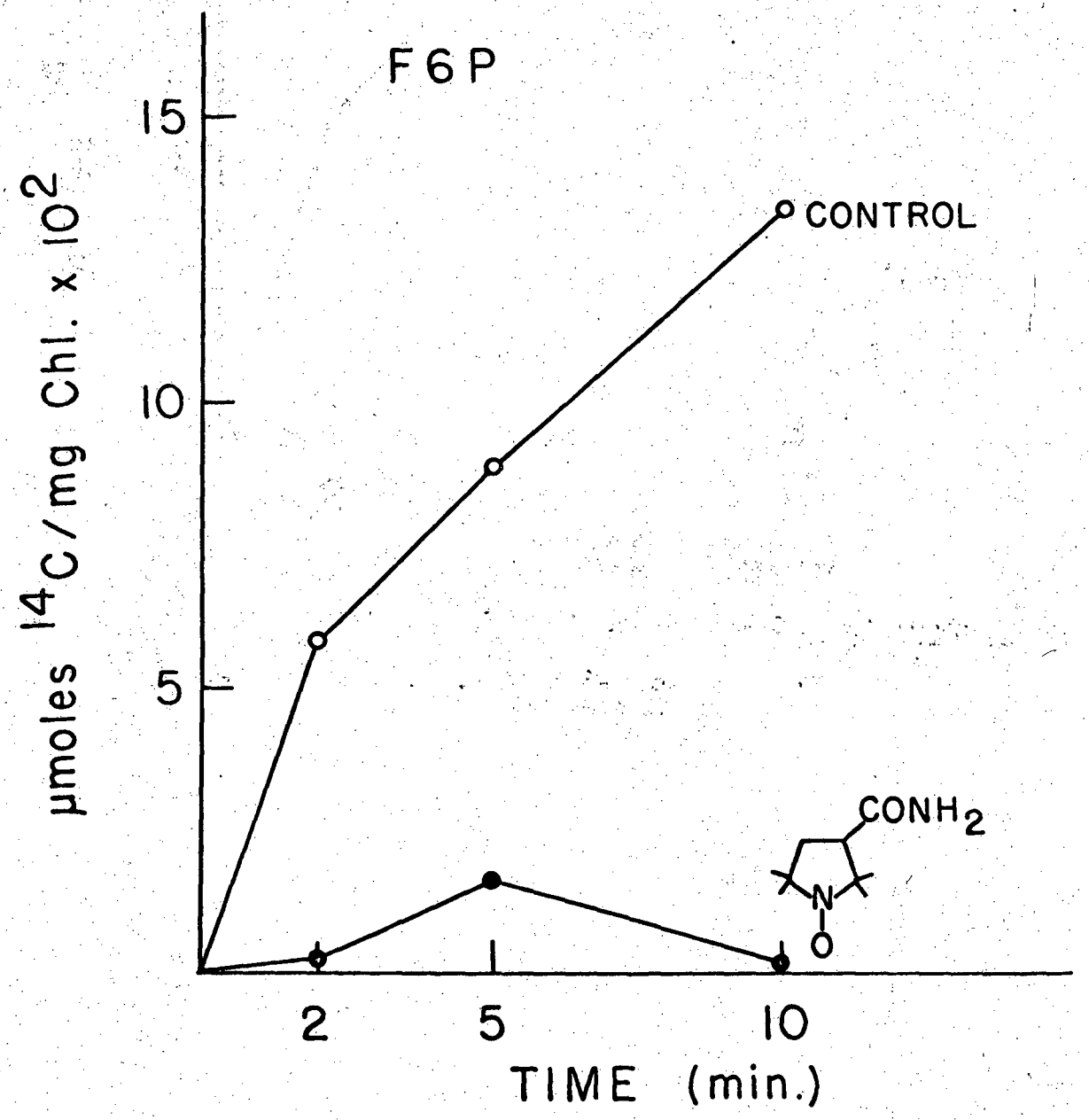
Figure 25. Effect of radical II on the rate of formation of phosphoglyceraldehyde by isolated chloroplasts.



XBL 708-5354

Figure 6-25

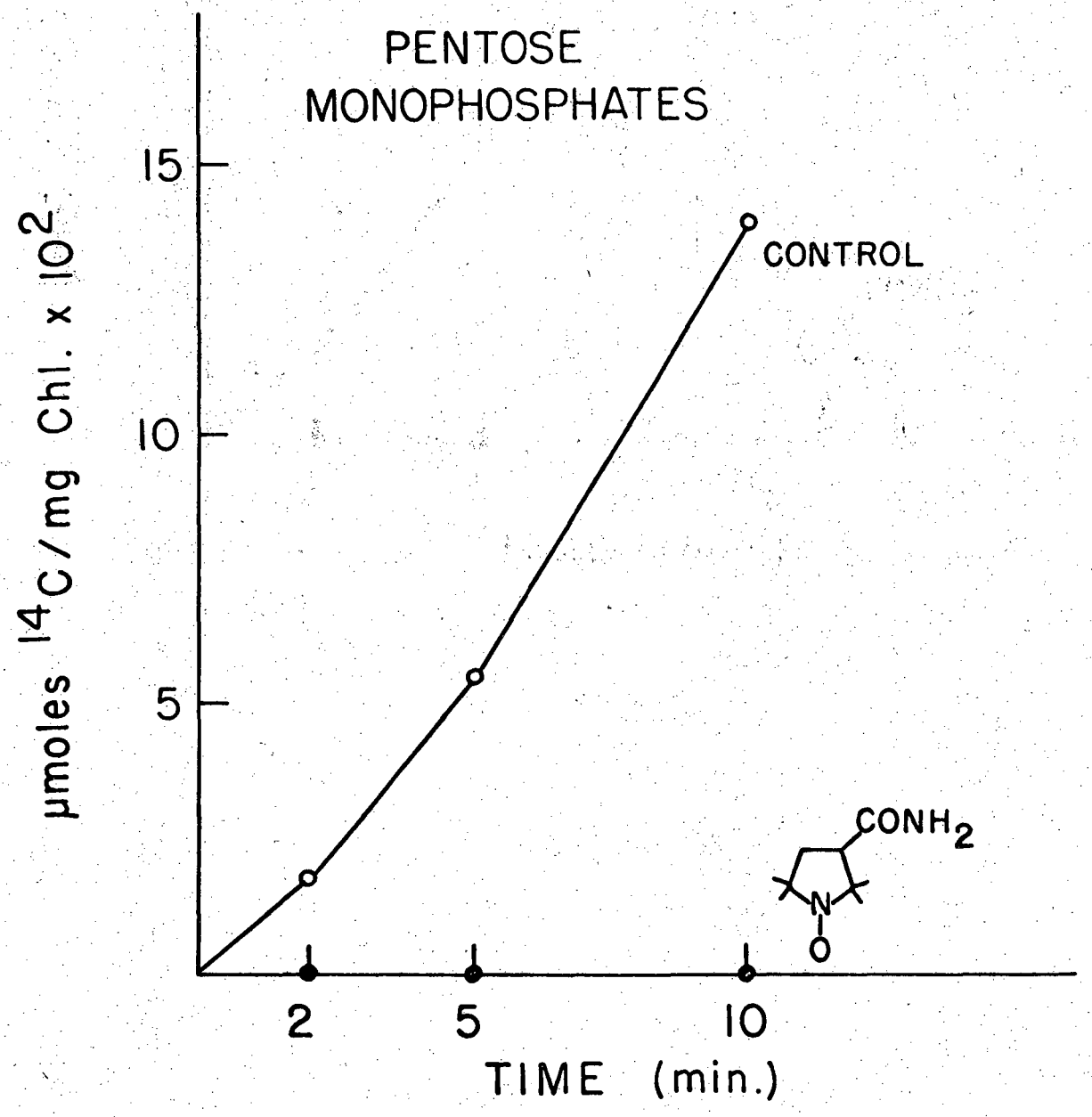
Figure 26. Effect of radical II on the rate of formation of fructose-6-phosphate by isolated chloroplasts.



XBL 708-5353

Figure 6-26

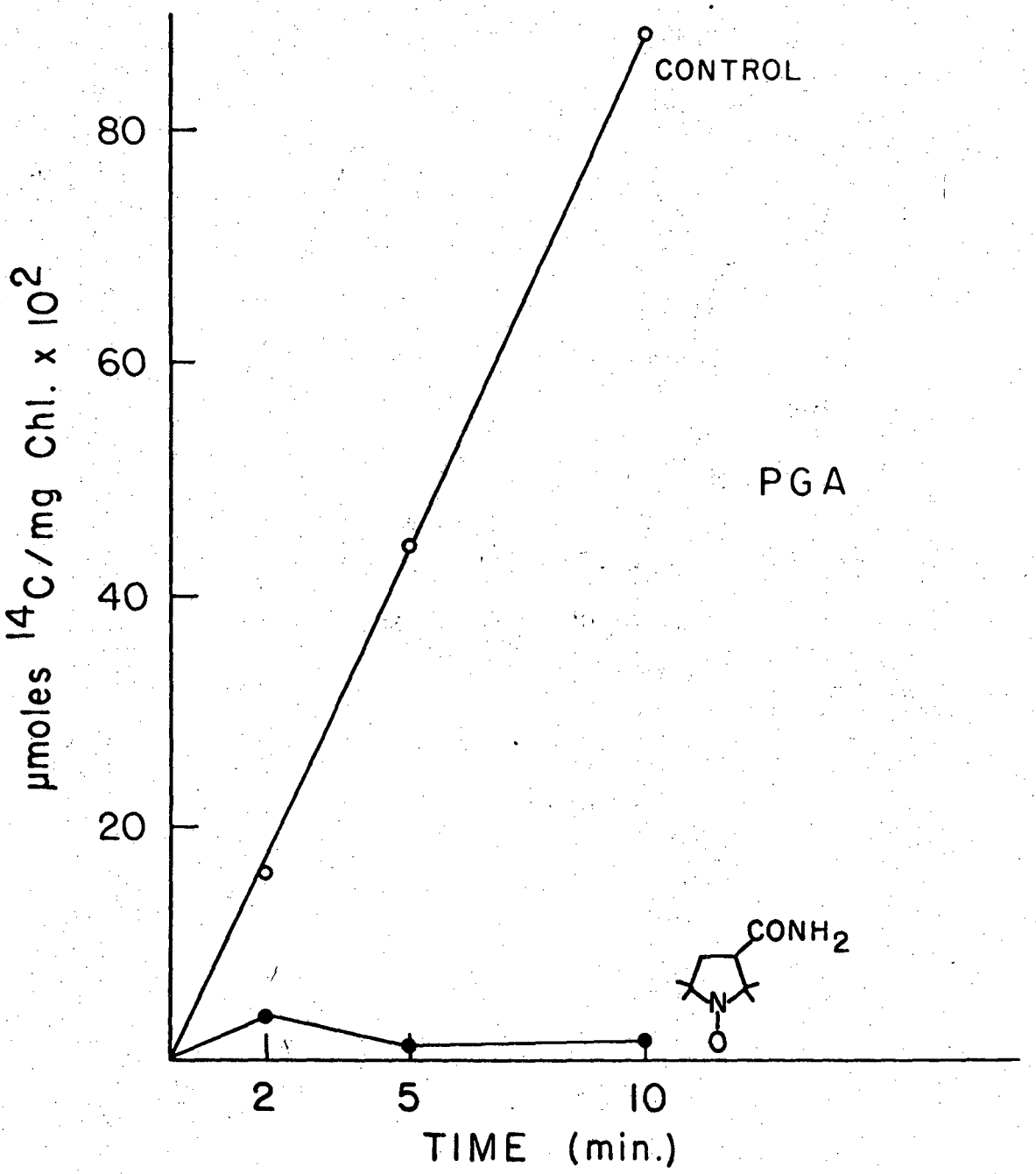
Figure 27. Effect of radical II on the rate of formation of pentose monophosphates by isolated chloroplasts.



XBL 708-5352

Figure 6-27

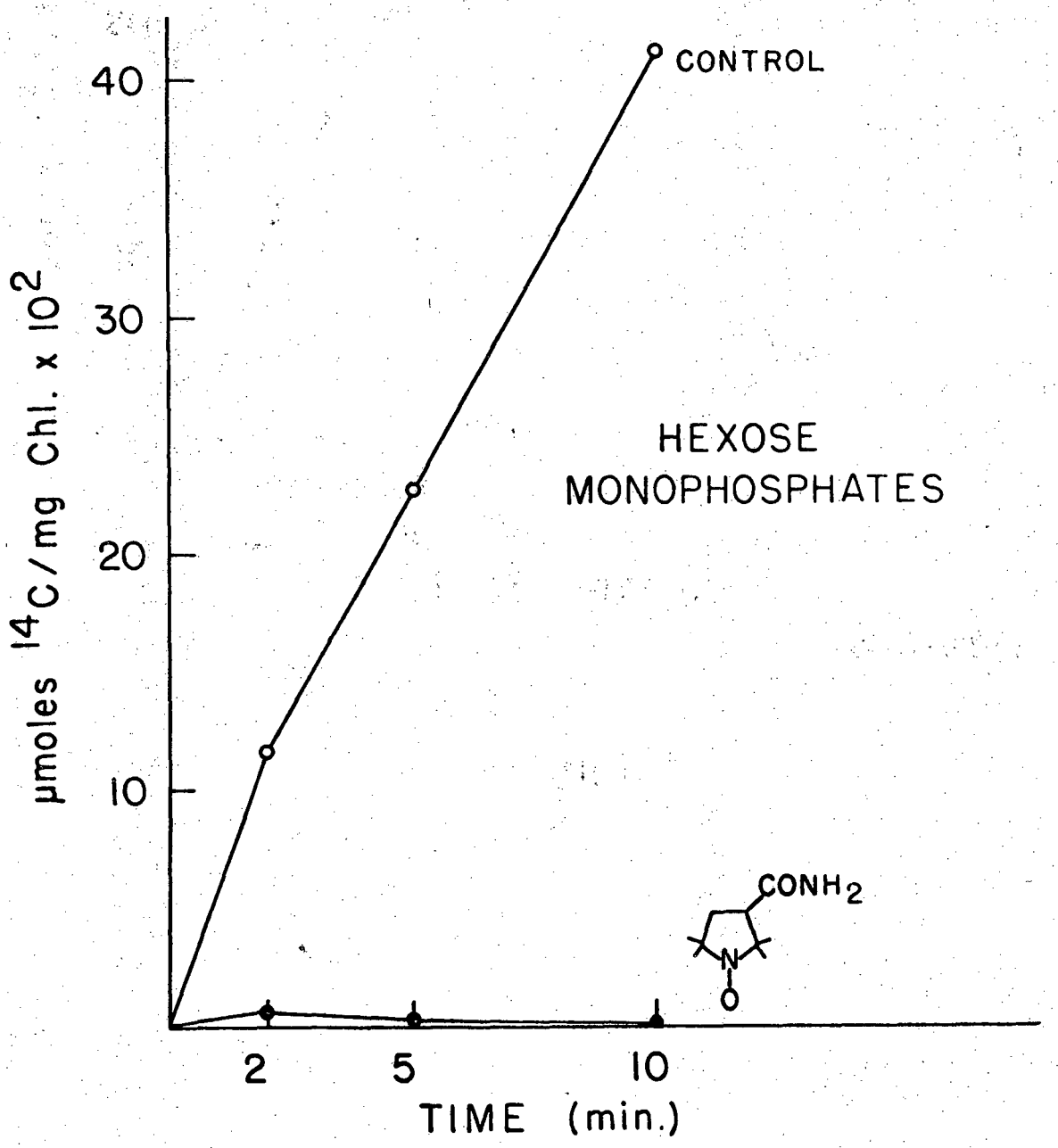
Figure 28. Effect of radical II on the rate of formation of phosphoglyceric acid by isolated chloroplasts.



XBL 708-5351

Figure 6-28

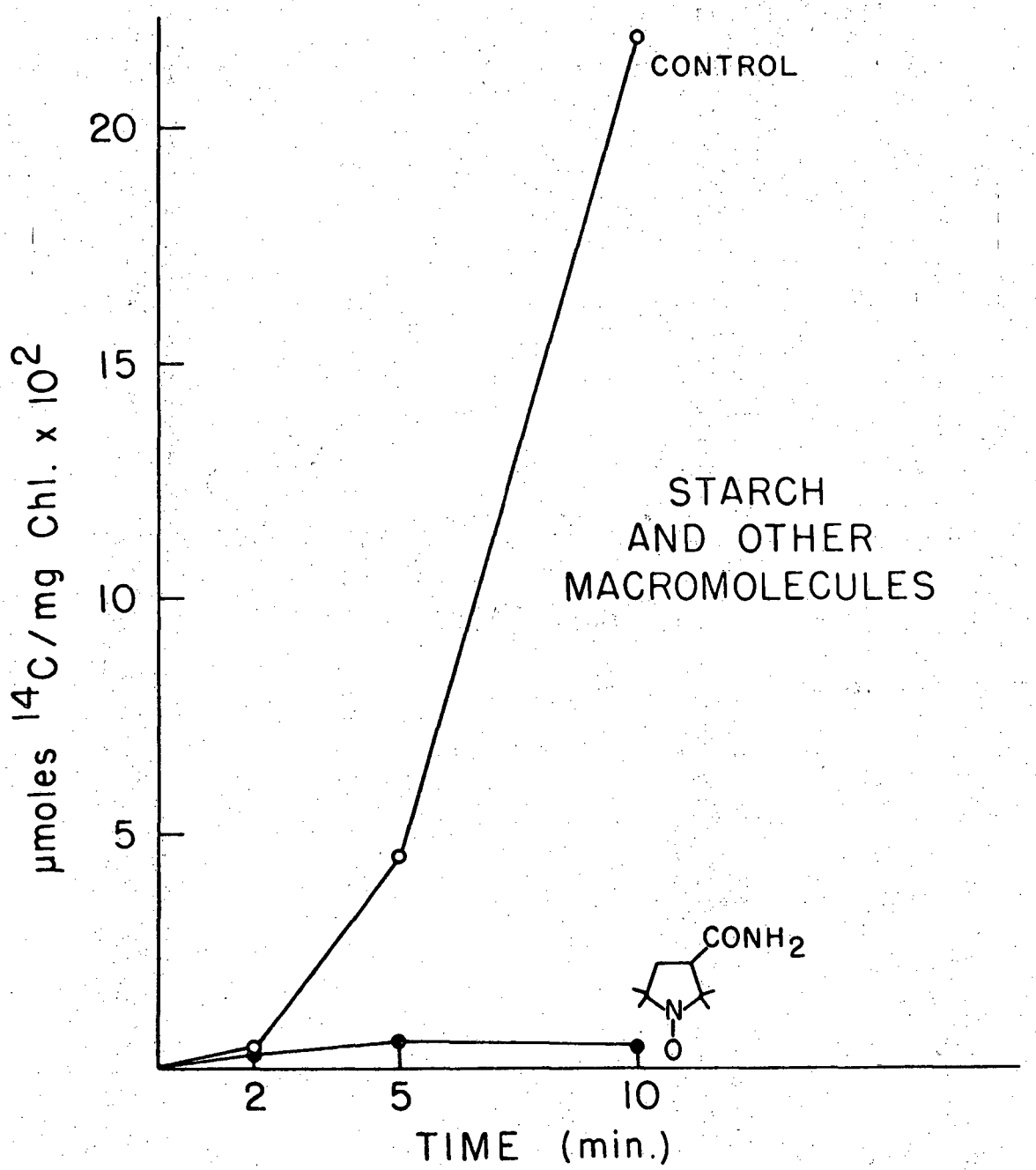
Figure 29. Effect of radical II on the rate of formation of hexosemonophosphates by isolated chloroplasts.



XBL 708-5350

Figure 6-29

Figure 30. Effect of radical II on the formation of starch and other macromolecules during photosynthesis by isolated chloroplasts.



XBL 708-5349

Figure 6-30

TABLE V

Effect of the Nitroxide Radicals on the Formation of the Carbon Reduction Cycle Intermediates

Radical	μmoles/mg Chl							
	Diphosphates	HMP	F6P	PMP	DHAP	PGA	Glycolate	Starch and Other Macromolecules
VIII	0.176	0.309	0.107	0.0477	0.316	2.420	0.097	0.198
VI	0.112	0.111	0.094	0.0183	0.115	0.045	0.0099	0.002
IV	0.048	0.055	0.017	0.004	0.041	0.243	0.005	0.001
Control	0.621	0.430	0.175	0.096	0.854	1.59	0.225	0.113

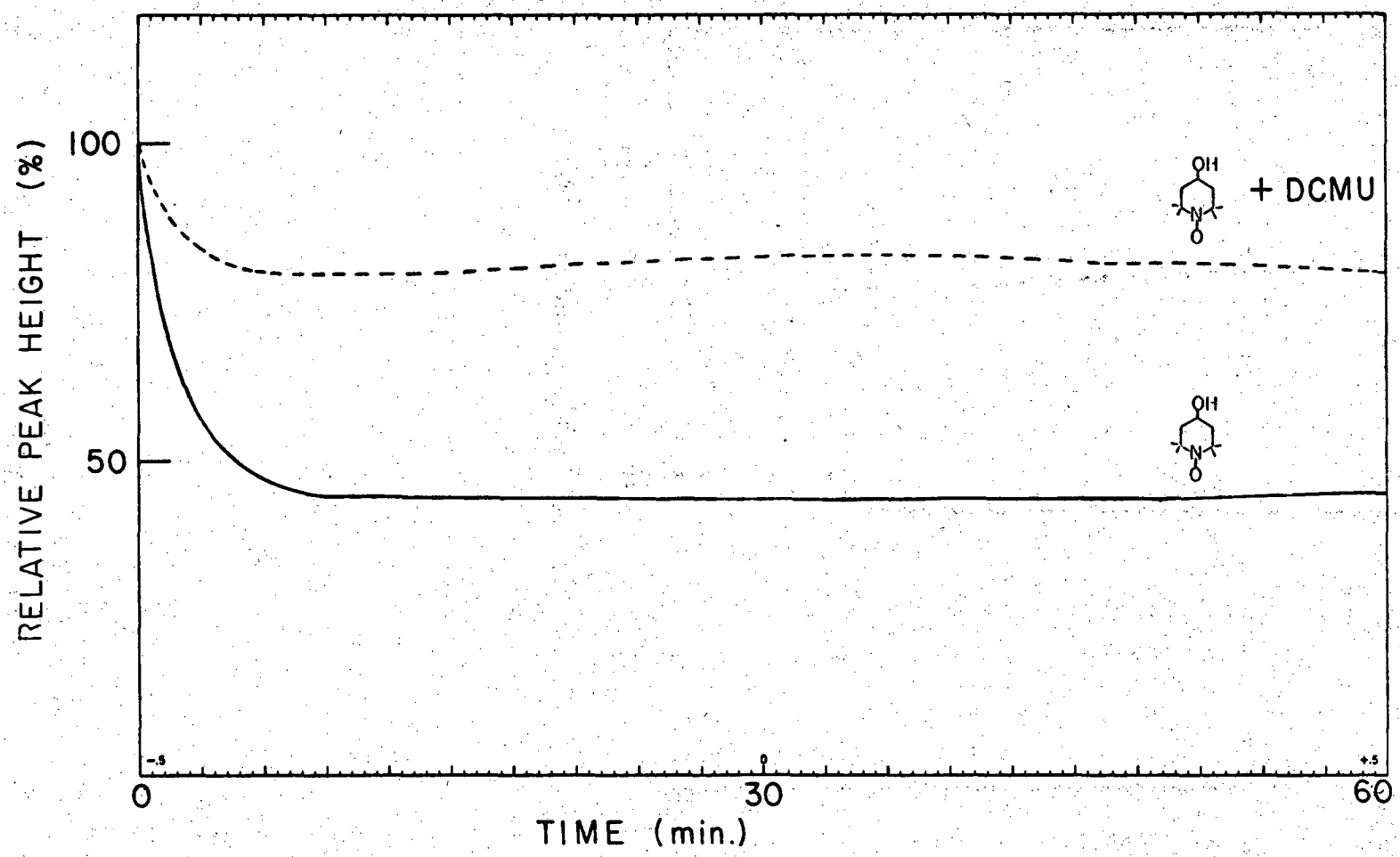
TABLE VI

Effect of a Weak Inhibitor of CO_2 Fixation on the Formation of the Carbon Reduction Cycle Intermediates

Radical was incubated with chloroplasts in the light for 5 minutes. Chloroplasts were spun down at 2000 x g for 50 sec, washed with Soln. C and centrifuged at 2000 x g for 50 sec, then used for the $^{14}CO_2$ fixation rate experiment.

Radical	μmoles/mg Chl							
	Diphosphates	HMP	F6P	PMP	DHAP	PGA	Glycolate	Starch and Other Macromolecules
III	0.089	0.138	0.051	0.013	0.213	0.200	0.038	0.003
Control (washed)	0.138	0.330	0.109	0.042	0.451	0.854	0.138	0.175
Control (unwashed)	0.621	0.430	0.175	0.096	0.854	1.59	0.225	0.113

Figure 31. Effect of DCMU on the decay of radical V upon illumination in the presence of chloroplasts.

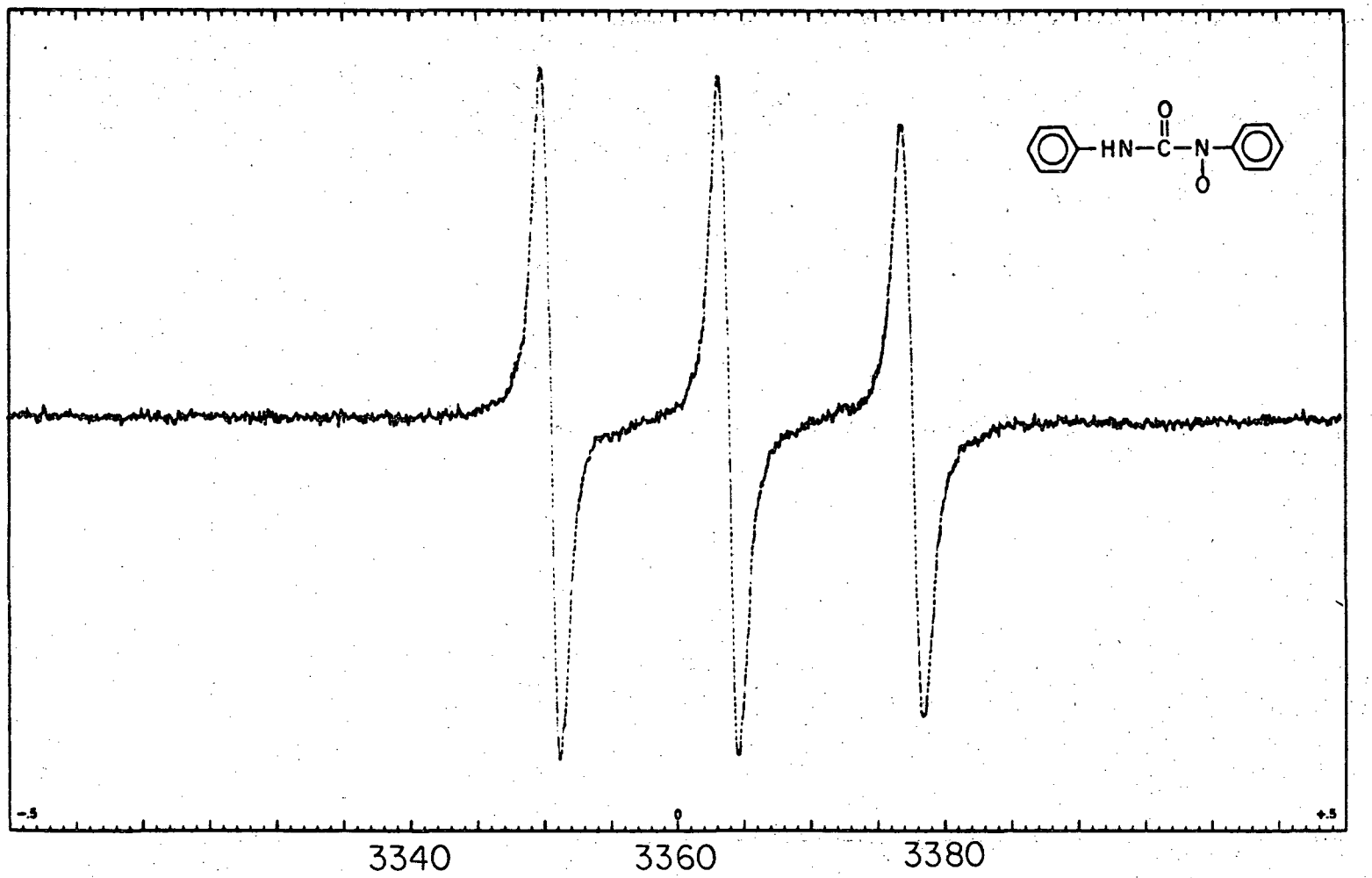


XBL 7012-4926

Figure 6-31

15. Studies with the nitroxide radical of diphenyl urea. Upon finding that DCMU inhibits the reduction of radical V by isolated chloroplasts, we tried to combine the nitroxide function with the structural features of DCMU in one molecule. An attempt was made to synthesize the nitroxide of DCMU, but we failed to detect the formation of this nitroxide. The nitroxide radical of diphenyl urea was more stable and it was synthesized by treating diphenyl urea with hydrogen peroxide in the presence of catalytic amounts of phosphotungestic acid in ethanol-water (9:1) solution. The product had the ESR spectrum shown in Fig. 32. However, we were unable to isolate the product in a crystalline form, in addition to the short lifetime of this nitroxide in solution; thus no quantitative data were possible.

Figure 32. ESR spectrum of diphenyl urea nitroxide radical.



XBL 7012-4941

Figure 6-32

D. CONCLUSION

We conclude from our results that the nitroxide radicals exert their action as photosynthetic inhibitors on the electron transport chain at a point before that at which ferricyanide ion acts. The action of nitroxide ions as CO_2-fixation inhibitors is a secondary effect to their action on the electron transport chain, which is the true biochemical lesion. This is apparent from their indifferent type of action (non-specific) on the product distribution of the Calvin cycle and on glycolate formation. Our evidence for their effect on the photosynthetic electron transport chain is further strengthened from the oxygen evolution data. Compounds which possess the features required for a potent Hill oxidant are also successful CO_2-fixation inhibitors.

In order to exert their effect at the site of action, nitroxide radicals must reach that site by penetrating the chloroplast membrane. Chloroplast membranes, like other biological membranes, are composed of lipoproteins. Thus, to penetrate this membrane a compound must possess a partition coefficient between the aqueous medium surrounding the chloroplast and the lipoprotein composing the chloroplast membrane favoring the latter. This clearly is the case; hydrophilic compounds possessing free carboxyl groups were shown to be poor Hill oxidants as well as poor CO_2-fixation inhibitors. Among these compounds containing an ionizable

group, the ratio of the size of the lipophilic moiety of the molecule to the number of carboxyl groups was directly proportional to their potency for inhibition of photosynthesis. Compounds which contain structural features favoring their solubilization in the lipoproteinatious chloroplast membrane were excellent photosynthetic inhibitors. We have also observed that the size of the nitroxide radical ring exerts no effect on the inhibitory power of the radical; and that the presence of a double bond in the ring renders the radical ineffective as a photosynthetic inhibitor. This may be due to facilitation of a detoxication mechanism working on the double bond analogous to those observed in microsomal oxidation of compounds containing unsaturations.

Since the action of the nitroxide radical depends on the accessibility of the active site to the radical through breaching the membrane, a delay in permeation would only result in the depletion of the radical by one of the "sites of loss," that is, storage, elimination, or chemical inactivation of the radical[23]. The most likely site of loss in our case would be chemical inactivation by a reducing agent which is produced by the chloroplast in the light and which diffuses out to the medium surrounding the chloroplasts where the reduction of the radical takes place. This is evident from the kinetics of the EPR signal decay which indicate that radicals which exhibit potent photosynthetic inhibition reach their site faster and thus bypass degradation through inactivation; on the other hand, poor photosynthetic inhibitors

are inactivated (as observed from the decay of the EPR signal) soon after illumination of the pretreated chloroplasts. Another possible mechanism of inactivation is through binding to nonfunctional proteins or other macromolecules where the radical is completely immobilized, resulting in partial loss of the signal, as seen in the reduction of intensity of all three lines of the EPR signal of the monoradicals tested. In the case of biradicals the increase in the intensity of the odd-numbered lines and the decrease in intensity of the even-numbered lines of the five-line spectrum may indicate immobilization through binding to a macromolecule. Thus the biradical becomes less flexible and the distance between the radical centers is increased, resulting in slow exchange between the two radical centers and consequently in the disappearance of the interpolated spectral lines which are absent on the monoradical spectra. The rate of exchange is denoted by J, and the frequency separation of the hyperfine splitting for ^{14}N is A(= 43 MH_z). Slow exchange implies $J/A < 1$ and fast exchange corresponds to $J/A > 1$. As more radical is bound to our hypothetical macromolecule (or particle, for that matter), J is decreased, resulting in a smaller value of J/A. Continued decrease in the value of $\frac{\Sigma J/A}{n}$ (where n is the total number of radical molecules, both those bound to the macromolecule and those free in the aqueous phase surrounding the chloroplast) as more biradical molecules bind to the macromolecule will eventually result in the complete disappearance of the interpolated spectral

lines arising from interaction of the two ^{14}N nuclei occupying different Zeeman levels; these lines are absent in the monoradical spectrum. Our data show that both mechanisms, the reduction of the radical and the deactivation, by binding to a macromolecule, contribute to the loss of the spin signal. If chloroplasts pretreated with biradical are illuminated in the EPR cavity, a shift from the five-line spectrum to the three-line spectrum is observed, with an increase in the intensity of the three monoradical lines. This favors the binding mechanism proposed above. Continued illumination, however, results in the eventual decrease of the three-line signal which was originally derived from the five-line signal of the biradical. This indicates that the reductive mechanism is operative at a much lower level when the radical is bound to the macromolecule. To eliminate the time element and avoid complications in interpreting the results due to aging of the chloroplasts, an experiment in which the untreated chloroplast suspension was illuminated with high intensity flashes for a short time (as described in the Methods section) was carried out. The results showed that if the reductive mechanism is allowed to work before the radical is given the time to bind to the macromolecule, the reduction of the biradical to yield a very low-intensity three-line signal occurs much more rapidly. It is clear that preillumination of the chloroplasts with high-intensity light resulted in the production of an abundant quantity of the reducing factor which acted on the radical at a faster

rate than the rate of binding of the radical to the macromolecule. The rate of diffusion of the reducing factor into the suspending medium around the chloroplasts depends on the composition of that medium. Sucrose-tricine medium retards the diffusion of the reducing factor while Sorbitol-type buffer facilitates its diffusion. The remaining fraction of the radical which bypasses inactivation reaches the active site where it acts as a Hill oxidant resulting in the evolution of O_2. A secondary effect of this action is the inhibition of CO_2 fixation by the carbon reduction cycle. Nitroxide radicals act not as electron acceptors, and their reduced form may act as electron donors, as is evident from the restoration of the signal by continued illumination. Our data suggest that ferricyanide ion forms a complex of varying stability with nitroxide compounds. This complex renders the radical less able to penetrate the membrane and results in a decrease of the potency of the radical as Hill oxidant.

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