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Conformation of Dinucleoside Monophosphates Modified with Benzo[*a*]pyrene-7,8-dihydrodiol 9,10-Oxide as Measured by Circular Dichroism†

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ABSTRACT: The conformational properties of GpU modified with the reactive derivative of benzo[*a*]pyrene, (±)-7β,8α-dihydroxy-9α,10α-epoxy-7,8,9,10-tetrahydrobenzo[*a*]pyrene, have been investigated utilizing circular dichroism spectroscopy. Binding of this carcinogen to the N^2 of G residues in GpU resulted in the formation of four compounds (I to IV) representing two pairs of diastereoisomers. The molar ellipticity values of the modified dimers were approximately twofold higher than those of the modified guanosine monomers. These values were decreased appreciably when the spectra of the dimers were obtained at 80 °C or in methanol rather than at 25 °C in water, suggesting that under the latter conditions there is a stacking interaction between the carcinogen and the neighboring uridine residue. Based on these results, a conformation is proposed for modified GpU. It includes insertion of the benzo[*a*]pyrene moiety, by rotation of the modified guanine residue about its glycoside bond, coplanar to the neighboring uridine and perpendicular to the phosphodiester backbone.

Benzo[*a*]pyrene, one of the most abundant environmental carcinogens (Committee on Biologic Effects of Atmospheric Pollutants, 1972), interacts with the genetic material of cells (Miller & Miller, 1974; Heidelberger, 1975). Recent studies indicate that the major reactive metabolite of benzo[*a*]pyrene with respect to covalent binding to nucleic acids, both in vitro and in vivo, is (±)-7β,8α-dihydroxy-9α,10α-epoxy-7,8,9,10-tetrahydrobenzo[*a*]pyrene (BPDE I)[1] (Sims et al., 1974; Daudel et al., 1975; King et al., 1976; Weinstein et al., 1976; Koreeda et al., 1976). The in vitro modification of DNA or poly[G] with BPDE yields derivatives which are the same as those found in cellular RNA and DNA obtained from bovine and human bronchial explants incubated with [^{3}H]BP (Jeffrey et al., 1976, 1977). More recently, BPDE adducts have also been detected in mouse skin RNA following topical application of [^{3}H]BP (Moore et al., 1977). It has been established that the major nucleic acid derivative results from the formation of a covalent bond between the 10 position of BPDE and the N^2 position of guanine (Weinstein et al., 1976; Jeffrey et al., 1976; Moore et al., 1977). Complete stereochemistry of the major guanine adduct present in bovine and human bronchial RNA and DNA has also been established (Nakanishi et al., 1977; Jeffrey et al., 1977; Yang et al., 1977).

The biochemistry of BPDE is complicated by the fact that it can exist as four isomers. In BPDE isomer I the 7-OH and 9,10-oxide groups are on opposite sides of the ring system and in isomer II they are on the same side. Both isomers I and II consist of enantiomeric pairs, designated 7α and 7β. All four of these have been prepared (Harvey & Cho, 1977; Yagi et al., 1977) and found to react with guanine and to a lesser extent with adenine and cytosine residues in nucleic acids (Weinstein et al., 1976; Jennette et al., 1977). It appears that both isomers I and II are formed in vivo, although the relative abundance of their corresponding in vivo nucleic acid adducts varies among cell cultures, tissues, and species (Weinstein et al., 1976; Jeffrey et al., 1977; Shinohara & Cerutti, 1977; Moore et al., 1977; Baird and Diamond, 1977). In addition, although trans addition to the 10 position appears to be the predominant reaction during nucleic acid modification, there is also evidence for cis addition products (Jeffrey et al., 1977; Moore et al., 1977).

The covalent attachment of this bulky carcinogen to nucleic acids presents steric and conformational problems which are associated with structural and functional changes in the modified nucleic acids and may be relevant to the biology of carcinogens (Pulkrabek et al., 1977; Gamper et al., 1977; Leffler et al., 1977). We have found that BPDE modification of calf thymus DNA produces small localized regions of destabilization of the native structure (Pulkrabek et al., 1977) and inhibits chain elongation when the modified DNA is used as a template for transcription by *E. coli* RNA polymerase (Leffler et al., 1977). To find out in greater detail how attachment of the bulky hydrocarbon molecule to the N^2 position of the guanosine residue alters the tertiary structure of nucleic acids, the present study on the conformation of BPDE modified

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[1] Abbreviations used: BP, benzo[*a*]pyrene; BPDE I, (±)-7β,8α-dihydroxy-9α,10α-epoxy-7,8,9,10-tetrahydrobenzo[*a*]pyrene; BPDE II, (±)-7β,8α-dihydroxy-9β,10β-epoxy-7,8,9,10-tetrahydrobenzo[*a*]pyrene; G*, guanosines containing a covalently bound hydrocarbon residue as a result of modification by BPDE; HPLC, high pressure liquid chromatography.

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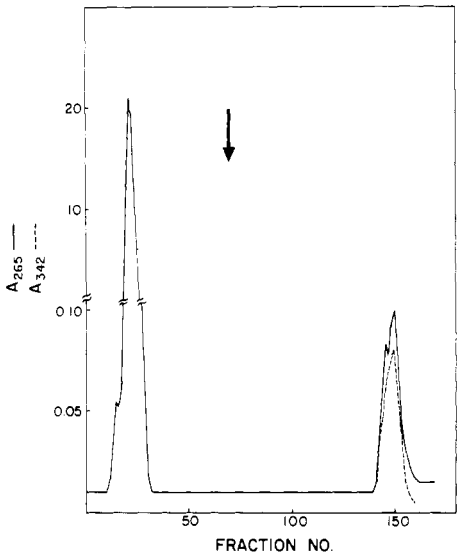

FIGURE 1: Chromatography of the products of reaction of GpU with BPDE I on Sephadex LH-20. Experimental conditions are described in Methods. The arrow indicates the start of a linear gradient of 20 to 100% methanol in 0.02 M sodium bicarbonate. The large peak eluted with aqueous solution in fractions 10 to 30 is unmodified GpU. The peak eluted with the methanol gradient in fractions 140 to 160 containing material with absorbance at 342 is a mixture of modified GpU compounds, designated G*pU.

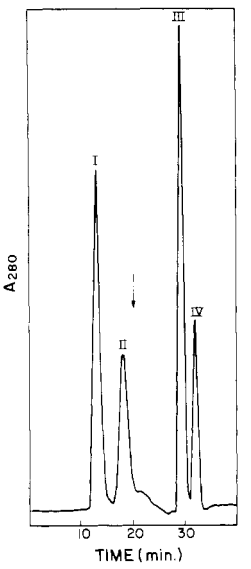

FIGURE 2: HPLC profile of the G*pU adducts isolated from the Sephadex LH-20 column. For details, see Figure 1 and Methods. The arrow indicates the start of a linear gradient of 5 to 10% methanol in water.

dinucleoside monophosphates was undertaken, utilizing circular dichroism as the method of analysis. One of the dimers, GpU, was chosen as a model compound because they are the smallest fragments of nucleic acid that retain some of the local interactions which characterize higher molecular weight nucleic acid polymers (Bloomfield et al., 1974). We have used isomer I of BPDE as the substrate because of the evidence that this isomer is the major one involved in the modification of DNA in human bronchial cells (Jeffrey et al., 1977).

Materials and Methods

Materials. Samples of BPDE I were kindly supplied by Dr. R. Harvey, Ben May Laboratories, University of Chicago, Chicago, Ill., and by the Chemical Repository of the National Cancer Institute, Bethesda, Md. Samples from both sources gave similar results. GpU, UpG, and alkaline phosphatase were purchased from Sigma Chemical Company, St. Louis, Mo. Sephadex LH-20 was obtained from Pharmacia Fine Chemicals, Uppsala, Sweden.

Methods: Preparation and Characterization of Modified Dinucleoside Monophosphates. GpU or UpG (8.3 and 15.7 pmol, respectively) was dissolved in 1 mL of H_2O plus 0.8 mL of acetone, and to this 16.6 pmol of BPDE I in 2.2 mL of acetone was added. After a 24-h incubation at 37 °C, the acetone was evaporated and the reaction mixtures were extensively extracted with ethyl acetate followed by ethyl acetate–*n*-butanol (4:1 v/v). The aqueous phase of each sample was then chromatographed on a Sephadex LH-20 column (1 × 40 cm). The unmodified dimers were eluted with about 100 mL of 0.02 M NH_4HCO_3 and the modified compounds with a 125 mL linear gradient of each of 20 to 100% methanol in 0.02 M NH_4HCO_3. Fractions containing modified dimers were concentrated by evaporation and further purified by HPLC on a Du Pont 830 instrument with an analytical reverse phase Zorbax ODS column (0.25 m length and 2.1 mm internal diameter). The column was operated at 36 °C and 3000 psi and modified dimers were eluted with 5% methanol, followed by a linear gradient of 5–10% in water.

For analysis of base compositions, the isolated dimers were hydrolyzed overnight with 0.3 M KOH at 37 °C, neutralized with 10% acetic acid, and then treated with 1 unit of alkaline phosphatase per 1 A_{260} unit of the dimer at 37 °C for 24 h. The released nucleosides were then separated by HPLC on the analytical ODS column (47 °C; 2000 psi; 40% methanol in water) after removal of the enzyme on a small LH-20 column.

Circular dichroism spectra were recorded on a Cary 61 spectropolarimeter using 0.5 cm thermostated Suprasil cell. The temperature was measured on a digital thermometer with a thermistor probe immersed into the sample. Ultraviolet spectra were recorded on a Gilford 240 spectrophotometer equipped with a 6040A recorder. Molar concentrations of the dimer were calculated from ultraviolet spectra using the sums (Cantor & Tinoco, 1965) of the extinction coefficients of the respective constituent monomers taken from the CRC Handbook of Biochemistry (1970). The ϵ_{278} values obtained for GpU and G*pU were 1.1×10^4, respectively. The ϵ_{278} for G* (5.4×10^4) was taken from Jeffrey et al. (1977) and was derived as previously described (Weinstein et al., 1976). Ellipticities were expressed on the molar basis $[\theta]$ [(deg/mol^{-1} cm^{-1})100] and were not corrected for the refractive index of the solvent.

Results and Discussion

The reaction of GpU with BPDE I resulted in the formation of multiple dinucleoside monophosphate–BPDE derivatives (G*pU). G*pU was separated from unmodified GpU and BPDE decomposition products on a Sephadex LH-20 column and eluted during a methanol–0.02 M NH_4HCO_3 gradient as one peak (Figure 1). This peak was further resolved into four compounds by HPLC (Figure 2) which were designated I, II, III, and IV, in their order of elution. To determine whether these components were actually dinucleoside monophosphates modified with BPDE, they were hydrolyzed to nucleosides and the hydrolyzates analyzed by HPLC as described in Methods. This established that all four compounds contained a uridine residue as well as a modified guanosine residue "G*".

Previous studies indicated that the reaction of poly(G) or RNA with BPDE I results in the formation of multiple gua-

TABLE I: HPLC of Modified G*s Obtained from Hydrolyzates of G*pU and Poly(G*).[a]

Compound	Retention time (min)
G* from G*pU I	17
G* from G*pU II	15
G* from G*pU III	26
G* from G*pU IV	26
G* 1 from poly(G)	15
G* 2 from poly(G)	17
G* 3 from poly(G)	26

[a] The poly(G) samples were provided by Dr. A. M. Jeffrey and have been described previously (Jeffrey et al., 1976). G* 4, corresponding to that obtained from G*pU IV, was not obtained in sufficient yield from poly(G) for further study.

TABLE II: Effects of Temperature and Solvent on the Molar Ellipticity Values of G*pU Compounds ($[\theta]_{282} \times 10^4$).

Compound	Water		Methanol at 25 °C	
	25 °C	80 °C	50%	100%
G*pU I	19.5	12.8	17.5	
II	15.8	10.0	13.6	8.6
III	18.6	13.6	15.5	13.6
IV	13.1	9.2	9.8	6.4

nosine adducts (Weinstein et al., 1976; Jeffrey et al., 1977). This is due to the fact that BPDE I is an enantiomeric mixture, as well as the fact that both cis and trans addition products with guanosine can be obtained. This means that there are four possible stereoisomers of G*.

Table I indicates that the G* compounds present in the modified dimers corresponded to four guanosine adducts previously detected and characterized in poly(G) and RNA modified by BPDE (Weinstein et al., 1976; Jeffrey et al., 1977; Jennette et al., 1977). The fourth component, retention time 26 min, is not well resolved as the free nucleoside (see below). Compounds I to IV contain different stereoisomers of G* (Table I) and this explains their separation into four distinct compounds by HPLC. In all four compounds the C-10 position of BPDE is covalently linked to the N^2 position of G. In G*pU II and IV the exocyclic amino group of G is trans to the C-9 hydroxyl of the hydrocarbon (Jeffrey et al., 1976, 1977; Nakanishi et al., 1977) and in G*pU I and III it is cis (Moore et al., 1977). Further evidence for this interpretation is provided below.

In an attempt to determine how the covalent binding of the BP residue to the N^2 position of guanosine residues in these dimers affects their conformation, we have measured their CD spectra. Figures 3A and B show the CD spectra of GpU and G*pU I through IV. These spectra indicate that the contribution of unmodified GpU to $[\theta]$ values is minimal (Figure 3A).

The CD spectra of G*pU I and III are almost exact mirror images (Figure 3A) and the same relationship is true of G*pU II and IV (Figure 3B). The near antipodal relationship between these two pairs of adducts, plus the fact that their CD spectra share several qualitative similarities with G* monomers (Jeffrey et al., 1976), provides further evidence that the four modified dimers differ from each other by their content of different stereoisomers of G* (Jeffrey et al., 1976; Moore et al., 1977). This conclusion is consistent with the above studies (see Table I) on hydrolyzates of the modified dimers. The molar ellipticity values at 282 nm of the dimers are, however, about 1.5- to 2.0-fold higher than those of the corresponding

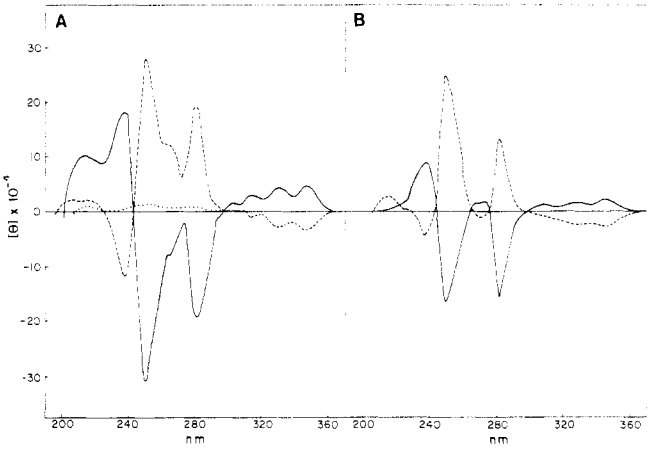

FIGURE 3: CD spectra of GpU and G*pU adducts. Adducts were purified by HPLC prior to the CD measurements, as described in Figure 2 and Methods. Spectra were determined in water, and $[\theta]$ was based on $\epsilon_{278} = 1.16 \times 10^4$ for GpU and $\epsilon_{278} = 5.84 \times 10^4$ for G*pU. (A) G*pU I (—); G*pU III (- - -); GpU (· · · ·). (B) G*pU II (—); G*pU IV (- - -).

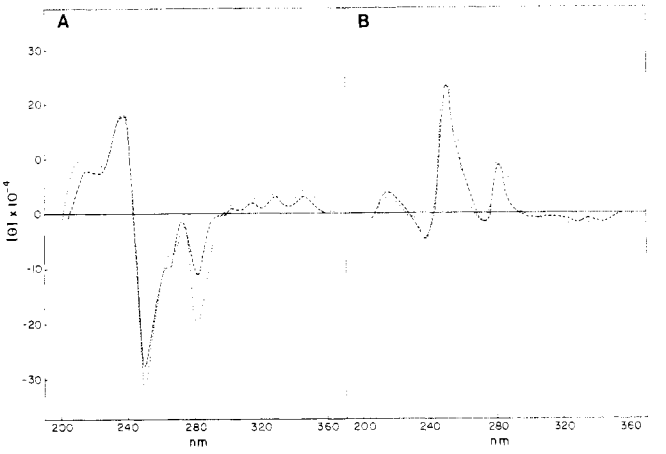

FIGURE 4: Temperature dependence of the CD spectra of G*pU adducts. (A) G*pU I; and (B) G*pU IV. Spectra were recorded in water at room temperature (· · · ·); and at 80 °C (- - - -).

G* monomers. Thus, the $[\theta]$ values at 282 nm of the monomers G* 1, 2, and 3 in 50% methanol were 6.6, 12.0, and 6.2×10^4, respectively (Jeffrey et al., 1976), whereas the $[\theta]$ values of the corresponding modified dimers II, I, and IV were 13.6, 17.5, and 9.8×10^4, respectively (Table II). The fourth G* monomer was not obtained in sufficient quantity for direct comparison of its CD spectrum with that of the dimer G*pU III.

The increased $[\theta]$ values of the modified dimers suggested a stacking interaction between the BPDE residue and the adjacent uracil base. It is known that this type of stacking can be disrupted by organic solvents or by elevated temperatures (Nelson et al., 1971; Topal & Warshaw, 1976; Brown & Shapiro, 1977). Therefore, we studied the CD spectra of all four G*pU isomers in water–methanol mixtures and also with heating up to 80 °C.

Figures 4A and B show the effects of heating to 80 °C on the CD spectra of G*pU I and IV. At intermediate temperatures (21 to 80 °C there was a gradual decrease of $[\theta]$ values [not shown]). Compound II showed changes with increased temperature similar to IV, and III showed changes similar to I. (The spectra are not shown.) The most pronounced and consistent change occurred at the 282-nm extremum. The effects of heating and methanol on $[\theta]$ values of modified dimers at 282 nm are summarized in Table II. These show that heating

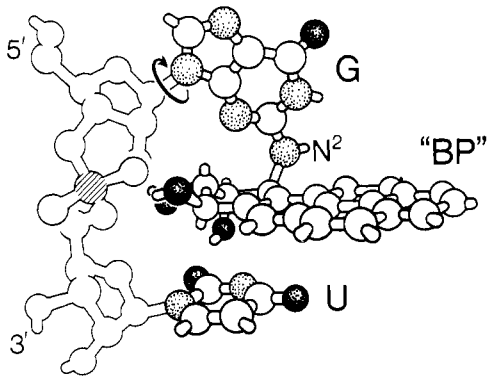

FIGURE 5: Schematic representation of the proposed conformation of G*pU II and IV (trans adducts). (○) Designates oxygen; (◎) nitrogen; (●) phosphorus; (BP) the BPDE residue following its covalent attachment to the N^2 of G. The G residue has been rotated about its glycosidic bond to allow "insertion" of the "BP" residue coplanar to the adjacent U.

or methanol produced a significant decrease in the $[\theta]$ values of all four compounds. The decrease varied from 27% for G*pU III to 50% for G*pU IV when samples were dissolved in 100% methanol rather than water. The $[\theta]$ values of all four G*pU samples dissolved in 100% methanol approach those of the corresponding G* monomers dissolved in water. These results show that an organic solvent or heating diminishes interactions between the BPDE residue and the adjacent base, but do not appear to change the basic conformation of the guanosine-BPDE adduct. Although there are changes in CD spectra of unmodified GpU at higher temperatures and methanol (Davis, 1966; Warshaw & Cantor, 1970), we must emphasize that the $[\theta]$ values obtained with modified dimers are about ten times greater and therefore the effect exerted by solvent and temperatures is mainly due to the interaction between BPDE and uracil, rather than between guanine and uracil residues.

The fact that the intensity of the CD extrema, particularly at 282 nm, was greater in the modified dimers than in the modified monomer, and that this intensity was appreciably reduced in the dimers by increased temperature or methanol as solvent, strongly suggests a stacking interaction between the hydrocarbon moiety and the uracil base adjacent to the modified G. Evidence for this type of stacking interaction has been presented for oligomers modified by other polycyclic aromatic compounds (Nelson et al., 1971; Boublik et al., 1975; Brown & Shapiro, 1977). Although this is the most likely interpretation, our data do not in themselves exclude other mechanisms which might enhance the CD extrema of the modified dimers.

The feasibility of the above interpretation was examined with CPK space-filling models. A plausible model for G*pU II or G*pU IV, the two trans products, is shown schematically in Figure 5. Starting with an oligomer having the usual parameters of RNA (Sundaralingam, 1975), a conformation which maximizes stacking interaction between the pyrene ring system and the neighboring uracil was achieved, largely by rotating the guanine residue about 45 degrees around its glycosidic bond, as shown in Figure 5. The plane of the pyrene ring is perpendicular to the long axis of phosphate-sugar backbone and the guanine is no longer coplanar to the neighboring uracil. At the same time, the hydrophilic hydroxyls of the BPDE residue face the ribose of the modified G. A similar model can be constructed with the cis adducts G*pU I and III.

We must stress, however, that our CD data, while providing evidence for a physical interaction between the BPDE residue and an adjacent uracil, do not in themselves establish that this is a stacking interaction. It is possible that the BPDE residue interacts with the adjacent base but is not actually coplanar to it. Definitive assignments of the conformations of the BPDE modified dimers, however, require further studies, such as NMR spectroscopy and x-ray crystallography.

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Modification of *Rhodospirillum rubrum* Ribulose Bisphosphate Carboxylase with Pyridoxal Phosphate. 1. Identification of a Lysyl Residue at the Active Site†

William B. Whitman and F. Robert Tabita*

ABSTRACT: Ribulose 1,5-bisphosphate carboxylase isolated from *Rhodospirillum rubrum* was strongly inhibited by low concentrations of pyridoxal 5′-phosphate. Activity was protected by the substrate ribulose bisphosphate and to a lesser extent by other phosphorylated compounds. Pyridoxal phosphate inhibition was enhanced in the presence of magnesium and bicarbonate, but not in the presence of either compound alone. Concomitant with inhibition of enzyme activity, pyridoxal phosphate forms a Schiff base with the enzyme which is reversible upon dialysis and reducible with sodium borohydride. Subsequent to reduction of the Schiff base with tritiated sodium borohydride, tritiated N^6-pyridoxyllysine could be identified in the acid hydrolysate of the enzyme. Only small amounts of this compound were present when the reduction was performed in the presence of carboxyribitol bisphosphate, an analogue of the intermediate formed during the carboxylation reaction. Therefore, it is concluded that pyridoxal phosphate modifies a lysyl residue close to or at the active site of ribulose bisphosphate carboxylase.

Ribulose bisphosphate carboxylase [3-phospho-D-glycerate carboxy-lyase (dimerizing), EC 4.1.1.39] is the primary carbon dioxide fixing enzyme in most photosynthetic and chemosynthetic organisms (McFadden & Tabita, 1974). Considering the importance of this enzyme in autotrophic metabolism, little is known about the structure of the active site. In part, this is due to the lack of reagents specific for the active site of this enzyme. While sulfhydryl directed reagents have long been known to inhibit enzymatic activity (Rabin & Trown, 1964), they also modify nonessential sulfhydryl groups and cause the dissociation of the quaternary structure (Sugiyama et al., 1967, 1968; Nishimura et al., 1973). More recently, the affinity label, 3-bromo-1,4-dihydroxy-2-butanone 1,4-bisphosphate, has been used to modify essential lysyl residues at the active site of the spinach and *Rhodospirillum rubrum* ribulose bisphosphate carboxylase (Norton et al., 1975; Schloss & Hartman, 1977). However, this reagent also modifies sulfhydryl groups and spontaneously decomposes under the conditions used for modification (Hartman et al., 1973). Therefore, it seemed worthwhile to investigate other reagents known to react with amino acids in proteins for their potential to specifically modify the active site of ribulose bisphosphate carboxylase.

Pyridoxal 5′-phosphate has been shown to selectively modify primary amino groups in or near phosphate binding sites of many proteins (Columbo & Marcus, 1974). Low concentrations of pyridoxal phosphate are inhibitory for the ribulose bisphosphate carboxylase from *Rhodospirillum rubrum, Chlamydomonas reinhardtii, Alcaligenes eutrophus* (*Hydrogenomonas eutropha*), and spinach leaf (Whitman & Tabita, 1976). The enzyme from *R. rubrum* is protected from pyridoxal phosphate inhibition by the substrate, ribulose bisphosphate (Whitman & Tabita, 1976). Pyridoxal phosphate is a competitive inhibitor with respect to carbon dioxide and noncompetitive with respect to ribulose bisphosphate for the spinach enzyme (Paech et al., 1977). The spinach enzyme also binds 2 mol of pyridoxal phosphate/mol of catalytic subunit, presumably 1 mol at the catalytic site and 1 mol at a regulatory site. Pyridoxal phosphate was also reported to inhibit the oxygenase activity of the spinach enzyme as well as the carboxylase activity (Paech et al., 1977).

The present study will further elucidate the inhibition by pyridoxyl phosphate of the *R. rubrum* ribulose bisphosphate carboxylase. Unlike other carboxylases hitherto purified, the enzyme from *R. rubrum* is a dimer of large, catalytic type subunits (Tabita & McFadden, 1974a), as compared with the spinach enzyme which is a hexadecamer of eight large and eight small subunits (Rutner, 1970). This relative structural simplicity may be used to advantage in determining the site of pyridoxal phosphate modification and the manner in which it interacts with the enzyme. In addition, comparisons of structure–function relationships between the ribulose bisphosphate carboxylase from photosynthetic microorganisms such as *R. rubrum* and the modern higher plant enzyme may provide useful insights into the evolutionarily conserved features of the carboxylase molecule.

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