drogenases, Schwert, G. W., and Winer, A. D., Ed., Louisville, Ky., The Univ. Press of Kentucky, p 179.
Fisher, H. F., and Cross, D. G. (1962), *Nature (London) 196*, 895.
Fisher, H. F., McGregor, L. L., and Power, U. (1962), *Biochem. Biophys. Res. Commun. 8*, 402.
Fiskin, A. M., and Beer, M. (1968), *Science 159*, 1111.
Foster, J. F. (1960), *in* The Plasma Proteins, Vol. 1, Putnam, F., Ed., New York, N. Y., Academic Press, p 179.
Frieden, C. J. (1962), *J. Biol. Chem. 237*, 2396.
Frieden, C. (1964), *Brookhaven Symp. Biol. 17*, 98.
Haber, J. E., and Koshland, D. E. (1967), *Proc. Nat. Acad. Sci. U. S. 58*, 2087.
Haine, M. E. (1961), The Electron Microscope; The Present State of the Art, Chapter IV, New York, N. Y., Interscience.
Harrington, W., Johnson, P., and Ottewill, R. (1956), *Biochem. J. 62*, 569.
Haydon, G. B. (1969), *J. Micros. 89*, 251.
Heidenreich, R. D. (1964), Fundamentals of Transmission Electron Microscopy, New York, N. Y., Interscience, p 139.
Highton, P. J., and Beer, M. (1968), *J. Roy. Micros. Soc. 88*, 23.
Horne, R. W., and Greville, G. D. (1963), *J. Mol. Biol. 6*, 506.
Jirgensons, B. (1961), *J. Amer. Chem. Soc. 83*, 3161.
Josephs, R. (1970), Pyridine Nucleotide-Dependent Dehydrogenases, Berlin, Springer-Verlag, p 301.
Kirtley, M. E., and Koshland, E. E. (1967), *J. Biol. Chem. 242*, 4192.
Kiselev, N. A., and Klug, A. (1969), *J. Mol. Biol. 40*, 155.
Klotz, I. M., Langerman, N. R., and Darnall, D. W. (1970), *Annu. Rev. Biochem. 39*, 25.
Koshland, D. E., Nemethy, G., and Filmer, D. (1966), *Biochemistry 5*, 365.
Luzzati, V., Witz, J., and Nicolaieff, A. (1961), *J. Mol. Biol. 3*, 379.
Marks, R. W. (1960), The Dymaxion World of Buckminster Fuller, New York, N. Y., Reinhold, p 41.
Mellema, J. E., van Bruggen, E. F. J., and Gruber, M. (1967), *Biochim. Biophys. Acta 140*, 182.
Monod, J., Wyman, J., and Changeaux, J.-P. (1964), *J. Mol. Biol. 10*, 565.
Olsen, J. A., and Anfinsen, C. B. (1952), *J. Biol. Chem. 197*, 67.
Smith, E., Landon, M., Piszkiewicz, D., Bratton, W. J., Langley, T. J., and Melamed, M. D. (1970), *Proc. Nat. Acad. Sci. U. S. 67*, 724.
Sund, H., and Burchard, W. (1968), *Eur. J. Biochem. 6*, 202.
Sund, H., Pilz, I., and Herbst, M. (1969), *Eur. J. Biochem. 7*, 517.
Valentine, R. C., Shapiro, B. M., and Stadtman, E. R. (1968), *Biochemistry 7*, 2143.
Whitehead, E. P. (1965), *J. Theor. Biol. 8*, 276.
Wolff, J. (1962), *J. Biol. Chem. 237*, 230.

Nature of the Binding Site of Pyridoxal 5′-Phosphate to Bovine Serum Albumin*

John A. Anderson,† Hsai-fei Wang Chang,‡ and Carter J. Grandjean§

ABSTRACT: In the reaction between equimolar amounts of pyridoxal 5′-phosphate (PLP) and bovine serum albumin only one pyridoxyl peptide was found following reduction at pH 4.2–4.4 and subsequent digestion with trypsin. The amino acid sequence of the purified peptide was Ser-Leu-Phe-Glu-Lys-Pro-ε-(pyridoxyl)Lys-Lys. The presence of three lysine residues at the PLP binding site is consistent with the substituted aldimine structure as the species responsible for the 336-nm absorption peak of the PLP–albumin complex. The predominate species which absorbs at 330 nm in PLP–albumin–urea solutions is a PLP–urea product. The absorbance peak at 330–336 nm is small and there is a large 413-nm peak immediately after addition of urea to the PLP–albumin mixture. It is concluded that the PLP–albumin product with absorbance maximum at 336 nm is unstable in urea. The fluorescence of the 336-nm peak of PLP–albumin is quenched. Reaction of the tryptophans of albumin with *N*-bromosuccinimide did not affect the affinity, absorbance spectrum, or quenching of fluorescence of the binding site. Nitration of exposed tyrosines with tetranitromethane (10.7 tyrosines/mole of albumin) caused reduced binding of PLP to albumin. Quenching of fluorescence may be due to interaction with tyrosine residues.

Pyridoxal 5′-phosphate binds to the ε-amino group of lysine in proteins (*e.g.*, Kent *et al.*, 1958). There are several types of bound forms. One of these is the protonated Schiff base, which has an absorption maximum in the 410- to 430-nm region of the spectrum. In aspartate aminotransferase (Hughes *et al.*, 1962; Polianovsky and Keil, 1963; Morino and Watanabe, 1969) the Schiff base may not be protonated and the absorption maximum is shifted to the region of 360 nm. Finally, PLP[1] is bound in a form which absorbs maximally in the 330-nm region. This last species occurs in the neutral pH region, is unaffected by carbonyl reagents and is

* From the Department of Chemistry, Texas Tech University, Lubbock, Texas 79409. *Received December 28, 1970.* This work was supported by NSF Grant No. GB-6434 and by Robert A. Welch Foundation Grant No. D-117. Part of this work was done in partial fulfillment of the M. S. degree of H.-F. W. C. and of the Ph.D. degree of C. J. G.

† Present address: Massachusetts General Hospital, Boston, Mass.

‡ Present address: The Eppley Institute for Research in Cancer, Omaha, Neb.

§ Author to whom correspondence should be addressed.

[1] Abbreviations used are: PLP, pyridoxal 5′-phosphate; PTH, phenylthiohydantoin; TMN, tetranitromethane.

nonreducible with sodium borohydride (Kent *et al.*, 1958). If the neutral solution in which the species is observed is adjusted away from neutrality, the solution becomes intensely yellow, with the concomitant appearance of a peak in the region of 410–430 nm (Kent *et al.*, 1958). The yellow color is due to the formation of a Schiff base. The Schiff base is reducible by sodium borohydride and the PLP moiety is thereby irreversibly bound to the protein. Structures which have been proposed for the product with absorbance maximum near 330 nm are (a) the substituted aldimine (Kent *et al.*, 1958), (b) carbinolamine (Dempsey and Christensen, 1962), and (c) nonionic H-bonded Schiff base in a hydrophobic environment (Shaltiel and Cortijo, 1970) (Figure 1).

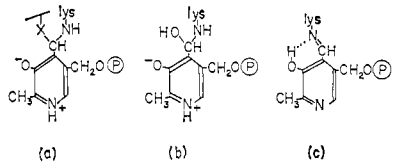

FIGURE 1: Proposed structures for PLP bound to protein in the form with absorbance near 330 nm. (a) Substituted aldimine, (b) carbinolamine, and (c) nonionic H-bonded Schiff base.

Dempsey and Christensen (1962) have demonstrated the presence in bovine serum albumin (hereafter simply referred to as albumin) of two sites with high affinity for PLP. One of these sites (site I) has an equilibrium constant that is at least an order of magnitude greater than the other site (site II) and has an absorption maximum at 332 nm in the neutral pH region. This paper reports the isolation and characterization of the primary binding site of PLP to albumin. The effect of urea on site I and the fluorescent properties of site I are also described.

Materials and Methods

ε-Pyridoxyllysine was prepared according to the method of Dempsey and Christensen (1962). *Escherichia coli* alkaline phosphatase, trypsin, albumin, PLP, carboxypeptidases A and B diisopropylfluorophosphate treated to eliminate tryptic and chymotryptic activities, and standards of PTH-glutamic acid and glutamine were all obtained from Sigma Chemical Co. 2,6-Dichloro-1,4-quinone-4-chloroimide was obtained from Chemical Procurement Laboratories, Inc. Dowex 50X-2 and Dowex 1X-2 (both 200–400 mesh) were obtained from J. T. Baker Chemical Co. The resins were cleaned according to the method of Schroeder (1967) prior to their use. Ultra Pure urea was from Mann Research Laboratories.

Borohydride reduction of equimolar PLP–albumin was performed as described by Dempsey and Christensen (1962, method 1, p 1114).

The trypsin digestion was accomplished by adjusting the pH of the reduced material to pH 8.5 with 10% ammonium hydroxide and adding 3% trypsin (w/w). The pH was brought to 8.5 by further additions of ammonium hydroxide during the first 5 hr at room temperature. After this time the reaction mixture was placed in an oven at 37° for 19 hr. After 24-hr incubation with trypsin the pH did not change appreciably, thereby indicating that the reaction was complete. Additional trypsin added after 24 hr did not bring about further degradation. Following digestion the material was lyophilized, redissolved in a minimal amount of water, centrifuged, and relyophilized.

Ion-exchange chromatography was carried out as follows. The purification of the site I peptide was done by using successively Dowex 50-X2 (200–400 mesh) and Dowex 1X-2 (200–400 mesh). In all of following operations light was excluded as much as possible in order to minimize photodecomposition.

For cation column chromatography (Dowex 50-X2) the lyophilized crude trypsin digest (7.7 μmoles) was dissolved in a minimal amount of 0.2 M ammonium acetate buffer (pH 4.3). The sample was applied to the column (1.9 × 80 cm) which had been equilibrated with the starting buffer (NH_4OAc, 0.2 M, pH 4.75). The column was then developed using a stepwise elution of the following buffer systems: (1) ammonium acetate, 0.2 M, pH 4.75, 200 ml; (2) ammonium phosphate 0.3 M, pH 6.7, 200 ml; and (3) ammonium bicarbonate, 1.0 M, pH 8.0, 200 ml. Fractions from Dowex 50 column chromatography which showed significant absorbance at 330 nm were pooled, lyophilized, and the residue redissolved in 0.1 M triethylamine acetate at pH 9.4. The solution was then applied to a column (1.3 × 56 cm) of Dowex 1X-2 which had been equilibrated with 0.1 M triethylamine acetate (pH 8.9). The column was subsequently developed using a linear gradient of 0.4 M acetate (pH 5.0) into the 0.1 M buffer at pH 8.9. Fractions of 2 ml were collected and again each fraction was checked for absorbance at 280 and 330 nm.

The final purification of the pyridoxyl peptide was achieved by Strausbauch's and Fischer's (1970) method using *E. coli* alkaline phosphatase on the 5′-pyridoxyl peptide. The pooled fractions from the Dowex 1 column were lyophilized and the dried material was redissolved in 3 ml of 0.05 M ammonium bicarbonate (pH 8.2). The amount of 5′-P-pyridoxyl peptide was approximately 2 μmoles. This solution was treated with 125 μg of phosphatase for 2.5 hr at room temperature and subsequently lyophilized. This sample was then fractionated on Dowex 1X-2 exactly as before.

Amino acid analysis was performed on a Beckman 116 amino acid analyzer according to Spackman *et al.* (1958). The samples were hydrolyzed *in vacuo* in constant-boiling HCl at 110° for 24 hr. During this time there is a 6% loss of serine and a 28% loss of ε-pyridoxyllysine. Upon hydrolysis the ε-pyridoxyllysine decomposes into lysine and other unidentified products (Forrey, 1963). However, the decomposition products do not interfere with the analyses. The above corrections were used in all subsequent hydrolysate analyses.

The analysis of ε-pyridoxyllysine was performed on the standard column for basic amino acids (0.9 × 7 cm) using Beckman's custom resin PA-35. The flow rate of the sodium citrate buffer (0.13 M, pH 5.30) was 68 ml/hr. Under these conditions the ε-pyridoxyllysine is eluted after lysine in the same position where histidine is eluted.

The subtractive Edman procedure by Konigsberg and Hill (1962) was modified and used for all N-terminal amino acid analyses. All analyses were done in triplicate, and all reagents were purified prior to their use. The phenyl isothiocyanate was redistilled just prior to each set of degradations. The modified procedure as used in this investigation was as follows. Approximately 10–20 nmoles of the pyridoxyl peptide was allotted for each degradation. A suitable amount of dried sample was dissolved in 0.3 ml of buffer which contained 50% aqueous pyridine and 2% triethylamine (pH 9.2). The solution was flushed with nitrogen and nitrogen was left above the solution. To this mixture was added 10 μl of freshly distilled phenyl isothiocyanate and the mixture was flushed again with nitrogen. The sealed system was then allowed to react for 2 hr at room temperature after which time the solution was evaporated almost to dryness under vacuum. The material was

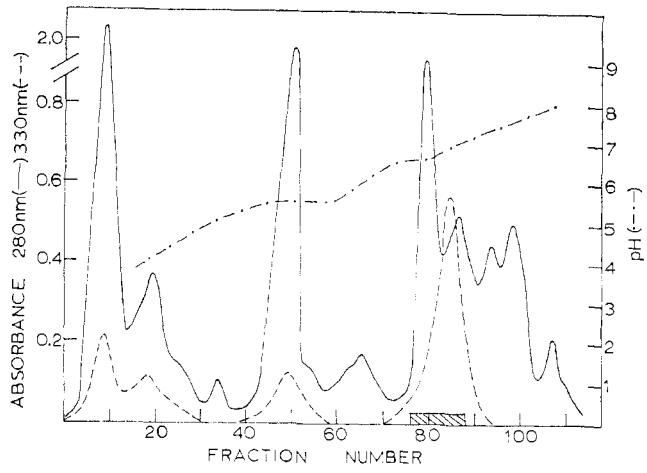

FIGURE 2: Elution pattern for column chromatography of the trypsin digest of the albumin–PLP complex (1:1, v/v), reduced with sodium borohydride at pH 4.4. Column is 1.9 × 80 cm of Dowex 50X-2. Elution is described under Methods. Fractions of 5 ml were collected (——) Absorbance at 280 nm; (---) absorbance at 330 nm.

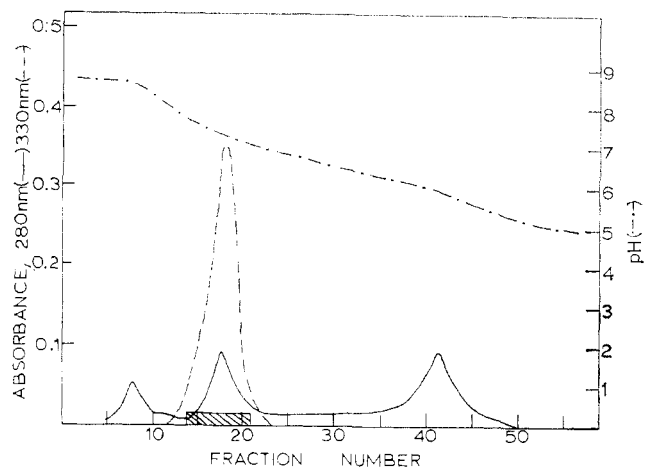

FIGURE 4: Elution pattern for column chromatography of the collected material from anion-exchange column and subsequently digested with alkaline phosphatase as described under Methods. The column and its development are identical as described for Figure 3.

extracted four times with benzene, and the last traces of benzene were removed by a high vacuum for 5 min. The phenylthiocarbamyl peptide was reacted with anhydrous trifluoroacetic acid at room temperature for 1 hr. Precautions were again taken to exclude oxygen from the system. The trifluoroacetic acid was removed by vacuum and the resulting material was exposed to 1 ml of 0.2 M acetic acid at 40° for 10 min. The final products were extracted three times with ethyl ether and twice with ethyl acetate. The excess ethyl acetate was evaporated from the aqueous phase and a suitable aliquot was taken for amino acid analysis.

Digestion with carboxypeptidases A and B was used to determine the amino acid sequence from the C-terminal portion of the peptide. The incubation proceeded at 37° in 0.5 ml of 0.2 M collidine buffer. At different periods of time a single tube was taken, the buffer removed and the dried material was redissolved in water and run directly on the amino acid analyzer.

Spectrophotometric and Fluorometric Determinations. All determinations were at room temperature. The PLP–albumin and PLP–albumin–urea mixtures were incubated 6 or more hr at room temperature before use to ensure that reaction was complete. The buffer was 0.1 M sodium phosphate (pH 7.5).

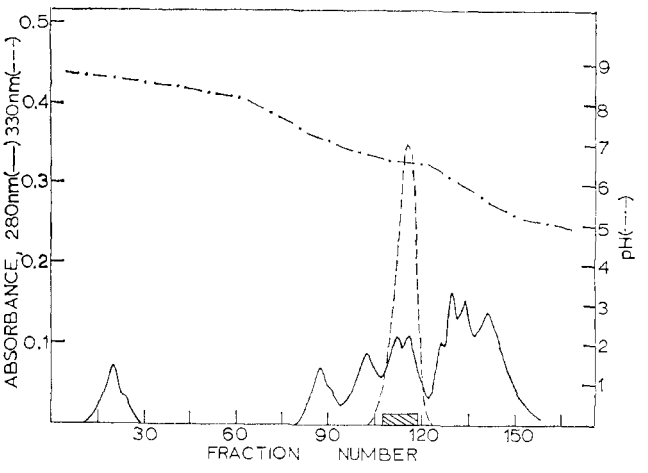

FIGURE 3: Elution pattern for column chromatography of the collected, lyophilized and redissolved material from cation exchange. Column is 1.3 × 56 cm of Dowex 1X-2. Elution is described under Methods. Fractions of 2 ml were collected.

Results

Trypsin digestion of the reduced albumin–PLP complex resulted in only one peptide which contained the pyridoxyl moiety. A small sample of the trypsin digest was analyzed by high-voltage electrophoresis on Whatman No. 3MM paper in a buffer system consisting of pyridine–acetic acid–water (1:2:250, v/v) at pH 4.4. Detection of the pyridoxyl peptide was made by observing its characteristic blue fluorescence and its reaction with 2,6-dichloro-1,4-quinone-4-chloroimide.

Purification of the Pyridoxyl Peptide. The observation of only one pyridoxyl peptide after electrophoresis is supported by the appearance of only one peak that has any significant absorption in the 330-nm region. In Figure 2 is shown a typical elution pattern for the cation column chromatography. It is apparent that only one major peak has any significant absorbance at 330 nm and the fractions that were pooled are indicated. A partial pH gradient is also shown which shows that the pyridoxyl peptide is eluted at about pH 6.9.

The combined material from the cation-exchange column was applied to the anion exchange column and developed. Figure 3 shows a typical elution pattern for this chromatography. Again only one major peak which has any significant 330-nm absorbance is indicated. However, the absorbance at 280 nm indicates appreciable contamination from other peptides.

After digestion with phosphatase, the lyophilized sample was developed on the anion-exchange column as described above. A typical elution pattern of the phosphatase-treated peptide mixture is shown in Figure 4. Since the pyridoxyl-containing peptide has lost the negatively charged phosphate group, it is eluted at a higher pH (8.0). The now obvious contaminating peptide emerges at approximately the same pH (6.7) as it did previously. The fractions that were collected as indicated were subsequently lyophilized and the purity of the final product was determined.

Upon high-voltage electrophoresis of the final product only one ninhydrin-positive spot was observed, and upon amino

TABLE I: Digestion of Pyridoxyl Peptide with Carboxypeptidase (A and B).[a]

Residue[b]	Acid Hydrolysate[c] (Integrals)	Residues in Integrals hr of Incubation with Carboxypeptidase 0.3	1.0	1.5	6
Lys	1.99[d]	0.22	0.62	1.07	0.97
ϵ-(Pyridoxyl)lys	0.89[d]	0.00	0.36	1.01	1.00

[a] Digestion was carried out using 5 μg each of carboxypeptidases A and B per 10 nmoles of peptide. [b] All other residues in the peptide were not released at all and are omitted from the table. [c] Represents 10 nmoles of the peptide which has been hydrolyzed in constant-boiling HCl for 24 hr at 110°. [d] Corrected for conversion of ϵ-(pyridoxyl)Lys into Lys and other products (see Methods). Integrals were calculated dividing the number of moles for each acid as taken from the analyzer by the average number of moles per residue in the peptide.

acid analysis an integral number could be assigned to each amino acid present in the peptide. These results indicated that the peptide was almost free from contamination.

The amino acid composition of the pyridoxyl peptide showed it to be rich in lysine. The composition was found to be Lys[2], ϵ-(pyridoxyl)Lys,Ser,Glu,Pro,Leu,Phe. Hydrolysis from the C-terminal end of the peptide using the carboxypeptidases was limited to 1 mole each of lysine and ϵ-pyridoxyllysine. The results of this digestion are shown in Table I. A possible explanation of the inability of carboxypeptidases A and B to release any more amino acids is that the C terminus, proline is adjacent to a lysine residue (Shepherd *et al.*, 1956; Hirs *et al.*, 1960). The results show that the sequence at the C-terminal end H_2N-Pro(?)-ϵ-(pyridoxyl)Lys-Lys-COOH.

Analysis from the amino-terminal end of the peptide by the subtractive Edman procedure is shown in Table II. The amino acids in italics were those which were presumed to be N terminal at each stage of the procedure. The amino acid analyses for the peptides remaining after each stage of the degradation were normalized by assuming that the average amount of amino acid (in nanomoles) represented an integral number of each residue. The entire amino acid sequence was determined with seven steps of the Edman procedure. The sequence is therefore H_2N-Ser-Leu-Phe-Glx-Lys-Pro-ϵ-(pyridoxyl)Lys-Lys-COOH.

Because the "subtractive" Edman procedure was used, and the use of carboxypeptidases A and B was halted after releasing only two residues, it was not possible to assign glutamic acid or glutamine positively to the sequence of the peptide. Therefore, the PTH derivative from the fourth step in the Edman degration was saved and purified further. The purified PTH derivative was then compared with authentic samples of PTH-Glu and PTH-Gln in the following way. The unknown, along with the two standards were chromatographed on fluorescent silica gel using solvent E devised by Edman and Sjöquist (1956). Following development of the silica gel plate, the spots were located by observing the fluorescence quenched regions The R_F value of the unknown turned out to be almost identical with that for PTH-Glu (0.70) as opposed to that for PTH-Gln (.30).

TABLE II: Edman Degradation of Pyridoxyl Peptide.[a]

Step No.	Ser	Glu	Pro	Leu	Phe	Lys	ϵ-(Pyridoxyl)-Lys
0	0.89	1.00	0.93	1.00	1.06	2.17	0.97
1	*0.14*	1.07	1.02	1.00	0.99	2.09	0.87
2	0.19	0.92	1.06	*0.06*	1.00	2.20	1.03
3	0.10	1.01	0.96	0.05	*0.16*	2.12	1.02
4	0.05	*0.00*	0.89	0.00	0.10	1.98	1.00
5	0.04	0.03	1.00	0.04	0.11	*1.18*	0.93
6	0.00	0.03	*0.00*	0.00	0.11	1.09	1.01
7	0.00	0.01	0.00	0.02	0.05	1.11	*0.15*

[a] Integrals were calculated as described under footnote *d* in Table I.

From the data resulting from the Edman degradation and digestion with carboxypeptidases A and B there appears to be no ambiguity in the amino acid sequence of the pyridoxyl peptide as being H_2N-Ser-Leu-Phe-Glu-Lys-Pro-ϵ-(pyridoxyl)-Lys-Lys-COOH.

Albumin with PLP bound at site I by reduction with borohydride was digested with pepsin according to the procedure of Weber and Young (1964), and then fractionated according to the procedure of Peters and Hawn (1967). The fractions containing "Asp" fragment and "Phe" fragment were negative for the pyridoxyl moiety. Since these two fragments are from the N-terminal and C-terminal portions of albumin, site I must be located in the interior of the polypeptide chain.

The difference spectrum of 1:1 PLP–albumin *vs.* albumin is shown in Figure 5. Besides the absorbance peak at 336 nm there is an additional peak at 253 nm. With varying PLP concentration the ratio A_{253}/A_{336} was constant with a value of 2.2. The peak at 253 nm is therefore due to site I.

A shoulder in the difference spectrum appeared consistently between the 253- and 336-nm absorbance peaks. When these peaks were subtracted from the difference spectrum with the DuPont curve resolver, a peak was obtained with absorbance maximum at 280 nm.

The absorbance at 413 nm was consistently more in 6.0 M urea than in the absence of urea at pH 7.5 (Figure 5). When

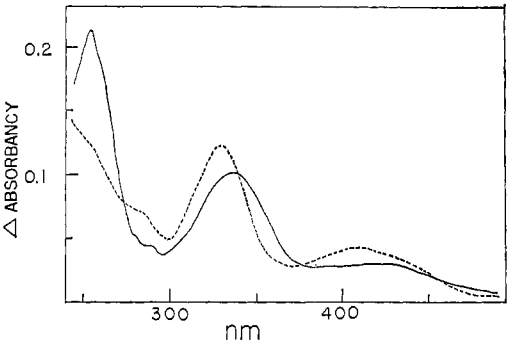

FIGURE 5: Difference spectra of PLP–albumin mixtures. Spectra were measured with the Cary 14 spectrophotometer with 1-mm path cells. (——) Reference cell, PLP–albumin; sample cell, albumin· (-----) Reference cell, PLP–albumin–urea; sample cell, albumin–urea. Concentrations were 3.3×10^{-4} M albumin and PLP and 6.0 M urea in 0.1 M sodium phosphate (pH 7.5).

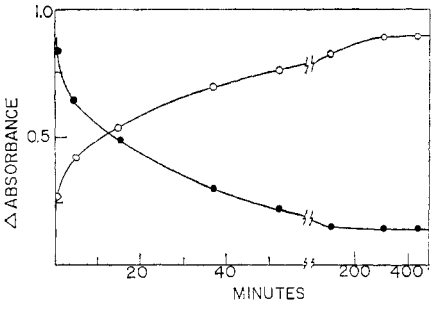

FIGURE 6: Change in absorbance with time after addition of urea to 1:1 albumin–PLP. At zero time, 1.7 ml of 9.3 M urea in 0.05 M sodium phosphate (pH 7.5) was mixed with 0.3 ml of 8.3×10^{-4} M albumin, 8.3×10^{-4} M PLP, in 0.05 M sodium phosphate (pH 7.5). Final concentrations were 1.2×10^{-4} albumin, 1.2×10^{-4} M PLP, and 7.9 M urea. The PLP–albumin mixture was incubated overnight at room temperature before addition of urea. Absorbance was measured against a reference sample which contained 1.2×10^{-4} M albumin and 7.9 M urea. The instrument was a Bausch & Lomb Spectronic 600 spectrophotometer. Temperature was 25°.

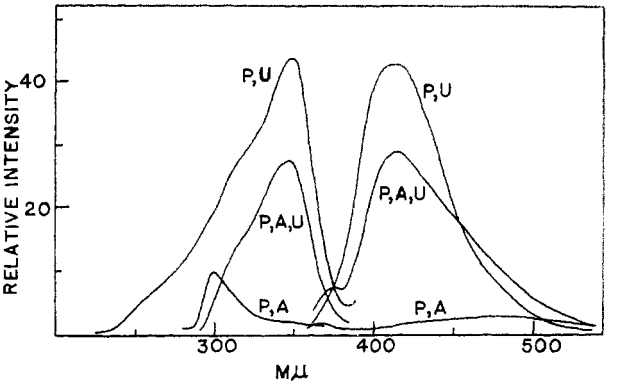

FIGURE 7: Fluorescence spectra of PLP–albumin mixtures. Excitation (left) and emission (right) spectra were measured with the Aminco-Bowman spectrofluorometer. The spectra were not corrected for variation in lamp intensity with wavelength. Concentrations were 3.3×10^{-4} M albumin, 2.0×10^{-4} M PLP, and 8.0 M urea in 0.1 M sodium phosphate (pH 7.5). P, PLP; A, albumin; U, urea.

the absorbance at 413 nm was determined as a function of pH, it was found that maximum absorbance at 413 nm was at pH 9 and that there was little absorbance at this wavelength below pH 7. When absorbance at 413 nm was measured as a function of urea concentration at pH 7.5, an increase in absorbance was observed as the concentration of urea increased from 3 to 5 M. This is about the concentration range of urea which would cause denaturation of albumin.

If the absorbance was measured immediately after mixing urea and equimolar PLP–albumin, a large peak at 413 nm was observed. The 330-nm peak appeared as a small shoulder on the 413-nm peak. The absorbance at 330 nm rapidly increased and the absorbance at 413 nm rapidly decreased (Figure 6). The change in absorbance was complete in 5 hr. If the PLP–albumin mixture was diluted with phosphate buffer instead of urea solution, the increase in absorbance at 413 nm was not observed. The increase in absorbance at 413 nm and decrease at 336 nm occurred within 15 sec after addition of urea before the first measurement of absorbance was made.

A 2:1 PLP–albumin mixture was reduced with borohydride at pH 7.5. This results in binding of PLP to secondary sites (site II) on albumin (Dempsey and Christensen, 1962). A 5.4:1.0 PLP–albumin mixture in 8.0 M urea at pH 7.5 was also reduced with borohydride. After trypsin digestion and fingerprinting (Ingram, 1958) the pattern of pyridoxyl-containing peptides was determined. There were three site II peptides. In urea, there were approximately ten peptides. The pattern was similar if the ratio of PLP to albumin in urea was decreased. None of the peptides corresponded to the peptide containing the primary binding site.

A mixture of PLP and urea form a product with absorbance maximum at 326 nm. The product does not have an absorbance peak at 253 nm. This is consistent with the absence of a 253-nm peak in the PLP–albumin–urea *vs.* albumin–urea spectrum shown in Figure 5. The association constant of PLP for urea at pH 7.5 was measured from the decrease in the PLP absorbance at 388 nm. The result was $K = 2.2$ M, ϵ 6900 $M^{-1} cm^{-1}$.

The fluorescence spectrum of the 1:1 PLP–albumin conjugate is shown in Figure 7. There was very little fluorescence when the solution was excited in the region of the 336-nm absorbance peak. In 8.0 M urea, there was an excitation maximum at 350 nm and an emission maximum at 410 nm. Urea plus PLP had excitation and emission maxima at the same wavelengths. With the same amount of added PLP and albumin, the fluorescence intensity for PLP plus albumin in urea was 70% of the intensity for PLP plus urea in the absence of albumin. The results suggest that most of the PLP in 8.0 M urea is bound to urea rather than to albumin.

The polarization of fluorescence was determined. The results were PLP plus urea (excitation 350 nm), +0.078; PLP plus albumin plus urea (excitation 350 nm), +0.100; and PLP plus albumin plus urea (excitation 422 nm), +0.248. The polarization of fluorescence with excitation at 422 nm is assumed to be due to PLP bound as Schiff base to denatured albumin. The polarization is high because of the larger size of the fluorescing molecule. The polarization with excitation at 350 nm for the PLP–albumin–urea mixture is much lower. The fluorescence is therefore due to a smaller molecule, *i.e.*, the urea–PLP compound. The polarization of the 350-nm excitation peak is slightly higher in the presence of albumin. This may be due to binding of some of the urea–PLP compound to albumin, to a small amount of PLP bound to site I of denatured albumin, or to the greater viscosity of urea–albumin solution compared to urea alone.

The quenching of fluorescence of PLP bound to site I indicates that the pyridoxyl phosphate group is interacting with an amino acid side chain. The effect of compounds related to side-chain groups which would be expected to quench the fluorescence of the pyridoxyl moiety on the fluorescence of pyridoxamine was determined. Compounds containing the side-chain groups of amino acids were added to 3.3×10^{-3} M pyridoxamine in 0.1 M sodium phosphate (pH 7.5). The concentrations and effects on fluorescence intensity (excitation 350 nm, emission 390 nm) were: none, 0.42; histidine, 0.42 (0.10 M); mercaptoethanol, 0.37 (0.10 M); tryptophan, 0.23 (0.10 M); and phenol, 0.23 (0.10 M). Phenol and tryptophan partially quenched the fluorescence of PLP bound at site I.

The possibility that the side chain was tryptophan was investigated. The two tryptophan residues of albumin were oxidized with *N*-bromosuccinimide (Blackburn, 1968). The reaction was proportional to the amount of *N*-bromosuccinimide added up to 70% reaction. With 15:1 *N*-bromosuccinimide–albumin, oxidation of tryptophan was 93% complete by color with *p*-dimethylaminobenzaldehyde and decreased in

fluorescence. The difference spectrum of PLP plus *N*-bromosuccinimide-treated albumin *vs.* *N*-bromosuccinimide-treated albumin was determined. The absorbance maximum was 336 nm and the absorbance was 98% of the absorbance for untreated albumin. Fluorescence of the 336-nm peak was still quenched. The results indicate that PLP does not interact with tryptophan at site I.

To study the effect of modification of tyrosine on site I, albumin was nitrated with TNM (TNM–albumin, 100:1). From the absorbance at 428 nm at pH 10.6 (Malan and Edelhoch, 1970) the number of tyrosine residues modified per molecule of albumin was found to be 10.7. Reaction of TNM with albumin in the presence of a 1:1 molar ratio of PLP to albumin had no effect on the number of tyrosine residues modified. The difference spectrum of PLP plus TNM-treated albumin *vs.* TNM-treated albumin had absorbance maxima at 388 nm and at 334 nm. PLP had absorbance maxima at pH 7.5 at 388 and 330 nm. From the absorbance spectrum it was determined that 70% of the added PLP was free in solution. The binding of PLP to albumin was therefore markedly decreased as a result of nitration of the tyrosine residues of albumin.

Discussion

Structure at the Binding Site. The proximity of three lysine residues suggests that the structure of PLP bound at site I of albumin is the substituted aldimine (Figure 1, structure a). With the lysine attached to the pyridoxyl phosphate group in the isolated peptide involved in the substituted aldimine, two structures are possible. One of the structures is shown in Figure 8, in which the lysine side chains on either side of proline form the substituted aldimine and the C-terminal lysine forms an ionic bond with the phosphate group. The reactions involved in forming the reduced product are also shown. Alternatively, the substituted aldimine could form between the adjacent lysines at the C-terminal end of the peptide, *i.e.*, -Lys-Pro-Lys′-Lys′- where the primed lysines form the substituted aldimine. The Corey–Pauling–Koltun models show no appreciable steric hindrance in either substituted aldimine. The results with analogs of PLP by Dempsey and Christensen (1962) suggested that the 5′-phosphate is necessary for binding to site I. The models indicate that the phosphate group can contribute to the binding of PLP to albumin by the formation of an ionic bond between one of its negatively charged oxygens and the protonated ε-amino group of the lysine that is not involved in the substituted aldimine structure.

Upon acidification of the solution in order to form the aldimine (Schiff base), one of the C–N bonds of the substituted aldimine is broken. Only one peptide is produced that carries the pyridoxyl moiety. Both of the C–N bonds cannot be equally labile to acid because this will result in the production of two different pyridoxyl peptides upon digestion with trypsin. The unidirectional breakage of the C–N bond suggests that stearic hindrance, ionic bond formation, and possible interaction with tyrosine residues favor formation of the Schiff base with the penultimate lysine residue to the exclusion of the other two lysine residues. The alternate interpretation, that PLP is bound to the penultimate lysine at pH 7.5 in the form of structures b or c of Figure 8, cannot be ruled out, however.

Glutamate decarboxylase (Strausbach and Fischer, 1970) has adjacent histidine and lysine residues and tryptophanase (Kagamiyama *et al.*, 1970) adjacent lysine residues at the PLP binding site. Both enzymes have absorbance maxima near

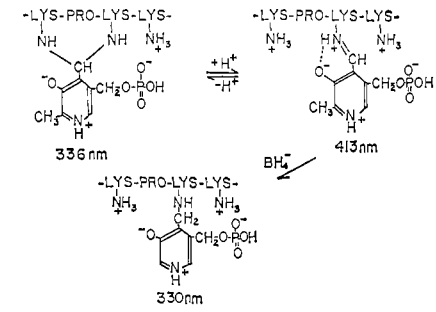

FIGURE 8: Proposed structure at the primary PLP binding site to BSA. The structure involving covalent linkage of PLP with the ε-amino groups of lysines on either side of the proline residue is shown. The other structure would involve the C-terminal and penultimate lysine ε-amino groups in covalent linkage to PLP.

335 nm at pH 7. The formation of a substituted aldimine at the active sites of these enzymes is therefore possible. Glycogen phosphorylase which has an absorbance maximum near 335 nm at pH 7, lacks a nucleophilic side chain adjacent to the lysine to which PLP is attached (Nolan *et al.*, 1964). PLP bound to phosphorylase appears to be present as a Schiff base with an intramolecular H bond between OH at position 3 and Schiff base nitrogen (Shaltiel and Cortijo, 1970) (Figure 1, structure c). It was proposed that bound PLP is buried in a nonpolar environment, since glycogen phosphorylase at pH 7 and *n*-hexylamine–PLP in organic solvents have absorbance maxima near 335 nm and fluorescence excitation and emission maxima near 335 and 535 nm, respectively.

Glutamate decarboxylase at pH 7 has an absorbance and fluorescence excitation maximum at 335 nm and an emission maximum at 420 nm (Shukuya and Schwert, 1960). The fluorescence and absorbance spectra are similar to those of pyridoxamine. Pyridoxamine and the substituted aldimine have the pyridoxyl moiety and a single-bonded nitrogen at the 4′ position. Since pyridoxamine and the substituted aldimine are structurally similar, the fluorescence spectrum of glutamate decarboxylase is consistent with the substituted aldimine structure for PLP at the binding site. Fluorescence of PLP bound to albumin is quenched. Consequently, no comparisons can be made with the fluorescence spectra of glycogen phosphorylase, glutamate decarboxylase, or model compounds such as pyridoxamine. Christensen (1958) suggested that the PLP-Leu-Gly-Gly product which absorbs at 330 nm is the carbinolamine, structure b of Figure 1. The PLP–albumin product is different from the PLP-peptide product since the PLP peptide lacks an absorbance peak at 253 nm.

The substituted aldimine was first proposed for PLP bound to glycogen phosphorylase by Kent *et al.* (1958). The recent results of Shaltiel and Cortijo (1970) suggest that this is not the correct structure. However, the substituted aldimine must still be considered as a strong possibility for glutamate decarboxylase, PLP bound to albumin, and tryptophanase. Further studies will be necessary to clarify the structure at the binding site of these proteins.

Quenching of Fluorescence. The absorbance maxima at 335 and 253 nm of PLP bound at site I are characteristic of PLP derivatives which lack a double bond at the 4′ position (Peterson and Sober, 1954). The spectrum is consistent with the substituted aldimine structure. Pyridoxamine, which has an absorption spectrum similar to that of PLP bound to albumin, and a fluorescence spectrum similar to glutamate decarboxylase (Shukuya and Schwert, 1960), was used as a model for

PLP bound to albumin. Phenol and tryptophan quenched the fluorescence of pyridoxamine. Tyrosine and tryptophan residues are therefore implicated in the quenching of fluorescence of PLP bound to site I.

The increase in absorbance at 280 nm in the PLP–albumin *vs.* albumin difference spectrum may be related to the peak at 280 nm in the difference spectrum of biotin plus albumin *vs.* albumin and of fatty acid anions plus albumin *vs.* albumin (Reynolds *et al.*, 1968). Reynolds *et al.* (1968) concluded that the increased absorbance near 280 nm was due to a red shift in the tyrosine spectrum and that the results indicated interaction between tyrosine and the ligand in the absence of interaction with tryptophan. PLP does not interact with tryptophan at the binding site since oxidation of tryptophan in albumin with *N*-bromosuccinimide had no effect on the binding of PLP to albumin or the quenching of fluorescence. Half of the tyrosine residues in albumin were exposed to the reagent TNM. Nitration of the tyrosine residues resulted in a marked decrease in binding of PLP to albumin. The decreased affinity suggests that tyrosine residues are involved in binding of PLP to albumin. However, the effect could also result from change in conformation at the binding site due to modification of the tyrosine residues. The results suggest that the pyridoxyl phosphate group interacts with tyrosine groups of albumin and that this interaction could contribute to the stabilization of the PLP–albumin product and also account for the quenching of fluorescence.

Effect Urea. The change in absorbance of 1:1 PLP–albumin at pH 7.5 above 300 nm when urea is added (Figure 5) is not dramatic. This led Dempsey and Christensen (1962) to conclude that the PLP–albumin product is stable in urea. However, there are a number of properties of the PLP–albumin–urea system which are inconsistent with this conclusion and favor the interpretation that the large absorbance peak at 330 nm is due to a urea–PLP product rather than an albumin–PLP product. (1) The 253-nm peak of the PLP–albumin product is absent in urea. (2) The 330-nm peak is essentially absent soon after addition of urea to the PLP–albumin mixture. (3) PLP reacts with urea to form a product with absorbance maximum at 326 nm. (4) The fluorescence of the 330-nm peak in albumin–PLP–urea is similar to the fluorescence of the PLP–urea product. The PLP–albumin product in the absence of urea is quenched. (5) The polarization of fluorescence of the 330-nm peak in the PLP–albumin–urea mixture is similar to that of the PLP–urea compound and is lower than the polarization of PLP bound as the Schiff base to albumin in urea.

The presence primarily of the PLP–urea compound rather than PLP at the primary binding site of albumin cannot be due to competition between urea and albumin for PLP for the following reasons. (1) With affinity constants for binding to PLP of 2.2 and $>10^6$ M (Dempsey and Christensen, 1962) for urea and albumin, respectively, the system in Figure 7 should contain >50% PLP bound at site I. This is clearly inconsistent with the above observations, which indicate that PLP bound to site I could not comprise more than 15% of the absorbance at 330 nm. (2) Within 15 sec after addition of urea to the PLP–albumin product, there is a large peak at 413 nm and a very small 330-nm peak. PLP is therefore bound as the Schiff base to urea-denatured albumin and not to urea or to site I which both absorb near 330 nm. This establishes the instability of site I in urea solutions. The progressive increase in absorbance at 330 nm and decrease at 413 nm results from formation of the urea–PLP product at the expense of the PLP-denatured albumin product. The spectrum of the PLP–albumin–urea mixture at equilibrium represents the urea–PLP product absorbing at 330 nm and PLP bound to albumin as the Schiff base absorbing at 413 nm. The fingerprint of the borohydride reduced PLP–albumin–urea mixture indicates that PLP is bound as the Schiff base to approximately ten sites on denatured albumin. The loss of binding to site I with urea denaturation suggests that the stearic requirements are strict. The effect of denaturation could either be to separate groups necessary for binding on different segments of the polypeptide chain, *e.g.*, tyrosine and the three lysines, or to change the conformation at the binding site so that the groups, *e.g.*, the two lysines proposed to be involved in formation of the substituted aldimine, are not oriented properly for formation of the 336-nm structure.

Acknowledgments

The assistance of Mr. Thomas Moore with the polarization measurements is gratefully acknowledged.

References

Blackburn, S. (1968), Amino Acid Determination. Methods and Techniques, New York, N. Y., Marcel-Dekker, p 190.

Christensen, H. (1958), *J. Amer. Chem. Soc. 80*, 99.

Dempsey, W. B., and Christensen, H. N. (1962), *J. Biol. Chem. 237*, 1113.

Edman, P., and Sjöquist, J. (1956), *Acta Chem. Scand. 7*, 447.

Forrey, A. W. (1963), Ph.D. Thesis, University of Washington, Seattle, Wash.

Hirs, C. H. W., Moore, S., and Stein, W. H. (1960), *J. Biol. Chem. 235*, 633.

Hughes, R. C., Jenkins, W. T., and Fisher, E. H. (1962), *Proc. Nat. Acad. Sci. U. S. 48*, 1615.

Ingram, V. M. (1958), *Biochim. Biophys. Acta 28*, 539.

Jenkins, W. T., and Sizer, I. W. (1957), *J. Amer. Chem. Soc. 79*, 2655.

Kagamiyama, H., Morino, Y., and Snell, E. E. (1970), *J. Biol. Chem. 245*, 2819.

Kent, A. B., Krebs, E. G., and Fischer, E. H. (1958), *J. Biol. Chem. 232*, 549.

Konigsberg, W., and Hill, R. J. (1962), *J. Biol. Chem. 237*, 2549.

Malan, P. G., and Edelhoch, H. (1970), *Biochemistry 9*, 3205.

Morino, Y., and Watanabe, T. (1969), *Biochemistry 8*, 3411.

Nolan, C., Jenkins, W. T., and Fischer, E. H. (1964), *Biochemistry 3*, 542.

Peters, T., and Hawn, C. (1967), *J. Biol. Chem. 242*, 566.

Peterson, E. A., and Sober, H. A. (1954), *J. Amer. Chem. Soc. 76*, 169.

Polianovsky, O. L., and Keil, B. (1963), *Biochimia 28*, 372.

Reynolds, J., Herbert, S., and Steinhardt, J. (1968), *Biochemistry 7*, 1357.

Schroeder, W. A. (1967), *Methods Enzymol. 11*, 351.

Shaltiel, S., and Cortijo, M. (1970), *Biochem. Biophys. Res. Commun. 41*, 594.

Shaltiel, S., and Zaidenzaig, Y. (1970), *Biochem. Biophys. Res. Commun. 39*, 1003.

Shepherd, R. G., Wilson, S. D., Howard, K. S., Bell, P. H., Davies, P. S., Davis, S. B., Eigner, E. A., and Shaperspeare, N. E. (1956), *J. Amer. Chem. Soc. 78*, 5067.

Shukuya, R., and Schwert, G. W. (1960), *J. Biol. Chem. 235*, 1653.

Spackman, D. H., Stein, W. H., and Moore, S. (1958), *Anal. Chem. 30*, 1190.

Strausbauch, P. H., and Fischer, E. H. (1970), *Biochemistry 9*, 233.

Weber, G., and Young, L. B. (1964), *J. Biol. Chem. 239*, 1424.

On Self-Interacting Oligoribonucleotides. I. Absorption and Optical Rotatory Dispersion of 2′–5′- and 3′–5′-Oligoguanylic Acids*

S. K. Podder

ABSTRACT: A series of 2′–5′-oligoguanylic acids are prepared by reacting G(cyclic)p with takadiastase T_1 ribonuclease and separating the products chromatographically. The 3′–5′-oligoguanylic acids are obtained by separating the products of alkaline degradation of 3′–5′-poly(G). The optical rotatory dispersion and hypochromism of both 2′–5′- and 3′–5′-oligoguanylic acids are studied at two different pH. The optical rotatory dispersion spectrum of 2′–5′-GpG is significantly different from that of 3′–5′-GpG. The magnitude of rotation of the long-wavelength peak of 2′–5′-GpG is larger than that of 3′–5′-GpG. This finding contradicts the explanation that the extra stability and more intense circular dichroism band of other 3′–5′-dinucleoside monophosphates is due to H-bond formation between 2′-OH and either the base or the phosphate oxygen. The end phosphate group has a marked effect on the spectrum of GpG between 230 and 250 mμ. In addition the optical rotatory dispersion spectra of 2′–5′ exhibit strong pH, temperature, and solvent dependence between 230 and 250 mμ. ΔH and ΔS for order $\rightleftharpoons$ disorder transition is estimated to be 9.7 kcal/mole and 35.2 eu, respectively. The optical rotatory dispersion spectra of guanine-rich oligoribonucleotides, GpGpC, GpGpU, GpGpGpC, and GpGpGpU are compared to the calculated optical rotatory dispersion from the semiempirical expression of Cantor and Tinoco, using measured optical rotatory dispersion of dimers. Contrary to previous studies, agreement is found not at all satisfactory.

However, optical rotatory dispersion of 3′–5′-GpGpGpC and GpGpGpU can be estimated from the semiempirical expression, if a next-nearest interaction parameter is introduced empirically. Such interaction parameter can be calculated from the measured properties of trinucleotide sequences like GpGpG, GpGpC, and GpGpU, assuming that only the nearest-neighbor interaction is important. The optical rotatory dispersion of single-stranded poly(G) is also predicted. The importance of syn–anti equilibrium and next-nearest-neighbor interaction in oligoguanylic acids is suggested as a probable explanation.

The stacking interaction is shown to be major source of conformational stability in helical polynucleotides (Van Holde *et al.*, 1965; Brahms *et al.*, 1966). Tinoco and his coworkers have shown that the optical properties such as absorption and optical rotatory dispersion of polynucleotides, particularly in the single-stranded conformation, is mainly influenced by its nearest-neighbor residue. Thus optical rotatory dispersion and absorption spectra of any single-stranded polynucleotide of known sequence can be empirically calculated from the corresponding measured properties of 16 dinucleotides (Cantor and Tinoco, 1965). Similarly the optical properties of double helices of any defined sequence can be expressed in terms of measured properties of the different dimer pairs (Cantor *et al.*, 1966). Jaskunas *et al.* (1968) have attempted to obtain the optical rotatory dispersion of different dimer pairs from the study of complexes formed between complementary trinucleotides in 10^{-2} M solutions in 0.1 M phosphate buffer containing 0.5 M NaCl (pH 7.0). Under those conditions, among the various trinucleotides studied, only GpGpC interacts with itself to form large aggregates as well as with its complementary GpCpC to form a series of higher order complexes with an average stoichiometry of 2:1. These approaches were then extended to the study of complexes between complementary tetranucleotides as one would expect that decrease in the extent of aggregation with increasing chain length might favor the formation of well-defined complexes containing complementary base pairs. Contrary to our expectation, the optical rotatory dispersion of GpGpGpU and GpCpCpA alone and of the mixture of the two indicated no complex formation to have occurred under those conditions. It has been shown later that GpGpGpU associates themselves strongly even in 10^{-5} M solution. The rates of formation and of dissociation of such self-associated complexes of GpGpGpU are extremely slow (compare rates of A–U and G–C base-pair formation, Podder, 1971) and thereby limits the formation of complementary base pairs. When this work was undertaken it was not known whether such slow rates are characteristics of G–G base pairs or intercalated complexes. The presence of uracil residue at the end might cause unstacking thereby favoring intercalation (Chan and Nelson, 1969). To comment further, knowledge of physicochemical properties

* From the Max-Planck Institut für Physikalische Chemie, 3400 Göttingen, West Germany. *Received June 19, 1970.* The work was done in part at the Department of Chemistry, University of California, Berkeley, Calif. The National Institutes of Health supported this work through a research grant to Professor Tinoco. Present address: Department of Biochemistry, Indian Institute of Science, Bangalore-12, India.