Reaction of α^5-Pyridoxal Methyl Chloride, an Analog of Pyridoxal 5′-Phosphate, with the B Protein of *Escherichia coli* Tryptophan Synthetase†

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ABSTRACT: α^5-Pyridoxal methyl chloride [5-(2-chloroethyl)-3-hydroxy-2-methyl-4-pyridinecarboxaldehyde], an analog of pyridoxal-P, irreversibly inactivates the apo-B-protein of *Escherichia coli* tryptophan synthetase. Pyridoxal-P decreases the rate and extent of modification. Loss of activity is stoichiometric with the incorporation of approximately 1 mol of chromophore per mol of B-monomer. The chromophoric residue was isolated from the acid hydrolysate of the modified enzyme and shown to be identical with the cyclic imino acid derivative of pyridoxal which had been previously prepared from α^5-pyridoxal methyl chloride and N^α-acetyl-L-lysine and identified as 6-(5-amino-5-carboxypentyl)-7,8-dihydro-3-methyl-2,6-naphthyridin-4-ol (Miles, E. W., Fales, H. M., and Gin, J. B. (1972), *Biochemistry 11*, 4945). The reaction of α^5-pyridoxal methyl chloride with the apo-B-protein occurs in two steps. First the carbonyl group forms a Schiff base with an ϵ-amino group of a lysyl residue at the active site of the apo-B-protein; then the chloroethyl group alkylates the imino nitrogen to form a very stable, cyclic imino acid derivative. This highly colored and fluorescent chromophore may prove to be a useful optical probe at the active site of pyridoxal-P enzymes. The modified B protein showed no structural changes which could be detected by circular dichroism and optical rotation measurements. The bound chromophore caused the same degree of quenching of tryptophan fluorescence as did the normal pyridoxal-P cofactor, but exhibited a much weaker ellipticity band in the region of its visible absorption than did the bound cofactor. The modified B-protein did not form an active $\alpha_2\beta_2$ complex with the A-protein of tryptophan synthetase.

Tryptophan synthetase of *Escherichia coli* is a multienzyme complex composed of two nonidentical, readily separable proteins, the A- and B-proteins (Crawford and Yanofsky, 1958). The B protein is normally a dimer and combines with two A-monomers to form an AB complex (or $\alpha_2\beta_2$ complex) (Wilson and Crawford, 1965). The B-protein contains two molecules of pyridoxal-P per dimer and catalyzes a number of pyridoxal-P-dependent reactions (Miles *et al.*, 1968; Kumagai and Miles, 1971). In the present study the reaction of α^5-pyridoxal methyl chloride[1] at the active site of the B-protein is investigated. The synthesis and model reactions of this analog of pyridoxal-P were reported in the preceding paper (Miles *et al.*, 1972).

Methods and Materials

Pyridoxal Derivatives. Pyridoxal-P and pyridoxamine were products of Sigma Chemical Co. α^5-Pyridoxal methyl chloride was synthesized as described in the preceding paper (Miles *et al.*, 1972). The cyclic imine derivatives of pyridoxal, compounds Y and Z, were prepared from α^5-pyridoxal methyl chloride and N^α-acetyl-L-lysine as described in the preceeding paper (Miles *et al.*, 1972). Tritium-labeled compound Z was prepared by using 5 μmol of NaB^3H_4 (New England Nuclear), 250 Ci/mol, for the reduction of 5 μmol of the synthetic reaction mixture. The same solution of NaB^3H_4 was used for the reduction of modified B-protein in 0.1 M potassium phosphate (pH 7.8). ϵ-Pyridoxyl-L-lysine was prepared as described in the preceding paper (Miles *et al.*, 1972). An aliquot of the synthetic reaction mixture equivalent to 2 μmol of product was reduced with 2 μmol of NaB^3H_4. The concentration of the isolated product was determined from its molar extinction coefficient at 325 nm in 0.05 M potassium phosphate (pH 7.0) ($\epsilon_{325\,nm}$ 7600) (Forrey *et al.*, 1971). An aliquot of this solution counted in 10 ml of Aquasol (New England Nuclear) gave a specific activity of 15,500 cpm/nmol. The efficiency of counting tritium under these conditions was about 17%. The specific activity of tritium-labeled compound Z was assumed to be the same as that of tritium-labeled ϵ-pyridoxyl-L-lysine which was prepared with the same solution of NaB^3H_4. Using this specific activity, the molar extinction coefficient of compound Z in 0.1 M potassium phosphate (pH 7.8) was determined to be 8800 M^{-1} cm^{-1} at 313 nm from the absorbance and radioactivity of a solution of tritium-labeled compound Z. Since the absorbance of a solution of compound Y in 0.1 M potassium phosphate (pH 7.8) at 438 nm is the same as the absorbance of the solution at 313 nm after treatment with NaB^3H_4 to form compound Z (see preceding paper, Miles *et al.*, 1972, Figure 2A), the molar extinction coefficient of compound Y at 438 nm in 0.1 M potassium phosphate (pH 7.8) is also 8800 M^{-1} cm^{-1}.

Enzyme Preparations and Assays. The apo-B-protein, the holo-B-protein, and the A-protein of tryptophan synthetase were prepared and assayed by standard methods as reported previously (Miles, 1970). The molecular weight of the B-monomer or chain used for calculations was 44,500 (Hathaway and Crawford, 1970). The B-protein was treated with *N*-ethylmaleimide (NEM)[2] under conditions which have been

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[1] The systematic name for α^5-pyridoxal methyl chloride is 5-(2-chloroethyl)-3-hydroxy-2-methyl-4-pyridinecarboxaldehyde. The structure of compound Y is 6-(5-amino-5-carboxypentyl)-7,8-dihydro-3-methyl-2,6-naphthyridin-4-ol. The structure of compound Z is 6-(5-amino-5-carboxypentyl)-3-methyl-5,6,7,8-tetrahydro-2,6-naphthyridin-4-ol. These structures are shown in Scheme I, where R– = $-(CH_2)_4-CHNH_2COOH$.

[2] Abbreviations used are: NEM, *N*-ethylmaleimide; NEM-apo-B, B-protein of tryptophan synthetase which was modified with 1 mol of NEM per mol of B monomer in the presence of pyridoxal-P and then resolved.

shown to result in the modification of one specific sulfhydryl residue (Miles, 1970). Holoenzyme (5–6 mg/ml) which had been dialyzed against 1 M NH_4HCO_3 (pH 8.3), containing 0.01 mM pyridoxal-P and 0.1 mM EDTA, was treated with 0.5 mM NEM in the presence of 0.1 mM pyridoxal-P and 1 mM EDTA for 1 hr at 25°. This modified enzyme was resolved by dialysis for 3 days against 0.1 M potassium phosphate (pH 7.8), containing 0.05 M mercaptoethanol and 0.1 M DL-serine, and was designated NEM-apo-B. NEM-apo-B-protein was assayed by its serine deaminase activity in the absence of the A-protein (Crawford and Ito, 1964) or by its stimulation of the conversion of indoleglycerol phosphate to indole in the presence of A-protein and hydroxylamine (Miles, 1970).

Optical Measurements. All enzyme solutions on which optical measurements were to be made were dialyzed in tubing which had been previously boiled in 5% $NaHCO_3$ and in two changes of distilled water, then stored at 4° in 50% ethanol. Absorption spectra were made in a Cary No. 11 or in a Cary No. 14. Circular dichroism and optical rotation measurements were made in a Cary No. 60 equipped with a circular dichroism attachment (Model No. 6001). Fluorescence measurements (uncorrected) were made in an Aminco-Bowman spectrofluorimeter equipped with an X–Y recorder. Measurements were made on 0.1-ml volumes of solutions in a microcell with a path length of 3 mm using 2-mm slits. The absorbance of the solutions used was kept below 0.1 unit in a 1-cm cell at the wavelength of excitation except for measurements of tryptophan emission where the absorbance at 278 nm was 0.4–0.6. Although some pyridoxyl derivatives (especially pyridoxamine) are extremely light sensitive under these instrumental conditions and require light attenuation (Chen, 1965), the compounds in this study were less light sensitive, and measurements were made with an exposure to the light beam of less than 1 min without light attenuation.

Total sulfhydryl residues were determined spectrophotometrically by the method of Ellman (1959). B-protein at about 1 mg/ml in 0.1 M potassium phosphate (pH 7.8), containing 1 mM EDTA, was diluted with 3 volumes of 10.5 M urea in the same buffer at 37°. The absorbance at 412 nm was recorded in a Gilford spectrophotometer before and after the addition of 0.2 M 5,5′-dithiobis(2-nitrobenzoic acid) and the number of sulfhydryl groups reacting was calculated from the molar extinction coefficient ($\epsilon_{412\ nm}$ 13,600 M^{-1} cm^{-1}).

Amino Acid Analysis and High-Voltage Electrophoresis. Amino acid derivatives of pyridoxal or α^5-pyridoxal methyl chloride were analyzed on the short column of a Beckman-Spinco Model No. 120C amino acid analyzer using the standard accelerated (120 ml/hr) system (Spackman, 1967).[3] When these derivatives contained tritium label, the effluent from the analyzer was collected at 1 tube/min. High-voltage electrophoresis was carried out on Whatman No. 3MM paper in pyridine–acetate buffer at pH 3.6 for 1 hr at 3200 V according to Bennett (1967). Radioactive areas on paper strips were located with a Vanguard Autoscanner 880.

Results

Effects of α^5-Pyridoxal Methyl Chloride on the Activity of the B-Protein. Since the aim of these studies was to modify the apo-B-protein at its cofactor binding site with the cofactor analog, α^5-pyridoxal methyl chloride, it seemed advantageous to prevent possible reactions of the analog with reactive residues outside of the cofactor binding site. Previous studies had shown that the holo-B-protein contains one sulfhydryl residue which is highly reactive with sulfhydryl reagents but which is not necessary for cofactor binding (Miles, 1970). This residue can be selectively modified with *N*-ethylmaleimide with no consequent loss in the serine deaminase activity of the B-protein. Since preliminary studies indicated that α^5-pyridoxal methyl chloride did react with one or more sulfhydryl residues, we decided to use NEM-apo-B-protein for subsequent studies in order to avoid any reactions of the α^5-pyridoxal methyl chloride with the sulfhydryl residue which was not required for cofactor binding.

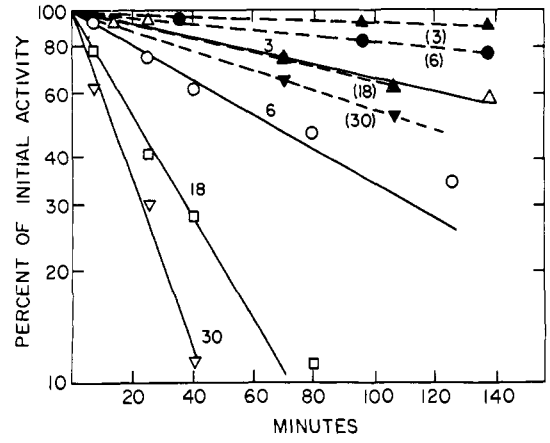
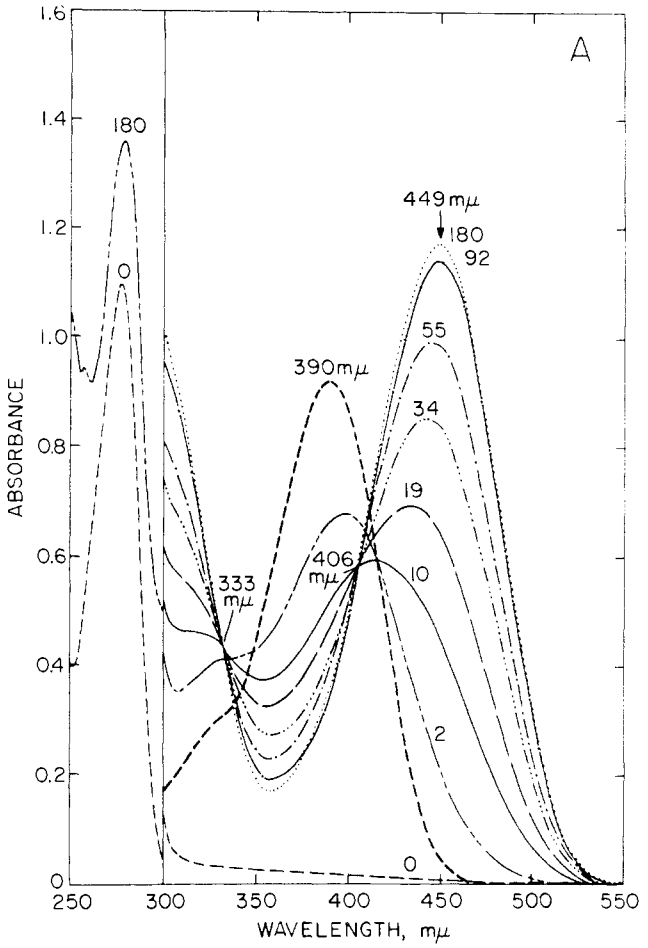


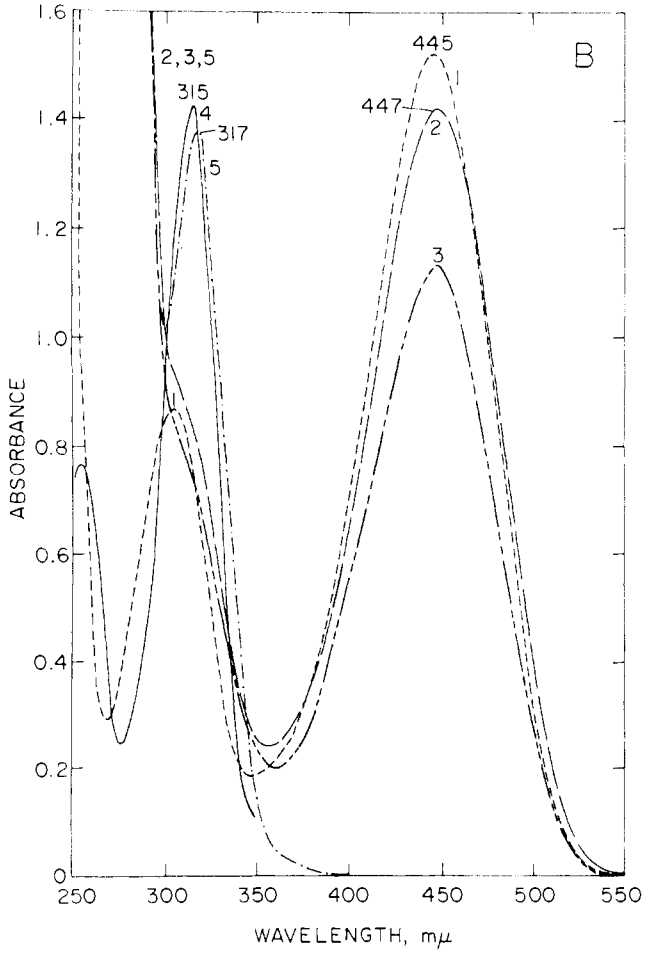
FIGURE 1: Effects of α^5-pyridoxal methyl chloride on the serine deaminase activity of the NEM-apo-B-protein. NEM-apo-B-protein (2.8×10^{-5} M in 0.1 M potassium phosphate, pH 7.8) was incubated with α^5-pyridoxal methyl chloride (3, 6, 18, or 30×10^{-5} M) at 22° in the presence or absence of pyridoxal-P (10×10^{-5} M). Aliquots (0.01 ml) were removed at intervals and diluted into assay mixtures (0.89 ml) containing excess pyridoxal-P and all other components for the assay of serine deaminase activity except L-serine. Assays were started by the addition of 0.1 ml of 1 M L-serine 10–30 min after dilution. Percent of initial activity is plotted against time of reaction. Initial concentrations of α^5-pyridoxal methyl chloride are shown by the following symbols: 3×10^{-5} M (▲), 6×10^{-5} M (●), 18×10^{-5} M (■), and 30×10^{-5} M (▼). Data in the presence of pyridoxal-P is shown by solid symbols, dashed lines, and concentrations in parentheses. Data in the absence of pyridoxal-P is shown by open symbols and solid lines.

Figure 1 shows the effect of various concentrations of α^5-pyridoxal methyl chloride on the rate of inactivation of the NEM-apo-B-protein. Pyridoxal-P (0.1 mM) decreased the rate of inactivation about 9-fold. Enzyme reaction mixtures from Figure 1 were dialyzed after 2.5 hr against the resolution buffer for 2 days to remove any bound pyridoxal-P and then against 0.1 M potassium phosphate buffer (pH 7.8). The dialyzed solutions had absorption peaks centered at 450 nm. The absorption at 450 nm was reduced by about one-third by the presence of pyridoxal-P during the incubation period. The enzyme which had been treated with a 10-fold molar excess of α^5-pyridoxal methyl chloride had an absorbance at 450 nm which was about six times higher than the absorbance of the holo-B-protein at 420 nm. This finding suggests that the analog reacts at more than one site per monomer when used in excess.

Therefore, it was attempted to increase the selectivity of the reagent by treating a higher concentration of enzyme with only a small excess of α^5-pyridoxal methyl chloride (Figure 2A). A new chromophore absorbing maximally at 442–449 nm and at 300 nm was generated over a period of 2–3 hr. A similar family of curves was generated over 2 hr when α^5-

[3] These analyses were kindly performed by Mr. George Poy, Arthritis and Rheumatism Branch, National Institute of Arthritis, Metabolism, and Digestive Diseases.





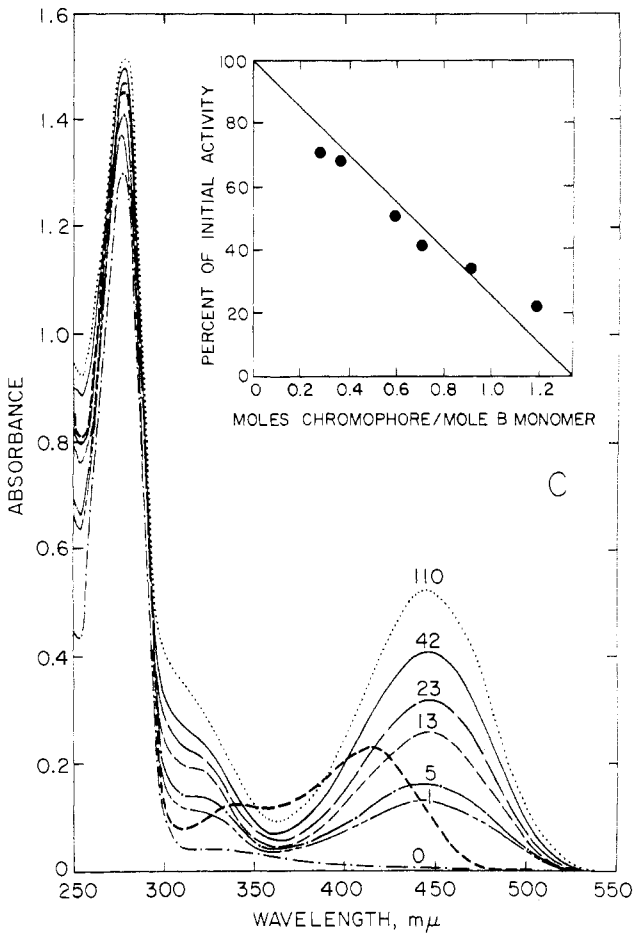


FIGURE 2: Spectra of α^5-pyridoxal methyl chloride and its reaction products. (A) Reaction with NEM-apo-B-protein. α^5-Pyridoxal methyl chloride (0.163 mM) was added to 0.1 M potassium phosphate (pH 7.8) containing 1 mM EDTA (unnumbered curve, -----) or to 0.089 mM NEM-apo-B-protein in the same buffer at 23°. Spectra of the enzyme solution were recorded at the indicated number of minutes. The two curves below 300 nm are spectra of 2-fold dilutions of NEM-apo-B-protein before and 180 min after addition of α^5-pyridoxal methyl chloride. (B) Reaction products with L-serine and NEM-apo-B-protein before and after treatment with $NaBH_4$. Curve 1: spectrum of product of reaction of 0.163 mM α^5-pyridoxal methyl chloride with L-serine (0.1 M in 0.1 M potassium phosphate (pH 7.8), containing 1 mM EDTA) at 23° after 100 min. Curve 2: spectrum of product of reaction of 0.23 mM α^5-pyridoxal methyl chloride with 0.13 mM NEM-apo-B-protein after 100 min as in part A. Curve 3: spectrum of enzyme treated in curve 2 after dialysis for 16 hr against the same buffer. Curve 4: spectrum of reaction mixture in curve 1 after treatment with 0.1 mM $NaBH_4$. Curve 5: spectrum of reaction mixture in curve 2 after treatment with 0.1 mM $NaBH_4$. (C) Effect of α^5-pyridoxal methyl chloride treatment of NEM-apo-B-protein for different times on its spectrum and activity after dialysis. During the treatment described in part B (curve 2), 0.2-ml aliquots were removed at 0, 1, 5, 13, 23, 42, and 110 min, diluted to 0.5 ml with cold buffer (0.1 M potassium phosphate (pH 7.8), containing 1 mM EDTA), and dialyzed for 16 hr against this buffer. Spectra were recorded after dialysis and serine deaminase activity was assayed. The spectrum of an equal concentration of holo-B-protein is shown by the unnumbered, dashed line for comparison. The amount of analog incorporated was estimated from the absorbance at 445 nm using a molar extinction coefficient for the chromophore of 8800 M^{-1} cm^{-1} (see text).

pyridoxal methyl chloride was treated with 0.1 M L-serine (data not shown). Figure 2B compares the final spectra obtained in similar reaction mixtures of α^5-pyridoxal methyl chloride with L-serine (curve 1) or NEM-apo-B (curve 2). Curves 1 and 2 are very similar in shape above 300 nm where the protein does not contribute appreciably to the absorption. Curve 2 shows a shoulder above 300 nm which corresponds to a peak at 300 nm in curve 1. Curve 3 shows that dialysis of the enzyme reaction mixture for 16 hr resulted in a 25% reduction in the absorption at 450 nm. Further dialysis for 3 days against 8 M urea in phosphate buffer (pH 7.8) at room temperature resulted in only a small decrease in this absorbance. Curves 4 and 5 show the spectra after addition of 0.1 mM $NaBH_4$ to reaction mixtures shown in curves 1 and 2, respectively. These two curves are closely similar above 300 nm with peaks at 315 and 317 nm, respectively. Figure 2C shows spectra of solutions of NEM-apo-B-protein which had been treated for various times with α^5-pyridoxal methyl chloride under the conditions given in Figure 2B and then diluted and dialyzed. These spectra had peaks centered at 450 nm and shoulders above 300 nm. They also exhibited increases in absorbance at 278 and 250 nm. The spectrum of the holo-B-protein, shown in Figure 2C by the dashed curve for comparison, shows quite different absorption peaks above 300 nm (340 and 412 nm) but similar absorption increases below 300 nm.

The spectral changes observed in Figure 2A–B are closely similar to those previously observed (Miles *et al.*, 1972) during the reaction of α^5-pyridoxal methyl chloride with N^α-acetyl-L-lysine. The product of this reaction was shown to be a cyclic imino acid derivative of homopyridoxal, compound Y.[1] The amount of chromophore bound to the enzyme was estimated from the absorbance at 445 nm on the basis of the assumption that the extinction coefficient of the bound chromophore at 445 nm is the same as the extinction coefficient of compound Y at 438 nm (ϵ_{438} 8800 M^{-1} cm^{-1}; see Methods and Materials). The activity of the dialyzed solutions in the serine deaminase reaction was determined and is plotted against the estimated number of moles of chromophore bound per mole of B-monomer in the inset of Figure 2C. The results show that under these conditions inactivation of the enzyme results from the incorporation of 1.3 mol of chromophore/mol of B-monomer.

Evidence for the Covalent Reaction of α^5-Pyridoxal Methyl Chloride with NEM-B-Protein and Isolation of a Ninhydrin-Positive Derivative. The chromophore produced by the reaction of α^5-pyridoxal methyl chloride with the B-protein appeared to be covalently bound since it was not removed from the protein by dialysis against buffer or 8 M urea or by treatment with cold trichloroacetic acid. Isolation of a chromophoric derivative from acid hydrolysates of the modified enzyme was therefore attempted. NEM-apo-B-protein (0.064 μmol in 0.8 ml of potassium phosphate buffer, pH 7.8) was treated with 0.15 μmol of α^5-pyridoxal methyl chloride under conditions similar to those shown in Figure 2A. After 77 min the enzyme was dialyzed for 16 hr against phosphate buffer (pH 7.8). Incorporation of chromophore was estimated to be 0.097 μmol/0.064 μmol of protein from the absorbance at 450 nm. The yellow protein was precipitated by cold trichloroacetic acid (10% final concentration) and subjected to acid hydrolysis for 22 hr at 110°. Acid was removed *in vacuo* and a spectrum of the neutralized aqueous solution was recorded between 300 and 550 nm. This spectrum was closely similar to that before hydrolysis except that the absorption maximum of the peak in the visible region had shifted from 450 to 435 nm. This solution was fractionated on a 0.9 × 15 cm column of Amberlite CG-50 with a 500-ml linear gradient between water and 1 N acetic acid. A single fluorescent peak (excitation at 435 nm; emission at 516 nm) was eluted at 0.7 M acetic acid. A spectrum of the combined and concentrated fractions in potassium phosphate buffer (pH 7.0) showed a peak in the visible region at 435 nm containing 50% of the initial absorbancy units.

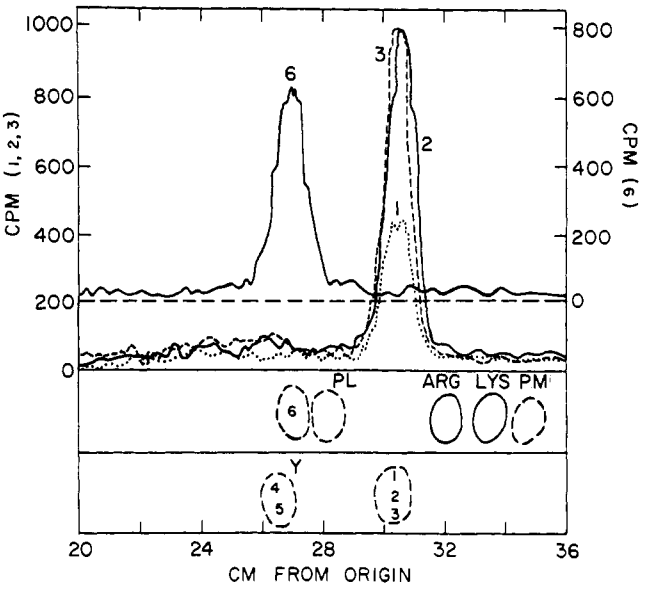


FIGURE 3: Paper electrophoresis of derivatives of α^5-pyridoxal methyl chloride and of pyridoxal. Electrophoresis at pH 3.6 and scans were carried out as described in Methods and Materials. The preparation of enzyme derivatives is described in the text. Curve 1 and spot 1: an aliquot of the total acid hydrolysate NEM-apo-B treated with α^5-pyridoxal methyl chloride and NaB^3H_4. Curve 2 and spot 2: radioactive derivative purified from the total acid hydrolysate by chromatography on Amberlite CG-50. Curve 3 and spot 3: synthetic compound ^{3}H-labeled Z. Spot 4: chromophoric derivative purified from an acid hydrolysate of NEM-apo-B which had been treated with α^5-pyridoxal methyl chloride. Spot 5: synthetic compound Y. Curve 6 and spot 6: synthetic [^{3}H]-ε-pyridoxyl-L-lysine. Standards (10 μg each): PL, pyridoxal; PM, pyridoxamine; ARG, arginine; and LYS, lysine.

A radioactive derivative was also isolated from NEM-apo-B-protein which was first treated with α^5-pyridoxal methyl chloride and then reduced with NaB^3H_4. NEM-apo-B-protein (0.075 μmol in 1.0 ml of 0.1 M potassium phosphate, pH 7.8) was treated with 0.10 μmol of α^5-pyridoxal methyl chloride for 2 hr and then treated with about 0.2 μmol of NaB^3H_4. The protein was precipitated with cold trichloroacetic acid (10% final concentration) and hydrolyzed for 40 hr at 110°. Aliquots of the total hydrolysate were removed for electrophoresis (Figure 3) and amino acid analysis (Figure 5). The remaining hydrolysate was fractionated on an Amberlite CG-50 column as above. A radioactive peak (2.9×10^5 cpm) was eluted at 0.7 M acetic acid.

The chromophoric derivative and the radioactive derivative which had been isolated from acid hydrolysates of the modified enzyme before and after NaB^3H_4 reduction, respectively, were further characterized by their electrophoretic mobility at pH 3.6 (Figure 3), amino acid analysis (Figure 4), and fluorescence (Figure 5). Tritium labeled ε-pyridoxyl-L-lysine was used as a marker. The chromophoric protein derivative and the synthetic compound Y each showed a single yellow fluorescent, ninhydrin positive spot at an identical position on electrophoresis (Figure 3, spots 4 and 5). These two derivatives also showed identical emission and excitation spectra (Fig-

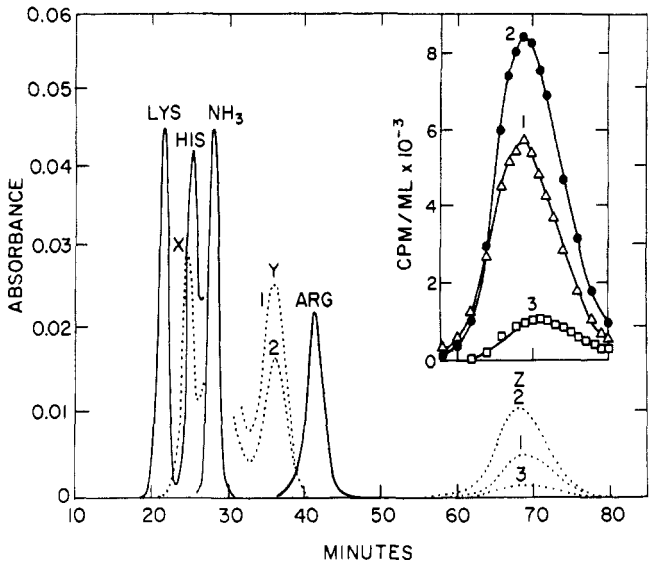


FIGURE 4: Amino acid analyses of derivatives of α^5-pyridoxal methyl chloride. Peaks Y: synthetic compound Y (peak Y-1) and the chromophoric derivative isolated from an acid hydrolysate of NEM-apo-B which had been treated with α^5-pyridoxal methyl chloride (see text; peak Y-2). Peaks Z: synthetic tritium-labeled compound Z (peak Z-1), radioactive derivative isolated from an acid hydrolysate of NEM-apo-B which had been treated with α^5-pyridoxal methyl chloride and NaB^3H_4 (see text; peak Z-2) and an aliquot of the total acid hydrolysate equivalent to about 2 nmol of enzyme monomer (peak Z-3). The latter analysis also showed the presence of lysine (35 nmol), histidine (26 nmol), arginine (36 nmol), and NH_3, but these peaks are not shown in the figure. The effluents from the analyses of the three tritium-labeled samples were collected and counted as shown in the inset. Elution times of the inset have been adjusted for a 5 min lag between the time when the ninhydrin peak was observed on the analyzer chart and in the waste effluent (see Methods and Materials). Standards: LYS (lysine), HIS (histidine), ARG (arginine), and X (ε-pyridoxyl-L-lysine).

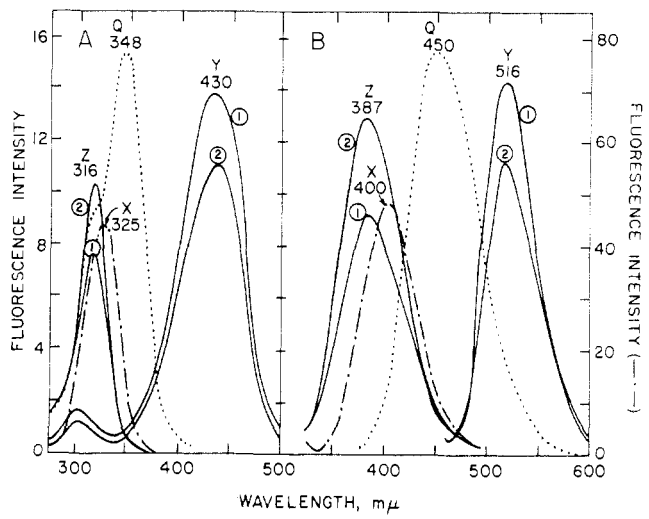


FIGURE 5: Fluorescence spectra of derivatives of pyridoxal and α^5-pyridoxal methyl chloride. Spectra were recorded as described in Methods and Materials. Spectra of synthetic compounds Y and Z are shown by curves Y-1 and Z-1. Spectra of the chromophoric and radioactive derivatives which were isolated from acid hydrolysates of NEM-apo-B which had been treated with α^5-pyridoxal methyl chloride (see text) are shown by curves Y-2 and Z-2, respectively. Spectra of a quinine standard solution Q and of an ε-pyridoxyl-L-lysine standard solution X are shown for comparison. X and Z were diluted with 0.05 M potassium phosphate (pH 7.0) and Y was diluted with 1 N acetic acid. (A) Excitation spectra with emission set at 387 nm (Z), 400 nm (X), 450 nm (Q), and 516 nm (Y). (B) Emission spectra with excitation set at 318 nm (Z), 325 nm (X), 348 nm (Q), and 430 nm (Y).

ure 5) and gave ninhydrin-positive peaks eluting at 36 min on amino acid analysis (Figure 4). The radioactive protein derivative and the synthetic tritium-labeled compound Z each showed a single ninhydrin-positive, Gibbs-positive, radioactive spot at identical positions on electrophoresis (Figure 3, curves and spots 2 and 3). Electrophoresis of an aliquot of the total hydrolysate also yielded a single radioactive spot at this position (Figure 3, curve and spot 1). Amino acid analyses of the total acid hydrolysate of tritium-labeled protein, the purified tritium-labeled protein derivative, and the synthetic tritium-labeled compound Z each yielded a radioactive and ninhydrin-positive peak eluting at 68 min (Figure 4, curves 1–3). The recovery of radioactivity in these three peaks ranged from 68 to 82%. The color yield of the synthetic compound relative to leucine on a molar basis was 0.96.

Effects of α^5-Pyridoxal Methyl Chloride Incorporation on the Activity, Sulfhydryl Content, and Fluorescence of the B-Protein. Figure 6 shows the effects of treating the NEM-apo-B-protein with a range of concentrations of α^5-pyridoxal methyl chloride which results in the incorporation of 0.1–4.3 mol of chromophore/mol of NEM-B-monomer. As shown previously in Figure 2C, the loss of activity is approximately stoichiometric with the incorporation of the first mole of chromophore. The total number of sulfhydryl residues titratable in urea does not decrease significantly when less than 1 mol of chromophore is incorporated, but about 1 sulfhydryl residue has disappeared in the enzyme which has incorporated 4 mol of chromophore.

The binding of pyridoxal-P to a number of apoenzymes has been shown to result in quenching of the tryptophan fluorescence of the enzymes (Bertland and Kaplan, 1968; Tate and Meister, 1969). Figure 6B shows that pyridoxal-P also causes a decrease in the tryptophan fluorescence of the NEM-apo-B-protein. There is a sharp break in the titration curve when 1 mol of pyridoxal-P is added per mol of NEM-B-monomer, although some additional quenching occurs at higher concentrations of pyridoxal-P. The incorporation of α^5-pyridoxal methyl chloride also results in quenching of tryptophan fluorescence (Figure 6B). The data for the incorporation of 1 or fewer mol of analog fall on the pyridoxal-P titration curve. The incorporation of 1.2 mol of analog results in the same decrease in tryptophan fluorescence as does the binding of 1 mol of pyridoxal-P. When more than 1 mol of analog is incorporated, there is some additional quenching of tryptophan fluorescence.

Physical Properties of the B-Protein in the Presence and Absence of Chromophores. Optical rotatory dispersion curves of the tryptophan synthetase B-protein have a trough with a minimum at 233 nm (E. W. Miles and I. P. Crawford, unpublished observations). The amplitude of the 233-nm trough has been used as a measure of the α-helix or ordered structure of proteins and thus as an indicator of gross conformational changes. Measurements of optical rotation at 233 nm were made on enzyme solutions at 233 nm in a cell with a 1.0-cm path length. Apo-B-protein, NEM-apo-B-protein, and the NEM-B-protein containing one mole of bound analog all had the same optical rotation at 233 nm ($[\alpha]_{233}^{27} = -5760°$). The addition of pyridoxal-P (0.01 mM) caused a small (4%) increase in the optical rotation at 233 nm of the two apo-B-proteins but not of the enzyme which contained bound analog. These data indicate that modification of the enzyme by NEM,

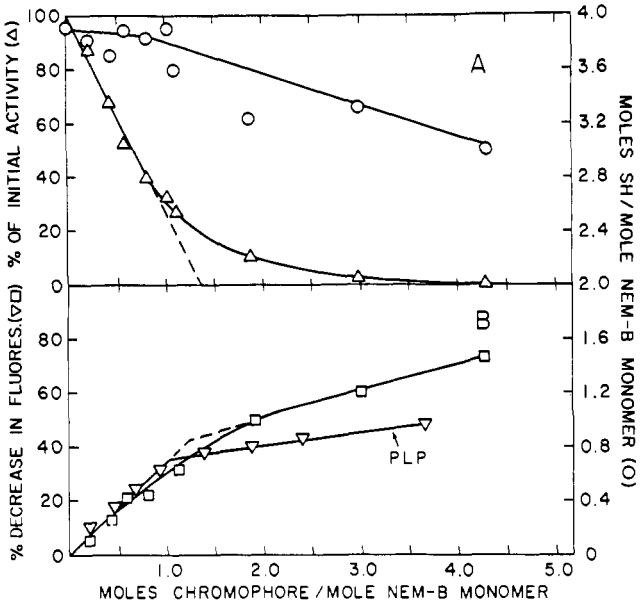


FIGURE 6: The effect of incorporation of α^5-pyridoxal methyl chloride on the serine deaminase activity, tryptophan fluorescence, and total sulfhydryl content of the NEM-apo-B-protein. NEM-apo-B-protein (0.128 nM in 0.1 M potassium phosphate (pH 7.8), containing 1 mM EDTA) was treated with eight different concentrations of α^5-pyridoxal methyl chloride to give ratios of analog to B-monomer ranging from 0.25 to 7.5 and with eight similar concentrations of pyridoxal-P. After 2 hr at 22° the 0.1-ml reaction mixtures were diluted to 0.6 ml with the above buffer. The fluorescence emission at 335 nm (with excitation at 280 nm) was measured and the solutions containing analog were dialyzed for 16 hr against the same buffer. The extent of incorporation of the analog was determined from absorption spectra of the dialyzed solutions as in Figure 2C. Aliquots were used for assay of serine deaminase activity (Δ) and total SH determination in urea (○) (see Methods and Materials). Fluorescence measurements on the dialyzed solutions gave values closely similar to those obtained before dialysis. Results are plotted against the amount of α^5-pyridoxal methyl chloride incorporated (□) or of pyridoxal-P added (▽).

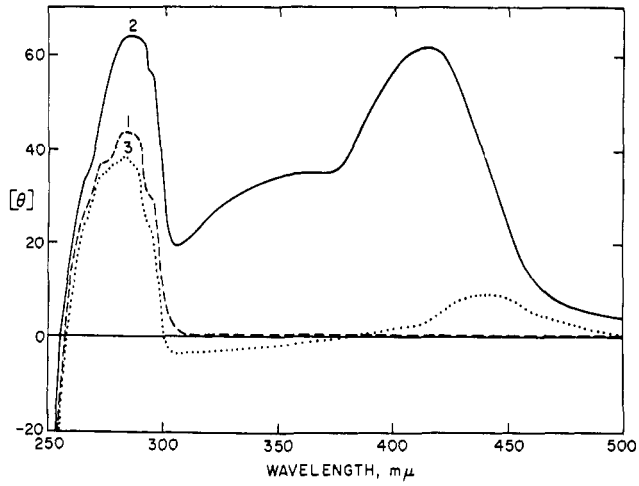


FIGURE 7: Circular dichroism of NEM-apo-B-protein at 25° in 0.1 M potassium phosphate (pH 7.8), containing 1 mM EDTA in the presence and absence of chromophores. Measurements were made in a rectangular cell with a 1-cm path length at a protein concentration of 3.9 mg/ml from 500 to 300 nm and of 1.95 mg/ml from 300 to 250 nm. Curve 1: NEM-apo-B. Curve 2: pyridoxal-P (0.1 mM) was added to NEM-apo-B 30 min before the spectrum was recorded. Curve 3: α^5-pyridoxal methyl chloride (0.16 mM) was added to NEM-apo-B 100 min before the spectrum was recorded (Figure 2A).

by analog, or by both, results in no conformational change which can be detected by this method.

Optical rotatory dispersion and circular dichroism measurements at higher wavelengths can give information about the conformation and environment of the aromatic residues and of bound chromophores (Ulmer and Vallee, 1965). Pyridoxal-P and other optically inactive chromophores may become optically active when bound to an asymmetric site on a protein. The binding of pyridoxal-P and its analogs to a number of apoenzymes has been studied by these techniques (Fasella, 1967). Figure 7 shows circular dichroism curves for different forms of the NEM-B-protein. The NEM-apo-B-protein (curve 1) shows positive ellipticity bands between 260 and 300 nm with a maximum at 285 nm and a shoulder at 295 nm. Addition of pyridoxal-P (curve 2) results in an increased amplitude of these positive ellipticity bands but in no obvious change in the shape of the bands. The circular dichroism spectrum between 300 and 500 nm corresponds closely in shape to the absorbance spectrum in this region (see Figure 2D); it exhibits a positive ellipticity band centered at 410 nm and a positive shoulder around 340 nm.

NEM-apo-B-protein treated with α^5-pyridoxal methyl chloride (curve 3) shows a small positive ellipticity band centered at 440 nm which is near the absorption maximum of the modified enzyme (Figure 2D). There is also a broad negative ellipticity band of low but significant amplitude at lower wavelengths which corresponds to a shoulder observed between 300 and 350 nm in the absorption spectrum of the modified enzyme (Figure 2C).

The circular dichroism spectrum of the modified enzyme (below 300 nm) is close to that of the NEM-apo-B-protein. This indicates that the conformation of aromatic amino acids is not greatly affected by modification and thus extends the similar findings of the optical rotatory dispersion data. However, the bound analog does not produce the enhancement in the "aromatic region" which is produced as a result of pyridoxal-P binding.

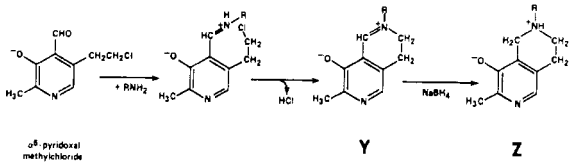
Effects of Modification by α^5-Pyridoxal Methyl Chloride on the Association of B-Protein with A-Protein. Previous studies (Miles, 1970) have shown that B protein which has been modified with NEM under the conditions used in this paper forms a normal $\alpha_2\beta_2$ complex with the A-protein of tryptophan synthetase. The apparent association constant for the formation of this complex is unaltered, the complex is active in the catalysis of the conversion of indoleglycerol phosphate to indole and glyceraldehyde 3-phosphate in the presence of hydroxylamine, and the complex sediments normally at 6.4 S in sucrose gradients containing pyridoxal-P and serine.

In the present study the serine deaminase and indoleglycerol phosphate cleaving activities of NEM-B-protein were measured before and after modification with various concentrations of α^5-pyridoxal methyl chloride. The conditions of modification were very similar to those shown in Figure 2C. The indoleglycerol phosphate cleavage was assayed using [^{14}C]-indole-3-glycerol phosphate in the presence of A-protein and hydroxylamine (Miles, 1970). The results showed that modification resulted in identical losses in the serine deaminase and indoleglycerol phosphate cleaving activities.

Discussion

α^5-Pyridoxal methyl chloride irreversibly inactivates the B-protein of tryptophan synthetase. This inactivation is prob-

SCHEME I



ably due to a reaction at the active site since the rate of inactivation is greatly decreased by the presence of pyridoxal-P. Since pyridoxal-P binding is reversible and the final step of analog binding is irreversible, pyridoxal-P would not be expected to completely prevent analog binding at the active site. However, partial protection by pyridoxal-P is not conclusive evidence that the analog reacts at the active site; pyridoxal-P could stabilize a conformation in which some other site is unavailable for modification.

α^5-Pyridoxal methyl chloride is not a highly specific reagent since several moles react with 1 mol of B-monomer when excess reagent is used. However, the reaction is much more specific when a higher concentration of enzyme is treated with a small excess of the analog. Under these conditions, loss of activity is stoichiometric with incorporation of about 1 mol of analog/mol of B-monomer. The incorporation of 1 mol of analog results in approximately the same extent of quenching of the tryptophan fluorescence as that which is produced by the binding of 1 mol of pyridoxal-P. This is further evidence that pyridoxal-P and the analog bond at the same site.

The product of the reaction of α^5-pyridoxal methyl chloride with the enzyme has been isolated after acid hydrolysis and shown to be identical to the cyclic imino acid derivative of homopyridoxal, compound Y, which was previously synthesized from α^5-pyridoxal methyl chloride and N^α-acetyl-L-lysine (Miles *et al.*, 1972). The reaction with the enzyme probably proceeds through two steps (see Scheme I). The carbonyl group of the analog first forms a Schiff base with the ϵ-amino group of a lysyl residue, presumably the same amino group which normally forms a Schiff base with the carbonyl group of pyridoxal-P. Then the imino nitrogen is slowly alkylated by the 5-chloroethyl side chain to form a stable six-membered ring. It is not surprising that a relatively high concentration of the analog is required for inactivation (0.1 mM), since pyridoxal-P enzymes have a low affinity for derivatives of pyridoxal which lack the 5′-phosphate moiety. Although the tryptic peptide containing the cyclic imine derivative has not been isolated and compared with the "active-site peptide" of Fluri *et al.* (1972) which contains pyridoxal-P covalently bound by reduction with $NaBH_4$, this is feasible and would constitute final proof of the active site location. It seems likely that other accessible ϵ-amino groups of lysine react with α^5-pyridoxal methyl chloride when excess reagent is used.

Studies of the optical rotation and circular dichroism of the modified and untreated enzymes show that no change in the total ordered structure or in the environment of the aromatic residues which can be detected by these methods accompanies modification. The optical activity of the covalently bound analog is much weaker than that of the reversibly bound pyridoxal-P. This is probably due to the fact that the analog does not have a second point of attachment to the enzyme through the electrostatic interaction of the 5′-phosphate side chain and is therefore more free to rotate on the enzyme surface.

The binding of analog and of pyridoxal-P both increase the absorption of the B-protein below 300 nm (see Figure 2C). Schiff bases formed between vitamin B_6 derivatives and amino acids absorb in this region (Johnson and Metzler, 1970). Therefore, it is not clear whether enzyme-bound pyridoxal-P itself contributes to the ellipticity bands observed below 300 nm, or whether the increased amplitude of these bands is due to a change in the environment of the aromatic residues when pyridoxal-P is bound. Martinez-Carrion *et al.* (1970) have observed large changes in the amplitude, sign, and fine structure of ellipticity bands below 300 nm when apo-(aspartate transaminase) binds pyridoxal-P and have attributed these changes to changes in the conformation of the aromatic amino acids. One interpretation of the data of Figure 7 is that bound pyridoxal-P exhibits positive ellipticity bands at 410, 350, and 280 nm, whereas the bound analog shows only weak bands in these regions and that the ellipticity bands in the aromatic region are essentially identical in the three forms of the enzyme. It is not possible to decide from these data whether this is the correct interpretation.

It is surprising that the modified enzyme does not facilitate the cleavage of indoleglycerol phosphate by the A-protein. Both NEM-apo-B-protein and $NaBH_4$-reduced holo-B-protein carry out this function. It is possible that modification has resulted in some critical conformational change which was not detected by the optical rotation and circular dichroism measurements or that the presence of cyclic derivative at the active site is sufficient to interfere with the interaction of the A- and B-proteins.

Possible Uses of α^5-Pyridoxal Methyl Chloride in the Study of Pyridoxal-P Enzymes. A promising use for this reagent is for the introduction of a covalently attached, environmentally sensitive chromophore into the active site of the enzyme. The model cyclic derivative, compound Y (Miles *et al.*, 1972), is very stable, has an intense yellow color over a wide range of pH, and has a strong fluorescence below pH 6.0. Studies of the effects of pH and other factors on the fluorescence of this group and on its interaction with neighboring tryptophan residues might give useful information on the active-site region.

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Mode of Action of Vitamin K. Calcium Binding Properties of Bovine Prothrombin†

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ABSTRACT: Due to recent observations which suggest that the calcium binding property of prothrombin results from the action of vitamin K, the binding of calcium to prothrombin has been examined more carefully. The binding curve is sigmoidal, and at 1 mM calcium, 4 mol of calcium is bound per mol of prothrombin. There was a strong dependence of calcium binding on pH with maximum binding occurring between pH's 7.5 and 9.5. The loss of calcium binding on either side of this range is very marked, suggesting an ionization event at these pH's. A biologically inactive prothrombin produced by dicumarol-treated cows has a lower calcium binding affinity, and at 1 mM less than 1 mol of calcium is bound per mol. In contrast to normal prothrombin, this abnormal prothrombin does not adsorb onto barium citrate, and this property appears to be related to the same sites that cause calcium binding. Modification of prothrombin with 8 M urea or 8 M urea plus reduction of disulfide bonds revealed that tertiary structure and disulfide bonds are necessary for maximum calcium binding. However, if the proteins were allowed to refold and reoxidize, calcium binding was fully restored. Similar treatment of the abnormal prothrombin did not result in any detectable calcium binding. These observations indicate that the abnormal prothrombin differs from the normal protein in some way other than its disulfide-bond arrangement or tertiary structure.

The hepatic biosynthesis of prothrombin and three other blood-clotting enzymes (factors VII, IX and X) is dependent upon the vitamin K status of the animal. Evidence has been presented to suggest that the vitamin acts to convert a preformed polypeptide chain to active prothrombin (Suttie, 1970; Shah and Suttie, 1971). In support of this general mechanism, Stenflo (1970) has reported the existence, and partial purification of an inactive prothrombin which is found in the circulatory system of cows treated with the vitamin K antagonist, dicumarol. There have been a number of other reports of new proteins or prothrombin of low specific activity in the plasma of vitamin K deficient or dicumarol-treated animals (Dulock and Kolmen, 1968; Hemker and Muller, 1968; Ganrot and Niléhn, 1968; Josso *et al.*, 1968; Malhotra and Carter, 1971; Morrissey *et al.*, 1972; Johnson *et al.*, 1972; Pereira and Couri, 1971, 1972). What relationship all of these have to the protein described by Stenflo is not yet clear.

We have reported the purification of the protein described by Stenflo, and have demonstrated that its amino acid and carbohydrate content is similar to that of normal prothrombin (Nelsestuen and Suttie, 1972b). Of the many properties tested the only differences observed between this abnormal prothrombin[1] and prothrombin are the biological activity, the failure of abnormal prothrombin to adsorb quantitatively onto barium citrate, and, as indicated by Stenflo (1970) and Josso *et al.* (1970), the alteration of the electrophoretical mobility of prothrombin in the presence of calcium ions.

The observations on the properties of normal and abnormal prothrombin indicate that the major difference between the two proteins is in the calcium binding site. The available data also suggest that formation of this calcium binding site, which is required to bind phospholipids to prothrombin (Bull *et al.*, 1972), may result from vitamin K action. We report here studies on the calcium binding by the prothrombin proteins.

Materials and Methods

Prothrombin. Bovine prothrombin was purified by a modification (Nelsestuen and Suttie, 1972a) of the method of Ingwall and Scheraga (1969) and by acrylamide gel electrophoretic analysis contained no single contaminating protein which accounted for as much as 1% of the total protein. The abnormal prothrombin was purified as previously described (Nelsestuen and Suttie, 1972b) from the plasma of dicumarol-treated cows. The purity of this protein approached that of normal prothrombin as judged by a quantitative antibody assay, by acrylamide gel electrophoretic analysis, and by

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[1] The inactive protein produced by dicumarol-treated cows will be referred to simply as abnormal prothrombin while the normal enzyme will be referred to as prothrombin or normal prothrombin.