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Title: DETERMINATION OF PYRIDOXAL PHOSPHATE AND PYRIDOXAL BY THE CYANOHYDRIN METHOD

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Pyridoxal phosphate is a coenzyme in about 50 known enzymatic reactions. A simple and accurate method for the determination of pyridoxal phosphate would be desirable because it could provide a means to assess the nutritional status of vitamin B_6 in the human.

The cyanohydrin methods to determine pyridoxal phosphate appear to be simple and promising. Cyanohydrin methods have been devised by Bonavita and Scardi, and Bonavita, and applied to biological materials by Yamada et al.

The cyanohydrin procedure of Yamada et al. was investigated. In this procedure, the pyridoxal phosphate and pyridoxal in a deproteinized sample are separated with the use of a column of SM-cellulose (1 gm., equilibrated with 0.01 N acetic acid). Pyridoxal phosphate is eluted from SM-cellulose with 0.01 N acetic acid, and

pyridoxal is eluted with 0.1 M sodium phosphate buffer, pH 7.4. Pyridoxal phosphate and pyridoxal are converted to their respective cyanohydrin derivatives by reaction with potassium cyanide. These cyanohydrin derivatives are measured fluorometrically at their activation and fluorescence maxima.

In preliminary studies on the procedure by Yamada et al., the activation and fluorescence spectra of the cyanohydrin derivatives of pyridoxal phosphate and pyridoxal were obtained to determine the appropriate activating and fluorescent wavelength settings to use for subsequent fluorometric analyses. Pyridoxal phosphate cyanohydrin at pH 3.8 in 0.2 M sodium phosphate buffer had an activation maximum at 325 mμ and a fluorescence maximum at 415 mμ; and pyridoxal cyanohydrin at pH 10 in 0.2 M sodium phosphate buffer had an activation maximum at 355 mμ and a fluorescence maximum at 435 mμ. To obtain maximum fluorescence of the cyanohydrin derivatives, pyridoxal phosphate had to be reacted with potassium cyanide at 50°C for 60 minutes, and pyridoxal had to be reacted for 150 minutes.

Following these preliminary studies, the elution pattern of pyridoxal phosphate and pyridoxal from a column of SM-cellulose was investigated. Pyridoxal phosphate was eluted with 0.01 N acetic acid; and pyridoxal, with both 0.01 N acetic acid and 0.1 M sodium phosphate buffer, pH 7.4.

The recovery of pyridoxal phosphate from SM-cellulose was

93.5% when pyridoxal phosphate alone was applied to the column, and that of pyridoxal was 108.8% when pyridoxal alone was applied. When a mixture of pyridoxal phosphate and pyridoxal was applied to SM-cellulose, the recovery of pyridoxal phosphate was 105.5% and that of pyridoxal was only 59.8%.

When either standard alone was added to blood, the recovery of pyridoxal phosphate in blood from SM-cellulose was 85.0%, and that of pyridoxal was only 29.1%. When a mixture of pyridoxal phosphate and pyridoxal was added to blood, the recovery of pyridoxal phosphate in blood from SM-cellulose was 62.6%, and that of pyridoxal was 52.1%. This lower recovery of pyridoxal phosphate in blood was due mainly to the high readings of the blanks. This higher recovery of pyridoxal phosphate in blood may be explained by the low concentration of pyridoxal in the buffer fractions from a column of SM-cellulose to which a mixture of pyridoxal phosphate and pyridoxal had been applied that was used to calculate the recovery. Determining the recovery of standards added to the supernatant after the precipitation of the proteins in blood, rather than to the hemolyzed blood before precipitation, would indicate whether pyridoxal phosphate and pyridoxal were lost by adsorption on the protein precipitate.

The modified procedure of Yamada et al. is not sensitive enough to determine the pyridoxal phosphate and pyridoxal content of human blood.

Determination of Pyridoxal Phosphate and Pyridoxal by the Cyanohydrin Method

by

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DETERMINATION OF PYRIDOXAL PHOSPHATE AND PYRIDOXAL BY THE CYANOHYDRIN METHOD

INTRODUCTION

Pyridoxal phosphate is a coenzyme in about 50 known enzymatic reactions (13). It plays an important role in protein metabolism and appears to be involved in the metabolism of carbohydrate and fat.

A simple and accurate method for the determination of pyridoxal phosphate in biological materials would be helpful in assessing the nutritional status of vitamin B_6 in the human. Several chemical, enzymatic, and microbiological methods to determine pyridoxal phosphate are available. The application of these methods to standards has been satisfactory, but their application to biological materials has not been entirely satisfactory.

The cyanohydrin methods to determine pyridoxal phosphate, and pyridoxal, appear to be simple and promising. Cyanohydrin methods have been devised by Bonavita and Scardi (10) and Bonavita (9), and applied to biological materials by Yamada et al. (104).

The purpose of this thesis is to investigate the determination of pyridoxal phosphate and pyridixal by the cyanohydrin procedure of Yamada et al. In this procedure the proteins in hemolyzed blood are precipitated by trichloroacetic acid, and the pyridoxal phosphate and

pyridoxal in the supernatant are separated with the use of a column of SM-cellulose (1 gm., equilibrated with 0.01 N acetic acid). Pyridoxal phosphate is eluted from SM-cellulose with 0.01 N acetic acid, and pyridoxal with 0.1 M sodium phosphate buffer, pH 7.4. Pyridoxal phosphate and pyridoxal are converted to their respective cyanohydrin derivatives by reaction with potassium cyanide. The cyanohydrin derivatives of pyridoxal phosphate and pyridoxal are measured fluorometrically at their activation and fluorescence maxima.

Reported herein are studies on the elution pattern of pyridoxal phosphate and pyridoxal from SM-cellulose, the recovery of pyridoxal phosphate and pyridoxal from SM-cellulose, and the recovery of pyridoxal phosphate and pyridoxal in blood from SM-cellulose. An attempt was made to determine the pyridoxal phosphate and pyridoxal content of blood from a 24-year old female according to the modified procedure of Yamada et al.

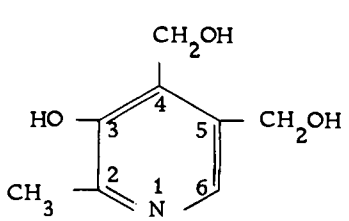
REVIEW OF THE LITERATURE

Historical Background

In 1934 György discovered that the lack of a component of the vitamin B_2 complex caused pellagra-like dermatitis in rats. György named this factor "vitamin B_6" (30, 81).

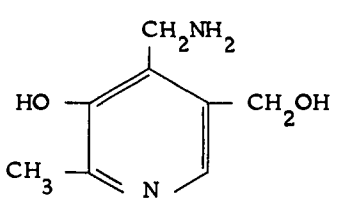
Crystalline vitamin B_6 was isolated from rice bran by Lepkovsky (54, 55) in 1938. That same year Keresztesy and Stevens (50), György (31, 32), Kuhn and Wendt (52), and Ichiba and Michi (47) also isolated vitamin B_6 (17, p. 75).

The structure of vitamin B_6 as elucidated by Stiller et al. (84), Harris et al. (42), and Kuhn and Wendt (52) was 2-methyl-3-hydroxy-4, 5-(dihydroxymethyl)-pyridine (Formula I). Vitamin B_6 was synthesized by Harris and Folkers (39) in 1939. György and Eckhardt (33) suggested that vitamin B_6 be named pyridoxine. Pyridoxine was the term adopted by the American Medical Association Council on Pharmacy and Chemistry (3) in 1940.



(I)

In 1942 substances more biologically active than pyridoxine were found by Snell et al. (32; 81; 82; 98, p. 170) in yeast extracts and extracts of rat brain, heart, kidney, liver, and leg muscle. Snell (78, 79) suggested that these substances could be the amine and aldehyde analogs of pyridoxine (17, p. 84). These two analogs were later synthesized by Harris et al. (40, 41). The structures of these newly synthesized compounds were similar to the one for pyridoxine except for the functional group at position 4. Harris et al. (40, 41) named the compound with $-CH_2NH_2$ at position 4 pyridoxamine (Formula II) and the compound with -CHO at position 4 pyridoxal (Formula III). Pyridoxamine and pyridoxal greatly exceeded pyridoxine in promoting growth of lactic acid bacteria, such as Lactobacillus casei and Streptococcus lactis R (40, 41, 79). Pyridoxamine and pyridoxal were found mainly in animal tissues and yeast; and pyridoxine in plant materials (73).



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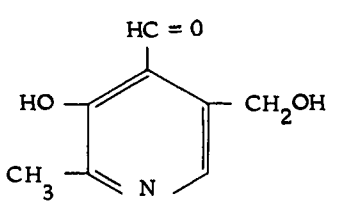
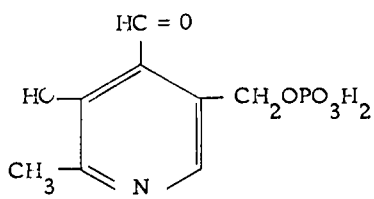


(III)

Phosphorylated derivatives of these three pyridine analogs also

occur naturally. A bound form of vitamin B_6 necessary for amino acid decarboxylation (24, 81) was identified as pyridoxal-5-phosphate (Formula IV) by Gunsalus et al. (27, 28). Another bound form of vitamin B_6 discovered by Rabinowitz and Snell (72) and Snell (81) was pyridoxamine-5-phosphate. Pyridoxine-5-phosphate has also been identified (81, 97).



(IV)

In 1949 the American Institute of Nutrition (1) recommended that the term vitamin B_6 should be used as the group name for pyridoxine, pyridoxamine, and pyridoxal. In 1960 the Commission on the Nomenclature of Biological Chemistry of the International Union of Pure and Applied Chemistry (48) suggested that the group name of the three naturally occurring forms of vitamin B_6 should be pyridoxine. The Commission also suggested the following names, depending on the functional group at position 4, for the components of pyridoxine: pyridoxol ($-CH_2OH$), pyridoxamine ($-CH_2NH_2$), and pyridoxal (-CHO). In current literature the analog with the hydroxymethyl group at position 4 is still called pyridoxine rather than pyridoxol. In this thesis

pyridoxine will be used to designate the 4-hydroxymethyl analog, and vitamin B_6 to designate the group name for the three naturally occurring forms.

Functions of Pyridoxal Phosphate

Protein Metabolism

Pyridoxal phosphate, the active form of vitamin B_6, functions as a coenzyme in many reactions involved in the metabolism of amino acids (17, p. 88; 61, p. 341-348; 87; 91; 98, p. 176-178). These reactions include:

Transamination

In transamination the α-amino group of an amino acid is transferred to the α-carbonyl group of a keto acid to produce the corresponding α-keto and α-amino acids. The keto acid may be either ketogenic or glycogenic. An example of a transamination reaction is:

$$\begin{array}{ccc} \text{glutamic acid} & \xrightarrow[\text{transaminase}]{\text{aspartic-glutamic}} & \alpha\text{-ketoglutaric acid} \\ + & & + \\ \text{oxaloacetic acid} & & \text{aspartic acid} \end{array}$$

Pyridoxal phosphate catalyzes this reaction by the formation of a Schiff base with glutamic acid. Transaminases from yeast (29, 45, 75), animals (53), and plants (99) are activated by pyridoxal phosphate.

Decarboxylation

Pyridoxal phosphate catalyzes the removal of the carboxyl group from some amino acids. Pyridoxal phosphate is required for the formation of the biologically active amines, histamine, norepinephrine, and serotonin (89).

Racemization

D- or L-amino acids can be converted through the formation of the α-keto acid to the corresponding L- or D-amino acids, respectively, by the catalysis of pyridoxal phosphate (44).

Elimination

Pyridoxal phosphate catalyzes α, β-elimination reactions in amino acid metabolism, including the following: dehydration and subsequent deamination of serine to pyruvic acid, and of threonine to α-ketobutyric acid; desulfhydration and subsequent deamination of cysteine to pyruvate; and cleavage of tryptophan by tryptophanase to indole and pyruvic acid (61, p. 345; 98, p. 176).

Pyridoxal phosphate catalyzes α, γ-elimination reactions, such as desulfhydration and subsequent deamination of homocysteine, and dehydration and subsequent deamination of homoserine (61, p. 345; 98, p. 178).

Tryptophan Synthesis

Pyridoxal phosphate catalyzes the condensation of serine and indole to form tryptophan (61, p. 346).

Dealdolization of Serine

The dealdolization of serine to glycine requires pyridoxal phosphate. The formaldehyde produced in this reaction is accepted by tetrahydrofolic acid (61, p. 347).

Oxidative Deamination of Amines

Certain amine oxidases require pyridoxal phosphate as a coenzyme for the oxidative deamination of amines. Oxidative deamination is an important mechanism for the detoxification of several amines (13, 38, 44).

Carbohydrate Metabolism

Pyridoxal phosphate is a component of muscle glycogen phosphorylase, an enzyme which catalyzes the breakdown of glycogen. Although the function of pyridoxal phosphate in glycogen phosphorylase is unknown, the enzyme is inactive if pyridoxal phosphate is removed. The most concentrated source of pyridoxal phosphate in the mammalian body is muscle (53), where vitamin B_6 is probably stored

as muscle phosphorylase (4, 51).

Fat Metabolism

Whether pyridoxal phosphate is essential for the metabolism of fat is not known. Nutritional evidence suggests that pyridoxal phosphate may be necessary for the formation of arachidonic acid from linoleic acid (65, p. 452; 102).

Chemical and Physical Properties of Pyridoxal Phosphate

Pyridoxal phosphate is stable in the crystalline form, and unstable in solution. When dissolved, pyridoxal phosphate decomposes at room temperature at a rate of 5-7% per month; and at 0°C at a rate of 2-4% (6, p. 1026). Fujita et al. (21) found that pyridoxal phosphate was stable at 40°C for 30 days in a solution containing 0.1 M Sorensen's buffer at pH 7.0 and 1-4 moles of $Na_2S_2O_4$. Pyridoxal phosphate is easily hydrolyzed in an acid solution (6, p. 1026).

Pyridoxal phosphate is very unstable to light. It is stable to heat, even when heated at 40°C for 30 minutes (106).

The absorption spectrum of pyridoxal phosphate depends on the pH of the solution. Peterson and Sober (68) reported that the absorption of pyridoxal phosphate in an acidic medium was at 295 mμ; in a

neutral medium, at 330 and 388 mμ (absorption maximum); and in an alkaline medium, at 305 and 388 mμ (absorption maximum). Recent spectrophotometric studies by Matsushima and Martell (62) showed that the absorption of pyridoxal phosphate in acidic methanol occurred at 230 and 295 mμ (absorption maximum); in neutral methanol, at 249, 289 (absorption maximum) and 340 mμ; and in alkaline methanol, at 232 (absorption maximum), 306, and 390 mμ.

Of the known vitamin B_6 compounds, pyridoxal phosphate is most weakly fluorescent. Storvick et al. (85) reported that pyridoxal phosphate in 0.1 M phosphate buffer, pH 7.0, had activation and fluorescence maxima at 330 and 375 mμ, respectively.

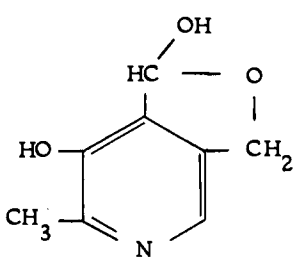
Chemical and Physical Properties of Pyridoxal

Pyridoxal dissolved in a 0.02 M phosphate buffer at pH 6.8 is rapidly destroyed by light, and is destroyed even more rapidly by light when oxygen is present (15, 80). Shiroishi and Hayakawa (76) suggested that oxygen participates in the photolysis of pyridoxal. Pyridoxal is completely destroyed by 30% hydrogen peroxide (14, 15) or exposure to ultraviolet light for five minutes (14).

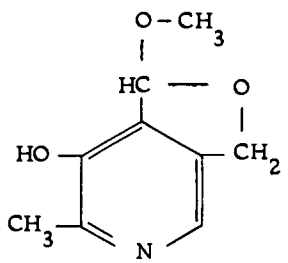
Pyridoxal is stable to heat in the presence of hydrochloric, sulfuric, or nitrous acid (80), but not in the presence of nitric acid, which is an oxidizing agent (15). Other oxidizing agents, such as manganese oxide and potassium permanganate, destroy pyridoxal at

room temperature in an acidic medium but not in an alkaline one (15).

Bolliger (7) stated that pyridoxal hydrochloride in hot methanol was not stable and that it formed two or three spots on a thin layer chromatogram. Bolliger suggested that the pyridoxal in hot methanol may have been in the form of a hemiacetal (Formula V) or an acetal (Formula VI).



(V)



(VI)

The absorption characteristics of pyridoxal depend on pH. Peterson and Sober (68) found that absorption of pyridoxal in an acidic medium occurred at 288 mμ; in a neutral medium, at 318 (absorption maximum) and 390 mμ; and in an alkaline medium, at 300 (absorption maximum) and 393 mμ. Recently Matsushima and Martell (62) found that the absorption peaks of pyridoxal in acidic methanol were at 230 and 290 mμ (absorption maximum); in neutral methanol, at 220 (absorption maximum), 280, and 328 mμ; and in alkaline methanol, at 234 (absorption maximum), 303, and 389 mμ.

Pyridoxal also exhibits fluorescence. Storvick et al. (85) reported that the fluorescence characteristics of pyridoxal in 0.1 M

phosphate buffer, pH 7.0, are 320 mμ activation and 385 mμ fluorescence. Pyridoxal is more fluorescent than pyridoxal phosphate (85).

Pyridoxal forms colored products when reacted with diazotized sulfanilic acid, concentrated sulfuric acid, sulfuric acid and thiophene, acetone and sodium hydroxide, ethanolamine, and phenylhydrazine. These reactions will be discussed further in the section "Determination of Pyridoxal", which follows.

Determination of Pyridoxal Phosphate

Chemical and Physical Methods

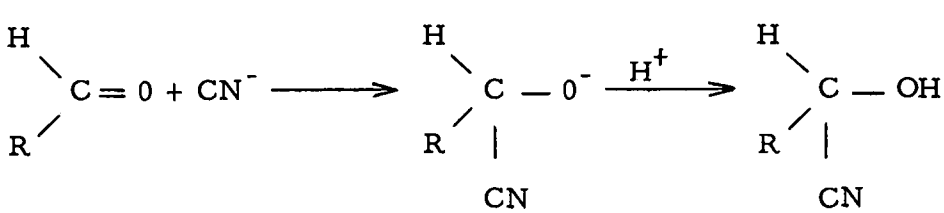
Spectrophotometric Methods

Spectrophotometric measurements of pyridoxal phosphate are based on the characteristic absorption of pyridoxal phosphate at around 388 mμ; all of the other components of vitamin B_6 have a characteristic absorption band at around 320 mμ. By measuring the absorption of pyridoxal phosphate at 388 mμ, Oike et al. (66) determined the recovery of pyridoxal phosphate eluted from a paper chromatogram, and Hayashi (43) determined the pyridoxal phosphate extracted from cerebral tissue.

Gaudiano and Polizzi-Sciarrine (25) proposed a method to determine pyridoxal phosphate (10-20 μg./ml.) in the presence of a high concentration of pyridoxal. At 395 mμ pyridoxal phosphate in 0.1 M

glycine at pH 5.4 absorbed maximally, and pyridoxal in 0.1 M glycine at pH 5.4 absorbed negligibly. Pyridoxal in a solution with or without glycine had a maximum absorption at 315 mμ. Gaudiano and Polizzi-Sciarrine (26) suggested that the suitable wavelengths for the measurement of pyridoxal and pyridoxal phosphate at pH 7.0 were, respectively, 315 and 389 mμ.

When pyridoxal phosphate was reacted with potassium cyanide at pH 7.4 in a sodium phosphate buffer, absorption occurred at 320 mμ and the characteristic absorption maximum of pyridoxal phosphate at 385 mμ was completely leveled off (74). Similarly, when pyridoxal was converted to its cyanohydrin derivative by reaction with potassium cyanide at pH 7.4 in a sodium phosphate buffer, absorption occurred at 350 mμ and not at the characteristic absorption band for pyridoxal, 315 mμ (9). In these reactions, potassium cyanide reacted with the 4-formyl group of pyridoxal phosphate or pyridoxal to form the respective cyanohydrin derivative (10):



R = the pyridine ring of pyridoxal phosphate or pyridoxal.

When pyridoxal phosphate standards were reacted with an excess of potassium cyanide, the decrease in absorbance at 385 mμ was directly proportional to the concentration of pyridoxal phosphate (0-25 μg./ml.)(10). The wavelength 385 mμ was chosen because at pH 7.0 all of the vitamin B_6 components except pyridoxal phosphate have a characteristic absorption maximum at around 320 mμ. Pyridoxal phosphate and its cyanohydrin derivative have different electrophoretic mobilities (10).

Fluorometric Methods

Fasella and Baglioni (18) described a method in which the components of vitamin B_6 were separated by paper chromatography and detected by exposure to ultraviolet light. Pyridoxal phosphate showed a blue fluorescence when untreated, or after having been exposed to ammonia vapors. This method, however, does not give a satisfactory resolution between pyridoxal phosphate and pyridoxamine phosphate. Only high concentrations of the vitamin B_6 components could be measured by this method.

Fasella et al. (19) developed a fluorometric method to determine pyridoxal phosphate in the presence of pyridoxamine phosphate. Only standards were studied. At pH 7 and at the characteristic wavelengths for pyridoxal phosphate, 330 mμ activation and 390 mμ fluorescence, pyridoxamine was 60 times more fluorescent than pyridoxal

phosphate. At a higher pH and at 410 mμ activation and 525 mμ fluorescence, pyridoxamine showed no fluorescence, while pyridoxal phosphate was five times more fluorescent than at pH 7 and at 330 mμ activation and 390 mμ fluorescence. The advantages of this method are speed, simplicity, specificity, and only a small sample is required.

The fluorescence spectra of pyridoxal phosphate cyanohydrin and pyridoxal cyanohydrin are different from those of pyridoxal phosphate and pyridoxal, respectively. At 313 mμ activation and 420 mμ fluorescence, pyridoxal phosphate cyanohydrin was 25 times more fluorescent than pyridoxal phosphate, pyridoxal, or pyridoxal cyanohydrin; at 358 mμ activation and 430 mμ fluorescence, pyridoxal cyanohydrin was 45 times more fluorescent than pyridoxal, pyridoxal phosphate or pyridoxal phosphate cyanohydrin (9). A fluorometric method based on these properties of the cyanohydrin derivatives of pyridoxal phosphate and pyridoxal was developed by Bonavita (9) to determine pyridoxal and pyridoxal phosphate in the presence of each other.

When pyridoxal phosphate was reacted with an excess of potassium cyanide, fluorescence was proportional to the concentration of pyridoxal phosphate (0.01 to 0.12 μg./ml.)(9). The fluorometric procedure for measuring pyridoxal phosphate as a cyanohydrin derivative is more sensitive than the spectrophotometric one.

The cyanohydrin method of Bonavita (9) was used by Bonasera

et al. (8) to determine the concentration of pyridoxal phosphate in the brains of mice, rats, rabbits, and cats.

Toepfer et al. (86) adapted the cyanohydrin method of Bonavita (9) to determine both pyridoxal and pyridoxamine. Pyridoxamine was oxidized to pyridoxal by reaction with sodium glyoxylate and potassium alum. After pyridoxal cyanohydrin was obtained, the pH was adjusted to 9.5, and fluorescence readings were made at the activating and fluorescent wavelengths, 358 mμ and 435 mμ, respectively. This method has not been applied to biological materials.

Polansky et al. (70) utilized the cyanohydrin method of Bonavita (9) to determine pyridoxine after it was oxidized to pyridoxal by manganese oxide. Fluorescence readings were made at the activating and fluorescent wavelengths, 356 and 435 mμ, respectively. A linear relationship was found over the range of 0.005 to 0.5 μg. of pyridoxine per ml.

Yamada et al. (104) modified the method of Bonavita (9) to determine pyridoxal phosphate and pyridoxal in hemolyzed human blood and in homogenates of mouse liver, brain, and kidney. Pyridoxal phosphate and pyridoxal in the deproteinized sample were separated before assay by column chromatography with SM-cellulose. The recovery of pyridoxal phosphate from hemolyzed human blood was 97.4%; and from mouse liver, 99.1%. The recovery of pyridoxal from hemolyzed human blood and mouse liver was, respectively,

95.9% and 97.4%. The cyanohydrin procedure of Yamada et al. may hold promise for the determination of pyridoxal phosphate and pyridoxal in biological materials.

Enzymatic Methods

Tyrosine Decarboxylase

In 1945 Umbreit et al. (90) found that a derivative of pyridoxine in yeast was the coenzyme of tyrosine decarboxylase. This coenzyme was determined by a manometric procedure in which the amount of carbon dioxide produced from the decarboxylation of tyrosine by tyrosine decarboxylase was measured.

Boxer et al. (12) modified the manometric method of Umbreit et al. (90) to determine the pyridoxal phosphate in whole blood and isolated leukocytes of man and animals. The method of Boxer et al. is also based on the measurement of carbon dioxide produced from the decarboxylation of tyrosine by tyrosine apodecarboxylase. The amount of carbon dioxide produced depends upon the amount of pyridoxal phosphate in the medium. Pyridoxal phosphate in blood occurs in a form that is either free or readily available for tyrosine apodecarboxylase. The concentration of pyridoxal phosphate in whole blood of most human adults is, however, lower than the minimum concentration detectable in biological materials by this method of Boxer

et al., 10 mμg./ml.

The manometric method of Boxer et al. was used by Wachstein et al. (92) to determine the pyridoxal phosphate content of plasma and leukocytes in pregnant women and non-pregnant controls who had received a load dose of vitamin B_6. The non-pregnant controls had higher levels of plasma pyridoxal phosphate than women in the last trimester of pregnancy or at delivery. Wachstein et al. suggested that the level of pyridoxal phosphate in plasma after the subject had ingested 100 mg. of pyridoxine could be used to determine vitamin B_6 nutriture.

The manometric method of Boxer et al. (12) was also used by Wachstein et al. to determine the pyridoxal phosphate levels in leukocytes of human maternal and cord blood (95), and in organs, leukocytes, and blood of rats deficient in vitamin B_6 (94). Baysal et al. (5) used the manometric method of Boxer et al. (12) to study the effect of vitamin B_6 depletion in man on plasma pyridoxal phosphate.

Hamfelt and deVerdier (37) proposed a tyrosine decarboxylase method in which the formation of $^{14}CO_2$ from tyrosine-1-^{14}C was measured. Later Hamfelt (34) developed a procedure in which the radioactivity of tyramine-^{14}C(U) formed from the decarboxylation of tyrosine-^{14}C(U) by tyrosine decarboxylase was measured. Tyramine-^{14}C(U) was separated from tyrosine-^{14}C(U) by paper chromatography.

Apotryptophanase

Tryptophan is converted to indole, pyruvic acid, and ammonia by tryptophanase, another pyridoxal phosphate-dependent enzyme (103). In the method by Wada et al. (96) pyridoxal phosphate in blood is determined by the amount of indole formed from tryptophan by apotryptophanase. The standard curve for pyridoxal phosphate was not linear because the enzyme was inhibited by the accumulation of indole.

A modification of the apotryptophanase method has been devised by Storvick et al. (85). Donald and Ferguson (16) developed a micro-procedure of the method by Wada et al. (96) to determine pyridoxal phosphate in rat blood and liver, and in leukocytes of human blood.

Gailani (23) described a reproducible method for the determination of pyridoxal phosphate in tissues and isolated leukocytes by using apotryptophanase obtained from Escherichia coli. Gailani found that maximum extraction of pyridoxal phosphate from the tissues was achieved when the tissues were heated at pH 4. The mean value of pyridoxal phosphate in the isolated leukocytes of the 18 patients with cancer studied by Gailani was 0.021 μg. per 100 million cells. Boxer et al. (12) reported that the mean value of pyridoxal phosphate in leukocytes of normal subjects was 0.015 μg. per 100 million cells.

Transaminase

Holzer et al. (46), and Holzer and Gerlach (45) reported a method for the determination of pyridoxal phosphate in yeasts and animal tissues by using apotransaminase prepared from brewer's yeast. Two enzymatic reactions are involved in this method. The transaminase reaction:

α-ketoglutarate + aspartate $\rightleftharpoons$ glutamate + oxaloacetate,

is coupled with the malic dehydrogenase reaction:

oxaloacetate + reduced nicotinamide adenine dinucleotide
$\rightleftharpoons$ malate + oxidized nicotinamide adenine dinucleotide

Pyridoxal phosphate becomes the limiting factor when an excess of malic dehydrogenase and apotransaminase are present in the assay mixture. Pyridoxal phosphate is measured by the oxidation of nicotinamide adenine dinucleotide, which is followed spectrophotometrically at 340 mμ. Pyridoxamine phosphate is also active as a coenzyme for apotransaminase (69).

Walsh (99) used glutamic-aspartic apotransaminase from wheat germ to determine pyridoxal phosphate in plasma. In this method by Walsh the transaminase reaction was also coupled with the malic dehydrogenase one, and the amount of pyridoxal phosphate in the assay

mixture was measured by the oxidation of nicotinamide-adenine dinucleotide.

The total pyridoxal phosphate and pyridoxamine phosphate content of human plasma has been determined with apoaspartic aminotransferase from brewer's yeast by Schreiber et al. (75) and with aspartate transaminase from baker's yeast by Gvozdova et al. (29).

Determination of Pyridoxal

Chemical Methods

Colorimetric Methods

Color reactions that depend on the presence of a phenolic hydroxyl group include:

Diazotized Sulfanilic Acid. Ormsby et al. (67) reported that pyridoxal reacts with diazotized sulfanilic acid to form a bright yellow compound with a maximum absorption at 440 mμ. In addition to the phenolic hydroxyl group, this color reaction depends on the unsubstituted para position of pyridoxal. The color obtained is very unstable. In addition, this reaction lacks sensitivity and specificity. This procedure has been applied only to standard solutions of pyridoxal.

Concentrated Sulfuric Acid. Pyridoxal forms a yellow complex when treated with concentrated sulfuric acid. This color formation

is based on a reaction involving the aldehyde and phenolic hydroxyl groups of pyridoxal with concentrated sulfuric acid. The color is stable for two days under refrigeration. Levine and Sass (58, 59), who described this method, did not study pyridoxal phosphate which could also form a colored complex with concentrated sulfuric acid because of its aldehyde and phenolic hydroxyl groups. This method was studied with standard solutions of pyridoxal only.

Color reactions that depend on the reactivity of the 4-formyl group of pyridoxal:

Thiophene. Pyridoxal and pyridoxal phosphate react with thiophene and sulfuric acid to form a stable jade-green colored product with a maximum absorption at 615 mμ. The other components of vitamin B_6 do not give this color reaction because they do not possess an aldehyde group. Levine and Hansen (56, 57) suggested that pyridoxal phosphate and short chain aliphatic aldehydes and ketones, which also give a color reaction with thiophene and sulfuric acid, should be removed before measuring pyridoxal. This method has not been applied to biological materials.

Ethanolamine. Pyridoxal reacts with ethanolamine to produce a highly colored complex with an absorption peak at 365 mμ. Metzler and Snell (63) devised a method based on this property of pyridoxal to measure pyridoxal in a transamination reaction mixture composed of

keto acid plus pyridoxamine, or an amino acid plus pyridoxal. According to Metzler and Snell, only p-hydroxyphenylpyruvic acid interferes in this color reaction. A correction can be made for this interference.

Acetone. In the presence of a base, pyridoxal condenses with acetone to form an intensely yellow product with an absorption maximum at 420 mμ. Siegel and Blake (77) found that pyridoxine, pyridoxamine, alanine, glutamic acid, pyruvic acid, α-ketoglutaric acid, and other similar amino and keto acids do not produce a color complex with acetone. Only standard solutions of pyridoxal were studied. Whether pyridoxal phosphate, which also possesses a 4-formyl group, gives a similar color reaction was not reported by Siegel and Blake.

Phenylhydrazine. Both pyridoxal and pyridoxal phosphate react with phenylhydrazine to form an intensely yellow hydrazone with an absorption maximum at 410 mμ. (97). The advantage of the phenylhydrazine method is that pyridoxal or pyridoxal phosphate can be measured in the presence of the other forms of vitamin B_6. This method is less sensitive than the cyanohydrin method of Bonavita (9) and the apotransaminase method of Wada et al. (96), but more sensitive than the method based on the color reaction with ethanolamine or the direct spectrophotometric measurement of pyridoxal or pyridoxal phosphate. Wada and Snell (97) used the phenylhydrazine method to

determine either pyridoxal or pyridoxal phosphate in enzyme reaction mixtures.

Fluorometric Methods

A direct fluorometric method for measuring the pyridoxal content of whole blood was devised by Coursin and Brown (14). Pyridoxal was extracted from blood with acetone and was measured at the activating and fluorescent wavelengths, 330 mμ and 385 mμ, respectively. To obtain a blank that contained no pyridoxal, the extract was treated with 30% hydrogen peroxide or exposed to ultraviolet light for five minutes.

Pyridoxal can be measured indirectly by fluorometry after its conversion to the lactone of 4-pyridoxic acid. Pyridoxal is oxidized to 4-pyridoxic acid, and is subsequently converted to the lactone of 4-pyridoxic acid. The lactone of 4-pyridoxic acid is more fluorescent than either 4-pyridoxic acid or pyridoxal (85). Pyridoxine and pyridoxamine can also be estimated by the lactonization method after conversion to the lactone of 4-pyridoxic acid. The lactone of 4-pyridoxic acid at pH 10.5 has maximum fluorescence at the activating wavelength 355 mμ and the fluorescent wavelength 445 mμ (85).

Fujita et al. (20) were the first to devise a method to measure pyridoxal as the lactone of 4-pyridoxic acid. Pyridoxal was oxidized with ammonia and silver nitrate to form 4-pyridoxic acid. 4-Pyridoxic

acid was then converted to its lactone by hydrochloric acid. To determine pyridoxal as the lactone of 4-pyridoxic acid, MacArthur and Lehmann (60) modified the lactonization procedure of Fujita et al.

Storvick et al. (85) modified the lactonization step of the method by Fujita et al. (20) to develop a microprocedure for the determination of pyridoxal as the lactone of 4-pyridoxic acid. Storvick et al., however, used standard solutions of pyridoxal to evaluate this method and did not apply this microprocedure to biological substances. The desirability of a microprocedure is that pyridoxal can be measured in biological substances which are available only in limited amounts.

The cyanohydrin method to determine pyridoxal was discussed above under "Determination of Pyridoxal Phosphate".

Microbiological Methods

Snell (80) found that Lactobacillus casei responds to pyridoxal only, and can be used as the test organism to determine pyridoxal in the presence of pyridoxamine and pyridoxine. Other organisms used to determine vitamin B_6 include Streptococcus faecalis which responds to pyridoxal and pyridoxamine, and Saccharomyces carlsbergensis which responds to pyridoxal, pyridoxamine and pyridoxine (80).

Rabinowitz et al. (71) improved the procedure for determining pyridoxal in animal or plant tissues with L. casei as the test organism by adding an enzymatic digest of casein to the medium, and

autoclaving the tissue in 180 ml. of 0.055 N hydrochloric acid at 20 pounds pressure for 5 hours. Jirsak (49) used the method of Rabinowitz et al. to determine pyridoxal in blood and serum.

Fukui (22) devised a differential determination of the vitamin B_6 group in animal and plant tissues by using S. carlsbergensis as the test organism. Total vitamin B_6 content in an acid hydrolysate of the tissue was determined. Pyridoxamine was then removed by adsorption on a cationic exchange resin, KH4B (Na^+), and pyridoxine and pyridoxal present in the effluent were measured. Pyridoxal was destroyed by treatment with acetone and alkali, and pyridoxine was determined. The content of each component of vitamin B_6 was estimated by difference.

Snyder and Wender (83) separated the three components of vitamin B_6 by paper chromatography, and determined each component with S. carlsbergensis as the assay organism. Only standards were studied.

Pyridoxal Phosphate and Pyridoxal Content of Human Blood

Pyridoxal Phosphate

The pyridoxal phosphate content of human blood as reported in the literature is summarized in Table 1.

Pyridoxal phosphate in whole blood has been determined only

Table 1. Pyridoxal phosphate content of human blood.

Method of determination and reference	No. of subjects	Sex	Age in years	Sample	Content
Tyrosine Decarboxylase					
Boxer et al. (12)	113	-	17-62	blood	102 subjects below 10 mμg./ml. 11 subjects above 10 mμg./ml. highest value 37 mμg./ml.
	111	-	25-75	blood	90 subjects below 10 mμg./ml. 21 subjects above 10 mμg.. ml. highest value 36 mμg./ml.
	8	-	premature infants	blood	all subjects above 10 mμg./ml. av. 32 ± 8 mμg./ml. highest value 46 mμg./ml.
	10	-	0-18 mo.	blood	all subjects above 10 mμg./ml. av. 30 ± 9 mμg./ml. highest value 45 mμg./ml.
	15	-	5-13	blood	all subjects below 10 mμg./ml.
	-	-	adults	leukocytes	0.15 ± 0.07 mμg./million cells
Wachstein et al. (95)	60	women	19-45	leukocytes	0.11-0.79 mμg./million cells av. 0.32 ± 0.02 mμg./million cells
Wachstein et al. (92)	27	men	-	leukocytes	0.15-0.36 mμg./million cells av. 0.23 ± 0.05 mμg./million cells
	20	women	-	leukocytes	0.14-0.30 mμg./million cells av. 0.22 ± 0.05 mμg./million cells
	27	men	-	plasma	5.2-16.2 mμg./ml. av. 10.5 ± 2.5 mμg./ml.
	20	women	-	plasma	5.2-12.0 mμg./ml. av. 8.4 ± 2.5 mμg./ml.
	19	women	-	leukocytes of maternal blood at term	0.02-0.19 mμg./million cells av. 0.09 mμg./million cells
	19	-	-	leukocytes of cord blood	0.11-1.22 mμg./million cells av. 0.47 mμg./million cells
	19	women	-	plasma of maternal blood at term	2-8.6 mμg./ml. av. 4.3 mμg./ml.
	19	-	-	plasma of cord blood	10.8-49.2 mμg./ml. av. 23.2 mμg./ml.
Wachstein et al. (93)	40	-	-	plasma	5.2-33.0 mμg./ml. av. 9.6 ± 2.8 mμg./ml.
	40	-	-	leukocytes	0.14-0.36 mμg./million cells av. 0.22 ± 0.05 mμg./million cells
Hamfelt (34)	12	-	-	plasma	5.0-16.8 mμg./ml. av. 9.9 ± 3.3 mμg./ml.

Table 1. Continued.

Method of determination and reference	No. of subjects	Sex	Age in years	Sample	Contents
Hamfelt (36)	14	women	-	plasma of maternal blood at term	2.4 mμg./ml.
	10	-	-	plasma of cord blood	13.9 mμg./ml.
	37	-	22-27	plasma	11.7 mμg./ml.
	9	-	-	erythrocytes of cord blood	1.46 mμg./10^9 cells
	37	-	22-27	erythrocytes	0.452 mμg./10^9 cells
	9	-	-	leukocytes of cord blood	0.285 mμg./million cells
	20	-	22-27	leukocytes	0.27 mμg./million cells
Hamfelt (35)	14	-	0-3	plasma of cord blood; plasma of child	6.5-57.1 mμg./ml. av. 16.3 ± 16.7 mμg./ml.
	13	-	20-29	plasma	3.8-21.6 mμg./ml. av. 11.3 ± 5.7 mμg./ml.
	11	-	30-59	plasma	2.4-12.4 mμg./ml. av. 7.1 ± 3.0 mμg./ml.
	21	-	over 60	plasma	0.13.5 mμg./ml. av. 3.4 ± 3.0 mμg./ml.
Tryptophanase					
Wada et al. (96)	-	-	-	serum	20-27 mμg./ml. av. 23 mμg./ml.
Donald and Ferguson (16)	8	-	-	leukocytes	0.22-0.38 mμg./million cells av. 0.30 ± 0.02 mμg./million cells
Apotransaminase					
Walsh (99)	-	-	3-10	plasma	13-11 mμg./ml.[1]
	-	-	10-20	plasma	11-10 mμg./ml.
	-	-	20-30	plasma	12-8 mμg./ml.
	-	-	30-40	plasma	9-8 mμg./ml.
	-	-	40-50	plasma	8-6 mμg./ml.
	-	-	50-60	plasma	7-4 mμg./ml.
	-	-	60-70	plasma	4-3 mμg./ml.
	-	-	70-80	plasma	3-0.5 mμg./ml.
Walsh et al. (100)	20	-	-	plasma	"67-130% normal for the subject's age" (100, p. 380)

[1] Values were obtained from a curve in which the levels of plasma pyridoxal phosphate in 24 normal persons were plotted against age.

by Boxer et al. (12). Premature infants and babies up to 18 months had pyridoxal phosphate levels above 10 mμg. per ml. of blood. Eighty percent of the adults studied by Boxer et al. had pyridoxal phosphate levels in blood that were less than 10 mμg. per ml.

Wachstein et al. (92) found a slightly higher average plasma level of pyridoxal phosphate in men (10.5 mμg./ml.) than in women (8.4 mμg./ml.). Both Hamfelt (34) and Wachstein et al. (93) reported comparable amounts of pyridoxal phosphate in plasma, 9.9 and 9.6 mμg. per ml., respectively. Later Hamfelt (36) reported a slightly higher value, 11.7 mμg. of pyridoxal phosphate per ml. of plasma.

Human serum, as reported by Wada et al. (96), contained 23 mμg. of pyridoxal phosphate per ml.

Both Hamfelt (35) and Walsh (99) found that the level of pyridoxal phosphate in plasma varied with age. Older subjects had lower levels of plasma pyridoxal phosphate (approximately 3 mμg./ml.) than younger ones (approximately 12-16 mμg./ml.).

Maternal blood at term contains significantly less pyridoxal phosphate than cord blood. Wachstein et al. (92) found that in maternal blood, the plasma contained 4.3 mμg. of pyridoxal phosphate per ml.; and the leukocytes, 0.09 mμg. per million cells. In cord blood, the plasma contained 23.2 mμg. of pyridoxal phosphate/ml. and the leukocytes, 0.49 mμg./million cells. Hamfelt (36) reported that the

pyridoxal phosphate content of plasma of the mother at term was 2.4 mμg./ml. and plasma of cord blood was 13.9 mμg./ml. The leukocytes in cord blood contained 0.285 mμg. of pyridoxal phosphate per million cells (36). The values reported for pyridoxal phosphate in the blood of the mother at term were much lower than those reported for the blood of non-pregnant women.

Average levels of pyridoxal phosphate in leukocytes of blood from men and non-pregnant women were reported by several groups of workers. Wachstein et al. (92, 93) reported 0.22 mμg. of pyridoxal phosphate per million cells; Hamfelt (36), 0.27 mμg. per million cells; Boxer et al. (12), 0.15 mμg. per million cells; Donald and Ferguson (16), 0.30 mμg. per million cells; and Wachstein et al. (95), 0.32 mμg. per million cells. Wachstein et al. (92) found that the changes in the level of pyridoxal phosphate in leukocytes paralleled those in the plasma.

Hamfelt (36) found that the level of pyridoxal phosphate in the erythrocytes of cord blood (1.46 mμg./10^9 cells) was much higher than that in the venous blood of normal controls (0.452 mμg./10^9) cells).

Pyridoxal

Jirsak (49) determined the pyridoxal content of blood and serum according to the microbiological methods of Rabinowitz et al. (71). In 30 male and 30 female subjects, 25 to 50 years of age, pyridoxal

in blood ranged from 2.4 to 3.0 (avg. 2.85) μg. per 100 ml.; and in serum, from 1.4 to 2.5 (avg. 1.98) μg. per 100 ml.

Fluorescence

In fluorescence a substance is excited to a higher energy state by absorption of energy in the ultraviolet region of the spectrum; and when it returns to a normal state, a portion of this absorbed energy is emitted as radiant light in the visible portion of the spectrum. The term "fluorescence" was derived from "fluorspar", the first mineral observed to produce a visual radiation after excitation by a high-energy source (101).

All compounds that absorb light exhibit fluorescence. Some fluorescence, however, is either very weak or is decreased by quenching processes, and is difficult to detect by the fluorometers presently available (88).

At lower concentrations fluorescence intensity is proportional to the concentration of the fluorescent substance and the amount of monochromatic light absorbed. At higher concentrations a significant amount of the exciting light is absorbed and no linear response between the concentration of the fluorescent substance and fluorescence is obtained. With a sensitive fluorometer concentrations as low as 0.0001 μg./ml. can be measured. Linearity between concentration and fluorescence can be obtained up to 10 μg./ml., or higher. Lower

concentrations can be measured by fluorometric methods than by colorimetric or spectrophotometric ones (88).

Fluorescence of a substance can be affected by: temperature; pH; purity of solvents; isolation procedures; interference by scattered light; contamination due to stopcock grease, cleansing agents, chemical reagents, and filter paper; adsorption of the fluorescent substance on the surface of glassware; oxidation; and photodecomposition. Fluorescence of a substance in solution can be quenched by other light absorbing species or by solutes which interact with the fluorescent compound (88).

EXPERIMENTAL

Introduction

Preliminary studies were made on the cyanohydrin methods to determine pyridoxal phosphate and pyridoxal. These studies included:

1. Determination of the absorption spectra of pyridoxal phosphate, pyridoxal, and their respective cyanohydrin derivatives.
2. Determination of the activation and fluorescence spectra of the cyanohydrin derivatives of pyridoxal phosphate and pyridoxal.
3. Effect of incubation time on the fluorescence of the cyanohydrin derivatives of pyridoxal phosphate and pyridoxal.

Following these preliminary studies, the cyanohydrin procedure of Yamada et al. (104) was studied. In the procedure of Yamada et al., the pyridoxal phosphate and pyridoxal in a deproteinized sample are separated by chromatography with SM-cellulose. Pyridoxal phosphate is eluted from SM-cellulose with 0.01 N acetic acid; and pyridoxal, with 0.1 M sodium phosphate buffer, pH 7.4. Pyridoxal phosphate and pyridoxal are converted to their respective cyanohydrin derivatives by reaction with potassium cyanide. These derivatives are measured fluorometrically at their activation and fluorescence maxima. Studies on the cyanohydrin procedure of Yamada

et al. included:

1. Elution pattern of pyridoxal phosphate and pyridoxal from a column of SM-cellulose.
2. Recovery of pyridoxal phosphate and pyridoxal from SM-cellulose.
3. Recovery of pyridoxal phosphate and pyridoxal in human blood from SM-cellulose.
4. Determination of pyridoxal phosphate and pyridoxal in human blood.

Equipment

1. Aminco-Bowman spectrophotofluorometer, No. 4-8106
   All fluorescence measurements were made with this instrument at a sensitivity setting of 50, and with a slit arrangement of 1/8, 3/16, 1/8, 1/8, 3/16, 1/8, and 1/16. This fluorometer has been described by Bowman et al.(11).
2. Beckman Model DU spectrophotometer
3. Beckman Model G pH meter
4. Misco fraction collector, No. 6500; and Misco drop counter, No. 6720
5. Ion-exchange columns, 1.2 (outside diameter) x 20 cm.
   A glass tube with a fine tip and a Teflon stopcock with a needle valve was joined to one end of the column and a 200 ml.

round bottom flask, which served as a reservoir, was fused to the other end. A 24-inch length of fine Tygon tubing was used to connect the fine tip of the column to the glass drip of the drop counter.

6. International centrifuge, Size 1, Type SB, No. W9601

7. Graduated centrifuge tubes, 15 ml., with stoppers; without stoppers

8. Graduated cylinders, 25 ml., with stoppers; without stoppers

9. Test tubes, without lip, 10 x 75 mm., 13 x 100 mm., 15 x 125 mm., and 17 x 150 mm.

10. Test tube stoppers

    Linear high-density polyethylene stoppers, Size No. 9. These were used for covering the test tubes during incubation.

11. Pipets

    10, 20, 100, and 200 µl. micropipets

    2 ml. constriction pipet

12. Syringe pipets

    1, 2, 5, and 10 ml. hypodermic syringe

    Syringe holders, adjusted to repeatedly deliver a predetermined amount of fluid. Manufactured by Northern Tool Company, Flushing, N.Y.

13. Hamilton gas-tight syringe and dispenser

    Model 1005 syringe with a capacity of 5 ml., fitted with a PB600-10 repeating dispenser which delivers 0.1 ml. with each click. This syringe was used to dispense potassium cyanide.

14. Variable speed mixer

15. Water bath with automatic temperature control

16. V-21 Vacoset, disposable blood donor set and Plasma-Vac, sterile, nonpyrogenic evacuated container; Don Baxter, Inc., Glendale, California.

    These were used to obtain and collect venous blood.

Standard Solutions

Pyridoxal Phosphate, Calbiochem, Los Angeles, California, Lot 40020. 10.7 mg. of pyridoxal phosphate monohydrate dissolved and diluted to 100 ml. with redistilled water (1 ml. = 100 μg. pryidoxal phosphate). Immediately after preparation, 0.5 ml. portions of this standard were placed in 10 x 75 mm. test tubes, covered with parafilm, and stored at - 10°C.

Pyridoxal, Sigma Chemical Company, St. Louis, Missouri, Lot P1128-90. Stock I: 121.2 mg. of pyridoxal hydrochloride dissolved and diluted to 200 ml. with 10% (w/v) acetic acid (1 ml. = 500 μg. pyridoxal). This standard was stored in a red bottle and kept

under refrigeration. Stock II: Two ml. of pyridoxal standard Stock I were diluted to 10 ml. with 0.2 M sodium phosphate buffer, pH 7.4 (1 ml. = 100 μg. pyridoxal). Pyridoxal standard Stock II was also stored in 0.5 ml. portions at -10°C.

The frozen standards were thawed and diluted just before use. They were protected from light at all times.

Reagents

1. Acetic acid, 1 N

   11.5 ml. of concentrated acetic acid diluted to 200 ml. with redistilled water.

2. Acetic acid, 0.01 N

   20 ml. of 1 N acetic acid diluted to 2 liters with redistilled water.

3. Ether, purified grade

4. Hydrochloric acid, 2 N

   16.5 ml. of concentrated hydrochloric acid diluted to 100 ml. with redistilled water.

5. Potassium oxalate, 15% (w/v)

   15 gm. of potassium oxalate dissolved and diluted to 100 ml. with redistilled water (1 ml. = 150 mg.). One ml. of this oxalate solution was placed in the Plasma-Vac container used to collect 100 ml. of venous blood.

6. Potassium cyanide, 0.03 M

195 mg. of potassium cyanide dissolved and diluted to 100 ml. with 0.2 M sodium phosphate buffer, pH 7.4.

7. Potassium cyanide, 0.05 M

325 mg. of potassium cyanide dissolved and diluted to 100 ml. with 0.1 M sodium phosphate buffer, pH 7.4. These two solutions of potassium cyanide were stored in red bottles and kept under refrigeration.

8. SM-cellulose, Brown Company, Berlin, New Hampshire, Lot No. 5464

SM-cellulose is a sulfomethylated derivative of cellulose which exhibits strongly acidic cationic exchange properties. Morris and Morris (64, p. 239-241) state that the concentration of the ionic groups in SM-cellulose is 0.40 mM/gm., and the pK' in 0.5 M sodium chloride is 2.5. The capacity of this lot of SM-cellulose, as stated on the label, was 0.1 meq. per gm. of cellulose.

9. Sodium carbonate, 0.6 M

31.8 gm. of sodium carbonate dissolved and diluted to 500 ml. with redistilled water.

10. Sodium hydroxide, 2 N

8 gm. of sodium hydroxide dissolved and diluted to 100 ml. with redistilled water.

11. Sodium hydroxide, 0.29 N

20 ml. of 2 N sodium hydroxide were added to 120 ml. of redistilled water.

12. Sodium phosphate buffer, 0.1 M, pH 7.4

Na_2HPO_4, 0.1 M (Solution A)

14.2 gm. of Na_2HPO_4 dissolved and diluted to 1 liter with redistilled water.

NaH_2PO_4, 0.1 M (Solution B)

3.45 gm. of $NaH_2PO_4 \cdot H_2O$ dissolved and diluted to 250 ml. with redistilled water.

The buffer was made by the following procedure:

One liter of Solution A was poured in a 1500 ml. beaker containing a Teflon coated magnet. The beaker was placed on a magnetic stirrer and Solution B was slowly added to Solution A until pH 7.4 was reached. The pH of the buffer was measured with a Beckman Model G pH meter which had been calibrated with a Beckman buffer at pH 6.86 (25°C).

13. Sodium phosphate buffer, 0.2 M, pH 7.4

Na_2HPO_4, 0.2 M (Solution A)

28.4 gm. of Na_2HPO_4 dissolved and diluted to 1 liter with redistilled water.

NaH_2PO_4, 0.2 M (Solution B)

6.9 gm. of $NaH_2PO_4 \cdot H_2O$ dissolved and diluted to 250 ml.

with redistilled water.

This buffer was prepared by the same procedure as given above for 0.1 M sodium phosphate buffer.

14. Tartaric acid, 0.5 N

15 gm. of tartaric acid dissolved and diluted to 100 ml. with redistilled water.

15. Trichloroacetic acid, 10% (w/v)

50 ml. (1 ml. = 1 gm.) of fluorometric grade trichloroacetic acid (Hartman Leddon Company, Philadelphia, Pennsylvania) were diluted to 500 ml. with redistilled water.

16. Trichloroacetic acid, 5% (w/v)

25 ml. (1 ml. = 1 gm.) of fluorometric grade trichloroacetic acid were diluted to 500 ml. with redistilled water.

Procedures

Since pyridoxal phosphate and pyridoxal are destroyed by light, all procedures in which these two compounds and/or blood were used were carried out in subdued light.

Preliminary Studies

Absorption Spectra of Pyridoxal Phosphate, Pyridoxal, and Their Respective Cyanohydrin Derivatives

The cyanohydrin derivatives of pyridoxal phosphate and

pyridoxal were prepared according to the method of Bonavita and Scardi (10).

Two ml. of pyridoxal phosphate standard (1 ml. = 100 μg.) were diluted to 10 ml. with 0.2 M sodium phosphate buffer, pH 7.4 (1 ml. = 20 μg.). From this solution two 3 ml. portions were taken. To one portion, 0.1 ml. of 0.03 M potassium cyanide was added (pyridoxal phosphate cyanohydrin); and to the other, 0.1 ml. of 0.2 M sodium phosphate buffer, pH 7.4 (pyridoxal phosphate). The two samples were incubated in a water bath at 50°C for 45 minutes. The samples were then cooled in cold water.

The absorption spectra of pyridoxal phosphate and its cyanohydrin derivative were obtained with a Beckman Model DU spectrophotometer. Absorption was measured at intervals of 5 mμ between 270 and 420 mμ. Readings were made against a blank of 0.2 M sodium phosphate buffer.

To obtain pyridoxal cyanohydrin, the procedure as given above was followed except that pyridoxal was substituted for pyridoxal phosphate. The absorption spectra of pyridoxal and its cyanohydrin derivative were obtained at 5 mμ intervals between 280 and 380 mμ.

Activation and Fluorescence Spectra of Pyridoxal Phosphate Cyanohydrin and Pyridoxal Cyanohydrin

The cyanohydrin derivatives of pyridoxal phosphate and

pyridoxal were prepared according to the method of Bonavita (9).

Two tenths ml. of pyridoxal phosphate standard (1 ml. = 100μg.) was diluted to 10 ml. with 0.2 M sodium phosphate buffer, pH 7.4 (1 ml. = 2 μg.). From this solution two 3 ml. portions were taken. To one portion, 0.1 ml. of 0.03 M potassium cyanide was added (pyridoxal phosphate cyanohydrin); and to the other, 0.1 ml. of 0.2 M sodium phosphate buffer (pyridoxal phosphate). The samples were incubated in a water bath at 50°C for 45 minutes. After the samples were cooled in cold water, they were adjusted to pH 3.0-3.8 with 2 N hydrochloric acid.

To obtain pyridoxal cyanohydrin, the procedure as given above was followed except that pyridoxal was substituted for pyridoxal phosphate, and the pH was not adjusted to 3.0-3.8 after incubation.

The activation and fluorescence maxima of the cyanohydrin derivatives of pyridoxal phosphate and pyridoxal were obtained by following the procedures as given in the instruction and service manual accompanying the Aminco-Bowman spectrophotofluorometer (2).

Pyridoxal phosphate cyanohydrin at pH 3.8 in 0.2 M sodium phosphate buffer absorbed maximally at 325 mμ and fluoresced maximally at 415 mμ. The fluorescence spectra of pyridoxal phosphate and its cyanohydrin derivative at pH 3.8 in 0.2 M sodium phosphate buffer with the activating wavelength set at 325 mμ are presented in Figure 1.

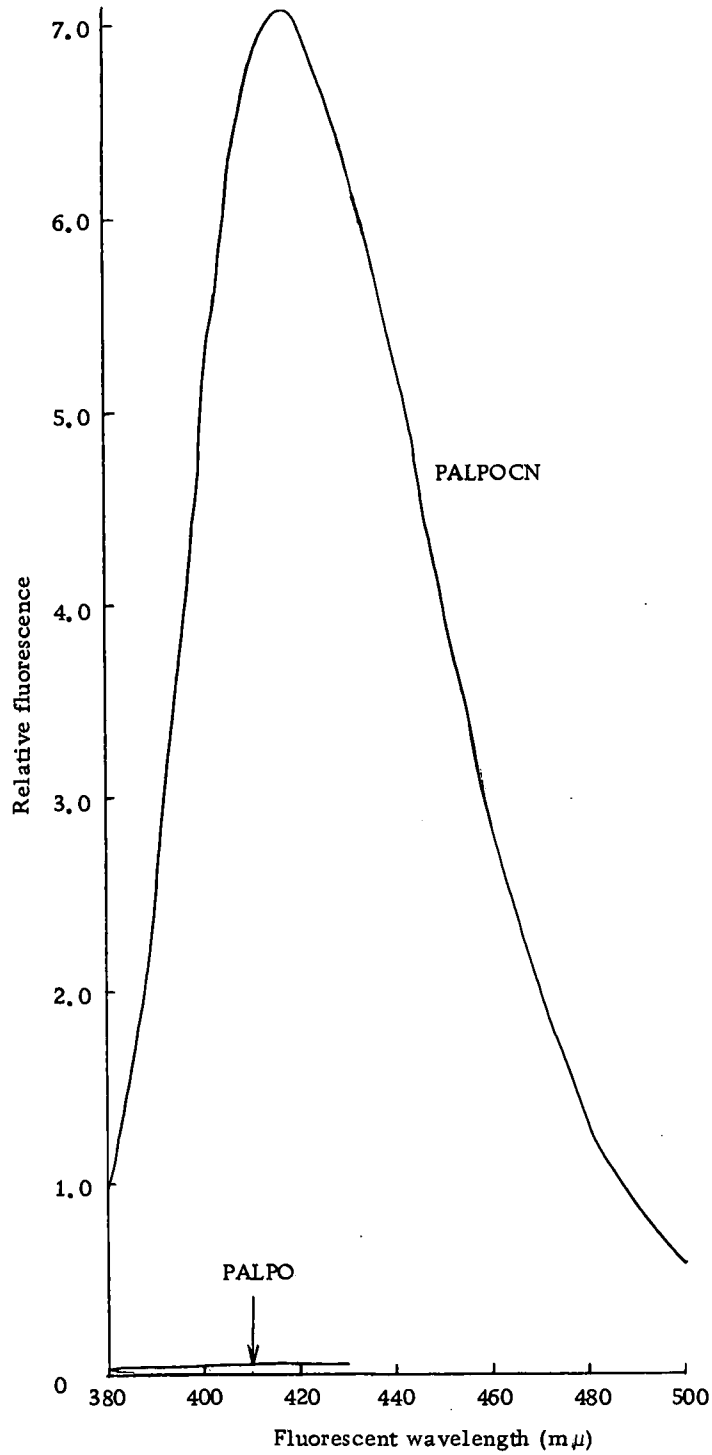


Figure 1. Fluorescence spectra of pyridoxal phosphate (PALPO) and its cyanohydrin derivative (PALPOCN) (1 ml. = 2 μg.) at pH 3.8 in 0.2 M sodium phosphate buffer. The cyanohydrin derivative was prepared according to the method of Bonavita (9). Activation set at 325 mμ.

Pyridoxal cyanohydrin at pH 7.5 in 0.2 M sodium phosphate buffer had an activation peak at 355 mμ and a fluorescence peak at 435 mμ. The fluorescence spectra of pyridoxal and its cyanohydrin derivative at pH 7.5 in 0.2 M sodium phosphate buffer with the activating wavelength set at 355 mμ are presented in Figure 2.

The activation and fluorescence maxima of the cyanohydrin derivatives of pyridoxal phosphate and pyridoxal prepared according to the procedure of Yamada et al. (104) were compared to those obtained for the cyanohydrin derivatives prepared by the method of Bonavita (9). The method by Yamada et al. differed slightly from that by Bonavita.

To prepare pyridoxal phosphate cyanohydrin, 100 μl. of pyridoxal phosphate standard (1 ml. = 100 μg.) were diluted to 100 ml. with 0.2 M sodium phosphate buffer, pH 7.4 (1 ml. = 0.1 μg.). To 2 ml. of the diluted pyridoxal phosphate standard, 2 ml. of 0.2 M sodium phosphate buffer and 0.1 ml. of 0.05 M potassium cyanide were added. The samples were incubated in a 50°C water bath for 60 minutes. After the samples were cooled in cold water, 0.55 ml. of 0.5 N tartaric acid was added to adjust the pH to around 3.8.

To prepare pyridoxal cyanohydrin, 100 μl. of pyridoxal standard (1 ml. = 100 μg.) were diluted to 100 ml. with 0.2 M sodium phosphate buffer (1 ml. = 0.1 μg.). To 2 ml. of the diluted standard solution of pyridoxal, 5 ml. of 0.2 M sodium phosphate buffer and

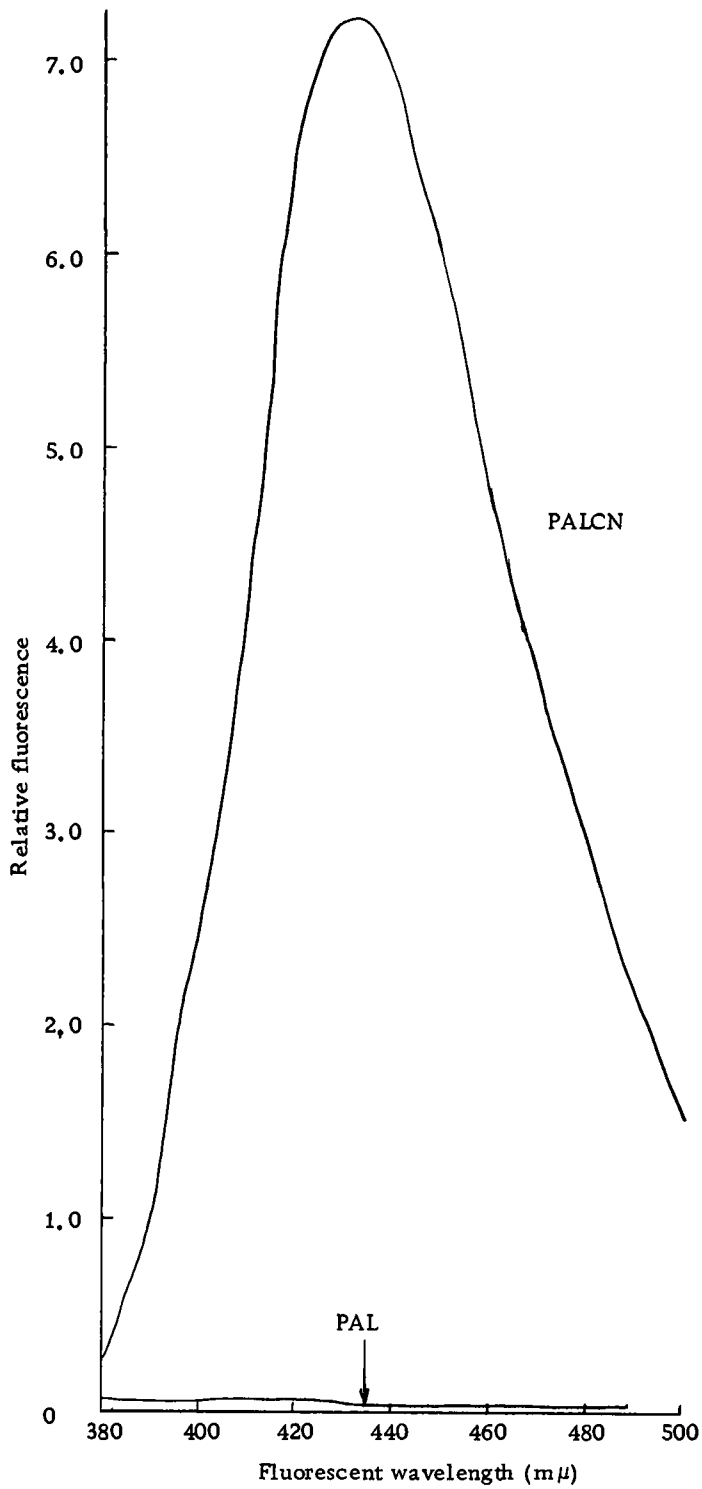


Figure 2. Fluorescence spectra of pyridoxal (PAL) and its cyanohydrin derivative (PALCN) (1 ml. = 2 μg.) at pH 7.5 in 0.2 M sodium phosphate buffer. The cyanohydrin derivative was prepared according to the method of Bonavita (9). Activation set at 355 mμ.

0.1 ml. of 0.05 M potassium cyanide were added. The samples were incubated in a 50°C water bath for 150 minutes. After the tubes were cooled, 1 ml. of 0.6 M sodium carbonate was added to adjust the pH to around 10.

The activation and fluorescence maxima of the cyanohydrin derivatives of pyridoxal phosphate and pyridoxal prepared according to the procedure of Yamada et al. were the same as those obtained for the cyanohydrin derivatives prepared according to Bonavita (9).

Effect of Incubation Time on the Fluorescence of the Cyanohydrin Derivatives of Pyridoxal Phosphate and Pyridoxal

The cyanohydrin derivatives of pyridoxal phosphate and pyridoxal were prepared according to the procedure of Yamada et al. (104).

To prepare pyridoxal phosphate cyanohydrin, 100 µl. of pyridoxal phosphate standard (1 ml. = 100 µg.) were diluted to 100 ml. with 0.2 M sodium phosphate buffer, pH 7.4 (1 ml. = 0.1 µg.). From this solution sixteen 2 ml. portions were taken. To each portion 2 ml. of 0.2 M sodium phosphate buffer were added. To half of the 2 ml. portions, 0.1 ml. of 0.05 M potassium cyanide was added (pyridoxal phosphate cyanohydrin); and to the other half, 0.1 ml. of 0.1 M sodium phosphate buffer was added (blank). The samples were incubated in a water bath at 50°C. At 15 minute intervals, a tube containing pyridoxal phosphate cyanohydrin and another containing the

blank were removed from the water bath and cooled. The pyridoxal phosphate cyanohydrin and blank were acidified with 0.55 ml. of 0.5 N tartaric acid to obtain a pH of around 3.8. The fluorescence of each pair was measured at the activating and fluorescent wavelengths, 325 and 415 mμ, respectively. The difference in fluorescence between each pair was plotted against time of incubation.

To obtain pyridoxal cyanohydrin, 100 μl. of pyridoxal standard (1 ml. = 100 μg.) were diluted to 100 ml. with 0.2 M sodium phosphate buffer (1 ml. = 0.1 μg.). From this solution twenty-eight 2 ml. portions were taken. To half of these portions, 5 ml. of 0.2 M sodium phosphate buffer and 0.1 ml. of 0.05 M potassium cyanide were added (pyridoxal phosphate cyanohydrin); to the other half, 5 ml. of 0.2 M sodium phosphate buffer and 0.1 ml. of 0.1 M sodium phosphate buffer were added (blank). The samples were incubated in a 50°C water bath. At 15 minute intervals, a tube containing pyridoxal cyanohydrin and another containing the blank were removed and cooled in cold water. The pH of the mixture was adjusted to 10 by the addition of 1 ml. of 0.6 M sodium carbonate. The fluorescence readings of each pair were obtained at the activating and fluorescent wavelengths, 355 and 435 mμ, respectively. The difference in fluorescence of each pair was plotted against the time of incubation.

To obtain maximum fluorescence, pyridoxal phosphate had to be reacted with potassium cyanide at 50°C for 60 minutes; and pyridoxal,

for 150 minutes (Figure 3).

Chromatography Studies

Preparation of SM-Cellulose Columns

A cotton plug was placed at the bottom of the ion-exchange column. This plug was prepared by laying a piece of cotton gauze about 5 cm. square in a crystallizing dish containing about 3 ml. of redistilled water. A small piece of loose cotton was placed in the center of this gauze, and the cotton was gently pressed with a stirring rod to remove any air bubbles. The corners of the gauze were folded over the cotton with the stirring rod to form a ball. The column was filled with redistilled water and the gauze-wrapped plug was gently pushed with a glass rod to the bottom of the column.

One gram of SM-cellulose was dispersed in 50 ml. of redistilled water in a 50 ml. beaker and was stirred occasionally for 5 minutes. The cellulose was allowed to settle for 30 minutes. The supernatant, containing fine particles, was removed by aspiration. Twenty-five ml. of 0.01 N acetic acid were added, and the cellulose and acetic acid were mixed thoroughly.

The water in the column was allowed to drain to the level of the cotton plug. The suspension of SM-cellulose in acetic acid was poured all at one time into the column. Any cellulose particles that adhered

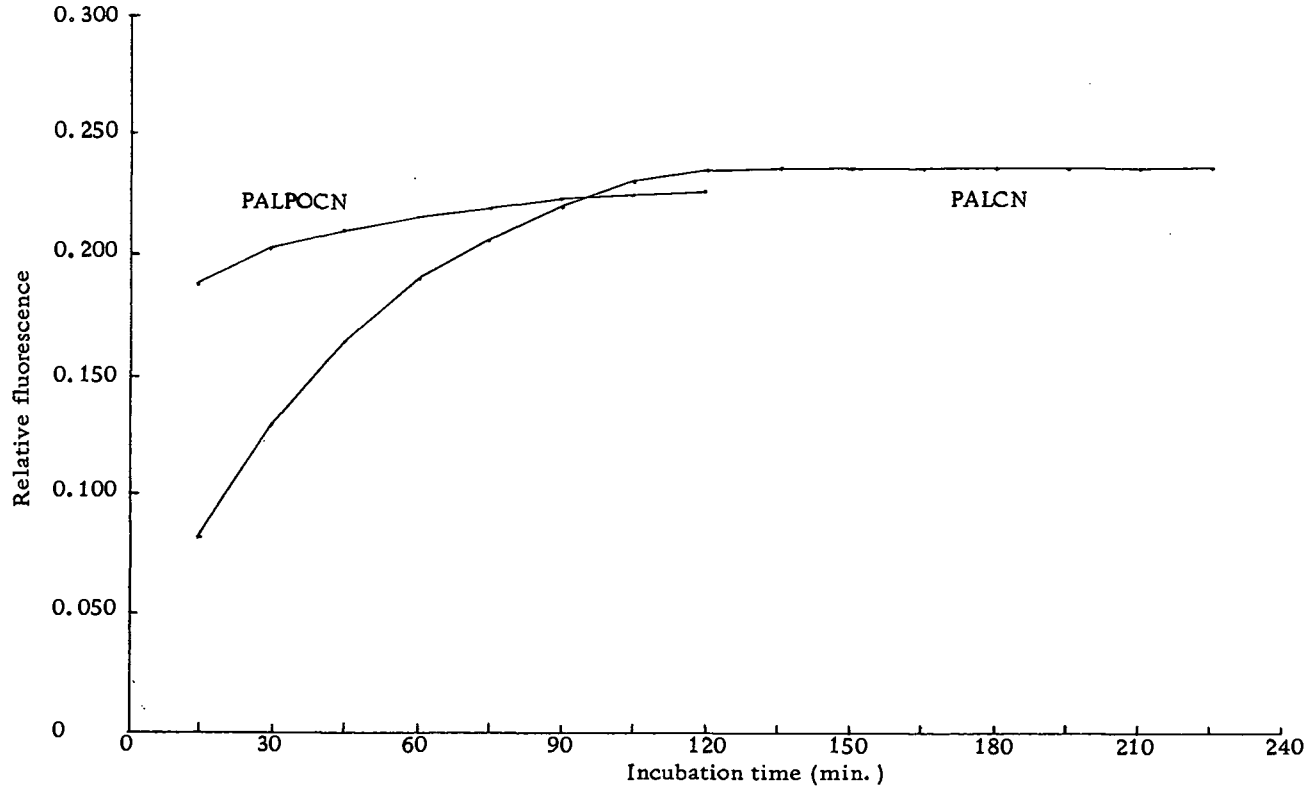


Figure 3. Effect of incubation time at 50°C on fluoresence of the cyanohydrin derivatives of pyridoxal phosphate (PALPOCN, 1 ml. = 0.1 μg.) and pyridoxal (PALCN, 1 ml. = 0.1 μg.). The cyanohydrin derivatives were prepared according to the method of Yamada et al. (104).

to the walls of reservoir were washed down with 0. 01 N acetic acid. The SM-cellulose in the column was allowed to settle, and the suspension of cellulose in the reservoir was stirred occasionally. When a column of SM-cellulose had formed, the supernatant was allowed to issue from the column at a flow rate of one drop every two seconds. To equilibrate and to remove the fluorescent material from SM-cellulose, it was necessary to pass 500 ml. of 0. 01 N acetic acid through the column. Later it was found that additional fluorescent material could be removed from SM-cellulose by washing the column with the following reagents, in order of addition after 500 ml. of 0. 01 N acetic acid: 5 ml. of 0. 29 N sodium hydroxide, 50 ml. of redistilled water, 150 ml. of 0. 01 N acetic acid, 25 ml. of 5% trichloroacetic acid, and 150 ml. of 0. 01 N acetic acid. The SM-cellulose column had to be washed just before use with at least 50 ml. of 0. 01 N acetic acid to remove the fluorescent materials that had leached from the cellulose during standing.

A Modification of the Procedure by Yamada et al.

Into a 15 ml. centrifuge tube were placed 2 ml. of oxalated blood and 4 ml. of redistilled water. The blood and water were thoroughly mixed. Six ml. of 10% trichloroacetic acid were added to the hemolyzed blood and mixed thoroughly with a glass stirring rod. The sample was incubated in a 50°C water bath for 15 minutes. After the

sample was cooled in cold water, it was centrifuged at 5000 r. p. m. for 30 minutes. About 9 ml. of supernatant were transferred to a 25 ml. stoppered graduated cylinder. The trichloroacetic acid was removed by extracting the supernatant three times with an equal volume of ether. [1] The ether dissolved in the aqueous phase was removed by bubbling air through the solution for 15 minutes.

The sample was divided into two-3 ml. portions. One-3 ml. portion of the prepared sample was applied to a previously prepared SM-cellulose column. The flow rate of the column was adjusted to 24 drops per minute. The effluent and eluate were collected in 65-drop fractions. One and one-half fractions of effluent were obtained. When the level of the sample was down to the surface of the SM-cellulose, 25 ml. of 0. 01 N acetic acid were added. The eluate was collected in 13 x 100 mm. test tubes (HAc fractions). When the acetic acid had passed through the column, 25 ml. of 0. 1 M sodium phosphate buffer, pH 7. 4, were added to the column. The eluate was collected in 15 x 125 mm. test tubes (buffer fractions). When the sample and elutriants were added to the column, care was taken to prevent disturbing the cellulose bed. The fractions from this column were analyzed for pyridoxal phosphate or pyridoxal by the cyanohydrin method.

To determine pyridoxal phosphate, 2 ml. of 0. 2 M sodium

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[1] Since no change in the volume of the supernatant occurred during this extraction, water-saturated ether was not used.

phosphate buffer, pH 7.4, and 0.1 ml. of 0.05 M potassium cyanide were added to each of the HAc fractions. The contents of each tube were mixed thoroughly. The tubes were covered with stoppers and incubated in a 50°C water bath for 60 minutes. After cooling the tubes in cold water, 0.55 ml. of 0.5 N tartaric acid was added to each fraction to bring the pH to around 3.8. After mixing, the fluorescence reading of each HAc fraction was obtained at 325 mμ activation and 415 mμ fluorescence.

To determine pyridoxal, 5 ml. of 0.2 M sodium phosphate buffer, pH 7.4, and 0.1 ml. of 0.05 M potassium cyanide were added to each of the buffer fractions. The contents of each tube were mixed thoroughly. The tubes were covered with stoppers and incubated in a 50°C water bath for 150 minutes. After cooling, 1 ml. of 0.6 M sodium carbonate was added to each fractions to adjust the pH to around 10. The fluorescence reading of each fraction was obtained at the activating and fluorescent wavelengths, 355 and 435 mμ, respectively.

The other 3 ml. portion of the sample was applied to another prepared column of SM-cellulose. The second column was run about one hour after the first and was operated the same as presented above. The fractions from the second column served as blanks for the fractions from the first column.

To prepare the blanks for pyridoxal phosphate, the procedure for the determination of pyridoxal phosphate was followed as given

above, except that 0.1 ml. of 0.1 M sodium phosphate buffer was added in place of 0.1 ml. of 0.05 M potassium cyanide to each of the HAc fractions from the second SM-cellulose column.

To prepare the blanks for pyridoxal, the procedure for the determination of pyridoxal was followed as given above, except that 0.1 ml. of 0.1 M sodium phosphate buffer was added in place of 0.1 ml. of 0.05 M potassium cyanide to each of the buffer fractions from the second SM-cellulose column.

Pattern of Elution of Pyridoxal Phosphate and Pyridoxal from SM-Cellulose

In these experiments the procedure was almost the same as given above under "A Modification of the Procedure by Yamada et al." The centrifugation step was omitted because there was no precipitate. The experiments, and how each differed from the modified procedure of Yamada et al., were:

Elution of Pyridoxal Phosphate from SM-Cellulose. In place of blood, 2 ml. of pyridoxal phosphate standard (1 ml. = 0.4 μg.) were used. Pyridoxal phosphate was determined in both the HAc and buffer fractions from the first column; the blanks for pyridoxal phosphate were prepared from the HAc and buffer fractions from the second column. Another sample containing pyridoxal phosphate standard was prepared, and 3 ml. of this sample were applied to a third prepared

column of SM-cellulose. The HAc and buffer fractions from this column were analyzed for pyridoxal.

Elution of Pyridoxal from SM-Cellulose. In place of blood, 2 ml. of pyridoxal standard (1 ml. = 0.8 μg.) were used. Pyridoxal was determined in both the HAc and buffer fractions from the first column; the blanks for pyridoxal were prepared from the HAc and buffer fractions from the second. Another sample containing pyridoxal standard was prepared, and 3 ml. of this sample were applied to a third prepared column of SM-cellulose. The HAc and buffer fractions from this column were analyzed for pyridoxal phosphate.

Elution of a Mixture of Pyridoxal Phosphate and Pyridoxal from SM-Cellulose. In place of blood, 2 ml. of pyridoxal phosphate standard (1 ml. = 0.4 μg.) and 2 ml. of pyridoxal standard (1 ml. = 0.8 μg.) were used. To dilute the sample before the addition of trichloroacetic acid, 2 ml. of redistilled water, rather than 4 ml., were added. Pyridoxal phosphate was determined in both the HAc and buffer fractions from the first column; and pyridoxal in both the HAc and buffer fractions from the second. No blanks were prepared for either column.

Recovery Experiments

About 100 ml. of venous blood were obtained from a healthy and

adequately nourished female subject, age 24 years. The blood was obtained with a V-21 Vacoset disposable blood donor set and collected in a Plasma-Vac sterile, nonpyrogenic evacuated container to which 1 ml. of 15% potassium oxalate solution had been added. The blood was drawn by a laboratory technician at the Student Health Service.

Fifty ml. of this oxalated blood were hemolyzed in 50 ml. of redistilled water (1:1 dilution). Four ml. portions were pipetted into 15 ml. centrifuge tubes. The tubes were covered with parafilm and stored at -10°C.

In these experiments the procedure was almost the same as given under "A Modification of the Procedure by Yamada et al." given above. The experiments, and how each differed from the modified procedure of Yamada et al., were:

Separation of Pyridoxal Phosphate and Pyridoxal in Blood. To 4 ml. of hemolyzed blood 2 ml. of redistilled water were added.

Recovery of Pyridoxal Phosphate in Blood from SM-Cellulose. To 4 ml. of hemolyzed blood 1 ml. of diluted pyridoxal phosphate standard (1 ml. = 0.4 μg.) and 1 ml. of redistilled water were added.

Recovery of Pyridoxal in Blood from SM-Cellulose. To 4 ml. of hemolyzed blood 1 ml. of diluted pyridoxal standard (1 ml. = 1.6 μg.) and 1 ml. of redistilled water were added.

Recovery of Pyridoxal Phosphate and Pyridoxal in Blood from SM-Cellulose. To 4 ml. of hemolyzed blood 1 ml. of diluted pyridoxal phosphate standard (1 ml. = 0.4 μg.) and 1 ml. of diluted pyridoxal standard (1 ml. = 1.6 μg.) were added.

Recovery of Pyridoxal Phosphate from SM-Cellulose. One ml. of pyridoxal phosphate standard (1 ml. = 0.4 μg.) was diluted with 5 ml. of redistilled water.

Recovery of Pyridoxal from SM-Cellulose. One ml. of pyridoxal standard (1 ml. = 1.6 μg.) was diluted with 5 ml. of redistilled water.

Recovery of a Mixture of Pyridoxal Phosphate and Pyridoxal from SM-Cellulose. One ml. of pyridoxal phosphate standard (1 ml. = 0.4 μg.) and 1 ml. of pyridoxal standard (1 ml. = 1.6 μg.) were diluted with 4 ml. of redistilled water.

The experiments on the recovery of pyridoxal phosphate, pyridoxal, and a mixture of pyridoxal phosphate and pyridoxal from SM-cellulose were repeated. The procedures were the same as given above, except that the samples were not heated before they were applied to prepared columns of SM-cellulose.

To determine the pyridoxal phosphate and pyridoxal content of the fractions collected from the columns in these recovery

experiments, standard curves for pyridoxal phosphate and pyridoxal were prepared.

To prepare the standard curves, appropriate amounts of each standard were diluted with 0.2 M sodium phosphate buffer, pH 7.4, to obtain concentrations of 0.01, 0.03, 0.05, 0.1, 0.3, 0.5, and 1.0 μg. per ml. For pyridoxal, an additional dilution was made to obtain 2.0 μg. per ml.

One ml. of each diluted standard was pipetted into each of a series of quadruplet 15 ml. stoppered centrifuge tubes. To each tube 2 ml. of redistilled water and 3 ml. of 10% trichloroacetic acid were added. The contents of each tube were mixed thoroughly and incubated in a 50°C water bath for 15 minutes. After cooling, the trichloroacetic acid was extracted three times with an equal volume of ether. The ether dissolved in the aqueous phase was removed by bubbling air through the solution for 15 minutes. These samples were not applied to SM-cellulose.

For pyridoxal phosphate, 3 ml. of each treated standard were diluted to 10 ml. with 0.01 N acetic acid. To two 2 ml. portions of each dilution, 2 ml. of 0.2 M sodium phosphate buffer, pH 7.4, and 0.1 ml. of 0.05 M potassium cyanide were added. To the other two 2 ml. portions of each dilution, 2 ml. of 0.2 M sodium phosphate buffer and 0.1 ml. of 0.1 M sodium phosphate buffer were added to obtain the blanks. All of the tubes were incubated in a water bath at

50°C for 60 minutes, cooled, and adjusted to pH 3.8 by adding 0.55 ml. of 0.5 N tartaric acid. The fluorescence readings were made at the activating and fluorescent wavelengths, 325 mμ and 415 mμ, respectively. The difference in fluorescence between each standard and its corresponding blank was plotted against the concentration (Figure 4).

For pyridoxal, 3 ml. of each treated standard were diluted to 10 ml. with 0.1 M sodium phosphate buffer, pH 7.4. To two 2 ml. portions of each dilution, 5 ml. of 0.2 M sodium phosphate buffer, pH 7.4, and 0.1 ml. of 0.05 M potassium cyanide were added. To the other two 2 ml. portions of each dilution, 2 ml. of 0.2 M sodium phosphate buffer and 0.1 ml. of 0.1 M sodium phosphate buffer were added to obtain the blanks. All of the tubes were incubated in a water bath at 50°C for 150 minutes, cooled, and adjusted to pH 10 with 1 ml. of 0.6 M sodium carbonate. The fluorescence readings were made at the activating and fluorescent wavelengths, 355 mμ and 435 mμ, respectively. The difference in fluorescence between each standard and its corresponding blank was plotted against the concentration (Figure 5).

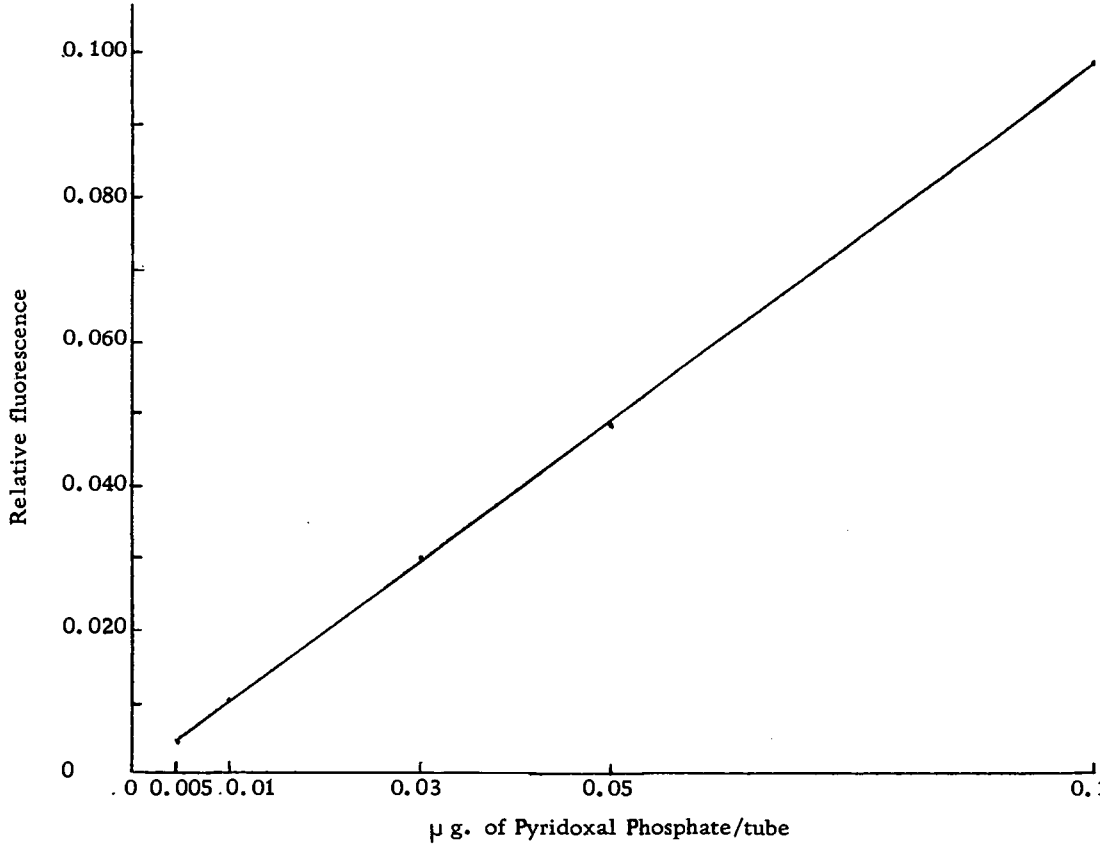

Figure 4. Standard curve for pyridoxal phosphate. Pyridoxal phosphate cyanohydrin was prepared according to the method of Yamada et al. (104).

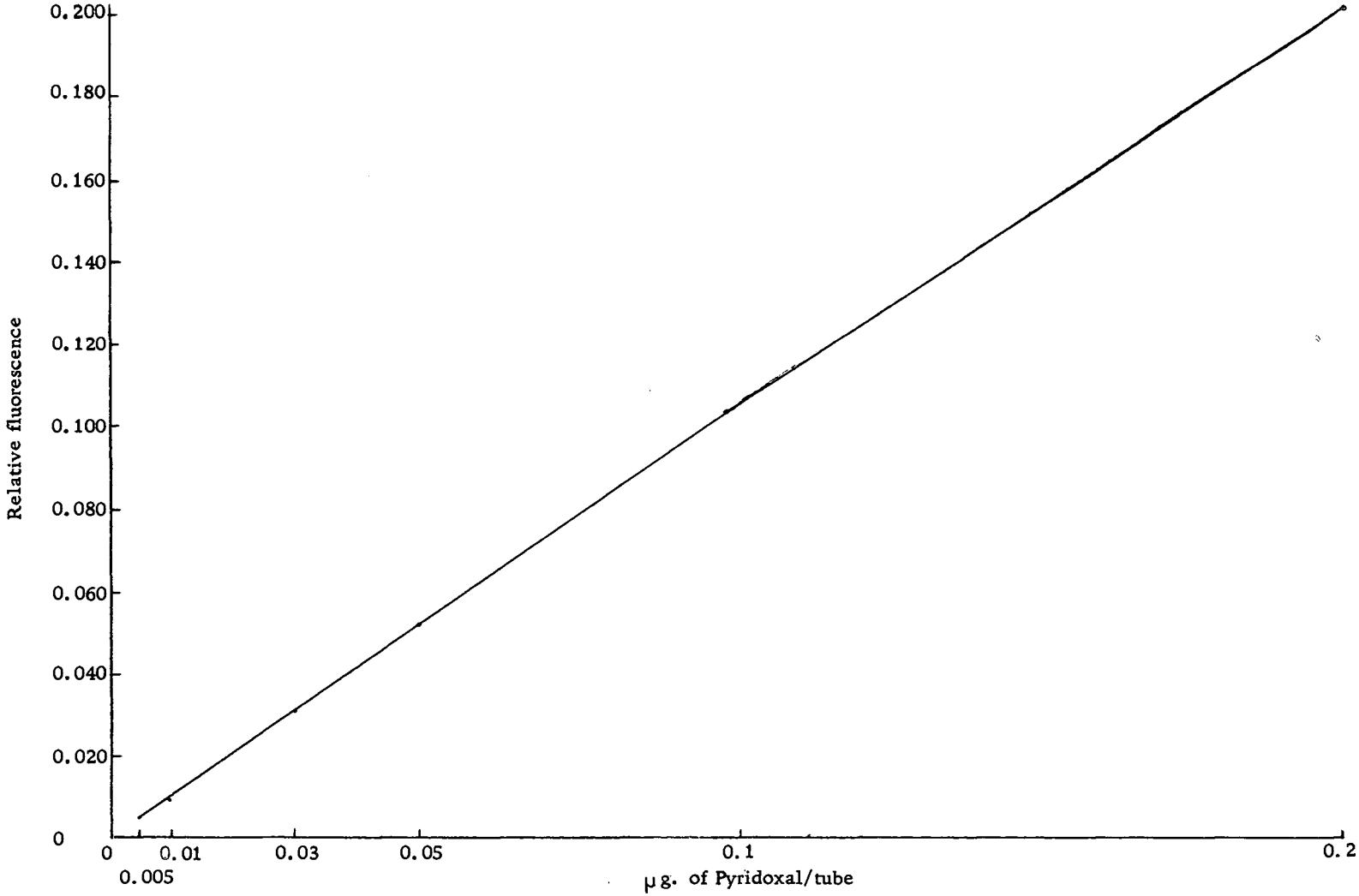

Figure 5. Standard curve for pyridoxal. Pyridoxal cyanohydrin was prepared according to the method of Yamada et al. (104).

RESULTS AND DISCUSSION

Preliminary Studies

Absorption Spectra

When pyridoxal phosphate was converted to its cyanohydrin derivative, maximum absorption occurred at 322 mμ instead of at the characteristic absorption maximum for pyridoxal phosphate, 385 mμ (Figure 6). Maximum absorption of pyridoxal occurred at 315 mμ, and absorption of pyridoxal cyanohydrin occurred at 320 and 350 mμ (Figure 7). Absorption of pyridoxal cyanohydrin at 320 mμ was not reported by Bonavita and Scardi (10), and Bonavita (9). This uncharacteristic absorption at 320 mμ may have been due to pyridoxal which had not been reacted with potassium cyanide. It takes longer for potassium cyanide to react with pyridoxal than with pyridoxal phosphate. In the preliminary work on the cyanohydrin method, 150 minutes of incubation at 50°C were required to obtain maximum fluorescence of pyridoxal reacted with cyanide, while 60 minutes were required for pyridoxal phosphate. In the method of Bonavita and Scardi both pryidoxal phosphate and pyridoxal were reacted with potassium cyanide for 45 minutes at 50°C.

Activation and Fluorescence Spectra

In the present study the fluorescence characteristics of the

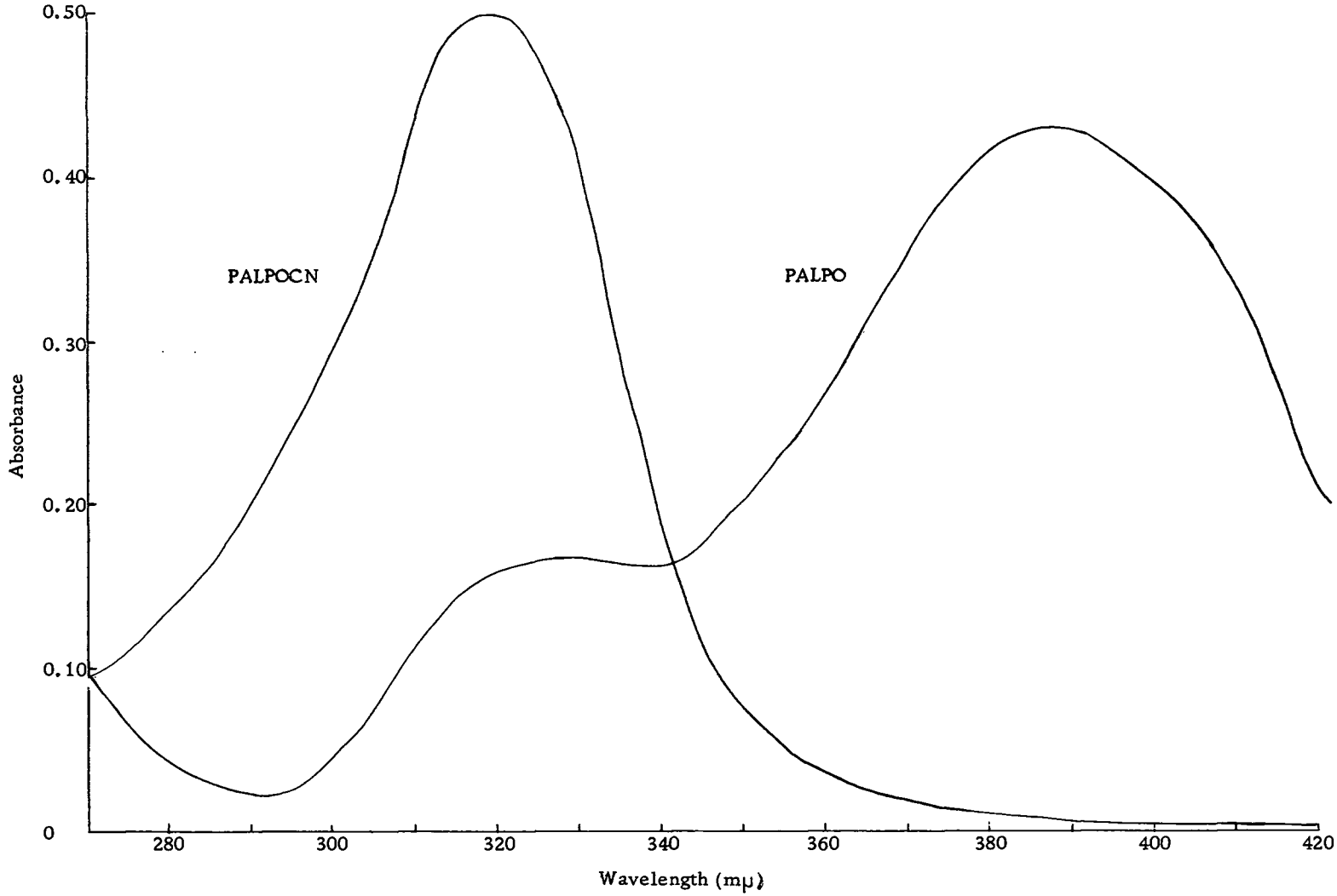

Figure 6. Absorption spectra of pyridoxal phosphate (PALPO) and its cyanohydrin derivative (PALPOCN) (1 ml. = 20 μg.). Pyridoxal phosphate cyanohydrin was prepared according to the method of Bonavita and Scardi (10).

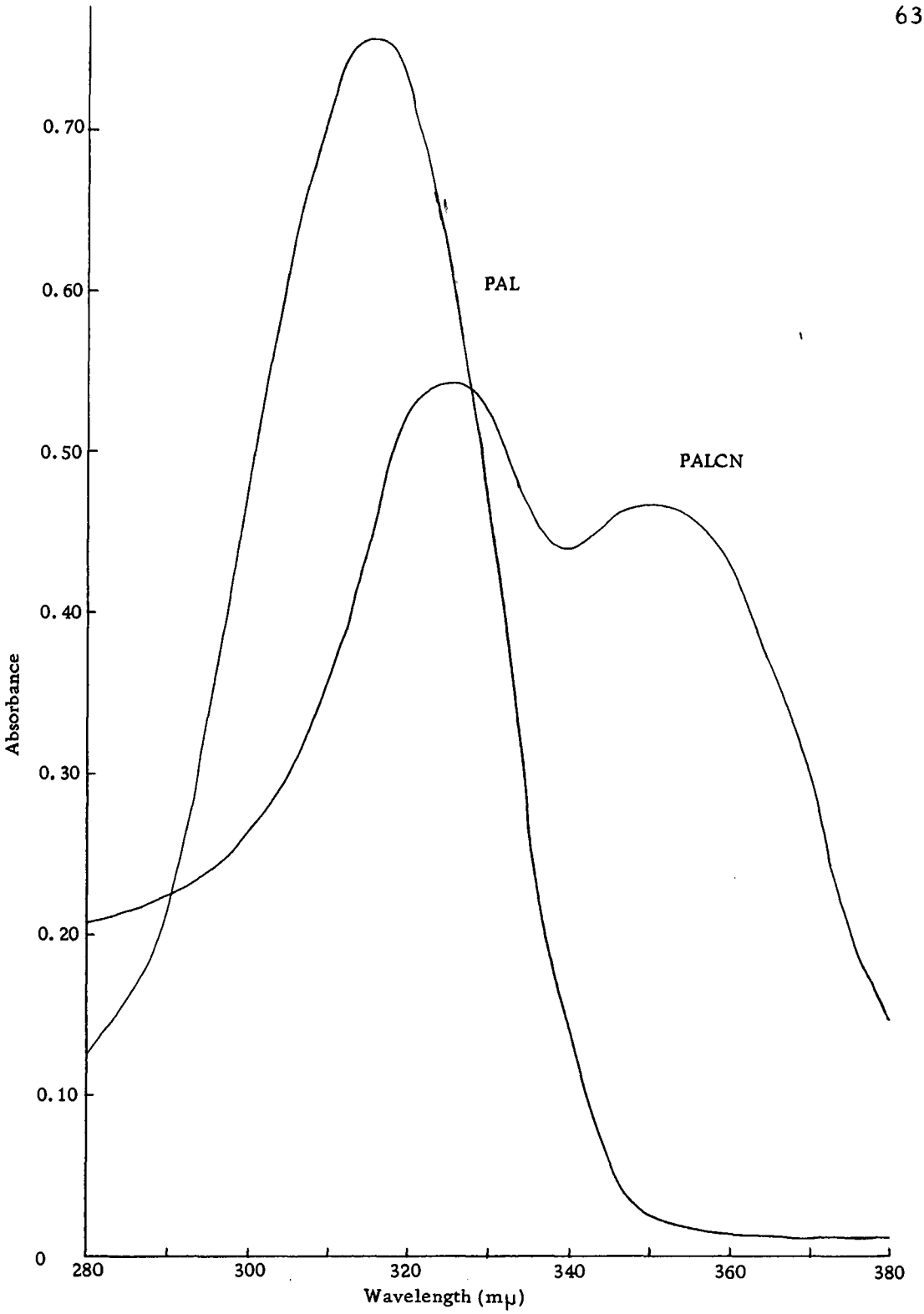

Figure 7. Absorption spectra of pyridoxal (PAL) and its cyanohydrin derivative (PALCN) (1 ml. = 20 μg.). Pyridoxal cyanohydrin was prepared according to the method of Bonavita and Scardi (10).

cyanohydrin derivatives were not affected by the procedure used to prepare them. The fluorescence characteristics of pyridoxal phosphate cyanohydrin prepared according to the method of either Bonavita (9) or Yamada et al. (104) were 325 mμ activation and 415 mμ fluorescence (Figure 1). Bonavita reported that the fluorescence characteristics of pyridoxal phosphate cyanohydrin were 313 mμ activation and 420 mμ fluorescence; Yamada et al. reported, 325 mμ and 420 mμ, respectively.

Pyridoxal cyanohydrin prepared by either method showed maximum fluorescence at the activating and fluorescent wavelengths, 355 and 435 mμ, respectively (Figure 2). In the procedure of Yamada et al., the pH of pyridoxal cyanohydrin was adjusted to 10 before reading; in the method of Bonavita, the pH of pyridoxal cyanohydrin was at 7.4. Bonavita reported that pyridoxal cyanohydrin had an activation peak at 358 mμ and a fluorescence peak at 430 mμ; Yamada et al. reported, 358 mμ and 438 mμ, respectively.

In the studies on the fluorescence characteristics of the cyanohydrin derivatives prepared according to the method of Bonavita, pyridoxal cyanohydrin exhibited little fluorescence at the characteristic wavelengths for pyridoxal phosphate cyanohydrin, and conversely, pyridoxal phosphate cyanohydrin exhibited little fluorescence at the characteristic wavelengths for pyridoxal cyanohydrin. The fluorescence characteristics of either cyanohydrin derivative in the presence

of the other cyanohydrin derivative were not studied. Pyridoxal phosphate and pyridoxal showed little fluorescence at the characteristic wavelengths for the cyanohydrin derivative of either compound.

Incubation Time

When pyridoxal phosphate in 0.2 M sodium phosphate buffer at pH 7.4 was reacted with potassium cyanide, fluorescence increased between 15 minutes and 60 minutes of incubation, but not much after 60 minutes. Thus one hour of incubation at 50°C was chosen in this study to allow pyridoxal phosphate to react with potassium cyanide (Figure 3).

Fluorescence intensity of pyridoxal cyanohydrin was also found to be affected by length of incubation. Fluorescence intensity increased up to 150 minutes of incubation at 50°C, and thereafter it remained relatively constant (Figure 3). In the present study 150 minutes of incubation at 50°C were allowed for pyridoxal to react with potassium cyanide.

Yamada et al. (104) specified that pyridoxal phosphate should be allowed to react with potassium cyanide at 50°C for 30 minutes, and pyridoxal for 2 hours. Bonavita (9) stated that both pyridoxal phosphate and pyridoxal should be incubated with potassium cyanide at 50°C for 45 minutes. The difference between the results obtained in the present study and those obtained by Bonavita and Yamada et al.,

may have been due to the differences in the size of test tubes used to contain the sample, the size and type of incubator, and number of samples incubated at one time.

Chromatography Studies

Preparation of SM-Cellulose Columns

Preparation of the SM-Cellulose Bed

By suspending the SM-cellulose in water before making the column, the fine particles of cellulose which remained suspended in the supernatant could be removed. Removal of the fine particles of SM-cellulose prevented the formation of columns with extremely slow flow rates. The retention of fine cellulose particles in the cotton plug at the base of the column may have caused the very slow flow rate of some of the prepared columns. Columns with extremely slow flow rates were not used.

In preliminary work the SM-cellulose sometimes settled in layers. This layering could be removed by backwashing. Layering of the SM-cellulose bed was later prevented by keeping the cellulose remaining in the reservoir suspended while the column was forming.

Washing and Equilibration of the SM-Cellulose Column

To equilibrate and to remove fluorescent materials from

SM-cellulose, it was necessary to pass 500 ml. of 0.01 N acetic acid through the column of SM-cellulose. This treatment, however, was not consistently satisfactory for the removal of fluorescent materials from the cellulose.

To study the factors causing fluorescent material to be leached from SM-cellulose, the procedure as given under "A Modification of the Procedure by Yamada et al." was followed, except that in the preparation of the influent, 0.2 M sodium phosphate buffer was used in place of blood, heating and centrifugation were omitted, and the ether dissolved in the aqueous phase was not removed. Three ml. of influent were applied to a column of SM-cellulose that had previously been washed with 500 ml. of 0.01 N acetic acid.

During the collection of eluate fractions, it was observed at times that the flow rate of the column increased and the drop size of the eluate decreased simultaneously. The fractions collected at the time these changes occurred smelled of ether. When the HAc fractions were treated as for the determination of pyridoxal phosphate by the cyanohydrin method, and the buffer fractions as for the determination of pyridoxal (treated HAc and buffer fractions), it was also observed that higher fluorescence was found in the fractions smelling of ether. Thus it was thought that the ether dissolved in the influent may have leached fluorescent materials from the SM-cellulose.

Removing the ether in the influent or omitting the extraction of

trichloroacetic acid from the sample with ether before application to the SM-cellulose column did not reduce the fluorescence in the treated HAc and buffer fractions. However, throughout the operation of the columns to which ether-free samples were applied, the flow rate of the columns and the drop size of eluate fractions remained constant.

Washing the SM-cellulose column with 25 ml. of ether after 500 ml. of 0.01 N acetic acid had passed through it did not reduce the fluorescence in the treated HAc and buffer fractions.

A satisfactory reduction in fluorescence of the treated HAc and buffer fractions was not consistently obtained when the influent in which the ether had been removed was applied to a SM-cellulose column that had been prepared by washing with 500 ml. of 0.01 N acetic acid, followed by 25 ml. of 5% trichloroacetic acid and 150 ml. of 0.01 N acetic acid. Minimum fluorescence was found in the treated HAc and buffer fractions when the column of SM-cellulose was prepared by washing with 500 ml. of 0.01 N acetic acid, followed by 5 ml. of 0.29 N sodium hydroxide, 50 ml. of redistilled water, 150 ml. of 0.01 N acetic acid, 25 ml. of 5% trichloroacetic acid, and 150 ml. of 0.01 N acetic acid.

Before the column was used, it was washed with 50 to 100 ml. of 0.01 N acetic acid to remove the fluorescent material that had leached from the SM-cellulose during standing.

Pattern of Elution of Pyridoxal Phosphate and Pyridoxal from SM-Cellulose

Elution of Pyridoxal Phosphate from SM-Cellulose

Pyridoxal phosphate was eluted with 25 ml. of 0.01 N acetic acid, and was found in tubes 5 through 9 of the HAc fractions (Figure 8a).

When the same amount of pyridoxal phosphate was applied to a second column of prepared SM-cellulose and 0.1 M sodium phosphate buffer was added to the HAc fractions in place of potassium cyanide to obtain the corresponding blanks for the first column, a slight increase in fluorescence was found in tube 6 (Figure 8a). This higher fluorescence in tube 6 of the HAc fractions was probably due to the variation in fluorescence among the HAc fractions. In preliminary work not reported, comparable variation in fluorescence was observed among the HAc fractions from a SM-cellulose column to which a sample containing 0.2 M sodium phosphate buffer in place of pyridoxal phosphate or pyridoxal had been applied.

Of the buffer fractions from these two columns to which pyridoxal phosphate had been applied, tubes 4 and 5 showed slightly more fluorescence than the other tubes (Figure 8a). This variation may have been due to the variation in fluorescence among the fractions. Comparable variation in fluorescence was observed among the buffer

fractions from a SM-cellulose column to which a sample containing 0.2 M sodium phosphate buffer in place of a standard had been applied.

The fluorescence of the blanks prepared with the buffer fractions from the second column was higher than that of the buffer fractions from the first column which were reacted with potassium cyanide (Figure 8a). These results could be due to differences between the two columns. To eliminate the variation caused by the use of different columns, it would have been desirable to obtain the cyanohydrin derivative and blank from the same fraction. However, to obtain higher fluorescence readings, the whole fraction (about 2 ml.) had to be used to prepare the cyanohydrin derivative.

When the HAc fractions from the third column to which pyridoxal phosphate had been applied were analyzed for pyridoxal, no increase in fluorescence was seen in tubes 5 through 9 (Figure 8b). This indicates that the presence of pyridoxal phosphate in the HAc fractions did not interfere with the determination of pyridoxal. In the preliminary studies and the studies reported by Bonavita (9), it was found that pyridoxal phosphate cyanohydrin showed little fluorescence at the activating and fluorescent wavelengths pyridoxal cyanohydrin was most fluorescent.

When the buffer fractions from the third column to which pyridoxal phosphate had been applied were analyzed for pyridoxal, more

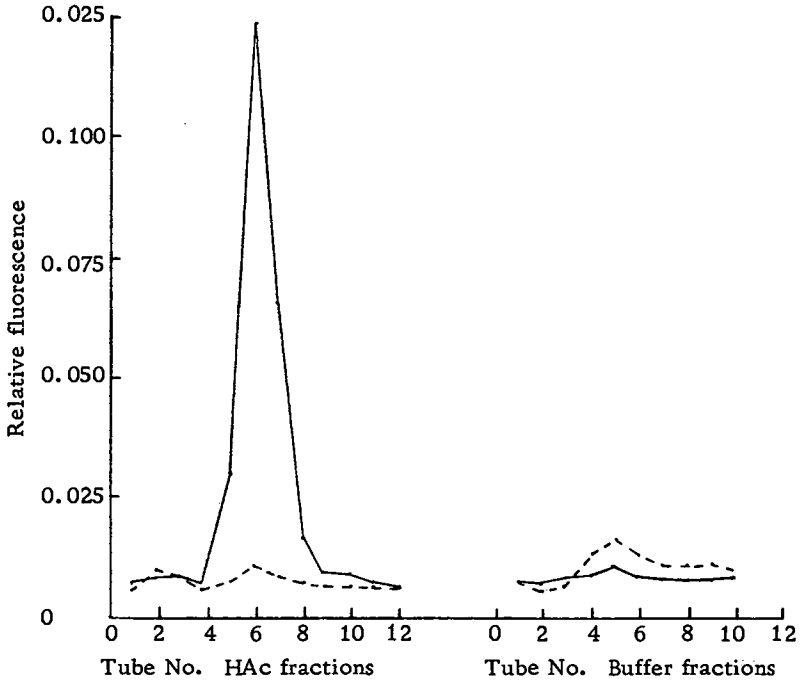

Figure 8a. Elution pattern of pyridoxal phosphate from SM-cellulose to which 0.2 μg. of pyridoxal phosphate had been applied. HAc and buffer fractions were analyzed for pyridoxal phosphate by the cyanohydrin method (solid line). To provide blanks (dashed line), fractions from a second column were treated similarly except for the substitution of 0.1 M sodium phosphate buffer for potassium cyanide.

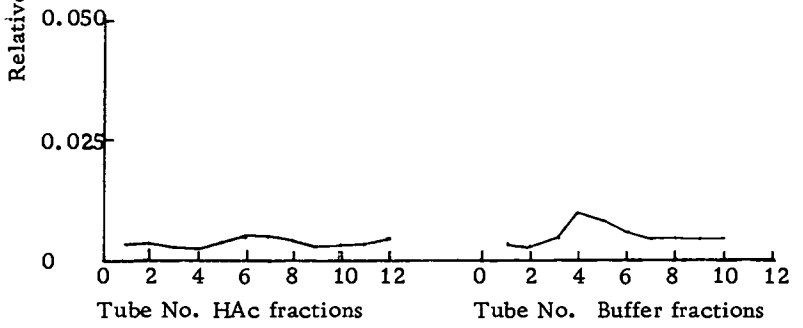

Figure 8b. Effect of pyridoxal phosphate on the assay of pyridoxal by the cyanohydrin method. 0.2 μg. of pyridoxal phosphate was applied to SM-cellulose and the HAc and buffer fractions were analyzed for pyridoxal by the cyanohydrin method.

fluorescence was found in tubes 4 and 5 than in the other tubes (Figure 8b). This could indicate that some pyridoxal phosphate had been converted to pyridoxal. Since pyridoxal phosphate is easily hydrolyzed in an acidic medium (6, p. 1026), it is possible that hydrolysis of pyridoxal phosphate may have occurred during the preparation of the sample or during the operation of the column. Whether this occurred would have to be confirmed by applying aliquots of the influent and of the buffer fractions showing the highest fluorescence to thin layer or paper chromatography. This variation in fluorescence could also be due to the variation among the buffer fractions.

Elution of Pyridoxal from SM-Cellulose

When pyridoxal was applied to a column of prepared SM-cellulose, pyridoxal was found in tubes 6 through 12 of the HAc fractions and tubes 4 through 8 of the buffer fractions (Figure 9a). Since pyridoxal in solution can exist as several species (62), it is possible that the pyridoxal eluted from SM-cellulose with 0.01 N acetic acid may have been a different species than the one eluted with 0.1 M sodium phosphate buffer. No other explanation for the presence of the high fluorescence in the HAc fractions treated as for the determinations of pyridoxal can be given.

When the same amount of pyridoxal was applied to a second column and 0.1 M sodium phosphate buffer was added to the HAc and

buffer fractions to obtain blanks, no increase in fluorescence was found in any of the HAc fractions, and a slight increase in fluorescence was found in buffer fractions 4 and 5 (Figure 9a). The higher fluorescence in tubes 4 and 5 of the buffer fractions from the second column could be due to variation among the individual buffer fractions.

Under the conditions for the determination of pyridoxal phosphate, a slight increase in fluorescence was found in tube 6 of the HAc fractions and in tubes 5 and 6 of the buffer fractions from the third column to which pyridoxal alone was applied (Figure 9b). The fluorescence in HAc fraction 6 was only slightly higher than that of the other HAc fractions. Thus it appears that if another species of pyridoxal was eluted with 0.01 N acetic acid, this species did not interfere with the determination of pyridoxal phosphate. Pyridoxal was not likely to be the cause of the increase in fluorescence in buffer fractions 4 and 5, because pryidoxal cyanohydrin shows little fluorescence under the conditions for the determination of pyridoxal phosphate by the cyanohydrin method.

Elution of a Mixture of Pyridoxal Phosphate and Pyridoxal from SM-Cellulose

When a mixture of pyridoxal phosphate and pyridoxal was applied to a column of prepared SM-cellulose, pyridoxal phosphate was eluted with 0.01 N acetic acid (Figure 10a). The elution pattern of

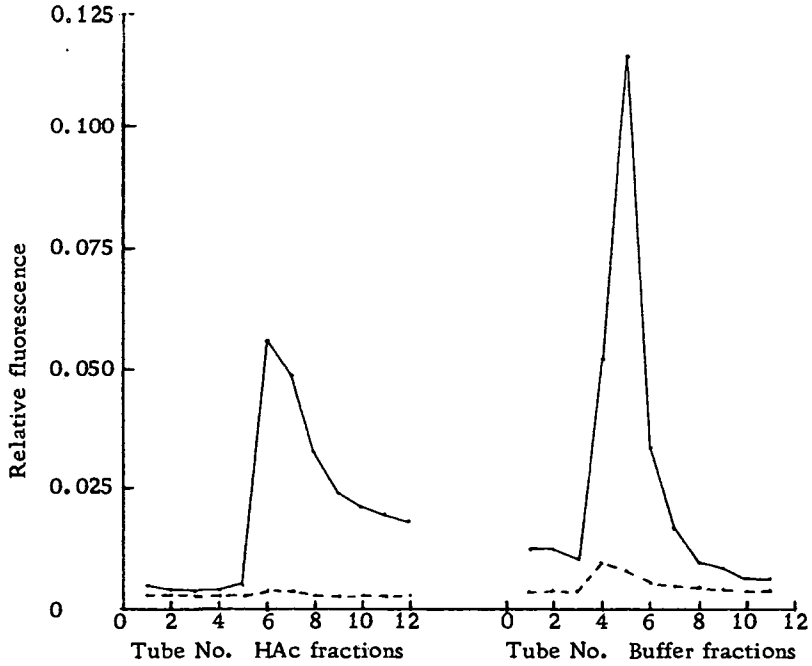

Figure 9a. Elution pattern of pyridoxal from SM-cellulose to which 0.4 μg. of pyridoxal had been applied. HAc and buffer fractions were analyzed for pyridoxal by the cyanohydrin method (solid line). To provide blanks (dashed line), fractions from a second column were treated similarly except for the substitution of 0.1 M sodium phosphate buffer for potassium cyanide.

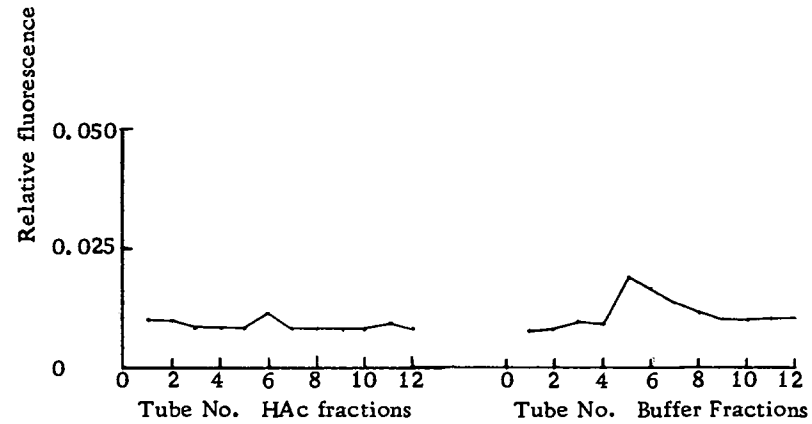

Figure 9b. Effect of pyridoxal on the assay of pyridoxal phosphate by the cyanohydrin method. 0.4 μg. of pyridoxal was applied to SM-cellulose and the HAc and buffer fractions were analyzed for pyridoxal phosphate by the cyanohydrin method.

pyridoxal phosphate in the HAc fractions from this SM-cellulose column was similar to that in the HAc fractions from the column to which pyridoxal phosphate alone had been applied (Figure 8a). The presence of pyridoxal did not change the elution pattern of pyridoxal phosphate from the SM-cellulose column.

When the buffer fractions were analyzed under the conditions for pyridoxal phosphate, the slight increase in fluorescence found among some of them was probably due to variation in fluorescence among the fractions (Figure 10a). This slight increase in fluorescence was also observed in the buffer fractions from a column to which pyridoxal phosphate alone was applied (Figure 8a).

When the HAc and buffer fractions from a column to which pyridoxal phosphate and pyridoxal had been applied were analyzed for pyridoxal, more fluorescence was found in the HAc fractions than in the buffer fractions (Figure 10b). The fluorescence in the HAc fractions was higher, and the fluorescence in the buffer fractions was lower than the fluorescence found in the corresponding HAc and buffer fractions from a column to which pyridoxal alone had been applied (Figure 9a). The fluorescence in the HAc fractions analyzed for pyridoxal is not likely to be caused by the presence of pyridoxal phosphate. No increase in fluorescence was observed when the HAc fractions from a column to which pyridoxal phosphate had been applied were analyzed for pyridoxal (Figure 8b). These results could indicate

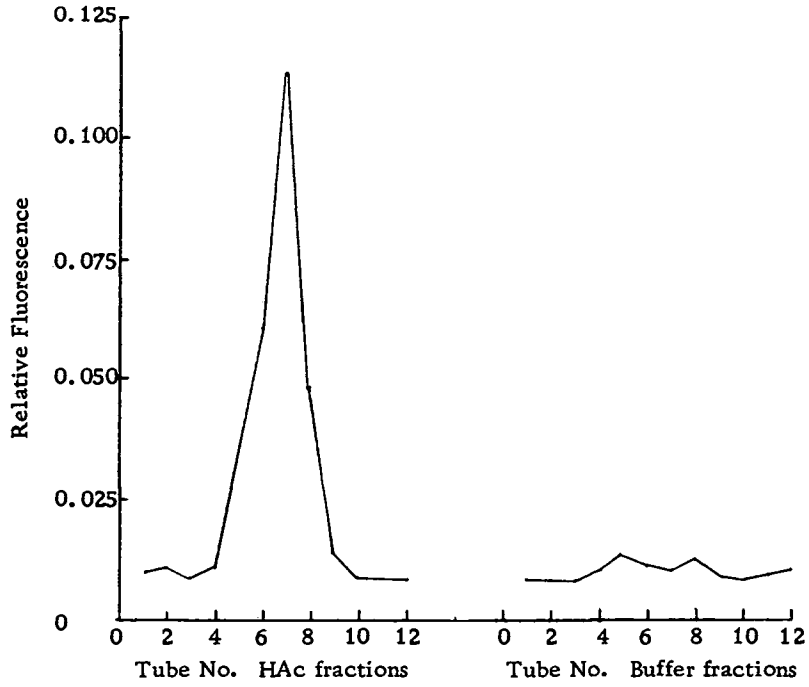

Figure 10a. Elution pattern of pyridoxal phosphate from SM-cellulose to which 0.2 μg. of pyridoxal phosphate and 0.4 μg. of pyridoxal had been applied. HAc and buffer fractions were analyzed for pyridoxal phosphate by the cyanohydrin method.

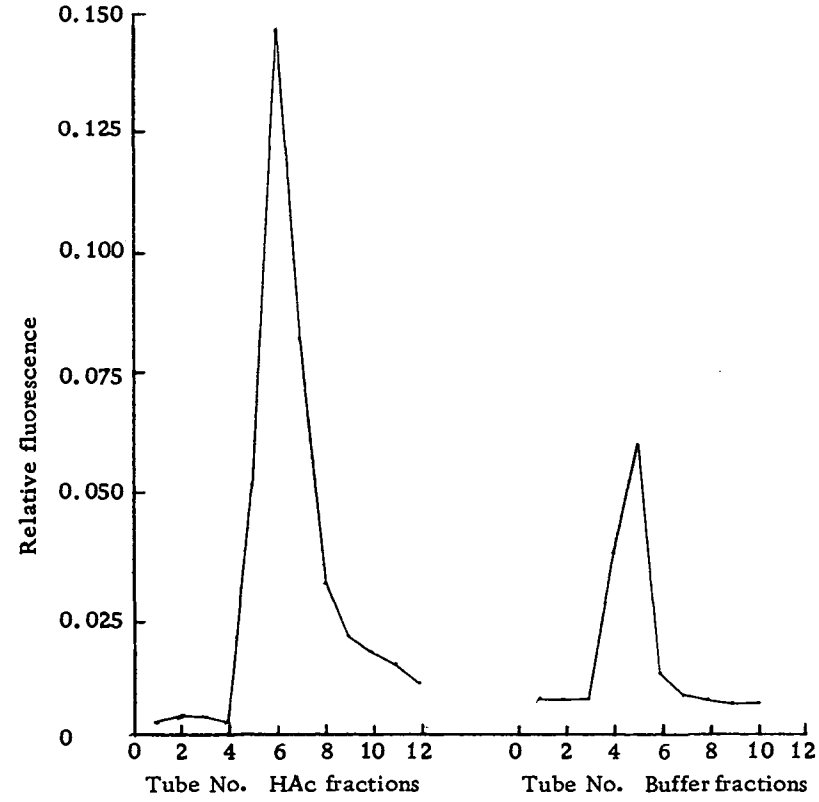

Figure 10b. Elution pattern of pyridoxal from SM-cellulose to which 0.2 μg. of pyridoxal phosphate and 0.4 μg. of pyridoxal had been applied. HAc and buffer fractions were analyzed for pyridoxal by the cyanohydrin method.

that the conversion of pyridoxal to another species which was eluted with 0.01 N acetic acid from SM-cellulose was increased when a mixture of pyridoxal phosphate and pyridoxal was applied to SM-cellulose.

Recovery Experiments

Standard Curves for Pyridoxal Phosphate and Pyridoxal

After the series of pyridoxal phosphate and pyridoxal standards had been reacted with potassium cyanide, a linear relationship was found between fluorescence and concentration except for the standards containing 0.21 and 0.63 mμg. of pyridoxal phosphate per ml., and 0.12 and 0.36 mμg. of pyridoxal per ml. The deviation of the fluorescence of these two lowest concentrations from linearity may indicate that the cyanohydrin method is not sensitive enough to determine concentrations lower than 1.05 mμg. of pyridoxal phosphate per ml. (Figure 4) and 0.60 mμg. of pyridoxal per ml. (Figure 5) after conversion to their respective cyanohydrin derivatives.

Similar standard curves for pyridoxal phosphate were obtained when pyridoxal phosphate cyanohydrin was prepared by other procedures. The other procedures for preparing the cyanohydrin derivative of pyridoxal phosphate were: (a) A series of standards were diluted with 0.2 M sodium phosphate buffer. Three ml. of each

standard were reacted with 0.1 ml. of 0.03 M potassium cyanide. After incubation at 50°C for 45 minutes, the pH was adjusted to 3.8 with 2 N hydrochloric acid. (b) Same as "a" except that 0.5 N tartaric acid was used to adjust the final pH. (c) A series of standards were diluted with 0.01 N acetic acid. To 2 ml. of each pyridoxal phosphate standard, 2 ml. of 0.2 M sodium phosphate buffer, pH 7.4, and 0.1 ml. of 0.05 M potassium cyanide were added. After incubation at 50°C for 60 minutes, the pH was adjusted to 3.8 with 0.5 N tartaric acid.

Similar standard curves of pyridoxal were also obtained when pyridoxal cyanohydrin was prepared by other procedures. The other procedures used to prepare the cyanohydrin derivative of pyridoxal were: (a) A series of standards were diluted with 0.2 M sodium phosphate buffer. Three ml. of each standard were reacted with 0.1 ml. of 0.03 M potassium cyanide and incubated at 50°C for 45 minutes. (b) A series of standards were diluted with 0.1 M sodium phosphate buffer, pH 7.4. To 2 ml. of each standard, 5 ml. of 0.2 M sodium phosphate buffer and 0.1 ml. of 0.05 M potassium cyanide were added. After incubation at 50°C for 150 minutes the pH was adjusted to 10 with 0.6 M sodium carbonate.

Calculation of the Pyridoxal Phosphate and Pyridoxal Content of the Eluate Fractions

For each prepared sample, two comparable columns were run. The HAc fractions from the first column were analyzed for pyridoxal phosphate by the cyanohydrin method. To obtain the corresponding blanks for this column, the HAc fractions from the second column were treated with 0.1 M sodium phosphate buffer in place of potassium cyanide.

Pyridoxal phosphate was found in the HAc fractions 5 through 9. The fluorescence readings of the blanks for these fractions were subtracted from the readings of the fractions containing pyridoxal phosphate cyanohydrin. The pyridoxal phosphate content in each tube was estimated by reference to the standard curve for pyridoxal phosphate. The sum of pyridoxal phosphate in these fractions represented the total pyridoxal phosphate eluted from the SM-cellulose with 0.01 N acetic acid.

Pyridoxal in the buffer fractions from the first column was determined by the cyanohydrin method. Corresponding blanks were obtained by adding 0.1 M sodium phosphate buffer in place of potassium cyanide to the buffer fractions from the second column.

Pyridoxal was found in the buffer fractions 4 through 8. The fluorescence readings of the blanks for these fractions were subtracted from the readings of the fractions containing pyridoxal cyanohydrin.

The pyridoxal content in each tube was estimated by reference to the standard curve for pyridoxal. The sum of the pyridoxal in fractions 4 through 8 represented the total pyridoxal that had been eluted with 0.1 M sodium phosphate buffer from the column of SM-cellulose.

Recovery of Pyridoxal Phosphate and Pyridoxal Standards from SM-Cellulose

Results from the experiments on the recovery of pyridoxal phosphate and pyridoxal from SM-cellulose are presented in Tables 2 and 3, and Figures 11, 12, and 13.

The recovery of pyridoxal phosphate from SM-cellulose was 93.5% when pyridoxal phosphate alone was applied to the column (Figure 11).

The recovery of pyridoxal was 108.8% when pyridoxal alone was applied to the column of SM-cellulose (Figure 12). In a comparable experiment presented above under "Pattern of Elution of Pyridoxal Phosphate and Pyridoxal from SM-Cellulose" (Figure 9a) in which the same amount of pyridoxal was applied to SM-cellulose, less fluorescence was found in the buffer fractions than in the buffer fractions from the column in the present experiment. This, and the high recovery of pyridoxal from SM-cellulose may indicate that more pyridoxal was eluted with 0.1 M sodium phosphate buffer in the present experiment than in the comparable experiment presented under the

Table 2. Recovery of pyridoxal phosphate and pyridoxal in blood and from SM-cellulose.

(A)	(B)	(C)	(D)		(E)	(F)	
		PALPO[1] in	Recovery of PALPO		PAL[2] in	Recovery of PAL	
Sample	Amount	HAc fractions	Blood	SM-cellulose	Buffer fractions	Blood	SM-cellulose
		μg.	percent	percent	μg.	percent	percent
PALPO	0.1 μg.	0.0935[a]		93.5[3]	0.005		
PAL	0.4 μg.	0.005			0.435[d]		108.8[4]
PALPO	0.1 μg.	0.1055[b]		105.5			
+ PAL	0.4 μg.				0.239[e]		59.8
Blood	0.5 ml.	0.0135[c]			0.0105[f]		
Blood	0.5 ml.						
+ PALPO	0.1 μg.	0.093	85.0[5]		0.007		
Blood	0.5 ml.						
+ PAL	0.4 μg.	0.003			0.137	29.1[6]	
Blood	0.5 ml.						
+ PALPO	0.1 μg.	0.0795	62.6[7]				
+ PAL	0.4 μg.				0.135	52.1[8]	

[1] Pyridoxal phosphate.

[2] Pyridoxal.

[3] Recovery of PALPO from SM-cellulose was calculated by (C)/(B) x 100.

[4] Recovery of PAL from SM-cellulose (E)/(B) x 100.

[5] (C-c)/a x 100.

[6] (E-f)/d x 100.

[7] (C-c)/b x 100.

[8] (E-f)/e x 100.

Table 3. Recovery of unheated pyridoxal phosphate and pyridoxal from SM-cellulose.

Sample	Amount	PALPO[1] in HAc fractions	Recovery of PALPO	PAL[2] in Buffer fractions	Recovery of PAL
		μg.	percent	μg.	percent
PALPO	0.1 μg.	0.0895	89.5[3]	0.0045	
PAL	0.4 μg.	0.007		0.4325	108.1[4]
PALPO	0.1 μg.	0.0945	94.5		
+ PAL	0.4 μg.			0.22	55.0

[1]Pyridoxal phosphate.

[2]Pyridoxal.

[3]Recovery of PALPO from SM-cellulose was calculated by (C)/(B) x 100.

[4]Recovery of PAL from SM-cellulose was calculated by (E)/(B) x 100.

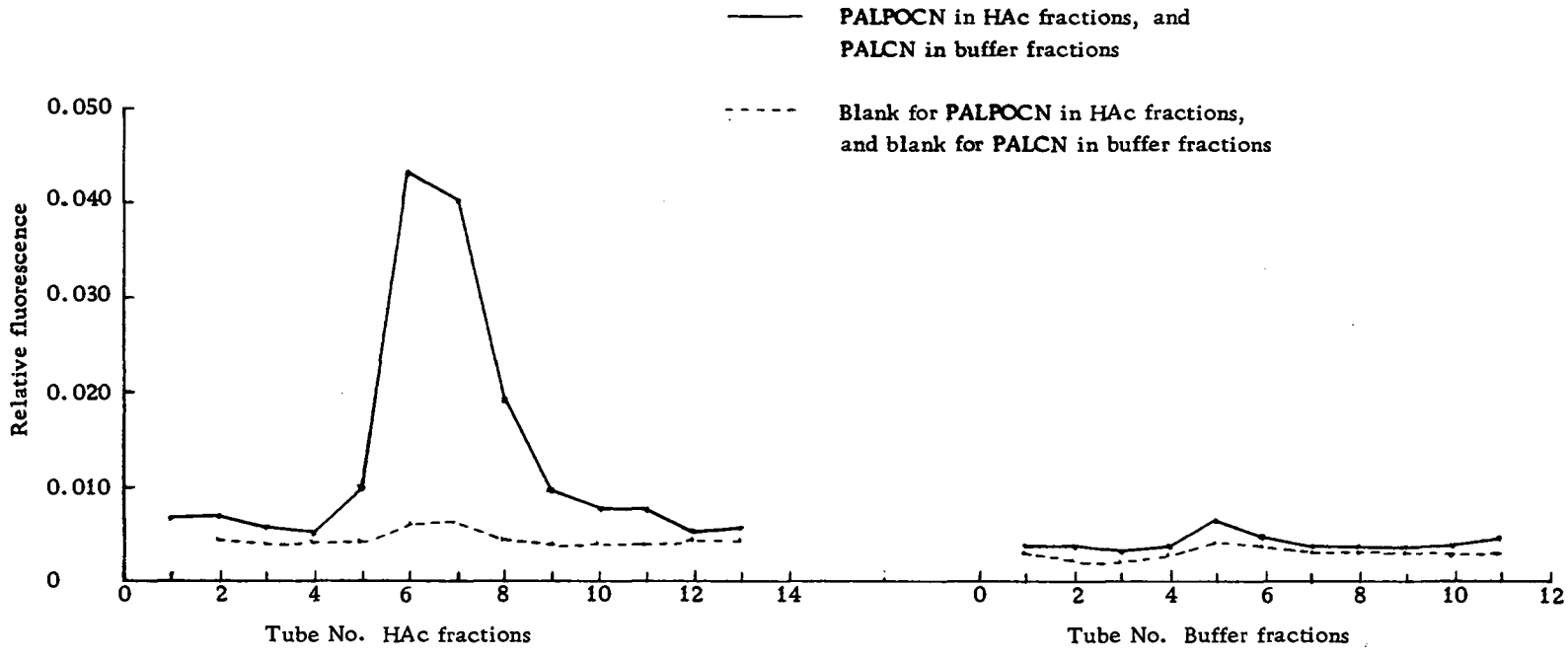

Figure 11. Recovery of pyridoxal phosphate (0.1 μg.) from SM-cellulose.

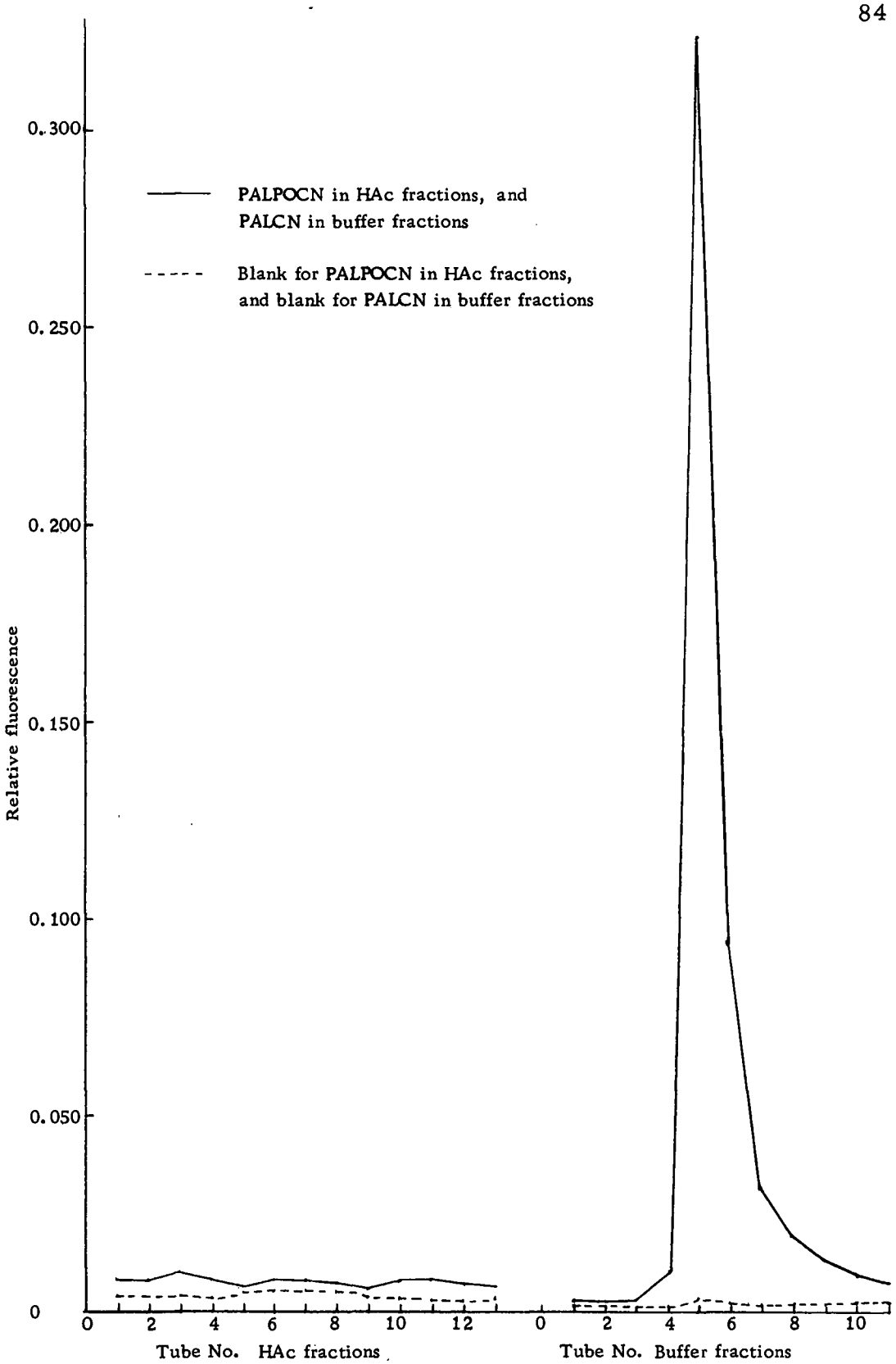

Figure 12. Recovery of pyridoxal (0.4 μg.) from SM-cellulose.

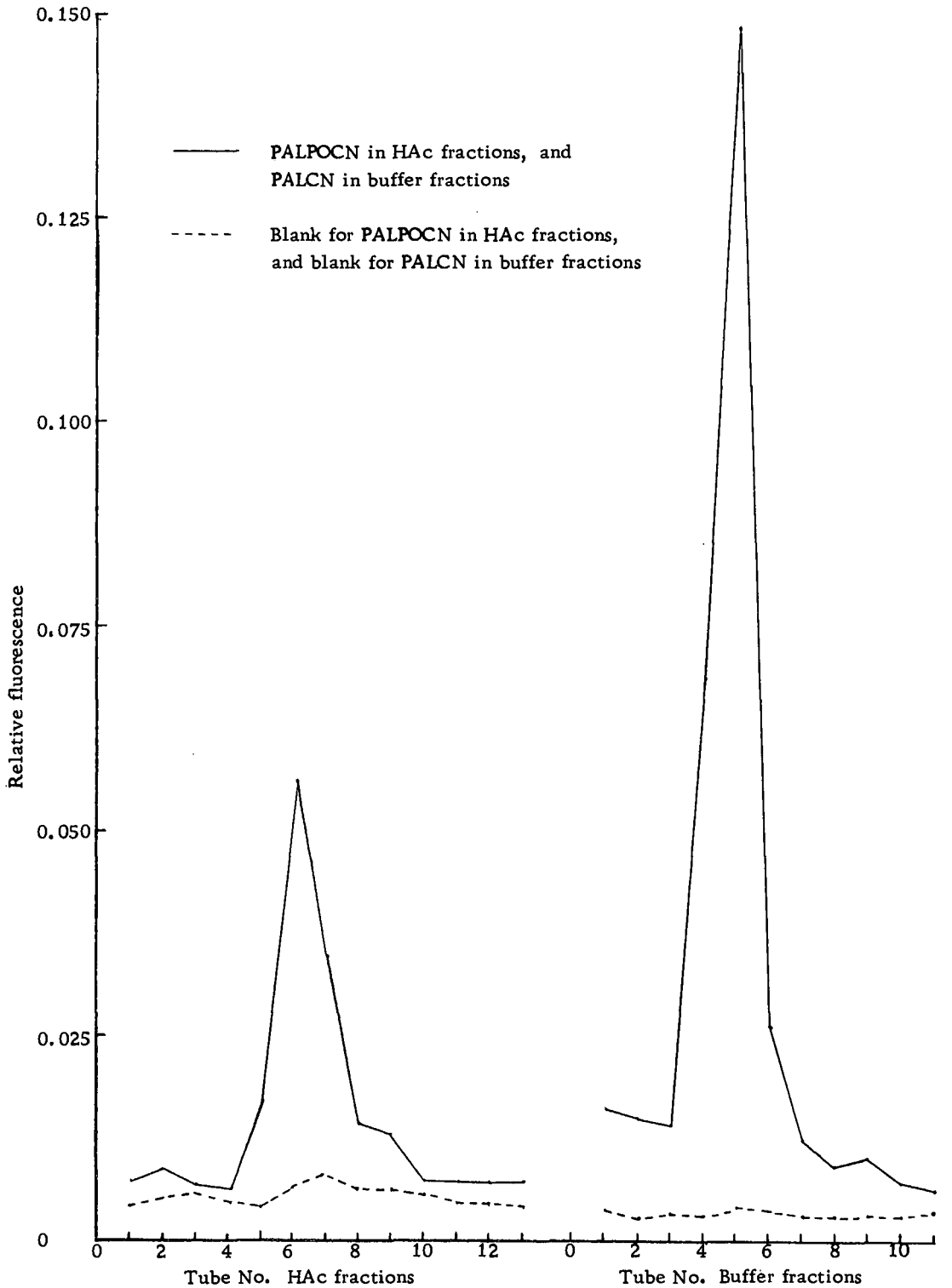

Figure 13. Recovery of pyridoxal phosphate (0.1 μg.) and pyridoxal (0.4 μg.) from SM-cellulose.

"Pattern of Elution". Whether pyridoxal was eluted with 0.01 N acetic acid in the "Recovery Experiments" was not determined. No explanation for these conflicting results can be given. The differences between the "Pattern of Elution" experiment and the "Recovery Experiments" were:

	"Pattern of Elution"	"Recovery Experiments"
Date	July 10, 12, 19, 1967	Oct. 25; Nov. 1, 1967
Standard	Stock standard I (1 ml. = 500 μg.) stored in refrigerator.	Stock standard II (1 ml. = 100 μg.) prepared from stock standard I, and stored at -10°C.
Preparation of influent	2 ml. of standard (1 ml. = 0.8 μg.) + 4 ml. of redistilled water + 6 ml. 10% trichloroacetic acid.	1 ml. of standard (1 ml. = 1.6 μg.) + 5 ml. of redistilled water + 6 ml. 10% trichloroacetic acid.
Delay before addition of potassium cyanide	30 minutes after elution.	5 minutes after elution.

When a mixture of pyridoxal phosphate and pyridoxal was applied to SM-cellulose, the recovery of pyridoxal phosphate was 105.5%, and the recovery of pyridoxal was 59.8% (Figure 13). In a comparable experiment presented under "Pattern of Elution," pyridoxal was found in the HAc and buffer fractions from a column to which a mixture of pyridoxal phosphate and pyridoxal had been applied. Thus this lower recovery of pyridoxal may have been caused by the elution of some of the pyridoxal from SM-cellulose with 0.01 N acetic

acid, and as a result, less pyridoxal would be eluted with 0.1 M sodium phosphate buffer.

That the recovery of pyridoxal phosphate was higher from SM-cellulose to which a mixture of pyridoxal phosphate and pyridoxal was applied than from SM-cellulose to which pyridoxal phosphate alone was applied could be caused by variations between the two experiments rather than by the presence of pyridoxal in the HAc fractions. In the "Pattern of Elution" experiments, the presence of pyridoxal in the HAc fractions did not affect the determination of pyridoxal phosphate (Figure 9b).

Omitting the step in which pyridoxal phosphate and pyridoxal were warmed at 50°C for 15 minutes in the preparation of influent did not alter the recovery of pyridoxal phosphate and pyridoxal from SM-cellulose (Table 3). This would indicate that under the conditions of the present experiments pyridoxal phosphate and pyridoxal were not destroyed during the time the samples were heated.

Recovery of Pyridoxal Phosphate and Pyridoxal in Blood from SM-Cellulose

The results for the experiments on the recovery of pyridoxal phosphate and pyridoxal in blood from SM-cellulose are presented in Table 2 and in Figures 14, 15, 16, and 17.

The recovery of pyridoxal phosphate in blood from SM-cellulose

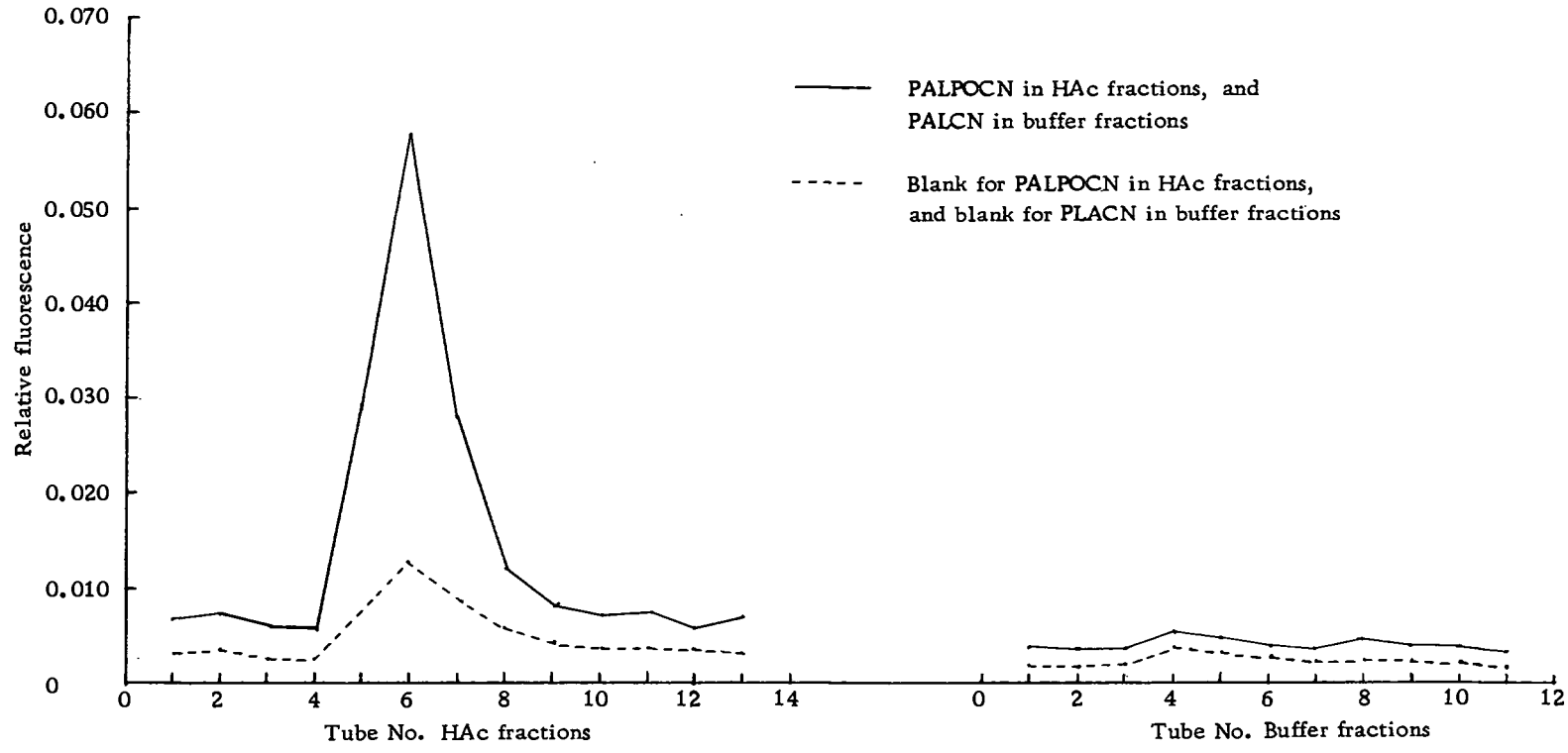

Figure 14. Recovery of pyridoxal phosphate (0.1 μg.) in blood (0.5 ml.) from SM-cellulose.

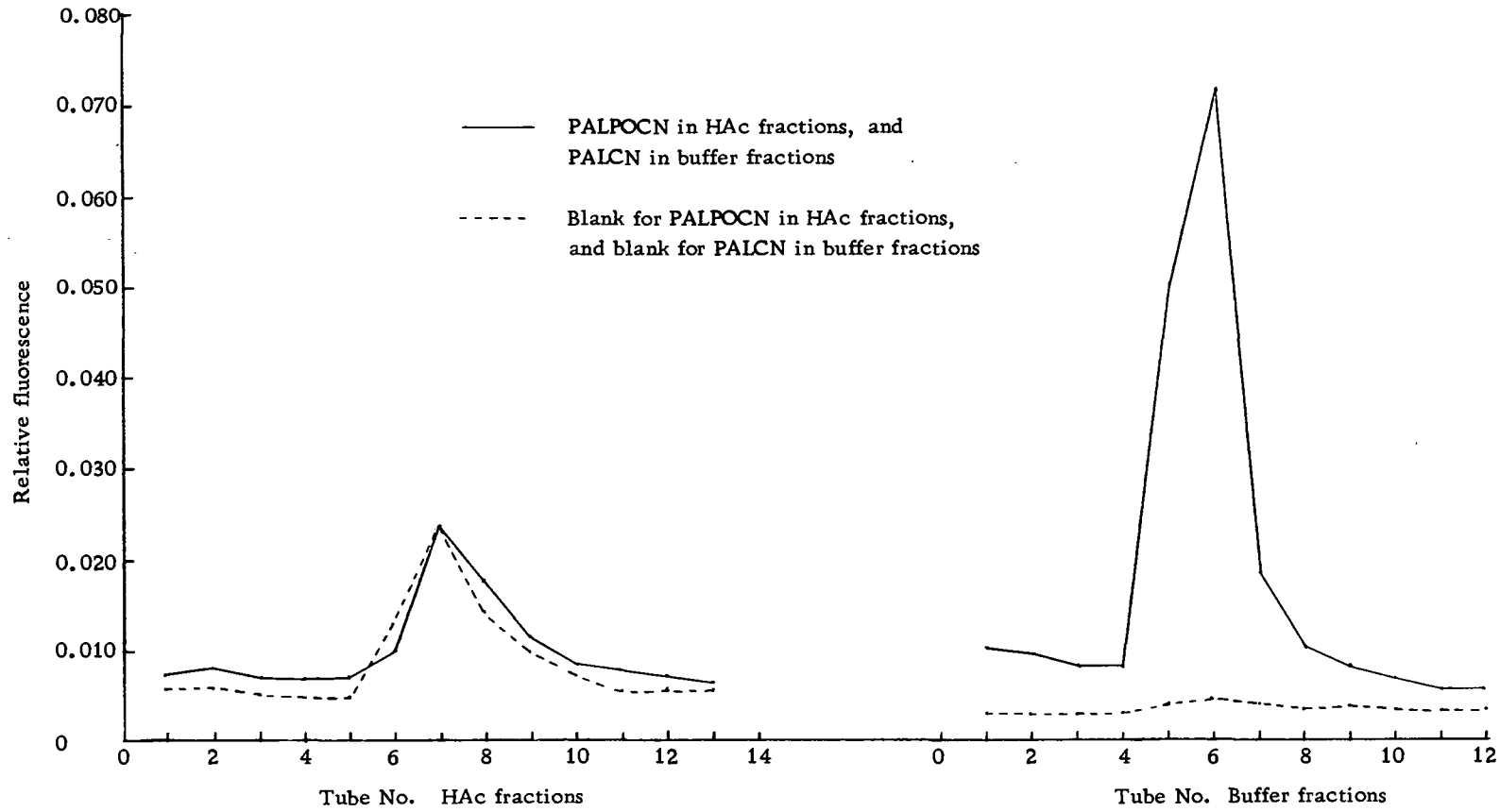

Figure 15. Recovery of pyridoxal (0.4 μg.) in blood (0.5 ml.) from SM-cellulose.

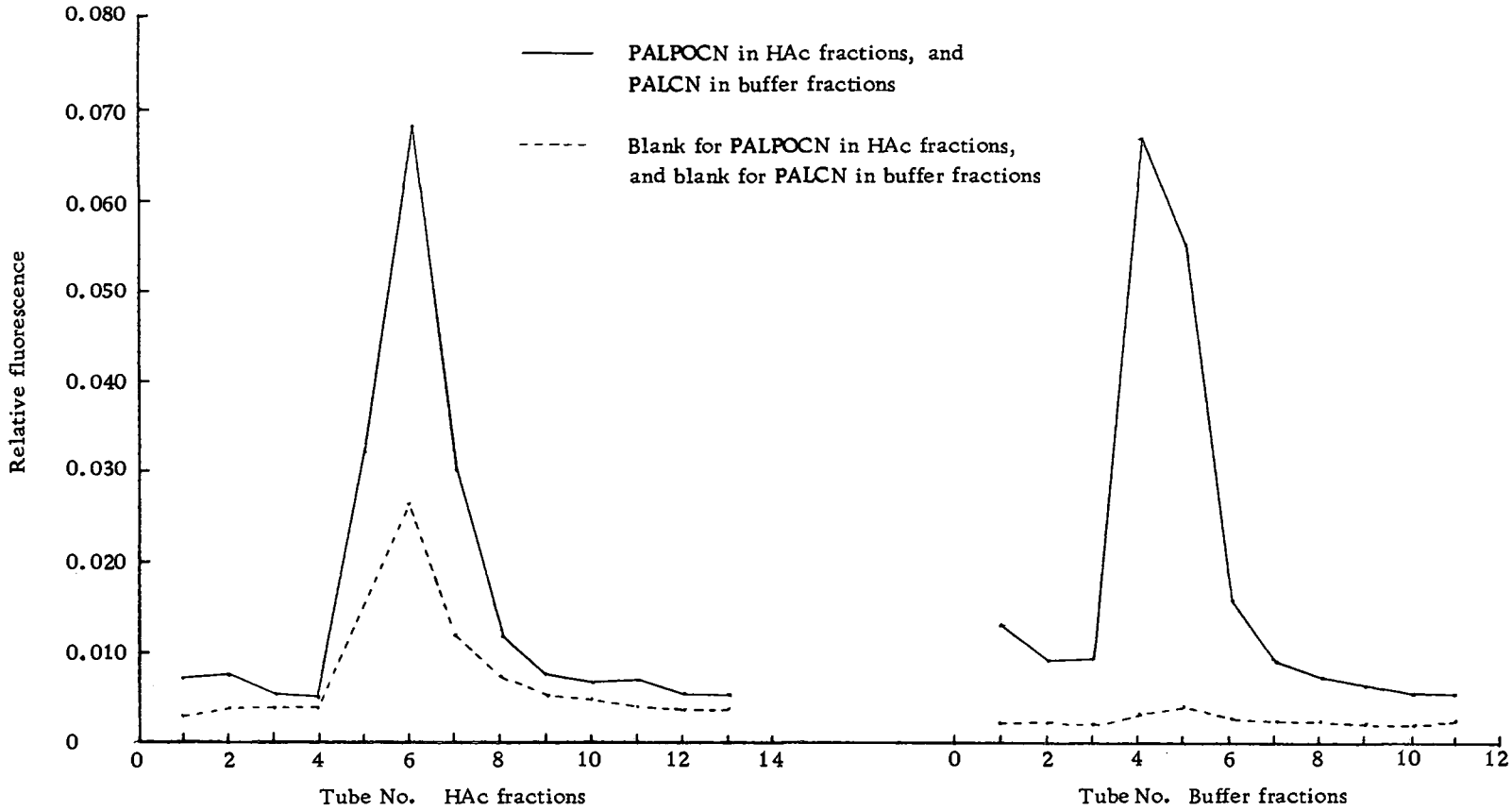

Figure 16. Recovery of pyridoxal phosphate (0.1 μg.) and pyridoxal (0.4 μg.) in blood (0.5 ml.) from SM-cellulose.

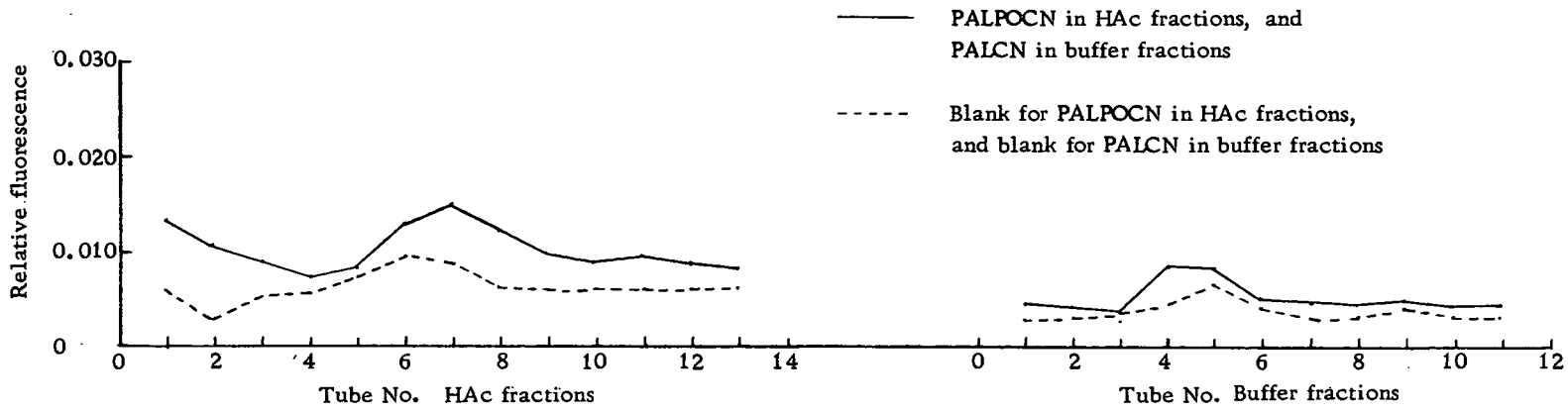

Figure 17. Separation of pyridoxal phosphate and pyridoxal in blood with SM-cellulose.

was 85.0% when pyridoxal phosphate alone was added to the blood (Figure 14). The recovery of pyridoxal when pyridoxal alone was added was only 29.1% (Figure 15).

When both pyridoxal phosphate and pyridoxal were added to the blood, the recovery of pyridoxal phosphate was 62.6% and that of pyridoxal was 52.1% (Figure 16). The low recovery of pyridoxal phosphate in blood when a mixture of pyridoxal phosphate and pyridoxal had been added to blood was due mainly to the high readings of the blanks for the HAc fractions. Probably the presence of pyridoxal in the HAc fractions from the SM-cellulose column to which blood plus pyridoxal phosphate and pyridoxal had been applied caused the high fluorescence of the blanks. High fluorescence readings were also obtained for the blanks of the HAc fractions from a column of SM-cellulose to which a sample containing blood and pyridoxal was applied (Figure 15). When blood and pyridoxal phosphate had been applied to a column of SM-cellulose (Figure 14), the fluorescence of the blanks for the HAc fractions was lower than that for the HAc blanks from a column to which blood and pyridoxal had been applied. When pyridoxal alone was added to blood, the recovery of pyridoxal was lower than when both pyridoxal phosphate and pyridoxal were added to blood. The higher recovery of pyridoxal in blood to which pyridoxal phosphate and pyridoxal had been added (Figure 16) can be explained by the low concentration of pyridoxal in the buffer fractions from a

column of SM-cellulose to which pyridoxal phosphate and pyridoxal had been applied (Figure 13) that was used to calculate the recovery.

The low recovery of both pyridoxal phosphate and pyridoxal from blood may have been caused by the adsorption of these compounds on the protein precipitate. Determining the recovery of standards added to the supernatant after the precipitation of the blood proteins would indicate whether pyridoxal phosphate and pyridoxal were lost by adsorption on the protein precipitate.

Pyridoxal Phosphate and Pyridoxal Content of Blood

The pyridoxal phosphate and pyridoxal content of blood from one female subject was determined by the modified procedure of Yamada et al. The data obtained on the separation of pyridoxal phosphate and pyridoxal in hemolyzed human blood by chromatography with SM-cellulose are presented in Table 4.

The modified procedure of Yamada et al. is not sensitive enough to determine the pyridoxal phosphate and pyridoxal content of blood. Only a small difference in fluorescence was obtained for the HAc fractions 5 through 9 and the buffer fractions 4 through 8 (Figure 17), the fractions in which pyridoxal phosphate and pyridoxal, respectively, had been found in the "Pattern of Elution" experiments. In addition, the difference in fluorescence for these fractions was not very different from that obtained for the other HAc and buffer

Table 4. Fluorescence data on the separation of pyridoxal phosphate and pyridoxal in blood according to the modified procedure of Yamada et al.

		Column 1[1]			Column 2[2]			
Fraction		Meter multiplier setting	Reading	Relative fluorescence	Meter multiplier setting	Reading	Relative fluorescence	Difference in fluorescence
Effluent	1[3]	.001	13.5	0.014	.001	6.0	0.006	0.008
	2	.001	11.0	0.011	.001	3.0	0.003	0.008
HAc	3[3]	.001	9.0	0.009	.001	5.5	0.006	0.003
	4	.001	7.5	0.008	.001	6.0	0.006	0.002
	5[4]	.001	8.5	0.009	.001	7.5	0.008	0.001
	6	.001	13.0	0.013	.001	10.0	0.010	0.003
	7	.001	15.0	0.015	.001	10.0	0.010	0.005
	8	.001	12.5	0.013	.001	9.0	0.009	0.004
	9	.001	10.0	0.010	.001	6.5	0.007	0.003
	10	.001	9.0	0.009	.001	6.5	0.007	0.002
	11	.001	10.0	0.010	.001	6.5	0.007	0.003
	12	.001	9.0	0.009	.001	6.5	0.007	0.002
Buffer	1[5]	.001	5.0	0.005	.001	3.5	0.004	0.001
	2	.001	4.5	0.005	.001	3.5	0.004	0.001
	3	.001	4.0	0.004	.001	3.5	0.004	0.000
	4[6]	.001	9.0	0.009	.001	5.0	0.005	0.004
	5	.001	8.5	0.009	.001	7.0	0.007	0.002
	6	.001	5.5	0.006	.001	4.5	0.005	0.001
	7	.001	5.0	0.005	.001	3.5	0.004	0.001
	8	.001	5.0	0.005	.001	3.5	0.004	0.001
	9	.001	5.5	0.006	.001	4.5	0.005	0.001
	10	.001	5.0	0.005	.001	3.5	0.004	0.001
	11	.001	5.0	0.005	.001	3.5	0.004	0.001

[1] Fractions from column 1 were reacted with potassium cyanide to obtain the cyanohydrin derivative.

[2] To obtain blanks for column 1, fractions from column 2 were treated with 0.1 M sodium phosphate buffer in place of potassium cyanide.

[3] Effluent fractions 1 and 2, and HAc fractions 3 through 12 were analyzed for pyridoxal phosphate.

[4] Pyridoxal phosphate content of blood was obtained by referring the difference in fluorescence in each of the HAc fractions 5 through 9 to a standard curve.

[5] Buffer fractions 1 through 11 were analyzed for pyridoxal.

[6] Pyridoxal content of blood was obtained by referring the difference in fluorescence in each of the buffer fractions 4 through 8 to a standard curve.

fractions which supposedly did not contain any prydioxal phosphate or pyridoxal.

The cyanohydrin method is probably not sensitive enough to accurately determine the low concentration of pyridoxal phosphate and pyridoxal found in these fractions. When standard curves for pyridoxal phosphate and pyridoxal were prepared, fluorescence was proportional to concentration except for the two lowest concentrations, containing 0.21 and 0.63 mμg. of pyridoxal phosphate per ml., and 0.12 and 0.36 mμg. of pyridoxal per ml.

To calculate the pyridoxal phosphate and pyridoxal content of blood, the concentration of pyridoxal phosphate or pyridoxal in each fraction was obtained by extrapolation from the standard curve. The difference in fluorescence in each of the HAc fractions 5 through 9 and the buffer fractions 4 through 8 was in the range in which the fluorescence was not proportional to the concentration of pyridoxal phosphate or pyridoxal.

The pyridoxal phosphate content of blood in this subject was calculated to be 27 mμg./ml.; and the pyridoxal content, 21 mμg./ml. This calculation for pyridoxal does not include the pyridoxal that may have been eluted with 0.01 N acetic acid.

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