The purpose of this thesis was to evaluate the cyanide method proposed by Contractor and Shane (1968) for the determination of vitamin B₆ compounds and 4-pyridoxic acid in human blood. These compounds in a concentrated protein-free extract of blood were separated by column chromatography with phosphocellulose. After application of the sample, the column was washed successively with 0.01 N acetic acid to remove pyridoxal phosphate and 4-pyridoxic acid, with 0.1 M acetate buffer at pH 4.7 to remove pyridoxal and pyridoxamine phosphate, and with 0.1 M phosphate buffer at pH 7.4 to remove pyridoxamine. The vitamin B₆ compounds and 4-pyridoxic acid in the eluates were determined by the cyanide and lactone methods, respectively.

The elution patterns of the vitamin B₆ analogues and 4-pyridoxic acid from phosphocellulose were studied in order to determine the volume of eluatriants needed to
remove the compounds from the column and to determine which eluate fractions to collect batchwise. These results were utilized in the procedures for the determination of the vitamin B₆ compounds and 4-pyridoxic acid in human blood, and the recovery of these compounds from phospho-cellulose and from blood.

Two series of experiments were conducted. Blood from the same lot was used throughout each series. For each vitamin B₆ compound, or 4-pyridoxic acid, three columns were run: standard, blood, and blood plus added standard. The two series were the same except in Series 1 untreated standard was applied to the column, and in Series 2 the standard was treated in the same manner as the blood.

Recovery of the standard from the column in Series 1 was good, indicating that all of the compound had been collected in the eluate. In Series 2 the lower recovery of vitamin B₆ compounds and 4-pyridoxic acid from phospho-cellulose might have been due to losses during evaporation or quantitative transfer, or to incomplete collection of the samples in the eluate.

The recovery of pyridoxal phosphate, pyridoxamine phosphate, pyridoxal, pyridoxamine, and 4-pyridoxic acid from blood in Series 1 was 86, 100, 17, 37 and 82 percent, respectively, while in Series 2 it was 74, 150, 0, 78, and 100 percent, respectively. Reasons for the erratic recovery of some compounds were discussed.
The concentrations of these compounds in the blood used in Series 1 were 12.0, 16.3, 8.4, 26.5, and 13.3 ng per ml, respectively; in the blood used in Series 2 the concentrations were 9.6, 19.3, 0, 21.7, and 21.7 ng per ml, respectively. These values are within the ranges found by Contractor and Shane except for pyridoxal, which was lower. The values for pyridoxal phosphate were close to those obtained by most enzymatic methods.

With the exception of pyridoxal this method appears to be sensitive and specific. Problems with the determination of pyridoxal need to be solved.
DETERMINATION OF VITAMIN B₆ COMPOUNDS IN HUMAN BLOOD
BY THE CYANIDE METHOD

by

Martha Ann Hammond

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Typed by Erma McClanathan for Martha Ann Hammond
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DETERMINATION OF VITAMIN B\textsubscript{6} COMPOUNDS IN HUMAN BLOOD

BY THE CYANIDE METHOD

INTRODUCTION

Pyridoxal phosphate serves as a coenzyme for many enzymes involved in the interconversion, degradation, and biosynthesis of amino acids. It also appears to be necessary for the metabolism of carbohydrates and fats (Beaton and McHenry, 1964). Pyridoxamine phosphate functions as a coenzyme for transaminases.

Since the other vitamin B\textsubscript{6} compounds, pyridoxal, pyridoxine, pyridoxamine, and pyridoxamine phosphate, can be converted to pyridoxal phosphate, specific and sensitive methods for measuring all of these compounds, as well as pyridoxal phosphate, in blood would be helpful in assessing the nutritional status of vitamin B\textsubscript{6} in the human. The various enzymatic, microbiological, and chemical methods for determining the components of vitamin B\textsubscript{6} in biological materials have been reviewed by Storvick and associates (Storvick and Peters, 1964; Storvick et al., 1964; and Chang, 1968).

Recently Contractor and Shane (1968) proposed a sensitive and specific procedure for the measurement of vitamin B\textsubscript{6} compounds and 4-pyridoxic acid in blood and urine. These compounds were separated on a phosphocellulose ion
exchanger and determined by the cyanide and the lactone methods, respectively.

The purpose of this thesis is to review the literature on the determination of vitamin B₆ compounds by the cyanide method, and to evaluate the procedure of Contraction and Shane for the determination of vitamin B₆ compounds and 4-pyridoxic acid in blood.
Vitamin B₆ is composed of several compounds: pyridoxal (PAL), pyridoxine (PIN), pyridoxamine (PAM), pyridoxal phosphate (PALPO), and pyridoxamine phosphate (PAMPO). All of them have the 2-methyl-3-hydroxy-pyridine ring structure in common (Table 1). At position 5, the free forms have a hydroxymethyl group and the phosphorylated forms have a methyl phosphoric acid group. The functional group on carbon 4 differs among these compounds: PAL and PALPO have a formyl group at position 4; PAM and PAMPO, an aminomethyl group; and PIN, a hydroxymethyl group (Snell, 1958).

PALPO, PAL, PAM, and PAMPO are found in animal tissues and yeast. PIN is found chiefly in plant materials (Snell, 1945). The chief metabolite of vitamin B₆, 4-pyridoxic acid (PIC) (Table 1), is found in both blood and urine (Contractor and Shane, 1968).

The main active form of vitamin B₆ is PALPO. It serves as a coenzyme in the transamination, deamination, decarboxylation, and desulfhydration of amino acids. It also appears to be involved in the metabolism of carbohydrates and fats (Beaton and McHenry, 1964). PALPO is converted to PAMPO

1. Pyridoxine (PIN), according to the 1966 Commission on the Nomenclature of Biological Chemistry of the International Union of Pure and Applied Chemistry, as cited by Pike and Brown (1967), is a designated alternate for pyridoxol (POL).
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in reversible transamination or deamination reactions (Snell, 1958).

**Chemical and Physical Properties of Vitamin B₆ Compounds**

PIN, PAL, and PAM are readily soluble in water, alcohol, or acetone, but only slightly soluble in ether or chloroform. The hydrochloride salts of these vitamin B₆ compounds are readily soluble in water, but sparingly so in alcohol or acetone (Association of Vitamin Chemists, 1951).

All of the vitamin B₆ compounds, including the phosphorylated forms, are markedly unstable to light, particularly in a neutral or alkaline medium (Storvick et al., 1964). These compounds are also destroyed by light in the absence of oxygen (Cunningham and Snell, 1945). Oxidizing agents, such as nitric acid, manganese dioxide, potassium permanganate, and hydrogen peroxide, rapidly destroy the vitamin B₆ compounds (Cunningham and Snell, 1945).

Peterson and Sober (1954) studied the stability of PAMPO and PALPO after 54 days of storage under various conditions by measuring the release of inorganic phosphate. When stored at room temperature (25°C), 15 percent of PALPO, but no PAMPO, was lost. Both compounds were stable when stored either frozen or refrigerated. Only 2.1 percent of PALPO was destroyed when refrigerated, and none when frozen. Less than a 0.2 percent loss of PAMPO
occurred under either condition.

Hamfelt (1967) studied the stability of PALPO in standard solutions by spectrophotometric measurements, and in plasma by determination with tyrosine decarboxylase. PALPO in a solution containing 1 mg per 100 ml was the most stable in an acid medium and stored frozen in darkness. No noticeable decrease in the concentration of PALPO occurred in plasma samples that had been stored directly or after precipitation with trichloroacetic acid at -20°C or 4°C for ten days. No PALPO was lost when the protein-free extract of blood was shaken with ether to remove the trichloroacetic acid.

Absorption and Fluorescence Characteristics of PAL and PALPO

All of the vitamin B₆ compounds exhibit characteristic light absorption maxima as well as different fluorescence characteristics.

The characteristic absorption maxima of PAL and PALPO depend upon pH. At neutrality, the absorption maxima of PAL are at 318 μμ and 390 μμ, and those of PALPO are at 330 μμ and 388 μμ (Storvick et al., 1964).

At pH 7, the fluorescence characteristics of PAL are 320 μμ (activation) and 385 μμ (fluorescence), while those of PALPO are 330 μμ and 375 μμ, respectively. According to Storvick et al. (1964), PALPO is the most weakly
fluorescent of the vitamin B\textsubscript{6} compounds.

**Reaction of PALPO and PAL with Cyanide**

Reaction with cyanide caused marked changes in the ultraviolet absorption spectrum of PALPO (Bonavita and Scardi, 1959). The characteristic absorption maximum of PALPO at 385 m\textmu was completely leveled off while the maximum at 320 m\textmu was intensified. Reaction with cyanide also caused changes in the ultraviolet absorption spectrum of PAL. The absorption maximum at 315 m\textmu decreased while the one at 353 m\textmu increased sharply. Bonavita and Scardi (1959) suggested that the complete disappearance of the maxima at 385 m\textmu and 315 m\textmu for PALPO and PAL, respectively, was due to the reaction of the formyl group on carbon 4 with cyanide to form the cyanohydrin derivative.

According to Bonavita (1960), changes in the fluorescence characteristics of PALPO and PAL also occur after reaction with cyanide. The activation and fluorescence maxima of the reaction product of PAL with cyanide are 358 m\textmu and 430 m\textmu, respectively, and of PALPO are 313 m\textmu and 420 m\textmu, respectively. Bonavita proposed the following as the mechanism of this reaction.

$$\text{R} \begin{array}{c|c} \text{C}=\text{O} & + \text{CN}^- \\ \hline \text{R} \end{array} \xrightarrow{\text{slow}} \begin{array}{c|c} \text{C}^- & \text{CN}^- \\ \hline \text{R} \end{array} \xrightarrow{\text{fast} \text{H}^+} \begin{array}{c|c} \text{C}^- & \text{OH}^- \\ \hline \text{R} \end{array}$$

R represents the pyridine ring of PALPO or PAL. This
reaction, according to Bonavita and Scardi (1959), goes to completion only when the reactants are present in equimolar concentrations or when cyanide is in excess.

Bonavita and Scardi found that the optimal pH for the reaction of PALPO or PAL with cyanide is at 7.4. At this pH the formyl group of PALPO or PAL is in the free aldehyde form, which favors interaction with cyanide. At pH 9.0, for example, Heyl and associates (1951) found that PALPO favors the anionic structure given below which does not permit the formation of an addition product with cyanide.

\[
\begin{array}{c}
\text{H-C-OH} \\
\text{CH}_2\text{OH} \\
\text{H}_3\text{C} \\
\text{N}
\end{array}
\]

The reaction between PALPO and cyanide is complete after incubation at 50°C for 30 minutes; whereas the reaction between PAL and cyanide is complete after 120 minutes (Yamada, Saito and Tamura, 1966). A longer incubation time is required for PAL in order to break the cyclic hemiacetal structure of this vitamer which is 80 times greater than the free form in a near neutral solution (Snell, 1958). This formula is given on the following page.
Bonavita found that the reaction product of PALPO with cyanide was most fluorescent at pH 3.8. He also reported that the reaction product of PAL with cyanide was most fluorescent at pH 7.55, while Yamada *et al.* (1966) found that it was most fluorescent at pH 10.

The reaction products of PALPO and PAL with cyanide are not stable. When left standing in an acidic medium, the reaction product of PALPO and cyanide decomposed at a rate of one percent and five percent after 20 and 45 minutes, respectively. In an alkaline medium the reaction product of PAL with cyanide decomposed at a rate of one percent within one hour (Yamada *et al.*, 1966).

**Determination of Other Vitamin B₆ Compounds by the Cyanide Method**

PAM can be determined by the cyanide method after conversion to PAL. In a procedure proposed by Toepfer, Polansky and Hewston (1961), PAM is transaminated to PAL by reaction with glyoxylate. This reaction, according to Metzler and Snell (1954), who originally studied this conversion, is as follows:
Potassium alum serves as a catalyst in this reaction. Metzler and Snell found that the disappearance of one mole of glyoxylate was accompanied by the formation of 0.6 to 0.7 mole of PAL. Toepfer and associates, however, reported complete conversion of PAM to PAL. Contractor and Shane (1968) converted PAMPO to PALPO by the same procedure Toepfer et al. had proposed for the transamination of PAM.

A procedure whereby PIN can be determined by the cyanide method has been reported by Polansky, Camarra and Toepfer (1964). Before reacting PIN with cyanide, it was oxidized to PAL by reaction with MnO₂ or KMnO₄. PIN is almost completely oxidized in this reaction.

Identification of the Reaction Products of PAL and PALPO with Cyanide

Ohishi and Fukui (1968) found that the reaction products of PAL and PALPO with cyanide were not the cyano-hydrin derivatives, but instead the lactone of 4-pyridoxic acid and 4-pyridoxic acid-5-phosphate, respectively. They based their conclusion on the fact that the reaction products of PAL and PALPO with cyanide exhibited the same chromatographic and ionophoretic behavior, as well as the same absorption and fluorescence spectra, as PIC lactone and 4-pyridoxic acid-5-phosphate, respectively.
Takanashi and associates (1968) isolated an intermediate in the reaction between PAL and cyanide. (This intermediate is indicated with an asterisk in Figure 1.) This compound decomposed at 130°C with the liberation of cyanide. The structure of the final product, as determined by infrared spectroscopy, x-ray crystallography, and elementary analysis, was the same as that of PIC lactone. Takanashi and associates proposed the mechanism of the reaction between PAL and cyanide to be that shown in Figure 1.

Determination of PIC by the Lactone Method

PIC and its lactone are highly fluorescent. The lactone at pH 9.0 ± 0.3, however, is 25 times more fluorescent than PIC at pH 3.4. The fluorescence characteristics of PIC are (activation) 325 μm and (fluorescence) 425 μm; and of PIC lactone, 350 μm and 434 μm, respectively (Storvick et al., 1964).

PIC is delactonized by heating in alkali and lactonized by heating in acid. Woodring, Fisher and Storvick (1964) developed a microprocedure for the determination of PIC by converting it to its lactone. They combined the column chromatographic procedure of Reddy, Reynolds and Price (1958) to separate PIC from other fluorescent compounds in urine and adapted the method by Fujita and Fujino (1955) to convert PIC to its lactone.
Figure 1. Mechanism proposed by Takanashi et al. (1968) for the reaction of PAL with cyanide. Asterisk indicates intermediate isolated by Takanashi and associates.
Chromatography

Thin Layer Chromatography

A few procedures for the separation of vitamin B₆ compounds by thin layer chromatography have been developed during the last few years. Stahl (1965) separated PIN, PAL, and PAM, and the 5-phosphoric acid esters of PAL and PAM on silica gel. The plates were developed with acetone, dried, and developed a second time with acetone:dioxane:25 percent ammonia (45:45:10). In a second method proposed by Stahl (1965) PIN, PAL, and PALPO were separated on silica gel by using either 0.2 percent ammonia or water as the developing solvent. More compact spots were obtained with dilute ammonia than with water. By exposing the developed chromatogram to ultraviolet light, he was able to detect as little as 3 μg of PIN or PAM. Stahl also detected the vitamin B₆ compounds with diazotized 2,6-dichloroquinonechloroimide (the indophenol or Gibbs' test), followed by treatment with ammonia vapor. The smallest quantities he could detect by this method were 0.1 μg of PIN or PAM, and 0.5-1.0 μg of PAL.

Yamada and Saito (1965) separated PALPO from a mixture of vitamin B₆ compounds by using cellulose powder as the adsorbent and dioxane:water (7:3) as the developing solvent. Yamada and Saito observed PALPO by fluorescence under ultraviolet light, either directly or after exposure to
ammonia vapors. They also used the Gibbs' test, followed by exposure to ammonia to detect PALPO. When spotted on the thin layer adsorbents without development, the smallest quantity of PALPO they detected was $10^{-4}$ M. To detect PALPO after development, a concentration above $10^{-3}$ M was required.

Since the concentration of PALPO in biological materials is generally less than $10^{-5}$ M (Storvick et al., 1964), these thin layer chromatographic procedures for the determination of vitamin B$_6$ compounds are unsatisfactory.

**Column Ion Exchange Chromatography**

Column ion exchange chromatography has been used in recent years to separate the components of vitamin B$_6$ in synthetic mixtures and in biological materials.

Peterson and Sober (1954) satisfactorily separated a 125-mg mixture of vitamin B$_6$ standards with Amberlite XW-64 ($H^+$), a weak cation exchange resin. PALPO, PINPO, PIC, and PAMPO were eluted with water, in the order represented, followed by PAL, PIN, and PAM, which were eluted successively with five percent acetic acid. Each compound was identified in both basic and acidic media by spectrophotometry. Storvick et al. (1964) questioned whether this method would be suitable for determining vitamin B$_6$ in biological materials due to the presence of interfering substances and to the minute quantity of the vitamin present.
Fujita, Matsuura and Fujino (1955) separated PIN, PAL, PAM, and PIC in hydrolysates of urine, tissue, and blood by using several columns and resins. PIN was adsorbed on Permutit and removed with boiling 0.1 N H$_2$SO$_4$. PIC was adsorbed on Amberlite IRA-410 (Ac$^-$), a strongly basic anionic exchanger, and eluted with boiling 25 percent KCl in 2 N acetic acid. To determine PAM, it was converted to PIN before it was applied to the Permutit column. PAL was retained on IR-112 (H$^+$), a strongly acidic cationic exchanger, and was eluted with 1 N NaOH.

They separated PIC and PAL in acid hydrolyzed blood by using two columns. Before application to the first column of IRA-410 (Ac$^-$), which retained PIC, NaOH was added to the supernatant to delactonize the PIC. The effluent and washings were then transferred to the second column of IR-112 (H$^+$) on which PAL was adsorbed. PIC was removed from the column of IRA-410 (Ac$^-$) with boiling 25 percent KCl in 2 N acetic acid, and PAL was eluted from the column of IR-112 (H$^+$) with 1 N NaOH. All of the compounds studied by Fujita et al. were converted to PIC and measured as its lactone. These methods from Fujita's laboratory are complicated due to the use of several resins and columns, but they were the first successful attempts to separate the vitamin B$_6$ compounds in biological materials.

Reddy et al. (1958) used two resins to separate PIC from other fluorescent compounds in urine. They applied
a sample of urine, which had been adjusted to pH 10.6, to
dowex 1 (Cl\(^-\)) column and eluted it with 0.05 N HCl. The
eluate was then applied directly to a Dowex 50 (H\(^+\)) column
from which it was eluted with 2 N HCl. PIC was determined
by the lactone method.

Toepfer and Lehmann (1961) separated the three ana-
logues of vitamin B\(_6\), PAL, PIN, and PAM, in tissues by
applying a filtered acid hydrolysate to a column of Dowex
50 WX-8 (K\(^+\)). The column was washed successively with
boiling solutions of 0.04 M potassium acetate at pH 6.0
to remove PAL, 0.1 M potassium acetate at pH 7.0 to remove
PIN, and KCl-K\(_2\)HPO\(_4\) solution at pH 8.0 to remove PAM. The
vitamin B\(_6\) compounds were determined in the eluates with
the test organism, *Saccharomyces carlsbergensis*, which
responds to all three vitamin B\(_6\) compounds. Results were
not checked with other organisms which are sometimes used
for the assay of individual forms. This method appears to be
satisfactory for the determination of vitamin B\(_6\) in animal
and plant tissues. It has been applied to foods (Toepfer
and Lehmann, 1961) and to human blood (Kelsey *et al.*, 1968).

Storvick and associates (1964) separated PIC from the
free forms of vitamin B\(_6\) on Dowex 1 (Ac\(^-\)) which retained
PIC. The effluent from this column was applied to a
second column of Dowex 50 (K\(^+\)), on which the other com-
pounds of vitamin B\(_6\) were retained. They were removed by
a uniform increase in pH and molarity which was achieved
by gradient elution with 0.1 M potassium acetate, pH 7.0, and 0.02 M potassium acetate, pH 5.5. Each component was eluted at a pH range which was distinct from the pH ranges required for the elution of the other two analogues. PAL was removed from pH 5.66 to 6.10; PIN, from pH 6.20 to 6.38; and PAM, from pH 6.60 to 6.73. PIC was removed from the Dowex 1 (Ac⁻) with 25 percent KCl in 2 N acetic acid. Storvick et al. (1964) determined these compounds in the eluate fractions by direct fluorescence.

Yamada and Saito (1965) studied the chromatographic separation of PALPO from the other components of vitamin B₆ on DEAE-(diaminoethyl), TEAE-(Triaminoethyl), SM-(sulfomethyl), and SE-(sulfoethyl) cellulose ion exchangers. They found that the most suitable elutriants for the separation of PALPO (10⁻³ M) from the other B₆ analogues on DEAE- and TEAE-celluloses were 0.01 N HCl and 0.01 M acetate buffer at pH 4.4, respectively. They found that 0.01 N acetic acid and 0.01 M acetate buffer, pH 4.4, eluted PALPO from SM-cellulose and 0.001 M acetate buffer at pH 4.7 eluted it from SE-cellulose. The recovery of PALPO from the DEAE-cellulose was 97-99 percent, while that from TEAE-cellulose was 90-98 percent.

Yamada et al. (1966) separated PAL and PALPO in protein-free extracts of animal tissues on SM-cellulose. The column was washed successively with 0.01 N acetic acid to remove the PALPO and 0.1 M phosphate buffer to
elute PAL. Both compounds were determined by reaction with cyanide. This procedure was not sensitive enough to measure the small amounts of PALPO and PAL in human blood (Chang, 1968).

Contractor and Shane (1968) separated PIC, PAL, PAM, PALPO, and PAMPO in a protein-free extract of human blood by applying it to a column of Whatman P-11 phosphocellulose ion exchanger. The column was washed successively with 0.01 N acetic acid to remove PALPO and PIC, 0.1 M acetate buffer at pH 4.7 to remove PAL and PAMPO, and 0.1 M phosphate buffer at pH 7.4 to remove PAM. All of the vitamin B6 compounds were measured by the cyanide method. Before reaction with cyanide, PAM and PAMPO were converted to PAL and PALPO, respectively, by transamination with glyoxylate, and PIN was oxidized with manganese dioxide to PAL. PIC was determined as its lactone by a slight modification of the microprocedure of Woodring et al. (1964), which is described on page 33 of this thesis.

**Vitamin B6 Compounds and PIC in Human Blood**

Studies on the determination of the PALPO in human blood were recently reviewed by Chang (1968). The reported concentrations of PALPO in blood, summarized according to method of determination, are as follows: by tyrosine decarboxylase, from less than 10 to 37 ng per ml of blood (80 percent of the subjects in one study had PALPO values
of less than 10 ng per ml), from 2.4 to 33.0 ng per ml of plasma, and from 0.11 to 0.79 ng per million leukocytes; by tryptophanase, an average of 23 ng per ml of serum and 0.30 ng per million leukocytes; and by apotransaminase, from 0.5 to 13.0 ng per ml of plasma (Chang, 1968).

More recent studies include the one by Contractor and Shane (1968), who separated the vitamin B₆ compounds and PIC in blood on a phosphocellulose ion exchanger and determined these compounds in the eluates by the cyanide and lactone methods, respectively. The blood of the four normal women they studied contained from 8 to 18 ng of PALPO and 0 to 17 ng of PAMPO per ml. The concentrations of PAL and PIC were the highest, ranging from 30 to 80 ng and 15 to 40 ng per ml, respectively. From 15 to 30 ng of PAM per ml were found. More recently, however, Contractor (1969) reported that the concentrations of the phosphorylated forms of vitamin B₆ in blood were higher than that of PAL, and that the true values for PAL were actually lower than those he and Shane had published in 1968. Contractor and Shane detected no PIN in blood, even in subjects who were given an oral dose of 100 mg of PIN. They suggested that PIN may be metabolized in the intestinal wall, blood or kidneys, or stored in the tissues.

Hines and Love (1969) determined PALPO in human blood and serum through the use of purified rabbit muscle
apophosphorylase b. They found that serum PALPO ranged from 23.0 to 55.0 ng per ml in subjects less than 40 years old, and from 15.8 to 31.0 ng per ml in subjects 48 to 80 years old. The concentration of PALPO in whole blood of 45 normal adults was from 80 to 250 ng per ml (average 168 ng). They found no correlation between whole blood values and the age of the subjects in this control group.

Kelsey, Baysal and Linkswiler (1968) utilized the column chromatographic procedure of Toepfer and Lehmann (1961) to separate PAL, PAM, and PIN in acid hydrolysates of blood. They studied six men who were receiving a diet containing 150 g of protein, and adequate or inadequate amounts of vitamin B₆. The only vitamin B₆ compound they detected in their subjects' blood while they were on this regimen was PAL. When the subjects received 1.66 mg of vitamin B₆ daily, their blood contained an average of 0.66 µg of PAL per 100 ml. During two consecutive 15-day periods, their subjects received, respectively, 0.16 mg and 0.76 mg of vitamin B₆ daily. At the end of each of these two periods their blood contained an average of 0.20 µg and 0.26 µg of PAL per 100 ml, respectively. After receiving 50 mg of PIN daily for two consecutive days, the subjects' blood contained from 4.0 to 5.5 µg of PAL and from 0.5 to 0.8 µg of PIN per 100 ml. Kelsey and associates were unable to detect PAM in their subjects' blood.
Jirsak (1963) determined PAL in blood by the microbiological method of Rabinowitz et al. (1948) and found that the values ranged from 24 to 30 (average 28.5) ng per ml. He reported that the total vitamin $B_6$ content of blood ranged from 50.0 to 65.0 (average 57.3) ng per ml.
EXPERIMENTAL

Studies were made to evaluate the procedure proposed by Contractor and Shane (1968) for the determination of vitamin B$_6$ compounds and PIC in blood. In this procedure these compounds in a protein-free extract of blood were separated by column chromatography with phosphocellulose. The vitamin B$_6$ compounds and PIC in the eluates were determined by the cyanide and lactone methods, respectively.

Experiments carried out in this study include:

1. Standard curves for each vitamin B$_6$ compound and PIC.

2. The elution of vitamin B$_6$ compounds and PIC from phosphocellulose. The dimensions of the column used in this study were different from those of the column used by Contractor and Shane, but the volume of phosphocellulose was the same in both studies.

3. The recovery of vitamin B$_6$ compounds and PIC from phosphocellulose and from blood.

All experiments were conducted in subdued light to protect the vitamin B$_6$ compounds and PIC from decomposition by light.
Procedure

Preparation of Standard Solutions of Vitamin B₆ Compounds and PIC

These standard solutions were diluted to give intermediate standards from which the desired concentrations were prepared. Redistilled water was used in the preparation of these and other reagents.

Pyridoxal Phosphate Monohydrate, Calbiochem, Los Angeles, California. 53.56 mg of pyridoxal phosphate monohydrate were dissolved and diluted to 100 ml with water (1 ml = 535.6 µg). 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 Hydrochloride, Sigma Chemical Company, St. Louis, Missouri. 100 mg of pyridoxal hydrochloride were dissolved and diluted to 200 ml with ten percent (v/v) acetic acid (1 ml = 500 µg). This standard was stored in a low actinic glass bottle at 4°C.

Pyridoxine Hydrochloride, United States Pharmacopoeia Reference Standard. 100 mg of pyridoxine hydrochloride were dissolved and diluted to 200 ml with ten percent (v/v) acetic acid (1 ml = 500 µg). This standard was stored in a low actinic glass bottle at 4°C.

Pyridoxamine Dihydrochloride, Merck and Company, Rahway, New Jersey. 100 mg of pyridoxamine hydrochloride
Blood Collection and Preservation

About 200 ml of venous blood were obtained on four occasions, twice from each of two women. The blood was drawn by technicians at Good Samaritan Hospital and the Student Health Center. Either heparin or citrate served as the anticoagulant.

Without delay the blood was transported to the laboratory, and was pipetted in 10-ml portions into 90-ml centrifuge tubes. Before each pipetting, the blood was mixed by slowly inverting the container two or three times. The tubes were covered with parafilm and stored at -10°C until analysis.

Preparation of Samples

The blood was thawed just before use. The 10-ml sample of blood was diluted with 20 ml of water. In experiments with added standard, 1 ml of standard and 19 ml of water were added to the thawed blood in place of 20 ml of water; in experiments using standards only (treated standard) 1 ml of standard was diluted with 29 ml of water. To the diluted blood or standard 30 ml of ten (w/v) percent trichloroacetic acid were added gradually with stirring. The tube was covered, the mixture was warmed in a water bath at 50°C for 20 minutes, and centrifuged at 4000 rpm for 20 minutes. Fifty milliliters of the supernatant were
transferred to a 250-ml separatory funnel which was fitted with a Teflon stopcock. Most of the trichloroacetic acid was removed by extracting the supernatant twice with approximately 50-ml volumes of analytical grade ether.

The aqueous phase was placed in a 250-ml round bottom flask. Each ethereal extract was washed with approximately 3 ml of water. The aqueous extract plus washings were evaporated down to approximately 1 ml under reduced pressure at 46 to 48°C in a Buchler Flash Evaporator. Full suction was not applied during the first few minutes of evaporation to prevent the formation of bubbles. The approximate length of evaporation was 30 to 40 minutes.

**Ion Exchange Chromatography**

Phosphocellulose P-11, control number 21112, was obtained from Reeve Angel Company, Clifton, New Jersey. Nominal total capacity of phosphocellulose is 7.4 meq per g. Phosphocellulose is a strong cation exchanger in which the major functional group is dihydrogen phosphate. In an acid medium it behaves as a monofunctional exchanger, while in a slightly alkaline medium it acts as a bifunctional exchanger.

**Precycling and Equilibration**

In precycling, aggregates of cellulose chains are broken so that the functional groups of the ion exchange
cellulose will be more accessible during chromatography. The procedure outlined in Whatman Technical Bulletin IE2 (1968) was followed.

To 150 ml of 0.5 N NaOH were added 10 g of phosphocellulose. The mixture was stirred gently for about five minutes and allowed to stand undisturbed for one hour. After decanting most of the NaOH, the cellulose was washed with redistilled water in the Buchner funnel lined with Whatman #541 filter paper until the wash was neutral to litmus. This procedure was repeated except that the phosphocellulose was treated with 0.5 N HCl in place of 0.5 N NaOH.

The precycled cellulose was equilibrated with 0.01 N acetic acid to prevent changes from occurring in the cellulose during chromatography. The precycled phosphocellulose was dispersed in about 100 ml of 0.01 N acetic acid and was allowed to stand undisturbed for 10 to 15 minutes. The supernatant, which contained fines, was removed by aspiration. After the eighth wash the pH of the supernatant was the same as that of the 0.01 N acetic acid, pH 3.2. The prepared cellulose was stored under an equal volume of 0.01 N acetic acid in the refrigerator. When the cellulose was stored for periods longer than one week, it was preserved with toluene or benzene.
Preparation of Columns

The dimensions of the glass columns used for chromatography were 1.2 (outside diameter) x 20 cm. The bottom of the column was drawn out to a fine tip and contained a Teflon stopcock with a metering valve (Kimble Art. No. 41575-F, size 1½ mm). A 200-ml round bottom flask, which served as a reservoir, was fused to the top of the column.

A cotton plug was placed at the bottom of the column to support the cellulose. The plug was prepared under water by wrapping a piece of cotton gauze, about 5 cm square, around a small piece of loose cotton. The column was filled with water and the plug was inserted gently to prevent packing.

The water was allowed to drain to the level of the plug, and the column was filled with about 10 ml of 0.01 N acetic acid. A suspension of phosphocellulose sufficient to give a final bed height of 6 cm or more was pipetted in one pass into the column. The stopcock was immediately opened, and as the supernatant was issuing from the column, any cellulose that adhered to the walls was washed down with 0.01 N acetic acid. When the supernatant was down to about 2 cm above the cellulose bed, the height of the column was measured. Any cellulose above 6 cm was removed. Just before the sample was applied, the column was washed with 20 ml of 0.01 N acetic acid. At this time the flow
rate was adjusted to 17-19 drops per minute. No further adjustment in flow rate was made during development of the column.

**Application of Sample**

The concentrated protein-free extract was transferred quantitatively with 0.01 N acetic acid by means of a funnel with a capillary stem to the previously prepared column of phosphocellulose. The sample and rinsings (5 ml total) were allowed to drain into the phosphocellulose.

In some experiments 1 ml of untreated standard or a mixture of untreated standards, followed by 4 ml of 0.01 N acetic acid were applied to the previously prepared column of phosphocellulose.

**Development of the Column**

Development of the column will be presented in the sections "Elution Patterns" and "Determination of Vitamin B₆ and PIC in Blood and Recovery of these Compounds from Phosphocellulose and Blood."

**Determination of Vitamin B₆ Compounds and PIC**

**PALPO and PAL**

The procedures for the determination of PAL and PALPO by the cyanide method are similar and are outlined in
Table 2. Four tubes were prepared for each sample; 2 ml of sample and 2 ml of phosphate buffer, pH 7.4, were placed in each tube. To tubes 1 and 2, 0.1 ml of 0.05 M KCN in 0.1 M phosphate buffer at pH 6.9 was added; and to tubes 3 and 4, the blanks, 0.1 ml of 0.1 M phosphate buffer at pH 6.9 was added. The cyanide was dispensed with a Hamilton No. 1005 automatic syringe which was fitted with a fixed needle and a Hamilton repeating dispenser PB-600-10. Each time the button was pressed 0.1 ml was dispensed. The tubes containing PAL were warmed in a 50°C water bath for 120 minutes, and those containing PALPO were warmed for 30 minutes. After the reaction was complete, the pH was adjusted to obtain the optimum fluorescence of each reaction product.

PAMPO and PAM

Before PAM and PAMPO (Table 3) were reacted with cyanide, they were converted to PAL AND PALPO, respectively, by transamination with sodium glyoxylate. Four tubes were prepared for each sample; 2 ml of sample, 2 ml of phosphate buffer, pH 7.4, and 0.1 ml of potassium alum (K₂Al₂O₄·3H₂O) were placed in each tube. To tubes 1 and 2, 0.1 ml of sodium glyoxylate was added; and to tubes 3 and 4, the blanks, 0.1 ml of water was added. The tubes were mixed and heated in a 100°C water bath for 20 minutes. Following transamination, 0.1 ml of 0.5 M KCN
Table 2. Procedure for the Determination of PAL and PALPO by the Cyanide Method

<table>
<thead>
<tr>
<th>Sample</th>
<th>PAL</th>
<th>PALPO</th>
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<tbody>
<tr>
<td>Sample</td>
<td>2 ml</td>
<td>2 ml</td>
</tr>
<tr>
<td>0.2 M PO₄ buffer, pH 7.4</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1.0 M PO₄ buffer, pH 7.4</td>
<td>2 ml</td>
<td>2 ml</td>
</tr>
<tr>
<td>0.05 M KCN in 0.1 M PO₄ buffer, pH 6.9</td>
<td>0.1 ml</td>
<td></td>
</tr>
<tr>
<td>0.1 M PO₄ buffer, pH 6.9</td>
<td></td>
<td>0.1 ml</td>
</tr>
<tr>
<td>Mix, cover, warm at 50°C</td>
<td>120 min</td>
<td>120 min</td>
</tr>
<tr>
<td>Cool</td>
<td>10</td>
<td>10</td>
</tr>
<tr>
<td>Adjust pH to by adding 0.33 N HCl</td>
<td></td>
<td>3.5-4</td>
</tr>
<tr>
<td>1.5 N NH₄OH</td>
<td>1 ml</td>
<td>1 ml</td>
</tr>
<tr>
<td>Mix</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Read at activation</td>
<td>350 μμ</td>
<td>350 μμ</td>
</tr>
<tr>
<td>fluorescence</td>
<td>434 μμ</td>
<td>434 μμ</td>
</tr>
</tbody>
</table>

1 Blanks
Table 3. Procedure for the Determination of PAM and PAMPO by the Cyanide Method.

<table>
<thead>
<tr>
<th></th>
<th>P A M</th>
<th>P A M P O</th>
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<tbody>
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<td></td>
<td>Tubes 1 &amp; 2</td>
<td>Tubes 3 &amp; 4</td>
</tr>
<tr>
<td>Sample</td>
<td>2 ml</td>
<td>2 ml</td>
</tr>
<tr>
<td>1.0 M PO₄ buffer, pH 7.4</td>
<td>2 ml</td>
<td>2 ml</td>
</tr>
<tr>
<td>0.05 M sodium glyoxylate</td>
<td>0.1 ml</td>
<td>-</td>
</tr>
<tr>
<td>Redistilled water</td>
<td>-</td>
<td>0.1 ml</td>
</tr>
<tr>
<td>0.005 M potassium alum</td>
<td>0.1 ml</td>
<td>0.1 ml</td>
</tr>
<tr>
<td>Mix, cover, heat in 100°C water bath</td>
<td>20 min</td>
<td>20 min</td>
</tr>
<tr>
<td>Cool</td>
<td></td>
<td></td>
</tr>
<tr>
<td>0.5 M KCN in 0.1 M PO₄ buffer, pH 6.9</td>
<td>0.1 ml</td>
<td>0.1 ml</td>
</tr>
<tr>
<td>Mix, cover, warm at 50°C</td>
<td>120 min</td>
<td>120 min</td>
</tr>
<tr>
<td>Cool</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Adjust pH to</td>
<td>10</td>
<td>3.5-4</td>
</tr>
<tr>
<td>by adding</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1.6 N HCl</td>
<td>-</td>
<td>1 ml</td>
</tr>
<tr>
<td>1.5 N NH₄OH</td>
<td>1 ml</td>
<td>-</td>
</tr>
<tr>
<td>Mix</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Read at</td>
<td></td>
<td></td>
</tr>
<tr>
<td>activation</td>
<td>350 μµ</td>
<td>318 μµ</td>
</tr>
<tr>
<td>fluorescence</td>
<td>434 μµ</td>
<td>417 μµ</td>
</tr>
</tbody>
</table>

1 Blanks
in 0.1 M phosphate buffer at pH 6.9, which had been prepared less than 30 minutes beforehand, was added to each tube. The KCN was dispensed with the Hamilton syringe described above. The samples of PAM and PAMPO were warmed in a 50°C water bath for 120 minutes and 30 minutes, respectively. After the reaction with cyanide the pH was adjusted to obtain the optimum fluorescence of each product.

**PIN**

To 15-ml centrifuge tubes were added 2 ml of sample and 0.02 g of MnO₂, which had been prepared according to the procedure of Polansky et al. (1964). This mixture was mixed, shaken for 30 minutes, and centrifuged at 4000 rpm for 20 minutes. The supernatant was removed and the precipitate was washed twice with 1-ml portions of 1.0 M phosphate buffer, pH 7.4. The washings were added to the supernatant, and 0.1 ml of 0.05 M KCN was added. The procedure was continued as given for PAL in Table 2.

**PIC**

PIC was determined as its lactone, according to the procedure of Woodring, Fisher, and Storvick (1964), as adapted by Contractor and Shane (1968).

PIC was first delactonized by heating in an alkaline medium. Into 13 x 100 mm test tubes were pipetted 200 μl
of sample, 1600 µl of water, and 400 µl of 2 N NaOH. After the contents of each tube were mixed, the tubes were covered with Teflon caps, heated in a boiling water bath for five minutes, and cooled to room temperature. The PIC in two tubes was lactonized by adding 400 µl of 6 N HCl to each, followed by mixing, capping, and heating in a boiling water bath for 20 minutes. After cooling the tubes to room temperature, the lactone was stabilized by the addition of 1 ml of 6 N NH₄OH. Unlactonized samples, which served as blanks, were prepared by adding 400 µl of 6 N HCl and 1 ml of 6 N NH₄OH to each of the two remaining tubes. All tubes were read at an activation wavelength of 350 mÅ and a fluorescent wavelength of 434 mÅ.

**Fluorescence Measurements**

Fluorescence measurements were made with an Aminco-Bowman Spectrophotofluorometer, No. 4-8106. The sensitivity was set at 50 and the slit arrangement was 1/8, 3/16, 1/8, 1/8, 3/16, 1/8, and 1/16. The spectrophotofluorometer was equipped with a RCA IP21 photomultiplier tube and an Osram XBl65 xenon light source.

**Standard Curves**

Two sets of standard curves for vitamin B₆ compounds and PIC were prepared with unchromatographed standards. In the first set the concentrations of the compounds
were calculated from the ranges Contractor and Shane found in 10 ml of blood and in the volume of the eluate in which the compound was found. Dilutions were made as follows: PAL·HCl standard was diluted with 0.1 M acetate buffer at pH 4.7, and PALPO with 0.01 N acetic acid, to obtain concentrations of 0.01, 0.02, 0.04, 0.06, and 0.10 µg per ml; PAM·2HCl was diluted with 0.1 M phosphate buffer at pH 7.4 to obtain concentrations of 0.005, 0.008, 0.01, 0.02, and 0.04 µg per ml; PAMPO was diluted with 0.1 M acetate buffer at pH 4.7 to obtain concentrations of 0.004, 0.008, 0.01, 0.02, and 0.04 µg per ml; and PIC was diluted with 0.01 N acetic acid to obtain concentrations of 0.004, 0.008, 0.016, 0.024, 0.06, and 0.08 µg per ml. After dilution the vitamin B₆ compounds were determined by the cyanide method, and PIC by the lactone method. The standard curves obtained from the above dilutions are presented in Figure 2.

Another set of standard curves was prepared the same as above, except that higher concentrations were used. The vitamin B₆ standards were diluted to give concentrations of 0.01, 0.02, 0.04, 0.08, and 0.10 µg per ml. For PAL·HCl, PAM·2HCl, PALPO, and PAMPO an additional dilution was made to obtain a concentration of 0.06 µg per ml. The standard of PIC was diluted to obtain concentrations of 0.08, 0.24, 0.48, and 0.64 µg per ml. These concentrations were later converted to µmoles per ml in order to
Figure 2. Standard curves for PALPO, PAL, PAMPO, PAM, and PIC.

The concentrations for these compounds were based on data by Contractor and Shane (1968), that is, the range of concentration of these substances in blood, and in the eluates after chromatography.
compare the fluorescence of the reaction products.

**Elution Patterns of the Vitamin B₆ Compounds and PIC from Phosphocellulose**

The elution patterns of the vitamin B₆ compounds and PIC from phosphocellulose were studied in order to determine the volume of elutriants needed to remove the compounds from the column and to determine which eluate fractions to collect batchwise. The dimensions of the phosphocellulose column used in this study differed from those of the column used by Contractor and Shane. They used a phosphocellulose bed of 1.2 x 4 cm. In this laboratory, a phosphocellulose bed of 1.0 (estimated) x 6 cm was used.

To collect the eluate fractions a Misco fraction collector, No. 6500, and drop counter, No. 6720, were used. The fine tip of the column was connected to the glass tube in the drop counter with a 24-inch length of fine Tygon tubing. Sixty-five drop (2-ml) fractions were collected.

Standards of the vitamin B₆ compounds and PIC were applied either individually or as a mixture to the previously prepared phosphocellulose column. The approximate quantities of vitamin B₆ compounds and PIC that Contractor and Shane found in 10 ml of blood were used: 0.20 μg of PALPO, 0.40 μg of PIC, 0.50 μg of PAL·HCl, 0.20 μg of PAMPO, and 0.25 μg of PAM·2HCl. PIN was not determined since Contractor and Shane did not find it in human blood.
One milliliter of standard or mixture of standards was applied to the previously prepared column of phosphocellulose, followed by 4 ml of 0.01 M acetic acid.

For studies on the separation of the vitamin B₆ compounds and PIC in blood, or of individual compounds added to blood, the sample was prepared as described under "Preparation of Samples." The quantities of vitamin B₆ compounds and PIC added to blood were the same as given above. The sample, after it was reduced to 1 ml, was quantitatively transferred to the prepared column with 4 ml of 0.01 M acetic acid.

When the elution patterns of a mixture of standards or of the vitamin B₆ compounds and PIC in blood were determined, two columns were needed. This was necessary because two compounds were eluted in the same fractions. The fractions were collected in the sample size (2 ml) needed for the determination of the vitamin B₆ compounds with cyanide.

The sample (sample plus 4 ml of 0.01 M acetic acid, 5 ml total) was allowed to drain into the phosphocellulose. To develop the column, it was washed successively with 20 ml of 0.01 M acetic acid to elute PIC and PALPO; with 55 ml of 0.1 M acetate buffer, pH 4.7, to elute PAL and PAMPO; and with 35 ml of 0.1 M phosphate buffer, pH 7.4, to elute PAM. The concentrations of the vitamin B₆ compounds and PIC in the eluate fractions were determined as described previously. The results of these studies
are seen in Figures 3, 4, and 5.

**Determination of Vitamin B\textsubscript{6} Compounds and PIC in Blood and the Recovery of these Compounds from Phosphocellulose and Blood**

The procedure for the determination of the vitamin B\textsubscript{6} compounds and PIC in blood, and the recovery of these compounds from blood and phosphocellulose, was based on the results of the elution studies presented in Figures 4 and 5.

Two series of experiments were conducted. Blood from the same lot was used throughout each series. For each vitamin B\textsubscript{6} compound or PIC, three columns were used: standard, blood, and blood plus added standard. The quantities of standards applied to the column and added to blood were the same as those used in the elution studies. In the first set of experiments (Series 1), 1 ml of untreated standard was applied directly to the column, followed by 4 ml of 0.01 N acetic acid. The samples of blood and blood plus standard were prepared as described under "Preparation of Samples" except that the supernatant was extracted four times with water-saturated ether, the ethereal extracts were not washed with water, and the protein-free extract was evaporated in approximately 7-ml portions in a 100 ml round bottom evaporating flask. Evaporation took from two to three hours, depending upon
Figure 4. Elution patterns of vitamin $B_6$ compounds and PIC in blood from phospho-cellulose.

The blood was prepared for chromatography as described under "Preparation of Samples." Two columns were developed. PIC, PAMPO, and PAM were determined in the eluate fractions from the first column, and PALPO and PAL were determined in the eluate fractions from the second. Two-ml fractions were collected. No blanks were prepared.
Relative Fluorescence

- PALPO
- PAL
- PIC
- PAMPO
- PAM

<table>
<thead>
<tr>
<th>Eff.</th>
<th>Tube no. HAc fractions</th>
<th>Tube no. of 0.1 M acetate buffer fractions</th>
<th>Tube no. of 0.1 M PO₄ buffer fractions</th>
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<td>42</td>
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the water pressure. The sample after evaporation was transferred quantitatively to the column with 4 ml of 0.01 N acetic acid.

In the second set of recovery experiments (Series 2) 1 ml of standard was combined with 29 ml of water and treated as described under "Preparation of Samples." This sample, after it had been reduced to 1 ml, was quantitatively transferred to the column with 4 ml of 0.01 N acetic acid (5 ml total). The blood and blood plus added standard were prepared the same as for the first series of experiments.

The sample and rinsings were allowed to sink into the phosphocellulose column. The first 4 ml of effluent were discarded. The column was washed with 20 ml of 0.01 N acetic acid. If PALPO was being determined, the fifth ml of the effluent and the next 12 ml of the 0.01 N acetic acid wash were collected (13 ml total); for the determination of PIC the fifth ml of the effluent and the next 13 ml were collected (14 ml total). The column was then washed with 55 ml of 0.1 M acetate buffer, pH 4.7. The first 28 ml of this eluate were discarded and the next 18 ml were collected for the determination of either PAL or PAMPO. The column was finally washed with 35 ml of 0.1 M phosphate buffer, pH 7.4. The first 16 ml of the phosphate buffer were discarded, and the next 16 ml, which contained PAM, were collected. Eluates which contained vitamin B₆
compounds and PIC were collected batchwise in stoppered low actinic glass cylinders.

At the same time the vitamin B₆ compound or PIC in the eluate was being determined, a standard curve was prepared from the unchromatographed standard. Of the intermediate standard solution, 0.5, 1.0, and 2.0 ml were diluted to the same volume as the eluate fraction in which the desired compound was found. This intermediate standard solution had been prepared earlier, and was the one from which the treated or untreated standard, and standard added to blood had been taken. The diluent was the same as the elutriant for that compound. The vitamin B₆ compounds were determined by the cyanide method as described in Tables 2 and 3, and PIC was determined by the lactone method.
RESULTS AND DISCUSSION

Problems with the Determination of PAMPO and PAM

In the preliminary studies on the determination of PAMPO and PAM erratic results, including high blanks and wide differences in the fluorescence of samples of the same concentration were obtained. Experiments to resolve these problems were carried out. They were done on PAMPO because the incubation time of this compound plus cyanide was shorter than that for PAM. It was assumed that the results obtained with PAMPO would apply to PAM also.

The effect of heating on the transamination step was studied. To each of 16 tubes were added 2 ml of PAMPO standard containing 0.08 µg per ml, 2 ml of 1.0 M phosphate buffer at pH 7.4, and 0.1 ml of 0.005 M potassium alum. To each of eight tubes 0.1 ml of 0.05 M sodium glyoxylate was added; 0.1 ml of water was added to each of the eight remaining tubes (blanks). The tubes were mixed, capped, and heated in a 100°C water bath. Two tubes containing sodium glyoxylate and two tubes containing the blank were removed from the bath after 12, 15, 20, and 25 minutes of heating. To each tube 0.1 ml of 0.5 M KCN was added. After mixing, the tubes were heated at 50°C for 30 minutes. One milliliter of 1.5 N HCl was added to each tube to adjust the pH from 3.5 to 4.0. The results of this experiment were:
<table>
<thead>
<tr>
<th>Time of heating in minutes at 100°C</th>
<th>Average corrected fluorescence</th>
</tr>
</thead>
<tbody>
<tr>
<td>12</td>
<td>0.075</td>
</tr>
<tr>
<td>15</td>
<td>0.078</td>
</tr>
<tr>
<td>20</td>
<td>0.100</td>
</tr>
<tr>
<td>25</td>
<td>0.102</td>
</tr>
</tbody>
</table>

The fluorescence of the samples that had been heated for 20 and 25 minutes during the transamination step was higher than that of the samples heated for 15 minutes, the time recommended by Contractor and Shane. This probably indicates that under the conditions of this laboratory transamination was complete after 20 minutes of heating, but not after 15.

Incubating PAMPO, after conversion to PALPO, with 0.5 M KCN at 50°C beyond the 30 minutes recommended by Contractor and Shane, did not increase the fluorescence of the reaction product.

The cause of the high blanks was determined by checking the fluorescence of each reagent that was used in the reaction mixture. The 0.5 M KCN in 0.1 M phosphate buffer at pH 6.9, which turned yellow within one to two hours after preparation, showed considerable fluorescence. When the 0.5 M KCN was added to the reaction mixture after transamination within one-half hour of its preparation, lower blanks were obtained. As a result of the lower blanks, the corrected readings of the transamination product with cyanide were higher.
Studies on the Relative Fluorescence of PIC Lactone and the Vitamin B₆ Compounds Reacted with Cyanide

Since the reaction product of PAL with cyanide was PIC lactone (Ohishi and Fukui, 1968; Takanashi et al., 1968), and since PAM and PIN were converted to PAL before reaction with cyanide, the fluorescence of graded equimolar concentrations of PIC lactone and the vitamin B₆ compounds after reaction with cyanide were compared (Figure 6).

The higher fluorescence of PIC lactone may indicate that PAL was not completely converted to the lactone. Possibly some or all of the many reactions involved in the conversion of PAL to PIC lactone via PAL cyanohydrin (Figure 1) might not have gone to completion in these experiments. The fluorescence of the reaction products of PAM and PIN with cyanide was close to that of the product with PAL, suggesting that the conversion of these compounds to the aldehyde was complete.

In view of the fact that recent findings indicate that the final reaction product of PAL is PIC lactone, there is now a discrepancy in the pH suggested for optimum fluorescence of this compound. Huff and Perlzweig, as cited in Woodring et al. (1964), reported that the optimum fluorescence of PIC lactone was at pH 9.0 ± 0.3. Yamada (1960) found, however, that the optimum fluorescence of the reaction product of PAL with cyanide was at pH 10. In their studies Contractor and Shane (1968) and Ohishi and Fukui
Figure 6. Comparative fluorescence of PAL, PAM, and PIN after reaction with cyanide and PIC after conversion to its lactone. All reaction products were read at 350 m\(\mu\) (activation) and 434 m\(\mu\) (fluorescence).
(1968) adjusted the reaction product of PAL with cyanide to pH 10. In this study the pH of the reaction mixture containing PIC lactone ranged from 9.27 to 9.30. The pH of the reaction mixture of PAL ranged from 9.55 to 9.60; of PAM from 9.42 to 9.64; and of PIN from 9.58 to 9.70.

The reaction products of PALPO and PAMPO with cyanide (Figure 7) were less fluorescent than the reaction products of free forms (Figure 6). This difference in fluorescence between the reaction products of PALPO and PAL with cyanide was observed earlier by Bonavita (1960).

The fluorescence of the reaction products of PAM and PAMPO was higher than that of PAL and PALPO (Figures 6 and 7), respectively. This may have been due to the higher concentration of cyanide in the reaction mixture containing PAM and PAMPO. The excess cyanide added to the reaction mixtures after transamination could by the law of mass action cause more complete conversion of PAL to PIC lactone, and of PALPO to 4-pyridoxic acid-5-phosphate.

The differences in the pH of the final reaction mixtures of PAM and PAL were not significant, nor were those of PAMPO and PALPO. There may have been a difference, however, in the pH of the reaction mixtures of PAL or PAM with cyanide before the final adjustment in pH was made to obtain optimum fluorescence. This was possible because KCN is a salt of a strong base and, even though it was dissolved in 0.1 M phosphate buffer at pH 6.9, the
Figure 7. Comparative fluorescence of PALPO and PAMPO after reaction with cyanide. Both reaction products were read at 318 m\textmu (activation) and 417 m\textmu (fluorescence).
0.5 M KCN solution was much more alkaline than the 0.05 M one.

The higher fluorescence was not due to the excess KCN in the samples, since it was also present in the blanks. The 0.05 M sodium glyoxylate, which was not in the blank, contributed little fluorescence.

Recovery of Vitamin B₆ Compounds and PIC from Phosphocellulose and from Blood

The good recovery of the vitamin B₆ compounds and PIC from phosphocellulose in Series 1 (Table 4) indicates that these compounds were completely removed from the column and collected in the eluate. In this series all of the standards had not been treated before application to the column. In Series 2 (Table 5), the lower recovery of vitamin B₆ compounds and PIC from phosphocellulose might have been due to losses during evaporation or quantitative transfer, or to the compounds not being completely collected in the eluate.

The recovery of vitamin B₆ compounds and PIC from blood was erratic. The zero percent recovery of PAL from phosphocellulose in Series 2, and its poor recovery from blood in both series, may indicate that chemical changes took place in the compound during evaporation. Possibly, the aldehyde group attached to carbon 4 of PAL was oxidized to form PIC. Whether this occurred could be checked
Table 4. Recovery of Vitamin B₆ Compounds and 4-Pyridoxic Acid from Phospho-cellulose and Blood (Series 1).

<table>
<thead>
<tr>
<th>Compound</th>
<th>Standard¹</th>
<th>Chromat'd Untreated Standard²</th>
<th>Recovery from P. Cell</th>
<th>Standard added to Blood</th>
<th>Blood plus Standard</th>
<th>Recovery from Blood³</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>µg</td>
<td>µg</td>
<td>percent</td>
<td>µg</td>
<td>µg/8.3ml of blood</td>
<td>percent</td>
</tr>
<tr>
<td>PALPO</td>
<td>0.25</td>
<td>0.26</td>
<td>104</td>
<td>0.21</td>
<td>0.10</td>
<td>0.28</td>
</tr>
<tr>
<td>PAMPO</td>
<td>0.20</td>
<td>0.18</td>
<td>90</td>
<td>0.17</td>
<td>0.14</td>
<td>0.31</td>
</tr>
<tr>
<td>PAL</td>
<td>0.41</td>
<td>0.41</td>
<td>100</td>
<td>0.34</td>
<td>0.07</td>
<td>0.12</td>
</tr>
<tr>
<td>PAM</td>
<td>0.18</td>
<td>0.20</td>
<td>112</td>
<td>0.15</td>
<td>0.22</td>
<td>0.27</td>
</tr>
<tr>
<td>PIC</td>
<td>0.40</td>
<td>0.41</td>
<td>103</td>
<td>0.33</td>
<td>0.11</td>
<td>0.38</td>
</tr>
</tbody>
</table>

1. Amount applied to column. Standard received no treatment prior to application to column.

2. Amount in eluate from column.

3. \[
\left[\text{Blood plus standard} - \text{(blood)}\right] \div \text{(standard added to blood)} \times 100.
\]
Table 5. Recovery of Vitamin B₆ Compounds and 4-Pyridoxic Acid from Phospho-cellulose and Blood (Series 2).

<table>
<thead>
<tr>
<th>Compound</th>
<th>Standard¹</th>
<th>Chromat'd Standard²</th>
<th>Recovery from P. Cell</th>
<th>Blood</th>
<th>Blood plus Standard</th>
<th>Recovery from Blood³</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>µg</td>
<td>µg</td>
<td>percent</td>
<td>µg/8.3ml of blood</td>
<td>percent</td>
<td></td>
</tr>
<tr>
<td>PALPO</td>
<td>0.21</td>
<td>0.19</td>
<td>90</td>
<td>0.08</td>
<td>0.22</td>
<td>74</td>
</tr>
<tr>
<td>PAMPO</td>
<td>0.17</td>
<td>0.06</td>
<td>35</td>
<td>0.16</td>
<td>0.25</td>
<td>150</td>
</tr>
<tr>
<td>PAL</td>
<td>0.34</td>
<td>0.00</td>
<td>0</td>
<td>0.00</td>
<td>0.18</td>
<td>0</td>
</tr>
<tr>
<td>PAM</td>
<td>0.15</td>
<td>0.16</td>
<td>109</td>
<td>0.18</td>
<td>0.30</td>
<td>78</td>
</tr>
<tr>
<td>PIC</td>
<td>0.33</td>
<td>0.26</td>
<td>79</td>
<td>0.18</td>
<td>0.44</td>
<td>100</td>
</tr>
</tbody>
</table>

1. Amount applied to column. Standard was treated in same manner as blood prior to application to the column.

2. Amount in eluate from column.

3. \[
\frac{[(\text{Blood plus standard}) - (\text{blood})]}{(\text{chromat'd treated standard})} \times 100.
\]
by treating the PAL standard and applying it to the column, as had been done in this series, and determining PIC in the 0.01 N acetic acid eluate. Chemical changes in PAL during evaporation could also be determined by subjecting the treated standard to thin layer or paper chromatography, and by determining its absorption maxima and fluorescence characteristics.

Poor recovery of PAL from blood suggests that it might have been adsorbed on the protein precipitate. Results from the elution studies obtained two to four months earlier, however, showed a sharp peak for PAL in the acetate buffer fractions (Figures 4 and 5). This indicates that treatment of the protein-free extract of blood or of the extract to which PAL standard had been added before protein precipitation did not destroy most of the PAL during evaporation and that most of this vitamer was not adsorbed on the protein precipitate.

Because of the poor results with PAL, recovery of a mixture of vitamin B₆ compound and PIC added to blood was not determined.

In Series 2, the high recovery of PAMPO from blood (150 percent) can be explained by the low recovery (35 percent) of this compound from phosphocellulose. The standard curve for PAMPO was quite flat (Figure 2), and hence the probability for error was great. PAMPO
determined by the cyanide method was read near the lower limits of fluorescence. Duggan et al. (1957) reported that some vitamin \( \text{B}_6 \) compounds could be detected by spectrophotofluorometry in concentrations as low as 0.001 \( \mu \text{g} \) per ml. The lowest concentration of PAMPO in the reaction mixture (Figure 2) was 0.0015 \( \mu \text{g} \) per ml.

Recovery of PAM from blood was poor in the first series (33 percent), but was better in the second series (78 percent). The reasons for improvement in Series 2 might have been due to the shortened period of evaporation and to calculating recovery using treated standard.

Recovery experiments on PIN were not conducted since this compound was not detected in blood by Contractor and Shane. Elution patterns for PIN should be determined and an attempt to find PIN in blood should be made. Kelsey et al. (1968) detected PIN in blood of subjects who had received 50 mg of PIN orally for two consecutive days, whereas Contractor and Shane were unable to detect it in the blood of their subjects who had taken an oral dose of 100 mg.

Experiments were conducted in order to determine why the recovery of PALPO from blood was not complete.

The possibility of the hydrolysis of PALPO standard added to blood was investigated. A concentrated protein-free extract of blood was applied to one column and a
concentrated protein-free extract of blood to which 0.25 μg of PALPO had been added before protein precipitation was applied to another. PALPO was eluted with 0.01 N acetic acid and PAL was eluted with 0.1 M acetate buffer, pH 4.7. The concentrations of PAL in the acetate buffer eluates from the two columns were the same, showing that there had been no hydrolysis of the PALPO standard added to blood.

A second experiment was performed to determine whether PALPO was lost by adsorption on the protein precipitate. Two samples of protein-free extract of blood were prepared. Their preparation was the same except that in one sample 1 ml of PALPO standard (0.25 μg per ml) was added to the blood before the addition of trichloroacetic acid, and in the other, after the addition of trichloroacetic acid. The trichloroacetic acid was removed from both samples and the procedures followed as described under "Preparation of Samples." The samples were applied to two columns, respectively. Analysis of the 0.01 N acetic acid eluates from both columns indicated that 29 percent of the PALPO was lost by adsorption on the protein precipitate.

The effect of the long evaporation process, from two to three hours, was also studied. One milliliter of PALPO standard (0.25 μg per ml), 29 ml of water, and 30 ml of ten percent trichloroacetic acid were pipetted into a 90-ml centrifuge tube and warmed at 50°C for 20 minutes. The trichloroacetic acid was removed with water-saturated
ether and the 20 ml of aqueous solution were evaporated in 7-ml portions to approximately 1 ml. This 1 ml of concentrated sample was quantitatively transferred to a 10-ml volumetric flask with 0.01 N acetic acid. One milliliter of standard (0.25 µg of PALPO per ml) was pipetted into another 10-ml volumetric flask and made to volume with 0.01 N acetic acid. These samples were not chromatographed before determination with cyanide. Results showed that approximately 17 percent of PALPO was lost during evaporation and quantitative transfer. The effect of ether extraction was not determined because Hamfelt (1967) reported no loss of PALPO due to this treatment.

Concentration of Vitamin B₆ Compounds and PIC in Blood

The values for PALPO, PAMPO, PAL, PAM, and PIC in the blood used in Series 1 were 12.0, 16.3, 8.4, 26.5, and 13.3 ng per ml, respectively. The values obtained for the blood used in Series 2 were 9.6, 19.3, 0.0, 21.7, and 21.7 ng per ml, respectively.

Except for PAL, the values for vitamin B₆ compounds and PIC in blood obtained in this study were within the range reported by Contractor and Shane. Contractor (1969), however, stated recently that the true values for blood PAL are lower than those he and Shane had reported earlier (26.4 ng per ml). Jirsak (1963), using a microbiological
assay, found 24 to 30 ng of PAL per ml of blood. Kelsey et al. (1968) reported 6.6 ng of PAL per ml of blood in subjects who were consuming a diet containing 150 g of protein and 1.66 mg of vitamin B₆.

The values for blood PALPO which were obtained in this study were close to the one that had been obtained with tyrosine decarboxylase, less than 10 ng to 37 ng per ml (Chang, 1968).

With the exception of PAL, the procedure of Contractor and Shane appears to be sensitive and specific for the estimation of the vitamin B₆ compounds and PIC in blood. When the problems with PAL have been solved, all five compounds should be determined in blood at one time. In these studies each vitamin B₆ compound or PIC was determined individually.

Several interesting applications of this method could be made. One would be to compare the procedures by Contractor and Shane, and Woodring et al. (1964) for the determination of PIC in urine. The microprocedure of Woodring et al. utilized two resins and two columns to remove the fluorescent materials in urine. The procedure by Contractor and Shane would be simpler since it utilized only phosphocellulose to separate PIC from other compounds in urine. The procedure by Contractor and Shane could also be applied to leukocytes. Analysis of the latter
might give a more accurate estimation of vitamin B₆ nutriture because the chemical composition and enzyme systems in leukocytes are similar to tissue cells in general. It would also be interesting to study vitamin B₆ metabolism in women of different ages, and especially in those who are using steroid hormones to prevent ovulation.
BIBLIOGRAPHY


