CHROMATOGRAPHIC SEPARATION AND FLUOROMETRIC DETERMINATION OF PYRIDOXAL, PYRIDOXAMINE AND PYRIDOXINE IN FOOD SYSTEM

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YEN-PING CHIN
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ABSTRACT

PROCEDURE FOR CHROMATOGRAPHIC SEPARATION AND FLUOROMETRIC DETERMINATION OF PYRIDOXAL, PYRIDOXAMINE AND PYRIDOXINE IN FOOD SYSTEMS

By

YEN-PING CHIN

Various procedures have been developed for the separation and determination of vitamin B_6. Although no one assay procedure has been satisfactory for the determination of all three forms of vitamin B_6, the microbiological assay using Saccharomyces carlsbergensis has been widely used for measuring the vitamin B_6 content in foods.

The fluorometric procedure reported here is a combination and modification of numerous chemical methods for the determination of vitamin B_6. Separation of pyridoxal, pyridoxamine and pyridoxine was carried out by Dowex AG 50 ion exchange chromatography. Quantitation of these purified fractions was accomplished by fluorometry following their conversion to 4-pyridoxic acid lactone. Pyridoxal was converted to 4-pyridoxic acid lactone using potassium cyanide. Pyridoxine and pyridoxamine were first converted to pyridoxal by manganese dioxide and sodium glyoxalate, respectively, and then oxidized to 4-pyridoxic acid lactone. Fluorescence

was measured at 355 nm (excitation) and 436 nm (emission) at pH 9-10.

Selected food samples were chosen to evaluate the applicability of the fluorometric method for the determination of vitamin B_6 in foods. The microbiological method of Toepfer et al. (A.O.A.C., 1961) was used as a basis of comparison for the proposed fluorometric method. Recovery values for the three vitamin B_6 fractions varied for each food sample assayed. The percent recovery of pyridoxal and pyridoxamine for the selected foods using the fluorometric assay varied between 83 to 110 with the standard deviation ranging from 2 to 13. The percent recovery for the same food products analyzed by the microbiological method exhibited a range of 50 to 110 with the standard deviation ranging from 7 to 23. Using the fluorometric assay the recovery values for pyridoxine from the selected foods varied from 55 to 74 with standard deviation ranging from 3 to 7, while the recovery values for the microbiological method varied from 99 to 132 with standard deviation ranging from 8 to 31.

Comparisons of total vitamin B_6 values for various food products indicated that values obtained by the fluorometric method exhibited almost twice as much total vitamin B_6 as those obtained by the microbiological method.

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YEN-PING CHIN

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INTRODUCTION

Pyridoxal, pyridoxamine and pyridoxine, the three biologically active forms of vitamin B_6, have been shown to occur in the free form or combined with phosphate groups or with proteins. Although numerous procedures have been developed for the separation and determination of the various forms of vitamin B_6, no single assay procedure has been found satisfactory for the quantitative determination of vitamin B_6 in foods.

The Saccharomyces carlsbergensis microbiological assay developed by Atkin et al. (1943), is generally the recognized method for the determination of total vitamin B_6 in foods and biological materials, even though it does not respond equally to the three biologically active forms of vitamin B_6.

Chemical methods utilizing colorimetric, spectrophotometric and fluorometric techniques have been found satisfactory with relatively pure and concentrated solutions of vitamin B_6, Attempts to adapt these chemical methods to the measurement of vitamin B_6 in foods have not been successful, because of interferring substances within the food products.

The purpose of this study was to develop a chemical method for the quantitative determination of vitamin B_6 in

food products and to reduce the time required for analysis. The S. carlsbergensis microbiological method was used as a basis of comparison for the proposed chemical procedure.

LITERATURE REVIEW

Vitamin B_6: History

The existence of vitamin B_6 as a dietary essential was first recognized by Gyorgy in 1936. The isolation and identification of pyridoxine from several natural sources facilitated the elucidation of its structure and chemistry, which led directly to its synthesis (Lepkovsky, 1938; Keresztesy and Stevens, 1938; Gyorgy, 1938; Kuhn and Wendt, 1938; Ichiba and Michi, 1938; Harris and Folkers, 1939).

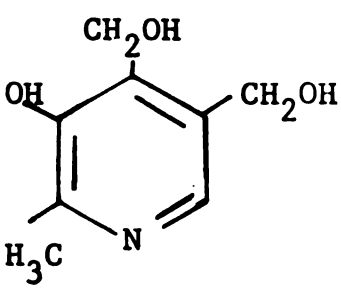
The terms "vitamin B_6" and "pyridoxine" were synonymous until Snell et al. (1942) found two related substances in food materials that surpassed pyridoxine in growth-promoting activity for certain species of lactic acid bacteria. Further work by Snell (1944a,b) and Harris et al. (1944a,b) demonstrated that the related substances were pyridoxamine and pyridoxal.

Concurrent with these studies, research by other workers indicated that vitamin B_6 functioned as a coenzyme in the amino acid metabolism of lactic acid bacteria (Gale, 1944; Bellamy and Gunsalus, 1944). This led to the discovery of the metabolically active phosphorylated forms of the vitamin; pyridoxal phosphate and pyridoxamine phosphate (Gunsalus and Bellamy, 1944; Heyl et al., 1951; Baddiley and

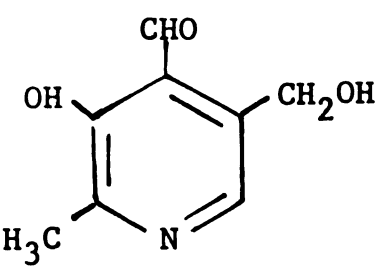
Mathias, 1952; Rabinowitz and Snell, 1947).

Vitamin B_6: Chemical and Physical Properties

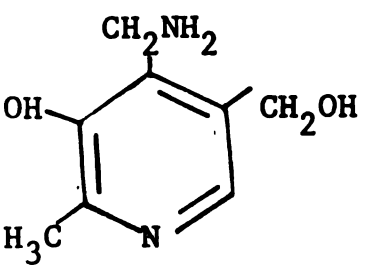
The various forms of vitamin B_6 are widely distributed in a large number of foods of animal and plant origin. The major portion, existing in the free form, is associated primarily with protein and starch. The naturally occurring forms of vitamin B_6 differ only by the substitute groups at the C-4- position.



Pyridoxin



Pyridoxal



Pyridoxamine

Pyridoxine (PIN), pyridoxal (PAL), and pyridoxamine (PAM), which are colorless and odorless crystals, exist as free bases or as the commonly available hydrochlorides. PAL, PAM, PIN are resistant to heat and acid but are decomposed by alkali, ultraviolet light, and oxidizing agents, such as nitric acid, potassium permanganate, and hydrogen peroxide (Cunningham and Snell, 1945).

The three biologically active forms of vitamin B_6 exhibit different light absorption maxima, as well as, different fluorescence characteristics. These latter two properties were the basis for several chemical methods of

analysis, which will be discussed later in detail.

Vitamin B_6: Methods of Determination

Pyridoxal, pyridoxamine and pyridoxine, which exhibit equal biological availability for man and higher animals, have been shown to occur in the free form or combined with phosphate groups and/or proteins. Thus, the estimation of total vitamin B_6 in foods requires the quantitative determination of these three components.

No single assay procedure has been reported satisfactory for the determination of all three forms. Thus, procedures for the separation and determination of the active forms of vitamin B_6 were essential to the development of a quantitative vitamin B_6 assay.

Methods available for the determination of vitamin B_6 were divided into four categories; microbiological, physical, chemical and animal. Microbiological procedures were the preferred method for determining the vitamin B_6 content of foods and biological materials. However, each species of microorganism responded differently to the various forms of vitamin B_6. This made it difficult to evaluate and compare assay results using different microorganisms. Physical and chemical methods were satisfactory with only relatively pure and concentrated vitamin B_6 preparations. The rat growth assay, on the other hand, gave erroneously high values if the samples analyzed contained growth promoting factors in addition to vitamin B_6.

Microbiological Methods

Microbiological methods have been used more frequently than any other method in assaying foods and biological materials for vitamin B_6. These methods employed microorganisms such as lactic acid bacteria (Rabinowitz and Snell, 1947), yeast (Atkin et al., 1943) and a mold mutant (Stokes, 1943).

Since the three forms of vitamin B_6 could elicit different activities for different microorganisms, the following three factors should be evaluated before choosing a method of assay: the form of the vitamin to be determined, the microorganism which would give the maximum response, and the material being assayed.

To date, no unqualified recommendations have been made for any one microbiological assay method for vitamin B_6 in complex biological substances. This is primarily due to the inherent nature of the assay organisms and the inadequacies of extraction procedures.

The Saccharomyces carlsbergensis assay, developed by Atkin et al. (1943) and modified by Woodring and Storvick (1960), had been generally accepted for the estimation of total vitamin B_6. The assay, based on the growth of the microorganism, was measured turbidimetrically. It was chosen for its simplicity, convenience, speed, and the ability of S. carlsbergensis to respond similarly to all three forms of vitamin B_6. However, Parrish et al. (1955)

demonstrated that on occasion certain strains of the organism gave lower response to pyridoxamine than to pyridoxine or pyridoxal.

Rabinowitz and Snell (1948) developed a differential technique for assaying the three components of vitamin B_6, as well as total vitamin B_6. Their procedure was based on the observation that Streptococcus faecalis R. responded to pyridoxal and pyridoxamine; Lactobacillus casei responded only to pyridoxal; and S. carlsbergensis responded to all three forms of the vitamin. Individual forms of vitamin B_6 were determined by subtraction. However, the pyridoxine value obtained from this differential method was probably higher than its actual content in the product, due to the inability of any organism to measure pyridoxine and the lack of sensitivity of S. carlsbergensis toward pyridoxamine.

Gregory (1959) reported that the differential assay technique of Rabinowitz and Snell (1948) was improved by the use of S. faecium Ø 51. This organism did not respond to DL-alanine or D-alanine, which was shown to replace pyridoxal or pyridoxamine as a growth factor for S. faecalis R. The primary function of vitamin B_6 in S. faecalis R. appeared to be that of a coracemase in the conversion of L- to D-alanine. Therefore, if D-alanine was present in the medium, the requirement for the vitamin was eliminated. DL-alanine, however, was not shown to replace pyridoxal or pyridoxamine as a growth factor for S. faecium Ø 51.

Diding (1955) proposed a method utilizing a biochemical mutant of Escherichia coli, strain 154-59 L, for the determination of total vitamin B_6. This procedure offered the advantage of simplicity with regard to medium preparation, but was limited to assaying pure compounds, such as multivitamin preparations.

Baker et al. (1962, 1963) proposed a method using Tetrachymena pyriformis, a protozoan, for the determination of vitamin B_6 in tissue, urine, blood, serum, and cerebrospinal fluid. This procedure was slower to perform than the S. carlsbergensis method, requiring an incubation period of 5 days. The T. pyriformis method was also limited by the fact that pyridoxine appeared to have considerably less activity than pyridoxamine on the assay organism. Thus, significant errors would have been encountered in using this organism for the assay of vitamin B_6 content of plant products or other materials that contained an appreciable amount of pyridoxine.

A plate method using the mold mutant, Neurospora sitophila, was described by Haenel and Muller-Beuthow (1956). Although not sensitive, this method had the advantage of requiring only a six hour incubation period.

An additional method utilizing the mold mutant, N. sitophila, received limited attention for the determination of total vitamin B_6 in biological materials (Stokes et al., 1943). Although the organism appeared to respond equally

well to all forms of the vitamin, the assay was complicated, time consuming, and required an incubation period of 5 days.

Extraction of Vitamin B_6

An adequate extraction method for the liberation of vitamin B_6 from its multiple bound forms has not been reported. To some degree this problem has been shown to account for many of the discrepancies in vitamin B_6 values reported in foods and biological materials, especially blood and tissues.

Birch and Gyorgy (1936) first noted the bound state of vitamin B_6. Siegel et al. (1943), using acid hydrolysis, demonstrated the necessity for the liberation of these bound forms for accurate chemical or microbiological assays. Since 1943 many diverse types of hydrolysis procedures were developed. Generally three types of hydrolysis; acid hydrolysis, enzymatic hydrolysis, and the combination of acid and enzymatic hydrolysis, have been employed to release bound vitamin B_6.

Acid Hydrolysis

Atkin et al. (1943) found that efficient acid extraction depended not only upon the strength of the acid but also upon the volume of acid used. They recommended using a sample which contained the equivalent of 2-4 µg of vitamin B_6 and hydrolyzing in 180 ml of 0.055 N H_2SO_4 for 1 hour at 20 psi steam. Later, many investigators modified Atkin's method by changing both the concentration of acid and

the length of hydrolysis. Rabinowitz and Snell (1948) improved the extraction method of Atkin et al. (1943) by increasing the hydrolysis time from 1 hour to 5 hours, the pressure from 15 psi to 20 psi and substituting H_2SO_4 for HCl. Fujita et al. (1955a,b) reported that pyridoxine and pyridoxamine were best extracted by preheating the sample homogenate, pH 4.5, at 80°C for 15 minutes, adjusting the supernatant to 0.6 N H_2SO_4 with 3 N H_2SO_4 and autoclaving at 130° C for 1 hour. However, better results were obtained for pyridoxal and 4-pyridoxic acid when the homogenate was heated directly in 0.1 N H_2SO_4 at 130° for 1 hour.

In 1959, Gregory demonstrated that samples heated in 0.55 N HCl under steam for 30 minutes gave results which were similar to those obtained by autoclaving and adopted this process for extraction.

Benson et al. (1964) studied the effect of hydrolysis time on vitamin B_6 recovery using 0.055 N HCl and 15 psi. Hydrolysis time was varied from 30 minutes to 72 hours. The results showed that both L. casei and S. faecium increased in growth rate after 30 minutes of hydrolysis and reached a peak between 10 and 24 hours. After 24 hours, growth decreased until leveling off at 72 hours at approximately the same value obtained between 2 and 5 hours. Tests with S. carlsbergensis showed significantly different results. The highest value was obtained after 2 hours of hydrolysis. A slight decrease was observed from 2 to 5 hours. Followed

by a sharp decrease to an almost unreadable minimum after 10 hours.

Another vitamin B_6 extraction method required the removal of protein from the sample with trichloroacetic acid or perchloric acids, however, this resulted in the removal of the phosphorylated and protein bound vitamin B_6 complex from the sample extract. Storvick and Peters (1964) studied this phenomenon and reported that blood subjected to acid hydrolysis and assayed with S. carlsbergensis, gave vitamin B_6 values 1 to 1½ times higher than the protein free filtrate.

Enzyme Hydrolysis

Most of the vitamin B_6 in food and biological materials has been found to be tightly bound to protein (Birch and Gyorgy, 1936). Therefore, it was advisable to hydrolyze protein rich materials, such as, meat and blood, before acid extraction. This prevented occlusion of the vitamin in the protein precipitate resulting from heat denaturation. Preliminary protein breakdown was usually accomplished with papain, pepsin, Taka-diastase, and alkaline or acid phosphatase.

The utilization of enzymes for the extraction of vitamin B_6 was for the most part unsatisfactory, since many of the enzyme preparations contained a considerable amount of vitamin B_6 (Rabinowitz and Snell, 1947; Hopkins and Pennington, 1947).

Williams et al. (1942) reported low vitamin B_6 values for milk using enzymatic hydrolysis with microbiological assay methods. Siegel et al. (1943) postulated that the value obtained by Williams et al. (1942) represented only free vitamin B_6 and not bound vitamin B_6. Swaminathan (1940) reported higher vitamin B_6 values for milk using a combination of pepsin digestion and acid hydrolysis before chemical determination.

Gregory and Mabbitt (1961) reported pyridoxamine phosphate was more resistant to hydrolysis than pyridoxal phosphate. In both milk samples and standard solution alkaline phosphatase was used after acid hydrolysis to achieve complete release of pyridoxamine from pyridoxamine phosphate.

Storvick and Peters (1964) reported that samples subjected to prolonged heat treatment exhibited a decrease in microbial growth. It was theorized that this decrease was a result of a rebinding or condensation of free vitamin B_6 to protein or phosphate groups, which rendered the vitamin biologically unavailable. This theory was supported by the results of Storvick and Peters (1964) who reported no destruction of standard solutions of pyridoxal, pyridoxamine and pyridoxine after 72 hours of acid hydrolysis. However, when milk was subjected to the same hydrolytic conditions, a decrease in the vitamin B_6 content of the milk resulted after 5 hours of autoclaving. In this same report,

a recovery study was performed in which standard solutions of vitamin B_6 were added to both milk and blood prior to hydrolysis. They found that in the presence of protein, prolonged heat treatment resulted in losses of vitamin B_6. Results from this study supported the findings of Fujita et al. (1955a,b), who reported that maximum liberation of vitamin B_6 depended on acid concentration, pressure, and the length of hydrolysis. Fujita et al. (1955a,b) further suggested that strong acid, high pressure and short extraction time would provide the best conditions for the liberation of vitamin B_6 and minimized rebinding.

To date, no method is available for determining if vitamin B_6 is totally liberated following acid hydrolysis. Therefore, the hydrolysis procedure which would result in the highest vitamin B_6 recoveries was used as a guideline for determining the completeness of hydrolysis. Storvick et al. (1964) further suggested that acid hydrolyzates should be subjected to treatment with acid or alkaline phosphatase to determine if vitamin B_6 was hydrolyzed from its phosphorylated forms.

Column Chromatography

Storvick and Peters (1964) reported that biological materials could contain substances which were inhibitory to vitamin B_6 assay organisms. They found that a 20-hour hydrolyzate of blood, which had no vitamin B_6 activity for S. carlsbergensis, gave a value of approximately 25 ng per

milliliter of blood after the hydrolyzate had been passed over Dowex 50 (K^+) column. This substantial increase indicated that a substance inhibitory to the yeast had been removed from the hydrolyzate by ion-exchange chromatography.

Peterson and Sober (1954) reported the first complete column chromatographic separation of pyridoxal, pyridoxamine, pyridoxine, the phosphate forms, and 4-pyridoxic acid. A weak cation exchange resin, Amberlite XE-64 (H^+), with water as the eluant was used for the separation of pyridoxal-5'-phosphate, pyridoxic acid and pyridoxamine-5'-phosphate. Acetic acid (5%) was used to elute pyridoxal, pyridoxine, and pyridoxamine. Fractions were collected and examined by spectrophotometry and paper chromatography. This procedure required high concentrations of pure vitamin B_6 and did not prove satisfactory for separating small amounts of the vitamin B_6 fractions from biological materials containing significant levels of interfering substances.

Fujita et al. (1955a,b) proposed a procedure for the chromatographic separation of the various components of vitamin B_6 present in biological materials. The various forms of B_6 were recovered as follows: 4-pyridoxic acid, Amberlite IRA-410 (Ac^-); pyridoxine, Permutit; pyridoxal, IRC-50 (H^+) or IR-112 (H^+). Pyridoxamine was deaminated to pyridoxine, which was then adsorbed on a Permutit column. Although their method was complicated, it represented the first successful attempt to isolate pure vitamin B_6 fractions

from biological materials and was fundamental to the development of separation schemes in recent years.

A less complicated method using Dowex AG 50W-X8 and differential elution was proposed by Toepfer and Lehman (1961) for the separation of the three forms of vitamin B_6. Pyridoxal, pyridoxine, and pyridoxamine were eluted with 100 ml aliquots of 0.04 potassium acetate, pH 6.0; 0.1 M potassium acetate, pH 7.0; and 1.0 M KCl-0.1 M K_2HPO_4, pH 8.0, respectively. Eluates were assayed microbiologically using S. carlsbergensis, however, it was not possible to determine whether complete separation had been attained, because S. carlsbergensis could not differentiate the three forms of the vitamin. Later, studies by Storvick et al. (1964) indicated that distinct separation of the components of vitamin B_6 was not achieved with the procedure of Toepfer and Lehmann (1961). Based on analyses of the eluate fractions with chemical and differential microbiological assays, Storvick et al. (1964) found that the pyridoxal fraction also contained pyridoxine, and the eluant for pyridoxine contained pyridoxamine.

Bain et al. (1960, 1962) reported a unique method for the separation of the six biologically active forms of vitamin B_6 using a single column, containing two resins separated from each other by a disk of filter paper. The upper layer contained Dowex 50 (K^+) and the lower layer Dowex 1-formate. When a perchloric acid tissue extract, adjusted to pH 4.25, was applied to the column, the three

unphosphorylated forms passed through the Dowex 50 (K^+) layer and were bound by the Dowex 1-formate. Using a continuous gradient elution method, pyridoxamine phosphate, pyridoxine phosphate, and pyridoxal phosphate, were eluted with a binary system of 0.05 M potassium citrate containing a 0.15 M KCl, pH 4.25 and 0.05 M potassium formate, pH 4.25. Elution of pyridoxal and pyridoxine was accomplished with a solution of 0.05 M potassium citrate and 0.5 M potassium chloride, pH 4.25. Finally, pyridoxamine was eluted with a 0.05 M potassium citrate and 0.5 M potassium chloride solution, pH 6.8.

Bain's method held considerable promise from the standpoint of separating all forms of vitamin B_6. However, S. carlsbergensis assay procedure, which was the best microbiological method available, was inadequate to identify each component eluted.

Chemical Methods

Microbiological methods have not proven completely acceptable for the measurement of total vitamin B_6 in foods and biological materials. This led to attempts to develop a chemical method for the determination of vitamin B_6.

Colorimetric

Following the synthesis of vitamin B_6 in 1939, a number of colorimetric methods were devised for the determination of pyridoxine. Most of these methods lacked sensitivity, specificity, stability of the color complexes formed,

and were only applicable to relatively pure solutions. Recognition of other forms of vitamin B_6 also invalidated many of these early colorimetric methods.

Spectrophotometric

Direct spectrophotometric determination of pyridoxal, pyridoxamine and pyridoxine were based on their ultraviolet absorption spectra, which showed marked and characteristic perturbations with changes in hydrogen ion concentrations. Melnick et al. (1945) studied the absorption spectra of pyridoxine, pyridoxal and pyridoxamine in aqueous solutions and reported that each form showed two absorption maxima (254 and 325 nm for pyridoxine, 251 and 316 nm for pyridoxal and 250 and 325 nm for pyridoxamine at pH 7.5). Although pyridoxal exhibited a maximum absorption at 316 nm, its absorption at 325 nm was approximately the same as that of pyridoxine and pyridoxamine.

The ultraviolet absorption spectra of the phosphates of vitamin B_6 were reported by Heyl et al. (1951) and Peterson and Sober (1954). The characteristic absorption spectrum of pyridoxal phosphate differentiated it from the other phosphorylated and free forms of vitamin B_6. This permitted the quantitative spectrophotometric estimation of pyridoxal phosphate in pure solutions or vitamin B_6 mixtures at pH 7.0 by its optical density at 388 nm.

An earlier method of Snell (1945) determined pyridoxamine phosphate spectrophotometrically after its conversion to pyridoxal phosphate with α-ketoglutarate.

The spectrophotometric method of Snell (1945) was only suitable for the analysis of a nearly pure solution of vitamin B_6. It required that test solutions be free of light-absorbing substances unless adequate blanks were used to correct for their presence. Although no absorption maximum was common to all three components of vitamin B_6, the absorption spectra were so close that individual components could not be differentiated. These limitations made the spectrophotometric method unapplicable to the analysis of foods or biological materials.

Fluorometry

Fluorometry was the most successful analytical tool used for the chemical determination of vitamin B_6. Its sensitivity has a distinct advantage over other chemical methods, but until recently its application had been restricted to relatively few compounds.

Fluorometric assay methods were classified according to whether the fluorescence of vitamin B_6 was measured directly or as a chemical derivative. Due to the high sensitivity of spectrophotofluorometry and the problem of interference, applications of the first method were restricted to relatively pure solutions. Thus, most of the fluorometric analyses for foods and biological materials were developed for fluorometric derivatives of vitamin B_6.

Lactone Method

Singal and Sydenstricker (1941) discovered that 4-pyridoxic acid, a fluorescence compound found in urine,

formed a lactone in the presence of acid which at pH 9 was 25 times more fluorescent than 4-pyridoxic acid. This B_6 metabolite was the first of the vitamin B_6 analogs to be determined fluorometrically.

Fujita et al. (1955) adapted the 4-pyridoxic acid lactone procedure to the fluorometric estimation of pyridoxal, pyridoxamine, and pyridoxine. The procedure included a method for extraction, hydrolysis, chromatographic separation and measurement of the individual forms of vitamin B_6. Quantitative measurement was based on the oxidative conversion of the isotels to 4-pyridoxic acid and then to the highly fluorescent lactone form. Pyridoxamine, which could not be oxidized directly, was converted to pyridoxine with nitrous acid, before oxidation to pyridoxic acid. Both Fujita et al. (1955a) and MacArthur and Lehman (1959) reported that the yield of 4-pyridoxic acid lactone derived from pyridoxamine was comparatively low.

MacArthur and Lehman (1959) simplified the lactonization procedure of Fujita et al. (1955a,b) after separation of the three forms of vitamin B_6 by differential elution from Dowex 50 (Na^+). The separation technique yielded a pyridoxamine fraction which was free of pyridoxine, however, this method was not satisfactory for biological extracts.

Later Storvick et al. (1964) found that if one form of vitamin B_6 greatly predominated in the extract, the separation of the various components of the vitamin prior to

lactonization was not necessary. For example, pyridoxine could be determined in the presence of pyridoxal with minimal interference unless pyridoxal greatly exceeded pyridoxine. This observation eliminated the need to separate the three forms of vitamin B_6 provided the vitamin B_6 content was predominated by one form of the vitamin and the sample preparation was relatively pure.

The lactone method was highly specific and much more sensitive than spectrophotometric or colorimetric methods. However, the existence of interference from other highly fluorescent materials of biological origin or from foodstuffs has limited the usefulness of this assay.

Cyanohydrin Method

Bonavita and Scardi (1959a,b) reported that the product of the reaction between cyanide and pyridoxal-5-phosphate exhibited fluorescent properties distinct from pyridoxal-5-phosphate. A year later Bonavita (1960) discovered that a similar reaction occurred between cyanide and pyridoxal. Subsequently, a method based on this reaction was developed specifically for the determination of pyridoxal and its phosphorylated form.

According to the study of Bonavita (1960), pyridoxal-5'-phosphate cyanohydrin exhibited maximum fluorescence at pH 3.8 with excitation at 315 nm and emission at 420 nm, while pyridoxal cyanohydrin exhibited fluorescence with excitation at 358 nm and emission at 430 nm. Studies concerning the spectral characteristics of the cyanohydrin

drivatives by Bonavita (1960) and Yamada et al. (1968) confirmed that pyridoxal cyanohydrin and pyridoxal phosphate cyanohydrin exhibited totally different responses with a change in pH. They concluded that more reproducible results were obtained if pyridoxal cyanohydrin was measured at pH 10 and pyridoxal phosphate cyanohydrin was measured at pH 3.5.

Pyridoxal or pyridoxal phosphate were also able to be measured in the presence of other components of vitamin B_6 because pyridoxamine and pyridoxine and their phosphates lacked the double bound in the C-4 position. This prevented them from reacting with cyanide to form the lactone. Moreover, pyridoxal phosphate was able to be measured in the presence of pyridoxal unless the latter greatly exceeded the former in concentration.

Toepfer and his associates (1960) adapted the method of Bonavita (1960) to the determination of pyridoxal and pyridoxamine. Determination of pyridoxamine required the quantitative conversion of pyridoxamine to pyridoxal via a nonenzymatic transamination reaction with glyoxylic acid (GOA) (Metzler et al., 1954). After conversion to pyridoxal, it was treated according to the procedure of Bonavita (1960).

Polansky et al. (1964) showed that pyridoxine could also be determined fluorometrically by the cyanohydrin method. Pyridoxine was quantitatively oxidized to pyridoxal over a range of 0.001 to 0.5 µg/ml with manganese dioxide and converted to the cyanohydrin form via the procedure of

Bonavita (1960). Conversion of pyridoxine to pyridoxal was greater than 90%.

Toepfer et al. (1961, 1964) reported reproducible results with standard solutions of pyridoxal, pyridoxamine and pyridoxine. The fluorescence intensity of the cyanohydrin derivative was of the same order of magnitude as the lactone of 4-pyridoxic acid. Later, Ohishi and Fukui (1968) and Takanashi (1968) reported that the spectral changes of pyridoxal and pyridoxal phosphate treated with potassium cyanide were not due to the formation of cyanohydrin derivatives, but the formation of 4-pyridoxic acid lactone and 4-pyridoxic acid phosphate lactone, respectively. These researchers proved that the cyanohydrin form was merely an intermediate in the reaction between pyridoxal and potassium cyanide and in the presence of oxygen was converted to 4-pyridoxic acid lactone. It was the final product, 4-pyridoxic acid lactone, which provided the desired fluoroscent intensity of vitamin B_6.

The advantages of the fluorometric determination of vitamin B_6 were its relative simplicity, sensitivity and specificity for the aldehyde forms of vitamin B_6. The use of this procedure for the determination of vitamin B_6 in foods and biological materials required the chromatographic separation of the biologically active components of vitamin B_6 from the extracts of food and biological materials because of the presence of naturally fluorescing substances

which interfered with the determination.

Despite the numerous advantages of the cyanide method, very few applications were reported for food and biological materials. Yamada (1970) modified and adapted the cyanide method to the assay of pyridoxal and pyridoxal phosphate in blood and tissue homogenates. Their method was quantitative and reproducible.

Takanshi et al. (1970) used the cyanide method to determine pyridoxal and pyridoxal phosphate content in biological materials. Homogenates of serum, plasma, and tissues were deproteinized with TCA prior to separation and concentration of the vitamin B_6 components from sample extracts with Dowex IX 8 and Amberlite CG 120 columns. The recoveries varied from 93-100% for pyridoxal and 68-90% for pyridoxal phosphate.

In 1971 Masukawa and his coworkers modified Takanashi's procedure and included pyridoxamine and pyridoxamine phosphate in their study. Again individual components were separated by ion exchange chromatography. Pyridoxamine, pyridoxal and their phosphate forms were determined as 4-pyridoxic acid lactone and pyridoxic acid-5-phosphate, respectively. Recoveries for pyridoxal, pyridoxamine and pyridoxamine phosphate in milk and plasma were 95-104% while pyridoxal phosphate was consistently 90%.

BIOLOGICAL ASSAYS

Procedures were developed to measure the vitamin B_6 that was biologically available to animals. One of the earliest biological tests was the cure of rat acrodynia. This was replaced by the rat growth test, in which the vitamin content was determined by growth resulting from the addition of graded doses of vitamin B_6 to a diet deficient in that vitamin. Chicken assays were similar but were used less frequently than those employing rats.

The method of Sarma et al. (1947), a modification of the method of Conger and Elvehjem (1941), is one of the principle bioassay methods used today. Sarma and his associates developed a basal ration which permitted minimum growth without vitamin B_6 and maximum growth with optimum levels of B_6. Food or biological materials to be assayed were mixed in the diet, usually at two levels, and weight gained was recorded for four weeks. Weight gain values were obtained and compared to a standard curve. Sarma et al. (1947) established that pyridoxal and pyridoxamine incorporated in the diet gave slightly lower values than when fed as a supplement with a medicine dropper or injected intraperitoneally. This presumably accounted for the somewhat lower values which were obtained by bioassay methods when compared to the S. carlsbergensis method.

Henderson et al. (1941) and Sarma et al. (1947) made use of the rat bioassay method in their estimation of

the vitamin B_6 content of various food products. Tomarelli et al. (1955) also employed the rat bioassay to determine the biological availability of vitamin B_6 of heated milk products. However, they did not use the bioassay as a means of estimating total vitamin B_6 in milk. Tomarelli et al. (1955) found that the overall effect of heat sterilization on the vitamin B_6 of milk resulted in a decrease in biological activity from one third to one sixth the original content. Assay the same product using S. carlsbergensis showed only fifty percent reduction in biologically availability. This clearly indicated that the bioassay methods generally have lower values than the microbiological assay.

Nutritionally, the rat bioassay method was advantageous, since its primary concern was to determine the amount of vitamin B_6 which was available for use by the animal, rather than the total amount present. However, one must also consider that not all biological systems were identical and results obtained for one species of animal could not be extrapolated to other animals. It was necessary to know how much of the total vitamin B_6 consumed was available to the rat, the chick and the human being. The method also had the advantage that no hydrolytic or extractive procedures were required, which simplified the procedure and eliminated a common source of error inherent in the chemical and microbiological procedures. According to a study by Toepfer et al. (1963) on the vitamin B_6 values of some selected food samples,

the bioassay showed 95% confidence limit which were $\pm$ 25% of the mean value.

METHOD

Reagents for Chemical and Microbiological Determination

1. Potassium acetate buffers- (a) 0.01 M, pH 4.5- Dissolve 0.981 g KOAc in H_2O and adjust pH with concentrated HOAc. Dilute to 1 liter. (b) 0.02 M, pH 5.5- Dissolve 1.96 g KOAc in H_2O and adjust pH with concentrate HOAc. Dilute to 1 liter. (c) 0.04 M, pH 6.0- Dissolve 3.92 g KOAc in H_2O and adjust pH with concentrated HOAc. Dilute to 1 liter. (d) 0.1 M, pH 7.0- Dissolve 0.815 g KOAc in H_2O and adjust pH with concentrated HOAc or 6 N KOH. Dilute to 1 liter.

2. Potassium chloride-phosphate buffer- pH 8.0. Dissolve 74.6 g KCl and 17.4 g K_2HPO_4 in 800 ml H_2O and adjust pH with concentrated HOAc. Dilute to 1 liter.

3. Ion exchange resin- Dowex AG 50WX-8 100-200 mesh.

4. Phosphate buffer, 0.4 M, pH 7.5- Dissolve 69.67 g of K_2HPO_4 in 800 ml H_2O. Adjust pH with concentrated H_3PO_4 and dilute to 1 liter.

5. Pyridoxine (PIN), pyridoxal (PAL), and pyridoxamine (PAM) standard solutions. Prepare separate solutions for each as follows: (a) Stock solution-10.0 µg/ml. Dissolve 12.16 mg pyridoxine HCl, 12.18 mg pyridoxal HCl, and 14.34 mg pyridoxamine HCl, respectively, in 1 N HCl and dilute to 1 liter with 1 N HCl. Store in brown glass bottles

at 4°C.

6. Potassium cyanide 1.0 M. Dissolve 6.512 g of KCN in H_2O and dilute to 100 ml.

7. Sodium carbonate, 0.4 M. Dissolve 4.24 g of Na_2CO_3 in H_2O and dilute to 200 ml.

8. Glyoxylic Acid[a], 0.5M. Dissolve 480 mg of GOA in H_2O and dilute to 10 ml.

9. Manganese dioxide. Prepared as described by Mancera et al. (1953).

Apparatus for Fluorometric Determination

Technicon Fluorometer Model No. II[b] was used for continuous flow analysis. Turner Spectrophotofluorometer[c] was used for manual analysis.

Samples used for Analysis

One food sample was chosen from each of four major food products, namely: dairy (infant formula), meat (ham), cereal (wheat flakes), and legume (lima bean).

Commercially processed samples were chosen with the same lot numbers for proper replication on different days of analysis. Two identical samples and recoveries from each category of food were analyzed during each run and were

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[a]Sigma Chem. Co., Detroit, Michigan.

[b]Technicon Instruments Corp., Tarrytown, N.Y.

[c]G. K. Turner Associates, Palo Alto, California.

repeated at least three different times. Both chemical and microbiological assays were performed on the same day.

Sample Preparation for Fluorometric Determination of Vitamin B_6

Sample extracts and chromatographic separation of the three components of vitamin B_6 were carried out as described in A.O.A.C. (1961, 1970). Sufficient sample was accurately weighed into a 300 ml Erlenmeyer flask, so that the concentration of total vitamin B_6 was 4-10 µg. One hundred and eighty milliliters of 0.44 N HCl was added to samples of plant origin, and 180 ml of 0.055 N HCl was added to samples of animal origin. Plant products were autoclaved for 2 hours at 121°C. and animal products for 5 hours at 121°C. The samples were cooled to room temperature, adjusted to pH 4.5 with 6 N KOH, quantitatively transferred to 250 ml volumetric flasks and diluted to mark with distilled water. The samples were filtered through a Whatman No. 42 filter paper and a 40-200 ml aliquot of filtrate was placed on the ion exchange column, which had been previously equilibrated with 0.01 M KOAc buffer (pH 4.5). Pyridoxal (PAL, pyrodixine (PIN) and pyridoxamine (PAM) were eluted from the column with 100 ml portions of 0.04 M KOAc (pH 6.0); 0.1 M KOAc (pH 7.0); and 1.0 M KCl-0.1 M K_2HPO_4 (pH 8.0), respectively.

Individual components eluted from the column were treated chemically as described below.

Pyridoxal

The eluted PAL fraction was diluted with 0.4 M phosphate buffer (pH 7.5) to obtain a final concentration of 0.01-0.05 μg/ml. Four milliliters of the diluted sample were placed in each of two test tubes A and B (B was for blank). One-tenth of a milliliter of 1.0 M KCN and 0.1 ml of water were added to test tubes A and B, and the tubes were placed in a 50°C water bath for 2 hours. After cooling, 2 ml of 0.4 M Na_2CO_3 were added to each test tube and the fluorescence was measured at 355 nm excitation and 436 nm emission.

Pyridoxamine

The eluate obtained from the ion-exchange column was diluted with 0.4 M phosphate buffer (pH 7.5) to a final concentration of 0.01-0.05 μg/ml. Four milliliters of the sample were pipetted into each of the two test tubes, A and B (B was for blank). One-tenth milliliter of 0.5 M GOA and 0.1 ml of water were added to test tubes A and B. The tubes were heated in a 100°C water bath for 15 minutes. After cooling, 0.1 ml of 1.0 M KCN was added to each test tube which were then placed in a 50°C water bath for 2 hours. After cooling, 1.9 ml of 0.4 M Na_2CO_3 was added to each tube. Fluorescence was measured in the same manner as described for PAL.

Pyridoxine

Sample eluate (10-40 ml) was pipetted into a 125 ml Erlenmeyer flask and adjusted to pH 5-6 with 0.1 N HCl.

One-tenth gram of MnO_2 was added to each sample and stirred continuously or shaken on a rotary shaker for 30 minutes at room temperature. Sample was centrifuged and the supernatants were filtered through Whatman No. 42 paper into a 100 ml volumetric flask. Manganese dioxide residue was washed with distilled water, centrifuged and the washing decanted into the original supernatant. The residue was discarded. The pH of the supernatant was adjusted to 7.2-7.5 with 0.4 M K_2HPO_4 and diluted to mark with distilled water. Four milliliters of the diluted solution were pipetted into a test tube and 0.1 ml of 1.0 M KCN was added to each sample. Samples were heated in a 50°C water bath for 2 hours. Fluorescence was determined in the same manner as described for PAL. Blank samples were treated as described above except that MnO_2 was omitted.

Standards

Ten milliliters each of 10 µg/ml standard PAL, PAM, and PIN stock solutions were mixed together and adjusted to pH 4.5 with 6 N KOH and concentrated HOAc. The standard mixture was applied to the ion-exchange column and the three fractions were eluted from the columns and treated as described above.

It was found that there was negligible difference between chromatographed and unchromatographed standards. For convenience, therefore, all standards were not chromatographed and were prepared directly from the stock solution.

Recovery Standards

Recovery standards were prepared by adding 1.0 ml of each of the Standard stock solutions of PAL, PAM and PIN (10 μg/ml) to the sample prior to acid hydrolysis.

Continuous Flow Analysis

For the determination of PAL, 2-8 ml of eluate were pipetted into each of the two test tubes A and B. Sufficient 0.4 M phosphate buffer was added to each of the test tubes to obtain a pH of 7.2-7.5 and a final volume of 10 ml. Twenty-five hundredth milliliter of 1.0 M KCN and 0.25 ml of water were added to test tubes A and B respectively, and the tubes were placed in a 50° C water bath for 2 hours. After cooling, the samples were placed in sample cups for continuous flow analysis. The flow diagram of vitamin B_6 analysis is shown in Figure 4.

A procedure similar to that employed for PAL was followed for the determination of PAM, except samples were treated with 0.25 ml of 0.5 M GOA at 100°C for 15 minutes prior to the addition of KCN.

PIN values were determined by the procedure previously described for pyridoxal, except 2.5 ml of 1.0 M KCN was added to the 100 ml sample prior to the two hour heat treatment.

The autoanalyzer system was assembled as shown in Figure 4. Water was pumped through all tubes and the baseline adjusted to 5 using a sample aperature of 3 and a

reference aperature of 1. After pumping Na_2CO_3 through the system for 20 to 30 minutes, the high standard of one of the three forms of B_6 was then used to adjust its maximum fluorescent response to 95. The sample probe was placed in water and the recorder allowed to return to the baseline before standards and samples were analyzed. Blanks for each sample were determined as described previously.

Calculations

Each form of vitamin B_6 was treated and measured separately. Fluorescence was proportional to the concentration of the lactone of 4-pyridoxic acid. Blanks were determined for each sample to measure the concentration of non-B_6 compounds exhibiting fluorescence. The difference between sample fluorescence and blank fluorescence was used to calculate the vitamin B_6 concentration in each sample using the following equation:

$$\frac{\text{Fluorescence sample-Blank}}{\text{Fluorescence of 1 } \mu\text{g } B_6/\text{ml}} \times \frac{100}{\text{Wt. of sample}} \times \text{dilution factor}$$

= ug of PAL or PAM or PIN/g of sample

Microbiological Assay for vitamin B_6

The procedure for the chromatographic separation and determination of vitamin B_6 using S. carlsbergensis was the revised method of Toepfer and Polansky (1970) with the following slight modifications. The innoculum, S. carlsbergensis, were suspended in 500 ml of sterilized water after the second rinse of the cells. One milliliter of the

assay innoculum was then aseptically added to each sample tube using an auto-pipet. Screw caps were used instead of caps with holes. In order to maintain an aerobic condition, free of contamination, caps were loosely fitted on to the test tubes. Samples were incubated at 30^{o}C in a NBS Gyrotory at a speed of 250 RPM.

RESULTS

Extraction

Acid hydrolysis and enzymatic hydrolysis of vitamin B_6 in various food products were examined in this study. Samples treated with 0.1 g each of Taka-diastase and papain for 4-6 hours at 37°C prior to acid hydrolysis were found to have total vitamin B_6 values higher than those samples receiving only acid hydrolysis. However, analysis of the enzymes indicated the presence of vitamin B_6 in these enzymes (Table I).

The acid hydrolysis described by Toepfer et al. (1961) was adapted for all samples studied. No significant differences were observed between the samples with recovery standards added prior to autoclaving and those with recovery standards added after autoclaving. Therefore, the recovery standards were added prior to autoclaving for all the samples reported in this study.

Separation of Pyridoxal, Pyridoxamine, and Pyridoxine by Column Chromatography

Dowex AG 50W-X8 was used for separation of PAL, PAM and PIN from food extracts (Toepfer et al., 1961). After elution each form was chemically and microbiologically analyzed.

Mixture of PAL, PAM, and PIN standards were combined and treated similarly to the sample. Chemical determination of the standards after separation on ion-exchange columns indicated that the columns provided nearly perfect separation of the three forms of the vitamin. Recovery of PAL, PAM and PIN from the column were 102.5%, 95.0% and 104.1%, respectively.

Fluorometric Determination of PAL, PAM and PIN Standards

Standard curves for PAL, PAM and PIN using the fluorometric method are shown in Figure 1. Fluorescence was linearly related to the concentration of PAL, PAM and PIN over a range of 0.01-0.08 µg/ml of solution.

Microbiological Determination of PAL, PAM and PIN Standards

Standards curves of PAL, PAM and PIN were determined from the relative growth of S. carlsbergensis, and normally varied from analysis to analysis. Freshly prepared standards were, therefore, analyzed along with each sample. Figure 2 shows representative standard curves for the microbiological determination of PAL, PAM, and PIN in ham. The concentrations of standard PAL, PAM and PIN (ng/ml) were plotted against percent transmittance (%T) which was obtained from an average of triplicate readings.

An analysis of the slopes showed that PAM exhibited the least sensitivity with a slope of 0.074. PAL was only slightly more sensitive than PAM with a slope of 0.075, while PIN exhibited the greatest sensitivity with a slope of 0.089.

Vitamin B_6 Contents in Selected Food Products

The content of pyridoxal, pyridoxamine, and pyridoxine determined in infant formula, ham, cereal, and frozen and canned lima beans using both microbiological and chemical methods are shown in Tables IIa and b, IIIa and b, IVa and b.

Pyridoxal

Data in Table IIa and b represents the PAL contents measured in various food products. Chemically determined PAL values were approximately 3-12 times higher than those measured using the microbiological method. Recoveries determined by the fluorometric method varied between 83 -97% with a standard deviation ranging from 3-13 for the different food products. Recoveries for the microbiological method varied from 53-106% with a standard deviation ranging from 7-19. Values corrected for percent recovery showed a slightly lower standard deviation than the uncorrected values.

Pyridoxamine

Data in Table IIIa and b showed the pyridoxamine content in various food products. PAM values obtained by the chemical method were approximately 2-3 times greater than those obtained with the microbiological assay. With the exception of ham, the percent recoveries for most of the samples using the chemical method varied from 86 -111, with a standard deviation ranging from 2 -11 depending on the product. Ham had a constant recovery of approximately 66%.

The percent recoveries using the microbiological method exhibited a range of 74-113 with the standard deviation varying from 7-23. Values corrected for percent recovery exhibited a slightly lower standard deviation for most of the food samples analyzed.

Pyridoxine

The PIN values of various food products are shown in Table IVa and b, Ham and frozen lima beans had only a small amount of PIN, while canned lima beans contained a negligible amount of PIN in the sample size used for analysis. High PIN values were found in the cereal and infant formula.

Values obtained from the chemical determination of PIN were 1-2 times as great as those values obtained from the microbiological method. The percent recovery for the various food products analyzed using the chemical method varied from 55-74 with a standard deviation ranging from 3-7. The percent recovery from the microbiological method varied from 99-132 with a standard deviation ranging from 8-31.

Comparisons of Fluorometric and Microbiological Total Vitamin B_6 Values

Table V shows the comparisons of total vitamin B_6 values for various food products obtained by the microbiological and chemical methods. The comparison also includes published values which were determined using the

S. carlsbergensis assay organisms. Values obtained by the chemical method exhibited almost twice as much total vitamin B_6 as those obtained by the microbiological method. However, the values for lima beans determined by the chemical method were 5 times greater than when determined microbiologically.

A bar graph representation for various food products comparing the fluorescent response of PAL and its blanks is shown in Figure 3.

Sample Blank Study

Table VI is a study of Vitamin B_6 standards and sample blanks which have been treated with ultraviolet light or $KMnO_4$ prior to the addition of KCN. Since ultraviolet light and $KMnO_4$ reacted with substances other than PAL, PAM and PIN in the food extract, thus, no conclusions could be made from this study.

Another study was conducted in this laboratory to determine the interference of thiamin in the fluorometric determination of vitamin B_6. Results indicated that KCN caused thiamin to partially fluoresce at the same wavelength of pyridoxal. However, when thiamin and pyridoxal were chromatographed over a Dowex AG 50W-X8 column, thiamin was not detected in the pyridoxal eluate. This would indicate that thiamin was not an interfering substance in the fluorometric determination of pyridoxal provided the sample extract was passed over the ion exchange column. Thiamin

interference in determination of PAM or PIN was not of concern because sample blanks would account for any fluorescence due to thiachrome formation.

TABLE I. A comparison of enzymatic and acid hydrolysis for the determination of vitamin B_6 in infant formula.

Treatment	CONCENTRATION (µg/g)		
	PAL	PAM	PIN
Sample (acid hydrolyzed)	0.10	0.19	0.14
Sample(acid & enzyme hydrolyzed)	0.19	0.34	0.37
Enzyme Blank[a]	0.11[b]	0.13[b]	0.25[b]
Difference between sample (acid & enzyme hydrolyzed) and enzyme blank	0.08	0.21	0.12

[a]Enzyme blanks contained 100 mg of Taka-diastase and 100 mg of papain.

[b]The concentration of the enzyme is µg B_6/.2g enzyme.

TABLE IIa. Pyridoxal values in selected food samples determined by the fluorometric and microbiological methods.

Product	Sample #	µg/g of sample Exper. Value Chem.	Micro	% Recovery Chem.	Micro	Corr. Value Chem.	Micro	Ratio of Chem/Micro Uncorr.	Corr.
Lima Beans (Canned)	1	1.33	0.38	89	119	1.50	0.32	3.6	4.7
	2	1.50	0.38	107	110	1.40	0.34	4.0	4.1
Mean		1.42	0.38	98	115	1.45	0.33	3.8	4.4
Stand.Dev.		0.12	0.0	13.0	6	0.07	0.01	0.1	0.4
Lima Beans (Frozen)	1	1.22	0.23	88	51	1.60	0.43	5.4	3.2
	2	1.33	0.20	83	54	1.47	0.38	6.5	4.2
Mean		1.28	0.22	86	53	1.54	0.41	6.0	3.7
Stand.Dev.		0.07	0.02	4	2	0.09	0.04	0.8	0.7
Ham	1	0.67	0.10	97	110	0.69	0.09	6.5	7.3
	2	0.67	0.10	97	110	0.67	0.09	6.5	7.3
	3	0.50	0.11	98	98	0.51	0.11	4.5	4.5
	4	0.46	0.15	94	99	0.49	0.15	3.0	3.2
Mean		0.58	0.12	97	104	0.59	0.11	5.1	5.6
Stand.Dev.		0.11	0.02	3	7	0.10	0.03	1.7	2.1

TABLE 11b. Pyridoxal values in selected food samples determined by the fluorometric and microbiological methods.

Product	Sample #	µg/g of sample Exper. Value Chem.	Micro.	% Recovery Chem.	Micro.	Corr. Value Chem.	Micro.	Ratio of Chem/Micro Corr.	Uncorr.
Cereal	1	3.91	0.41	97	118	4.05	0.35	9.5	11.6
	2	4.06	0.36	112	124	3.61	0.29	11.3	12.6
	3	3.78	0.37	90	90	4.20	0.42	10.2	10.0
	4	3.67	0.36	87	90	4.27	0.39	10.3	10.8
Mean		3.85	0.37	97	106	4.03	0.37	10.3	11.3
Stand.Dev.		0.27	0.03	11	18	0.30	0.06	0.7	1.1
Infant Formula	1	0.07	0.02	71	81	0.10	0.02	4.6	4.7
	2	0.07	0.02	86	81	0.08	0.02	4.6	5.5
	3	0.09	0.01	86	47	0.11	0.02	4.6	5.5
	4	0.09	0.01	90	47	0.09	0.02	7.7	4.1
Mean		0.08	0.01	83	64	0.10	0.02	6.1	4.7
Stand. Dev.		0.01	0.03	8	19	0.01	0.00	2.1	0.6

TABLE IIIa. Pyridoxamine values in selected food samples determined by the fluorometric and microbiological methods.

Product	Sample #	µg/g of sample Exper. Value Chem.	Micro.	% Recovery Chem.	Micro.	Corr. Value Chem.	Micro.	Ratio of Chem/Micro Corr.	Uncorr.
Lima Beans (Canned)	1	2.00	0.22	150	106	1.33	0.23	8.1	5.7
	2	1.30	0.29	98	116	1.33	0.24	4.4	5.6
Mean		1.65	0.26	124	111	1.33	0.24	6.3	5.7
Stand. Dev.		0.50	0.05	37	7	0.00	0.01	2.6	0.1
Lima Beans (Frozen)	1	2.28	0.18	103	113	2.27	0.16	12.9	14.2
	2	2.32	0.18	118	97	1.96	0.19	12.9	10.6
Mean		2.30	0.18	111	105	2.00	0.18	12.9	12.4
Stand. Dev.		0.07	0.0	11	11	0.10	0.02	0.0	2.5
Ham	1	3.67	1.39	67	61	5.52	2.26	2.6	2.4
	2	3.67	1.78	67	61	5.52	2.91	2.1	1.9
	3	3.47	1.78	66	91	5.30	2.91	1.7	2.3
	4	3.73	2.21	67	83	5.58	2.67	1.7	2.1
Mean		3.63	1.86	66	74	5.48	2.53	2.0	2.2
Stand. Dev.		0.11	0.36	1	15	0.12	0.32	0.5	0.3

TABLE IIIb. Pyridoxamine values in selected food samples determined by the fluorometric and microbiological methods.

	µg/g sample	

TABLE IIIb. Pyridoxamine values in selected food samples determined by the fluorometric and microbiological methods.

Product	Sample #	Exper. Value µg/g sample Chem.	Micro.	% Recovery Chem.	Micro.	Corr. Value Chem.	Micro.	Ratio of Chem/Micro Uncorr.	Corr.
Cereal	1	5.06	2.71	113	72	4.49	3.75	1.9	1.2
	2	4.92	2.78	101	75	4.92	3.73	1.8	1.3
	3	4.50	1.85	101	83	4.45	2.25	2.4	2.0
	4	4.50	2.25	101	105	4.45	2.15	2.0	2.0
Mean		4.74	2.40	104	105	4.47	2.96	2.0	1.6
Stand. Dev.		0.29	0.43	6	15	0.02	0.89	0.3	0.5
Infant Formula	1	0.19	.10	88	79	0.21	.07	1.9	2.9
	2	0.18	.11	86	122	0.21	.08	1.7	2.8
	3	0.18	.13	89	125	0.20	.10	1.4	2.0
	4	0.18	.10	89	127	0.20	.08	1.9	2.7
Mean		0.18	.11	88	113	0.21	.08	1.7	2.6
Stand. Dev.		0.00	.01	2	23	0.01	.01	0.1	0.4

TABLE IVa. Pyridoxine values in selected food samples determined by the fluorometric and microbiological methods.

Product	Sample #	µg/g of sample Exper. Value Chem.	Micro.	% Recovery Chem.	Micro.	Corr. Value Chem.	Micro.	Ratio of Chem/Micro Uncorr.	Corr.
Lima Beans (Canned)	1	0.17	0.29	58	126	0.29	0.23	0.8	1.3
	2	0.17	0.32	58	137	0.29	0.23	0.6	1.2
Mean		0.17	0.30	58	132	0.29	0.23	0.7	1.3
Stand. Dev.		0.00	0.01	0.00	8	0.00	0.00	0.1	0.0
Lima Beans (Frozen)	1	cannot be measured		75	123	--	--	--	--
	2	cannot be measured		71	137	--	--	--	--
Mean		--	--	73	130	--	--	--	--
Stand. Dev.		--	--	3	10	--	--	--	--
Ham	1	0.17	0.20	58	141	0.28	0.14	0.8	2.0
	2	0.17	0.22	58	129	0.28	0.74	0.7	1.6
	3	cannot be measured		51	80	--		--	--
	4	cannot be measured		51	82	--		--	--
Mean		0.17	0.15	55	108	0.28	0.45	0.8	1.8
Stand. Dev.		0.00	0.07	4	31	0.00	0.35	0.1	0.3

TABLE IVb. Pyridoxine values in selected food samples determined by the fluorometric and microbiological methods.

		µg/gm. of sample							
		Exper. Value		% Recovery		Corr. Value		Ratio of Chem/Micro	
Product	Sample #	Chem.	Micro.	Chem.	Micro.	Chem.	Micro.	Uncorr.	Corr.
Cereal	1	17.19	21.06	70	112	24.67	19.34	0.8	1.3
1	2	17.19	21.00	70	112	24.67	19.34	0.8	1.3
	3	31.69	36.67	83	122	38.41	30.50	0.9	1.3
	4	32.22	37.92	67	84	47.12	44.90	0.9	1.1
Mean		24.82	29.16	72	109	33.72	28.52	0.8	1.2
Stand. Dev.		8.24	9.40	7	17	11.03	12.12	0.03	0.1
	1	0.26	0.24	70	118	0.37	0.20	0.1	1.9
	2	0.25	0.20	64	77	0.39	0.26	1.5	1.5
	3	0.20	0.19	82	75	0.24	0.24	1.0	0.9
	4	0.18	0.19	78	125	0.23	0.15	1.0	1.5
Mean		0.22	0.20	74	99	0.31	0.22	1.2	1.5
Stand. Dev.		0.04	0.02	8	26	0.08	0.05	0.3	0.4

TABLE V. Comparison of total vitamin B_6 values for selected food products using the microbiological and fluorometric methods.

Product	Published Total	Exp. Total µg/gm of sample Chem.	Micro.	PAL Chem.	Micro.	PAM Chem.	Micro.	PIN Chem.	Micro.	RATIO Chem./Micro
Lima(Canned)	1.1[a]	2.78	.56	1.45	.33	1.33	.236	--	--	4.92
Beans(Frozen)	1.3[a]	3.87	.80	1.49	.40	2.09	.171	.29	.23	4.80
Ham	3.19[b]	6.34	3.09	.59	.11	5.48	2.53	.28	.45	2.05
Cereal	23.6[c]	42.22	31.85	4.03	.37	4.47	2.96	33.72	28.52	1.33
Infant Formul.	.37[d]	.61	.32	.10	.02	.21	.08	.31	.22	1.93

[a]Cook, B.B., Gunning, B and Uchimoto, D., J. Agr. Food Chem. 9, 316, 1961.

[b]Polansky and Toepfer, E.W., J. Agr. Food Chem. Vol. 17, 6, 1394, 1969.

[c]Obtained from the labeling of Post Grape Nuts.

[d]Obtained from the labeling of Similac infant formula.

TABLE VI. Study of sample blanks using the fluorometric method.

	(% Fluorescence)								
	PAL			PAM			PIN		
Treatment	W/CN	CTP* W/O CN	W/O CTP W/O CN	W/CN	CTP W/O CN	W/O CTP W/O CN	W/CN	CTP W/O CN	W/O CTP W/O CN
Sample	10.0	--	5.5	25.0	3.5	5.5	30	--	4.0
Stand.	2.2	--	0.0	25	0.0	1.0	20	--	0.0
Blank	10.0	--	5.5	7.5	5.5	3.5	8.5	--	4.0
Stand.	22.0	--	0.0	3.0	0.5	1.0	1.0	--	0.0
U.V. treat.	7.0	--	4.0	11.0	--	1.5	26.0	--	4.0
U.V. treat. Stand.	1.0	--	0.0	3.0	--	0.0	13.0	--	0.0
$KMnO_4$ treat.	10.0	--	5.0	10.0	--	6.0	30.0	11.0	--
$KMnO_4$ treat. Stand.	1.0	--	0.0	.15	--	0.0	11.0	9.0	--

*CTP: converted to PAL.

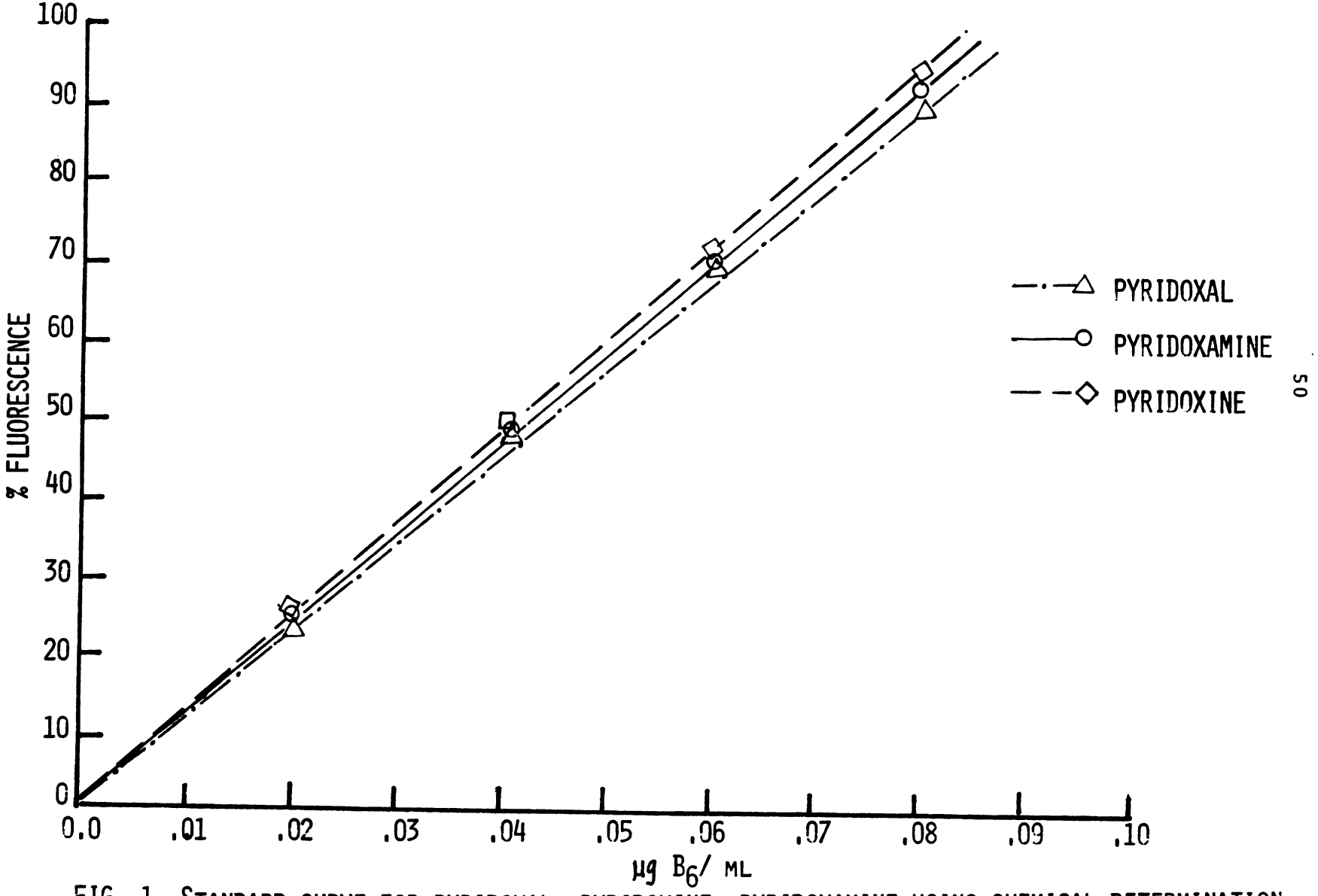


FIG. 1. STANDARD CURVE FOR PYRIDOXAL, PYRIDOXINE, PYRIDOXAMINE USING CHEMICAL DETERMINATION

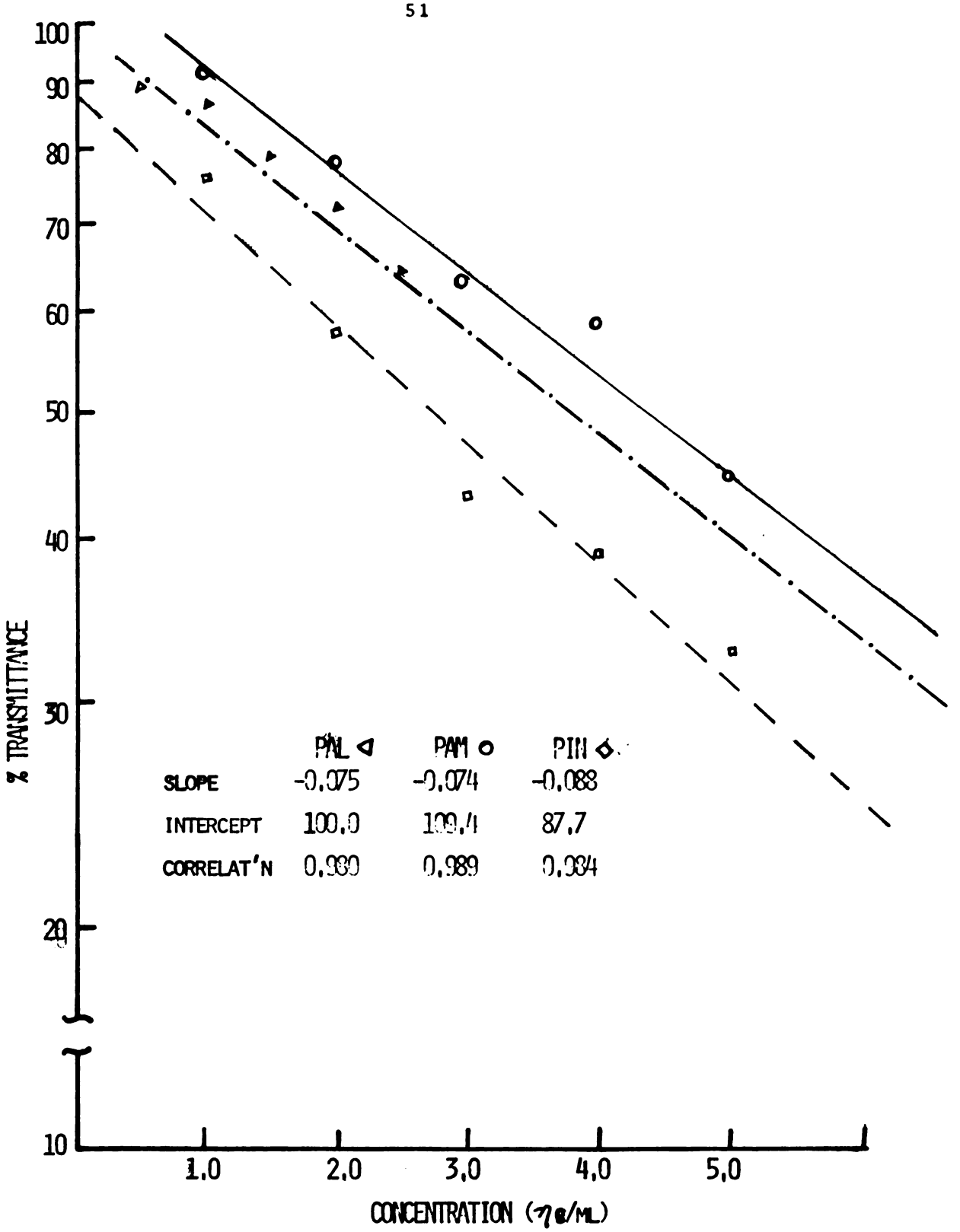


FIG.2. STANDARD CURVES OF PYRIDOXAL, PYRIDOXAMINE, AND PYRIDOXINE DETERMINED BY S. CARLSBERGENSIS.

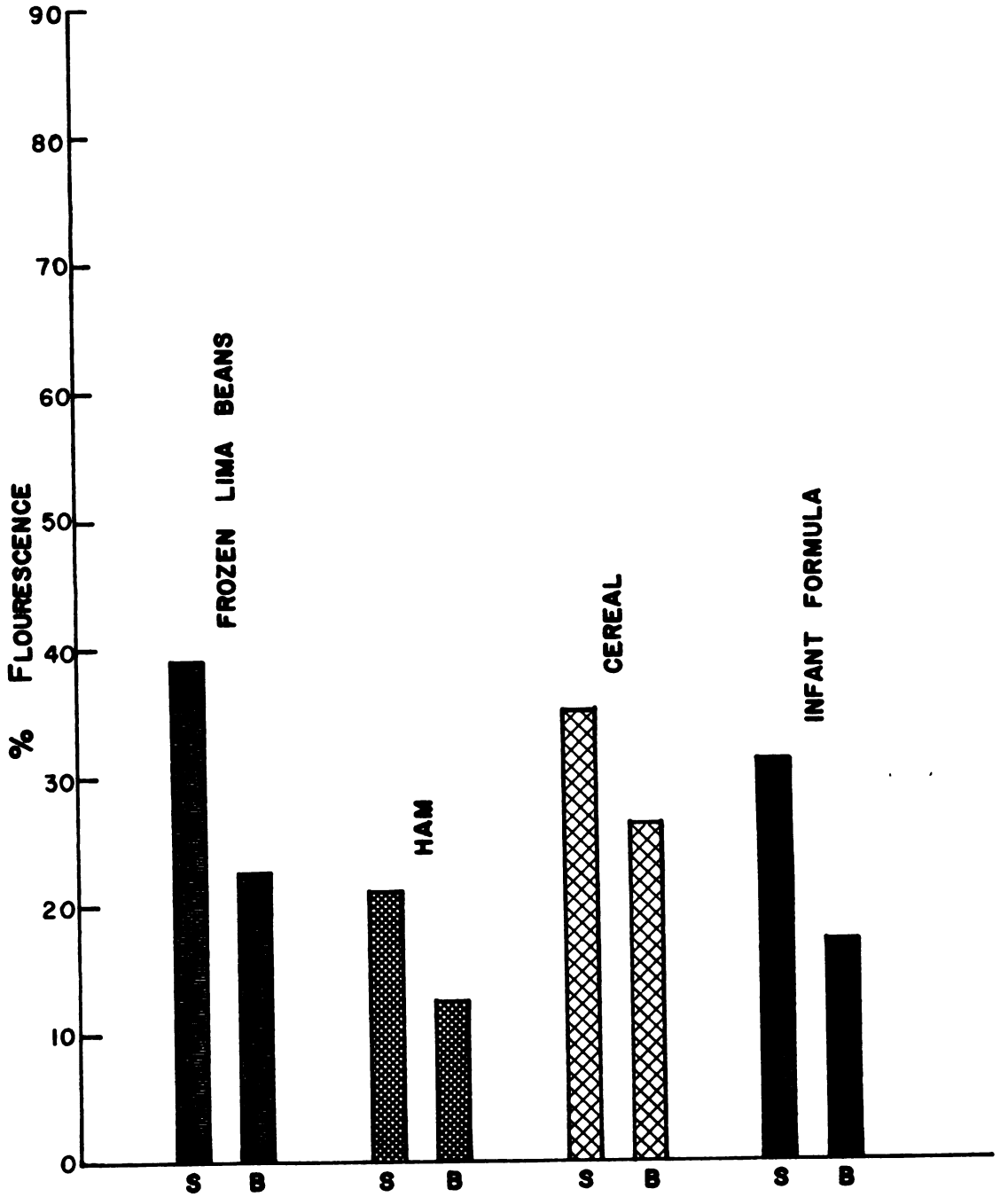


FIG3. COMPARISON OF PYRIDOXAL FLOURSCENCE RESPONSE FOR SAMPLE AND SAMPLE BLANK IN SELECTED FOOD SAMPLES

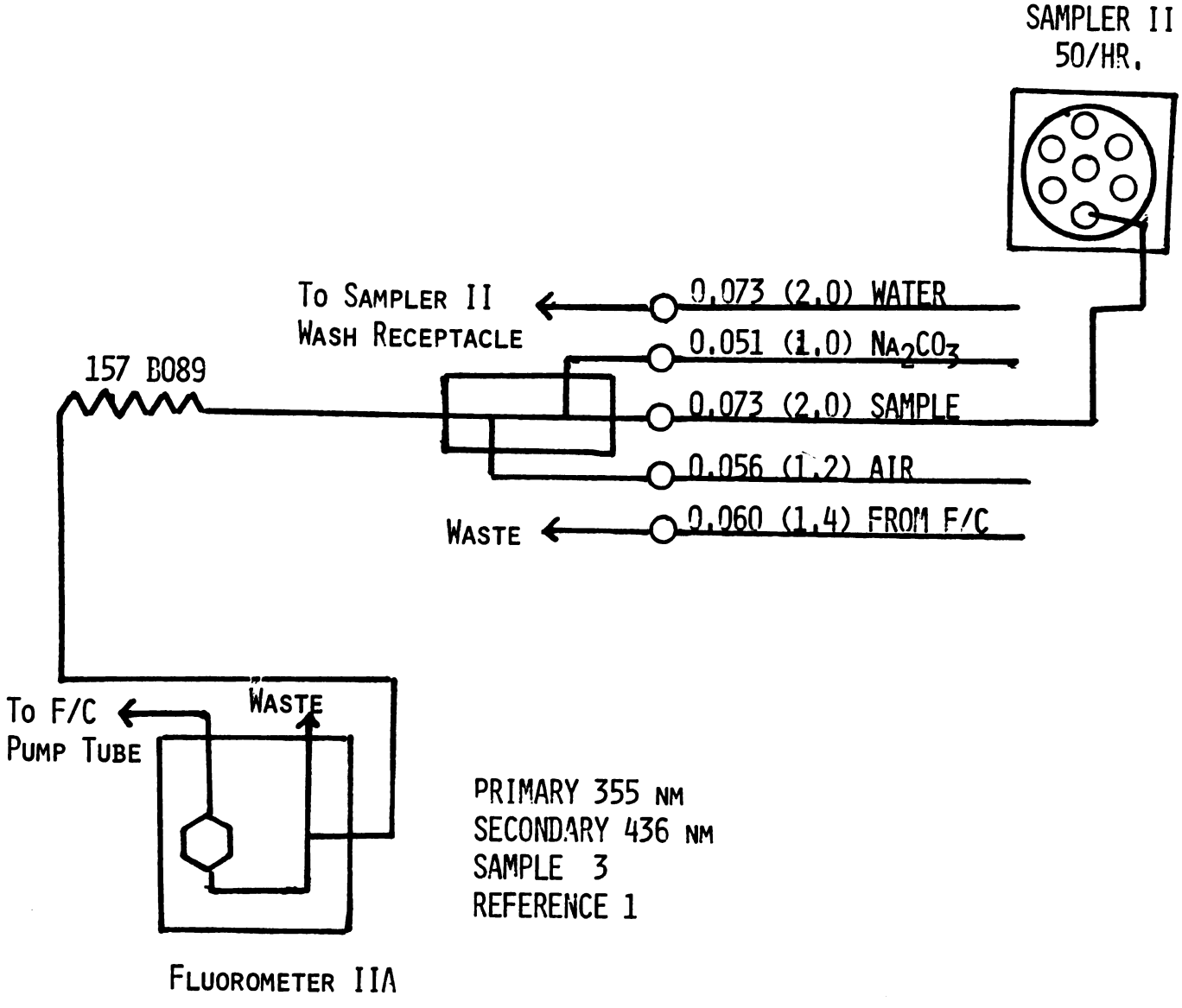


FIG. 4 Continuous flow automated analysis system for determining vitamin B_6 in foods.

DISCUSSION

Extraction

Researchers have studied the various factors that may influence quantitative liberation of vitamin B_6 during hydrolysis. Factors, such as: the type of enzymes, sample size, hydrogen ion concentration, time and pressure of autoclaving, were all shown to be important in the extraction of the vitamin. To date, no one extraction method has been reported to quantitatively release the bound forms of vitamin B_6 from foods and biological materials. This lack of an adequate method for the extraction of bound forms of vitamin B_6 has been one of the most difficult problems encountered in the development of a quantitative chemical or microbiological assay.

Enzymatic Extraction

Various extraction procedures were examined during the development of this fluorometric determination for vitamin B_6. Samples treated with the enzymes, Takadiastase and papain, prior to acid hydrolysis had a higher vitamin B_6 value than those samples receiving only acid hydrolysis. This was shown to be due to the presence of a considerable amount of vitamin B_6 (2 μg/g) in these enzymes. It should not be implied that enzymatic hydrolysis can not be used as

a method of vitamin B_6 extraction, since enzyme blanks could be determined and the true vitamin B_6 value of the sample obtained from the difference of the observed sample value and the sample and enzyme blanks.

Enzymatic extraction had several advantages that should not be overlooked. Unlike acid hydrolysis, enzymatic hydrolysis was performed without high heat. Therefore, thermal degradation of the sample did not occur during the extraction procedure. Also, non-enzymatic browning and protein degradation, which were observed to occur during acid hydrolysis and interfered with fluorometric determination of vitamin B_6, was not encountered with enzymatic hydrolysis. This method was not applied to this study because it was time consuming and complicated.

Acid Extraction

The most widely accepted extraction procedure is the use of acid hydrolysis at 15 or 20 psi steam pressure (Rabinowitz and Snell, 1947; Rubin et al., 1947; Polansky and Toepfer 1969). The concentration of the acid used for the extraction procedure has been examined by numerous researchers. According to a study by Polansky and Toepfer (1969) extraction of meat products with 0.44 N HCl instead of 0.055 N HCl gave approximately the same pyridoxine and pyridoxal values, but only about half of the pyridoxamine value. Therefore, 0.055 N HCl was recommended for the extraction of animal products.

Toepfer and Polansky (1969) also reported that commercial infant formula hydrolyzed with both 0.055 N HCL and 0.44 N HCl gave comparable results.

Based on the above information, the extraction procedure of Toepfer and Polansky (1961, 1970) was adapted for all food samples. The concentration of acid used for the extraction was 0.44 N HCl and 0.055 N HCl for plant and animal products, respectively.

Limitation of Acid Hydrolysis

Despite the simplicity of the acid extraction method, there were many limitations to this method.

Many reactions have been shown to occur during hydrolysis which changed some of the chemical and physical properties of the product being analyzed. At acid pH, proteins were denatured during autoclaving. Transamination and deamination reactions have also been reported to occur between pyridoxal and pyridoxamine (Toepfer and Polansky, 1969). Also, under these conditions, products containing free carbonyl groups and free amino groups could result in non-enzymatic browning. These chemical and physical changes which have been shown to occur during hydrolysis must be carefully examined, because of their possible interference in the fluorometric determination of vitamin B_6 content in the food products.

During hydrolysis, proteins were denatured and may undergo transamination and/or deamination reactions which

could effect the concentration of pyridoxal and pyridoxamine in the product. In a study of vitamin B_6 content of raw pork loin and ham, Toepfer and Polansky (1969) found that ham contained 73% pyridoxamine and 16% pyridoxal, while pork loin contained 61% pyridoxal and 22% pyridoxamine. These data indicated a dramatic change in the concentrations of pyridoxal and pyridoxamine in ham and fresh pork loin which could have been the result of transamination and deamination reactions occurred during processing and/or extraction of vitamin B_6 from the ham.

Studies in our laboratory showed that pyridoxamine was indeed the predominant form in ham, accounting for 82% of the total vitamin B_6 content (Table IIa,b, IIIa, b).

Another problem encountered in this research was the possible interaction between the added standards and the various components of the food product during acid hydrolysis. Storvick and Peters (1964) reported that prolonged heat treatment in the presence of protein resulted in either vitamin B_6 destruction or rebinding of the free vitamin B_6 with the protein. If either hypothesis were correct, then, recovery standards added before hydrolysis should be lower than those added after the hydrolysis. However, experiments conducted in this laboratory, showed that there was no difference in the concentration of standards added prior to or after acid hydrolysis. The reason for this discrepancy between previously reported results could

have been due to differences in hydrolysis time and samples analyzed. Storvick and Peters (1964) hydrolyzed some of their samples, as long as 72 hours whereas samples analyzed in this experiment were hydrolyzed for 2-4 hours. This could indicate that longer hydrolysis times are needed for destruction or rebinding of vitamin B_6 to occur during autoclaving.

Column Chromatography

The eight columns used for this research were not all identical. Some were purchased from chemical companies while others were made by the glass shop at M.S.U. Therefore, there were some slight differences in their dimensions, which could have influenced elution and adsorption rates. The packing of the column, which improved with experience, was also very important for proper adsorption and elution of the vitamin B_6.

Functions of the Ion-exchange Column

The function of the Dowex AG 50W-X8 was twofold; separation of the three active forms of vitamin B_6 and the removal of interferring substances from the food extracts. These interferring compounds were partially removed by washing the column with 0.02 M acetate buffer (pH 5.5) after adsorption of the three vitamin B_6 components to the ion-exchange resin. Pyridoxal fraction, which was the first of the three components to be eluted from the Dowex column, had the most noticeable interference. Products

containing a large amount of carbohydrates showed greater levels of interfering substances than products containing a low carbohydrate level. In this study, a yellow tinge was observed in all vitamin B_6 fractions from cereal extract, with pyridoxal fraction having the strongest color and pyridoxamine fraction the weakest. Such observation indicated that perhaps 100 ml of acetate rinse was not sufficient to remove all of the interfering substances from the column. The amount of washing buffer was increased from 100 ml to 125 ml to increase the removal of the interfer substances. A further study with pyridoxal standard showed that increasing the volume of buffer rinse to as much as 350 ml would not interfere with the recovery of pyridoxal. However, in this study the volume of the buffer rinse was maintained at 125 ml.

Criticism of the ion exchange column

The failure to completely remove interfering fluorescent substances was not the only criticism reported of the Dowex AG 50W-X8 ion exchange column. Storvick et al. (1964) reported that distinct separation of the three forms of vitamin B_6 was not achieved using this column. Examination of the effectiveness of the Dowex AG 50W-X8 column in this laboratory, using the lactone method of Bonavita (1960) to measure the eluted vitamin B_6 standards, showed that the column was capable of completely separating the three forms. The poor recovery of Storvick et al. (1964) could have been

due to the use of the lactone method of Fujita et al. (1955a, b), which exhibited extremely low yields of pyridoxine (26%) and pyridoxal (67%). These yields would have been insufficient to evaluate the separational capabilities of the Dowex AG 50W-X8 column.

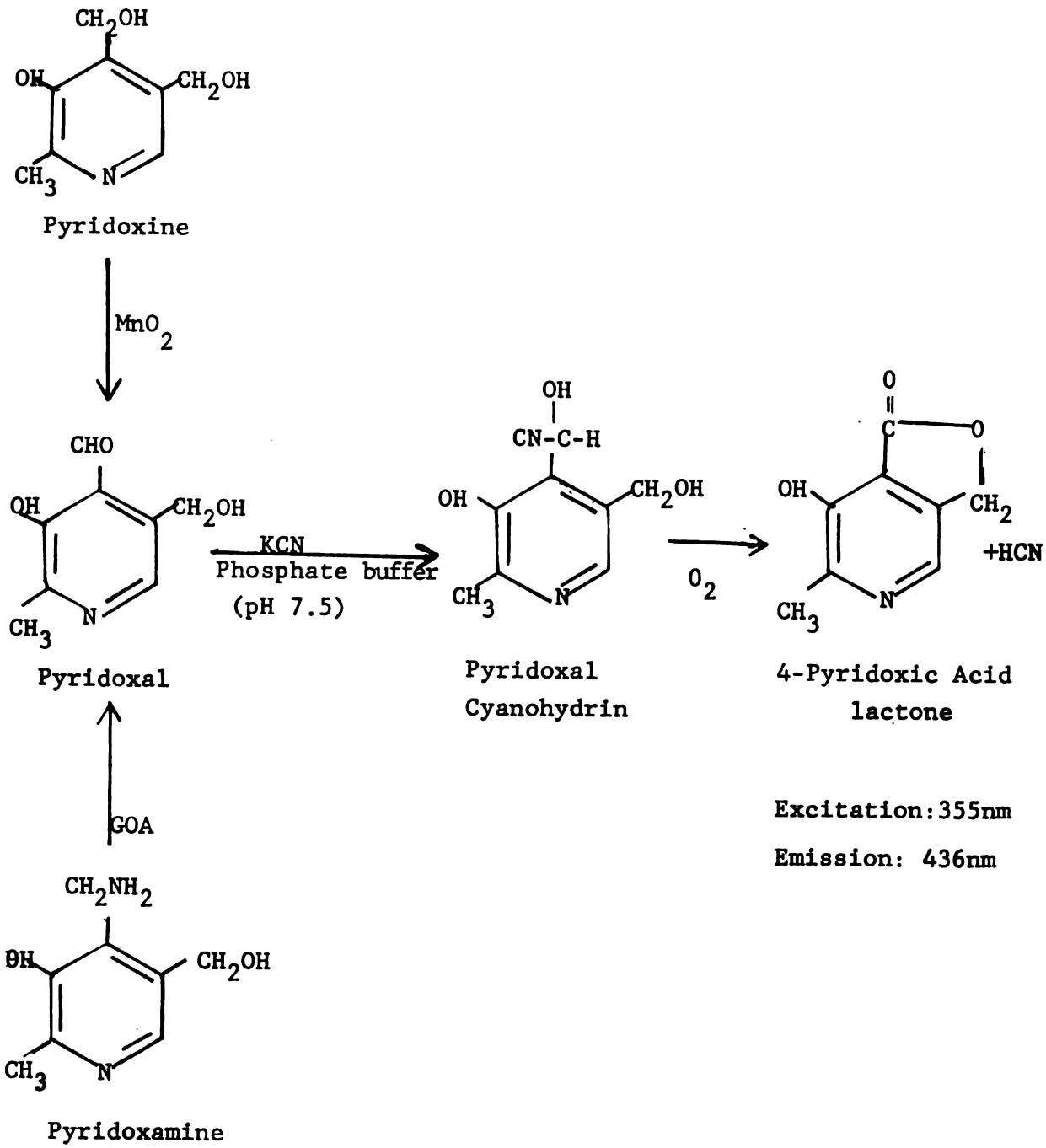
Another criticism of the ion-exchange column was the use of boiling acetate buffers, which were essential for the removal of the vitamin B_6 components from the resin. The boiling acetate buffers were hard to handle and lengthened the time required for the analysis.

Fluorometric Determination of PAL, PAM and PIN Standards

The procedure reported in this study was a combination and modification of numerous chemical methods (Metzler et al., 1954; Bonavita, 1960; Toepfer et al., 1961; Toepfer and Polansky, 1964, 1970). This method was adapted for the determination of the three biologically active components of vitamin B_6 in foods. The separation of pyridoxal, pyridoxamine and pyridoxine from acid hydrolyzed food extracts was carried out by Dowex AG 50W-X8 ion exchange chromatography and quantitated by fluorometry following their conversion from pyridoxal to 4-pyridoxic acid lactone (Bonavita, 1960). Pyridoxine and pyridoxamine were converted to pyridoxal by reacting with manganese dioxide (Polansky et al., 1964) and sodium glyoxalate (Toepfer et al., 1961), respectively. Fluorescence was measured at 355 nm (excitation) and 436 nm (emission) at pH 9-10.

Mechanisms of Reactions

The fluorometric determination of vitamin B_6 was based on the following reactions:



The relationship between fluorescence and the concentration of pyridoxal, pyridoxine or pyridoxamine was linear as shown in Figure 1. Comparison of the linear response of pyridoxine and pyridoxamine indicated quantitative conversion of these components to pyridoxal. The quantities of pyridoxine and pyridoxamine recovered as 4-pyridoxic acid lactone, were not statistically different from pyridoxal. The slightly lower response for pyridoxamine was due to a higher dilution in the final reaction mixture.

Analysis of the Blanks for the Vitamin B_6 Standards

Analysis of the blanks for the vitamin B_6 standards showed no fluorescent interfering substances present. There was no difference between blanks of pyridoxamine and pyridoxine standards treated with KCN and those treated without KCN. This indicated that pyridoxamine and pyridoxine exhibited no fluorescing properties of their own and would not react with KCN in the pyridoxine or pyridoxamine forms.

Microbiological Determination of PAL, PAM and PIN Standards

Pyridoxal, pyridoxamine and pyridoxine standards were determined without chromatography, since the standards used for the fluorometric method were determined without chromatography. Analyses of these vitamin B_6 standards indicated that the microorganism, S. carlsbergensis, gave less response to pyridoxamine than to pyridoxal or pyridoxine (Figure 2). This lack of equal response by the microorganism stressed the need of separating the three biologically active forms of vitamin B_6 and determining them

individually.

Standard curves determined by the microbiological method using S. carlsbergensis were not transferable from one determination to another. Growth of the microorganism in response to vitamin B_6 varied from analysis to analysis, depending on the amount of cells inoculated, and the time and temperature of the incubation. Therefore, a standard curve was determined with each set of samples.

The growth of S. carlsbergensis increased in an exponential manner as the concentration of vitamin B_6 increased (Fig. 2). Since there was considerable growth of S. carlsbergensis in the basal broth without presence of vitamin B_6, inoculated basal broth was used as a blank for the vitamin B_6 determination. The spectrophotometer was adjusted to 100% transmittance with a mixture of 9 tubes of inoculated basal broth. Therefore, at low concentration of vitamin B_6, if the growth of S. carlsbergensis is less or equal to the blank, the percent transmittance would be equal to or greater than 100% T. This phenomenon was shown in the standard curves of pyridoxal and pyridoxamine whose y-intercepts were 100%T and 109%T, respectively (Figure 2).

Microbiological Determination of Vitamin B_6 in Selected Food Products

The vitamin B_6 values of the food products used in this microbiological study correlated well with those values reported previously (Toepfer and Polansky, 1970; Polansky and Toepfer, 1969; Polansky et al., 1964), Table V.

Canned lima beans showed a lower value than the published value. Exclusion of packing brine in the determination and the difference in the variety of the lima beans could account for the difference in values obtained in these two studies. The slightly higher vitamin B_6 value in frozen lima beans indicated that a shorter heat processing could result in higher vitamin B_6 retention.

Ham showed an almost perfect correlation between the experimental value and the reported vitamin B_6 value. The high vitamin B_6 value in ham indicated that it is an excellent source of vitamin B_6.

The experimental value of vitamin B_6 in wheat flake showed a greater value than what was reported on the package label. This was probably due to the overfortification of the cereal with pyridoxine hydrochloride.

A slightly lower vitamin B_6 value was found in the infant formula than what was reported on the label. Since vitamin B_6 is extremely sensitive to ultraviolet light, and the formula was commercially prepared and packaged in glass jars, vitamin B_6 loss could have occurred during handling or storage.

Fluorometric Determination of vitamin B_6 in Selected Food Products

Ham

Pyridoxal and pyridoxamine were the predominant forms of vitamin B_6 in hydrolyzed extracts of animal products with only small amounts of pyridoxine present. Fully

cooked ham contained approximately 82% of its total vitamin B_6 as pyridoxamine (Table V). The percent recovery for pyridoxamine in ham was consistently low at approximately 66%, while percent recoveries of pyridoxamine for other products were $\geq$ 90%. The cause of a low recovery for ham could be the result of transamination and deamination reactions of pyridoxal and pyridoxamine during autoclaving.

Infant Formula

Bessey et al. (1957) reported that most of the inherent vitamin B_6 content in infant formula was destroyed during processing. This necessitated the fortification of all infant formula with pyridoxine hydrochloride. From the chemical and microbiological analysis used in this study, pyridoxine was found to be the predominant form of vitamin B_6 in infant formula which contained approximately .2µg/g of sample (Table IV).

Cereal

Plant products contained predominantly pyridoxine with small amounts of pyridoxamine and pyridoxal. Microbiological and chemical analyses of wheat flakes indicated that pyridoxine was the predominant form of vitamin B_6 (Table V). The extremely high pyridoxine value was in part due to the fortification of the cereal during processing. Vitamin fortification of cereals is usually accomplished by spraying a solution containing the vitamins on the cereals after it has left the drying oven. This normally results in an uneven distribution of the vitamins on

the product. This variance was evident from the vitamin B_6 analysis carried out on the same box of wheat flakes at different times. The microbiological and chemical analyses which were performed at the same time exhibited relatively close pyridoxine values between sample 1 and 2 of the wheat flakes. Samples 3 and 4 of the same product, which were analyzed on a different date than samples 1 and 2, were similar but statistically different from samples 1 and 2 (Table IIIb.)

Lima Beans

Some difficulties were encountered in the analysis of lima beans. Canned lima beans contained negligible amounts of pyridoxine as analyzed by both chemical and microbiological methods. Even with a three-fold increase in the suggested sample size, no measurable vitamin B_6 content could be determined. These results do not agree with a recent study by Toepfer and Polansky (1971), who reported that lima beans contained approximately 6.05 µg/g of total B_6, of which 4.12 µg/g was pyridoxine. Because the study did not specify the variety or processing variables, it was not possible to draw any conclusion from comparison of the two sets of data. In addition, processing and storage has been shown to cause diffusion of vitamin B_6 from the beans into the packing brine. Exclusion of the packing brine could account for the low vitamin B_6 values observed in this study (Table V). This theory was further supported

by the high values obtained with frozen lima beans.

Analysis of Sample Blank

The fluorescence of the sample blank was interpreted as quantitative measurement of the interfering fluorescent compounds in the sample assay mixture. As shown in Figure 3, the presence of the interfering substances varied with the food being analyzed. Food products studied, which had low levels of interfering substances, such as, ham and infant formula, exhibited small color changes during acid hydrolysis. High carbohydrate containing foods, such as, lima beans and wheat flakes, resulted in the formation of light and dark brown solutions during hydrolysis. These substances greatly increased the fluorescence of the sample blank, however, the recovery values for the individual vitamin B_6 components showed that the sample blank accounted for most of the interfering substances present in the eluates (Tables IIa,b, IIIa,b, IVa,b).

A study of the effect of KCN on interfering substances naturally present in the extract was carried out with the sample blanks. Treatment of sample blank with $KMnO_4$ and ultraviolet light, which has been shown to destroy vitamin B_6, was used to indicate whether other substances in the sample would react with KCN to produce fluorescing compounds.

To determine the effectiveness of $KMnO_4$ and ultraviolet light on vitamin B_6 destruction, vitamin B_6

standards were subjected to the same treatments as the sample blanks. The results showed that $KMnO_4$ and ultraviolet light destroyed $\geq$ 95% of the vitamin present. Thus, vitamin B_6 within the food extract should have decreased by the same factor.

Analysis of the sample blank treated with ultraviolet light and $KMnO_4$ showed a lower fluorescent response than those without treatments. However, addition of KCN to the $KMnO_4$ or ultraviolet treated sample blanks resulted in higher fluorescent readings than samples not treated with $KMnO_4$ or ultraviolet and reacted with KCN. This indicated that ultraviolet light and $KMnO_4$ did not selectively destroy vitamin B_6 in the food extract, but resulted in a reorientation of the chemical structures of some compounds in the sample extract to a form that reacted with KCN. Therefore, no definite conclusions could be made from this study based on the informations obtained.

Interfering Substances in the Fluorometric Determination of Vitamin B_6

NAD

Takanashi and Tamura (1969) observed that nicotinamideadeninedinucleotide (NAD) exhibited a fluorescent response with KCN. However, NAD interference would not appear to affect the results of this study, because acid hydrolysis at autoclaving temperature has been shown to destroy NAD.

Metal Ions

Toepfer et al. (1960) found that Dowex AG 50WX-8 ion-exchange column did not remove all of the salts and metallic ions which interferred in the fluorometric determination of vitamin B_6. Later Takanashi and Tamura (1969) confirmed Toepfer's study and reported that metallic ions such as Cu^{+2}, Zn^{+2}, Mg^{+2} and Fe^{+3} effected the fluorometric determination of vitamin B_6. This was of great importance, since most food products contained these interfering metal ions.

Manganese Dioxide

Most of the food samples in this study, analyzed by the fluorometric method, had a low recovery for pyridoxine (Table IVa,b), even though, the pyridoxine standards showed a $\geq$ 99% conversion of pyridoxine to pyridoxal (Fig. 1). The cause of this phenomenon was not known. However, in a system as complex as food, many reactions could have occurred in the presence of oxidizing agents. Manganese dioxide, which was an oxidizing agent used for the conversion of pyridoxine to pyridoxal, could cause oxidation of some substances other than pyridoxine present in the eluate. Thus, the oxidative strength of manganese dioxide would have been reduced. An increase of manganese dioxide concentration from 0.1 g to 0.2 g did not improve the percent recoveries. Further investigation is needed to show whether a greater increase of manganese dioxide will overcome this problem.

Thiamin

Strohecker and Henning (1968) observed that thiamin (vitamin B_1) interferred with the fluorometric determination of vitamin B_6, because thiachrome fluoresced at the same wavelengths as vitamin B_6. They also showed that thiamin in the presence of KCN was capable of partial oxidation to thiochrome. A study of pyridoxal and thiamin standards in this laboratory showed that thiamin was adsorpted to the Dowex AG 50WX-8 ion exchanger and was not eluted in the pyridoxal fraction. These data indicated that thiamin would not be an interfering substance in the fluorometric determination of vitamin B_6, if the ion-exchange chromatography procedure were used to separate the forms of vitamin B_6. The importance of thiamin interference was considered important only for the pyridoxal fraction, because pyridoxamine and pyridoxine blanks could be treated with KCN. Therefore, the presence of thiamin in these fractions and any interaction between thiamin and KCN would have been accounted for in the sample blanks.

Comparison of Vitamin B_6 Values in Selected Food Samples Determined by the Fluorometric Method and Microbiological Method

Pyridoxal values determined fluorometrically were 2-12 times higher than the microbiological values depending on the food sample (Table IIa,b). Analysis of variance of these pyridoxal values showed no difference in the individual products between runs, indicating that each method was

reproducible. Significant differences in pyridoxal values determined by the two methods could have been due to the presence of interfering fluorescent substances or microbial growth inhibitors in the pyridoxal eluate. Failure of the ion exchange column to quantitatively remove interfering fluorescent substances prior to fluorometric determination could have resulted in artificially high pyridoxal values, while vitamin B_6 growth inhibitors would decrease the growth rate of the microorganisms and lower pyridoxal values. Recovery studies of pyridoxal were consistent within each method. This would indicate that the differences were constant within each sample.

Pyridoxamine values obtained by the fluorometric method were approximately two times greater than those determined microbiologically (Table IIIa,b). The presence of interfering substances in the eluate should have been negligible, because pyridoxamine was the last vitamin B_6 fraction to be eluted from the column. The lack of sensitivity of S. carlsbergensis to pyridoxamine (Parrish et al., 1955) could have been a contributing factor to the difference in pyridoxamine values between the two methods.

Pyridoxine values obtained by the fluorometric method and the microbiological method showed an almost one to one ratio, with the fluorometric values slightly higher in some food products (Table IVa,b). The experimental ratio of chemical to microbiological methods was less than one,

however, when the pyridoxine values were corrected for percent recovery, the ratio of the two methods was greater than one.

Sample recoveries which were determined for each sample analyzed could also be responsible for high vitamin B_6 values using the chemical method. Experimentally determined values were corrected according to the percent of recovery obtained, therefore, experimental error occurring in either the sample or the sample recovery could result in erroneous vitamin B_6 values.

CONCLUSIONS

The proposed fluorometric method was shown to be a promising method for the quantitative determination of vitamin B_6 in food products. Based on the lactone formation of pyridoxal in the presence of potassium cyanide, a distinctive fluorophor was obtained. Pyridoxamine and pyridoxine were quantitatively converted to pyridoxal by sodium glyoxalate and manganese dioxide, prior to their conversion to 4-pyridoxic acid lactone.

Acid hydrolysis at autoclaving temperature was proven to be inadequate for samples containing large amounts of carbohydrate or proteins. Non-enzymatic browning and transamination-deamination reactions occurring during autoclaving could interfere with the determination of vitamin B_6.

Dowex AG 50W-X8 ion exchange resin used for the separation of the three biologically active forms of vitamin B_6 failed to remove all of the interfering substances encountered in the fluorometric determination when only 125 ml of rinse buffer was used. The pyridoxal fraction exhibited the greatest interference, because it was the first fraction eluted from the column.

There was no adequate way of determining the pyridoxal sample blank, which accounts for the fluorescence that could be formed from the interaction of components other

than pyridoxal in the food extract with potassium cyanide. This problem was not encountered in the pyridoxamine or pyridoxine sample blanks.

Recoveries of vitamin B_6 obtained by the chemical method showed a narrow range of variation among the same type of food from analysis to analysis.

Microbiological determination of vitamin B_6 in foods yielded recovery values which exhibited a wide range of variation from analysis to analysis. The standard deviation of recovery values obtained by using the fluorometric method was significantly lower than those obtained by microbiological method.

The total vitamin B_6 values obtained by the chemical method were almost twice as much as those obtained by the microbiological method. This phenomenon was observed in all food samples studied except lima beans. No reasonable explanation is presently available for the higher values of vitamin B_6 in food products. However, many shortcomings of the microbiological method resulting in low yields have been shown in previously published data. Chemical method may have increased vitamin B_6 values due to the interference of natural fluorescent substances in food products not accounted for by the blank samples.

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