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IMMUNOCHEMICAL METHODS FOR QUANTITATION OF VITAMIN B₆

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<p>A procedure is described which proposes schemes for determining the total of all B₆ vitamins in acid-hydrolyzed samples utilizing a radio-immunoassay (RIA) or an enzyme-immunoassay (EIA). Sample preparation is similiar for both RIA and EIA. Two specific antibodies (antipyridoxine and antipyridoxamine) are employed to determine pyridoxine, pyridoxamine and pyridoxal. After the determination of pyridoxine and pyridoxamine, a portion of the sample is reduced with sodium borohydride. Pyridoxal is determined by</p>		

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20. Abstract (cont'd)

→ difference between pyridoxine before and after reduction. The results indicate that two procedures have been developed which are selective for pyridoxamine (the fluorescent enzyme immunoassay and the spin immunoassay) and one assay which is equally sensitive to pyridoxine and pyridoxamine (the radio-immunoassay).

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PREFACE

Natick Project Order 78-159 was awarded to the United States Department of Agriculture, Western Regional Research Center (WRRRC) to develop specific methodology for the determination of pyridoxine in foods. At that time WRRRC indicated an interest in the development of a simple unequivocal analytical method for pyridoxine and the expertise to accomplish this formidable project.

Several procedures are available for the determination of pyridoxine which are applicable to pharmaceutical vitamin preparations or require the use of expensive, sophisticated equipment. Too often, methodology developed for pharmaceuticals is not suitable for food, and many laboratories are not equipped with the required instrumentation to obtain specific data after food analyses.

The US Army Natick Research and Development Laboratories (NLABS) have a continual interest in the development of new or the improvement of existing procedures that are specific to biologically available forms of nutrients and can be applied in a modest laboratory to result in reliable, reproducible, unquestionable data. It is hoped that the procedure described herein will respond to these needs.

The authors wish to acknowledge the assistance of Drs. Lawrence L. Layton and John J. Windle who provided guidance and electron spin resonance determinations, respectively, as well as the technical staff and Student Aids who worked on the project. The project was prodigiously assisted by the increased level of technical support. Special thanks are given to NLABS staff members; Ms Edna Albert, Technical Publishing Editor and Mrs. Joyce Barrett and Mrs. Judith Tamburro for preparing this document.

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IMMUNOCHEMICAL METHODS FOR QUANTITATION OF VITAMIN B₆

INTRODUCTION

The aim of this project is to develop an immunoassay for vitamin B₆, preferably a simple assay that could be used for research purposes (monitoring nutritional status of human subjects or experimental animals) as well as for routine analysis of foodstuffs.

The biochemistry and metabolism of vitamin B₆ has been reviewed by Coursin (1975).¹ This vitamin is critically important because of its function as a coenzyme (pyridoxal-5'-phosphate) in many metabolic pathways. Although acute deficiencies of this vitamin result in numerous clinical disorders, such extreme malnutrition is unlikely to be a major problem in the United States. However, recent surveys indicate that some sectors of the U.S. population may be particularly prone to inadequate B₆ nutriture: the elderly (Driskell, 1978)² and pregnant women (Dempsey, 1978),³ for example. Moreover, use of oral contraceptives (Rose, 1978)⁴ and excessive intake of alcohol (Li, 1978)⁵ may increase the requirement for vitamin B₆.

The problem of assaying vitamin B₆ (reviewed in Haskell, 1978),⁶ is complicated by the occurrence of six different vitamers (pyridoxine, pyridoxal, pyridoxamine, and their phosphate esters), as well as two biologically inactive derivatives (pyridoxic acid and its phosphate ester). Pyridoxal is abundant in animal products, whereas pyridoxine is chiefly ingested in plant products. Pyridoxal phosphate and pyridoxamine phosphate are the major stored forms of the vitamin in humans. To determine the quantity of bioavailable vitamin accurately, it is necessary to determine the amounts of each vitamer, or use a bioassay which is equally responsive to the vitamers which are bioavailable to humans.

¹D. B. Coursin, ^VVitamin B₆ and pantothenic acid. *Progr. in Fd. and Nutr. Sci.* 1, 183-190 (1975).

²J. A. Driskell, Vitamin B₆ status of the elderly. *Human Vitamin B₆ Requirements*, 252 (1978).

³W. B. Dempsey, Vitamin B₆ and pregnancy. *Human Vitamin B₆ Requirements*, 202 (1978).

⁴D. P. Rose, Oral contraceptives and vitamin B₆. *Human Vitamin B₆ Requirements*, 193 (1978).

⁵T. K. Li, Factors influencing vitamin B₆ requirement in alcoholism. *Human Vitamin B₆ Requirements*, 210 (1978).

⁶B. E. Haskell, Analysis of vitamin B₆. *Human Vitamin B₆ Requirements*, 61 (1978).



Factors affecting bioavailability of vitamin B₆

Some vitamins and other nutrients in the diet are not effectively metabolized. For example, in a typical American diet, vitamin B₆ (as determined microbiologically) is only 73% bioavailable compared to pure pyridoxine as a standard (Tarr et al., 1979).⁷ The presence of factors which bind vitamins covalently or noncovalently and the presence of substances that inhibit uptake or metabolism of vitamins will influence bioavailability.

The impact of commercial and domestic food processing on nutrient content and availability is dramatic. Schroeder (1971)⁸ reviewed losses of vitamin B₆ in food processing. Losses of 50% or more are common in canning or freezing various foodstuffs. Sterilization of milk products alters the ratio of B₆ vitamers, and different assays can give conflicting results (Davies et al., 1959).⁹ These observations are apparently explained by the conversion of pyridoxal into bis-4-pyridoxyl disulfide, which has a relatively low bioavailability in mammalian assays, compared to microbiological assays (Wendt and Bernhart, 1960; Bernhart et al., 1960).^{10,11} Loss of bioavailable B₆ can also occur through reduction of pyridoximino Schiff's base compounds during cooking and processing (Gregory and Kirk, 1978a).¹² Although the reduced compound is partially available as measured in a rat bioassay, phosphopyridoxyl-BSA exerts an anti-vitamin effect *in vivo* (Gregory and Kirk, 1978b).¹³ In addition to enzymes for which pyridoxal phosphate is a cofactor and the well-known binding capacity of serum albumin, low molecular weight factors that bind vitamin B₆ occur in foodstuffs (Nelson et al., 1977).¹⁴

⁷J. B. Tarr, T. Tamura, and E. L. R. Stokstad, Availability of vitamin B₆ and pantothenic acid in a typical American diet in man. *Fed. Proc.* **38**, 452 (1979).

⁸H. A. Schroeder, Losses of vitamins and trace minerals resulting from processing and preservation of foods. *Am. J. Clin. Nutr.* **24**, 562 (1971).

⁹M. K. Davies, M. E. Gregory, and K. M. Henry, The effect of heat on the vitamin B₆ of milk, II. A comparison of biological and microbiological tests of evaporated milk. *J. Dairy Res.* **26**, 215 (1959).

¹⁰G. Wendt and F. W. Bernhart, The structure of a sulfur-containing compound with vitamin B₆ activity. *Arch. Biochem. Biophys.* **88**, 270 (1960).

¹¹F. W. Bernhart, E. D'Amato, and R. M. Tomarelli. The vitamin B₆ activity of heat-sterilized milk. *Arch. Biochem. Biophys.* **88**, 267 (1960).

¹²J. F. Gregory and J. R. Kirk, Assessment of storage effects on vitamin B₆ stability and bioavailability in dehydrated food systems. *J. Food. Sci.* **43**, 1801 (1978a).

¹³J. F. Gregory and J. R. Kirk, Vitamin B₆ in foods: Assessment of stability and bioavailability. *Human Vitamin B₆ Requirements*, 72 (1978b).

¹⁴E. W. Nelson, C. W. Burgin, and J. J. Cerda, Characterization of food binding of vitamin B₆ in orange juice. *J. Nutr.* **107**, 2128 (1977).

Microbiological assays

Microbiological assays for nutrients have advantages and disadvantages. The advantages include:

1. They may detect all forms of a vitamin, including vitamin bound covalently to other compounds.
2. They may be adaptable to measure vitamins in a crude mixture, unsuitable for analysis by other means.

Among the drawbacks of microbiological assays are the following:

1. Even if an organism is able to use all forms of a vitamin, it may show an unequal growth response to the different forms. Microbes — especially prokaryotes — may not be sensitive to the same forms of a vitamin that are bioavailable to humans (Toepfer and Lehmann, 1961).¹⁵
2. The growth of microorganisms may be inhibited or stimulated by extraneous compounds, making the assay unreliable (e.g., Haskell and Wallnofer, 1967).¹⁶
3. Eukaryotes, although possessing metabolic pathways more similar to humans than prokaryotes, grow relatively slowly, and make microbiological assays time consuming.

The present official method of vitamin B₆ assay adopted by the AOAC (Horwitz et al., 1975)¹⁷ has two major drawbacks. It uses *Saccharomyces uvarum* as test organism, requiring a growth period of 22 hours, in addition to time required to grow the inoculating cultures. Because of the differing abilities of the B₆ vitamers to support growth of the organism (Toepfer et al., 1963),¹⁸ the assay requires prior acid hydrolysis and

¹⁵E. W. Toepfer and J. Lehmann, Procedure for chromatographic separation and microbiological assay of pyridoxine, pyridoxal and pyridoxamine in food extracts. J. Assoc. Off. Anal. Chem. 44, 426 (1961).

¹⁶B. E. Haskell and U. Wallnofer, D-alanine interference in microbiological assays of vitamin B₆ in blood. Anal. Biochem. 19, 569 (1967).

¹⁷W. Horwitz, A. Senzel, H. Reynolds, and D. L. Park, Official Methods of Analysis of the Association of Official Analytical Chemists. 12th ed. 849 (Washington, DC, Assoc. of Off. Anal. Chem.), 1975.

¹⁸E. W. Toepfer, M. M. Polansky, L. R. Richardson, and S. Wilkes, Comparison of vitamin B₆ values of selected food samples by bioassay and microbiological assay. J. Agric. Food Chem. 11, 523 (1963).

chromatographic separation of the three major vitamers. A recently reported modification of the yeast assay (Guilarte and McIntyre, 1979)¹⁹ uses an organism which apparently responds equally to the three biologically active, nonphosphorylated vitamers of B₆.

A microbial assay using *Tetrahymena pyriformis* offers another alternative which may be superior to the *Saccharomyces* assay, since it gives a nearly equal response to the three vitamers (Voigt and Eitenmiller, 1978).²⁰ However, food preservatives and neutralization salts interfered with this protozoan assay (Voigt et al., 1979a).²¹ It is apparent that sample preparation and choice of organism produce considerable variations in the assay results (Voigt et al., 1979b).²²

Chemical assays

Pyridoxal phosphate can be determined colorimetrically in a linked enzyme assay (Haskell and Snell, 1972),²³ affording sensitivity in the 10 ng range. Although fluorometric methods may offer greater sensitivity, biological samples often require purification to avoid interference with fluorometry. Tamura and Takanishi (1970)²⁴ have described a method that involves column chromatography, oxidation, and fluorometric determination of vitamin B₆ as pyridoxic acid. The development of new reagents (e.g., N-methyl-bis-trifluoroacetamide) to make volatile derivatives (Patzner and Hilker, 1977)²⁵ and new procedures for HPLC (e.g.,

¹⁹T. P. Guilarte and P. A. McIntyre, A radiometric assay for all active forms of vitamin B₆ in biological material. J. Nuclear Med. 20, 676 (1979).

²⁰M. N. Voigt and R. R. Eitenmiller, Comparative review of the thiochrome, microbial, and protozoan analyses of B-vitamins. J. Food Protec. 41, 730 (1978).

²¹M. N. Voigt, R. R. Eitenmiller, and G. O. Ware, Vitamin analysis by microbial and protozoan organisms: Response to food preservatives and neutralization salts. J. Food Sci. 44, 723 (1979a).

²²M. N. Voigt, R. R. Eitenmiller, and G. O. Ware, Comparison of protozoan and conventional methods of vitamin analysis. J. Food Sci. 44, 729 (1979b).

²³B. E. Haskell and E. E. Snell, An improved apotryptophanase assay for pyridoxal phosphate. Anal. Biochem. 45, 567 (1972).

²⁴Z. Tamura and S. Takanishi, Fluorometric determination of pyridoxal and pyridoxal-5'-phosphate in biological materials by the reaction with cyanide. Vitamins and Coenzymes 18, 471 (1970), D. B. McCormick and L. D. Wright (Eds.) NY Academic Press.

²⁵E. M. Patzner and D. M. Hilker, New reagent for vitamin B₆ derivative formation in gas chromatography. J. Chrom. 135, 489 (1977).

Wong, 1978),²⁶ suggest that more accurate and sensitive assays will be forthcoming to avoid problems encountered with existing assay systems (Gregory and Kirk, 1977).²⁷ Methods employing nuclear magnetic resonance (Hassan, 1978)²⁸ and electron spin resonance (see section 3, Immunoassays) offer alternative approaches, although the instrumentation is somewhat less widely available.

Use of binding proteins to assay vitamins

Many radioassays using naturally occurring binding proteins have been described, and some commercial kits are available. A few assays are listed below:

Vitamin	Binder	Reference
Biotin	Avidin	Horsburgh and Gompertz (1978) ²⁹
Riboflavin	Apoprotein from egg white	Bashor and Tillotson (1978) ³⁰
Cobalamin	Transcobalamin	Jacob et al. (1977) ³¹
	Toadfish serum	Kubiatowicz et al. (1977) ³²
Folacin	Milk protein (commercial kit)	Ruddick et al. (1978) ³³

²⁶F. F. Wong, Analyses of vitamin B₆ in extractives of food materials by high performance liquid chromatography. J. Agric. Food Chem. 26, 1444 (1978).

²⁷J. F. Gregory and J. R. Kirk, Improved chromatographic separation and fluorometric determination of vitamin B₆ compounds in foods. J. Food Sci. 42, 1073 (1977).

²⁸S. S. M. Hassan, Simultaneous determination of vitamin B₁ and B₆ by nuclear magnetic resonance of spectrometry. J. Assoc. Off. Anal. Chem. 61, 111 (1978).

²⁹T. Horsburgh and D. Gompertz, A protein-binding assay for measurement of biotin in physiological fluids. Clin. Chim. Acta. 82, 215 (1978).

³⁰M. M. Bashor and J. A. Tillotson, Isolation of a riboflavin binding apoprotein from chicken egg white and its use in a radioassay for urinary riboflavin. Fed. Proc. 37, 672 (1978).

³¹E. Jacob, K. T. J. Wong, and V. Herbert, A simple method for the separate measurement of transcobalamins I, II, and III: Normal ranges in serum and plasma in men and women. J. Lab. Clin. Med. 89, 1145 (1977).

³²D. O. Kubiatowicz, D. S. Ithakissios, and D. C. Windorski, Vitamin B₁₂ radioassay with oyster toadfish (*Opsanus tau*) serum as a binder. Clin. Chem. 23, 1037 (1977).

³³J. E. Ruddick, J. Vanderstoep, and J. J. Richards, Folate level in food: A comparison of microbiological assay and radioassay methods for measuring folate. J. Food Sci. 43, 1238 (1978).

However, there are complications in binding assays for vitamins. In some cases, the binding component is not homogeneous — as, for example, kits for B₁₂ which contain a mixture of proteins with differing specificities for various forms of the vitamin (Anon., 1979).³⁴ Naturally occurring binding components in the sample can also interfere with these assays. For example, human milk (Hansen et al., 1977)³⁵ and human serum (Holm et al., 1979)³⁶ both contain folacin-binding proteins. Vitamin-binding components are not limited to fluid tissues of animals, for folate binding is associated with the brush border membranes of rat kidney (Selhub and Rosenberg, 1978),³⁷ and vitamin B₆ binding is found in orange juice (Nelson et al., 1977).¹⁴

Antibodies for vitamin assay

Antibodies directed against folic acid and its closely related analogue, methotrexate, were described by Jatton and Ungar-Waron (1967).³⁸ The antimethotrexate antibodies bound this drug selectively, but antibodies elicited against folate discriminated poorly between the two compounds. It is possible, however, that more highly purified antibodies would show greater specificity. Endres et al. (1978)³⁹ have described a radioimmunoassay for vitamin B₁₂, using the [¹²⁵I]-tyrosine methyl ester of cobalamin as ligand in a competitive binding assay. The assay can measure 10 ng of B₁₂, or about 10⁻⁸ M. Other binding assays for this vitamin employ ⁵⁷Co as label, but the iodine permits higher specific activity with a lower level of radiated energy. A radioimmunoassay for pantothenic acid has recently been introduced (Howe

³⁴Anon. Pitfalls in the diagnosis of vitamin B₁₂ deficiency by radiodilution assay. *Nutr. Revs.* 37, 313 (1979).

³⁵S. I. Hansen, J. Holm, and J. Lyngbye, Folate binding by human milk protein. *Scand. J. Clin. Lab. Invest.* 37, 363 (1977).

³⁶J. Holm, S. I. Hansen, and J. Lyngbye, High and low affinity binding of folate in human serum. *Acta. Physiol. Scand.* 105, 64A (1979).

³⁷J. Selhub and I. H. Rosenberg, Demonstration of high affinity folate binding activity associated with the brush border membranes of rat kidney. *Proc. Natl. Acad. Sci. U.S.A.* 75, 3090 (1978).

³⁸J. C. Jatton and H. Ungar-Waron, Antibodies to folic acid and methotrexate obtained with conjugates of synthetic polypeptides. *Arch. Biochem. Biophys.* 122, 157 (1967).

³⁹D. B. Endres, K. Painter, and G. D. Niswender, A solid-phase radioimmunoassay for vitamin B₁₂ in serum, with use of radioiodinated tyrosine methyl ester of vitamin B₁₂. *Clin. Chem.* 24, 460 (1978).

et al., 1979),⁴⁰ and this assay compares favorably with the conventional microbiological assay for quantitating pantothenate in foodstuffs. The potential of immunoassay is not limited to the water-soluble vitamins. Indeed, radioimmunoassays have recently been reported for vitamin A (Westfall et al., 1979)⁴¹ and vitamin D (Clemens et al., 1978).⁴²

Antibodies directed against vitamin B₆

Ungar-Waron and Sela (1966)⁴³ elicited antibodies directed against pyridoxal, using as antigen pyridoxal coupled to a synthetic polypeptide, poly-DL-alanine on a poly-L-lysine backbone. The antibodies bound pyridoxal, pyridoxal phosphate, pyridoxamine, and pyridoxine. The antibodies inhibited glutamate-oxaloacetate transaminase, presumably by binding the pyridoxal phosphate cofactor. In contrast, Cordoba et al, (1966)⁴⁴ were unable to obtain inhibition of aspartate aminotransferase with anti-pyridoxal phosphate. However, Cordoba et al. (1966 and 1970)^{44,45} reported that their antisera bound phosphate esters of pyridoxal and pyridoxamine much more strongly than the nonphosphorylated vitamins, suggesting that the phosphate ester was immunodominant. Consequently, such antibodies would not be likely to interact with the coenzyme in the active site of its enzyme. Both groups of workers reported that their antisera bound pyridoxamine more strongly than pyridoxal. These results are consistent with our own, and are not surprising in view of the hapten — a reduced Schiff's base, which is a derivative of pyridoxamine, rather than pyridoxal.

⁴⁰J. Howe, B. W. Wyse, and R. G. Hansen, Measurement of pantothenic acid in nursing home meals using a microbiological assay and a radioimmunoassay. *Fed. Proc.* **38**, 452 (1979).

⁴¹S. S. Westfall and G. H. Wirtz, Vitamin A radioimmunoassay. *Fed. Proc.* **38**, 874 (1979).

⁴²T. L. Clemens, G. N. Hendy, R. F. Graham, E. G. Baggiolini, M. R. Uskokovic, and J. L. H. O'Riordan, A radioimmunoassay for 1, 25-dihydroxycholecalciferol. *Clin. Sci. Mol. Med.* **54**, 329 (1978).

⁴³H. Ungar-Waron and M. Sela, Pyridoxal-specific antibodies obtained with a synthetic pyridoxal-polypeptide conjugate. *Biochim. Biophys. Acta* **124**, 147 (1966).

⁴⁴F. Cordoba, C. Gonzalez, and P. Rivera, Antibodies against pyridoxal 5-phosphate and pyridoxamine 5-phosphate. *Biochim. Biophys. Acta* **127**, 151 (1966).

⁴⁵F. Cordoba, P. Rivera, J. Calderon, and C. Agundis, Immunological specificity of antibodies toward pyridoxal and pyridoxal 5-phosphate. *Immunochem.* **7**, 543 (1970).

Raso and Stollar (1975)⁴⁶ elicited anti-pyridoxyl antibodies against three derivatives of pyridoxamine phosphate, as part of a study of the mechanism of pyridoxal phosphate mediated catalysis. As found by the Cordoba group, the antibodies were specific for phosphorylated vitamers, but did not discriminate well between pyridoxal phosphate and pyridoxamine phosphate. Deoxypyridoxine phosphate bound to the antibodies only slightly more weakly. These results are consistent with the immunodominance of the phosphate ester, and indicate that phosphorylated antigens are unlikely to elicit antibodies which discriminate well among vitamers differing at the four position. This conclusion is consistent with the recent report by Thanassi and Cidlowski (1979),⁴⁷ who also found that anti-pyridoxal phosphate antibodies bind nonphosphorylated vitamers weakly.

ORGANIC CHEMISTRY

1. Derivatives of pyridoxine

Rationale. We hypothesized that hapten conjugates having their unique features most exposed to the immune system would be most likely to elicit specific antibodies. This has been the guiding principle of the organic chemistry component of this project.

We have prepared derivatives which enable pyridoxine and related vitamers to be coupled to protein or to small molecules (enzyme substrates or molecules which can be radiolabeled) through five of the positions on the pyridine ring of pyridoxine.

a. Derivatization of the 1 position

Pyridoxine derivatives containing a functional group in the 1 position are useful haptens since they can be coupled to immunogenic carriers with the biologically active end of the molecule unhindered. We have tried a number of approaches to the synthesis of N-carboxymethylpyridoxine (V, Figure 1). Our initial attempts to prepare N-carboxymethylpyridoxine betaine (CMP) resulted in impure product in low yield. We therefore undertook thorough consideration of the reaction conditions.

The only quaternary salt of pyridoxine reported in the literature is the methiodide (VII, Figure 2; Harris, et al., 1944).⁴⁸ Substituents in the 2 and 6 positions of the pyridine ring

⁴⁶V. Raso and B. D. Stollar, Antibody-enzyme analogy. Comparison of enzymes and antibodies specific for phosphopyridoxyltyrosine. *Biochem.* **14**, 584 (1975).

⁴⁷J. W. Thanassi and J. A. Cidlowski, Radioimmunoassay for phosphorylated forms of vitamin B₆ using a matrix bound antibody. *Fed. Proc.* **38**, 644 (1979).

⁴⁸S. A. Harris, D. Heyl, and K. Folkers, The vitamin B₆ group. II. The structure and synthesis of pyridoxamine and pyridoxal. *J. Am. Chem. Soc.* **66**, 2088, (1944).

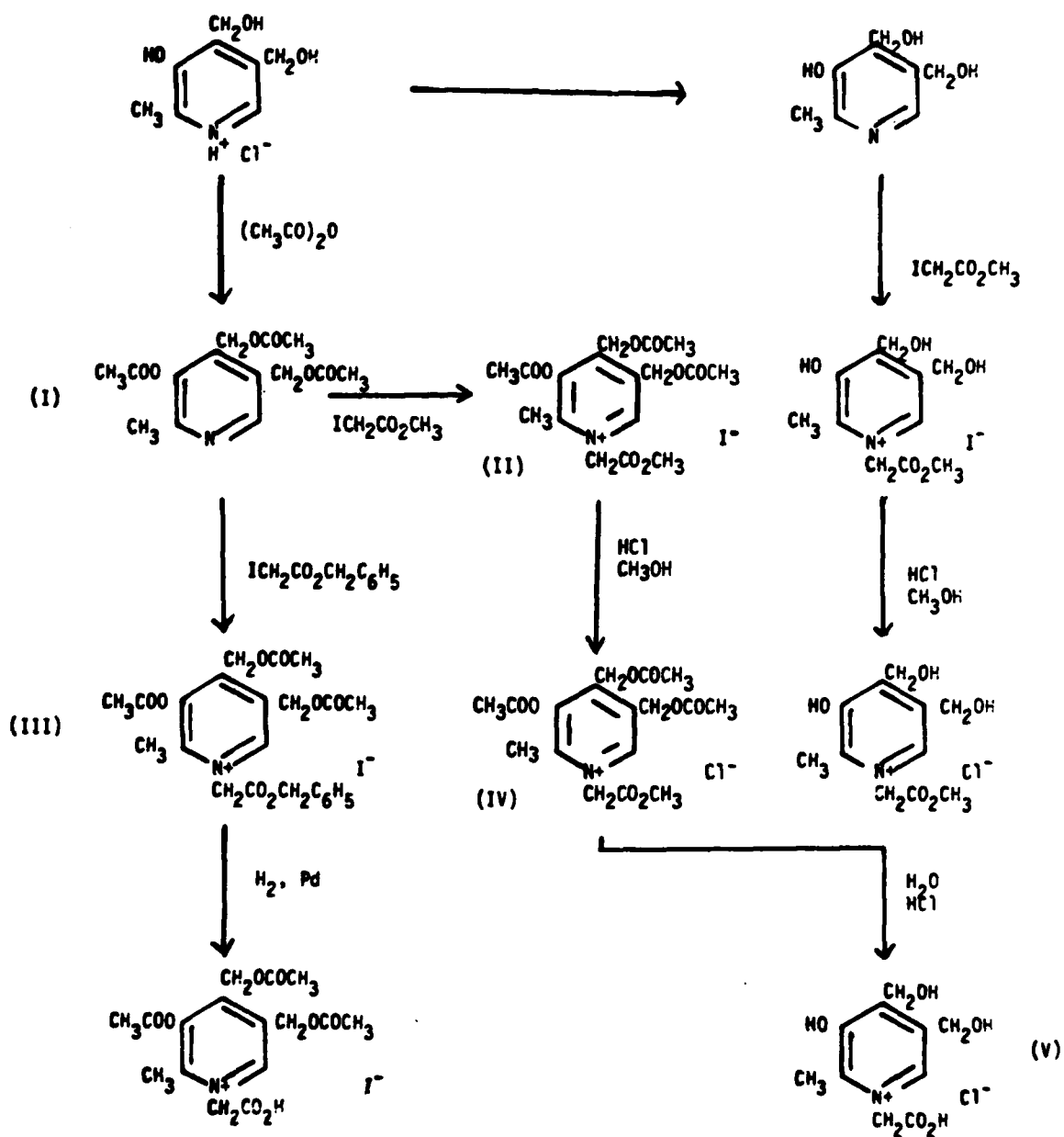


Figure 1. Synthesis of N-Carboxymethylpyridoxine

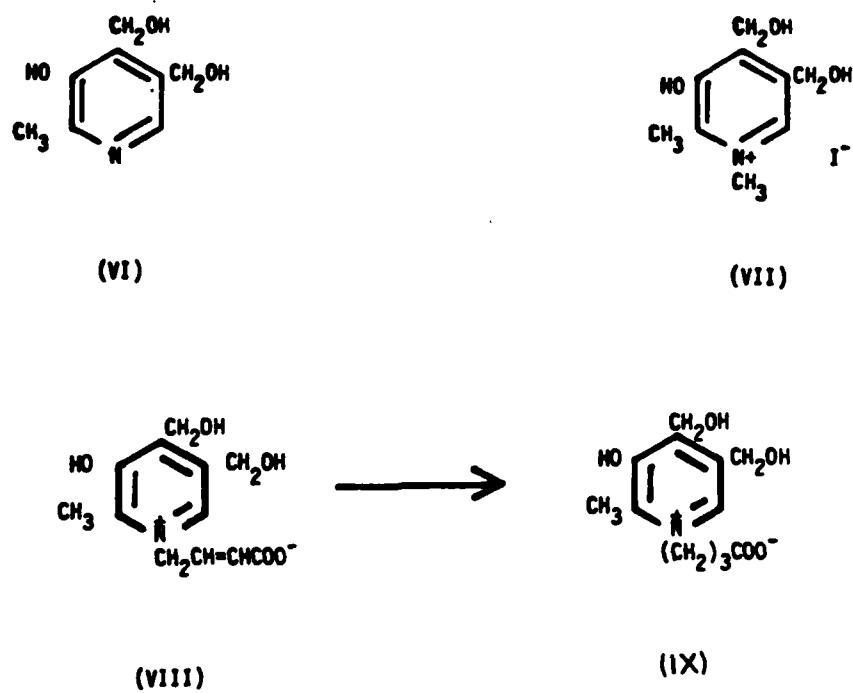


Figure 2. Pyridoxine and N-Substituted Derivatives

hinder the formation of quaternary salts (Kirpal, 1910).⁴⁹ We found this to be true with the B₆ vitamers, and believe that the poor yield of CMP can be attributed to steric hindrance. However, the alkylation of amines — the Menshutkin reaction — is very susceptible to solvent effects (Parker, 1969; Abraham, 1971).^{50,51} Since the intermolecular S_N2 reactions (like the alkylation of pyridoxine) are favored by the use of aprotic dipolar solvents, we repeated the reaction in dimethylformamide (DMF).

Pyridoxine and pyridoxine triacetate (I, Figure 1) synthesized essentially according to Harris, 1940,⁵² react reasonably well with methyl iodoacetate and benzyl iodoacetate in DMF to yield, respectively, N-carbomethoxymethylpyridoxonium iodide (II) and N-carbobenzyloxy-methyl-3,4,5-triacetylpyridoxonium iodide (III).

Synthesis of derivative V. A solution of 6.0 g (0.02 mole) of compound I and 10 g (0.05 mole) of methyl iodoacetate in 50 mL of DMF were allowed to stand at room temperature for two weeks. The volatiles were removed at 0.05 mm (50°C), and the residue was poured into one L of water (40°C). The oily material was removed by extraction with ethyl acetate, and the aqueous residue was evaporated to dryness (6.2 g). The iodide ion was conveniently exchanged with chloride ion by treatment with methanolic HCl (Kaminsky, et al., 1978).⁵³ Methanol (100 mL) containing anhydrous HCl (5.4 g) was added to the reaction mixture, and the solution was refluxed for 10 min on a steam bath. The condenser was removed and the methanol was allowed to evaporate. The small amount of iodine was removed by washing the gum with chloroform.

Hydrolysis of the acetyl and methyl ester group was effected by refluxing with dilute hydrochloric acid, as follows. The residue was dissolved in 500 mL water containing 25 mL concentrated HCl and heated on a steam bath overnight. The water was removed *in vacuo*, and the residue (3.6 g) was dissolved in 25 mL of water and applied to a 2.5- x 45-cm column of Dowex AG-50X8 (200 to 400 mesh, H⁺ form). The column was eluted with water until the effluent was neutral. The eluent was then changed to 0.17 N ammonium hydroxide. Fractions containing material absorbing at 275 nm were collected and evaporated to dryness. Trituration with alcohol gave crystals that were recrystallized from ethanol-ethyl acetate (m.p. 186°C).

⁴⁹A. Kirpal, Über Betainbildung und sterische Hinderung. Monatsh. 31, 969 (1910).

⁵⁰A. J. Parker, Protic-dipolar aprotic solvent effects on rates of bimolecular reactions. Chem. Rev. 69, 1 (1969).

⁵¹M. H. Abraham, Substitution at saturated carbon. Part VIII. Solvent effects on the free energy of trimethylamine, the nitrobenzyl chlorides, and the trimethylamine-nitrobenzyl chloride transition states. J. Chem. Soc. (B), 299, 1971.

⁵²S. A. Harris, Chemistry of vitamin B₆. II. Reactions and derivatives. J. Am. Chem. Soc. 62, 3203 (1940).

⁵³J. J. Kaminski, K. W. Kuntson, and N. Bodor. A convenient method of anion exchange in quaternary salts. Tetrahedron 34, 2857 (1978).

Results of elemental analysis:

Calculated for $C_{10}H_{13}NO_5$: C, 52.86; H, 5.77; N, 6.17.

Found: C, 52.5; H, 5.85; N, 6.06.

NMR (TFA): δ 2.7(s, 3H), 4.95(s, 2H), 5.33(s, 2H), 5.48(s, 2H), 8.33(s, 1H).

Synthesis of derivative III. A mixture of 1 g (0.0034 moles) of compound I, Figure 1, and 0.93 g of benzyl iodoacetate were dissolved in 20 mL of ether. Crystals began to appear after six days and the mixture was allowed to stand for another two weeks. The crystals were collected and washed with ether (m.p. 150°C).

Results of elemental analysis:

Calculated for $C_{23}H_{26}INO_8$: C, 48.35; H, 5.49; N, 2.45.

Found: C, 47.5; H, 4.73; N, 2.21.

NMR (DMSO): δ 2.0(s, 3H), 2.13(s, 3H), 2.5(s, 3H), 2.63(s, 3H), 5.29(s, 4H), 5.45(s, 2H), 6.07(s, 2H), 7.41(m, 5H), 9.32(s, 1H).

A related compound, pyridoxine methiodide (VII, Figure 2), was also synthesized. This synthesis served as a model for the preparation of N-carboxymethylpyridoxine described above. The methiodide will be useful in assessing the specificity of anti-pyridoxine antibodies.

Absorption spectra of CMP and the methiodide were obtained in phosphate-buffered saline, pH 7.0. Extinction coefficients were computed for the absorption maxima in the 330-nm region:

N-carboxymethylpyridoxine $\epsilon_{M, 333} = 8.45$

Pyridoxine methiodide $\epsilon_{M, 330} = 8.19$

Since the spacing of hapten from its protein carrier backbone can be important in influencing specificity of the immune response, it may be that a longer spacer is needed for an effective pyridoxine immunogen. To this end, we have synthesized N-pyridoxine-4'-crotonic acid (VIII, Figure 2) and are currently in the process of purification of the product. This compound may be reduced to the butyric acid derivative (IX). Either VIII or IX can be conjugated to carrier protein to prepare an immunogen.

Synthesis of derivative VIII, Figure 2. A mixture of 8.19 g (0.028 mole) of compound I and 14.3 g (0.074 mole) of ethyl 4-bromoacetate in 50 mL of dry dimethylformamide was allowed to stand one week at room temperature. The solvent was removed *in vacuo* (0.01 mm) and the residue was poured into 100 mL of water (50°C). The small amount of oily material was extracted with ethyl acetate. Ten milliliters of HBr (48%) was added to the aqueous layer and it was refluxed for 0.5 hr and evaporated to dryness. The dark residue was dissolved in 50 mL of water, treated with decolorizing carbon, filtered and applied to a 2.5 x 45 cm column of Dowex AG-50X8 (200-400 mesh, H^+ form). The column was eluted with water until the eluate was neutral, and then the elution was continued with 0.15 N NH_4OH . Fractions

containing the substituted betaine (material absorbing at 275 nm) were evaporated *in vacuo* and 100 mL 2N HCl was added and again evaporated. The resulting dark gum did not crystallize after several weeks. It was then dissolved in hot 100% alcohol, decolorized, and treated with ether. The resulting floc was collected and dried (yield 1.8 g). The NMR spectrum was complex and indicated that *cis-trans* isomers and an unknown contaminant were present.

b. Derivatization of the 2 position

2-Norpyridoxine. Before finding proper and suitable conditions for synthesis of CMP (described above), we proceeded with an alternate pathway to a useful hapten. Since the 2-methyl substituent of pyridoxine interferes with alkylation of the ring nitrogen, we expected that derivatives of 2-norpyridoxine (XI, Figure 3) would be more readily accessible synthetically. We reasoned that these derivatives would still possess the important immunological determinants needed to elicit specific anti-pyridoxine antibodies. We prepared 2-norpyridoxine essentially as described by Doktorova et al. (1969).⁵⁴ N-formylglycine ethyl ester was cyclized with phosphorus pentoxide to form compound X, which was reacted with dimethyl maleate and reduced with lithium aluminum hydride. Since we obtained CMP in sufficient yield and purity, we have not as yet found it necessary to use the norpyridoxine in chemical synthesis.

2-Carboxyvinyl-3-O-benzyl-4,5-acetonyl-norpyridoxine (XIII, Figure 4). The starting material for synthesis of this compound was 3-O-benzyl-4,5-acetonyl-2-formylpyridoxine (XII, Figure 4), which was prepared as described by Korytnyk, et al. (1973).⁵⁵

A mixture of 1.45 g (0.0046 mole) of compound XII, Figure 4, and 0.76 g of malonic acid in 5 mL of dry pyridine, to which a drop of piperidine had been added, was warmed overnight at 60°C. The solvents were removed in a rotary evaporator. The residue was dissolved in potassium bicarbonate solution, filtered, and the solution acidified to about pH 4.5. The crystals were collected and dried (Yield: 0.66 g; m.p. 163–165°C). The NMR spectrum was consistent with the indicated structure.

NMR (DMSO): δ 1.40(s, 6H), 4.81(s, 6H), 6.8(d, H, $J=13.5$ Hz) 7.42(m, 5H), 7.67(d, H, $J=15$ Hz), 8.24(s, 1H).

⁵⁴N. D. Doktorova, L. V. Ionova, M. Y. Karpeisky, N. S. Padyukova, K. F. Turchin, and V. I. Florentiev. *Tetrahedron* **25**, 3527 (1969).

⁵⁵W. Korytnyk, S. C. Srivastava, N. Angelino, P. G. G. Potti, and B. Paul. A general method for modifying the 2-methyl group of pyridoxol. Synthesis and biological activity of 2-vinyl and 2-ethynyl-pyridoxols and related compounds. *J. Med. Chem.* **16**, 1096 (1973).

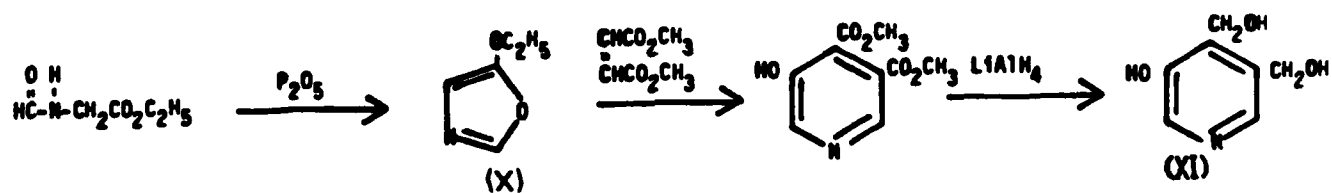


Figure 3. Synthesis of 2-Norpyridoxine

2-Carboxyvinyl-3-benzylorpyridoxine (XIV, Figure 4). A solution of 0.5 g (0.0014 mole) of compound XII in 10 mL of trifluoroacetic acid was allowed to stand at room temperature for three hours. The TFA was removed *in vacuo*. Twenty mL methanol was added, and it was removed *in vacuo*. This was repeated. The residue was taken up in 3 mL H₂O, and 5 mL 4N HCl was added. This was removed *in vacuo* and the crystals that separated were recrystallized from methanol-ether-petroleum ether (m.p. 141–151°C).

NMR (DMSO): δ 4.63(s, 2H), 4.77(s, 2H), 4.97(s, 2H), 6.97(d, H, J=15), 7.43(m, 5H), 7.70(d, H, J=15), 8.54(s, H).

α^2 -Aminopyridoxine (XV, Figure 4). The synthesis of this compound was performed essentially as described by Korytnyk et al. (1977)⁵⁶ as outlined in Figure 4. The amino group introduced will enable synthesis of immunogenic conjugates, iodinated ligands, and ligands for fluorogenic EIA and spin immunoassay.

c. Derivatization of the 3 position

The synthesis of 3-oxaliphatic acids derived from pyridoxal and pyridoxine was completed in accordance with the scheme in Figure 5. These compounds differ from N-carboxymethylpyridoxine in the position of substitution and in charge.

3-O-(3'-Carboxypropyl)pyridoxal ethyl acetal (XVI, Figure 5). A mixture of 3.48 g (0.015 mole) of pyridoxal ethyl acetal HCl, 3.66 g (0.03 mole) of potassium t-butoxide, and 100 mg of 18-crown-6-ether in 50 mL of dimethylformamide was stirred for 0.5 h at room temperature. A solution containing 3.45 g (0.015 mole) of ethyl 4-iodobutyrate was added and stirring was continued at 30°C overnight. The reaction mixture was no longer basic and the solvent was removed at 0.05 mm. The residue was treated with 100 mL of water and extracted twice with 50 mL of ether. The ether extracts were washed with water and the ether removed *in vacuo*. The residual oil was saponified with a solution of 15 mL 4N NaOH and 25 mL of ethanol by standing overnight at room temperature. The ethanol was removed *in vacuo*, the residual liquid was made up to about 20 mL with H₂O and then carefully adjusted to pH 5.5 with 4N HCl. After cooling, the crystals that formed were collected, washed with ice water, dried, and recrystallized from ethanol-ether (m.p. 144–146°C).

Results of elemental analysis:

Calculated for C₁₄H₁₉NO₅: C, 59.78; H, 6.81; N, 4.98.
Found: C, 59.7; H, 6.77; N, 4.97.

The NMR spectrum is indicative of the correct structure.

NMR (DMSO): δ 1.14(t, 3H), 2.0(m, 2H), 3.48–3.82(br m, 2H), 4.02–4.31(br m, 2H) 5.0(s, 2H), 6.42(s, 1H), 8.12(s, 1H).

⁵⁶W. Korytnyk and P. G. G. Potti, Antagonists of vitamin B₆. Simultaneous and stepwise modification of the 2 and 4 positions. J. Med. Chem. 20, 1 (1977).

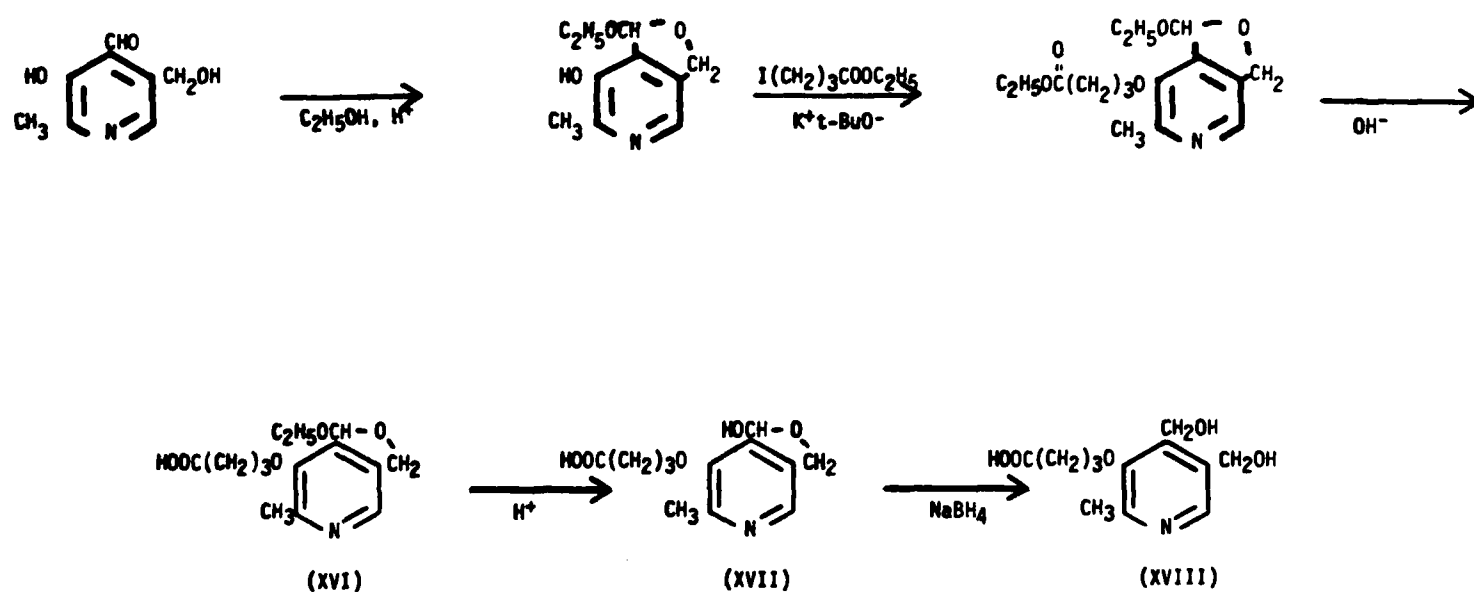


Figure 5. Derivatives of Pyridoxine Substituted in the 3 Position

3-O-(3'-Carboxypropyl)pyridoxal hydrochloride (XVII, Figure 5). Two grams (0.007 moles) of compound XVI — Figure 5, was heated on a steam bath with 10 mL N HCl for two hours and then allowed to evaporate to dryness. The crystals were recrystallized from methanol-ether (m.p. 105–108°C).

NMR (D_2O): 2.03–2.3(br m, 2H), 2.52(s, 1H), 2.58(s, 1H), 2.67(s, 3H),
4.27–4.6(br m, 2H), 5.23(d, 2H, $J=4.5$ Hz), 6.87(s, 1H),
8.26(s, 1H).

3-O-(3'-Carboxypropyl)pyridoxine hydrochloride (XVIII, Figure 5). A solution of 0.076 g (2 mmoles) of sodium borohydride in 5 mL of water was added slowly with stirring to a solution of 0.58 g (2 mmoles) of compound XVII — Figure 5 in 0.4 g potassium bicarbonate in 10 mL of H_2O . After 30 minutes, 5 mL of acetone was added and the solvents were removed *in vacuo*. Ten mL of N HCl was added and the solution evaporated to dryness *in vacuo*. The residue was extracted with two 20-mL portions of hot ethanol. The extract was filtered and evaporated. The resulting crystalline hydrochloride was recrystallized from methanolether (m.p. 150–158°C). The NMR spectrum is consistent with the structure.

NMR ($TFAH$): δ 2.36(m, 2H), 2.88(m, 5H), 4.2(m, 2H), 5.27(s, 4H),
8.67(s, 1H).

d. Derivatization of the 4 position

No derivatives were prepared, but two naturally occurring products — pyridoxal (and its phosphate ester) and pyridoxic acid — have functional groups which can be used for coupling (see below).

e. Derivatization of 5 position

α^5 -Pyridoxylformic acid (XIX, Figure 6). This compound was prepared by the method of Tomita et al. (1967),⁵⁷ as outlined in Figure 6.

NMR (DMSO): δ 8.10(s, 1H), 4.75(s, 2H), 2.92(s, 2H), 2.6(m, 5H)

α^5 -Pyridoxylacetic acid (XX, Figure 6). This compound was prepared by the method described for the analogous formic acid above.

NMR (DMSO): δ 7.90(s, 1H), 5.40(s, 2H), 3.73(s, 2H), 2.40(s, 3H).

⁵⁷ I. Tomita, H. G. Brooks and D. E. Mettler, Synthesis of vitamin B₆ derivatives. II. 3-Hydroxy-4-hydroxymethyl-2-methyl-5-pyridine. J. Heterocyclic Chem. 3, 178 (1966).

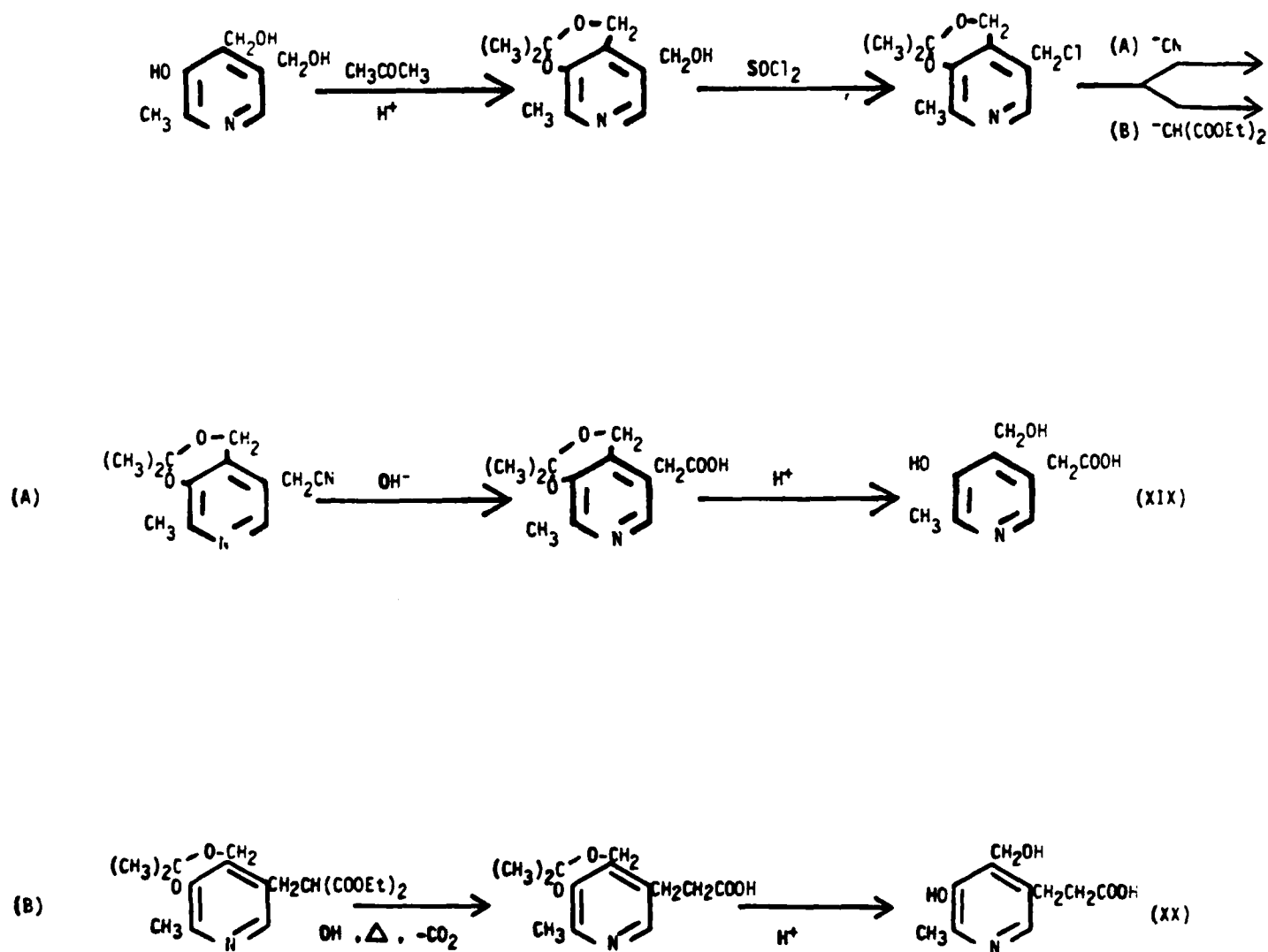


Figure 6. Synthesis of α^8 -Pyridoxylformic Acid and α^8 -Pyridoxylacetic Acid

2. Fluorogenic substrates for enzyme immunoassay

Although we have performed enzyme-linked immunoassays using labeled enzyme (see Immunoassays Section), there are reasons to prefer a substrate-labeled assay system. With an enzyme-labeled assay, protein-protein interactions between enzyme and antibody, favored by divalent binding, could limit the sensitivity. However, the sensitivity of the substrate-labeled assay depends solely on the antibody binding constant for the hapten. Consequently, we are in the process of preparing suitable substrates. The key compound, β -(7-(3-carboxycoumarinoxy))-D-galactoside (XXI, Figure 7) has been synthesized and purified (it is not presently commercially available). This compound has a carboxyl group available for coupling to amino compounds. We are proceeding by coupling the coumarin derivative to diaminopropane, thus introducing a free amino group. The final step will be to couple CMP or other haptens to the derivatized galactoside by means of an amide linkage (XXII).

A fluorogenic derivative of pyridoxamine (XXIII) was prepared using a carbodiimide reagent. Briefly, 50 μ moles of compound XXI as the potassium salt was activated with 50% molar excess of 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide (EDAC) in aqueous solution, pH 4. A two-fold molar excess of pyridoxamine (at pH 4.0) was added, and the mixture was stirred for two hours. A white precipitate formed gradually, and was recovered, washed with 0.05 M ammonium formate, and lyophilized. The product was dissolved in dimethylformamide and analyzed in appropriate thin-layer chromatography systems. The results indicated one major product, distinguishable from the starting materials and active as a substrate in the fluorogenic assay of β -galactosidase. The NMR spectrum indicated that the product was impure. Purification of the product and preparation of a larger batch of substrate is in progress. The preparation of a substrate synthesized using compound α^5 -aminopyridoxine (XV) is in progress.

3. Tritiated ligands for radioimmunoassay

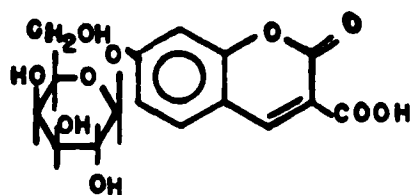
a. ^3H -pyridoxine

^3H -pyridoxine synthesized by catalytic exchange was purchased from a commercial supplier (Amersham). The compound was analyzed by thin-layer chromatography (TLC), and a considerable amount of radioactive impurities was found. The compound was used without purification in a radioimmunoassay for pyridoxine-specific antibodies (see below). It was later purified by preparative TLC for use in an RIA for pyridoxine (see below).

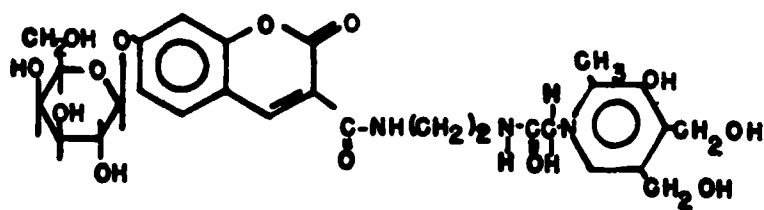
^3H -pyridoxine was also synthesized in our laboratory by reduction of pyridoxal with sodium borotritide (NaBT_4). The product was analyzed by TLC, which indicated greater than 90% radiochemical purity.

b. ^3H -pyridoxamine

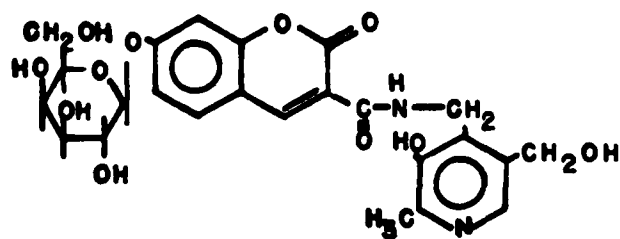
^3H -pyridoxamine was custom synthesized commercially by catalytic reduction of the oxime of pyridoxal.



(XXI)



(XXII)



(XXIII)

Figure 7. Fluorogenic Galactosides

4. Iodinated ligands for radioimmunoassay

Gamma-labeled ligands have some advantages over beta-labeled compounds for RIA. The sample preparation for gamma counting is less complex (assuming a gamma counter is available), and the radiation is less prone to quenching than weak beta emitters such as tritium. However, half-lives are generally shorter. Consequently, reagent shelf-life is generally limited. Also, radiological hazards are often greater, and shielding is required when gamma labels are used in quantity.

A derivative of pyridoxine suitable for coupling to an iodlatable compound (e.g., hydroxybenzoic acid) has been prepared (XV, Figure 4). Synthesis of an iodlatable ligand has not yet been completed.

5. Paramagnetic ligands for spin immunoassay

Pyridoxamino derivatives of two spin labels were prepared as outlined in Figure 8. The preparation of the piperidine-1-oxyl compound (XXIV) is described in detail.

Pyridoxylidene-4-amino-2,2,6,6-tetramethylpiperidine-1-oxyl. A mixture of 2.03 g (0.01 mole) of pyridoxal HCl, 1.72 g (0.01 mole) of 4-amino-2,2,6,6-tetramethylpiperidine-1-oxyl, 1.4 g of potassium carbonate, 20 mL of H₂O, 20 mL of ethyl acetate, and 100 mg of Adogen was stirred overnight at room temperature. The organic layer was separated, and the aqueous layer was extracted with an equal volume of ethyl acetate. The combined extracts were washed with water, dried, and evaporated. The resulting crystals were recrystallized from methyl acetate-petroleum ether (m.p. 161–163°C).

Results of elemental analysis:

Calculated for C ₁₇ H ₂₆ N ₃ O ₃ :	C, 63.72; H, 8.18; N, 13.11.
Found:	C, 63.8; H, 8.2; N, 13.0.

4-N-[4'-(2',2',6',6'-Tetramethylpiperidine-1-oxyl)]-pyridoxamine. A solution of 0.15 g (0.004 mole) of sodium borohydride in 3 mL of water was added to a solution of 0.64 g of pyridoxylidene-4-amino-2,2,6,6-tetramethylpiperidine-1-oxyl in 10 mL of 50% ethanol. After 30 minutes, 5 mL of acetone was added and the solvents were removed *in vacuo*. The product was recrystallized from methyl acetate-petroleum ether (m.p. 158–159°C).

Results of elemental analysis:

Calculated for C ₁₇ N _{2.8} N ₃ O ₃ :	C, 63.33; H, 8.75; N, 13.03.
Found:	C, 63.4; H, 8.69; N, 12.9.

IMMUNOCHEMISTRY

1. Preparation of Immunogens

a. Conjugates of pyridoxal

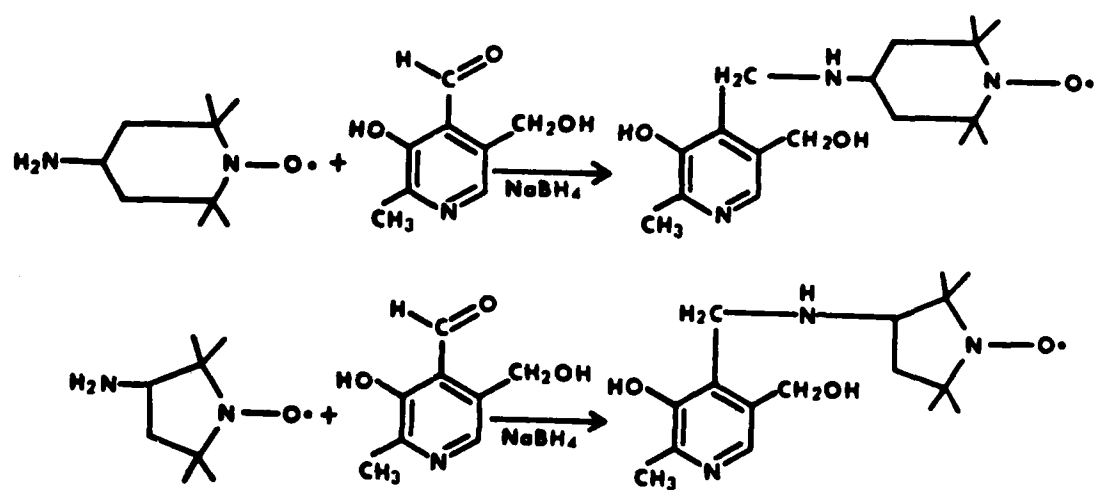


Figure 8. Synthesis of Pyridoxyl Spin Labels

Conjugates of keyhole limpet hemocyanin (KLH) or BSA were prepared as illustrated in Figure 9. The dependence of the degree of conjugation on reaction conditions has been studied. Test antigens for use in analysis of antisera by immunodiffusion have been prepared similarly. A spectrum of a typical conjugate is illustrated in Figure 10.

b. Conjugates of N-carboxymethylpyridoxine

The procedure for coupling CMP to proteins is illustrated in Figure 11. The extinction coefficients for CMP and pyridoxine methiodide were used to estimate the degree of conjugation of bovine serum albumin, KLH, and horse gamma globulin (HGG), following the carbodiimide-mediated reaction with CMP. A number of experiments, using 90–95% pure CMP produced degrees of conjugation in the range of 5 to 7 moles CMP per mole protein. Variation of reaction conditions (time, temperature, concentration) has little effect on the degree of coupling, and appearance of a new chromophore (perhaps a degradation product of CMP) was observed at temperatures above 35°C. When purified, crystalline CMP became available, the experiments were repeated, and a high degree of coupling (ca. 25 moles hapten per mole protein) was obtained reproducibly, with both BSA and HGG. A spectrum of the BSA conjugate (BSA–CMP) is shown in Figure 12.

c. Conjugates of α^5 –pyridoxylformic acid (PFA)

Typical conditions for conjugation to BSA or KLH are described. Fifty mg of crystalline PFA was dissolved in 3.5 mL of warm water, and the pH was adjusted to 4 with 1 N NaOH. Fifty mg of EDAC was added, and the solution was stirred for thirty minutes at room temperature. One mL of BSA or KLH solution in water (30 mg/mL) was added, and the mixture was stirred in the dark overnight. Saturated ammonium sulfate solution was added until a copious protein precipitate formed. After the mixture was stirred for about 15 minutes the precipitate was collected by centrifugation. The precipitate was dissolved in phosphate-buffered saline (PBS). The precipitation procedure was repeated, and the redissolved protein was dialyzed against PBS. The UV-visible absorption spectra were recorded on a Cary 15 spectrophotometer (Figure 13). This procedure results in a conjugation ratio of approximately 20 moles hapten per 100,000 g of protein.

d. Conjugates of pyridoxal with lipopolysaccharide (LPS)

Rationale. Although protein-hapten conjugates are immunogenic, we have also explored the use of mitogen-hapten conjugates as immunogens. Mitogen-hapten conjugates have been used in experimental studies of the immune response in the mouse (Moller et al., 1975; Jacobs and Morrison, 1975),^{58,59} but we are not aware of any practical application of the technique.

⁵⁸G. Moller, A. Coutinho, and U. Persson, Mechanism of B-lymphocyte activation: Failure to obtain evidence of a direct role of the Ig receptors in the triggering process. *Scand. J. Immunol.* 4, 37 (1975).

⁵⁹D. M. Jacobs and D. C. Morrison, Stimulation of a T-independent primary anti-hapten response *in vitro* by TNP–lipopolysaccharide (TNP–LPS). *J. Immuno.* 114, 360 (1975).

SYNTHESIS OF PROTEIN CONJUGATE FOR ANTIBODY DETECTION

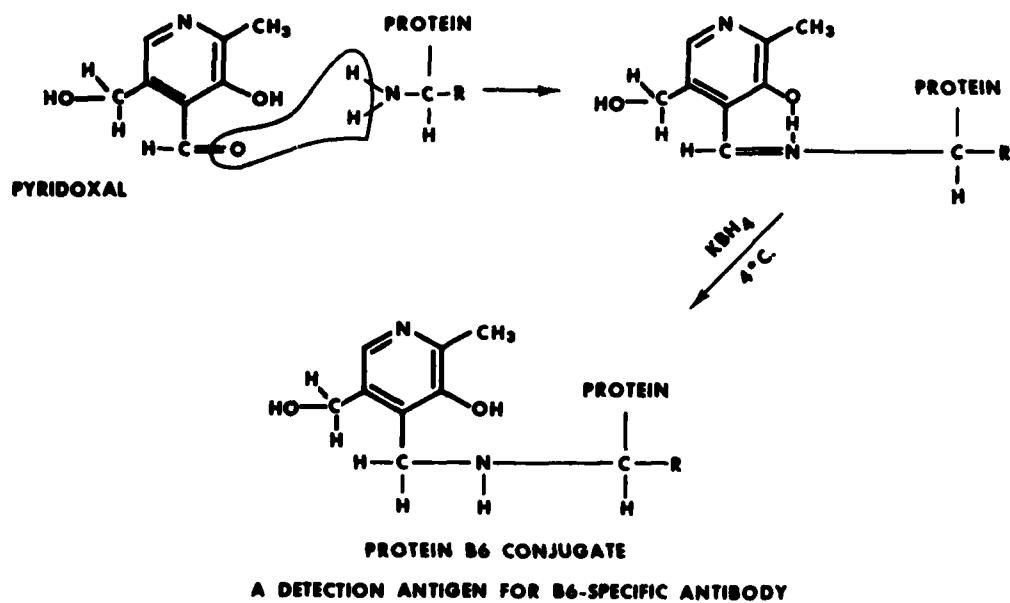


Figure 9. Synthesis of Conjugates of Pyridoxal

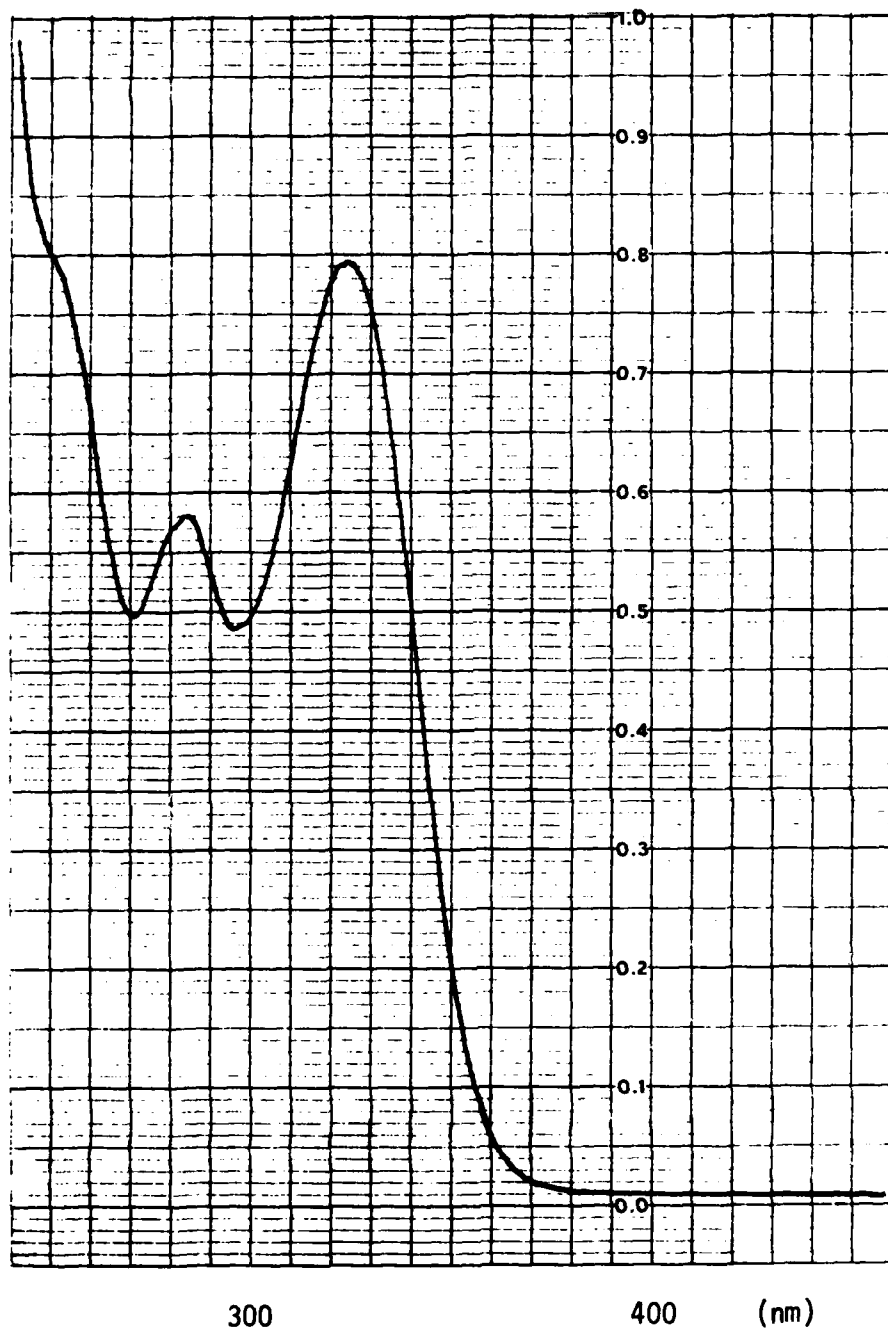


Figure 10. Spectrum of BSA-Pyridoxal Conjugate

SYNTHESIS OF PYRIDOXYL CONJUGATES

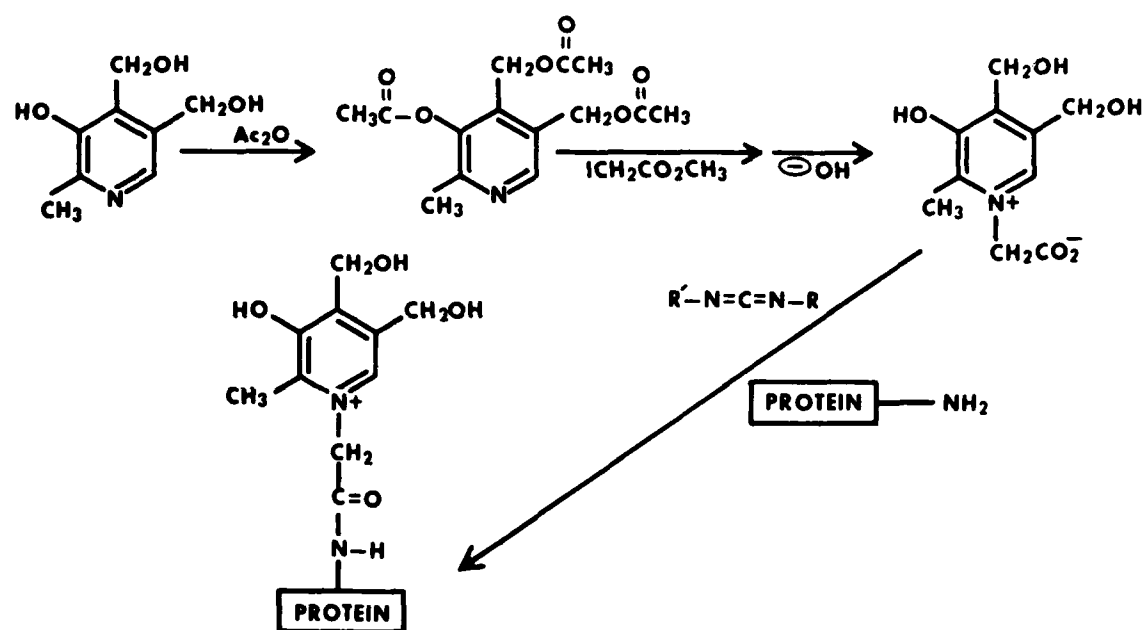


Figure 11. Synthesis of Pyridoxyl Conjugates

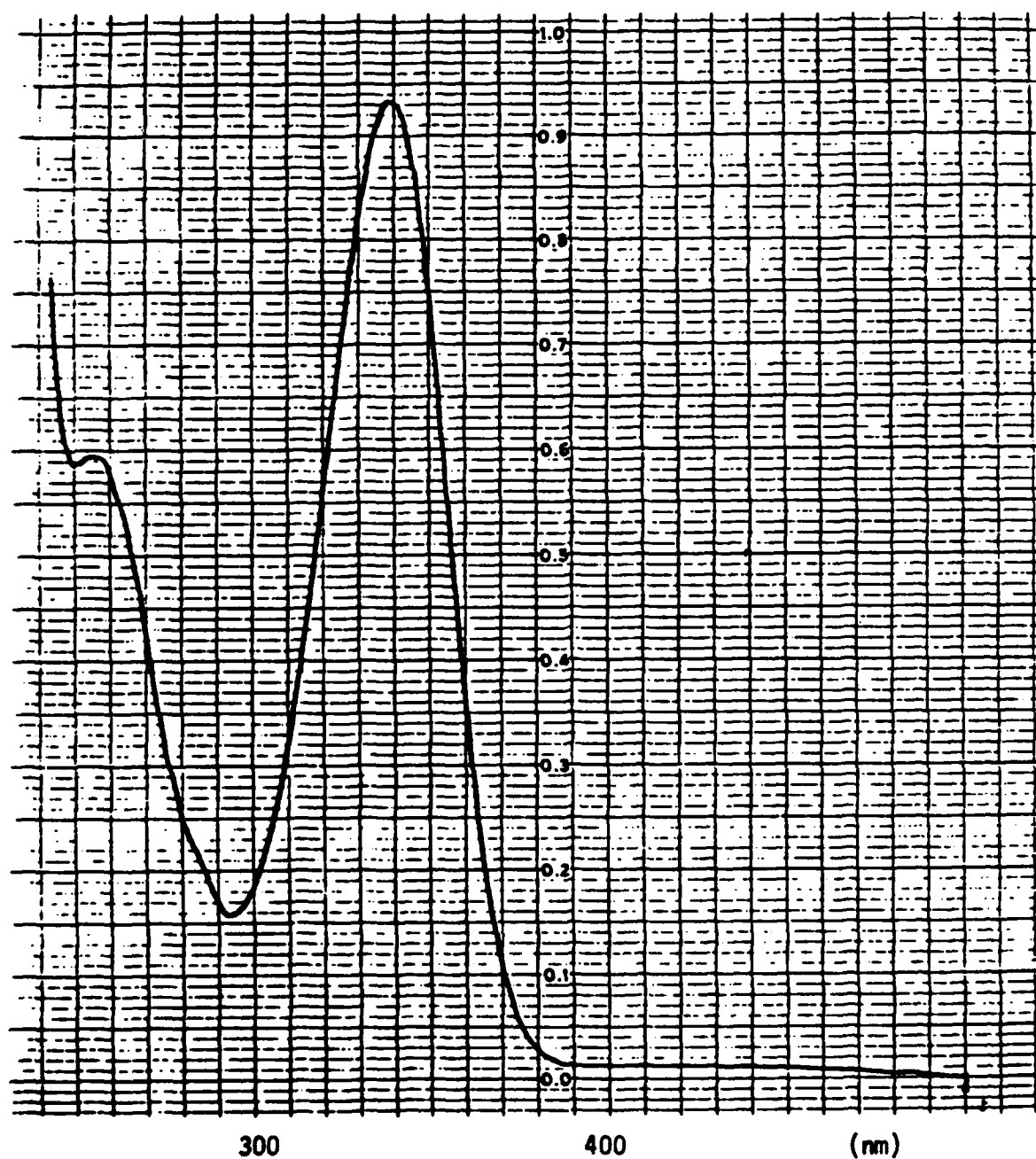
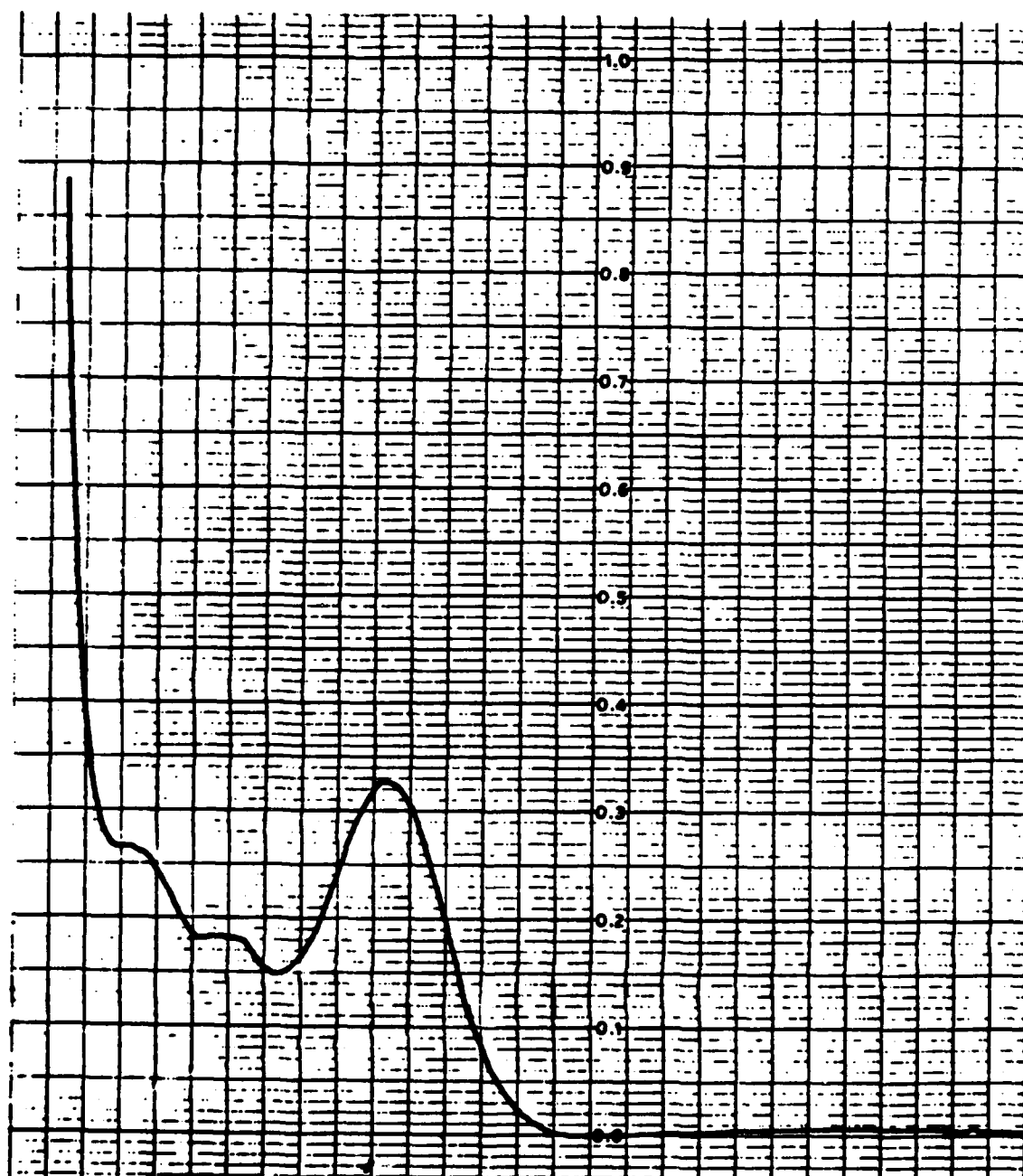


Figure 12. Spectrum of Carboxymethylpyridoxine Conjugate of BSA



320 nm

Figure 13. Spectrum of Pyridoxylformic Acid Conjugate of BSA

Although the rationale is straightforward, the detailed physiological mechanism is a subject of debate. Mitogens like bacterial lipopolysaccharide are able to bind to the surfaces of B lymphocytes and induce proliferation and differentiation into antibody-secreting cells (Moller, 1975).^{5,8} This nonspecific stimulation results in a polyclonal response against multiple determinants. Hapten conjugates of LPS, especially if used at a low concentration, can induce a very potent anti-hapten response. At low concentrations of hapten-LPS, we expect that cells bearing high affinity immunoglobulin receptors will bind the conjugates selectively. A high percentage of this cell population will be triggered, since the hapten is accompanied by a strong B-cell mitogen. Our plan is to couple haptens to amino groups on LPS (Jacobs and Morrison, 1975)^{5,9} and to investigate the use of such conjugates as a general method for efficient production of hybridomas that secrete antibodies directed against vitamins. Thus far, we have prepared one batch of LPS-PM conjugate for use in mice (see below).

2. Conventional antibodies

a. Radioimmunoassay for pyridoxine-specific antibody

We are using a sensitive radioimmunoassay for detection of antibodies that bind pyridoxine. The assay is performed in phosphate-buffered saline. The serum to be assayed is diluted serially and incubated with 0.1 μ C of ³H-pyridoxine for fifteen minutes. The tubes are then chilled in an ice-water bath, 1 mg of horse IgG is added to each tube (as carrier), and 2 mL of 50% saturated ammonium sulfate solution is added. After fifteen minutes, the tubes are centrifuged, the supernatants removed, and the precipitates dissolved in 0.3 mL formic acid. The formic acid solutions are transferred to scintillation vials and counted after addition of scintillator. The assay is sensitive, detecting anti-pyridoxine activity in anti-pyridoxamine sera at 1/100 dilution.

b. Immunodiffusion procedure

Immunodiffusion is performed in 0.4% agarose, containing 55 mM barbital buffer, pH 8.6, with 0.1% sodium azide as a preservative. Melted agar solution is poured onto standard microscope slides and stored at 4°C until used. Sera are tested against the homologous carrier, as well as against hapten coupled to heterologous carrier. Immunodiffusion slides are developed for 24 to 48 hours, and then washed with saline (24 h) and water (2 h). Slides are then stained with Amido Black solution and destained with acetic acid-95% ethanol (1:9).

c. Immunization of rabbits

The general immunization procedures are as follows. Female New Zealand white rabbits were immunized with two intramuscular injections of 1 mg of hapten-protein conjugate (e.g., KLH-PM), emulsified in complete Freund's adjuvant, at two-week intervals. Boosts were administered subcutaneously at two- to four-week intervals, using immunogen emulsified in incomplete Freund's adjuvant. Generally, the serum antibody titer reached a plateau after four injections. Rabbits were bled by puncturing the inner marginal ear vein. Test bleedings were done 5 to 7 days after boosting, and pools of positive sera were collected every 2 or 3 days, between days 5 and 12 after boosting. Serum was allowed to clot at room temperature

for one hour, then in the refrigerator for several hours or overnight. Serum was separated from particulates by centrifugation at 1500 g for 10 minutes. Small portions of serum were stored at -20°C . Large pools were lyophilized, stored at -20°C , and reconstituted, as necessary, by adding 1 mL of water per 85 mg of dry serum.

The titer and specificity of sera were assayed by immunodiffusion, radioimmunoassay, and spin immunoassay. Almost all the data described in this report were obtained with one pool of serum from rabbit #239, which had the highest titer of anti-pyridoxine antibodies (assayed by RIA as described above).

d. Immunization of mice

The immunization protocol for mice was as follows. Female Balb/c mice were immunized by intraperitoneal injection of protein conjugates emulsified in complete Freund's adjuvant. In general, 0.1 mL of emulsion containing protein at 1 mg/mL was used. A second immunization with complete Freund's adjuvant and additional boosts with incomplete Freund's adjuvant were administered at three-week intervals. Serum antibody titer was measured by immunodiffusion and RIA.

e. Antibodies elicited with conjugates of pyridoxal

Immunization of rabbits and mice with KLH-PM has elicited antibodies selective for pyridoxamine. These antisera have been used in three varieties of immunoassay (as indicated below).

f. Antibodies elicited with conjugates of carboxymethylpyridoxine

Rabbits. Several groups of rabbits were immunized with protein conjugates — BSA-CMP or KLH-CMP. None of these rabbits produced positive anti-pyridoxine sera.

Mice. Several groups of mice were immunized with CMP conjugates. A number of mice produced weakly positive sera (1 to 5% of positive control rabbit anti-pyridoxine titer). Several of these mice were used as spleen cell donors (see below).

g. Antibodies elicited with conjugates of pyridoxylformic acid

Rabbits. One group of Dutch rabbits was immunized with KLH-PFA. These rabbits have not produced positive sera, as assayed by immunodiffusion and RIA.

Mice. Several groups of mice were immunized with BSA-PFA or KLH-PFA. As with the CMP conjugates, a number of mice produced weakly positive sera, and several were used as spleen cell donors (see below).

3. Monoclonal antibodies

a. Rationale for use of monoclonal antibodies

The monoclonal antibody technique, developed just five years ago (Kohler and Milstein, 1975),⁶⁰ promises to revolutionize the field of immunology, as well as other disciplines to which immunochemical techniques may be applied. The technique (see Figure 14) involves the fusion of a myeloma cell and an antibody-secreting lymphocyte, to produce a hybridoma cell line, possessing the "immortality" of the myeloma line, and the specific antibody-secreting ability of the lymphocyte. The selection technique uses "HAT" medium (Littlefield, 1964),⁶¹ containing hypoxanthine, aminopterin, and thymidine. Aminopterin blocks normal *de novo* synthesis of purine and pyrimidines, and the cells are forced to salvage these compounds from the bases provided. Azaguanine-resistant myeloma cells, lacking hypoxanthine-guanine phosphoribosyltransferase, cannot utilize hypoxanthine, and consequently there is strong selective pressure for a cell hybrid containing the phosphoribosyltransferase of normal, lymphoid cells. Although there is no selection against the growth of normal lymphocytes, these cells have limited potential for proliferation *in vitro*.

Monoclonal antibodies offer major advantages over conventional antibody techniques, particularly relevant to the problem of vitamin immunoassay:

- (1) Cells secreting monoclonal antibodies can be screened or selected for desired specificity and affinity. Thereafter, the homogeneous antibody can be produced indefinitely, without variations from batch to batch.
- (2) Cell lines can be preserved by freezing; hence, desirable hybridomas can be provided to other laboratories and the standardization of vitamin immunoassays will be possible at the international level.
- (3) The capability to produce desired antibodies will be protected by safe storage of clones, eliminating the possibility that a chance infection in animal facilities could prevent production of desired antibody for an extended time.

Monoclonal antibodies are ideally suited for analyzing complex mixtures of antigens. In the brief time they have been widely accessible, these antibodies have been used to investigate

⁶⁰G. Kohler and C. Milstein, Continuous cultures of fused cells secreting antibody of predefined specificity. *Nature* 256, 495 (1975).

⁶¹J. W. Littlefield, Selection of hybrids from mating of fibroblasts *in vitro* and their presumed recombinants. *Science* 145, 709 (1964).

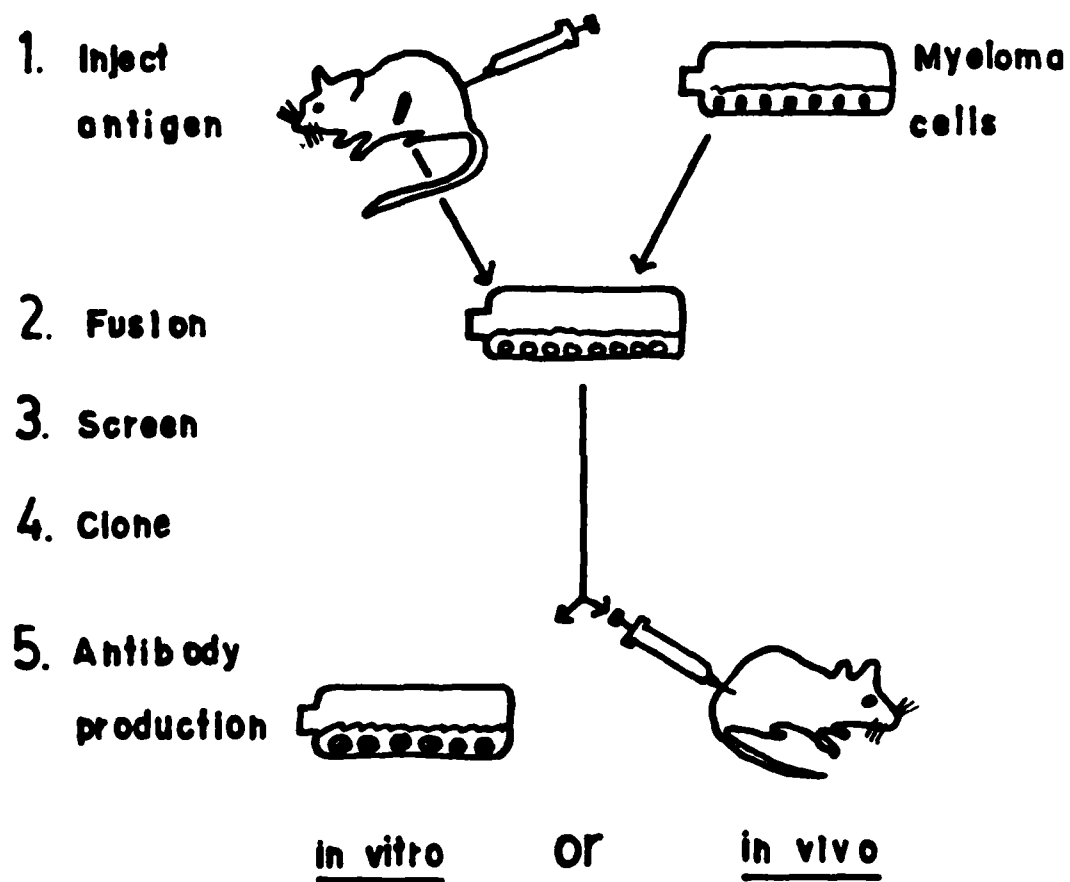


Figure 14. Monoclonal Antibody Technique

complex protein mixtures such as histocompatibility antigens (Parham and Bodmer, 1978)⁶² and have discriminated between strains of rabies virus hitherto considered a single strain (Wiktor and Koprowski, 1978).⁶³ The hybridoma technique can also provide large quantities of lymphocyte products otherwise rare — e.g., mouse IgE (Bottcher et al., 1978).⁶⁴

There is an important third category of problems for which the monoclonal antibody technique is ideally suited: the production of high affinity antibody specific for small haptens, including vitamins. Because small haptens may not completely fill the combining site of anti-hapten antibodies, maximal affinity and specificity are not generally achieved by conventional immunochemical techniques. Discriminating among a mixture of related small haptens (e.g., vitamins) with great sensitivity necessitates the powerful tool available in the hybridoma technique.

The combining sites of antibodies are, of course, heterogeneous, but the site may be as large as $35 \times 10 \times 7 \text{ \AA}$, and accommodate at least six saccharide units or amino acid residues (Kabat, 1966).⁶⁵ However, some antibodies have sites just big enough to accommodate one saccharide unit (Kabat, 1966).⁶⁵ It is therefore possible and advantageous to seek antibodies with small, "tailored" combining sites in order to achieve maximal affinity and specificity. Although selecting for or screening hybridomas with this feature may be tedious and time-consuming, it is essentially a one-time operation for a given hapten. This technique will be superior in efficacy and efficiency to conventional methodology that must be applied separately to each pool of immune serum.

b. Status of monoclonal antibody work

This laboratory has recently set up facilities for producing monoclonal antibodies (supported in part by USDA Competitive Research Grants). We have completed the screening phase of our work, and can now produce hybridoma cell lines. The procedures for screening cell cultures for desired antibodies have generated large numbers of RIA's. In

⁶²P. Parham and W. F. Bodmer, Monoclonal antibody to a human histocompatibility alloantigen. *Nature* 276, 397 (1978).

⁶³T. J. Wiktor and M. Koprowski, Monoclonal antibodies against rabies virus produced by somatic cell hybridization: Detection of antigenic variants. *Proc. Natl. Acad. Sci. USA* 75, 3938 (1978).

⁶⁴I. Bottcher, G. Hammerling, and J. F. Kapp, Continuous production of monoclonal mouse IgE antibodies with known allergenic specificity by a hybrid cell line. *Nature* 275, 761 (1978).

⁶⁵E. A. Kabat, The nature of an antigenic determinant. *J. Immunol.* 97, 1 (1966).

addition, the tissue culture work itself has demanded a lot of effort — from preparation of media and sterile materials to cell preparation and microscopic examination of cells to determine growth rates and viability. The procedures involved in development of a specific hybridoma cell line are outlined in Table 1.

c. Pyridoxine-specific monoclonal antibodies

We have produced hybridoma cell lines from spleen cells of mice immunized with KLH—CMP. These cell lines were, unfortunately, lost before stable clones were prepared. Presumably, the problem is caused by the more rapid growth of non-Ig secreting cell lines that arise either during the initial fusion or as Ig-secreting cells lose genetic material coding for Ig synthesis. We are currently exploring variations in tissue culture procedure which will be more favorable for maintaining desired Ig-secreting hybridomas.

d. Pyridoxamine-specific antibodies

Thus far, we have elicited pyridoxamine-specific antibodies in mice, using KLH—PM as immunogen. We have not as yet been able to produce a positive hybridoma. We have investigated immunization protocols and tissue culture procedures in order to increase the efficiency of hybridoma production.

e. Changes in immunization procedures

In contrast to the requirements for serum antibody production, immunization for the monoclonal antibody technique should be designed to maximize the production of *blast cells* (cells which are enlarged, heavily engaged in protein synthesis, and preparing for cell division). We have used *E. coli* LPS as carrier in immunizing mice (see above). LPS, a B cell mitogen in the mouse, elicits blastogenesis in over 30% of the B cells (cells of the antibody-producing lineage). We reasoned that this would increase the chances of a productive fusion, since hybridomas are thought to result when a blast cell fuses with the myeloma cell of choice. LPS conjugated to vitamin should focus itself on cells that bear (and secrete) antibodies specific for the vitamin. To this end, LPS—PM (described above) was prepared and injected into Balb/c mice, *i.p.* Three days later, spleen cells were prepared and fused with NS—1 myeloma cells. So far, the cultures have all been negative. These experiments will be repeated with more heavily conjugated LPS before rejecting this potentially powerful technique.

We reasoned that mice producing antibodies specific for vitamin could be boosted with mitogen, rather than with antigen. This would lead to increased blastogenesis and more efficient hybridoma production. We have done one pilot experiment to test this hypothesis. Three mice, previously injected with BSA—PFA or KLH—PFA and positive for antipyridoxine, were injected *i.p.* with 10 μ g of LPS. Four days later the mice were bled and the serum tested by RIA. One of the three mice had a four-fold increase in antibody titer and was used as spleen cell donor for fusion. The first fusion using this procedure has not succeeded in producing a pyridoxine-specific hybridoma cell line. We are continuing to try this technique, while modifying tissue culture conditions (see below).

Table 1**Typical Timetable for Monoclonal Antibody Preparation**

Step	Day Number	Procedure
1.	-45 and -24	Immunization of Balb/c mice (<i>i.p.</i>)
2.	-17	Bleed mice; assay sera
3.	-3	Boost positive mouse (<i>i.v.</i>)
4.	0	Obtain spleen cells from boosted mouse
5.	0	Fuse spleen cells with myeloma cells (NS-1 line)
6.	0	Culture cell fusion products in selective medium
7.	14	Test culture supernatant for antibody
8.	16	Transfer colonies from positive cultures to microculture dishes
9.	23	Test microcultures for antibody production
10.	24	Expand positive microcultures
11.	31	Clone positive cultures by limiting dilution
12.	38	Test presumptive clones for antibody production
13.	40	Expand positive clones
14.	47	Test expanded cultures for antibody production
15.	49	Freeze desired clones; store in liquid N ₂
16.	49	Characterize monoclonal antibody with regard to Ig class, specificity, and affinity
17.	60	Use monoclonal antibody in immunoassay

f. Changes in tissue culture procedures

There are many possible variations in the hybridoma technique. One which we are currently trying is the inclusion of macrophages in the initial hybridoma cultures. The procedure is thought to help detoxify cultures during the critical first days after fusion. During this period, most cells (except the hybridomas themselves — a small percentage of total cells) die in the selective medium. A second variation in our procedure, which we are trying, is the use of an alternate myeloma cell line (FO) and modified culture conditions — in accordance with the recommendations of Fazekas de St. Groth and Schneidegger (1980).⁶⁶

IMMUNOASSAYS

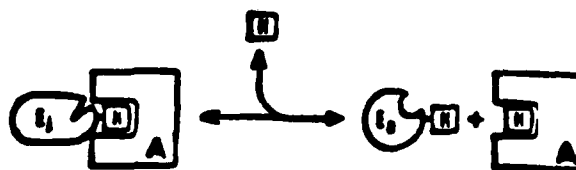
1. Fluorescent enzyme immunoassay

a. Rationale

We are developing homogeneous, fluorescent, enzyme-linked immunoassays for vitamins, to enable sensitive measurements with a minimum of sample processing. These assays depend on the inhibition of vitamin-conjugated β -galactosidase by anti-vitamin antibodies. β -galactosidase affords the greatest sensitivity because of its high turnover number — even single molecules of this enzyme can be assayed fluorometrically using a fluorogenic substrate. The assay depends on inhibition of the enzyme by anti-vitamin (anti-hapten) antibody molecules.

The principle of enzyme-labeled immunoassay is illustrated below:

HOMOGENEOUS ENZYME IMMUNOASSAY



A = antibody
H = hapten (e.g., vitamin)
E = enzyme

⁶⁶S. Fazekas de St. Groth and D. Schneidegger, Production of monoclonal antibodies: Strategy and tactics. J. Immunol. Methods 35, 1 (1980).

Our experience with precipitin analysis of anti-vitamin and anti-hormone anti-bodies and spin resonance immunoassays suggests that free vitamin will rapidly displace bound vitamin from the antibody combining site. Thus far, we have set up the enzyme assay system, using 4-methylumbelliferyl- β -galactopyranoside (MUG) as substrate and pyridoxal as hapten (via reduced Schiff's base linkage.)

b. Materials and Methods

Antisera. Preparation of anti-pyridoxyl antibodies has been described above.

Enzyme. β -Galactosidase from *E. coli* (320 μ /mg) was obtained from Sigma. First, 2.5 mg of lyophilized enzyme powder was dissolved in 1.25 mL 50 mM NaHCO_3 , pH 8.0. An equal volume of 0.15 M pyridoxal HCl (Sigma) in the same buffer was then added to the enzyme solution and incubated at 37°C for four hours. The mixture was then cooled to 0°C and 2 mL of sodium borohydride solution (15 mg/mL) was added. The sample was left for 15 hours at 0°C, dialyzed first against bicarbonate buffer, and then 0.1 M sodium phosphate buffer, pH 7.3. The enzyme solution was stored at -20°C.

Assays

(i) Colorimetric assays for β -galactosidase were performed at 37°C, in a final volume of 1.5 mL, by incubating enzyme in 0.1 M sodium phosphate buffer, pH 7.3, containing 1 mM MgCl_2 and 110 mM 2-mercaptoethanol. O-nitrophenylgalactoside (ONPG, Sigma) was added to a final concentration of 2.3 mM to initiate the reaction. One mL of 0.1 M Na_3PO_4 solution was added to terminate the reaction. The absorbance was read at 410 nm.

(ii) Fluorogenic assays were performed essentially as the colorimetric assays, but with a final volume of 400 μ L, using 0.02 M 4-methyl-umbelliferyl- β -galactopyranoside (Sigma) as substrate. Incubations were terminated by addition of 100 μ L 1 M Na_2CO_3 , and fluorescence at 450 nm was determined with a Fluoricord spectrofluorometer (Baird-Atomic), using excitation at 360-nm, and 8-nm entrance and exit slits.

c. Results

Conjugation of the enzyme. The absorbance peak at 320 nm was used to calculate a conjugation ratio of 20 moles of vitamin per mole of protein, or 12 molecules of vitamin per 100,000 daltons of protein (see Figure 15). Conjugation of the enzyme and antigenicity was confirmed using the double immunodiffusion technique. After conjugation, the enzyme forms a precipitin with specific antipyridoxyl antiserum.

Inhibition of enzyme activity by antibody-colorimetric assay. To determine whether the enzyme conjugate was suitable for EIA, the effect of normal rabbit serum and immune serum on enzymic activity was determined (Table 2). It is apparent that anti-pyridoxyl serum, but not normal serum, is capable of inhibiting the activity.

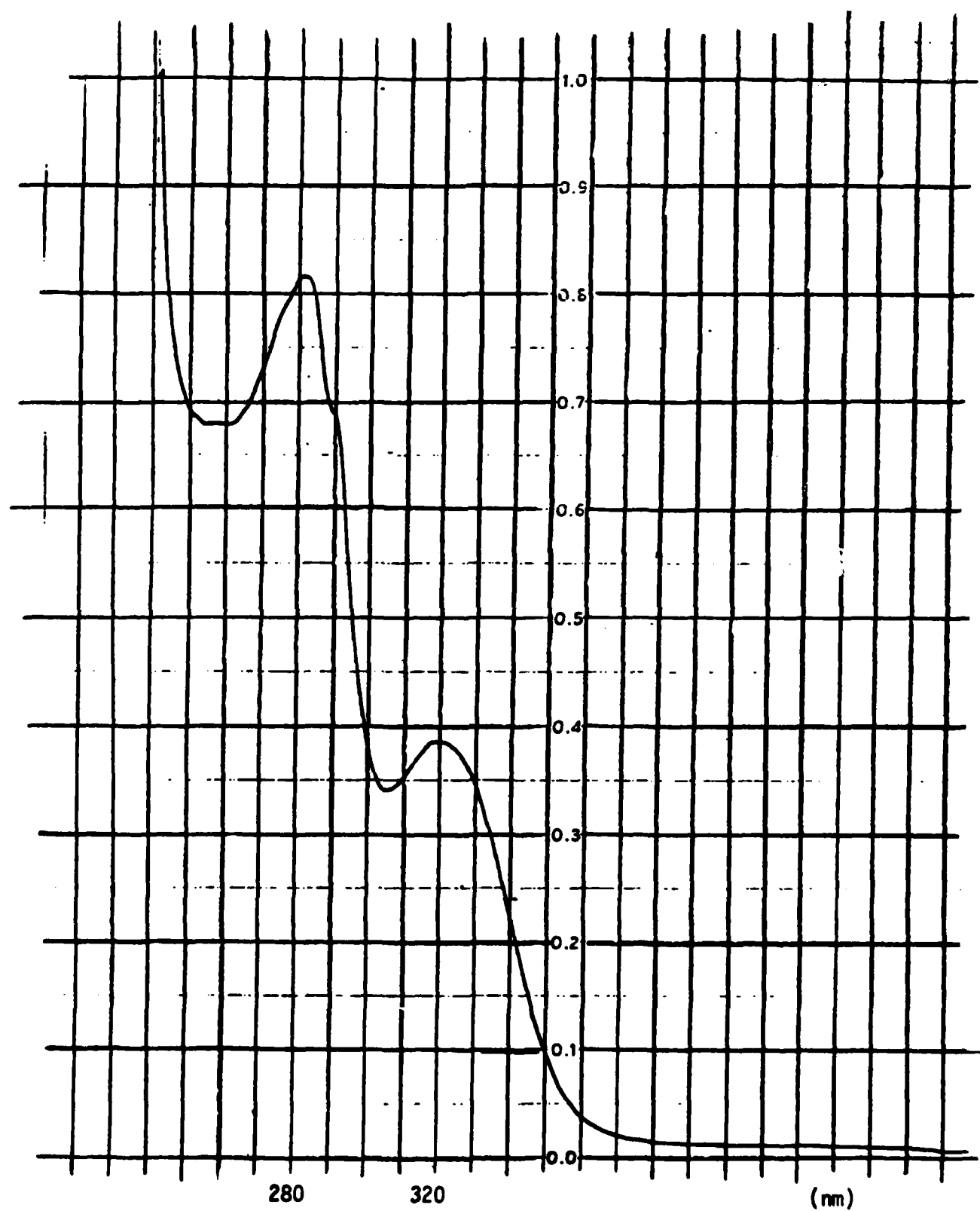


Figure 15. Spectrum of Pyridoxyl-Galactosidase Conjugate

Table 2. Inhibition of β -galactosidase activity by anti-pyridoxyl antibodies

Addition	Activity
---	0.565
normal serum	0.608
anti-pyridoxyl serum	0.225

Assays were conducted for 5 minutes at 37°C and terminated by addition of Na₃PO₄ solution, and the optical density at 410 nm was determined. The results shown were obtained by 1/30 final concentration of serum.

Dependence of enzyme activity on pyridoxamine and pyridoxine. The results in Table 3 indicate that the enzyme activity was sensitive to pyridoxamine concentration, and, to a lesser extent, pyridoxine concentration.

Table 3. Dependence of β -galactosidase activity on pyridoxamine and pyridoxine concentration, in the presence of anti-pyridoxyl antibodies

Addition	Activity (arbitrary units)
None	100
Pyridoxamine	
3×10^{-6} M	100
3×10^{-5} M	153
3×10^{-4} M	220
Pyridoxine	
3×10^{-5} M	100
3×10^{-4} M	133

Pyridoxyl β -galactosidase activity was determined in the presence of 1/30 final concentration antiserum, with or without added vitamin, using ONPG as substrate.

Fluorogenic assay. A series of experiments was performed to determine suitable conditions for a fluorescence EIA for vitamin B₆. We determined that the 10 μ l of enzyme at a concentration of 1 μ g/mL provides a convenient concentration for assays using MUG as substrate, with the photomultiplier at the highest sensitivity. Two antisera were titrated to determine suitable dilutions for the assay. The degree of inhibition was insensitive to duration of preincubation — periods of 30 minutes to 16 hours produced comparable inhibition of activity.

Potential of inhibition of enzyme activity by second antibody. Since rabbit anti-pyridoxyl antiserum inhibited enzyme activity up to 70%, we wished to determine whether a second antibody — goat anti-rabbit IgG — could potentiate this inhibition and reduce background activity in the absence of vitamin. The results in Table 4 indicate that goat anti-rabbit IgG decreases enzyme activity in the presence of rabbit anti-pyridoxyl serum. There was no inhibition of activity by the goat antibody, in the absence of anti-pyridoxyl serum.

Table 4. Potentiation of inhibition of pyridoxyl-galactosidase activity by second antibody

Additions	Activity (arbitrary units)
Buffer	96.0
Rabbit anti-pyridoxyl serum	59.5
Rabbit anti-pyridoxyl serum + goat anti-rabbit IgG	32.5

Enzyme activity was determined as described in Materials and Methods, using MUG as substrate.

Enzyme immunoassay of pyridoxamine. Figure 16 illustrates standard curves obtained for pyridoxine and pyridoxamine, in the double antibody assay system. In this assay, pyridoxine cross-reacts with pyridoxamine to the extent of one to 10%, depending on concentration.

2. Radioimmunoassay

Radioimmunoassay is the most widely used type of immunoassay. Although enzyme immunoassay offers advantages, there are some circumstances when RIA would be preferable:

- when sample turbidity, color, or autofluorescence unavoidably interferes with the enzyme assay.
- in laboratories not set up for fluorometry.

One of our goals has been a radioimmunoassay based on monoclonal antibodies, using either beta- or gamma-emitting labels. Thus far, we have developed a prototype RIA, using rabbit antisera and tritiated ligand. This assay demonstrates the feasibility of our approach, but further refinements are necessary to adapt the assay for routine use.

a. Protocol

- (1) A 50- μ L sample of vitamin-containing solution is pipetted into a 10- x 75- mm disposable culture tube.
- (2) 20 μ L of 3 H-pyridoxine is added to the tube.

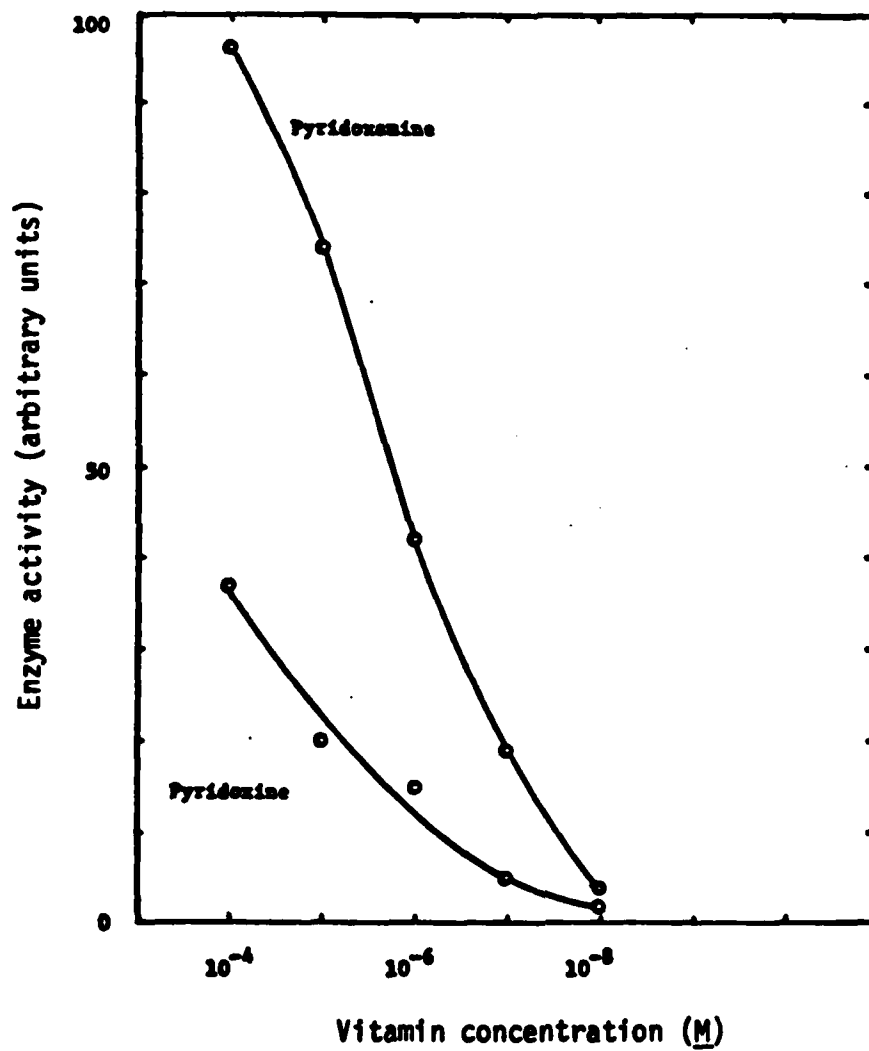


Figure 16. Fluorescence Enzyme Immunoassay of Pyridoxamine and Pyridoxine

- (3) 50 μ L of standard anti-pyridoxine antiserum is added to the tube.
- (4) Tubes are incubated at 22°C for 30 minutes.
- (5) Tubes are placed in an ice-water bath, and 2 mL of 50% saturated ammonium sulfate solution is added.
- (6) After 15 minutes, the tubes are centrifuged for 10 minutes at 30,000 *g*.
- (7) The supernatant is carefully removed with a water aspirator.
- (8) The precipitate is dissolved in 350 μ L 88% formic acid, and transferred to scintillation vials.
- (9) Scintillation fluid is added, and the samples are counted for two minutes.
- (10) The percentage of radioactivity bound relative to a control sample without added vitamin is computed, after subtraction of counts in samples with negative control serum. A standard curve is plotted on semi-log paper.

b. Results and Discussion

Standard curves obtained for pyridoxamine and pyridoxine are illustrated in Figure 17. It is apparent that, under these assay conditions, pyridoxine and pyridoxamine are detected nearly equally well. This is, in part, due to use of pyridoxine as labeled ligand in this assay. The use of labeled pyridoxamine and the use of more specific sera (especially monoclonal antibodies) would enable quantitation of individual vitamers. We have also done assays using labeled ligand of higher specific activity, and it appears that the assay is useful down to about 5×10^{-6} M vitamin — i.e., samples containing 5 ng of vitamin.

3. Spin Immunoassay

a. Rationale

In contrast to optical reporter groups which have a characteristic spectrum, the spectrum of a spin label depends largely on the medium, and is very sensitive to the anisotropy of the environment of the spin label. Changes in viscosity or rotational freedom dramatically alter the spectrum. In fact, this feature provides the basis for "spin immunoassay" of haptens, such as vitamin B₆. A competition between free hapten (B₆) and spin-labeled hapten (pyridoxamino-TEMPO) for anti-hapten antibody binding sites can be established. Spin-labeled hapten bound to antibody will have a broadened spectrum approaching the "rigid glass" limit, whereas unbound spin-labeled hapten will have a characteristically sharp spectrum. A standard curve can be prepared, relating vitamin concentration to a spectral parameter (usually the peak

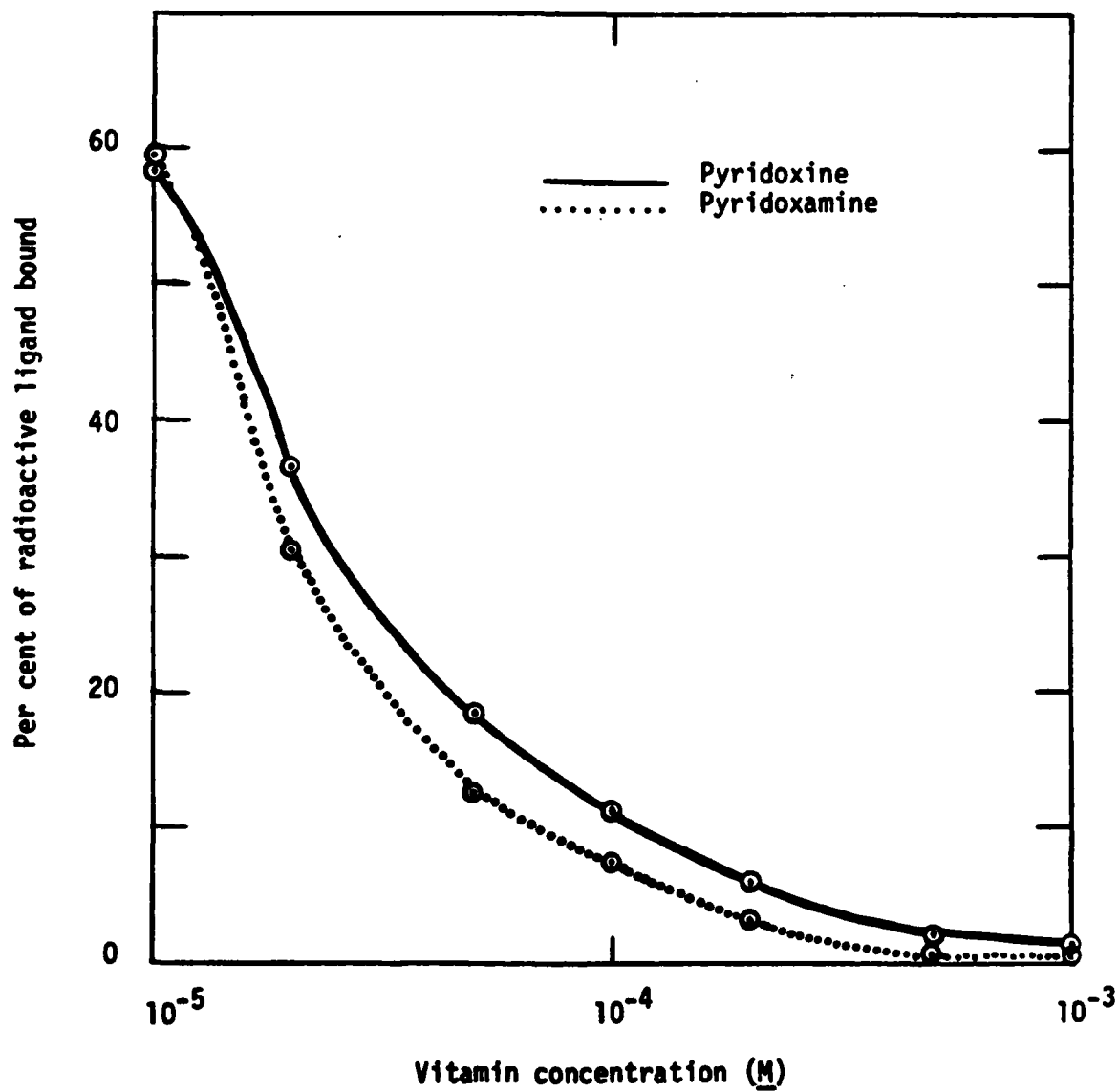


Figure 17. Radioimmunoassay of Pyridoxine and Pyridoxamine

height of the low-field line). Although the technique is theoretically complex, spin immunoassay offers the advantage of a homogeneous system, not requiring fractionation of the reaction mixture nor the use of radioisotopes.

b. Results

All assays were conducted using the pyridoxamine conjugate of TEMPO described above (XXIV, Figure 8). Incubations of vitamin, antibody, and paramagnetic ligand were conducted in a total volume of 100 μ l. Spin label was present at a final concentration of 5×10^{-6} M. Incubation times from approximately one minute to several hours did not affect the result. Spectra were recorded on a Varian E-3 spectrometer, at ambient temperature ($20^{\circ}\text{C} \pm 1^{\circ}\text{C}$). Generally eight-minute scans were used. Samples were drawn into 50- μ L disposable micropipettes, one end of which was then heat-sealed. Control experiments indicated that the immobilization of spin label depended on the antibody activity, and not on nonspecific binding or viscosity effects. A standard curve illustrating the peak height of the low-field line as a function of pyridoxamine concentration is shown in Figure 18. We also obtained a standard curve for pyridoxine, as illustrated in Figure 19. The system is less sensitive to this B₆ vitamer. Some experiments were performed with the aid of a minicomputer which stored and added repetitive scans ("CAT-ing"). Under these conditions, coefficients of variation were 2 to 5%, compared to 10% for single-scan experiments.

CONCLUSIONS AND RECOMMENDATIONS

1. Summary of status of vitamin-specific antibodies

We have tried to raise antibodies specific for pyridoxine and pyridoxamine in rabbits and mice. We have obtained antibodies which are selective for pyridoxamine, but which bind pyridoxine also. By manipulating conditions, we have set up two assays which are selective for pyridoxamine (the fluorescent EIA and the spin immunoassay) and one assay which is about equally sensitive to pyridoxine and pyridoxamine (the RIA).

Having produced hybridomas (lost before cloning) which secreted vitamin-binding antibodies, we feel close to achieving our goal of having monoclonal antibodies which can be used to quantitate individual vitamers or total vitamin B₆. We are currently making improvements in our tissue culture systems to enable more reliable hybridoma preparation. We are also developing better screening assays for secreted antibody. This will enable more rapid detection of desired hybridomas and will enable screening of a greater number of tissue culture supernatants. We will then exploit our battery of synthetic haptens more fully, in order to elicit the necessary vitamin-specific antibodies.

2. Method for quantitation of vitamin B₆

a. Proposed general procedure for exploiting EIA and RIA for vitamin B₆

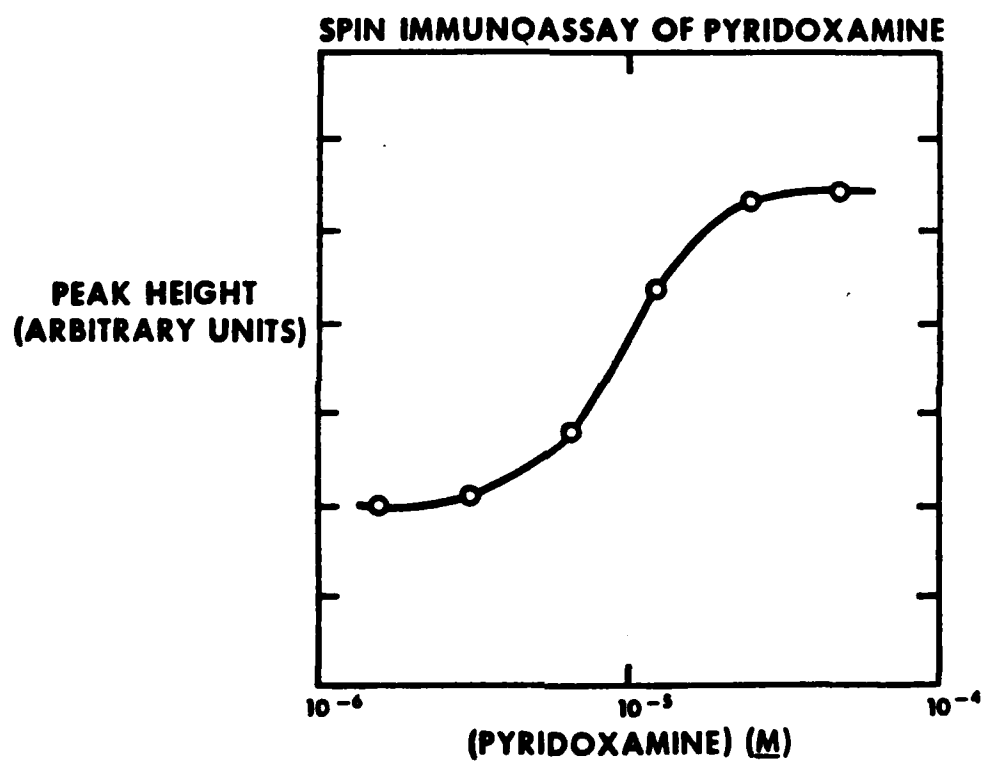


Figure 18. Spin Immunoassay of Pyridoxamine

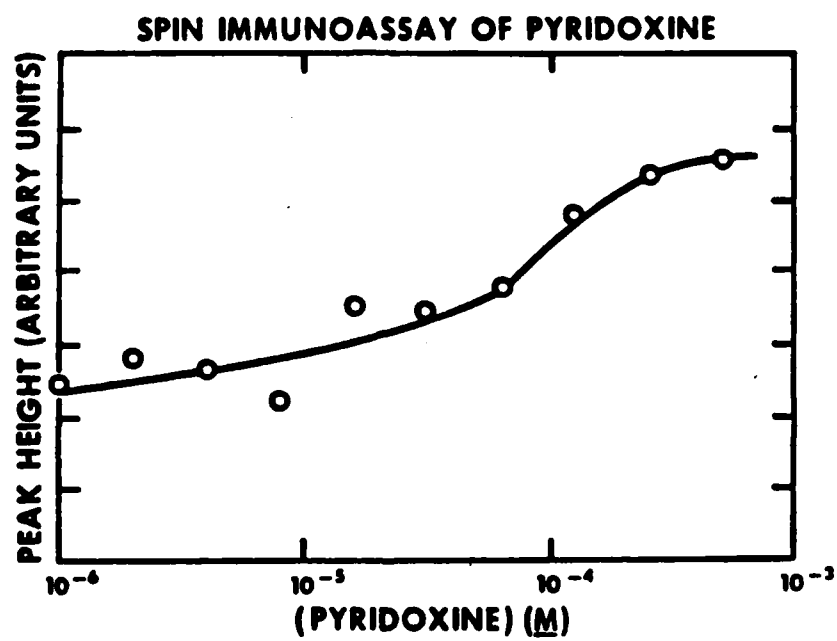


Figure 19. Spin Immunoassay of Pyridoxine

Sample preparation will be similar for both the RIA and EIA. Two specific antibodies (anti-pyridoxine and anti-pyridoxamine) will be employed to determine pyridoxine, pyridoxamine, and pyridoxal in samples hydrolyzed and neutralized essentially in accordance with the established method of the AOAC (Horwitz et al., 1975).¹⁷ The procedure will exploit the quantitative conversion of pyridoxal to pyridoxine by reduction with borohydride. The steps are summarized below:

- (1) Determine pyridoxine.
- (2) Determine pyridoxamine.
- (3) Reduce a portion of the sample with sodium borohydride, and determine pyridoxine.
- (4) Calculate pyridoxal as the difference between pyridoxine determined in steps 1 and 3.

b. Comments on enzyme immunoassay

The advantages of a homogeneous EIA for vitamins B₆, using a fluorogenic substrate are the following:

1. Homogeneous assay systems require the minimum sample processing.
2. Once developed, the assay can be performed without special training and is amenable to automation.
3. Fluorescent assays are more sensitive than colorimetric assays and do not generate radioactive waste.

Our results indicate that the essential requirements for an enzyme in the EIA are met by β -galactosidase from *E. coli*, with a fluorescent substrate, such as 4-methylumbelliferyl galactoside. The enzyme is relatively stable and its high turnover number enables high sensitivity. Even single molecules of the enzyme can be assayed under appropriate conditions (Rotman, 1961).⁶⁷

In a labeled-enzyme configuration of EIA, the following criteria for hapten-enzyme conjugates must be satisfied:

⁶⁷B. Rotman, Measurement of activity of single molecules of β -d-galactosidase. Proc. Natl. Acad. Sci. USA 47, 1981 (1961).

1. The enzyme must be inhibited by antibody in the absence of free vitamin. Insufficient conjugation will be revealed by poor inhibition.
2. The enzyme-antibody complex must be activated by low concentrations of vitamin. High avidity binding by antibody, requiring high concentrations of vitamin for displacement, will indicate excessive conjugation.

Our preliminary results have been encouraging, and we need only ascertain whether improvements can be made. In order to minimize the blank (enzyme activity in the absence of free vitamin) unconjugated or poorly conjugated enzyme should be separated from desired conjugate. The conjugated enzyme could be chromatographed using agarose-bound antibodies to vitamin B₆. Alternatively, charge differences could be exploited by ion exchange chromatography or preparative isoelectric focusing. β -Galactosidase, a very stable enzyme, should withstand these preparative procedures.

One area of concern is interference with the assay due to fluorescence of the sample and fluorescence quenching. If the umbelliferone fluorescence is subject to interference, a commercially available fluorescein derivative can be used. Fluorescein has different excitation and emission spectra, and a quantum yield close to one. Alternatively, colorimetric substrates are available and can be used when maximal sensitivity is not required.

As discussed above (Immunoassays), substrate-labeled EIA has some advantages over enzyme-labeled assays. We are therefore pursuing synthesis of the required ligands for this assay configuration.

c. Comments on radioimmunoassay

The RIA system described above is offered as a prototype to demonstrate the feasibility of this approach. We propose two changes in the assay system.

1. Use of solid-phase reagents to replace precipitation of Ig.
 - (a) Protein A. This protein is a naturally occurring IgG-binding protein from *S. aureus*. It is available as a solid-phase reagent in the form of formalinized bacterial cell walls or as an agarose bead conjugate.
 - (b) Derivatized agarose beads. These reagents can be coupled to appropriate antibodies and can then be used to bind to the anti-vitamin antibodies in the assay.
2. Use of ¹²⁵I-labeled ligand. The use of this gamma-emitting isotope will enable the assay to be performed in a single tube, without the use of scintillator.

d. Determination of pyridoxal

The extent of cross-reactivity among various vitamers must be determined for any immunoassay of vitamin B₆. It is possible that pyridoxal will not interfere with the determination of pyridoxine. However, if cross-reactivity is impossible to eliminate, a "trapping" agent for pyridoxal could be used — for example, penicillamine or dinitrophenhydrazine, which should form noncross-reacting derivatives of pyridoxal (Sauberlich, 1968).⁶⁸ If the trapping reaction is quantitative in dilute solution, one could consider using antibodies specific for the derivatized pyridoxal to assay this vitamer independently (rather than by difference).

e. Phosphorylated vitamin and protein-bound vitamin

These forms of vitamin B₆ provide special problems for immunoassay. It seems likely that antibody specific for the phosphopyridoxyl hapten could be developed.

The following are two proposed procedures for using immunoassay to quantitate protein-bound vitamin — vitamin which has relatively low bioavailability.

1. Pyridoxamino-protein could be estimated by immunoassay of the protein residue after mild acid hydrolysis of pyridoximino groups and trapping of the released pyridoxal, if necessary, as described above. The residue could be dissolved in a detergent such as deoxycholate, and the vitamin quantitated with a solid-phase RIA.
2. Pyridoxyllysine could be determined after complete acid hydrolysis and neutralization of samples.

f. Total vitamin B₆ determination

The following are proposed schemes for determining the total of all B₆ vitamers in acid-hydrolyzed samples:

1. Immunoassay using a broadly specific anti-pyridoxyl antibody, possibly elicited with a carboxymethyldeoxypyridoxine immunogen.
2. Conversion of B₆ vitamers to pyridoxic acid (essentially as summarized by Gregory and Kirk),²⁷ followed by immunoassay with anti-pyridoxic acid. Suitable correction for pyridoxic acid in the sample prior to oxidation must be made.
3. Determination as the sum of pyridoxine and pyridoxamine in acid-hydrolyzed, borohydride-reduced samples. This is the scheme which we are closest to achieving.

⁶⁸H. E. Sauberlick, Vitamin B₆ group. Active compounds and antagonists. *The Vitamins*, 2nd ed., Vol. II, NY Academic Press, 33 (1968).

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