The Separation of Pyridoxine, Pyridoxal, and Pyridoxamine by a Sulfonic Acid Ion Exchange Resin

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Procedures for the separation and determination of pyridoxine, pyridoxal, pyridoxamine, and their phosphorylated derivatives on a sulfonic acid ion exchange resin or on sulfonic acid ion exchange resin impregnated papers are described. Best separations for the free forms were observed with 0.1*M* phosphate buffer, pH 6.5. No movement occurred with dilute acetic acid solution, thus providing for concentration of dilute vitamin extracts and concomitant removal of interfering materials. Best separations for the phosphorylated derivatives occurred with 0.1*N* acetate buffer, pH 5.0. Analysis for the content of the free forms in a dried yeast sample indicated that the application of these procedures for determination of the vitamin B_6 content of foods and tissues appears feasible if the measurement is accomplished by a spectrophotofluorometric procedure.

An increased knowledge of the essential functions of vitamin B_6 has emphasized the need for reliable and rapid methods of the assessment of its various forms in foods and biological tissues. A relationship of vitamin B_6 to the metabolism of protein and fat has been established (*3*, *20*), and diets high in protein and fat—a pattern common in the American population—enhance the requirement for this vitamin. More recently, vitamin B_6 deficiency experimentally established in cats and rats evoked an increased excretion of endogenous oxalic acid and formation of calcium oxalate stones in the urinary tract (*1*, *5*).

Approximately 50% of the B_6 content of wheat is removed during the manufacture of flour, and losses are sustained in foods processed by thermal and radiation methods (*8*, *15*, *16*). Extended, high-temperature storage is responsible for additional decrement (*2*, *14*). Military studies in man have shown that a diet composed exclusively of processed foods stored at high temperature contains insufficient vitamin B_6 as judged by response to a tryptophan load test (*6*).

Present animal, microbiological, and chemical procedures are often unsatisfactory because they lack sensitivity or specificity and are cumbersome. The yeast, *S. carlsbergensis*, does not respond with comparable growth to the three forms of vitamin B_6 (*7*, *9*, *17*, *18*, *21*). The rat does not respond equally to the three forms when they are fed, but does when they are administered by injection (*13*). The chick does not show the three forms to be equally active when fed (*19*). *S. fecalis* responds to pyridoxal and pyridoxamine while *L. casei* responds to pyridoxal alone (*12*). The phosphorylated forms in general produce a smaller response than the related free forms (*12*).

Previously, chemical procedures were not sensitive enough to determine vitamin B_6 in foods and tissues or depended on a preliminary differential destruction, rather than a physical separation (*4*). Recently, fluorometric determination of the three free forms or the lactone of their oxidation product, 4-pyridoxic acid, has been shown to be very sensitive (*7*). The preliminary chromatographic separation of the three forms reported by these workers required the application of several hot, high ionic strength buffers, however. The development of a simpler and rapid physical procedure seemed desirable and is the subject of this report.

Experimental

Pyridoxine hydrochloride, pyridoxal hydrochloride, and pyridoxamine dihydrochloride were obtained commercially and recrystallized until they were pure chromatographically. The phosphorylated forms were prepared following the method of Peterson and Sober (*10*) who phosphorylated pyridoxamine with P_2O_5 and converted the resulting 5-pyridoxamine phosphate to 5-pyridoxal phosphate and 5-pyridoxine phosphate by oxidation with manganese oxide–Celite and by nitrosation with HNO_2, respectively. Each phosphorylated form was purified by chromatography on an acidified IRC-50 column, crystallized, and shown to possess the expected physical properties.

Commercial grade Amberlite IR-120 resin (−400 mesh) was treated with 1*N* HCl and 1*N* NaOH prior to pouring into columns as a slurry of desired buffers. Column effluents were collected with a volume principal fraction collector, adjusted to pH 6.5, and the absorbance was determined at 322 mμ. This wave length was chosen because it is midway between the peak absorptions of pyridoxal (318 mμ) and pyridoxine and pyridoxamine (325 mμ) in neutral solutions.

Screening to establish suitable resins and eluants was accomplished with the recently introduced Amberlite ion exchange resin impregnated papers. The papers were always prewashed with the eluting buffer and dried before spotting except where otherwise noted. All buffers were of 0.1*M* concentration. Location of the spots was accomplished for pyridoxal and pyridoxamine with an ultraviolet light, and for pyridoxine with an ultraviolet light after exposing the paper to ammonia vapor (*10*).

Results and Discussion

Paper Chromatographic Studies. Preliminary studies proved most successful with the sulfonic acid resin impregnated papers. Figures 1 and 2 illustrate some of the chromatograms which enabled selection of suitable conditions for application to column separations. These chromatograms suggest optimum separation of the phosphorylated forms from one another and from the free forms at approximately pH 5. For the free forms, the optimum pH is approximately 6.5. The papers were always prewashed with the eluting buffer and dried before spotting except for one innovation which is demonstrated in Figure 3.

In this instance, the chromatogram

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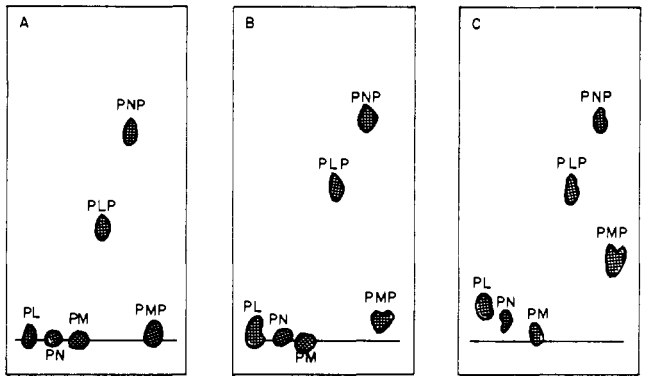

Figure 1. Separation of pyridoxal (PL), pyridoxine (PN), pyridoxamine (PM), and their related 5-phosphates on a sulfonic acid-type, resin-impregnated paper in the acid stage

(A) 2.5% acetic acid; (B) pH 4 acetate; (C) pH 5 acetate

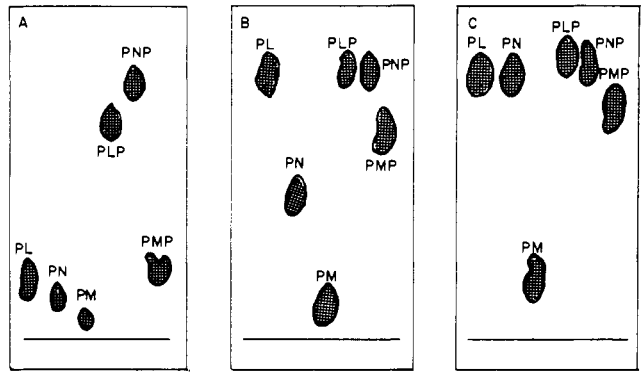

Figure 2. Separation of pyridoxal (PL), pyridoxine (PN), pyridoxamine (PM), and their related 5-phosphates on a sulfonic acid-type, resin-impregnated paper with near neutral buffers

(A) pH 6.0 phosphate; (B) pH 7.0 phosphate; (C) pH 8.0 phosphate

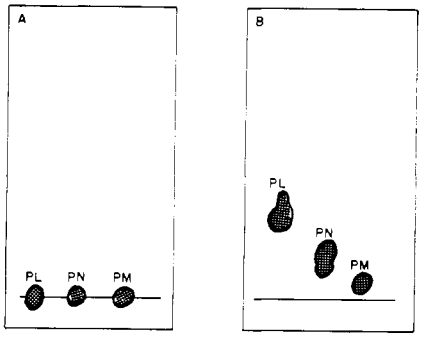

Figure 3. Separation of the three free forms with 0.1*N* phosphate buffer, pH 6.5, after a 2.5% acetic acid prewash

(A) 2.5% acetic acid; (B) 2.5% acetic acid plus pH 6.5 phosphate

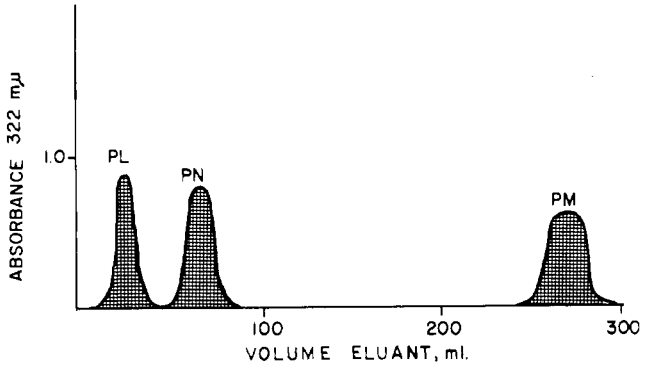

Figure 4. Separation and quantitative recovery of the three free vitamin B_6 forms from a synthetic mixture containing 250 μg. of each with pH 6.5 phosphate buffer

Recoveries: pyridoxal, 98%; pyridoxine, 101%; and pyridoxamine, 95%

was prewashed with 2.5% acetic acid solution, dried, spotted with the three free forms, and irrigated in the ascending direction with additional 2.5% acetic acid. As previously observed in Figure 1, no movement of any of the forms occurred. The paper was dried a second time, and finally irrigated with 0.1*M* phosphate buffer, pH 6.5, to achieve the desired separation. The development of this two-step separation was desired because it would provide for concentration of the vitamin B_6 in dilute solutions of natural products and for removal of that material which was soluble in dilute acetic acid prior to separation of the three forms.

Column Chromatographic Studies. Figure 4 shows the separation achieved where a mixture of 250 μg. each of pyridoxal hydrochloride, pyridoxine hydrochloride, and pyridoxamine dihydrochloride was applied to an IR-120 column (10 × 0.9 cm.), equilibrated with 0.1*M* phosphate buffer of pH 6.5 and eluted with the same buffer. The recoveries observed were routinely within 5% of theoretical. If desired, the elution of pyridoxamine could be hastened by employment of a more alkaline buffer after emergence of pyridoxal and pyridoxine, or alternatively by a gradient elution system.

In anticipation of column separation and quantitative determination of the three forms in foods and tissues, the IR-120 column was equilibrated with 2.5% acetic acid solution. Two hundred fifty micrograms of each form and 100 mg. each of vitamin-free casein and sucrose were autoclaved 4 hours with 200 ml. of 0.055*N* HCl at 20 pounds pressure, filtered, evaporated to dryness in vacuo, dissolved in the acetic acid solution, and applied to the column. As previously observed with the ion exchange resin impregnated papers (Figure 3), none of the three forms moved when the column was washed with the 2.5% acetic acid solution, thus providing for concentration of dilute solutions and simultaneous removal of considerable interfering material. Elution of the three forms was then achieved with 0.1*M* phosphate buffer, pH 6.5. As seen in Figure 5, the pyridoxal emerged shortly after breakthrough of the phosphate buffer. Pyridoxine and pyridoxamine followed in that order. Quantitative recoveries were again observed.

Finally, to effect a separation of a mixture containing the three free forms plus the three related phosphorylated derivatives, the previously described IR-120 10-cm. column was equilibrated with 0.1*M* acetate buffer, pH 5.0. A mixture of 250 μg. of each of the six forms plus 100 mg. each of vitamin-free casein and sucrose was autoclaved as previously described and applied in a solution buffered at pH 5.0. Upon emergence of pyridoxal phosphate and pyridoxine phosphate (Figure 6) after approximately 100 ml.

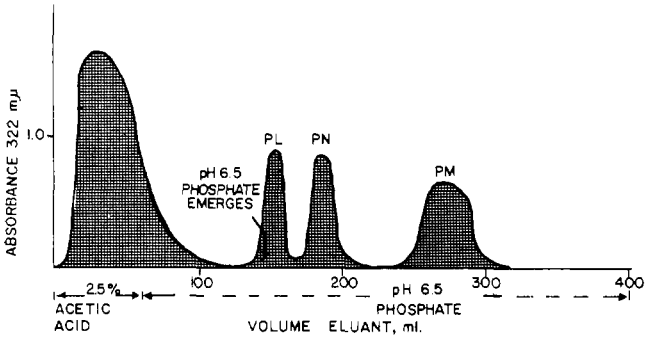

Figure 5. Recovery after autoclaving a mixture of 250 μg. each of the three free forms plus 100 mg. each of vitamin-free casein and sucrose for 4 hours in 0.055N HCl at 20 pounds pressure

Recoveries: pyridoxal, 97%; pyridoxine, 99%; and pyridoxamine, 96%

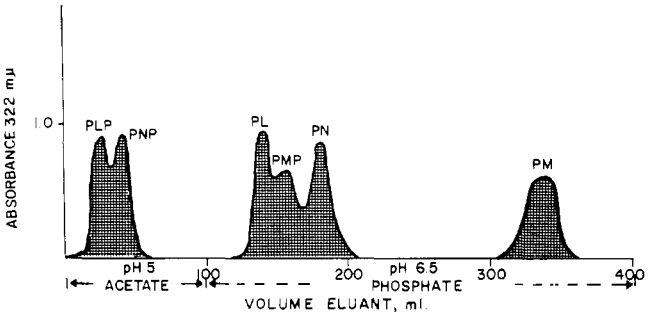

Figure 6. Elution pattern from a mixture of 250 μg. each of the three free forms and their phosphorylated derivatives plus 100 mg. each of vitamin-free casein and sucrose after autoclaving for 4 hours in 0.055N HCl at 20 pounds pressure

Table I. Recovery of Pyridoxal, Pyridoxine, and Pyridoxamine from Fortified Yeast Sample[a]

	Yeast, Amount Added, μG.	Recovered, μG.
Pyridoxal	400	430
Pyridoxine	0	5
Pyridoxamine	0	25

[a] Recovery measured as absorbance at 322 mμ in neutral solution after separation on an Amberlite IR-120 column.

elution with the acetate buffer, elution of the three free forms plus pyridoxamine phosphate was accomplished with phosphate buffer pH 6.5.

The elution pattern presented in Figure 6 shows that a separation with this system appears feasible although it was not complete under the specific conditions. No dilute acid prewash was possible in this instance because of the movement and acid-lability of the phosphorylated forms. However, at pH 5.0, pyridoxal phosphate, the fastest moving form, is sufficiently delayed to allow removal of most of the amino acids, protein, and other interfering material expected in foods and tissues. This procedure is pertinent to samples which have not been treated either with acid or alkali since the phosphorylated derivatives are stable only in neutral solutions. Application to biological systems is contingent upon development of enzymatic or other systems capable of freeing bound vitamin B_6 forms without concomitant dephosphorylation.

Analysis of a Fortified Dry Yeast Sample. While the investigation was devoted chiefly to separation of synthetic mixtures, the content of the free forms of a fortified dry yeast sample was determined (Table I). The conditions of hydrolysis were those presently in general use with microbiological assays (*11*). One gram of the dried, pyridoxal-fortified yeast was autoclaved 4 hours with 200 ml. of 0.055*N* HCl at 20 pounds pressure. The solution was filtered or centrifuged, adjusted to pH 2, and concentrated at reduced pressure prior to chromatography.

The recovery of pyridoxal from yeast was satisfactory because 1 gram of unsupplemented yeast contains approximately 40 μg. of pyridoxal (*9*). While small amounts of pyridoxine and pyridoxamine were also detected in yeast as expected, the related absorbances in the ultraviolet were too low for any degree of reliability. For greater sensitivity, the analysis should be accomplished spectrophotofluorometrically. Measurement in concentrations as low as 0.001 μg. per ml. have been achieved with a 330-mμ light source for excitation. The resulting fluorescence was determined at 390 mμ (*7*), thus avoiding major interference.

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