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Removal of Ferrous Ions by *N,N'*-Ethylenediaminetetraacetic Acid in Microbiological Assay of Pyridoxine

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Abstract □ The application of a general microbiological method for the determination of pyridoxine was satisfactory for most preparations containing pyridoxine, but there were discrepancies in some multivitamin products. The chief cause of these discrepancies was shown to be Fe^{+2}. A modification was made to the method by using *N,N'*-ethylenediaminetetraacetic acid to sequester Fe^{+2} before acid hydrolysis of the preparations. By using this modification, 100% of the pyridoxine can be detected compared with 75 or 77% for 20:1 or 12:1 ratios of Fe^{+2}–pyridoxine, respectively.

Keyphrases □ Pyridoxine, multivitamin combinations—bioassay, ferrous-ion removal with *N,N'*-ethylenediaminetetraacetic acid □ Multivitamin combinations—pyridoxine bioassay □ *N,N'*-Ethylenediaminetetraacetic acid—ferrous-ion complexing, pyridoxine bioassay

In 1943, Atkin *et al.* (1) reported a microbiological method which employed a yeast, *Saccharomyces carlsbergensis*, to determine pyridoxine. Other studies (2–5) further supported this method. In natural products and pharmaceutical preparations, pyridoxine exists partly as the combined form which must be hydrolyzed prior to assay. The method was satisfactory for most preparations containing pyridoxine, but there were discrepancies in some multivitamin products. The chief cause of these discrepancies was shown to be Fe^{+2}. A combination of Fe^{+2} with multivitamins is generally recommended for the treatment of iron-deficiency anemia (6). The interference of Fe^{+2} might be prevented by the addition of a compound such as *N,N'*-

Table I—Composition of Assay Medium

Dextrose	100.0 g.
KH_2PO_4	1.10 g.
KCl	0.85 g.
$CaCl_2$	0.25 g.
$MgSO_4 \cdot 7H_2O$	0.25 g.
$FeCl_3$ solution (1%)	0.50 ml.
$MnSO_4$ solution (0.1%)	0.50 ml.
Potassium citrate	10.00 g.
Citric acid	2.00 g.
Acid hydrolysate of casein[a]	10.00 g.
Thiamine hydrochloride solution (10 mcg. thiamine/ml.)	50 ml.
Inositol solution (1.0 mg./ml.)	50 ml.
Biotin solution (0.8 mcg./ml.)	20 ml.
Riboflavin solution (100 mcg./ml.)	10 ml.
Ca pantothenate solution (200 mcg./ml.)	25 ml.
Nicotinic acid solution (200 mcg./ml.)	25 ml.
$(HN_4)_2 HPO_4$	2.00 g.
L-Asparagine	1.50 g.
Agar	20.00 g.
Water to make	1000 ml.

[a] Sheffield Hy-case, Sheffield Chemical, Division of National Dairy Products Corp.

ethylenediaminetetraacetic acid, which is known to form a stable complex with Fe^{+2} (7) and to remove undesired metals from living organisms (8, 9).

EXPERIMENTAL

Organism—*Saccharomyces carlsbergensis*, Fleishman 4228 (ATCC 9080), was used throughout these studies. It was incubated for 48 hr. at 30° on modified Sabouraud dextrose agar. One loopful of this culture was transferred to a flask containing 100 ml. of sterilized yeast dextrose broth. The inoculated flask was incubated for 20 hr. at 30° on a mechanical shaker. The cells were harvested and centrifuged under sterile conditions and then resuspended in 100 ml. of sterilized saline solution. This suspension was stored at 4° and used as the stock inoculum for up to 1 month.

Assay Medium—The assay medium was prepared according to the formula shown in Table I.

Plate Preparation—On the day of assay, 100 ml. of freshly prepared and cooled (48°) assay medium was inoculated with 3 ml. of stock inoculum suspension. Eleven milliliters of inoculated assay medium was applied to each petri plate and allowed to solidify. The plates were covered and preincubated at 30° for 4 hr.

Stock Standard Solution and Standard Solution of Pyridoxine—For the stock standard solution, 25.13 mg. of 99.48% pyridoxine hydrochloride standard was weighed into a 250-ml. volumetric flask and made up to volume with distilled water; this stock solution had a potency of 100 mcg. pyridoxine hydrochloride/ml. The solution was stored in the refrigerator and used within 30 days. On the day of assay, a series of dilutions of the stock standard solution was prepared to contain 1.0, 0.5, 0.25, and 0.125 mcg. of pyridoxine activity/ml. The 0.5-mcg. standard was used as the reference solution.

Sample Preparation—An aliquot of sample, expected to contain approximately 2.0 mg. of pyridoxine hydrochloride, was transferred to a 250-ml. conical flask, and 100 ml. of 1 *N* HCl was added. The flask was covered with an aluminum foil cap and autoclaved at 15 lb. pressure for 60 min. The solution was cooled to room temperature, adjusted to pH 6.8 with 2 *N* NaOH, transferred to a 100-ml. volumetric flask, and made up to volume with distilled water. This solution containing approximately 0.5 mcg. pyridoxine hydrochloride/ml. was then filtered.

Table III—Effect of Component in Sample A on Stability of Pyridoxine to Acid Hydrolysis

Component	Weight of Component in 2 mg. Pyridoxine	Average Pyridoxine Concentration, mcg.	Recovery, %
Blank	—	2063	100
Ascorbic acid	750 mg.	2046	99.2
Benzoic acid	25 mg.	2080	100.8
Ferrous sulfate	525 mg.	1490	74.5
Glucose	10 g.	2050	99.4
Menthol	1.9 mg.	2053	99.5
Niacinamide	8.25 mg.	2065	100.1
D-Pantothenol	4.5 mg.	2016	97.7
Riboflavin	5.25 mg.	2003	97.1
Thiamine	6.75 mg.	2036	98.7
Sodium saccharin	2.5 mg.	2020	97.9
Sucrose	1.25 g.	2017	97.8
Cyanocobalamin	49.4 mcg.	2061	99.9

Assay Procedure—The pyridoxine content of each preparation was determined by a cylinder plate method. Six stainless steel cylinders were equally spaced on each agar plate, 2.8 cm. from the center. Three plates were used for each point in the standard series. On each of these plates, three cylinders were filled with the 0.5-mcg. reference solution; the remaining three at opposite positions were filled with a standard solution. Three plates were also used for each sample. Three of the cylinders on each of these plates were filled with the 0.5-mcg. reference solution and three with the sample solution. The filled plates were incubated at 30° for 18 hr. All determinations were run in duplicate and repeated six times. All chemicals were either USP, NF, or ACS grade.

Calculation of Results—The resultant zones of exhibition were read on a Fisher Lilly zone reader and recorded. Averages of the readings of each point for each set of three plates and all readings of the 0.5-mcg./ml. reference solution were calculated. The average diameter for the reference solution was used to correct the average obtained for each point in the standard series and the samples. The corrected values of the standard diameters were substituted in the following formulas to calculate the line of best fit:

$$l = \frac{7a + 4b + c - 2d}{10} \qquad \text{(Eq. 1)}$$

$$h = \frac{7d + 4c + b - 2a}{10} \qquad \text{(Eq. 2)}$$

where l is the calculated diameter (in mm.) of the lowest concentration (0.125 mcg./ml.); h is the calculated diameter of the highest concentration (1.0 mcg./ml.); and a, b, c, and d denote the corrected diameters for each concentration from the lowest to the highest in that order. The values l and h were plotted on one-cycle semilogarithmic graph paper, with the concentration in micrograms per milliliter on the logarithmic scale and the zone diameters on the arithmetic scale. The two points were joined by a straight line.

The potency of each sample could be read from the line or, preferably, calculated according to the following equation (10):

$$U = \text{antilog}\left[\frac{\log H - \log L}{h - l}(u - l) + \log L\right] \qquad \text{(Eq. 3)}$$

Table II—Pyridoxine Stability against Acid Hydrolysis

	Autoclaved Time, min.					
	0	10	20	40	60	80
Pure pyridoxine, mcg.	5000	5015	4975	5003	5020	4982
Recovery, %	100	100.3	99.5	100.1	100.4	99.6
Sample A pyridoxine, mcg.	5150	5037	4913	4160	3725	3240
Recovery, %	100	97.8	95.4	83.2	74.5	64.8

Table IV—Effect of Mineral Ions on Stability of Pyridoxine to Acid Hydrolysis (100 mg. Ion/5 mg. Pyridoxine Solution)

Component	Average Pyridoxine Concentration, mcg.	Recovery, %
Blank	5000	100
Ferrous sulfate	3730	74.6
Copper sulfate	4892	97.8
Magnesium sulfate	4934	98.7
Manganese oxide	4848	97.0
Zinc sulfate	4964	99.3
Cobalt chloride	4848	97.0
Potassium iodide	4822	96.4
Sodium citrate	4916	98.32
Calcium sulfate	5006	100.1

Table V—Effect of Amount of Fe^{+2} on Pyridoxine Stability to Acid Hydrolysis

Pyridoxine, mg.	2.0	2.0	2.0	2.0	2.0
Fe^{+2}, mg.	0.0	10.0	20.0	40.0	60.0
Fe–pyridoxine ratio	0:1	5:1	10:1	20:1	30:1
Average pyridoxine concentration, mg.	2.000	1.666	1.617	1.492	1.428
Recovery, %	100	83.3	80.86	74.60	71.40

where U is potency of sample solution in final dilution, u is diameter of exhibited zone of sample solution, log H is logarithm of highest concentration of standard, h is diameter of exhibited zone of highest standard, log L is logarithm of lowest concentration of standard, and l is diameter of exhibited zone of lowest standard.

Statistical Analysis of Results—Reproducibility of the method and the significance of the results obtained by the *N,N'*-ethylenediaminetetraacetic acid modification were calculated from the following equations:

$$\text{standard deviation } (SD) = \sqrt{\frac{\Sigma(\bar{X} - x_1)^2}{n}} \qquad \text{(Eq. 4)}$$

$$\text{coefficient of variation } (CV) = SD \times \frac{100}{\bar{X}} \qquad \text{(Eq. 5)}$$

$$\text{standard error } (S\bar{X}) = \sqrt{\frac{SD}{K}} \qquad \text{(Eq. 6)}$$

$$\text{Student's } t = \frac{\bar{X} - u}{S\bar{X}} \qquad \text{(Eq. 7)}$$

where $\bar{X}$ is mean of potency, x_1 is potency of each test, K is number of treatments, n is degrees of freedom $= K - 1$, and u is theoretical potency.

The theoretical t value of 2.776 was derived from statistical tables for Biological, Agricultural, and Medical Research (11) at the 5% probability level ($p = 0.05$) and the degrees of freedom $n = 5 - 1 = 4$ for five replicates of results.

Determination of Effect of Component Contained in Sample A and Various Mineral Ions on Stability of Pyridoxine to Acid Hydrolysis—The stability of pure pyridoxine solutions and multivitamin Sample A solution was tested by the described procedure with variations in the time of autoclaving. Studies of the factors affecting pyridoxine were made by the addition of each component in the multivitamin Sample A. Various mineral ions were also added, and their effect on pyridoxine assay was shown.

Determination of Effect of *N,N'*-Ethylenediaminetetraacetic Acid on Pyridoxine Stability in the Presence of Fe^{+2} during Acid Hydrolysis—Various quantities of *N,N'*-ethylenediaminetetraacetic acid disodium salt were added to solutions containing Fe^{+2}–pyridoxine in the ratio 20:1 before acid hydrolysis, and the effect on the assay of pyridoxine was determined.

Determination of Reproducibility and Precision of Pyridoxine Assay with and without *N,N'*-Ethylenediaminetetraacetic Acid Treatment—Four multivitamin samples were tested by the general method and the *N,N'*-ethylenediaminetetraacetic acid modified method, and the precision of the results was determined.

RESULTS AND DISCUSSION

In the stability studies, only 74.5% of pyridoxine was found in the multivitamin Sample A, whereas no decrease in pyridoxine alone was observed under the same conditions (Table II). This indicates that Sample A contains some factor that decomposed pyridoxine during acid hydrolysis in the autoclave.

The addition of individual components of the multivitamin mixture showed that only ferrous sulfate had an appreciable effect on the recovery of pyridoxine (Table III). In comparing the mineral ions, 100 mg. cation/5 mg. pyridoxine was used; the results showed that there was no appreciable effect apart from Fe^{+2} (Table IV). It is clear that the major factor affecting the stability of pyridoxine under acid hydrolysis is Fe^{+2}. The quantity of Fe^{+2} affecting the stability of pyridoxine was further investigated, and the degradation was increased by increasing the Fe^{+2} (Table V). The percentage recovery fell from 83.3 to 71.4% for solutions with Fe^{+2}–pyridoxine ratios of 5:1 to 30:1, respectively.

The interference of Fe^{+2} was eliminated by the use of *N,N'*-ethylenediaminetetraacetic acid as a sequestering agent (Table VI). It was necessary to add 10 g. *N,N'*-ethylenediaminetetraacetic acid to a sample containing 100 mg. Fe^{+2} to eliminate interference; this quantity of *N,N'*-ethylenediaminetetraacetic acid was also effective in the multivitamin Sample A.

There was no significant difference between the two methods in the reproducibility of results as expressed by the coefficient of variation (Table VII). The recovery of pyridoxine in samples that did not contain Fe^{+2} was fairly close to 100% by both methods; but in Samples A and B, only 74.85 and 77.23% of pyridoxine were detected, respectively, by the general method compared with complete recovery by the *N,N'*-ethylenediaminetetraacetic acid method.

The t tests showed that there was a significant reduction in the recovery of pyridoxine only in samples containing Fe^{+2} to which *N,N'*-ethylenediaminetetraacetic acid had not been added.

The following procedure is, therefore, proposed for the determination of pyridoxine in pharmaceutical preparations as well as in natural products that may or may not contain Fe^{+2}. The sample

Table VI—Effect of *N,N'*-Ethylenediaminetetraacetic Acid–2Na on Stability of Pyridoxine in the Presence of Fe^{+2} to Acid Hydrolysis

	1	2	3	4	5	6	7	8	9
Pyridoxine, mg.	5.000	5.000	5.000	5.000	5.000	5.000	5.000	5.000	5.000
Fe^{+2}, mg.	0	100	100	100	100	100	100	100	100
Fe–pyridoxine ratio	—	20:1	20:1	20:1	20·1	20:1	20:1	20:1	20:1
N,N'-Ethylenediaminetetraacetic acid, g.	0	0	1	2	3	4	6	8	10
A: Pure Pyridoxine Solution									
Average pyridoxine, mg.	5.000	3.378	3.491	4.037	4.264	4.440	4.643	4.754	4.994
Recovery, %	100[a]	67.56	69.82	80.74	85.28	88.80	92.86	95.08	99.99
B: Multivitamin Product A									
Average pyridoxine, mg.	2.022	1.507	—	—	—	—	1.884	1.985	2.016
Recovery, %	100[a]	74.53	—	—	—	—	93.18	98.17	99.70

[a] No heat treatment.

Table VII—Pyridoxine Products Assayed by *N,N'*-Ethylenediaminetetraacetic Acid Modified Method and General Method

	Sample A		Sample B		Sample C		Sample D	
Pyridoxine content, mg.	0.200		2.78		0.200		1.75	
Fe^{+2} content, mg.	4.00		33.3		0.0		0.0	
Fe^{+2}–pyridoxine ratio	20:1		12:1		0		0	
N,N'-Ethylenediaminetetraacetic acid–2Na added, g.	0	10	0	10	0	10	0	10
Assay performed								
1	0.1509	0.2051	2.216	2.820	0.2014	0.2180	1.775	1.775
2	0.1495	0.2034	2.095	2.846	0.2047	0.2035	1.781	1.798
3	0.1504	0.1977	2.185	2.910	0.2058	0.2118	1.787	1.745
4	0.1484	0.2028	2.156	2.799	0.2152	0.2185	1.703	1.752
5	0.1491	0.2015	2.131	2.843	0.2119	0.2158	1.746	1.703
Average ($\bar{X}$), mg.	0.14966	0.2021	2.157	2.844	0.2078	0.2135	1.756	1.755
Recovery, %	74.85	101.05	77.23	102.30	103.90	106.75	100.46	100.29
SD	0.001119	0.002779	0.04686	0.0417	0.0056	0.0062	0.0347	0.03557
$\bar{X} \pm 2SD$	0.14966± 0.002238	0.2021± 0.00556	2.157± 0.09372	2.844± 0.0834	0.2078± 0.0112	0.1135± 0.0124	1.758± 0.0694	1.755± 0.07114
Coefficient of variation, %	0.9418	1.375	1.726	1.47	2.69	2.90	1.97	2.02678
$S\bar{X}$	0.01496	2.02358	0.09681	0.09133	0.03347	0.03522	0.08332	0.08445
Theoretical *t* ($df = 4$)	2.776	2.776	2.776	2.776	2.776	2.776	2.776	2.776
t test	3.362	0.8902	6.4360	0.70076	0.2330	0.38330	0.09602	0.0592

containing approximately 2.0 mg. of pyridoxine is transferred to a 250-ml. conical flask. Then 100 ml. of 1 *N* HCl and 10 g. *N,N'*-ethylenediaminetetraacetic acid disodium salt are added and stirred until homogeneous. The flask is covered with an aluminum foil cap and autoclaved for 60 min. at 15 lb. pressure. The solution is cooled, adjusted to pH 6.8 with 2 *N* NaOH, transferred to a 1000-ml. volumetric flask, made to volume with distilled water, and then filtered. The filtrate is diluted with distilled water to the concentration that contains approximately 0.5 mcg. pyridoxine/ml. The bioassay is then carried out according to the general procedure.

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