Increased Aspartate Aminotransferase Activity of Serum after in Vitro Supplementation with Pyridoxal Phosphate

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We examined the effect of pyridoxal phosphate supplementation on the apparent aspartate aminotransferase (EC 2.6.1.1.) activity of human serum. Supplementation by 25 μmol/liter effected an average increase of 16% in the results for kinetic assay. The increase was not the result of increased enzymatic or nonenzymatic blanks, and, within a small range, sample dilution had no significant effect. Part of the increase was attributable to the enzyme being protected against the loss of activity that occurs during preincubation with L-aspartate. A similar increase was not demonstrated in a two-point colorimetric method, perhaps because of the short reaction time, without preincubation, and the initial presence of both substrates in the assay. We attempted to correlate such stimulation of aminotransferase activity and the patient’s diagnosis or treatment. Pyridoxal phosphate should be included in the reaction mixture when aspartate aminotransferase activity is being measured clinically.

Additional Keyphrases: differences in results by kinetic and colorimetric methods · serum enzyme activity

The diagnostic use of measurements of serum AST2 activity is well known. In 1955 Karmen et al. (1) demonstrated increased aminotransferase activity in sera from patients with various clinical diagnoses; and AST activity of serum is now known to be increased in a variety of physiological conditions (2, 3). The requirement of pyridoxal phosphate for aminotransferase activity is also well documented. O’Kane and Gunsalus (4) in 1947 and Meister et al. (5) in 1954 were able to activate AST in porcine heart preparations by incubating them with pyridoxal or pyridoxamine phosphate. Pyridoxal phosphate is now firmly established as the cofactor for AST and other aminotransferases (6-8).

Despite this knowledge, pyridoxal phosphate has not been incorporated into any commonly used clini-

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3 Nonstandard abbreviations used, and enzyme identification: AST, aspartate aminotransferase, EC 2.6.1.1, L-aspartate:2-oxoglutarate aminotransferase [formerly known as glutamic-oxaloacetic transaminase (GOT)]; malate dehydrogenase, EC 1.1.1.27, L-malate:NAD oxidoreductase; GD, glutamate dehydrogenase, EC 1.4.1.3, L-glutamate:NAD (P) oxidoreductase (deaminating); and lactate dehydrogenase, EC 1.1.1.27, L-lactate:NAD oxidoreductase.

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Human
L-Aspartic
acid; 2-oxoglutaric acid; pyridoxal phosphate; cis-oxalacetic acid (grade I); \(\beta\)-nicotinamide adenine dinucleotide, reduced form (\(\beta\)-NADA (Grade III)); 6-benzamido-4-methoxy-m-toluidine diazonium chloride [Fast Violet B salt (grade I)]; and crystalline glutamate dehydrogenase [bovine liver, glycerol suspension (Type II)] were all obtained from Sigma Chemical Co., St. Louis, Mo. 63178. Malate dehydrogenase (derived from porcine heart) was obtained from Boehringer Mannheim Corp., New York, N.Y. 10017; polyvinylpyrrolidone (PVP; av mol wt, 30,000) from Technicon Instruments, Tarrytown, N.Y. 10591; and sodium azide, purified, from Fisher Scientific Co., Pittsburgh, Pa. 15219. All other chemicals were of reagent grade. Distilled, de-ionized water (\(>15 \text{ Ml/cm}\)) was used throughout. Human erythrocyte AST was prepared by the method of Rej et al. (19).

Statistical equality was analyzed by use of the \(t\)-distribution (20).

Results

Kinetic Assay

Human sera, chosen without conscious bias from specimens collected for a hospital clinical laboratory, were kept at 4\(^\circ\)C and assayed within 24 h from the time of drawing. Aliquots of 0.2 ml of each specimen were added to control and to pyridoxal phosphate-supplemented substrates lacking 2-oxoglutarate, and were incubated for 1 h at 30\(^\circ\)C. The composition of the preincubation mixture was, per liter: 89 mmol of phosphate buffer, pH 7.5; 134 mmol of L-aspartate; 0.19 mmol of NADH; 2.7 U of malate dehydrogenase and, when supplemented, 26.8 \(\mu\)mol of pyridoxal phosphate.

When incubation was complete, 2-oxoglutarate was added and AST activity was measured at 30\(^\circ\)C. Each serum specimen was assayed in duplicate with each substrate preparation. We observed a mean increase of 16\% in AST activity with the supplemented substrate for 125 sera (\(P < 0.001\)). “Normal” serum specimens were also obtained from 16 healthy laboratory personnel, and assays were begun within 1 h. A 16\% increase was also observed for pyridoxal phosphate-supplemented sera in this population (\(P < 0.001\)).

A scatter diagram for the results obtained by these two methods for all sera tested with initial activities less than 52 U/liter (135 specimens) is shown in Figure 1. The ratio of AST activity measured using pyridoxal-phosphate-supplemented substrate, compared to control, for four groups of specimens is shown in Table 1. While a slight decrease in this ratio is observed for sera with elevated AST activity (>40 U/liter), no statistical difference can be demonstrated between the ratios of any two groups (\(P > 0.25\) in each case).

Two-Point Colorimetric Assay

The AST activities of 39 human sera were estimated by the assay method of Babson et al. (17), with and without pyridoxal phosphate supplementation (25 \(\mu\)mol/liter during incubation). The results are

![Fig. 1. Correlation between AST activity determined with and without pyridoxal phosphate supplementation in a kinetic assay](attachment:image.png)

Each point represents the mean of duplicate analyses by each method. Activities are reported at 30\(^\circ\)C; sample size was 0.2 ml. Pyridoxal concentration was 25 \(\mu\)mol/liter; (-----) represents a slope of 1.0; (-----) represents the regression fit of \(y = 1.16x - 0.26, r = 0.987\).
shown in Figure 2. The best linear model to describe the relationship between control (X) and supplemented (Y) substrates is \( Y = X + 0.7 \). This regression fit, with a slope of 1.0 and an intercept of 0.7, is consistent with increased blank because of addition of pyridoxal phosphate without stimulation of enzyme activity. Although the magnitude of the increased blank is small (<1 U/liter), a statistically significant difference can be demonstrated between results by the two conditions \( P < 0.05 \).

Factors Influencing the Effect of Pyridoxal Phosphate on AST Activity

**Blank.** Pyridoxal phosphate can artifically increase measured aminotransferase by stimulation of apo-AST, which may be present as a contaminant in the malate dehydrogenase suspension (21). In addition, the pyridoxal phosphate moiety is able to duplicate AST catalytic activity in the absence of apo-enzyme (22).

The blank activity of the kinetic AST assay was measured after 60-min incubation with and without pyridoxal phosphate (25 μmol/liter). Control substrate exhibited a 0.5 ± 0.1 U/liter blank activity; for the supplemented substrate the activity was 0.6 ± 0.2 U/liter. Measurements also include effects on spontaneous breakdown of NADH (23).

**Preincubation conditions.** One-hour preincubations at 30°C were used in these experiments. Previous data (5, 12) indicate that a 60-min incubation is sufficient for complete saturation of aminotransferase at pyridoxal phosphate concentrations in the range of 25 μmol/liter. Increasing incubation times to 120 and 180 min did not observably increase the enhancement of AST activity measured after 60-min incubation. Decreasing incubation time to 30 min lowered the stimulation observed to a mean of 10% for 12 human sera investigated.

Increasing temperature to 37°C for both 1-h preincubation and assay gave a mean increase of 15%, not statistically different from the increase observed with 30°C incubation and assay.

**Glutamate dehydrogenase activity.** Oxidation of NADH by 2-oxoglutarate and ammonium ion, catalyzed by GD, is a source of blank activity in the kinetic assay of AST (24):

\[
2\text{-oxoglutarate} + \text{NH}_3 + \text{NADH} \xrightarrow{\text{GD}} \text{L-glutamate} + \text{NAD}^+ + \text{H}_2\text{O}
\]

Pyridoxal phosphate might stimulate serum GD activity in the kinetic assay, thereby increasing the apparent aminotransferase activity. But this possibility is unlikely, since (a) GD is barely present in normal serum (25) and (b) the \( K_m \) of the human serum enzyme for ammonium ion is about 24 mmol/liter (26), while the final concentration of ammonium ion in the described assay is only 0.4 mmol/liter. Even with elevated serum GD activity and ammonium ion concentrations, an increase in GD blank activity with increased pyridoxal phosphate supplementation is unlikely.

GD blank activity was measured using conditions identical to those in the kinetic AST assay omitting L-aspartate. Five human sera were selected in which pyridoxal phosphate supplementation produced an increase of greater than 25% AST activity over control. Samples were incubated for 1 h at 30°C, both with and without supplementation. No increase could be demonstrated in GD blank activity as the result of increased pyridoxal phosphate concentration. Blank activity was <1 U/liter for all sera.

The effect of high amounts of GD activity on the kinetic AST assay was also measured. A glycerol suspension of bovine-liver GD was prepared to give an activity of 400 U/liter when measured at optimal pH and ammonium ion concentration (27). This preparation gave a blank activity of 100 ± 7 U/liter in the control assay and 103 ± 8 U/liter in the pyridoxal-phosphate-supplemented assay. While there was no difference between the two blank activities,
the magnitude of the blank activity at such low ammonium ion concentrations was unexpected. This may be explained by a lower $K_m$ (NH$_4^+$) for the bovine enzyme (28) than for the human serum.

Sample size. The sample size, and consequently serum dilution, varies considerably in clinically used AST assays: 60-fold dilution in the Eskalab method (29), 2.5-fold dilution in the Technicon SMA procedure (30, 31). For spectrophotometric assays, Henry et al. (16) use 0.2 ml of specimen in a total volume of 3.0 ml, while Bergmeyer and Bernt (25) recommend 0.5 ml of sample in the same total volume. We compared the AST activity of 67 human sera at both 0.5 ml per assay (sixfold dilution) and 0.2 ml per assay (15-fold dilution), with substrate concentrations and assay conditions identical to those described in "Materials and Methods," and with no exogenous pyridoxal phosphate. Specimens were equilibrated and assayed at 30°C. The ratio between the assays was 1.0, and no significant difference could be demonstrated between the results of these two procedures (Figure 3).

We also investigated the effects of pyridoxal phosphate supplementation on the measurement of AST activity, by use of a sample size of 0.5 ml. Samples of 69 human sera were added to control and supplemented substrates, incubated for 1 h at 30°C, and assayed after addition of 2-oxoglutarate. AST activity increased 15% over control with the supplemented substrate ($P < 0.001$). Figure 4 compares results by these two procedures.

AST stability. We have previously shown (19) that AST activity found in serum and erythrocytes is labile when incubated at 45°C in the presence of aspartate. Our experiments and the procedure of Henry et al. (16) call for incubation of AST in the presence of aspartate. Pyridoxal phosphate thus may exert its effect by preserving rather than enhancing AST activity. To examine this role of pyridoxal phosphate, we preincubated two aliquots from each of 17 human sera in control substrate at 30°C for 6 min and 60 min, respectively. A 6-min preincubation was required both for temperature equilibration and for completion of the side reaction (oxidation of NADH by pyruvate, catalyzed by lactate dehydrogenase). Aminotransferase activity measured after the 60-min incubation exhibited a decrease of 4% (4 ± 1%, mean ± standard error), which is slight but statistically significant ($P < 0.005$), from that measured after 6 min. However, this average decrease was not sufficient to explain the 16% increase in activity observed when pyridoxal phosphate was added to the preincubation mixture.

To further elucidate the effect of pyridoxal phosphate, we added human cytoplasmic AST prepared from erythrocytes (19) to pooled human serum. Of this highly active serum (95 U of AST activity per liter at 30°C), 0.2-ml samples were added to both control and pyridoxal-supplemented substrates (without 2-oxoglutarate). The samples were then incubated for various lengths of time at 45°C. After each sample was removed from the 45°C bath and cooled to 30°C, 2-oxoglutarate was added, and the mixture was assayed for AST activity. The results (Figure 5) show a dramatic decrease in AST activity with use of the control substrate, consistent with our previous findings (19), and a 13% increase over the first 20 min in the supplemented substrate. AST activity in the supplemented substrate remained near this elevated level throughout the experiment. Pyri-
doxal phosphate thus not only enhances AST activity but protects it from inactivation in the presence of aspartate as well.

Discussion

An added 25 μmol of pyridoxal phosphate per liter increases the activity of AST in serum as determined in a kinetic assay (Table 1, Figures 1 and 3). The results obtained show an average increase of 16% in measured AST activity for 141 human sera. This increase is attributable neither to pyridoxal phosphate stimulation of contaminant apo-AST nor to nonproteincatalysis by the coenzyme moiety alone. Pyridoxal phosphate stimulation of GD blank activity can also be dismissed as the cause of the observed increase. Since identical mean increases of 16% were observed in sera assayed within 24 h and within 1 h of sample collection, pyridoxal phosphate stimulation is not the result of coenzyme lability in specimens during the <24-h storage at 4°C.

As this study demonstrates, pyridoxal phosphate, at a concentration of 25 μmol/liter, protects serum cytoplasmic AST from loss in activity when incubated with L-aspartate (Figure 5). For us to examine the true stimulation of AST by pyridoxal phosphate, it was necessary to investigate this protective effect. Our experiments included 1-h preincubations at 30°C for control and supplemented substrates. While we have here and previously (19) shown a significant decrease in measured AST activity when sera are incubated at 45°C in the presence of L-aspartate, we did not expect large decreases with 30°C incubations. This was borne out when sera incubated at 30°C for 60 min exhibited 96% of the activity observed with 6-min incubations. While slight, this decrease was statistically significant, but it can account for only a portion of the 16% increase in AST activity observed with pyridoxal phosphate supplementation. This was confirmed by altering the order of substrate addition, allowing sera to incubate in the presence of 2-oxoglutarate and initiating the reaction by addition of L-aspartate. It has been shown that AST is protected from denaturation by incubation with 2-oxoglutarate (32). Here, pyridoxal phosphate was able to effect a 14 ± 2% increase on a sample that showed 17 ± 1% increase when incubated in a medium containing aspartate. As Figure 5 also demonstrates, this protective action of pyridoxal phosphate is increasingly important at higher incubation temperatures.

Bowers (33) has suggested that the effect of the ratio of the sample volume to total reaction volume in kinetic assays must be examined before the advisability of using pyridoxal phosphate in a reference method for AST can be decided. Arbitrary variations of 6- to 15-fold serum dilution are now used in such assays. It is tempting to theorize that these differing dilutions might explain the discrepancies previously reported (1, 9–12) in the degree of pyridoxal phosphate stimulation of AST. Our results, however, showed that pyridoxal phosphate stimulates AST activity to about the same degree with use of either serum volume (Figures 1 and 4). Furthermore, in the nonsupplemented assay, there is no significant difference when either 0.2 or 0.5 ml of serum is used in the 3.0-ml kinetic assay (Figure 3).

The stimulation of AST activity observed with pyridoxal phosphate supplementation indicates that this enzyme is not fully saturated with coenzyme in the kinetic assay medium. For a normal serum, with a pyridoxal phosphate concentration of 24 μg/liter (34), final concentrations of this coenzyme in a 3.0-ml total assay volume are 6 × 10⁻⁹ mol/liter with 0.2 ml of serum and 1.5 × 10⁻⁸ mol/liter with 0.5 ml of serum. Turano et al. (35) have shown that with modified preparations of porcine AST, pyridoxamine phosphate dissociates from the holoenzyme at low concentrations in the presence of the amino acid substrate. In addition, while the binding of coenzyme to the aminotransferase is the result of many factors, the contribution of the phosphate group in the binding is important. Inorganic phosphate buffer, the buffer most frequently used in AST assays, has been shown to inhibit competitively the recombination of coenzyme with apoenzyme (36). Albumin, present in serum at a molar concentration about 7 × 10⁸ times that of AST, has also been shown to have an affinity for pyridoxal phosphate (37). Dissociation of the coenzyme, perhaps in the more easily resolved pyridoxamine form (38), from the holoenzyme and inhibition of its recombination with apoenzyme is thus a likely possibility in the AST reaction mixture. Our data also suggest that the enzyme present in serum is not fully saturated with coenzyme.

In our experiments, no comparable stimulation of serum AST could be observed when the assay method of Babson et al. (17) was used. Recombination of pyridoxal phosphate with apo-AST has been shown to be retarded by the presence of either 2-oxoglutarate or (less effectively) L-aspartate (4). In the Babson assay method (unlike the kinetic method), both
these substrates are present in the assay mixture when serum is added. In addition, only 20 min is required for enzyme catalytic activity, without preincubation. These factors may explain the lack of observable effect of pyridoxal phosphate in this method, and may also account for the lack of effect observed by other authors (9-11) who used similar methods.

The reported lack of AST stimulation by pyridoxal phosphate may be due in some cases to oversupplementation of coenzyme. Holzer and Schreiber (39) have shown that higher concentrations of pyridoxal phosphate (150 μmol/liter) inhibit the recombination of the coenzyme with the apoenzyme obtained from yeast. This is also consistent with our observations regarding AST of human cytoplasmic origin. Gonnard and Nguyen-Philippon (9), who reported no pyridoxal phosphate stimulation of serum AST, used it in concentrations as high as 320 μmol/liter.

Pyridoxal phosphate addition also elicited a 15% mean increase when the preincubation and assay were done at 37°C. Thus the lack of effect in the Babson colorimetric assay is not due to the higher assay temperature.

We also attempted to correlate the diagnoses of the patients with large increases in AST activity produced by added pyridoxal phosphate. Four of 141 sera examined (Table 1, Figure 1) demonstrated increases greater than 45% in aminotransferase activity when they were supplemented with pyridoxal phosphate. One specimen, the activity of which increased by 46%, was obtained from a 41-year-old male subject. The day before the sample was obtained the patient had undergone 12 h of single-pass, warm hemodialysis, with regional heparinization. A recent note by Wolf et al. (40) discusses low serum AST activity in patients undergoing hemodialysis. These authors suggest that hemodialysis may result in depletion of plasma pyridoxal and pyridoxamine phosphate and consequent lowered AST activity. The large stimulation in AST activity we observed supports this hypothesis.

A specimen drawn from another patient (female, 62 years old), diagnosed as having squamous cell carcinoma of the liver, exhibited a 56% increase in serum aminotransferase activity when supplemented with pyridoxal phosphate. In various liver disorders, Hamfelt (12) has observed large increases in AST activity when serum is supplemented with pyridoxal phosphate. Our results agree substantially with those of Hamfelt, who measured an average 17% increase in the serum AST activity of normal subjects when the specimen was supplemented with pyridoxal phosphate (26-30 μmol/liter). We concur with Hamfelt that there is “a fundamental error in the methods for serum aminotransferase determination as currently used in clinical laboratories” (12).

Our data indicate that including pyridoxal phosphate in the kinetic determination of serum AST protects against a loss of activity during preincubation that becomes increasingly important as preincubation temperature is increased; (b) increases measured activity by about 16%; and (c) cancels the effects of age, nutritional state, and similar conditions that artifactually influence AST activity by varying the concentration of vitamin B6 in the plasma. Thus pyridoxal phosphate supplementation seems to be desirable in clinical and reference methods for the estimation of true aspartate aminotransferase activity in human serum.

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References