Glycation reaction of aspartate aminotransferase by various carbohydrates in an in vitro system

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Effects of various carbohydrates on cytosolic aspartate aminotransferase were studied in an in vitro system. When the purified aminotransferase was incubated with glucose or glucose-6-phosphate, the enzymatic activity and isoelectric points were essentially unaffected. On the other hand, fructose, fructose-6-phosphate, and ribose lowered both the enzymatic activities and isoelectric points in the absence of pyridoxal phosphate, and such alteration of the enzyme was partially prevented by the presence of pyridoxal phosphate. We suggest that these carbohydrates may bind to the Lys258 residue of the enzyme, which is the binding site of pyridoxal phosphate. Glyceraldehyde and dihydroxyacetone had powerful effects on the enzymatic activity and shifted the isoelectric points to the anodic side. In these cases, the addition of pyridoxal phosphate showed little effect on such alterations of the enzyme. Carbohydrates, except for glucose and glucose-6-phosphate, also resulted in fragmentation and polymerization of the enzyme. It was found that six and seven of the lysyl residues of the enzyme per subunit were modified with glyceraldehyde and dihydroxyacetone, respectively, based on the analysis of amino acid composition. (J. Nutr. Biochem. 5:485–489, 1994.)

Keywords: glycation; aspartate aminotransferase; VB6 deficiency; pI of aspartate aminotransferase; SDS-PAGE

Introduction

There are two isozymes of aspartate aminotransferase (EC2. 6. 1. 1) in animal cells, a cytosolic and a mitochondrial isozyme. Cytosolic aspartate aminotransferase (AspATc) has been separated by electrophoresis or chromatography into several forms designated as α, β, γ, etc. in increasing order of negative charge.[1,2] The origin of these multiple forms is still unknown, although several possibilities have been reported.[3–10] We reported an increase in subforms of the enzyme with low pI values and low specific activities in vitamin B6-deficient rat liver.[11,12] We also observed a similar phenomenon when the cytosolic fraction of rat liver was incubated in vitro.[13] During studies of the factors responsible for inactivation of AspATc, a glycation reaction was considered as one of the possible mechanisms for producing variants of the enzyme. In previous papers,[14] influences of glucose and fructose on AspATc were examined and fructose were found to be effective in decreasing the pI values and the enzymatic activity. Fructose was more effective on apoenzyme than holoenzyme, suggesting that it may bind to the Lys258 residue of AspATc, which is the binding site of pyridoxal phosphate (PLP).

In the present study, we examined effects of various glucose metabolites on the glycation reaction of AspATc in a purified enzyme system.

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Received November 9, 1993; accepted May 5, 1994.

Methods and materials

Preparation of AspATc from rat liver

Male Wistar strain rats, weighing about 200 g, were fed a 70% casein diet[15] for 1 week, then their livers were removed for purification of AspATc. The enzyme was purified essentially by the method of Huynh et al.[16] The final preparation of AspATc gave a single band on acrylamide gel electrophoresis with sodium dodecyl sulfate (SDS). Anti-rat AspATc antiserum was prepared in rabbits as described previously.[17]

Chemicals

All reagents used were of the highest grade available and were purchased from Wako Pure Chemicals (Osaka, Japan), Boehringer (Mannheim, Germany) or Sigma Chemicals Co (St. Louis, MO US). Ampholyte, pI, and weight markers were obtained from Bio-Rad Laboratory (Hercules, CA, USA).

Incubation of purified AspATc

AspATc was incubated with various carbohydrates in the presence or absence of 10^{-4} M PLP at 37° C for indicated periods. The incubation mixtures usually contained AspATc (1 mg/1 mL), carbohydrate of indicated concentration, and 0.1% NaN_3 in 0.1 M sodium phosphate buffer (pH 7.0).

Electrophoretic analyses

Isoelectric focusing (IEF) was carried out essentially as described by Reinhart and Malamud.[18] Acrylamide gels (5%) of 1 mm thickness containing 2% Ampholine were prepared on glass plates, and a current was applied in a horizontal IEF apparatus (Atto Co. Ltd., Tokyo, Japan) with 1 M NaOH as catholyte and 1 M H_3PO_4 as anolyte. Gels were chilled to 10° C and pretreated at 400 V for 1 hr. Samples were then loaded onto the gels with a plastic applicator and focused to equilibrium (overnight, 400 V). The gels were washed with a 5% salicylic acid-10% TCA solution overnight to remove ampholyte and then stained for protein with a 0.25% Coomassie Brilliant Blue (CBB) G 10% acetic acid-30% methanol solution. SDS polyacrylamide gel electrophoresis (SDS-PAGE) was carried out as described by Laemmli.[19] Slab gels (9 × 7 cm, 1 mm thick) containing 12% acrylamide were run for 2 hr at 10 mA. Protein was located by staining with 0.25% CBB R in a 10% acetic acid-30% methanol solution. The position of AspATc cross-

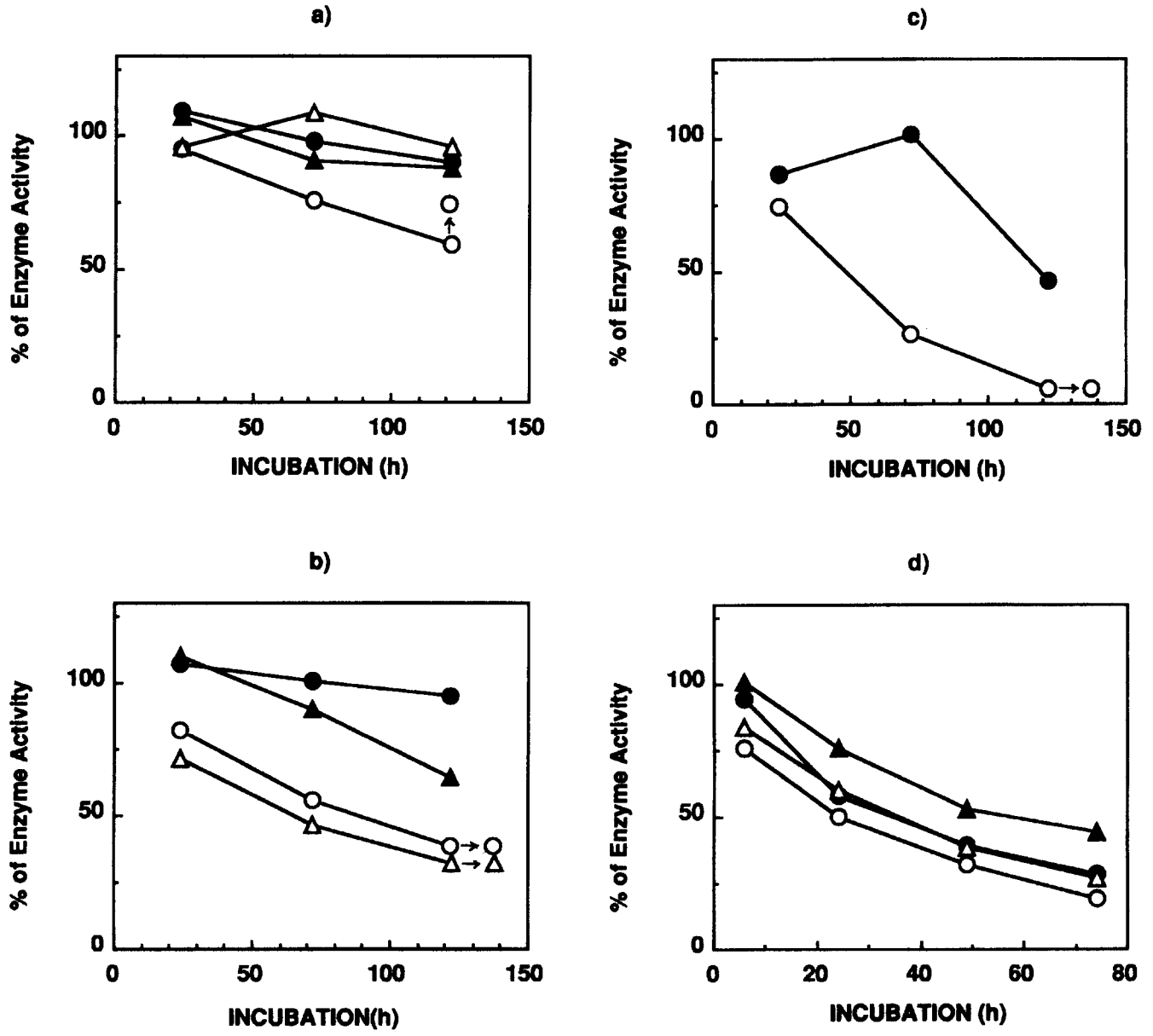

Figure 1 Effects of carbohydrate and PLP on AspATc activity. AspATc was incubated with various carbohydrates at 37° C for the periods indicated in the presence or absence of 10^{-4} M PLP. After the incubation, the remaining activities were determined. PLP was added to AspATc incubated without PLP at the end of incubation periods and then incubation was continued for 30 min. After the incubation periods, AspATc activities were determined as indicated by arrows. a) circles, 50 mM glucose; triangles, 50 mM G-6-P; b) circles, 50 mM fructose; triangles, 50 mM F-6-P; c) 50 mM ribose; d) circles, 1 mM DHA; triangles 1 mM GAD. Open symbols, no PLP; filled symbols, + PLP.

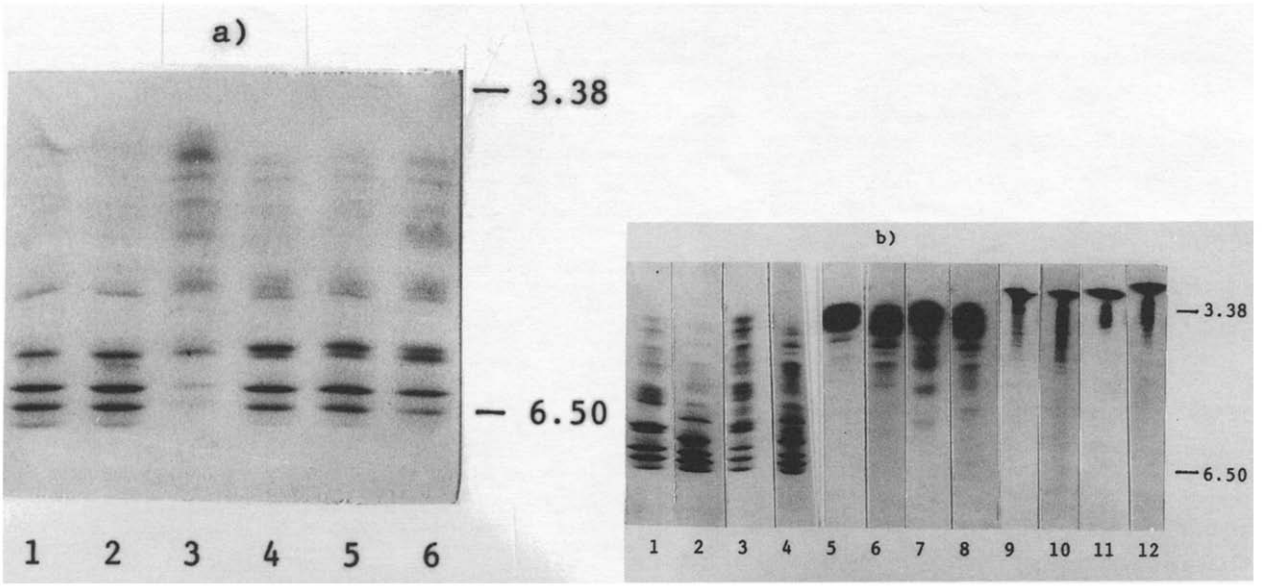

Figure 2 Effects of carbohydrates and PLP on IEF pattern of AspATc. AspATc was incubated with carbohydrate at 37° C for 5 days in the presence or absence of 10^{-4} M PLP, and 5 to 10 μg of each sample was applied to the gels. Additions are as follows: a) 1; none, 2; 50 mM glucose, 3; 50 mM fructose, 4; 10^{-4} M PLP, 5; 50 mM glucose + PLP, 6; 50 mM fructose + PLP, b) 1; none, 2; 10^{-4} M PLP, 3; 50 mM G6P, 4; G6P + PLP, 5; 50 mM F6P, 6; F6P + PLP, 7; 50 mM ribose, 8; ribose + PLP, 9; 50 mM GAD, 10; GAD + PLP, 11; 50 mM DHA, 12; DHA + PLP.

reactive material in the gel was determined by Western blot analysis.[20] After electrophoresis, protein was transferred from the gel to a nitrocellulose membrane at 2.0 mA/cm² for 60 min in the following buffer: 25 mM Tris-195 mM glycine-20% methanol. Then the membrane was briefly washed with buffered saline and used for immunoblotting. The immunoblotting was performed essentially by the method described previously.[13]

Analysis of amino acids

AspATc samples incubated with glyceraldehyde (GAD) or dihydroxyacetone (DHA) were reduced with 10 mM sodium borohydride (20° C, for 1 hr), extensively dialyzed against water, and hydrolyzed in 6N HCl at 110° C for 24 hr. Amino acids were automatically analyzed in an LKB Alpha Plus 4151 amino acid analyzer as described previously.[14]

Other methods

AspATc activity was measured by the modification of the method of Karmen[21] as described previously.[11]

Results

Influences of carbohydrates and PLP on AspATc activity

AspATc activity was determined after incubation with various carbohydrates. The enzymatic activity was generally decreased with an increase in the incubation period, but the extent of the decrease varied depending on the carbohydrate added. As shown in *Figure 1a,* glucose and glucose-6-phosphate (G6P) had almost no effect on AspATc activity during a 5-day incubation period at 37° C in the presence of PLP. Fructose, fructose-6-phosphate (F6P), and ribose decreased the AspATc activity, but the activity was protected from the inactivation by the presence of 10^{-4} M PLP to a variable extent *(Figures 1b and 1c).* On the other hand, the activity of enzyme inactivated by these compounds was not restored by the addition of PLP *(Figure 1b).* DHA and GAD were powerful inactivators of AspATc with or without PLP, and these were effective even in a lower concentration than the others examined *(Figure 1d).*

Influences of carbohydrates on isoelectric focusing (IEF) pattern of AspATc

The IEF pattern of AspATc incubated with various carbohydrates was studied. As shown in *Figure 2a,* pI values of AspATc were not altered by incubation with glucose regardless of the presence or absence of PLP. However, pI values were altered by incubation with fructose in the absence of PLP, and this alteration was prevented by the presence of PLP. G6P altered the IEF pattern of AspATc to a small extent in the presence or absence of PLP *(Figure 2b).* F6P, ribose, GAD, and GHA altered the pI values of AspATc extensively toward lower values (pI 3 to 4), suggesting that a glycation reaction of AspATc occurred under these conditions. The presence of excess PLP had little effect on the alteration of the pI values.

Influences of carbohydrates on molecular size of AspATc

The molecular size of AspATc incubated with various carbohydrates was examined by means of SDS-PAGE. Only one 47 kDa band, which corresponds to the subunit size of the enzyme, was observed for SDS-PAGE stained with CBB in all cases tested *(Figures 3a and 3b).* AspATc incubated with fructose and ribose without added PLP was found to have a slightly higher molecular size than the others, suggesting that these carbohydrates are possibly bound to the enzyme

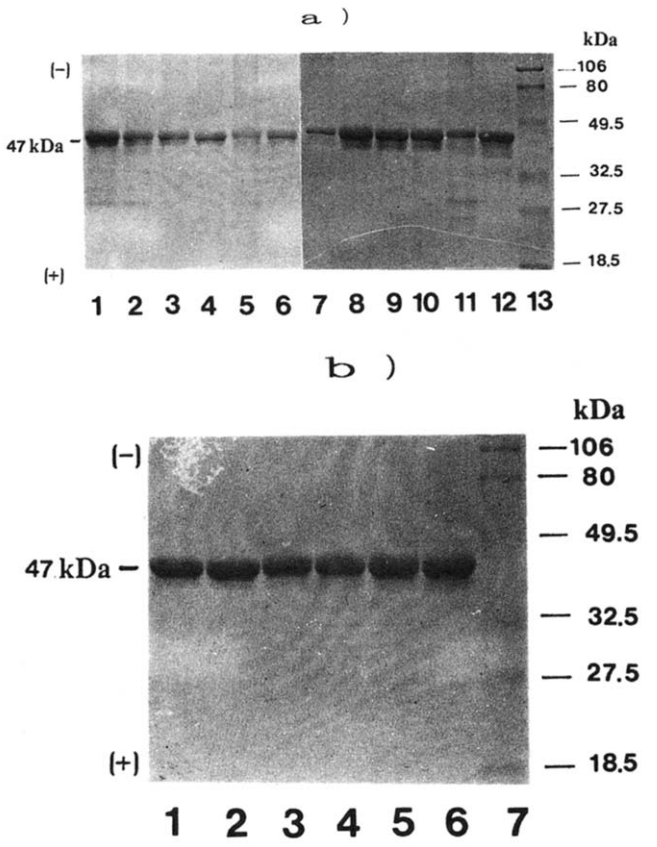

Figure 3 SDS-PAGE of AspATc incubated with carbohydrate. Incubation conditions were as described in *Figure 2*. Electrophoresis was carried out as described in the text. Protein was stained with Coomassie Brilliant Blue R. Additions are as follows; a) 1; none, 2; 10^{-4} M PLP, 3; glucose, 4; Glucose + PLP, 5; G6P, 6; G6P + PLP, 7; fructose, 8; fructose + PLP, 9; F6P, 10; F6P + PLP, 11; ribose, 12; ribose + PLP, 13; markers (106 kDa; phosphorylase B, 80 kDa; BSA, 49.5 kDa; ovalbumin, 32.5 kDa; carbonic anhydrase, 27.5 kDa; soybean trypsin inhibitor, 18.5 kDa; lysozyme). b) 1; none, 2; PLP, 3; GAD, 4; GAD + PLP, 5; DHA, 6; DHA + PLP, 7; markers.

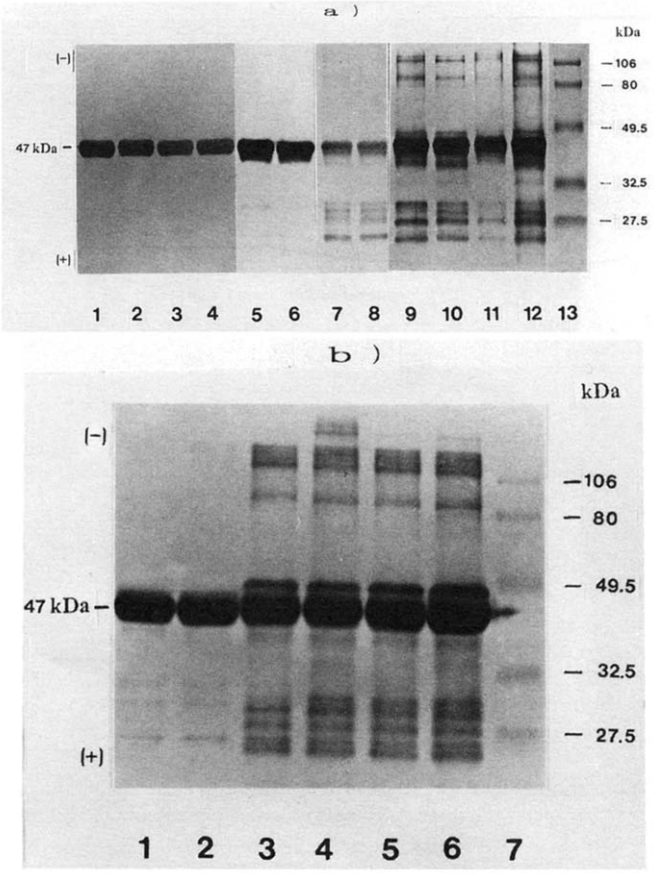

Figure 4 Immunoblotting analysis of AspATc incubated with carbohydrates. Experimental conditions were as described in *Figures 2 and 3* and in the text. Additions were the same as in *Figure 3*.

molecule. On the other hand, when the SDS-PAGE was immunologically stained, many bands other than the 47 kDa band were observed when sugars other than glucose or G6P were used. *Figures 4a and 4b* show that polymerization and fragmentation occurred by incubation of AspATc with various carbohydrates. Such alteration in the molecular size of AspATc was correlated with the alterations of the enzymatic activities and the pI values.

Amino acid composition of glycated AspATc

AspATc incubated in the presence of GAD or DHA, both of which are powerful effectors of the enzyme, was analyzed for amino acid composition. As shown in *Table 1*, lysyl residues were found to be modified with these carbohydrates; numbers of lysyl residue modified were calculated to be six for GAD and seven for DHA per subunit. When the enzyme was incubated with DHA, serine residues seemed to increase. This result was clarified by identification of a lysyl-DHA adduct that appeared at the serine position.

Table 1 Amino acid composition of AspATc incubated with GAD or GHA

Addition	none	GAD	DHA
Aspartic acid*	45	45	45
Threonine	22	22	22
Serine	26	26	33‡
Glutamic acid†	40	42	41
Glycine	30	29	33
Alanine	32	31	33
Valine	30	28	29
Isoleucine	17	18	17
Leucine	37	38	37
Tyrosine	12	11	11
Phenylalanine	25	26	25
Lysine	21	15	14
Histidine	8	10	9
Arginine	24	22	18
Proline	23	23	23

* and † contain asparagine and glutamine, respectively.
‡ included lysyl-DHA adduct.
Values are expressed as molar per subunit. Cysteine and tryptophan were not determined under the condition used. The experiment was repeated and the values are means for two determinations in the typical one. The experimental conditions are described in the text.

Discussion

Our results suggest that lysyl residue 258, which is the binding site of PLP, was modified by carbohydrates in the absence of PLP. Whereas DHA and GAD strongly inhibited AspATc with or without PLP, the lysyl residue 258 seemed to have a high affinity for these two carbohydrates rather than PLP. Such differences in the ability of inactivation of AspATc by various carbohydrates may depend on the number of open chain forms in the solution. The rate of Schiff base formation is directly proportional to the percentage of sugar in the open chain form. Thus, glucose has the slowest rate of Schiff base formation, whereas GAD (100% open chain) forms more than 200 times more glycated protein than equimolar amounts of glucose.[22] Immunobloting analysis showed that the glycation reaction of AspATc by those carbohydrates tested resulted in polymerization and fragmentation *(Figure 4)*.

Recently, glycation reactions of several proteins has been reported in vivo and in vitro.[23–25] The structural and functional alterations of such glycated protein have been observed not only in diabetes mellitus but in some other diseases and in aging. There are many PLP-dependent enzymes in living cells, and when they are subjected to a vitamin B_6-deficient state, the fraction of apoenzyme must be increased due to the lack of PLP. As PLP is known to be bound to lysyl residue of the enzyme protein in PLP-dependent enzymes, there will be a free ϵ-amino group of the lysyl residue and some open space will be present in the coenzyme site. It is conceivable that this space may be easily occupied by some other compound with a carbonyl group. In this study, we found that several lysyl residues besides Lys258 were glycated by some carbohydrates and this may have been caused by exposure of lysyl residues by an unstable conformation of the apoenzyme.

In our previous study,[17] we found that in vitamin B_6-deficient rat livers the activity of AspATc was decreased and was not restored by the addition of PLP to the assay medium, but that the antigenic activity was maintained at normal level. We also observed an increase in subforms of the enzyme with low isoelectric points in vitamin B_6-deficient rat liver.[12] It would be reasonable to consider that glycation of AspATc may occur in the liver of vitamin B_6-deficient rats. We therefore are investigating the possible presence of a glycated AspATc molecule in the livers of vitamin B_6-deficient rats.

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