SUMMARY

DL-Glyceraldehyde 3-phosphate in low concentrations has been found to be a time-dependent inhibitor of aspartate aminotransferase (EC 2.6.1.1) isozymes. The cationic isozyme is more susceptible than the anionic isozyme to this inhibition. The L-isomer of glyceraldehyde 3-phosphate was as effective as the D-isomer for each isozyme. Study of various glycolytic intermediates indicated that the con-joint presence of the free aldehyde group and the phosphoryl residue of glyceraldehyde 3-phosphate was necessary for inhibition.

The extent of inhibition was dependent on the duration of preliminary incubation, the concentration of glyceraldehyde-3-P, and the pH of the preliminary incubation mixture, but was independent of the concentration of enzyme. Half-maximum inhibition of each isozyme was obtained between pH 6.5 and 6.7. Maximum inhibition of the anionic isozyme was obtained at pH values of 8.4 and above, while the cationic isozyme was optimally inhibited at pH 7.4. The presence of α-ketoglutarate in the preliminary incubation mixture decreased inhibition while aspartate accentuated it.

After 30 min of preliminary incubation with glyceraldehyde-3-P, dialysis of the anionic isozyme against buffer, aspartate, α-ketoglutarate, or pyridoxal 5-phosphate resulted in a substantial release of inhibition. In the case of the cationic isozyme, substantial release of inhibition was obtained only by dialysis against α-ketoglutarate. The addition of α-ketoglutarate to either the anionic or cationic isozyme-inhibitor preliminary incubation mixtures released inhibition, but only in the case of the anionic isozyme was the extent of release dependent on the duration of preliminary incubation. Prolonged incubation of this isozyme with glyceraldehyde-3-P resulted in the appearance of a second phase of inhibition.

The mechanism of the inhibition of the isozymes by glyceraldehyde-3-P is discussed.
In the presence of sodium barbital-HCl, pH 7.4. Anionic isozyme was inhibited by the addition of substrate mixture at 37°C to the initially incubated isozyme solution. The final reaction volumes of 1.0 or 3.0 ml contained 16.7 mM aspartate, 6.7 mM α-ketoglutarate, 0.067 mg per ml of NADH, 0.064 unit per ml of malic dehydrogenase, and 6.7 × 10⁻³ unit per ml or 7.0 × 10⁻³ unit per ml of the anionic and cationic isozymes, respectively. Initial velocities were determined by following the rate of decrease in absorbance at 340 nm in a Beckman model DU spectrophotometer fitted with a Gilford 2000 automatic sample changer and recorder. Enzyme velocities were expressed as change in absorbance per 3 min.

Materials—The chemicals used were of the highest purity commercially available. All compounds were obtained as, or converted to, the sodium or potassium salts. Dipotassium dihydrogen 1-phosphate, disodium di-fructose 1-phosphate, disodium DL-glycerol 1-phosphate, dihydroxyacetone phosphate dimethylketal, barium DL-glyceraldehyde 3-phosphate diethylacetal, sodium D-3-phosphoglycerate, sodium 2-phosphoglycerate, trisodium phosphonoacetate, and D-glyceraldehyde were purchased from Sigma. Malic dehydrogenase (Sigma) was reconstituted in 0.04 M sodium barbital-HCl buffer, pH 7.4, containing 0.15% human serum albumin and dialyzed against 0.04 M sodium barbital-HCl, pH 7.4, to remove ammonium sulfate (17). Pyridoxal 5'-phosphate, α-ketoglutaric acid, disodium D-glucose 6-phosphate, and trisodium D-fructose 1,6-diphosphate were obtained from Calbiochem. Barium D-fructose 6-phosphate and rabbit muscle D-glyceraldehyde 3-phosphate dehydrogenase were purchased from Boehringer Mannheim. Aspartic acid was obtained from Ceylo Chemical Corporation or Calbiochem.

DL-Glyceraldehyde-3-P was prepared from fructose 6-phosphate by periodate oxidation according to the procedure of Szewczuk et al. (10). The product obtained was 65% D-glyceraldehyde-3-P and was further purified. A solution, 14 ml, that contained approximately 500 μmoles of organic phosphorus of which 316 μmoles were D-glyceraldehyde-3-P, as determined by enzymic analysis (20), was placed on a column, 1.3 × 10.0 cm, of Dowex AG-XS that had been equilibrated with 1 mM HCl. The column was eluted with a linear gradient between 35 ml of 1 mM HCl and 35 ml of 1 mM HCl-0.1 mM NaCl. This was followed by an additional 35 ml of 1 mM HCl-0.1 mM NaCl. Fractions of 3.7 ml were collected, and those tubes in which the total phosphorus and the alkali labile phosphorus (10 min at room temperature in 1 N NaOH) were the same were pooled. Both the D-glyceraldehyde-3-P and the impurities were eluted close to the upper limit of the gradient. Because of overlapping of the peaks only 161 μmoles (about 50%) of the D-glyceraldehyde-3-P could be recovered free of impurities. The compound was precipitated as the calcium salt and dried at room temperature, in a vacuum, over silica gel. The calcium salt was transformed to the hydrogen form with Dowex 50, and the stock solution was kept frozen at pH 2.5. Aliquots were adjusted to pH 7.4 immediately before use. A comparison of the phosphorus analysis with enzymic analysis by means of glyceraldehyde-3-P dehydrogenase showed 83 μmoles of organic phosphorus, 76 μmoles of alkali labile phosphorus, and 78 μmoles of enzymically active compound. Thus, the final preparation was at least 94% pure.

RESULTS

Effect of Glycolytic Intermediates on Activities of Aspartate Aminotransferase Isozymes—Table I shows the effect of preliminary incubation of aspartate aminotransferase isozymes with various glycolytic intermediates for 30 min at 37°C. Of the compounds tested, only DL-glyceraldehyde-3-P substantially inhibited both isozymes. The cationic isozyme was more sensitive than the anionic isozyme to this inhibition. The auxiliary enzyme in the assay of aspartate aminotransferase, malic dehydrogenase, was not affected by DL-glyceraldehyde-3-P. The inhibition of the cationic isozyme by dihydroxycetone-P was low but reproducible. Enzymic analysis of the preparation of dihydroxycetone-P used in these studies showed that it contained no more than 0.24% D-glyceraldehyde-3-P. This amount of contamination would not have affected the activity of either isozyme when dihydroxycetone-P was tested at 2 mM (Table 1). None of the other compounds had an effect at the concentrations used. With respect to each isozyme, D- and DL-glyceraldehyde-3-P were equally effective inhibitors (Fig. 2). Thus, the L-isomer was as inhibitory as the D-isomer of glyceraldehyde-3-P.

Time Dependence of Inhibition of Aspartate Aminotransferase Isozymes by DL-Glyceraldehyde-3-P—Fig. 3A shows that the

FIG. 1. The effect of chloride on the stability of aspartate aminotransferase isozymes at 37°C. Isozymes were diluted in either 0.04 M sodium barbital-barbituric acid, pH 7.4, or 0.04 M sodium barbital-HCl, pH 7.4. Each diluent also contained 0.15% human serum albumin. Samples of 0.2 ml were brought up to 2.5 ml with the appropriate buffer and incubated at 37°C for the indicated times. Enzyme activity was determined after adding 0.5 ml of previously warmed substrate mixture. The results are expressed as percentage of the activity at zero time for each isozyme preparation that had been kept in 5 ml of Tris-barbital-HCl buffer at pH 7.4, containing 0.15% human serum albumin. Samples of 0.2 ml were brought up to 2.5 ml of the anionic and cationic isozymes, respectively. Initial concentrations of chloride also maintained the activity of the diluted isozyme without chloride (○) and with 0.034 M chloride (●). Cationic isozyme without chloride (□) and with 0.034 M chloride (△).

1 Solutions of DL-glyceraldehyde-3-P were stable for at least 6 hours in sodium barbital-HCl buffer at pH 7.4 when kept on ice. At 37°C, however, this compound was destroyed at a rate of approximately 28% per hour over a 3-hour period.
inhibition of the anionic isozyme by DL-glyceraldehyde-3-P was time-dependent and attained maximal values within 30 min and remained constant for up to 1 hour. In the absence of either substrate in the preliminary incubation mixture, the maximal inhibitions were 18 and 47% at concentrations of 0.2 and 2.0 mM DL-glyceraldehyde-3-P, respectively. When 16.7 mM aspartate was included in the preliminary incubation mixture, the maximal values of inhibition increased to 51 and 70% respectively. In contrast, when 6.7 mM α-ketoglutarate instead of aspartate was included in the preliminary incubation mixture the maximal inhibitions were decreased to zero at 0.2 mM and 11% at 2.0 mM DL-glyceraldehyde-3-P.

Fig. 3 shows that essentially the same patterns were obtained for the cationic isozyme. In the absence of substrates in the preliminary incubation mixtures, maximal inhibitions of 14 and 67% were attained at 30 min with 0.06 and 0.6 mM DL-glyceraldehyde-3-P, respectively. In the presence of 16.7 mM aspartate, levels of inhibition were increased to 33 and 86% and were attained after about 15 min. On the other hand, the presence of 6.7 mM α-ketoglutarate during preliminary incubation led to a marked decrease of the inhibition of the cationic isozyme. Fig. 3 also shows that the time course of inhibition for each isozyme, whether or not substrate was present, was essentially the same at two concentrations of DL-glyceraldehyde-3-P that differed by 10-fold.

Dependence of Extent of Inhibition on Concentration of DL-Glyceraldehyde-3-P—In the absence of either substrate the extent of inhibition of the anionic isozyme after 30-min preliminary incubation increased with increasing concentration of DL-glyceraldehyde-3-P until it reached a maximum of 60% at 3 mM DL-glyceraldehyde-3-P (Fig. 4A). When 16.7 mM aspartate was present during preliminary incubation, maximal inhibition of 70% occurred at the same concentration of DL-glyceraldehyde-3-P. When 6.7 mM α-ketoglutarate was present during preliminary incubation, the inhibition was almost completely abolished. Fig. 4B shows that, for the cationic isozyme, the presence of aspartate during preliminary incubation did not affect the maximal extent of inhibition, 90%, but decreased the concentration of DL-glyceraldehyde-3-P required to attain this degree of inhibition from 2.0 to 0.6 mM DL-glyceraldehyde-3-P.

Table I

| Compound                          | Concentration in preliminary incubation mixture | Cationic isozyme | Anionic isozyme |
|----------------------------------|-----------------------------------------------|------------------|----------------|
| d-Glucose 1-phosphate            | 2.0 mM                                        | 1                | 0              |
| d-Glucose 6-phosphate            | 2.0 mM                                        | 2                | 1              |
| d-Fructose 1-phosphate           | 2.0 mM                                        | 0                | 1              |
| d-Fructose 6-phosphate           | 2.0 mM                                        | 1                | 0              |
| d-Fructose 1,6-diphosphate       | 2.0 mM                                        | 2                | 0              |
| DL-Glycerol 1-phosphate          | 4.0 mM                                        | 0                | 0              |
| Dihydroxyacetone phosphate       | 2.0 mM                                        | 12               | 1              |
| DL-Glyceraldehyde                | 4.0 mM                                        | 5                | 1              |
| DD-Glyceraldehyde 3-phosphate    | 1.0 mM                                        | 81               | 31             |
| 2-Phosphoglycerate               | 2.0 mM                                        | 0                | 0              |
| Phosphoenolpyruvate              | 2.0 mM                                        | 0                | 0              |

*Enzymic analysis showed this preparation to contain 0.24% DL-glyceraldehyde 3-phosphate, corresponding to 0.005 mM in the preparation mixture.

Fig. 2. Inhibition of aspartate aminotransferase isozymes by DL-glyceraldehyde-3-P and nN-glyceraldehyde-3-P. Cationic isozyme was incubated in 2.5 ml of sodium barbital- HCl, pH 7.4, that contained nN-glyceroldehyde-3-P ( ), or DL-glyceroldehyde-3-P ( ). Anionic isozyme was incubated in 0.83 ml of buffer that contained nN-glyceroldehyde-3-P ( ) or DL-glyceroldehyde-3-P ( ). Mixtures were initially incubated at 37° for 30 min. Enzyme activity was measured after addition of 0.5 or 0.17 ml of previously warmed substrate mixture to the cationic and the anionic preliminary incubation mixtures, respectively. Percentage inhibition values were calculated from controls that did not contain glyceroldehyde-3-P.

Fig. 3. Time course of inhibition of aspartate aminotransferase isozymes by DL-glyceraldehyde-3-P in the absence and presence of substrates. A, anionic isozyme was initially incubated at 37°, in 2.5 ml of sodium barbital-HCl, pH 7.4, with DL-glyceraldehyde-3-P at concentrations of 2.0 mM ( ), 0.62 mM ( ), and 0.2 mM ( ), in the presence of buffer ( ), or 16.7 mM aspartate ( ), or 6.7 mM α-keto glutarate ( ). B, cationic isozyme was initially incubated in 2.5 ml of sodium barbital-HCl, pH 7.4, with DL-glyceraldehyde-3-P at concentrations of 0.6 mM ( ) or 0.06 mM ( ), in the presence of buffer ( ), or 16.7 mM aspartate ( ), or 6.7 mM α-keto glutarate ( ). Enzyme activities were determined after addition of 0.5 ml of substrate mixture. The concentrations of substrates in these mixtures had been adjusted to give 16.7 mM aspartate and 6.7 mM α-keto glutarate in the final 3.0-ml volume. Percentage inhibition values were calculated from controls that did not contain glyceroldehyde-3-P.
in which DL-glyceraldehyde-3-P was omitted provided a measure was determined. This concentration of buffer was sufficient to HCl, pH 7.4, was added to each sample and enzyme activity 2.5 ml of complete substrate mixture in 0.04 M sodium barbital-
to the same pH. The chloride concentration was approximately 
substrate mixture. The concentrations of substrates in these mix-
tures had been adjusted to give 16.7 mM aspartate and 6.7 mM 
the final 3.0-ml volume. Percentage inhibition values were calculated from controls that did not contain glycer-
aldehyde-3-P.
mM. In agreement with the results obtained with the anionic isozyme, 6.7 mM  
aldose substantially protected the cationic isozyme against inhibition.

Inhibition of Aspartate Aminotransferase Isozymes by DL-
Glyceraldehyde-3-P as Function of Isozyme Protein Concentration—
It has been shown that the isozymes of aspartate aminotrans-
fase may dissociate into subunits in dilute solutions (21, 22). 
The possibility that such subunits might have different sensitivity  
to inhibition by glyceraldehyde-3-P was explored. Fig. 5A shows 
that the activity of the anionic isozyme was a linear function of 
its concentration between 2.6 and 260 ng of protein per ml after 
preliminary incubation for 30 min both in the absence and in 
the presence of 0.1 or 4.0 mM DL-glyceraldehyde-3-P. Similar 
results were obtained when the cationic isozyme at concentra-
tions between 1.16 and 116 ng of protein per ml was initially 
incubated with 0, 0.2, or 1.0 mM DL-glyceraldehyde-3-P (Fig.  
5B). The presence of either 16.7 mM aspartate or 6.7 mM  
ketoglutarate with or without DL-glyceraldehyde-3-P in the 
preliminary incubation mixture did not alter the linear relation-
ship between reaction velocity and protein concentration for 
either isozyme.

Effect of pH on Inhibition of Aspartate Aminotransferase 
Isozymes by DL-Glyceraldehyde-3-P—The isozymes were diluted in 
0.08 M NaCl-0.004 M sodium barbital-HCl-0.15% human serum albumin, adjusted to the desired pH. Diluted isozyme, 0.2 ml, 
was mixed with either 0.3 ml of DL-glyceraldehyde-3-P solution or 
0.3 ml of 0.004 M sodium barbital-HCl in controls, adjusted to 
the same pH. The chloride concentration was approximately  
0.004 M in the preliminary incubation mixture. The pH of each 
incubation mixture was measured before incubation. After 30 min at 37°,  
2.5 ml of complete substrate mixture in 0.04 M sodium barbital-
HCl, pH 7.4, was added to each sample and enzyme activity 
was determined. This concentration of buffer was sufficient to 
bring the pH in the reaction to 7.4 ± 0.05. Control experiments 
in which DL-glyceraldehyde-3-P was omitted provided a measure 
of the stability of the isozymes over the pH range studied (top curves, Fig. 6). The percentage inhibition of each isozyme was 
calculated at each pH value from the activity of the corresponding 
control. Fig. 6A shows that the anionic isozyme was maximally 
inhibited between pH 8.4 and 10.3 in the presence of either 0.4 
or 4.0 mM DL-glyceraldehyde-3-P. At pH values below 8.4 there 
was a progressive decrease of inhibition. At pH 5.4, the in-
hibition was less than zero, indicating a slight protection of the 
anionic isozyme against the inactivation observed in the control. 
Fig. 6B shows that, in the presence of 0.16 or 0.6 mM DL-glyceral-
dehyde-3-P, the inhibition of the cationic isozyme increased 
from zero at pH 5.4 to a maximum at pH 7.4 and decreased at 
higher pH values. The lowered levels of inhibition at pH values 
other than the optima were not the result of changes in the 
rates of interaction between the isozymes and the inhibitor, 
since it was found that maximal inhibition was attained within 
30 min at pH 6.4 and 9.2 as well as at pH 7.4. There was no relationship between the effects of pH on inhibition by DL-
glyceraldehyde-3-P and the effects of pH on the stability of the 
two isozymes.

Reversal of Inhibition—As has been noted (Figs. 3 and 4) 
the inhibition of both isozymes by DL-glyceraldehyde-3-P. In order to study the effect of adding either substrate after 
preliminary incubation of the isozymes with DL-glyceraldehyde- 
3-P, the anionic isozyme was initially incubated with 3.6 mM 
DL-glyceraldehyde-3-P and the cationic isozyme with 0.36 mM 
DL-glyceraldehyde-3-P for varying periods. Fig. 7A shows that, 
in the case of the anionic isozyme, addition at 15 min of α-
to be the result of the instability of DL-glyceraldehyde-3-P. When buffer instead of substrate was added to the preliminary incubation mixture, the extent of inhibition remained constant during the following 30 min. Thereafter a progressive loss of activity, which may be regarded as a second phase, was observed in all three types of incubation mixtures, albeit at differing rates. With regard to the cationic isozyme (Fig. 7B), the directions of changes in activity during the 30 min after addition of substrate or buffer were similar to those observed with the anionic isozyme. However, on further incubation and in contrast to the anionic isozyme, all three types of mixture showed progressive increases in activity.

This release of inhibition of the cationic isozyme was shown to be the result of the instability of DL-glyceraldehyde-3-P. Cationic isozyme was incubated for 30 min with aliquots of DL-glyceraldehyde-3-P that had been kept at 37° in buffer, pH 7.4, for varying periods of time. The longer the inhibitor was kept at 37°, the less the inhibition. In spite of the instability of glyceraldehyde-3-P, a second phase of inhibition of the anionic isozyme was observed during prolonged incubation with this inhibitor. As has been noted by Webb (23) for other enzymes and inhibitors, this second phase may be the result of increased instability of the isozyme when combined with glyceraldehyde-3-P, or to reaction of this compound with other, more slowly reacting groups.

In order to determine whether the release of inhibition, observed after the addition of α-ketoglutarate, and the potentiation of inhibition obtained after aspartate addition were dependent on the time of preliminary incubation of the isozyme with DL-glyceraldehyde-3-P, a mixture of these in buffer, pH 7.4, were initially incubated for 3 to 4 hours. At intervals during this period, aliquots were added to solutions of buffer, α-ketoglutarate, or aspartate to yield the concentrations described in the preceding sections. The mixtures were then incubated at 37° for an additional 30 min in the case of the anionic isozyme or 60 min for the cationic isozyme. The activity of the inhibited anionic isozyme initially incubated without substrate was constant between 30 and 75 min, then decreased (Fig. 8A). In contrast, the activity of the inhibited cationic isozyme showed a moderate increase after 30 min (Fig. 8B). Fig. 8A shows that the extent of reactivation of the anionic isozyme by α-ketoglutarate decreased markedly with increasing time of preliminary incubation with DL-glyceraldehyde-3-P. In contrast, the extent to which the cationic isozyme was reactivated by α-ketoglutarate decreased only slightly with increasing time of preliminary incubation with the inhibitor (Fig. 8B). The degree of potentiation of inhibition in the presence of aspartate was largely independent of the time of preliminary incubation with DL-glyceraldehyde-3-P for both isozymes.

Fig. 9A shows that it was also possible to reverse the inhibition of the anionic isozyme substantially by dialysis for 3 hours at 37° after it had been initially incubated for 30 min with 2.0

Fig. 6. Effect of pH on the inhibition of aspartate aminotransferase isozymes by DL-glyceraldehyde-3-P. Each isozyme was incubated with DL-glyceraldehyde-3-P for 15 min in a volume of 10 ml. The pH was then adjusted to 7.4 ± 0.05 by adding 2.5 ml of previously warmed substrate mixture, pH 7.4, in 0.01 M sodium barbital to start the reaction. Controls incubated without DL-glyceraldehyde-3-P (top curves) show the stability of the isozymes over the pH range studied. The lower curves show the inhibition as a function of pH. A, the anionic isozyme with 4.0 mM (□) and 0.4 mM (●) DL-glyceraldehyde-3-P. B, the cationic isozyme with 0.5 mM (□) and 0.16 mM (●) DL-glyceraldehyde-3-P. At each pH value, percentage inhibition was calculated from the controls.

Fig. 7. Effect of time on the inhibition of aspartate aminotransferase isozymes by DL-glyceraldehyde-3-P after the addition of substrates. The anionic isozyme (A) was incubated with 3.6 mM and the cationic isozyme (B) with 0.36 mM DL-glyceraldehyde-3-P for 15 min in a volume of 10 ml. At the end of this period, 0.66 ml of 0.04 M sodium barbital-HCl, pH 7.4, or substrate was added to 3.3-ml aliquots to give final concentrations of 16.7 mM aspartate (ASP) or 6.7 mM α-ketoglutarate (α-KG) and 3.0 and 0.3 mM DL-glyceraldehyde-3-P in A and B, respectively. At intervals, 0.6-ml aliquots were assayed for enzyme activity after the addition of 2.4 ml of previously warmed substrate mixture. The final substrate concentrations during assay were 16.7 mM aspartate and 6.7 mM α-ketoglutarate. Enzyme controls without DL-glyceraldehyde-3-P or substrate were incubated simultaneously. Control enzyme activity (X), activity with DL-glyceraldehyde-3-P and buffer (●), 16.7 mM aspartate (◆), or 6.7 mM α-ketoglutarate (□). All activities were expressed as percentage of the control activity at zero time.

Fig. 8. Effect of time on the inhibition of aspartate aminotransferase isozymes by DL-glyceraldehyde-3-P after the addition of substrate. The anionic isozyme (A) was incubated with 3.6 mM and the cationic isozyme (B) with 0.36 mM DL-glyceraldehyde-3-P for 15 min in a volume of 10 ml. At the end of this period, 0.66 ml of 0.04 M sodium barbital-HCl, pH 7.4, or substrate was added to 3.3-ml aliquots to give final concentrations of 16.7 mM aspartate (ASP) or 6.7 mM α-ketoglutarate (α-KG) and 3.0 and 0.3 mM DL-glyceraldehyde-3-P in A and B, respectively. At intervals, 0.6-ml aliquots were assayed for enzyme activity after the addition of 2.4 ml of previously warmed substrate mixture. The final substrate concentrations during assay were 16.7 mM aspartate and 6.7 mM α-ketoglutarate. Enzyme controls without DL-glyceraldehyde-3-P or substrate were incubated simultaneously. Control enzyme activity (X), activity with DL-glyceraldehyde-3-P and buffer (●), 16.7 mM aspartate (◆), or 6.7 mM α-ketoglutarate (□). All activities were expressed as percentage of the control activity at zero time.
isozymes incubated without \( \text{nN-glyceraldehyde-3-P} \) were also the same, from 35\% of the control activity before dialysis to 75\%.

0.1 ml of aspartate to give 16.7 mM (A), or 0.1 ml of \( \alpha\)-ketoglutarate measured at various time intervals (B). Activities are expressed initially incubated at 37°C with 3.6 mM \( \text{nN-glyceraldehyde-3-P} \) in 10 ml of 0.04 M sodium barbital-HCl, pH 7.4. At various time intervals 0.5 ml aliquots were withdrawn, added to 2.5 ml of previously warmed substrate mixture, and assayed (\( \Delta \)). Other 0.3-ml aliquots were also withdrawn at timed intervals and were incubated for an additional 30 min after the addition of 0.1 ml of buffer (\( \bullet \)), 0.1 ml of aspartate to give 16.7 mM (A), or 0.1 ml of \( \alpha\)-ketoglutarate to give 6.7 mM (B). At the end of this 30-min period the enzyme reactions were initiated by addition of 2.4 ml of substrate mixture. B, cationic isozyme was initially incubated in the same manner except that the concentration of \( \text{nN-glyceraldehyde-3-P} \) was 0.36 mM, and the incubation was continued for an additional 60 min (see text) after addition of buffer or substrate. The activities of isozymes incubated without \( \text{nN-glyceraldehyde-3-P} \) were also measured at various time intervals (G). Activities are expressed as percentage of the control activity at zero time.

100 mM \( \text{DL-glyceraldehyde-3-P} \). Dialysis against sodium barbital-HCl buffer or buffer that contained 8 mM \( \alpha\)-ketoglutarate, or 0.4 mM pyridoxal-5-P restored the activity from 35 to 41\% to only 80 to 85\% of that of a control enzyme solution without \( \text{DL-glyceraldehyde-3-P} \) dialyzed under the same conditions. In the presence of 20 mM aspartate the extent of recovery was essentially the same, from 35\% of the control activity before dialysis to 75\%.

In contrast, cationic isozyme that had been initially incubated for 30 min with 0.6 mM \( \text{DL-glyceraldehyde-3-P} \) when dialyzed against buffer, or buffer that contained 20 mM aspartate or 0.4 mM pyridoxal-5-P increased in activity from 22 to 29\% to only 51 to 55\% of the control levels (Fig. 9B). Dialysis against \( \alpha\)-ketoglutarate gave almost complete recovery of enzyme activity, to 93\% of the control value. These results suggest that, in the absence of \( \alpha\)-ketoglutarate, \( \text{DL-glyceraldehyde-3-P} \) was bound more tightly to the cationic isozyme than to the anionic isozyme. It is unlikely that pyridoxal-5-P was dissociated from either isozyme as a result of inhibition by \( \text{dl-glyceraldehyde-3-P} \) since dialysis against pyridoxal-5-P was no more effective in restoring activity than was dialysis against buffer alone.

The second phase of inhibition of the anionic isozyme (Figs. 7A and 8A) was apparently not reversible by dialysis. This isozyme was initially incubated at 37°C with 2.0 mM \( \text{DL-glyceraldehyde-3-P} \) for the usual period of 30 min as well as for a longer period of 2 hours, then dialyzed against 0.04 M sodium barbital-HCl, pH 7.4, for 3 hours at 37°C. Controls without \( \text{DL-glyceraldehyde-3-P} \) were treated in the same manner. As in the earlier experiment (Fig. 9A), enzyme activity rose substantially, from 46\% of that in the control after 30 min of preliminary incubation to 87\% after dialysis, when after 2 hours of preliminary incubation to 87\% after dialysis, whereas after 2 hours of 

DISCUSSION

Each isozyme of aspartate aminotransferase purified from rat liver was inhibited by \( \text{DL-glyceraldehyde-3-P} \). As noted previously, there was no stereospecific requirement with regard to the second carbon of glycerdehyde-3-P. A survey of the compounds tested showed that the conjoint presence of a free aldehyde and a phosphate group was necessary for inhibition. The \( pK_a \) for glycerdehyde-3-P is 6.45 (25). At pH 7.4, where about
95% glyceraldehyde-3-P exists as a monovalent ion, there was no inhibition of either isozyme (Fig. 6). Half-maximal inhibition of each isozyme occurred between pH 6.5 and 6.7, suggesting that the divalent ion of glyceraldehyde-3-P was the inhibitory species. As the pH was increased above 7.4 where glyceraldehyde-3-P exists completely as the divalent anion, maximal inhibition of the anionic isozyme was attained at approximately pH 8.4 and remained constant up to pH 10.3. The inhibition of the cationic isozyme was optimal at pH 7.4 and decreased substantially at higher pH values. Above pH 7.4 there is decreased ionization of the positively charged groups that are responsible for the electrophoretic behavior of the cationic isozyme, as indicated by the finding that this isozyme which is a cation near neutrality has no net charge at pH 8.8 (14). It would appear therefore that the change in net charge at high pH values renders the cationic isozyme less sensitive to inhibition by 2,3-glycer-aldehyde-3-P.

The active center of aspartate aminotransferase contains pyridoxal-5-P linked to an ε-aminolysyl residue through an internal Schiff base (1, 26). Hughes, Jenkins, and Fischer (26) have isolated from the enzyme, after reduction with sodium borohydride and subsequent digestion, a tetradecapeptide which contains one ε-pyridoxyllysine as well as a 2nd lysyl residue. Transamination is accomplished by transfer of the amino group of the lysine bound in the internal aldimine linkage of the pyridoxal enzyme and the possibility existed that it combined with the inhibited isozymes in a manner similar to that employed by Hughes et al. (26) for the isolation from holotransaminase of a peptide containing ε-pyridoxyllysine.

Our results raise the possibility that glyceraldehyde-3-P may be implicated in the regulation of gluconeogenesis in vivo by affecting the activity of the isozymes of aspartate aminotransferase. Such regulation would depend not only upon the intracellular concentration of glyceraldehyde-3-P and substrates but also upon their distribution between the mitochondria and the cytosol.

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