Factors Contributing to the Inhibition of Aspartate Aminotransferase by Dicarboxylic Acids*

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At pH 8.0 aspartate aminotransferase (L-aspartate:2-oxoglutarate aminotransferase, EC 2.6.1.1) reacts with the modified substrate, erythro-P-hydroxy-L-aspartate, to form a mixture of enzyme-substrate complexes absorbing at 492 nm. A variety of dicarboxylic acids were studied spectrophotometrically as competitive inhibitors of this reaction. All of the inhibitory dicarboxylic acids form a complex with the enzyme, absorbing at 362 nm. In addition, some of the dicarboxylic acids form a protonated complex absorbing at about 435 nm. This complex, which is the conjugate acid of that absorbing at 362 nm, is formed only by those dicarboxylic acids which can assume a configuration in which the two carboxyl groups are positioned as in maleic acid. Bulky substituents, such as aromatic rings or even methyl groups, prevent the formation of the protonated complex, presumably because of steric restrictions at the active site. Substitution of the central carbon atom of glutaric acid by heteroatoms of increasing charge density results in a progressive decrease in inhibitory effectiveness, at pH 8, primarily due to a loss of this pH-dependent stabilization of the enzyme-dicarboxylic acid complex.

Acids with an aromatic ring are among the most potent dicarboxylic acid inhibitors of this enzyme in spite of the fact that they do not undergo the pH-dependent stabilization of their enzyme complexes.

From these observations it was concluded that the affinity of aspartate aminotransferase for dicarboxylic acids is determined as much by the mechanism of binding as by the solvation and steric effects.

Structurally, dicarboxylic acids are among the simplest of enzyme inhibitors, and the study of the interactions of enzymes with different dicarboxylic acids has contributed much to an understanding of the factors which determine the selectivity exhibited towards ligands binding at an enzyme's active site (1-8). A principle shortcoming of many of these studies, however, has been that the affinities of the dicarboxylic acids for the enzyme have been derived indirectly from kinetic studies of their inhibition of the reaction catalyzed (1, 2, 4, 6). In contrast, the phosphopyridoxal enzyme, aspartate aminotransferase (L-aspartate:2-oxoglutarate aminotransferase, EC 2.6.1.1) provides an ideal system for the direct study of ligand affinities since the interactions with substrates and inhibitors cause marked changes in the visible absorption spectrum of the prosthetic group (3, 9). These spectral changes may be used to determine the dissociation constant from the active site with a high degree of precision (10). However, the unique advantage of direct spectrophotometric studies is that they frequently reveal that the ligand-macromolecule interaction produces more than one complex (3, 11, 12). Such studies have provided information regarding the mechanism of formation and the interrelationships between such multiple complexes (3, 9, 13).

As described previously (3), the reaction of the phosphopyridoxal form of aspartate aminotransferase (E) with many dicarboxylic acids (A) produces spectral changes which are completely described by the equilibria in Scheme I.

\[
\begin{align*}
K_1 & \quad E + A^+ \\
K_2 & \quad EH^+ \\
K_3 & \quad EHA \\
K_4 & \quad EHA^+ \\
K_5 & \quad EA \\
K_6 & \quad EA^+ \\
\end{align*}
\]

**Scheme I**

in which \(EH^+\) and \(EHA\) are the protonated forms of the free enzyme (E) and of its dicarboxylic acid complex (EA),
respectively. From this scheme it is obvious that the observed affinity of this enzyme for a dicarboxylic acid depends not only upon the ligand dissociation constant, $K_a$ = $(E)(A)/(EA)$, but also upon the protolytic dissociation constant, $K_d$ = $(EA)(H^+)/E$), for the initial enzyme-dicarboxylic acid complex. In fact, it can be shown that the observed inhibition constant, $K_i$, for a dicarboxylic acid as a competitive inhibitor of aspartate aminotransferase varies with the pH between two limiting values, $K_i$ and $K_a$, according to Equation 1.

$$K_i = \frac{K_d K_a}{K_a + K_i} = K_a \left[ \frac{[K_a]^3}{[K_a]^2 + [K_a]^2} \right]$$

Previous work showed that pimelic acid and fumaric acid are relatively weak inhibitors of aspartate aminotransferase even at low pH values. On the other hand, the high degree of inhibition by glutaric acid and maleic acid at low pH values has been demonstrated to be due to a low value of the dissociation constant for the protolytic step (3, 14).

In effect, these observations indicate that a dicarboxylic acid may inhibit by alternative mechanisms, that is, by forming two different inhibitory complexes which are the enzyme-dicarboxylic acid complexes, EA and EIA, described in Scheme I. The present paper further explores this concept by analyzing the factors contributing to the relative and absolute stabilities of EA and EHA. For this purpose, several dicarboxylic acids were studied as competitive inhibitors of the reaction of the modified substrate, erythro-$\beta$-hydroxy-L-aspartate, with aspartate aminotransferase. The observed differences in the extent of inhibition were further investigated in terms of the constants $K_i$ and $K_a$ of Scheme I for those dicarboxylic acids which are able to form a protonated EHA complex with this enzyme at pH 8.0.

**Materials and Methods**

**Enzyme Preparation, Dicarboxylic Acids, and Other Reagents.**—The $\alpha$ subform of extramitochondrial aspartate aminotransferase was prepared in the phosphopyridoxal form from pork heart as described by Martinez Carrion et al. (15). The various enzyme preparations which were used in these experiments had values for the ratio of the absorbance at 430 nm to that at 340 nm of 0.3 to 0.4. All enzyme solutions were dialyzed for 48 hours in several changes of Tris-acetate buffer at pH 8.0, which had an acetate concentration of 10 mM. Enzyme-bound pyridoxal phosphate concentrations were determined in 0.1 N NaOH. Pyridoxal phosphate has a molar extinction coefficient in this medium of $6.55 \times 10^3$.

Imino dicarboxylic and oxydicarboxylic acids were purchased from Aldrich. Thiodiglycolic acid, 2,5-furandicarboxylic acid, and 2,5-thiophenedicarboxylic acid were obtained from Eastman. 2,6-Pyridinedicarboxylic acid (dipicolinic acid), $\alpha$-methylglutaric acid, and $\beta$-dimethylglutaric acid were purchased from K & K Laboratories. $\beta$-Ketoglutaric acid was obtained from Chemical Procurement Laboratories. All dicarboxylic acids were recrystallized before use, and their solutions were neutralized with sodium hydroxide.

**Erythro-$\beta$-hydroxy-L-aspartate** was prepared by enzymatic transamination from the keto-tautomer of dihydroxyfumaric acid using the $\beta$ subform of aspartate aminotransferase. The three isomer of the product was identified by column chromatography on Dowex 1 formate resin (16). Tris base was obtained from Sigma. All other chemicals were of reagent grade and were used without further purification.

**Optical Density and pH Measurements**—A Cary 15 spectrophotometer was used to record the spectra of enzyme solutions containing various dicarboxylic acids or substrates, or both. A Zeiss PMQ 2 spectrophotometer was used to measure absorption changes at a single wavelength, 435 nm or 492 nm, produced by the addition of microliter aliquots of a solution of dicarboxylic acid or substrate to a solution of aspartate aminotransferase, at a concentration of around $4 \times 10^{-4}$ M, in a 1-ml cuvette at 25°C. Erythro-$\beta$-hydroxy-L-aspartate concentrations were increased in increments of $2 \times 10^{-3}$ up to a final concentration of 100 $\mu$M. Dicarboxylic acid concentrations were varied between 0 and 4 $\mu$M for the inhibition analyses and up to 40 $\mu$M for the direct binding studies. Higher concentrations were not used in the inhibition analyses in order to prevent binding of the dicarboxylic acids to the phosphopyridoxal form of the enzyme which otherwise is produced in insignificant small amounts from the reaction of erythro-$\beta$-hydroxy-L-aspartate with aspartate aminotransferase (12). All solutions were buffered with Tris-acetate buffers which were 10 mM with respect to the concentration of acetate.

The pH was always increased by adding to the cuvette small aliquots of a concentrated solution of Tris base; a constant ionic strength was thus maintained. The pH of all solutions was measured with a Corning, model 12, pH meter fitted with a Tris-insensitive Beckman electrode (No. 59030). The measured pH values agreed with those calculated from the experimental buffer ratio with the Henderson-Hassebalch equation and a $pK_a$ = 8.08 for Tris.

**Determination of Inhibition Constant, $K_i$, and of Ligand Affinity Ratio**—The reaction at pH 8.0, in Tris-acetate buffer, of phosphopyridoxal aspartate aminotransferase (E) with the substrate, erythro-$\beta$-hydroxy-L-aspartate (S), results in a stable mixture of enzyme-substrate complexes (ES) which are characterized by an intense absorption at 492 nm. It has been shown that, under these conditions, very little of the enzyme is converted to the pyridoxamine form in this reaction (9, 10, 12). In the presence of a dicarboxylic acid competitive inhibitor (A) which forms a complex or complexes (ESA) with the enzyme, this reaction may be described by Scheme II.

In Scheme II, $K_a$ = $(E)(S)/(ES)$ is the apparent substrate dissociation constant, and similarly $K_a$ = $(E)(A)/(EA)$ is the apparent inhibition constant for a particular dicarboxylic acid.

The observed maximum absorption at 492 nm ($A_{492}$) of the mixture of enzyme-substrate complexes depends upon both the absorption at 492 nm for each of the complexes and the stability of the complex relative to the other complexes. Experimental values for $K_a$, $K_i$, and $A_{492}$ for each of the dicarboxylic acids were obtained by measuring the absorption at 492 nm ($A_{492}$) produced by the addition of aliquots of erythro-$\beta$-hydroxy-L-aspartate to a solution of concentration ($E_i$) of aspartate aminotransferase, in a concentration (A) of dicarboxylic acid. The data points were fitted to the theoretical equation:

$$A_{492} = \frac{K_a E_i S_i K_p}{K_a E_i S_i + K_p A + K_a}$$

by a nonlinear least squares computer program which utilized a convergence procedure similar to that suggested by Wilkinson (47) and elaborated upon by Cieland (18). An essential difference in our treatment was that the total substrate concentration ($S$) was corrected for the amount bound to the enzyme ($E_i$) to give the free substrate concentration ($S_i$) by the expression:

$$S_i = S - \left( E_i \right) A_{492}$$

Values of $K_a$, $K_i$, and $A_{492}$ used to initiate the nonlinear analysis were provided by a series of linear least squares analyses which are identical with graphical methods described elsewhere (12) and illustrated in Fig. 1. Between 20 and 30 data points were used to determine the three constants for each dicarboxylic acid. All error determinations are in accord with the treatment presented by Cieland (18). The data for each dicarboxylic acid were reasonably well conditioned, exceptions being data for iminodiacetic acid and methylmaleic acid.

The ligand affinity ratio, as defined here, is equal to the ratio of

\[ \text{Ligand Affinity Ratio} = \frac{A_{492}}{A_{492}} \]
inhibitor dissociation constant to substrate dissociation constant, $K_i/K_s$. This ratio is a sensitive measure of the affinity of aspartate aminotransferase for a particular ligand relative to its affinity for erythro-$\beta$-hydroxy-$\gamma$-aspartate (19). A low value of the affinity ratio thus signifies a high affinity of the enzyme for that ligand. $K_i/K_s$ values which appear in Table I have been normalized by multiplying each ligand affinity ratio by the average value of $K_i$. This is an average value from four different enzyme preparations. It should be noted that these values of $K_i$ have very large standard deviations, as accounted for by the large deviation of $K_s$. However, the deviations in the “affinity ratio” ($K_i/K_s$) are quite modest, showing that the ratio is the most accurate measure of the relative affinities.

**Determination of Dicarboxylic Acid Aminotransferase Dissociation Constant, $K_s$, and of Protolytic Dissociation Constant, $K_p$—Since the $pK_s$ of Scheme I is equal to 6.3 (20), the binding of a dicarboxylic acid ($A$) to phosphopyridoxal aspartate aminotransferase ($E$) at pH 8.0 may be represented by Scheme III, which is a simplification of Scheme I. Note that the sum of $EA$ and $EHA$ in Scheme III corresponds to $\Sigma EA$ in Scheme II.

\[
K_1 \quad + \quad H^+ \quad \rightarrow \quad K_2
\]

**Scheme III**

In Scheme III, $K_1 = (E)(A)/(EA)$ is the ligand dissociation constant for the unprotonated enzyme-dicarboxylic acid complex ($EA$), and $K_2 = (EA)(H^+)/(EHA)$ is the protolytic dissociation constant for the acidic enzyme-dicarboxylic acid complex ($EHA$). The maximum absorption change at 435 nm ($D_{435}$) is produced by saturating the basic enzyme ($E$) with a dicarboxylic acid at a pH so that the enzyme is completely converted to the acidic complex ($EHA$).

Values of $K_1$, $K_2$, and $D_{435}$ could only be obtained by this analysis for those dicarboxylic acids which formed an appreciable amount of the acidic complex at pH 8.0 (i.e., for those with a $pK_a$ greater than about 7.7). For these few, the absorption change at 435 nm ($D_{435}$) produced by the addition of dicarboxylic acid to aspartate aminotransferase at several different pH values were fitted by a nonlinear least squares computer analysis similar to the one described above, to the function:

\[
D_{435} = \frac{(A)(H^+)(A)}{(A)(H^+)+K_1+K_2
\]

where ($H^+$) is the proton concentration, is fixed by the $Tris$ base to $Tris$-acetate ratio.

Initial values of $K_s$, $K_p$, and $D_{435}$ for the nonlinear computer analysis were obtained from linear least squares analyses such as shown graphically in Fig. 2, which have been described earlier (3). Again, the data were fairly well conditioned with respect to the experimental ligand concentrations and pH ranges.

**RESULTS**

**Inhibition of Aspartate Aminotransferase by Dicarboxylic Acids**—The results of the inhibition analysis according to Scheme II are presented in Table I. It is important for the validity of this simple analysis that the value of $A_{435}$ was found to be essentially the same regardless of the dicarboxylic acid investigated. This indicates, primarily, that none of the multiple enzyme-substrate intermediates ($\Sigma ES$ in Scheme II) has an appreciable affinity for dicarboxylic acid, because the distribution of the intermediary complexes was not dependent upon the type or concentration of dicarboxylic acid present. Furthermore, the phosphopyridoxamine form of this enzyme, which is produced by the reaction of the phosphopyridoxal form with amino acids, binds dicarboxylic acids (9). Formation of the phosphopyridoxamine enzyme with its complexes would also reduce $A_{435}$. However, the reaction of phosphopyridoxal aspartate aminotransferase with erythro-$\beta$-hydroxy-$\gamma$-aspartate produces very little of the phosphopyridoxamine form (9, 10) and with the low concentrations of dicarboxylic acid used in this study, there was negligible formation of the phosphopyridoxamine enzyme-dicarboxylic acid complexes. Consequently, the dicarboxylic acids were acting as simple competitive inhibitors of the amino acid in that their effect was empirically described by the familiar equation:

\[
K_o = K_s (1 + \frac{A}{K_i})
\]

where $K_o$ is the observed substrate dissociation constant in the presence of a concentration ($A$) of an inhibitor, and $K_s$ and $K_i$ have their meanings defined in Scheme II.
Table I also shows that, while there was negligible deviation in $A_{\text{max}}$, there was a large variation in the values of $K_a$, the substrate dissociation constant, in the absence of inhibitor. This variability may in part be due to slight differences among the enzyme preparations used for these experiments, as evidenced by an inconsistent ratio of the absorption of the enzyme at 430 nm to the absorption at 340 nm, as described under "Materials and Methods." Another possibility, not necessarily independent of the first, is that this large deviation in the substrate dissociation constants ($K_a$) may have been caused by the presence of variable amounts of an unknown inhibitor which was purified along with the enzyme or was added to the enzyme during the preparative procedure (e.g. sulfate, phosphate, bicarbonate, $\alpha$-ketoglutarate). Although all attempts to identify such an inhibitor have been inconclusive so far, the situation appears to be similar to that described by Kearney et al. (21), in which it was found that oxaloacetate bound tightly to succinate dehydrogenase caused irreproducible kinetic parameters.

Until the precise nature of this variation of $K_a$ is resolved, it seems best to use a measure of inhibitor affinity which is minimally dependent upon the effects discussed above and which would allow comparisons between experiments performed with different enzyme preparations and in different laboratories. The "affinity ratio," equal to the ratio of the inhibitor to substrate dissociation constants ($K_a/K_s$), is such an index since it measures the affinity of one ligand with respect to a second reference ligand: in this study the substrate, erythro-$\beta$-hydroxy-L-aspartate. As discussed by Webb (19) this affinity ratio, as well as being a sensitive measure of the relative binding affinities among several different ligands, is also thermodynamically interpretable in that it may be used to calculate the free energy change for the replacement of a substrate by an inhibitor.

Mechanisms of Dicarboxylic Acid Inhibition—Table II gives the results of the analyses of the ligand dissociation constants, $K_s$, and the protolytic dissociation constants, $K_p$, for the enzyme-dicarboxylic acid complex according to Scheme III. Also tabulated are values of the inhibition constant, $K_a$, which have been calculated from the equation:

$$K_a = \frac{K_1 K_2}{K_1^* K_2^*}$$

This equation is merely a simplified form of equation 1 that applies at pH 8.0 where $K_1 \gg (H^+)^2$ (20). Comparison of the calculated values of $K_a$ with those independent experimental values in Table I shows a reasonable agreement.

Table III, which presents a summary of data obtained in both this and previous studies (3, 9, 14), shows those dicarboxylic acids which have been found to interact with aspartate aminotransferase. This list separates the dicarboxylic acids into two categories: those that form a protonated complex at pH 8.0 with aspartate aminotransferase (EHA in Scheme I, with absorption maximum at about 435 nm), and those acids that do not form an acidic complex at that pH. Space-filling models demonstrated that those dicarboxylic acids which form a protonated complex with the enzyme at pH 8 are all able to assume the configuration of maleic acid, as shown in Fig. 3, in which the carboxyl groups are nearly adjacent. Those acids that do not form a protonated enzyme complex at pH 8.0 are either sterically hindered to prevent approximation of their carboxyl groups or else possess a ring or bulky substituents which protrude upward relative to the downward pointing carboxyl groups, as illustrated by Fig. 4.

**DISCUSSION**

The pK of the enzyme, even in the absence of dicarboxylic acids, is not constant but rather varies with the anion concentration. Bergami et al. (22) estimated that the limiting value of the pK in the absence of anions was near pH 5 and that monovalent anions bind to the protein to raise this pK to about 6.3 at an ionic strength of 0.1. In unpublished experiments we noted that formate will raise the pK even higher. However, no monovalent anions have been found which will raise the pK of the enzyme by guest on March 21, 2020 http://www.jbc.org/Downloaded from

<...>
The contribution of the yellow acidic complex to the binding affinity can be appreciated by consideration of Scheme I and of

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**Table III**

*Data on interaction of dicarboxylic acids and aspartate aminotransferase*

| Dicarboxylate anions which form a protonated EHA complex (Scheme I) at pH 8.0 with aspartate aminotransferase: |
|---|
| Maleate | Thiodiglycolate |
| Succinate | l-Malate, d-Malate |
| Glutarate | meso-Tartarate |
| α-Ketoglutarate | meso-Diaminomalonic acid* |
| β-Ketoglutarate | cis-1,2-Cyclohexanedicarboxylate |
| α-Methylglutarate | Adipate |

| Dicarboxylate anions that are unable to form a protonated EHA complex at pH 8.0 with aspartate aminotransferase: |
|---|
| Methylmaleate | dl-Tartarate |
| 2,3-Dimethylmaleate* | dl-Diaminomaleic acid* |
| Fumarate | Phthalate |
| Methylfumarate | Isophthalate |
| α-Oxalocarboxylic acid* | Terephthalate |
| β-Oxalocarboxylic acid | 2,5-Thiophenedicarboxylate |
| ββ-Dimethylglutarate | 2,5-Purindicarboxylate |
| Oxalate | 2,6-Pyrindicarboxylate |
| Pimelate | Acetylenedicarboxylate |
| Glutaconate* | 1,2,3-Benzenericarboxylate |
| Iminodiacetate* | trans-1,2-Cyclobutane-dicarboxylate |
| Oxydiglycolate | |

*Results of recent experiments.

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high enough to give a yellow color at pH 8. The choice of pH 8 for Table III was thus arbitrary but is satisfactory to distinguish dicarboxylic acids, capable of forming bidentate ligands, from monovalent anions and from those dicarboxylic acids capable of acting only, as though they were monovalent anions, to form monodentate complexes. Some of the acids which do not form an acidic complex at pH 8 may do so at a lower pH but it is not easy to identify them unequivocally.

The large number of dicarboxylic acid inhibitors of aspartate aminotransferase that are presented in this study allows the analysis of the factors that contribute to the affinity of this enzyme for a particular ligand. These contributing factors can be discussed as belonging to one of the following broad categories: steric effects, mechanistic effects, and solvation effects. However, it must be noted that none of these categories is wholly independent of the other two.

The data presented in Table I indicate that the aromatic ring dicarboxylic acids are the most potent inhibitors in this group. However, as revealed in Table III, none of these acids are able to form an acidic EHA complex with the enzyme at pH 8. In contrast, the straight chain dicarboxylic acids, glutaric and thioglycolic acids, are nearly as potent inhibitors, but these acids are able to form the acidic complex. These results suggest that the high affinity of the enzyme depends upon different factors for each of these two classes of dicarboxylic acid inhibitors. In the case of the aromatic ring acids, the high affinity is probably produced by the hydrophobic effect. On the other hand, the high affinity of the enzyme for the straight chain acids is most likely due to the formation of this additional yellow complex.

The contribution of the yellow acidic complex to the binding affinity can be appreciated by consideration of Scheme I and of

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**Fig. 3 (left).** Space-filling models of four dicarboxylic acids which are able to form a protonated complex with aspartate aminotransferase at pH 8.0. Model A, maleic acid; B, glutaric acid; C, cis-1,2-cyclohexanedicarboxylic acid; and D, mero-tartaric acid. The models are arranged so that the adjacent carboxyl groups are eclipsed and are in a conformation analogous to that of maleic acid.

**Fig. 4 (right).** Space-filling models of dicarboxylic acids that are able to form a protonated complex with aspartate aminotransferase at pH 8.0. These models are to be compared with those in Fig. 3. Model A, maleic acid; B, β-methylglutaric acid; C, isophthalic acid; and D, dl-tartaric acid.

Equations 1 and 6. These equations predict that the formation of a protonated enzyme-dicarboxylic acid complex (EHA) is responsible for a large amount of the binding energy for those acids that are able to form this complex with the enzyme. However, the data presented here further indicate that dicarboxylic acids must fulfill rather strict steric requirements in order to inhibit in this manner. The first requirement is that these dicarboxylic acids have as an allowable conformation one in which the carboxyl groups are close together. Maleic acid is a model for this particular conformation. A second requirement is that the dicarboxylic acid must be fairly compact. Apparently, steric restrictions at the enzyme site prevent methylmaleic and β-methyl and ββ-dimethyl glutaric acids from forming the acidic EHA complex (14).

This second steric restriction is, however, dependent upon the polarity of the substituent groups in a series of related dicarboxylic acids. This is shown in Table III, for various derivatives of succinic acid, which itself is able to form an acidic EHA complex at pH 8.0 (14). As shown, the presence of a methyl group on succinic acid prevents the formation of a protonated complex at pH 8; however, the addition of one polar group (e.g., hydroxy) - malic acid, either L or D) still permits tight binding because of formation of the protonated complex. Furthermore, two vicinal polar groups permit formation of the acidic complex but these groups must be in erythro conformation as can be appreciated by comparing meso-tartaric acid and meso-diaminobutane acid with their corresponding dl isomers in Table III. This must mean that the meso isomers bind in the maleic acid conformation without either hydroxyl group interacting unfavorably with the enzyme. On the other hand, the same conformation of the dl isomers is either too strained or, alternatively it does not result in a favorable interaction of a hydroxyl group with a group on the enzyme. It is interesting to note, in this regard, that the erythro isomer of β-hydroxyaspartate, which has the same stereochemistry of its polar groups as meso-tartaric and meso-diaminobutane, forms a unique covalent complex with the enzyme which was not observed with the threo isomer (14), nor, to any significant extent, with any other amino acid.

Returning to the consideration of the enzyme's high affinity for the aromatic dicarboxylic acids, which are sterically prohibited from forming a stabilized protonated EHA complex, it seems reasonable to conclude that these ligands derive much of their binding energy from solvation effects or, in other words, from "hydrophobic interactions" (23). This hypothesis is
further supported by consideration of the data in Table I that show the effect of replacing a central carbon atom by heteroatoms of increasing charge density (4). For the series of aromatic dicarboxylic acids, the order of the affinity for the enzyme is: thiophenedicarboxylic acid > isophthalic acid > furandicarboxylic acid > dipicolinic acid. A corresponding series of the straight chain glutaric analogue has the order of affinity: thioglycolic acid = glutaric acid > oxyglycolic acid > iminodiacetic acid. Thus the replacement of the central atom by sulfur, carbon, oxygen, and nitrogen results in a progressive decrease in the enzyme’s affinity for members of these two series.

In a recent paper, Rogers (4) examined the same series of glutaric acid analogues as inhibitors of glutamate dehydrogenase and found a good correlation between the sigma charge density at the central atom and the extent of inhibition of the enzyme. His data are compared in Table IV with the data presented in this paper. Rogers pointed out that the charge density is an index of the interaction between atoms of an organic compound and water. He thus suggested that the hydration of the dicarboxylic acid is an important factor in the binding reaction. The data presented in this paper also indicate that in the case of aspartate aminotransferase, the more hydrated ligands, iminodiacetic acid and oxyglycolic acid, have a different mechanism of binding in that they are not able to form an acidic complex (Table III). A probable explanation of this observation is that water molecules introduce a steric restriction by hydrating the oxygen and nitrogen atoms and thereby prevent formation of the protonated complex. That the water of solvation can act as a structural component to affect binding affinities and specificities has in fact been demonstrated in antibody-hapten reactions (24).

It is relevant at this point to mention other studies of phosphopyridoxal enzymes that have examined dicarboxylic acid inhibitors. Haarhoff (25) did a detailed computer kinetic analysis of the inhibition by dicarboxylic acids of the reaction catalyzed by aspartate aminotransferase. In general, his study showed trends which have been further delineated in the present study. Previous studies of aspartate aminotransferase also have shown that the extent of dicarboxylic acid binding increases with decreasing pH (3, 26) even with the phthalic acids (27). Another enzyme, kynurenine transaminase, was shown to be inhibited most strongly and specifically at low pH values, and it was found that adipic acid had the optimum chain length. From these observations, Mason (6) proposed that there must be two cationic sites about 11 A apart, only one of which binds the dicarboxylic acid at high pH. Finally, a study of glutamate decarboxylase found that glutaric, adipic, and pimelic acids were powerful inhibitors, especially at low pH. Fonda (5) therefore concluded that the positive binding sites must be about 6 A apart since neither maleic acid nor fumaric acid reacted. She also pointed out that the positive binding sites on aspartate aminotransferase must be less than 6 A apart since maleic acid is much more effective than fumaric acid as an inhibitor of this enzyme (14).

Despite these many studies, the nature of the protonated aspartate aminotransferase dicarboxylic acid complex has not been satisfactorily established. It has been suggested (3) that one of the carboxyl groups binds as a carboxylate anion, possibly to the same active site histidine residue that is reputed to bind monoanions (26), and that the second carboxyl group binds as an undissociated acid, probably to, or close to, the nitrogen of the pyridoxal phosphate-lysine Schiff base (28), as shown schematically in Fig. 5. Such a representation is consistent with the data presented in this paper. It is implied with this drawing that the dicarboxylic acid ligand undergoes a conformational change with protonation of the EHA complex to form EHA, and, as the data in this paper indicate, only certain dicarboxylic acids are sterically able to undergo this conformational change at the active site to form the protonated EHA complex.

In conclusion, when it is observed, as in many studies with dicarboxylic acid enzyme-inhibitors, that the relative potencies vary with the pH, it is plainly an invalid oversimplification to use inhibition data obtained at one pH to attempt to map the active site. This paper shows that with aspartate aminotransferase, this pH variation can be accounted for by assigning two dissociation constants to each dicarboxylic acid inhibi-

\[
\begin{array}{|c|c|c|c|}
\hline
\text{Dicarboxylate inhibitor} & \text{Charge density}^* & K^* & \text{Affinity ratio}^* \\
\hline
\text{Thiodiglycolate} & 0.058 & 1.3 & 25 \\
\text{Glutarate} & 0.086 & 1.3 & 26 \\
\text{Oxydiglycolate} & 0.276 & 10 & 135 \\
\text{Iminodiacetate} & 0.378 & 60 & 540 \\
\hline
\end{array}
\]

* According to Rogers (4).

* Data from Table I, this paper.

**Fig. 5.** Graphical representation of possible interactions of dicarboxylic acids with aspartate aminotransferase which cause the pH-dependent spectral changes of the protein-bound pyridoxal phosphate chromophore. This mechanism corresponds to that shown, schematically, in Scheme I.
The separate effects of structural variations on these two dissociation constants give much more information about the geometry and nature of the active site than do the inhibitor dissociation constants obtained at a single pH. With newer techniques of computerized analysis (10, 29), it is now possible to study such complex equilibria spectroscopically over a wide pH range.

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