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MOLECULAR INTERACTIONS OF SIX AROMATIC COMPETITIVE INHIBITORS WITH BOVINE LIVER GLUTAMATE DEHYDROGENASE

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SUMMARY

Six aromatic dicarboxylic acids (isophthalic acid, pyridine-3,5-dicarboxylic acid, and pyridine-2,6-dicarboxylic acid) and bromo-substituted aryl monocarboxylic acids (5-bromofuroic acid, *m*-bromobenzoic acid, and 5-bromothiophene-2-carboxylic acid) were tested with bovine liver glutamate dehydrogenase [L-glutamate: $NAD(P)^+$ oxidoreductase (deaminating) EC 1.4.1.3] for inhibition against L-glutamate as a function of pH (6.6 to 9.0). These compounds were competitive inhibitors at all pH values tested and the inhibition remained competitive when either $NADP^+$ or NAD^+ was coenzyme. Maximum inhibitor potency (pK_i) for the dicarboxylic acids occurred at pH 7.8 and for the monocarboxylic acids occurred at pH 8.7. The relative inhibitor potencies were correlated with the inhibitors' "central atom" absolute charge densities $|Q^T|$ calculated from molecular orbital theory. This indicated that desolvation of this atom may be important for combination of inhibitor with enzyme. A high degree of solvation as indicated by the magnitude of charge density could have decreased the interaction of inhibitor with enzyme. Similar results had been obtained previously for 4 aliphatic dicarboxylic acid competitive inhibitors [K. S. Rogers, *J. Biol. Chem.*, 246 (1971) 2004].

INTRODUCTION

In an abstract in 1962, GREVILLE AND MILDVAN[1] reported that logarithm plots of v_{max}, K_m, and v_{max}/K_m *versus* pH for the oxidative deamination of L-glutamate in the presence of a fixed level of NAD^+ by bovine liver glutamate dehydrogenase [L-glutamate: $NAD(P)^+$ oxidoreductase (deaminating, EC 1.4.1.3] indicated pK_a values for the rate limiting step(s) of 7.2 and for the enzyme–coenzyme complex of 7.9 and 8.6–8.9. This work was recently confirmed and extended to include effects of pH 6.6–8.6 on the deamination of L-glutamate in the presence of $NADP^+$ (ref. 2). In the latter instance, ionizable group or groups were observed in the enzyme complex to

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have a pK_a of about 7.8. It was suggested[2] that the observed pK_a may have reflected the microdissociation constant of a bound species of L-glutamate which had ionized α-amino and γ-carboxylic groups. In addition, the relative effectiveness of 4 aliphatic inhibitors (glutaric acid, thiodiglycolic acid, oxydiglycolic acid, and iminodiacetic acid) competitive with L-glutamate correlated inversely with the magnitude of charge density ($|Q^T|$) of the "meso" atom of the inhibitors. Charge density was calculated from molecular orbital theory considerations of the inhibitor's atoms. Since absolute charge density values had been shown to be an index of the interaction between atoms of organic compound and water in a partition system[3], then desolvation of the inhibitors was considered important for effective combination of inhibitor with enzyme.

Six aromatic compounds, isophthalic acid, 5-bromofuroic acid, *m*-bromobenzoic acid, pyridine-2,6-dicarboxylic acid, pyridine-3,5-dicarboxylic acid, and 5-bromothiophene-2-carboxylic acid were tested for inhibition as a function of pH. The first three compounds had been previously shown[4] to be competitive with L-glutamate at pH 8.5. Calculation of the π- and σ-electron atom densities of these molecules from molecular orbital theory has provided useful data pertinent to the compounds' effectiveness as inhibitors.

MATERIALS AND METHODS

Bovine liver glutamate dehydrogenase was obtained as a crystalline protein suspension from Sigma Chemical Company. The enzyme was collected by centrifugation at 4° and the protein was dissolved in 0.15 M Na_2HPO_4 buffer adjusted with 5 M H_2SO_4 to pH 7.80. The enzyme solution was dialyzed overnight at 4° against buffer. Denatured protein was removed by centrifugation at 12 000 × *g* for 20 min at 4°. Protein concentration was estimated by a biuret method[5]. Stock solutions of protein (6 mg/ml) were refrigerated at 4° prior to use. Water of $10^6\ \Omega$ resistance was used throughout the experiments.

Reagents

$NADP^+$, NAD^+, and L-glutamic acid were purchased from Sigma Chemical Company. Isophthalic acid was obtained from Eastman Chemical Company. Pyridine-3,5-dicarboxylic acid, pyridine-2,6-dicarboxylic acid and *m*-bromobenzoic acid were received from Matheson, Coleman and Bell Chemical Company. 5-Bromofuroic acid and 2-bromothiophene were obtained from Aldrich Chemical Company.

Synthesis of 5-bromothiophene-2-carboxylic acid from 2-acetyl-5-bromothiophene

The procedure of HARTHOUGH AND CONLEY[6] was used to synthesize 2-acetyl-5-bromothiophene. A mixture of 8.15 g (50 mmoles) of 2-bromothiophene, 4.7 ml (50 mmoles) of acetic anhydride and 0.5 g of 85% phosphoric acid was refluxed for 3 h. The black solution was then added to ether and water. The organic phase was washed serially with water and a saturated sodium chloride solution; the organic phase was then dried over anhydrous sodium sulfate. A dark brown semi-solid resulted when the solvent was removed under reduced pressure. The solid was dissolved in 100% ethanol and decolorized with activated charcoal. This afforded 4.43 g (a yield of 42%) of 2-acetyl-5-bromothiophene as a yellow solid with a melting point of 93–94° (reported[6] 94–95°).

The method of LEVINE AND STEPHENS[7] was used to synthesize 5-bromothiophene-2-carboxylic acid from 2-acetyl-5-bromothiophene. A solution of sodium hypobromite was prepared from 24.4 g (0.61 mole) NaOH and 100 ml of water. The above solution was cooled to 8°, then 7.8 ml (24.4 g = 0.16 mole) of bromine was added dropwise while the solution was stirred. Next, 8.6 g (42 mmoles) of 2-acetyl-5-bromothiophene dissolved in 70 ml of monoglyme was added to the above solution which then formed a yellow suspension. This suspension was stirred for 2 h at room temperature (about 25°). The mixture was added to ether and water. The basic aqueous phase was acidified with cold, conc. HCl. The precipitated solid was removed by filtration, then washed with water, and finally air dried to give 8 g (yield of 92%) of 5-bromothiophene-2-carboxylic acid as a yellow solid with a melting point of 139–140° (reported[7] melting point was 141–141.5°).

Methods

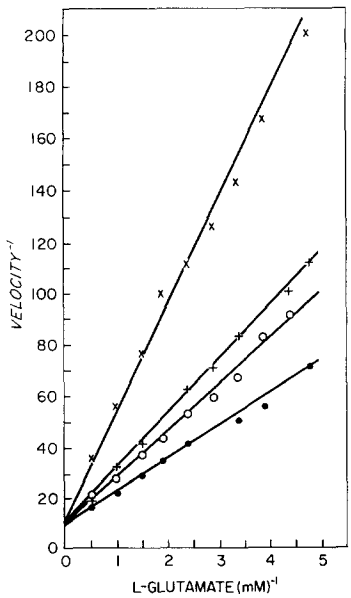
Enzyme assays were performed at 30 ± 0.1° using either a Beckman DU monochromator modified with a Gilford 222 photometer or a Gilford 300 spectrophotometer. Enzyme velocity was determined as the increase in absorbance per min at 340 nm for the reduction of $NADP^+$ or NAD^+. The pH was measured prior to and directly after enzyme assay with use of a Beckman research pH meter. The inhibitors were neutralized with NaOH and were kept at 4° in low actinic glass containers. Molar inhibition constants (K_i) were determined from double reciprocal plots[8] of velocity *versus* substrate concentrations, and from Dixon plots[8] of reciprocal velocity against inhibitor concentrations. In the former plot K_i values were calculated from differences in slopes of inhibited *versus* uninhibited enzyme reactions. In the latter plot K_i values were determined from the intersection point of lines extrapolated to the fourth quadrant. Varying the glutamate concentrations by 4-fold provided a large angle for accurate interpolation of K_i. Experimental error in the determination of apparent K_i values was ±5%.

Molecular orbital calculations

All calculations were performed on an IBM 1130 computer. The simple Huckel method[9] was used for the π-electron system calculations and the method of DEL RE and coworkers[10,11] as modified by BERTHOD AND PULLMAN[12] was used for the σ-electron system calculations. The atom parameters employed were those suggested by the references cited. Model linear least squares regression equations were employed for comparing relative inhibitor potencies with calculated molecular interaction indices.

RESULTS

Double reciprocal plots of velocity *versus* glutamate concentrations at fixed levels of $NADP^+$ and inhibitor showed that isophthalic acid, pyridine-3,5-dicarboxylic acid, pyridine-2,6-dicarboxylic acid, 5-bromofuroic acid, *m*-bromobenzoic acid, and 5-bromothiophene-2-carboxylic acids were competitive inhibitors of bovine liver glutamate dehydrogenases. Slopes with the inhibitors intersected with those of controls on the ordinate of the plots in Figs. 1 and 2. Thus these inhibitors were competitive with L-glutamate when either $NADP^+$ or NAD^+ (ref. 4) was the coenzyme. Apparent K_i values of the inhibitors were calculated from Dixon plots of velocity *versus* inhi-



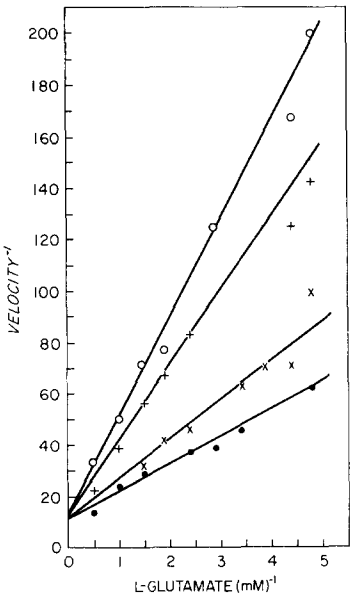


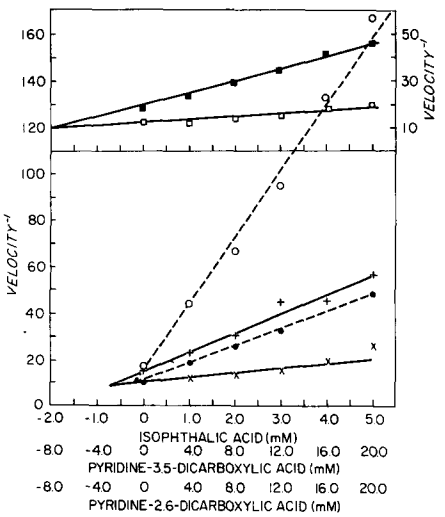
Fig. 1. Double reciprocal plots of velocity against glutamate concentrations in the presence of isophthalic acid, pyridine-3,5-dicarboxylic acid, or pyridine-2,6-dicarboxylic acid at a fixed level of $NADP^+$. Assays were conducted at 30° in 3.0 ml of 0.1 M Na_2HPO_4–0.1 M $Na_4P_2O_7$ buffer adjusted with 5 M H_2SO_4 to pH 7.95. The assay contained 0.2 mM $NADP^+$, enzyme (10 μg/ml), inhibitor, and varying amounts of L-glutamate. The reaction was started by addition of enzyme in buffer. Concentrations of inhibitors were 0.78 mM isophthalic acid (○), 10 mM pyridine-3,5-dicarboxylic acid (×), and 10 mM pyridine-2,6-dicarboxylic acid (+).

Fig. 2. Double reciprocal plots of velocity against glutamate concentrations in the presence of 5-bromofuroic acid, *m*-bromobenzoic acid, or 5-bromothiophene-2-carboxylic acid at a fixed level of $NADP^+$. Assays were conducted at 30° in 3.0 ml of 0.1 M Na_2HPO_4–0.1 M $Na_4P_2O_7$ buffer, pH 8.72. The assay system contained 0.2 mM $NADP^+$, enzyme (10 μg/ml), inhibitor, and varying amounts of L-glutamate. The reaction was started by addition of enzyme in buffer. Concentrations of inhibitors were 0.26 mM 5-bromofuroic acid (○), 1.55 mM *m*-bromobenzoic acid (+), and 2.07 mM 5-bromothiophene-2-carboxylic acid (×).

bitor concentrations (Figs. 3 and 4). The relative potency of inhibitors at pH 7.9 (Fig. 3) were isophthalic acid > pyridine-3,5-dicarboxylic acid > pyridine-2,6-dicarboxylic acid, and at pH 8.8 (Fig. 4) 5-bromofuroic acid > *m*-bromobenzoic acid > 5-bromothiophene-2-carboxylic acid, respectively. This relative order of inhibitor effectiveness was not changed when equivalent concentrations of NAD^+, 0.6 mM, were substituted for $NADP^+$ (not shown) at the two pH values investigated 7.9 and 8.8.

pH and inhibitor potency

The effect of pH on the relative potencies of the 6 competitive inhibitors is given in Fig. 5. The negative logarithms of apparent K_i values (pK_i), determined from Dixon plots similar to Figs. 3 and 4, were plotted as a function of pH. pH was determined immediately after enzyme assay. Maximum potency of dicarboxylic acid inhibitors occurred approximately at pH 7.8 and maximum potency of the monocarboxylic acid inhibitors occurred approximately at pH 8.6. The differences in pH maxima observed for the two classes of inhibitors were not related to differences



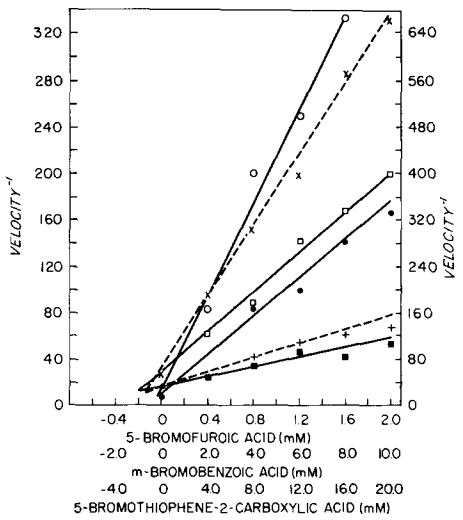


Fig. 3. Dixon plot of reciprocal velocity against inhibitor concentrations at fixed levels of L-glutamate and $NADP^+$. Assays were conducted at 30° in 3.0 ml of 0.1 M Na_2HPO_4–0.1 M $Na_4P_2O_7$ buffer, pH 7.9. The assay system contained 0.6 mM $NADP^+$, enzyme (10 μg/ml), varying amounts of inhibitor and L-glutamate. The reaction was started by addition of enzyme in buffer. The competitive inhibitors were isophthalic acid in the presence of 0.5 mM (○) and 2.0 mM L-glutamate (●); pyridine-3,5-dicarboxylic acid in the presence of 0.5 mM (×) and 2.0 mM L-glutamate (+), and pyridine-2,6-dicarboxylic acid in the presence of 0.5 mM (□) and 2.0 mM L-glutamate (■). Left ordinate, isophthalic acid and pyridine-3,5-dicarboxylic acid; right ordinate, pyridine-2,6-dicarboxylic acid. Extrapolation of slopes for two different substrate concentrations gave an intersection point in the fourth quadrant that represented the K_i value for the inhibitor.

Fig. 4. Dixon plot of reciprocal velocity against inhibitor concentration at fixed levels of L-glutamate and $NADP^+$. Assays were conducted at 30° in 3.0 ml of 0.1 M Na_2HPO_4–0.1 M $Na_4P_2O_7$ buffer, pH 8.8. The assay system contained 0.6 mM $NADP^+$, enzyme (10 μg/ml), varying amounts of inhibitor and L-glutamate. The reaction was started by addition of enzyme in buffer. The competitive inhibitors were 5-bromofuroic acid in the presence of 0.5 mM (○) and 2.0 mM L-glutamate (●), *m*-bromobenzoic acid in the presence of 0.5 mM (+) and 2.0 mM L-glutamate (×), and 5-bromothiophene-2-carboxylic acid in the presence of 0.5 mM (□) and 2.0 mM L-glutamate (■). Extrapolation of slopes for two different substrate concentrations gave an intersection point in the fourth quadrant that represented the apparent K_i value for the inhibitor.

between the intraproton distances[4] of the dicarboxylic molecules and the distances[4] between the bromide group and carboxylic group of the other series of inhibitors since both distances were equivalent as was required for maximum inhibition, *i.e.*, 7.5 Å (ref. 4).

Charge density and inhibitor effectiveness

The absolute charge density calculated from π and σ electron frameworks of inhibitors, on the "central" atom(s) (*cf.* ref. 2) of each inhibitor is presented in Table I together with values of pK_i that represented maximum potencies of the inhibitor recorded over the pH range studies, 6.6 to 9.0. The "central" atom of each inhibitor was designated in Table I by Geneva nomenclature. The "central" atom, with its attached hydrogen, was located midway between the substituted ring atoms of the inhibitor. The extent of inhibitory effectiveness (pK_i) was correlated with the magnitude of absolute charge density using a linear least squares regression equation.

TABLE I

COMPETITIVE INHIBITION CONSTANTS (pK_i) AND CALCULATED ABSOLUTE CHARGE DENSITIES ($|Q^T|$)

The inhibition coefficients of the inhibitors were expressed as negative common logarithms of K_i values from Figs. 3 and 4. Parenthesis values represented mM concentrations of K_i. Absolute charge densities ($|Q^T|$) are reported for the central atoms of the inhibitors.

Inhibitor	*Atoms*	$\|Q^T\|$	pK_i	
Isophthalic acid	C-2, H	0.081	3.60	(0.25)
Pyridine-3,5-dicarboxylic acid	C-4, H	0.128	2.52	(3.0)
Pyridine-2,6-dicarboxylic acid	N-1	0.452	2.10	(8.0)
5-Bromofuroic acid	O-1	0.016	4.46	(0.035)
m-Bromobenzoic acid	C-2, H	0.087	3.22	(0.60)
5-Bromothiophene-2-carboxylic acid	S-1	0.135	2.70	(2.0)

$$pK_i = 3.41 - 3.04 \; |Q^T| \quad (r = 0.79) \tag{1}$$

$$pK_i = 4.65 - 14.99 \; |Q^T| \quad (r = 0.99) \tag{2}$$

Eqn. 1 represented correlation of inhibitor potency of the aryl dicarboxylic acids at pH 7.9, and Eqn. 2 represented correlation of inhibitor potency of the aryl monocarboxylic acids at pH 8.8. The correlation coefficient r measured the fit of experimental points to the equation. A value of 1.0 for r would be a perfect fit. Eqn. 2 gave a better correlation with experimental data than did Eqn. 1. Inclusion of a second term, *e.g.* polarizability of "central" atom(s)[3] would improve the correlation of Eqn. 1 but on a statistical basis this would be meaningless as only 3 experimental values are examined. The negative sign of the coefficients of $|Q^T|$ indicated that this

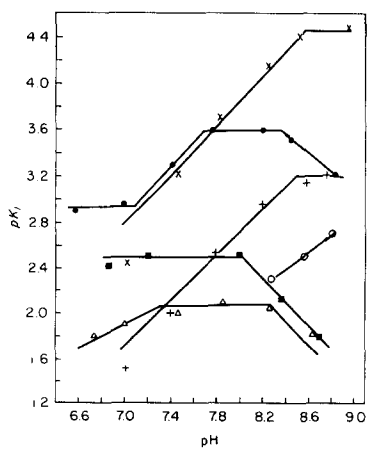


Fig. 5. Profile of inhibitor potency (pK_i) as a function of pH. Assays were conducted at 30° in 3.0 ml of 0.1 M Na_2HPO_4–0.1 M $Na_4P_2O_7$ buffer. The assay system contained 0.6 mM $NADP^+$, enzyme (10 μg/ml), 0.5 or 2.0 mM L-glutamate, and varying amounts of inhibitor. The reaction was started by addition of enzyme in buffer. Apparent K_i values were determined graphically from Dixon plots of reciprocal velocity against inhibitor concentrations (see typical examples in Figs. 3 and 4). Isophthalic acid (●), pyridine-3,5-dicarboxylic acid (■), pyridine-2,6-dicarboxylic acid (△), 5-bromofuroic acid (×), *m*-bromobenzoic acid (+), and 5-bromothiophene-2-carboxylic acid (○). Note the intersection points (pK_a) extrapolated from lines drawn through the experimental data for each inhibitor.

term was a repulsive quantity in Eqn. 1 and 2. This indicated that desolvation of the "central" atom may be important for combination of inhibitor with enzyme. A high degree of solvation as indicated by the magnitude of absolute charge density[3] could have decreased the interaction of inhibitor with enzyme. Similar results had been observed for 4 aliphatic inhibitors[2]. There was no apparent correlation between inhibitor potency of these aryl compounds and their pK_a values[13].

DISCUSSION

It is common practice[14] to compare at a single pH physicochemical properties of a series of structurally related compounds (congeners of a parent compound) with the compounds' relative biological potencies in order to gain some insight into the molecular events that occurred. Parameters employed may be electronic, steric, and/or physical[15]. Although extrapolation of *in vitro* experiments to *in vivo* or physiological conditions, may be proper at a single pH if that pH is 7.4[8], studies of enzyme inhibition *in vitro* at a single pH may not necessarily provide correct information about the interaction of inhibitor–enzyme combinations, when substrates, coenzymes, inhibitors or enzymes are capable of ionization[8]. This was exemplified in our studies of the inhibition of bovine liver glutamate dehydrogenase by 6 competitive aromatic inhibitors. The relative order of inhibitor potency (pK_i) in decreasing effectiveness at pH 7.0 was isophthalic acid > 5-bromofuroic acid = pyridine-3,5-dicarboxylic acid > pyridine-2,6-dicarboxylic acid > *m*-bromobenzoic acid and at pH 8.7 was 5-bromofuroic acid > isopthalic acid = *m*-bromobenzoic acid > 5-bromothiophene-2-carboxylic acid > pyridine-2,6-dicarboxylic acid (RESULTS, Fig. 5). Since the effect of pH change was to reduce the apparent K_i from the real K_i[8], only the highest observed pK_i value should be used for comparison purposes since this value may be equal to or at least approximate the real pK_i value. Thus the 6 aromatic inhibitors were separated into two classes: aryl dicarboxylic acids and bromo-substituted aryl monocarboxylic acids. The inhibitor potency of each group was inversely correlated with the magnitude of absolute charge density ($|Q^T|$) of the "central" atom for each inhibitor. This parameter derived from molecular orbital theory has been shown to be an index of the atom's preference for aqueous solvation in a partition system[3,16] and that atoms with greater values of $|Q^T|$ tended to be more solvated than atoms with smaller values of $|Q^T|$. The requirement that inhibitor potency was related to the central atom's hydration was not unexpected since it seems reasonable to assume that both enzyme and inhibitor are hydrated and that effective combination of inhibitor with "active site" of enzyme may depend to some extent upon displacement of their ("active site" and inhibitor) bound water[8].

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