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L-Tryptophan

A NON-ALLOSTERIC ORGAN-SPECIFIC UNCOMPETITIVE INHIBITOR OF HUMAN PLACENTAL ALKALINE PHOSPHATASE

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L-Tryptophan, but not D-tryptophan, inhibits human placental and intestinal alkaline phosphatases, but not those of liver and bone. The nature of this stereospecific organ-specific inhibition has been elucidated. Thus, from a study of the effect of substrate concentration on inhibition in which double-reciprocal plots of $1/v$ versus $1/s$ at various inhibitor concentrations were made, this inhibition is judged to be 'uncompetitive'. That the inhibition is non-allosteric is an opinion based on (1) hyperbolic curves obtained from plotting the percentage inhibition against inhibitor concentration; (2) the independence of the inhibition to heat denaturation and urea treatment; (3) the relatively low value of entropy change; and (4) a value close to unity for n, the number of L-tryptophan molecules that combine with one molecule of enzyme. Finally, a homosteric mechanism is further postulated for the inhibition by L-tryptophan based on the increase of optimum temperature for maximum velocity and the decrease of this inhibition with increasing temperature. The mechanism of this inhibition is discussed.

In searching for possible organ-specific inhibitors for mammalian alkaline phosphatase isoenzymes, Fishman, Green & Inglis (1962, 1963) found that L-phenylalanine inhibited rat and human intestinal alkaline phosphatase isoenzymes but had little or no effect on isoenzymes from several other organs. This inhibition has been characterized as 'uncompetitive and stereospecific' (Ghosh & Fishman, 1966). On the basis of results of kinetic and thermodynamic studies, a homosteric mechanism has been suggested for the inhibition by L-phenylalanine of rat intestinal alkaline phosphatase (Ghosh & Fishman, 1968*a*). Further, a study of the effect of L-phenylalanine on the active centre of this enzyme provides evidence for the hypothesis that the inhibitor combines not with the free enzyme but with the intermediate enzyme–substrate complex, to form a more stable enzyme–inhibitor–substrate complex (Fishman & Ghosh, 1967*a*).

A study on the organ-specific inhibitors for alkaline phosphatase has produced another stereospecific inhibitor, L-tryptophan, which specifically inhibits human placental and intestinal alkaline phosphatase isoenzymes (Fishman & Sie, 1971). Earlier workers (Cox & Griffin, 1967) reported inhibition by L-tryptophan of rat intestinal alkaline phosphatase.

In the present study, we wished to determine if the inhibition of human placental alkaline phosphatase by L-tryptophan exhibits the same uncompetitive and homosteric mechanism that has been demonstrated for the inhibition by L-phenylalanine. The results obtained show marked similarity between the two inhibitions in terms of kinetic data and thermodynamic parameters, as well as in their behaviour on heat or urea denaturation. It is therefore suggested that the inhibition by L-tryptophan is also uncompetitive and homosteric. Finally, an explanation is offered for the mechanism of this inhibition, which reconciles the ideas of Fishman & Ghosh (1967*a*) and Fernley & Walker (1970).

EXPERIMENTAL

The method of enzyme assay with and without L-tryptophan was described by Fishman & Ghosh (1967*a*). The incubation mixture contained 18mM-phenyl disodium phosphate as substrate and 50mM-sodium carbonate–bicarbonate buffer, pH 10.3. The digest was incubated for 15min at 37°C and the reaction was terminated by adding an equal volume of 1.5M-formaldehyde. The liberated phenol was measured as described by Stolbach, Nisselbaum & Fishman (1958). The enzyme unit is defined as μmol of substrate hydrolysed/min per ml of incubation mixture under the conditions specified.

A preliminary study showed that at pH 10.3, L-tryptophan exhibited the best differential inhibitory effect on placental isoenzyme in comparison with other isoenzymes (Fig. 2) and therefore this pH was used in all enzyme assays. Unless otherwise specified, concentration of the L-tryptophan in the enzyme assays was 3 mM, as was the concentration of D-tryptophan used in the control digest.

The percentage inhibition of alkaline phosphatase by L-tryptophan was calculated from the equation

$$\% \text{ inhibition} = \frac{D-L}{D} \times 100$$

in which L was the enzyme activity measured in the presence of L-tryptophan and D, that in the presence of D-tryptophan.

The placental alkaline phosphatase used in these experiments was prepared by the method of Ghosh & Fishman (1968*b*) from a butanol extract of human placental homogenate followed by ammonium sulphate precipitation (between 30 and 60% saturation). The preparation had a specific enzyme activity of 10 units/mg of protein.

RESULTS

Organ-specific and stereospecific inhibition. The organ-specific and stereospecific properties of inhibition by tryptophan of human alkaline phosphatase isoenzymes are shown in Table 1. Percentage inhibition was calculated against tryptophan-free controls. The results showed that L-tryptophan strongly inhibited placental isoenzyme activity (70% inhibition), had moderate effect on the intestinal isoenzyme (55% inhibition) and only slightly inhibited (less than 25%) liver and bone isoenzymes. Except for some activation of intestinal isoenzyme, D-tryptophan had little effect on any of the other isoenzymes examined. However, when percentage inhibition was calculated from $100 \times (D-L)/D$, the intestinal isoenzyme is inhibited as strongly as the placental by L-tryptophan.

Fig. 1 shows the effects of L-tryptophan concentration on placental and liver alkaline phosphatase isoenzymes. At 3 mM-L-tryptophan, the inhibitions were 70% for the placental and 20% for the liver isoenzymes. At higher L-tryptophan concentrations, although the degree of inhibition of placental isoenzyme increased to 80%, the inhibition of liver isoenzyme also was enhanced significantly.

Variation of the pH had little effect on inhibition by tryptophan (Fig. 2), except with D-tryptophan, which showed a 22% inhibition of the placental isoenzyme at pH 10.

Effect of substrate concentration. The effect of varying the substrate concentration on the extent of L-tryptophan inhibition of placental alkaline phosphatase was examined between 0.2 and 20 mM-phenyl phosphate with D-tryptophan in the control solutions. The plot of percentage inhibition against substrate concentration (Fig. 3) shows that at substrate concentrations up to 5 mM, the inhibition was dependent on the concentration of substrate. Saturation of the enzyme with a concentration of substrate higher than 5 mM did not decrease the inhibition by L-tryptophan. This indicates that L-tryptophan does not compete with the substrate for the substrate-binding site of the enzyme and that there is a definite requirement for the substrate for the inhibition by L-tryptophan.

Denaturation by heat and urea. To examine the effect of heat denaturation on inhibition by L-tryptophan, placental enzyme was diluted with water (final activity 0.02 unit/mg of protein) and heated at 70°C, a portion of the enzyme being removed at various time-intervals and immersed in an ice bath to stop the denaturation. The activity of these heated enzymes was then assayed along with the unheated control, with or without 3 mM-L- or D-tryptophan.

Fig. 4 shows that heat denaturation resulted in a progressive loss of enzyme activity, although the percentage inhibition by L-tryptophan (D-tryptophan in control solution) was maintained at a fairly constant value near 60%. Since heat denaturation is usually associated with the destruction of the allosteric site with a consequent effect on the inhibition pattern, our experimental results do not

Table 1. *Percentage inhibition by* D- *or* L-*tryptophan of isoenzymes of alkaline phosphatase*

The substrate was 20 mM-phenyl phosphate, the reaction was carried out at pH 10.3 in 0.05 M-carbonate–bicarbonate buffer, and 3 mM-D- or L-tryptophan was added as inhibitor. Other details are given in the Experimental section. The minus sign indicates activation.

Enzyme source	Inhibition (%) L-Tryptophan (L)	Inhibition (%) D-Tryptophan (D)	$\frac{D-L}{D} \times 100$
Placenta	77.6	1.7	77.1
Intestine	59.7	−22.2	67.5
Liver	26.1	−0.2	26.3
Bone	17.8	−1.9	20.2

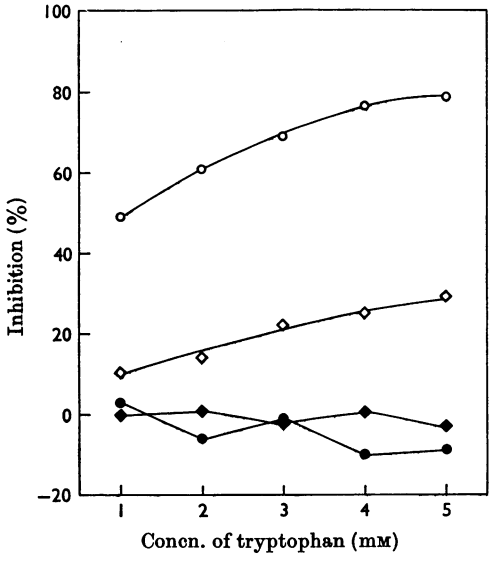


Fig. 1. Effect of inhibitor concentration on percentage inhibition of D- and L-tryptophan on placental and liver alkaline phosphatases. ○, L-Tryptophan, placental enzyme; ◇, L-tryptophan, liver enzyme; ●, D-tryptophan, placental enzyme; ◆, D-tryptophan, liver enzyme. The enzyme was assayed with 18mM-phenylphosphate as substrate in 0.05M-carbonate–bicarbonate buffer, pH 10.3.

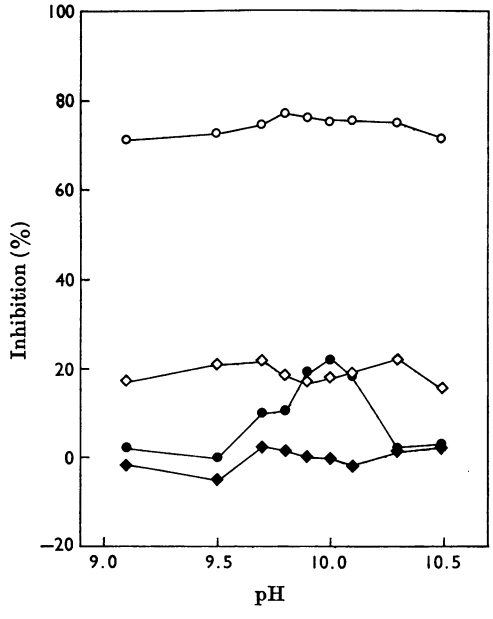


Fig. 2. Effect of pH on percentage inhibition of D- and L-tryptophan on placental and liver alkaline phosphatases. ○, L-Tryptophan, placental enzyme; ◇, L-tryptophan, liver enzyme; ●, D-tryptophan, placental enzyme; ◆, D-tryptophan, liver enzyme. The concentration of each inhibitor was 4mM.

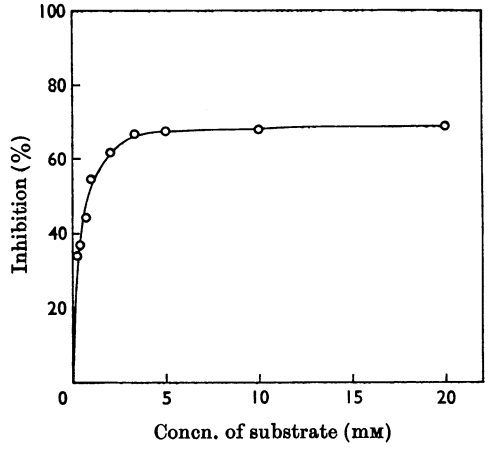


Fig. 3. Effect of substrate (phenyl phosphate) concentration on L-tryptophan inhibition of placental alkaline phosphatase. The concentration of the inhibitor was 3mM.

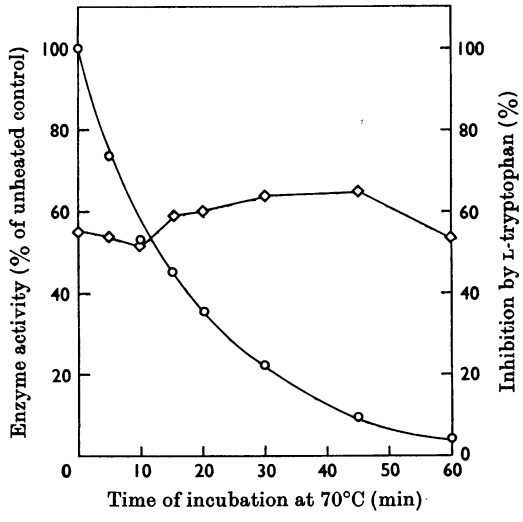


Fig. 4. Effects of heat denaturation on placental alkaline phosphatase activity (○) and its percentage inhibition (◇) by 3mM-L-tryptophan.

agree with those expected for heat denaturation of an allosteric enzyme.

The effects of urea on placental alkaline phosphatase and the inhibition by L-tryptophan were studied by incubating the diluted enzyme (0.25 unit/mg of protein) in 1M-urea at 37°C for various time-intervals. The action of urea was terminated at the end of the incubation by diluting the enzyme–urea mixture with cold water (1:5) and then immersing it in an ice bath. The enzyme activity was

assayed in the presence of 3mM-L-tryptophan with the same concentration of D-tryptophan in the control solutions. At the concentration used, urea actually increased the enzyme activity by a maximum of 45% after 3.5h of incubation with the enzyme (Fig. 5). This can be explained by the known action of urea in unfolding the enzyme molecule so that the active centre is further exposed and becomes more accessible to the substrate without destruction of the active centre. The inhibition by L-tryptophan again is unaffected.

Plots of $1/v$ versus $1/s$ at different concentrations of L-tryptophan. The effect of substrate concentration on placental alkaline phosphatase activity was examined at different concentrations of L-tryptophan. Enzyme assays were carried out with the same concentration of enzyme (0.52 unit/mg of protein) present in all test digests. The substrate (phenyl phosphate) concentration was varied from 0.25 to 4.0mM, and L-tryptophan concentrations were 0, 0.75, 1.5, 3.0 and 6.0mM. The initial-velocity results were organized in the form of double-reciprocal plots of $1/v$ versus $1/s$ at various L-tryptophan concentrations and these (Fig. 6) showed a series of parallel straight lines. This indicates that the inhibition of placental alkaline phosphatase by L-tryptophan is uncompetitive.

Effect of inhibition by L-tryptophan on maximum velocity as a function of temperature. The effect of inhibition by L-tryptophan on the optimum temperature for maximum velocity ($V_{max.}$) is shown in Fig. 7. By measuring the enzyme activities at various substrate concentrations and plotting $1/v$ versus $1/s$, the $V_{max.}$ values were obtained from the intercept where $1/s$ is zero. In this study, the $V_{max.}$ values were measured as a function of temperature, ranging from 26.5 to 70°C, in the presence of 3mM-D- or L-tryptophan. The result showed that inhibition by L-tryptophan caused a shift of the optimum temperature for $V_{max.}$ from 55 to 60°C. This enhancement of the optimum temperature for $V_{max.}$ by the inhibitor indicates that the binding of L-tryptophan to the enzyme–substrate complex changes the enzyme molecule to a more thermostable conformation.

Inhibition by L-tryptophan as a function of inhibitor concentration at different temperatures. The effect of inhibitor concentration on inhibition by L-tryptophan was studied by measuring the extent of enzyme inhibition at different L-tryptophan concentrations ranging from 0.02 to 20mM, with the same concentration of D-tryptophan present in the control. The study was conducted at four different temperatures (37, 42, 50 and 60°C) with a constant substrate concentration. The curves obtained (Fig. 8) by plotting percentage inhibition against L-tryptophan concentration are apparently hyperbolic. When the results are plotted as $\log[(V_0-v)/v]$ against log[L-tryptophan] (where v is the enzyme activity in the presence of L-tryptophan and V_0 is the control activity of the D-isomer at the same concentration), a series of parallel straight lines was obtained (Fig. 9). According to Taketa & Pogell (1965), the slopes of these lines are equal to the n value, which is the apparent number of inhibitor molecules combining with one enzyme molecule. In this study, the slopes at various temperatures studied were found to range from 0.82 to 0.89. This

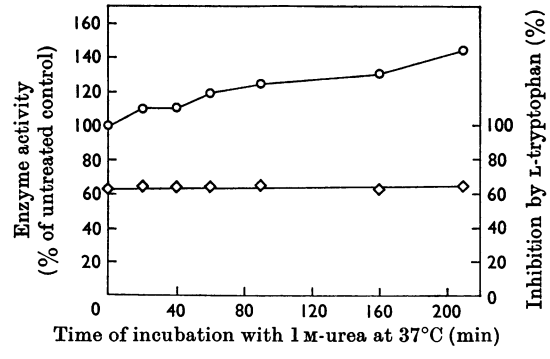


Fig. 5. Effect of urea on placental alkaline phosphatase activity (○) and its percentage inhibition by 3mM-L-tryptophan (◇).

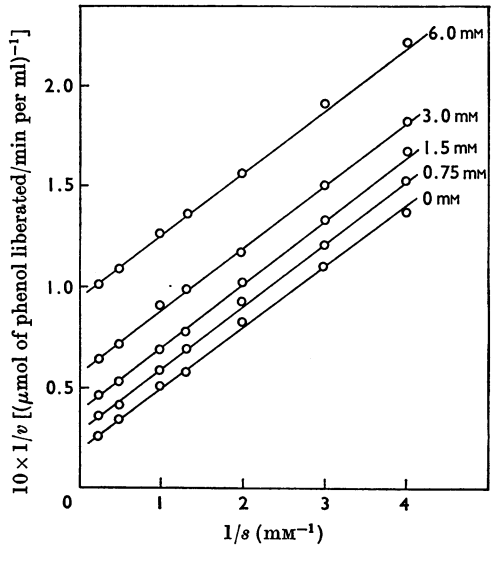


Fig. 6. Double-reciprocal plots of the velocity against substrate (phenyl phosphate) concentration at five different L-tryptophan concentrations (no inhibitor, 0.75mM, 1.5mM, 3.0mM and 6.0mM). The velocity of the reaction is expressed as μmol of phenol released/min per ml of enzyme on incubation at 37°C.

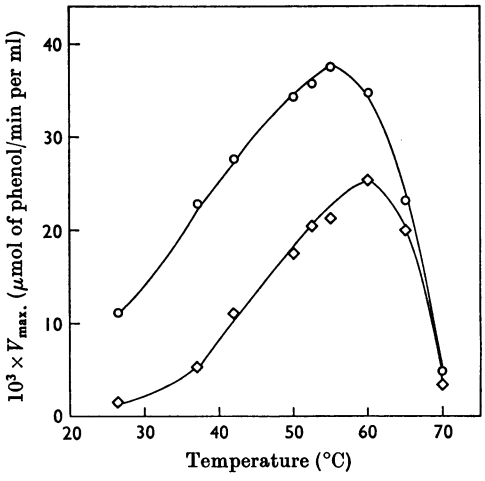


Fig. 7. Effect of L-tryptophan (3mM) on the optimum temperature of maximum velocity of placental alkaline phosphatase: ○, D-tryptophan; ◇, L-tryptophan. Enzyme assays were carried out with 18mM-phenyl phosphate as substrate and 0.05M-carbonate–bicarbonate as buffer, at pH 10.3.

indicates that approximately one molecule of L-tryptophan combines with one molecule of enzyme during the inhibition reaction.

Thermodynamic parameters of inhibition by L-tryptophan. The association constants (K), inhibition constants (K_i) and changes of free energy, enthalpy and entropy for the reaction of L-tryptophan inhibition on placental alkaline phosphatase were determined at four different temperatures, i.e. 37, 42, 50 and 60°C. The results are summarized in Table 2.

The association constant for the binding of enzyme and L-tryptophan was determined from Fig. 9, which shows plots of $\log[(V_0-v)/v]$ versus log[L-tryptophan]. From the overall reaction $E+I \rightleftharpoons EI$, $\log K = \log([EI]/[E]) - \log[I]$. At equilibrium, when $\log[I] = 0$, $\log K = \log([EI]/[E])$, which is measured by $\log[(V_0-v)/v]$. Thus, in the plot of $\log[(V_0-v)/v]$ versus log[L-tryptophan], the intercept where log[L-tryptophan] = 0 is the value of $\log K$.

The plot of the $\log K$ values obtained against $1/T$ is a straight line (Fig. 10) with slope 2.68×10^3. According to Taketa & Pogell (1965), the molecular enthalpy change ΔH can be calculated from this slope: $\Delta H = -\text{slope} \times 2.303R$ where R is the gas constant. The ΔH for the L-tryptophan inhibition thus calculated was −12.6kcal/mol.

The changes in free energy and entropy were calculated from the equations $\Delta F = -2.303RT \log K$ and $\Delta S = (\Delta H - \Delta F)/T$.

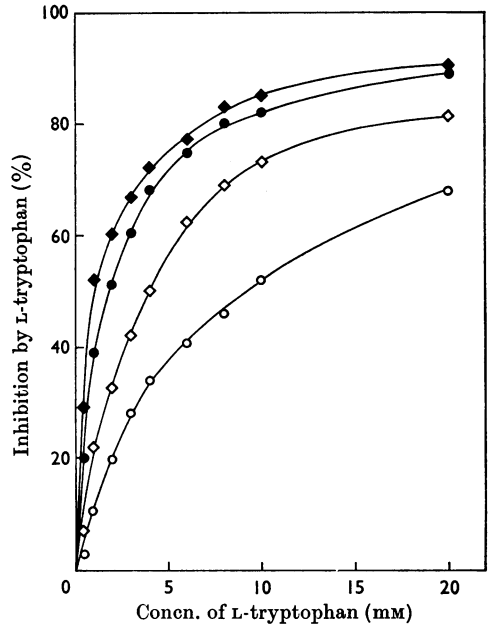


Fig. 8. Percentage inhibition of placental alkaline phosphatase by various concentrations of L-tryptophan at different temperatures: 37°C (♦), 42°C (●), 50°C (◇), 60°C (○). Enzyme activity was measured with 18mM-phenyl phosphate as substrate in 0.05M-carbonate–bicarbonate buffer, pH 10.3.

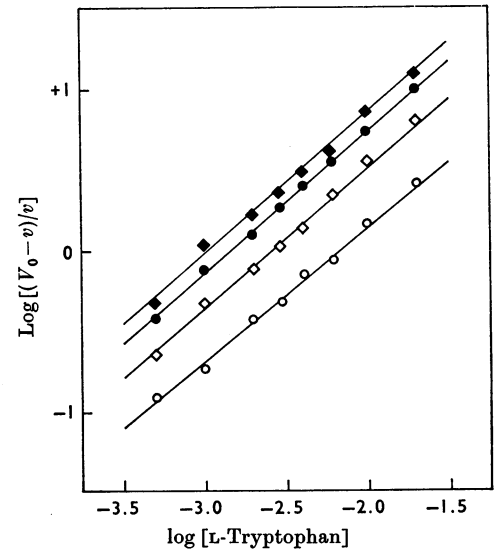


Fig. 9. Linear plots of $\log[(V_0-v)/v]$ versus log[L-tryptophan] at different temperatures (♦, 37°C; ●, 42°C; ◇, 50°C; ○, 60°C). V_0 is the control activity of the enzyme in the presence of D-tryptophan and v the activity with the equivalent concentration of L-tryptophan.

Table 2. *Kinetic and thermodynamic parameters for the binding reaction of* L-*tryptophan with placental alkaline phosphatase*

For experimental details see the text. $\Delta H = -12.26$ kcal/mol. K is the association constant between L-tryptophan and the enzyme; K_i is the inhibition constant (the concentration required for 50% inhibition).

Temperature (°C)	K	K_i (mM)	Inhibition by 5 mM-L-tryptophan (%)	ΔF (kcal/mol)	ΔS (cal/mol per degree)
37	412	0.6	67.0	−3.71	−27.59
42	395	1.1	61.0	−3.56	−27.62
50	191	4.0	42.0	−3.37	−27.53
60	98	9.0	28.0	−3.02	−27.75

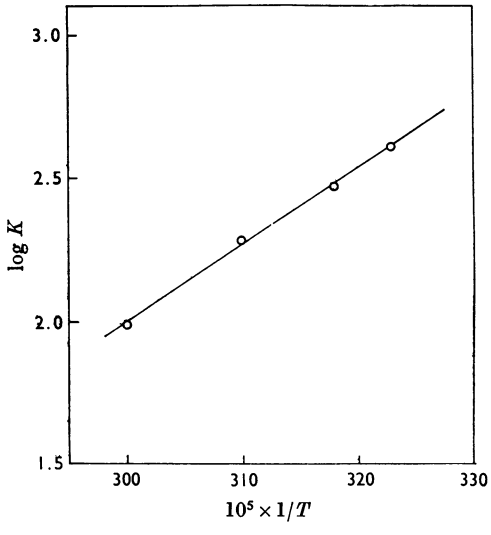


Fig. 10. Plot of $\log K$ against $1/T$. K is the association constant for the inhibitor to the enzyme and T the absolute temperature at which the inhibition reaction was carried out. The slope of this linear plot is 2.68×10^3, which is used to calculate the enthalpy change of the overall inhibition reaction.

With increasing temperature, the apparent association constant decreased whereas the inhibition constant increased, and the extent of inhibition by 5 mM-L-tryptophan decreased. ΔF values decreased from −3.71 to −3.02 kcal/mol as the temperature changed from 37 to 60°C. The overall reaction had an entropy decrease of 27.6 cal/mol per degree.

DISCUSSION

The series of parallel straight lines obtained by plotting $1/v$ against $1/s$ at various concentrations of L-tryptophan is evidence that the inhibition of placental alkaline phosphatase by L-tryptophan is 'uncompetitive'. The shift of the optimum temperature for the maximum velocity toward a higher temperature (from 55 to 60°C) in the presence of L-tryptophan indicates an enhancement of thermostability of the enzyme molecule resulting from the binding of the inhibitor. This phenomenon is consistent with the 'homosteric mechanism' defined by McElroy, DeLuca & Travis (1967) who suggested that a conformational change occurred in the protein as a result of the binding of a specific modifier to the active site and led to a modification of the reaction of the bound intermediate. This is in contrast with the allosteric mechanism where the modifier molecule combines at a site away from the active centre. In an analogy to the example given by McElroy *et al.* (1967) in which the specific substrate luciferin acts as a modifier for the enzyme luciferase, the stereospecific inhibitor L-tryptophan is the modifier for placental alkaline phosphatase. Further study is needed to search for a possible conformational change of the enzyme molecule resulting from L-tryptophan binding.

Results of the present study on the mechanism for L-tryptophan inhibition of human placental alkaline phosphatase are not consistent with a typical allosteric inhibition, although the inhibition is not a competitive one. The conclusion that the mechanism is non-allosteric results from the observations that denaturation by heat and urea, known to affect the allosteric site, have no effect on the inhibition. Further, the entropy change and the n value (number of molecules of modifier per enzyme molecule) are much smaller than those observed in most of the allosteric inhibitions.

The results of the present study markedly resemble those of Ghosh & Fishman (1966, 1968*a*) on the study of inhibition by L-phenylalanine of rat intestinal alkaline phosphatase in that both indicate an uncompetitive and homosteric inhibition mechanism. The noticeable difference between the results of these two studies seems mainly based on the fact that placental alkaline phosphatase is more stable in response to denaturation treatments.

Placental alkaline phosphatase is known to be highly heat stable, its activity being unchanged after 30min at 70°C in the presence of Mg^{2+}, as found by Neale, Clubb, Hotchkis & Posen (1965). However, because the butanol-extracted placental enzyme preparation used in our study was less heat-stable, it was possible to denature the enzyme partially at 70°C without Mg^{2+} in order to observe the effect of heat on the inhibition by L-tryptophan. The optimum temperature for $V_{max.}$ is higher for placental alkaline phosphatase (55°C) than for the intestinal isoenzyme (38°C) and the treatment of placental isoenzyme with urea enhanced the enzymic activity rather than inactivating it. The placental isoenzyme is also stable to digestion by papain. Incubation of the enzyme with papain (5mg/ml) at 37°C for up to 1h failed to affect the enzyme activity. Incubation with diethylstilboestrol at 0.06M also had no effect on the enzyme activity.

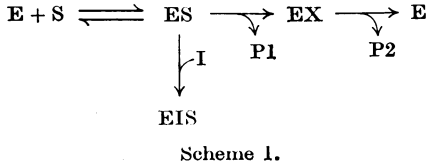
It was observed that inhibition by L-phenylalanine was highly pH-dependent. Human intestinal alkaline phosphatase exhibited maximum inhibition at pH9.2 whereas the placental isoenzyme had a peak at pH9.6 when phenyl phosphate was used as substrate (Fishman & Ghosh, 1967*b*), and the maximum inhibition was at pH8.8 for the intestinal isoenzyme with β-glycerophosphate as substrate (Fishman & Ghosh, 1967*a*). The inhibition by L-tryptophan, however, was pH-independent for both placental and liver alkaline phosphatase.

As with inhibition by L-phenylalanine, the present results show that the inhibition by L-tryptophan is dependent on both inhibitor and substrate concentrations before saturation of each is reached. On the basis of this observation and other kinetic data, Fishman & Ghosh (1967*b*) suggested the formation of a poorly dissociable enzyme–inhibitor–substrate complex (EIS) for the mechanism of L-phenylalanine inhibition of placental alkaline phosphatase as shown in the reaction sequence given in Scheme 1, where E is free enzyme, S the monoester substrate, ES the enzyme–substrate complex, EX the phosphoryl-enzyme intermediate, P1 the non-phosphate product and P2 the inorganic phosphate. In this proposed mechanism, L-phenylalanine was positioned between the metal (possibly zinc) site of the enzyme molecule and the phosphate group of the substrate. As a consequence, the ionic oxygen of the phosphate becomes separated from the thiol group (or possibly the hydroxyl group of serine) of the enzyme molecule, thus preventing the catalytic event, which is initiated by a nucleophilic attack by OH^- ions in the medium on the phosphorus atom of the substrate.

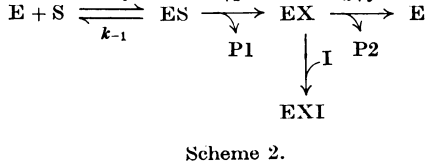
A report by Fernley & Walker (1970) suggested a different mechanism for the inhibition by L-phenylalanine of placental alkaline phosphatase. In their study, the effect of the inhibitor on the initial reaction was examined by a stopped-flow apparatus which measured the fluorescent product produced from the substrate 4-methylumbelliferyl phosphate. They proposed from their results that the inhibitor had little effect on the reactivity of the enzyme–substrate complex, but prevented the breakdown of the phosphoryl-enzyme intermediate, as indicated in Scheme 2. In this mechanism the inhibitor may combine with E, ES or EX, but only EXI is inactive.

Since in this mechanism the inhibitor does not react with the organic moiety of the substrate, Fernley & Walker (1970) suggest that the inhibition would be independent of the type of substrate used. However, there are reports showing that inhibition by L-phenylalanine is dependent on the type of substrate used, e.g. Watanabe & Fishman (1964) showed differences in the percentage of inhibition by L-phenylalanine of intestinal alkaline phosphatase when five different phosphate monoesters were compared as substrates. Keiding (1966) also showed that L-phenylalanine did not inhibit intestinal alkaline phosphatase when *p*-nitrophenyl phosphate was used, but did inhibit with phenyl phosphate as substrate. Since under alkaline conditions, k_{+3} is normally greater than k_{+2} (Fernley & Walker, 1967), the hydrolysis of the phosphoryl-enzyme complex is a faster step, and it is unlikely that the inhibitor can effectively combine by a random collision with EX before it is hydrolysed. It is more reasonable to expect that the inhibitor is fixed into position before the catalytic event.

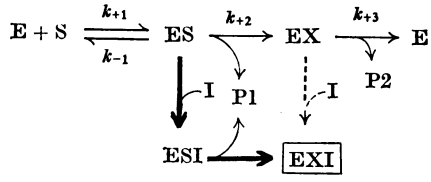
A reaction sequence (Scheme 3) is therefore proposed which conciliates the two mechanisms mentioned above and is in agreement with the known data. As shown in the reaction sequence, the inhibitor enters the reaction by combining with the ES complex to form the ESI complex, which is converted into the more stable complex EXI by



Scheme 1.



Scheme 2.



Scheme 3.

splitting off P1, the organic moiety of the substrate. The inhibitor may also combine with EX to form EXI, but this is considered of minor importance owing to the greater value of k_{+3}. This mechanism agrees with the facts that the inhibition is dependent on concentration of both substrate and inhibitor and on the type of substrate used. It also agrees with the result of Fernley & Walker (1970), who showed that the organic moiety is split off in the presence of the inhibitor to form a stable EXI complex. However, in order for the ESI complex to be converted into the EXI complex, the inhibitor must not be positioned at the active site to interfere with the hydrolysis of the organic moiety but it can be fixed out of the way to the phosphoryl moiety and the enzyme.

Thus, a modification of the original proposal by Fishman & Ghosh (1967*b*) for the position of the L-phenylalanine molecule in forming the enzyme–inhibitor–substrate complex is needed to fit the above requirements. The inhibitor could be situated close to the 'hydrophobic site' proposed by Fishman & Sie (1971) possibly located between the phosphate group of the substrate and the lysine side chain of the enzyme molecule, and thus accomplish the binding of the inhibitor to the phosphate moiety, probably at the hydroxyl group of serine. The idea that the inhibitor is bound near the hydrophobic site could also explain the requirement of a specific structure for the inhibitor and perhaps even the substrate.

Finally, it is recognized that more experimental results are needed to provide necessary support for this mechanism.

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