Note

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Shigeaki Kuwano, Kazuko Yamauchi, and Tchan Gi Bak : Further Studies on Competition of Berberine with Pyridoxal Phosphate in the Tryptophanase System of *Escherichia coli.*

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In a previous paper[1] it was reported that berberine competes with pyridoxal phosphate in the tryptophanase system of *Escherichia coli.* It was noticed at the same time that an irreversible inhibition occurred if the apoenzyme was incubated with berberine prior to the addition of pyridoxal phosphate and tryptophan to start the reaction. Since reversibility is generally regarded as the basis of competitive inhibition, such unusual occurrence of both competition and irreversibility in the same system seemed rather curious. The problem was therefore treated kinetically and a scheme is proposed which explains the mechanisms underlying the apparently contradictory phenomena.

According to the proposed scheme, the irreversible inhibition caused by preincubation of the enzyme with berberine should be eliminated if an excess of pyridoxal phosphate is simultaneously present during the preincubation. Despite many attempts, however, complete elimination of the inhibition could not be obtained even if the enzyme (cell-free preparations) was preincubated for 15 minutes with berberine in the presence of an excess of pyridoxal phosphate; the enzyme activity being measured by determining indole formed during the initial 30-minute period. This fact suggested that even in the presence of pyridoxal phosphate, some nonspecific inactivation not reversible by pyridoxal phosphate might occur in the cell-free system by preincubation of the enzyme with berberine or by contact of the enzyme with inhibitor during the time required for the initial velocity measurements.

It seemed desirable, therefore, to reinvestigate the previous work with special references to these situations. The results obtained in the present study indicate that berberine brings about two distinct types of inhibition toward tryptophanase; (a) an instantaneous and reversible inhibition, and (b) a time-dependent and irreversible inhibition as proposed by Hoch, *et al.*[2] for the inhibition of yeast alcohol dehydrogenase by chelating agents.

Materials and Methods

A cell-free tryptophanase preparation almost free from pyridoxal phosphate was obtained from Me_2CO-dried cells of *E. coli* K-12[1] by a modification of the method of Ichihara, *et al.*[3] The enzyme reaction was run at 37° without shaking in the same way as described in a previous paper,[1] but the substrate (L-tryptophan) and enzyme concentrations were changed to $5\times10^{-3}M$ and approximately 170 γ protein/cc., respectively. Protein was determined by the Folin-copper reagent.[4] Indole formed from L-tryptophan was measured by the method described previously[1] at 5-min. intervals for 15 min., and the initial reaction velocity v was estimated from the tangent at zero time of the time curve and expressed in terms of γ of indole formed per 10 min. Berberine hydrochloride and pyridoxal phosphate employed were the same preparations as those described previously.[1]

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Results and Discussion

Competition between Berberine and Pyridoxal Phosphate——In the previous work,[1] the enzyme was preincubated with berberine in the presence and absence of pyridoxal phosphate prior to the initiation of the reaction. In the present series of experiment, however, the reaction was started by the simultaneous addition of a mixture of temperature-equilibriated solutions of the coenzyme, substrate, and, if necessary, inhibitor into the reaction vessel containing the enzyme solution. Fig. 1 shows the $1/v-1/c$ *2 and

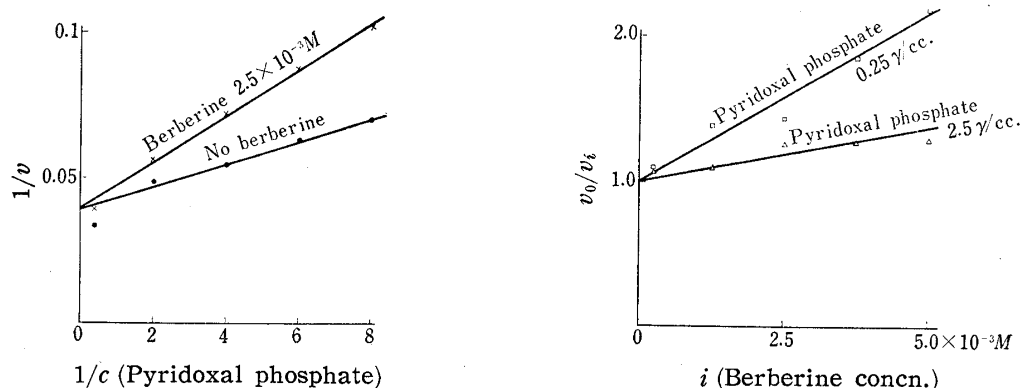

Fig. 1. Competition between Berberime and Pyridoxal Phosphate

v_0/v_i-i*2 plots, respectively, of the results obtained. These Lineweaver-Burk type graphs clearly prove the presence of a competition between berberine and pyridoxal phosphate[1] under the conditions employed. Attempts to reverse the inhibition by pyridoxal, not by pyridoxal phosphate, were unsuccessful as reported in the previous paper.[1]

Although it was very difficult to confirm the reversibility of this instantaneous inhibition directly by the usual "dilution" and "dialysis" methods, it seems possible to conclude that the instantaneous interaction between the enzyme and berberine is substantially reversible in nature for the following two reasons. First, the competitive nature of the enzyme-inhibitor relationship as elucidated above theoretically postulates the presence of reversibility in the interaction. Second, the fact that the formation of indole took place linearly even in the presence of the inhibitor at least for a 15-minute period also indicates the absence of irreversible inactivation of the enzyme during the initial phase of the reaction.

Time-dependent and Irreversible Inhibition by Berberine——When the enzyme was brought into contact with berberine for a certain period prior to initiation of the reaction by adding a mixture of the coenzyme and the substrate, an increase was observed in the inhibition. Table I shows that such increased inhibition was greater when the pre-

Table I. Time-dependent Inhibition by Berberine

	Berberine added ($2.5\times10^{-3}M$)	$v^{a)}$ (γ/cc.)		Incubation time (min.)	$v^{a)}$ (γ/cc.)
Ordinary inhibition test	none	42.0	Enzyme preincubated with berberine ($2.5\times10^{-3}M$)	5	26.0
	addition	34.5		15	23.0

a) Estimated by the method described in the text except the simultaneous addition of pyridoxal phosphate (final concentration : 2.5 γ/cc.).

*2 Where c is the concentration of pyridoxal phosphate in γ/cc., v_0 and v_i are the initial velocities in the absence and presence of the inhibitor, and i is the final concentration (M) of the inhibitor, berberine.

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incubation time was prolonged. This fact, together with the results reported in the previous paper,[1] may best be explained by assuming that an irreversible inactivation of the enzyme not reversible by pyridoxal phosphate occurs during preincubation in the absence of the coenzyme. Since this irreversible inactivation due to berberine could be prevented partly and competitively to a certain extent, as described previously,[1] by the co-existence of pyridoxal phosphate during the preincubation, it appears probable that the inactivation is caused by the attack of berberine at the site where the coenzyme also combines. A possible mechanism of the inactivation may, therefore, be as follows : When the enzyme (E) and the inhibitor (I) are brought into contact, a free, dissociable enzyme-inhibitor complex (EI) will instantaneously be formed. The formation of (EI) will of course be greatly diminished in the presence of a coenzyme, since the coenzyme competes with the inhibitor for the same site of the enzyme surface. At any rate, the complex (EI) will then slowly and irreversibly be converted to a catalytically inactive form (EI′) due, for example, to denaturation of the enzyme protein.

Detailed approach to such peculiar kinetics will be made by employing other enzyme systems and inhibitors, and will be reported in the following paper.

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