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86. Shigeaki Kuwano and Kazuko Yamauchi : Competition of Berberine with Pyridoxal Phosphate in the Tryptophanase System of *Escherichia coli*.

(*Kotaro Institute for Physiological Chemistry of Crude Drugs**1)

In a previous paper[1] it was reported that berberine inhibited the activity of tyrosine decarboxylase of *Streptococcus faecalis* under appropriate conditions. Evidence was also presented to show that the inhibition was irreversible in nature and berberine competed with the coenzyme, pyridoxal phosphate, for the apodecarboxylase. In the present investigation, similar experiments were conducted on the tryptophanase reaction of *Escherichia coli* to examine if the same type of inhibition was also observable with other pyridoxal phosphate-requiring enzymes. It is well known that tryptophanase is a typical pyridoxal phosphate-requiring enzyme.[2]

The present paper shows that the inhibition of tryptophanase by berberine can also be explained in terms of competition of the alkaloid with pyridoxal phosphate. Since the inhibition here was also irreversible, some kinetic considerations are made to reconcile the apparent contradiction between the irreversibility and competitive nature of the inhibition.

Materials and Methods

Berberine and Pyridoxal Phosphate—These were the same as preparations used in the previous work.[1]

Enzyme Preparations—*E. coli* strain K-12 was grown at 30° for 18 hr. with shaking in a medium composed of the following 10 g. of yeast extract, 5 g. each of meat extract, peptone, and K_2HPO_4, 2 g. of succinic acid, 100 mg. each of $MgCl_2 \cdot 6H_2O$ and DL-tryptophan, and 1000 cc. of tap water (pH adjusted to 7.5). After cultivation, the cells were harvested by centrifugation, washed twice with physiological saline, and suspended in the saline (ca. 0.3 mg. dry weight per cc.). Acetone-dried cells were obtained by treating the cell suspension with a large amount of Me_2CO at −45°. Both intact and acetone-dried cells retained most of their pyridoxal phosphate. The crude, cell-free extract was prepared according to the procedure described by Gunsalus, Galeener, and Stamer.[3] The partially purified tryptophanase was precipitated from the extract with $(NH_4)_2SO_4$ between 0.3~0.65 saturation and used as cell-free preparation. This preparation was almost free from pyridoxal phosphate and catalytically active only in the presence of pyridoxal phosphate added.

Enzyme Assay—The reaction with intact and acetone-dried cells was carried out as follows: The intact cells (approximately 0.15 mg. dry weight) suspended in 0.5 cc. of physiological saline or acetone-dried cells (2 mg.) in the same volume of distilled water were mixed with 3.0 cc. of 0.1*M* sodium or potassium phosphate buffer at pH 7.5 (Na salt for intact cells and K salt for dried cells*2) containing, if necessary, berberine hydrochloride or other additives. The mixture was incubated at 37° for 15 min. prior to initiation of the reaction which was effected by the addition of 0.5 cc. of *M*/160 DL-tryptophan or *M*/320 L-tryptophan. The reaction was continued for 30 min. under constant shaking and stopped by adding 0.2 cc. of trichloroacetic acid. 4.0 cc. of toluene was added to the reaction mixture and the indole formed was extracted into the toluene layer by gentle shaking. The indole in the toluene was determined by Ehrlich's *p*-dimethylaminobenzaldehyde method. The reaction with cell-free preparation was performed in the same way except that the cell-free preparation (approximately 0.15 mg. protein as determined by the Folin-Cu reagent[4]) was incubated with pyridoxal phosphate for 15 min. in potassium phosphate buffer prior to subsequent preincubation with berberine and/or others.

*1 Nakatsuhamadôri-1, Ohyodo-ku, Osaka (桑野重昭, 山内和子).

*2 The tryptophanase activity of acetone-dried cells is activated by K^+, but rather inhibited by Na^+.

1) S. Kuwano, K. Yamauchi : This Bulletin, 8, 491(1960).
2) W. A. Wood, I. C. Gunsalus, W. W. Umbreit : J. Biol. Chem., **170**, 313(1947).
3) I. C. Gunsalus, C. C. Galeener, J. R. Stamer : "Methods in Enzymology," **2**, 238(1955), Academic Press Inc., New York.
4) B. Hagihara : "Kôso Kenkyuhô," **1**, 166(1955), Asakura Publ. Co., Tokyo.

Results

Inhibition of Tryptophanase by Berberine—The tryptophanase activity of *E. coli* in both intact and acetone-dried cells was inhibited by berberine as illustrated in Fig. 1. Considerable difference

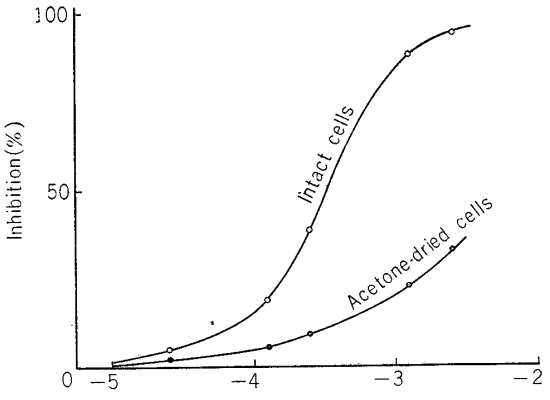

Fig. 1. Inhibition of Tryptophanase by Berberine in Intact and Acetone-dried Cells*

* The reaction conditions were as described under "Materials and Methods." No pyridoxal phosphate was added. Indole formation in the absence of berberine was 46.8 γ in intact cells and 49.0 γ in acetone-dried cells.

was, however, observed between the two types of cells, the inhibition in intact cells being much more significant than that in the acetone-dried cells. The reason for this difference is at present difficult to explain, but it seems likely that different mechanisms are functioning in inhibitions observed with the two types of cells. It may not be unreasonable to assume that the inhibition in acetone-dried cells is simpler in its mechanism than that in intact cells. For this reason and in view of the convenience of handling, the acetone-dried cells were used as the test system in most of the experiments, although, as mentioned above, the degree of inhibition is smaller in this system.

Reversal of Berberine Inhibition by Coenzyme—Inhibition of the tryptophanase activity of the acetone-dried cells by berberine was found to be reversed by pyridoxal phosphate if the coenzyme was added to the system prior to or simultaneously with the inhibitor. There was little or no restoration of the activity when pyridoxal phosphate was added after the cells had been incubated in the presence of berberine because of the irreversible nature of the inhibition (see below). When the inhibition experiments were performed at a fixed concentration of the substrate and with increasing concentration of added pyridoxal phosphate both in the absence and presence of $2.5\times10^{-3}M$ berberine, it was found that the inhibition became less pronounced as more pyridoxal phosphate was added to the system, a fact suggesting the competitive nature of the inhibition. This is more clearly indicated in Fig. 2 in which the data obtained are plotted according to the procedure of

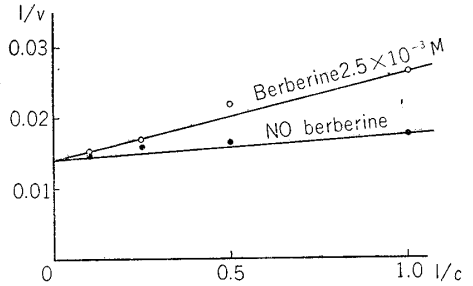

Fig. 2. Competition between Berberine and Pyridoxal Phosphate in Acetone-dried System (I)*

* The reaction conditions as in Fig. 1 except that pyridoxal phosphate(concn.indicated) or pyridoxal phosphate plus berberine ($2.5\times10^{-3}M$) was added during preincubation (Method I).
v : γ indole formed
c : γ pyridoxal phosphate added

Lineweaver and Burk.[5] As can be seen from this graph, two straight lines in the $1/v$—$1/c$ plot*3 corresponding to the presence and absence of berberine cut the $1/v$ axis at the same point; such a feature of the graph can only be expected from the assumption that berberine competes with the coenzyme. The kinetic equation for such a case will be discussed in the section under Discussion. The competitive nature is further supported by the data of another experiment shown in Fig. 3 in which v_0/v_i*4 is plotted against i, the inhibitor concentration. Two straight lines are also obtained

*3 v is the initial reaction velocity as expressed by γ of indole formed during 30 min., and c is the concentration of pyridoxal phosphate (γ per tube).
*4 v_0 and v_i are the initial velocities in the absence and presence of the inhibitor, respectively.
5) H. Lineweaver, D. Burk : J. Am. Chem. Soc., **56**, 658(1934).

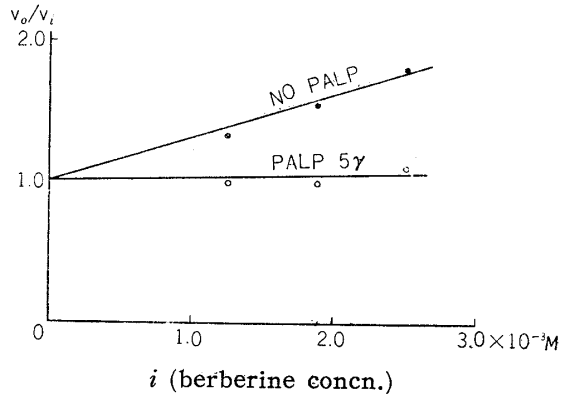

Fig. 3. Competition between Berberine and Pyridoxal Phosphate (PALP) in Acetone-dried System (II)

Reaction conditions as in Fig. 2 except that berberine (concn. indicated) was added later (Method II).

in this case corresponding to the presence and absence of pyridoxal phosphate and they cut the ordinate at the same point where $v_0/v_i=1.0$. Although the experiments described above were conducted at pH 7.5, the same type of competitive inhibition was also observed at pH 8.0.*5

The inhibition could not be reversed when pyridoxal instead of pyridoxal phosphate was added to the reaction mixture. As is shown in Table I, the addition of as much as 1000 γ of pyridoxal (hydrochloride) was ineffective in restoring the activity, while the addition of 10 γ of pyridoxal phosphate fully abolished the inhibition. It is therefore evident that pyridoxal is completely incapable of competing with berberine.

Table I. Effect of Pyridoxal on Berberine Inhibition in Acetone-dried System

Pyridoxal hydrochloride added (γ)	0		100		1000		(Pyridoxal phosphate 10)	
Berberine ($2.5\times10^{-3}M$)	−	+	−	+	−	+	−	+
Indole formed (γ)	61.4	44.0	65.0	42.0	66.8	44.2	78.7	78.7
Inhibition (%)	—	28.4	—	35.4	—	33.8	—	0

Attempts to reverse the inhibition due to berberine by the addition of pyridoxal (1 mg.) and adenosine triphosphate (ATP, 10 mg.) were unsuccessful with the acetone-dried cells. Definite, if not complete, restoration of the activity was, however, observed when the above two compounds were added to the intact cell system. It seems probable, therefore, that the enzyme catalyzing the conversion of pyridoxal and ATP to pyridoxal phosphate is inactivated in the dried cells. On the other hand, the addition of pyridoxal phosphate to the intact-cell system was not so effective as in the case of dried cell system. The reason for this is as yet to be clarified.

Table II. Effect of Pyridoxal Phosphate on Tryptophanase Activity and Its Inhibition by Berberine in Cell-free System

Pyridoxal phosphate added (γ)	Berberine ($2.5\times10^{-3}M$)	Indole formed (γ)	Inhibition (%)
0.8	−	28.8	—
	+	4.8	83.4
1.0	−	36.8	—
	+	9.6	73.9
1.5	−	39.4	—
	+	10.9	72.3
2.5	−	40.3	—
	+	17.1	57.5
5.0	−	41.0	—
	+	25.9	36.8
20.0	−	43.8	—
	+	38.4	12.3

Inhibition and Competition in Cell-free System—The tryptophanase activity of partially purified cell-free preparation was found to depend on the amount of pyridoxal phosphate with which the preparation was preincubated. As can be seen from Table II, the enzyme preincubated with 0.8 γ of pyridoxal phosphate produced 28.8 γ of indole in 30 min., while that preincubated with 20 γ of pyridoxal phosphate produced 43.8 γ of indole under the same condition. It was further shown that this system was also inhibited by berberine. The degree of inhibition, as shown in Table II, was found to decrease with increasing concentration of pyridoxal phosphate added to the system. When the

*5 The concentration of K^+ was the same as that at pH 7.5.

data of Table II were plotted in a $1/v$—$1/c$ graph (Method I), two straight lines cutting the $1/v$ axis at the same point was obtained as shown in Fig. 4. This again suggests, as in the case of acetone-dried cells, that pyridoxal phosphate acts also in the cell-free system as a competitor of berberine. This conclusion was further confirmed by Fig. 5 in which v_0/v_i is plotted against i (data taken from another experiment, Method II).

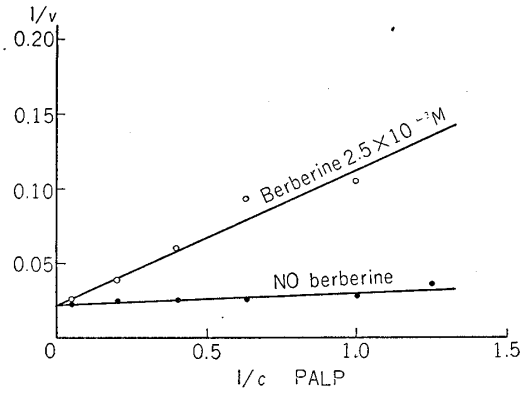

Fig. 4. Competition between Berberine and Pyridoxal Phosphate (PALP) in Cell-free System (I)

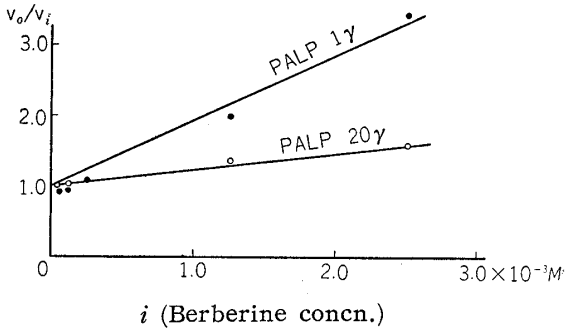

Fig. 5. Competition between Berberine and Pyridoxal Phosphate (PALP) in Cell-free System (II)

Furthermore, it will be noted from the data recorded in Table II that complete elimination of the inhibition due to $2.5\times10^{-3}M$ of berberine could not be observed even when the enzyme was preincubated with as much as $20\,\gamma$ of pyridoxal phosphate (the use of more than $20\,\gamma$ of pyridoxal phosphate was inhibitory rather than protective). Since this fact might be due to a nonspecific inactivation which cannot be reversed by pyridoxal phosphate, attempts were made to protect the enzyme from such nonspecific effect by adding various substances such as K^+,[6] NH_4^+,[6] glutathione,[6] yeast ribonucleic acid, or thymus desoxyribonucleic acid.[7] These substances, however, were unable to restore full activity even when added together with $20\,\gamma$ of pyridoxal phosphate.

Further feature noted in Table II is the very strong inhibition by berberine observed when the concentration of pyridoxal phosphate was low; the inhibition by $2.5\times10^{-3}M$ of berberine being 83% when $0.8\,\gamma$ of pyridoxal phosphate was added. This value is much higher than the inhibition of acetone-dried system by the same concentration of berberine (32%) and is comparable with the value in the intact cell system (93%). This may be explained by a higher concentration of pyridoxal phosphate retained in the acetone-dried cells. As mentioned already, the stronger inhibition in the intact cells may be due to a different inhibition mechanism.

Irreversibility of Inhibition—As in the case of tyrosine decarboxylase of *S. faecalis*,[1] inhibition of tryptophanase of *E. coli* by berberine was also found to be irreversible. As shown in Table III,

Table III. Irreversibility of Inhibition by Berberine

Preincubation condition	Pyridoxal phosphate added (γ)	Berberine ($2.5\times10^{-3}M$)	Indole formed (γ)	Inhibition (%)
Enzyme[a] + pyridoxal phosphate + berberine, 30 min.	5.0	−	50.2	—
		+	17.3	65.5
	20.0	−	58.0	—
		+	28.8	50.3
Enzyme[a] + berberine, 15 min., and then + pyridoxal phosphate, 15 min.	5.0	−	52.8	—
		+	9.0	82.9
	20.0	−	58.8	—
		+	11.0	81.3

a) Cell-free preparation

maximal inhibition was obtained only when the enzyme preparation was incubated with berberine for a certain period of time before pyridoxal phosphate was added to the system. This is also indicative of the irreversibility of the action of berberine. Experiments to prove the irreversibility by dialysis as conducted with tyrosine decarboxylase[1] were not successful because of the instability of tryptophanase to prolonged dialysis.

6) H. Wada, H. Yoshimatsu, T. Koizumi, F. Inoue, K. Ito, T. Morisue, H. Nasu, H. Ito, Y. Sakamoto, K. Ichihara: Proc. Intern. Symp. on Enzyme Chem., **2**, 148(1957).
7) M. Klímek, L. Hnilica: Arch. Biochem. Biophys., **81**, 105(1959).

Discussion

It was suggested in a previous paper[1] that the competition of berberine with pyridoxal phosphate towards pyridoxal phosphate-requiring enzymes is at least partly, involved in the bacteriostatic action of the alkaloid. In the present work efforts were made to give further quantitative account for this competition using the tryptophanase system of *E. coli*. The results obtained in the present study with both acetone-dried and cell-free preparations seem to be compatible with the postulated competition. Thus, these data can be explained by assuming the following equilibria and reaction for the inhibition mechanism :

$$E+C \overset{K_c}{\rightleftharpoons} EC \qquad (1)$$

$$EC+S \overset{K_s}{\rightleftharpoons} ECS \qquad (2)$$

$$E+I \overset{K_i}{\rightleftharpoons} EI \qquad (3)$$

$$ECS \overset{k}{\longrightarrow} E+\text{products} \qquad (4)$$

where E, C, S, and I are enzyme, coenzyme (pyridoxal phosphate), substrate (tryptophan), and inhibitor (berberine), respectively; K_c, K_s, and K_i are dissociation constants of the complexes, EC, ECS, and EI, respectively; and k is the velocity constant of the decomposition of ECS to the free enzyme and products, and thus represents the velocity constant of the overall reaction. If the total concentration of the enzyme is expressed as e, the concentration of free enzyme in the steady state is :

$$e-[EC]-[ECS]-[EI] \qquad (5)$$

The reaction velocity v can now be calculated from Eqs. (1) to (5) as follows :

$$v=\frac{ke\cdot\frac{K_i}{i}\cdot\frac{c}{K_c}\cdot\frac{s}{K_s}}{1+\frac{K_i}{i}+\frac{K_i}{i}\cdot\frac{c}{K_c}+\frac{K_i}{i}\cdot\frac{c}{K_c}\cdot\frac{s}{K_s}} \qquad (6)$$

where c, s, and i are the initial concentration of coenzyme, substrate, and inhibitor, respectively.

When Eq. (6) is put into a reciprocal form Eq. (7) is obtained.

$$1/v=\frac{K_cK_s}{kes}\left(1+\frac{i}{K_i}\right)\cdot\frac{1}{c}+\frac{1}{ke}\cdot\left(1+\frac{K_s}{s}\right) \qquad (7)$$

Eq. (7) shows that $1/v$ is proportional to $1/c$ provided that s and i are kept constant and that the slope of the straight line in the $1/v$—$1/c$ plot, i.e. $K_cK_s(1+i/k_i)/kes$, varies with the value of i (if s is kept constant). The equation also indicates that the straight lines cut the $1/v$ axis at the same point, i.e. $(1+K_s/s)/ke$, irrespective of the value of i including the case where $i=0$ if s is kept constant. These features are exactly in coincidence with those observed in Figs. 2 and 4, indicating the correctness of the assumptions made in Eqs. (1) to (4).

When, on the other hand, the reaction velocities in the absence and presence of the inhibitor are represented by v_0 and v_i, respectively, it is possible to obtain the following Eq. (8) from Eq. (6) or (7) :

$$v_0/v_i = 1 + \frac{1}{1+\left(1+\frac{s}{K_s}\right)\cdot\frac{c}{K_c}}\cdot\frac{i}{K_i} \qquad (8)$$

This equation shows that v_0/v_i is proportional to i provided that s and c are kept constant and the slope of the straight line in the v_0/v_i—i plot, i.e. $1/[1+(1+s/K_s)\cdot(c/K_c)]\cdot K_i$, is dependent on c (if s is constant). It is also indicated in Eq. (8) that when $i=0$, v_0/v_i takes the value of 1. Figs. 3 and 5 are precisely in accordance with these features, again proving the validity of the assumed mechanism.

There is, however, one fact which is apparently incompatible with the postulated mechanism, namely, the observation that the inhibition by berberine is an irreversible phenomenon. As is evident from Eq. (3), one of the basic assumptions made was that the combination of inhibitor with enzyme is freely reversible: the velocity equations (Eqs. (6), (7), and (8)) being valid only when this assumption is held. This difficulty can, however, be overcome by assuming that the complex EI formed by the reversible combination of E and I (as shown in Eq. (3)) is slowly converted in an irreversible manner to another inactive form designated as EI′ due, for example, to the denaturation of the enzyme protein. This implies that the following reaction takes place in addition to Eqs. (1) to (4):

$$EI \xrightarrow[\text{(irreversible)}]{k'} EI' \qquad (9)$$

where k′ is the velocity constant of the irreversible conversion. If it is assumed that $k \gg k'$, as is very likely the case, the concentration of EI′ will be negligible at the initial phase of reaction during which the initial velocity v is measured.*6 The reaction formula (9), therefore, does not interfere with the equilibria postulated in Eqs. (1) to (3) at the initial phase of the reaction and thus can be omitted in the derivation of the velocity equations. This equation (Formula (9)), on the other hand, provides a sufficient explanation for all the observations concerning the irreversible inhibition.

It was found in the present study that the behavior of apoenzyme towards berberine and pyridoxal phosphate is not different between pH 7.5 and 8.0. This is quite in contrast to the results obtained with tyrosine decarboxylase of *S. faecalis* in which both the inhibition and competition are markedly affected by changing pH from 7.4 to 8.0.[1] Although the reason for this difference is as yet to be studied, it is probable that this reflects differences in the nature of the apoenzyme proteins of the two enzymes.

The stronger inhibition of the intact cell system by berberine as compared to that of the acetone-dried system appears to be due, as already discussed, to different inhibition mechanisms in the two systems. The mechanism operating in the intact-cell system is, however, still unknown.

It was observed that the inhibition in the cell-free system is also stronger than that in the acetone-dried system. This seems to be accounted for by the almost complete absence of pyridoxal phosphate in the former system, since the degree of inhibition could be reduced to the level of the acetone-dried system by preincubating the cell-free system

*6 The data shown in Table III suggest that the conversion of EI to EI′ is fairly rapid if the enzyme is incubated with the inhibitor alone. This may be due to a relatively high steady-state concentration of EI under this condition. It is, however, very likely that the steady-state concentration of EI is greatly reduced in the presence of both the substrate and coenzyme, a condition under which the initial velocity is measured (actual concentration of EI under this condition can be computed only when the values of K_c, K_s, and K_i are known). Thus, the irreversible formation of EI′ seems to be negligible during 30 min. of reaction required for the initial velocity measurements.

with a large amount of pyridoxal phosphate (see Table II). The fact that the inhibition of the cell-free system by $2.5\times10^{-3}M$ of berberine could not be fully eliminated by the addition of 20γ of pyridoxal phosphate might suggest that there is some nonspecific inactivation not reversible by pyridoxal phosphate, but this does not seem to be very probable, since the addition of a variety of possible protecting agents failed to eliminate residual inhibition. It seems probable that there is no difference in the inhibition mechanism between the acetone-dried and cell-free systems.

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Summary

It was found that berberine inhibits the tryptophanase system of *Escherichia coli* by competing with the coenzyme, pyridoxal phosphate, when acetone-dried cells or partially purified cell-free extracts were used as the enzyme. The inhibition was also found to be of irreversible nature. A mechanism was proposed to account for both the competitive and irreversible nature of the inhibition. Some kinetic considerations were made on the proposed mechanism. The inhibition of tryptophanase by berberine in intact cells was stronger than that in acetone-dried cells and it was inferred that different mechanisms are functioning in the inhibitions of the two types of cells.

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