UDC 576.851.21.095.18:547.978.1

85. Shigeaki Kuwano and Kazuko Yamauchi: Effect of Berberine on Tyrosine Decarboxylase Activity of *Streptococcus faecalis*.

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Berberine is an alkaloid occurring in a number of plants such as *Coptis japonica* MAKINO and *Phellodendron amurense* RUPRECHT. It belongs to the group of isoquinoline derivatives and is a quaternary base. Studies reported from various laboratories have established that this alkaloid exhibits a potent bacteriostatic activity.[1~5] It has further been shown that it also acts as an antitumor agent.[6] There are evidences that suggest an interaction between berberine and desoxyribonucleic acids.[7,8]

The present paper deals with an investigation which was intended to shed light on the mechanisms underlying the bacteriostatic action of berberine. It was found that tyrosine decarboxylase of *Streptococcus faecalis* was inhibited by the alkaloid under appropriate conditions and that such inhibition was a result of the prevention by the alkaloid of the binding of pyridoxal phosphate, the coenzyme, with the apoprotein.

Materials and Methods

Berberine Hydrochloride—This was isolated from *Coptis japonica* MAKINO. Rhizomes of the plant were cut into small pieces and exhaustively extracted with EtOH in a Soxhlet extractor. Berberine was allowed to crystallize from the extract in the form of its hydrochloride ($C_{20}H_{17}O_4N \cdot HCl \cdot 2H_2O$), and recrystallized from EtOH, m.p. 204° (decomp.).[9] Since there was no difference in the biological effect between the hydrochloride and the free base, the former was used throughout the experiments.

Enzyme Preparations—*S. faecalis*, strain R, was cultivated in a pyridoxine-deficient medium, the cells were harvested, and suspended in distilled water as described by Gunsalus and Smith.[10] Acetone-dried cells were prepared by treating the cell suspension with Me_2CO at −45°. Most of the pyridoxal phosphate contained in the cells was removed by this treatment. Cell-free enzyme preparation was obtained from the acetone-dried cells as follows: A dispersion of 1 g. of the dried cells in 100 cc. of 0.022*M* phosphate buffer (pH 5.5) was frozen and then thawed. The thawed suspension was warmed to 37° and allowed to autolyse for 3 hr. at this temperature. Freezing, thawing, and autolysis were repeated three times and the final autolysate was centrifuged to remove the cell debris. The crude cell-free extract thus obtained was fractionated with $(NH_4)_2SO_4$ and the fraction precipitating between 0.4 and 0.7 saturation wa scollected. This fraction containing the apodecarboxylase catalysed the decarboxylation of L-tyrosine at the rate of 1150 μL. CO_2/hr./mg. protein when supplemented with an excess of pyridoxal phosphate.

Assay of Enzyme Activity—The enzyme activity was followed by measuring the evolution of CO_2 in a Warburg manometer. In the main compartment of the manometric vessel were placed 2.5 cc. of a solution containing 0.16*M* acetate buffer (pH 5.5), a few γ of pyridoxal phosphate,[*2]

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[*2] Disodium salt produced by the Sigma Chemical Company.

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enzyme preparation,*3 and if necessary, appropriate concentrations of berberine hydrochloride. The side arm contained 0.5 cc. of a suspension containing 0.03*M* L-tyrosine. After equilibrium at 37° for 15 min.,*4 the tyrosine suspension was tipped into the main compartment and the amount of CO_2 evolved during the subsequent 60 min. was measured. The results were usually expressed in terms of Q_{CO_2} (μL. CO_2/hr./mg. cell) or μL. CO_2/hr./vessel (cell-free preparation).

Growth Inhibition Experiments—Test organisms (*S. faecalis* and *E. coli*) were grown in test tubes containing 5 cc. each of a glucose-Tryptone medium (glucose 0.5%, yeast extract 1%, Difco Tryptone 1%). After cultivating the organisms at 37° for 16 hr. in the presence or absence of berberine, the cells were collected by centrifugation and suspended in a suitable amount of physiological saline solution. The growth was estimated by measuring optical density of the suspension at 600 mμ by a Coleman Junior spectrophotometer.

Other Measurements—Absorption spectra were measured with a Cary Model 14 recording spectrophotometer. pH was determined with a Horiba Model T pH-titrator. Protein was determined by the Folin-Cu method.[11]

Results

Experiments with Acetone-dried Cells—As shown in Table I, berberine was not at all inhibitory

Table I. Effect of Berberine on Tyrosine Decarboxylase Activity of Acetone-dried Cells at pH 5.5

Berberine added (*M*)	Tyrosine decarboxylase activity (Q_{CO_2})	
	without PO_4^{3-}	with PO_4^{3-} *a)*
0	173	168
1.7×10^{-4}	169	157
3.3×10^{-3}	185	183

a) NaH_2PO_4, final concn. $3.3\times10^{-2}M$.

but rather slightly stimulatory to the tyrosine decarboxylase activity of acetone-dried cells of *S. faecalis* when the experiments were run at pH 5.5 which is the optimum pH for the enzyme. Addition of phosphate ions to the reaction mixture was also ineffective in causing an inhibition at this pH. Powerful inhibitions by berberine could, however, be induced if the dried cells were incubated with the inhibitor and pyridoxal phosphate in neutral or slightly alkaline phosphate medium prior to initiation of the reaction at pH 5.5 (the pH of the preincubation mixture was adjusted to 5.5 with acetate buffer after preincubation). The degree of inhibition was found to be profoundly dependent on pH of the preincubation medium. As can be seen from Fig. 1, no inhibition could be observed if

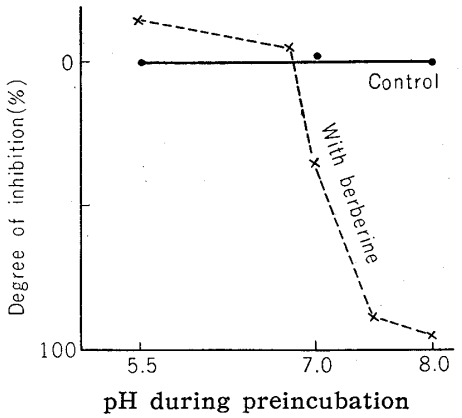

Fig. 1. Effect of pH of Preincubation Mixture* on Berberine Inhibition

* Acetone-dried cells with pyridoxal phosphate (1γ/3 cc.) and, if necessary, berberine ($3.3\times10^{-3}M$).

preincubation was carried out at pH 6.8 or lower. When, however, the pH of preincubation mixture was raised above this value, the degree of inhibition increased sharply until it reached as high as 95% at pH 8.0.

Tables II, III, and IV show the results of a series of experiments in which the effect of various preincubation conditions on the inhibitory action of berberine was compared. In all these experi-

*3 1 mg. of acetone-dried cells or 150 γ protein when cell-free enzyme was used.

*4 In some experiments equilibration was made for 30 min. as indicated in respective tables.

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Table II. Effect of Preincubation Condition on Tyrosine Decarboxylase Activity of Acetone-dried Cells

	Substances added[a]					
	Expt. 1			Expt. 2		
Preincubating conditions: 1st at pH 7.4 for 15 min.	PALP	PALP	PALP+B	[b]	[b]	B
Preincubating conditions: 2nd at pH 5.5 for 15 min.	[b]	B	[b]	PALP	PALP+B	PALP
Q_{CO_2}	170	172	20	181	178	14
Inhibition (%)	—	no	88.2	—	1.7	92.2

	Expt. 3	
Preincubating conditions: 1st at pH 5.5 for 30 min.	[b]	B
Preincubating conditions: 2nd at the same pH for 15 min.	PALP	PALP
Q_{CO_2}	198	196
Inhibition (%)	—	1.0

a) Final concn. of pyridoxal phosphate (PALP), 1 γ/3 cc.; berberine hydrochloride (B), $3.3\times10^{-3}M$. *b*) Incubated without addition.

Table III. Protective Effect of Pyridoxal Phosphate

pH of preincubation mixture[a]	Berberine added (M)	Pyridoxal phosphate added (γ/3 cc.) 1.0	2.5	5.0
		Q_{CO_2} recorded		
7.0	—	187	190	199
	3.3×10^{-3}	117	165	192
	(Inhibition %)	(37.5)	(13.2)	(3.5)
7.4	—	183	188	180
	1.7×10^{-3}	128	153	159
	(Inhibition %)	(30.0)	(18.6)	(11.7)
8.0	—	192	189	184
	3.3×10^{-4}	86	87	90
	(Inhibition %)	(55.2)	(54.0)	(51.0)

a) Acetone-dried cells incubated with pyridoxal phosphate and berberine for 30 min.

Table IV. Effect of Preincubation Time at pH 7.2

Substances added during preincubation[a] 1st at pH 7.2 for 15 min.	2nd at pH 5.5 for 15 min.	Q_{CO_2}	Inhibition (%)
PALP	[b]	156	—
PALP+B	[b]	68	56.5
[b]	PALP	156	—
B	PALP	17	89.1
for 30 min.	for 15 min.		
PALP	[b]	156	—
PALP+B	[b]	48	69.2
[b]	PALP	152	—
B	PALP	6	96.0

a) Final concn. of pyridoxal phosphate (PALP), 1 γ/3 cc.; berberine hydrochloride (B), $3.3\times10^{-3}M$. *b*) Incubated without addition.

ments the reaction was started at pH 5.5 by tipping L-tyrosine. It will be seen from Table II that berberine exerts its inhibitory action only when the cells were preincubated with the alkaloid at pH 7.4, but not at pH 5.5, regardless of the presence or absence of pyridoxal phosphate during that period. This fact suggests that at neutral or slightly alkaline pH berberine combines with the apoenzyme at a site where pyridoxal phosphate is to occupy under uninhibited conditions and that this combination is not dissociable when the pH was reduced to 5.5.

Such consideration leads to the expectation that the presence of pyridoxal phosphate at higher concentrations during preincubation may counteract the inhibitory action of berberine. The data recorded in Table III show that this is really the case. It was found that the presence of a higher concentration of pyridoxal phosphate (5 γ/3 cc.) in the preincubation mixture can either completely (at pH 7.0) or partly (at pH 7.4) protect the enzyme from inhibition by berberine. When the

preincubation was conducted at pH 8.0, however, no protection could be observed even in the presence of a higher concentration of pyridoxal phosphate.

Furthermore, it was found, as shown in Table IV, that the inhibition became more extensive when the preincubation was carried out (at pH 7.2) for a longer period of time. As expected from the protective effect of pyridoxal phosphate, the inhibition was also more pronounced when pyridoxal phosphate was absent during the preincubation.

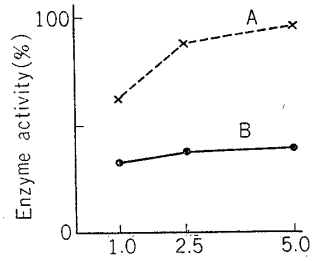

Fig. 2. Effect of Pyridoxal Phosphate Concentration on Acetone-dried Cells

A : Preincubated with berberine ($3.3\times10^{-3}M$) and pyridoxal phosphate at pH 7.0 for 30 min.
B : Preincubated with berberine ($3.3\times10^{-3}M$) alone at pH 7.0 for 30 min. and pyridoxal phosphate added after adjusting to pH 5.5.

Table V. Failure of Reversing Berberine Inhibition by Means of Dialysis[a)]

	Original activity		Activity after dialysis at pH 5.5		Activity after dialysis at pH 7.2	
	Control	Preincubated with berberine[b)]	Control	Preincubated with berberine[b)]	Control	Preincubated with berberine[b)]
Q_{CO_2} (μL.)	181	137	122	93	146	64
Inhibition (%)	—	24.3	—	23.8	—	56.2

a) At below 5° for 24 hr.
b) At 37° and pH 7.2 for 30 min., 10 mg. of dried cells plus $3.3\times10^{-3}M$ of berberine hydrochloride in a total volume of 3 cc.

An attempt was then made to test whether the addition of pyridoxal phosphate to the berberine-inhibited cells could reactivate the inhibited enzyme activity. The data shown in Fig. 2 indicate, however, that the inhibition once introduced into the dried cells by contact with berberine was very stable and could hardly be reversed by the addition of a large amount of pyridoxal phosphate. This finding suggesting the irreversible nature of berberine-apoenzyme interaction was further confirmed by dialysis experiments recorded in Table V. It is clearly shown in the table that the original activity could not be restored even if the dried cells, which had been brought into contact with berberine at pH 7.2, were extensively dialysed at either pH 5.5 or 7.2 to remove the alkaloid as thoroughly as possible.

Final examinations were made with the acetone-dried cells to see if pyridoxal itself can replace pyridoxal phosphate as a protecting agent for the berberine inhibition during the preincubation. However, only a slight protective effect was observed even if 10 to 100 times as much pyridoxal as pyridoxal phosphate employed above was added in the preincubation mixture.

Table VI. Effect of Preincubation with Berberine on Cell-free Tyrosine Decarboxylase Preparation and its Protection by Pyridoxal Phosphate

pH when preincubated for 30 min. with $3.3\times10^{-3}M$ of berberine	5.5		7.4		8.0	
	Control	With berberine	Control	With berberine	Control	With berberine
CO_2 (μL./hr.)	130	153	138	99	131	34
Inhibition (%)	—	accelerative	—	28.3	—	74.0

Pyridoxal phosphate (γ/3 cc.) preincubated with berberine ($3.3\times10^{-3}M$) and enzyme preparation at pH 7.4	1.0		2.5		5.0	
	Control	With berberine	Control	With berberine	Control	With berberine
CO_2 (μL./hr.)	118	85	114	100	116	103
Inhibition (%)	—	28.0	—	12.3	—	11.2

Experiments with Cell-free Enzyme Preparation—Table VI summarizes experiments with the cell-free tyrosine decarboxylase preparation. These data show that berberine inhibits the cell-free enzyme in almost the same manner as in the case of acetone-dried cells. The only difference observed is the fact that the degree of inhibition is less significant with cell-free preparation than that observed with the dried cells.

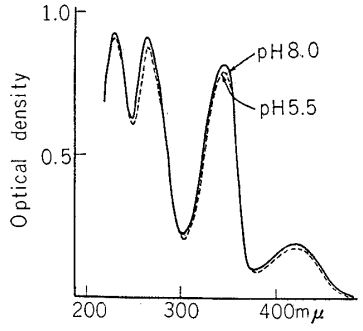

Fig. 3. Absorption Spectra of Berberine Hydrochloride ($3\times10^{-5}M$) at Various pH*

* The spectrum at pH 7.0 is between those at pH 5.5 and 8.0.

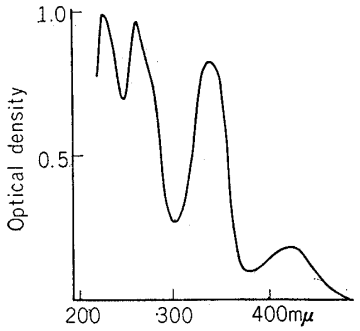

Fig. 4. Difference in Spectra of Pyridoxal Phosphate-Berberine Mixture minus Pyridoxal Phosphate alone at pH 7.4*

* The sample cell contained a mixture of pyridoxal phosphate (10γ/cc.) and berberine hydrochloride ($3\times10^{-5}M$), while the control cell contained only pyridoxal phosphate (10γ/cc.). The spectrum obtained therefore eliminates the contribution by pyridoxal phosphate alone.

Spectrophotometric Observations—In Figs. 3 and 4 are reproduced absorption spectra of berberine measured at pH 5.5, 7.0, and 8.0, as well as that of a mixture of berberine and pyridoxal phosphate at pH 7.4. As can be seen from these curves, no essential difference was observed in the spectra of berberine at the three pH values, except that the peaks at 262 and 344 mμ increased slightly at pH 8.0. The absorption spectrum of the berberine-pyridoxal phosphate mixture could be interpreted from Fig. 4 as a simple sum of the spectra of the two constituents. This fact may be taken as an indication that there is no interaction between berberine and pyridoxal phosphate, at least at this pH value.

Growth Inhibition by Berberine—If the inhibition of tyrosine decarboxylase activity by berberine is one of the main factors responsible for bacteriostatic action of the alkaloid, it is expected that the inhibition of growth may be more serious in slightly alkaline media than in acid media. As is shown in Table VII, the growth of both *S. faecalis* and *E. coli* was found to be inhibited with increasing initial pH of the growing culture.

Table VII. Growth Inhibition of *S. faecalis* and *E. coli* by Berberine

Bacterial strain	Berberine ($\times10^{-3}M$)	Initial pH of growing culture: 6.8			7.4			8.0		
		E_{600}[a]	%[b]	pH[c]	E_{600}	%	pH	E_{600}	%	pH
S. faecalis R	—	0.330	100	4.2	0.410	100	4.2	0.480	100	4.2
	0.5	0.344	104	4.2	0.390	95	4.2	0.380	79	4.2
	0.75	0.320	97	4.2	0.340	83	4.2	0.080	17	7.4
	1.0	0.306	93	4.2	0.080	20	7.2	0.050	10	7.4
E. coli K-12	—	0.340	100	4.6	0.360	100	4.6	0.400	100	4.6
	1.0	0.336	99	4.6	0.320	89	4.6	0.310	78	4.6
	1.25	0.320	94	4.6	0.304	84	4.6	0.190	48	7.0
	1.5	0.310	91	4.6	0.160	44	6.8	0.130	33	7.4

a) Optical density at 600 mμ
b) The degree of growth to control
c) Final pH after cultivation

Discussion

The fact that berberine inhibits the growth of certain microörganisms has been known, but no effort has ever been made to elucidate the underlying mechanism. The investigation reported in this paper suggests that a competition between pyridoxal phosphate, the coenzyme of a number of enzymes concerned in amino acid metabolism, and berberine plays an important rôle in the bacteriostatic action of the alkaloid. Although this conclusion was obtained with only one enzyme, tyrosine decarboxylase, it does not seem unreasonable to assume that this can be extended to other enzymes requiring pyr-

idoxal phosphate as a coenzyme.

The growth-inhibition experiments, though preliminary in nature, showed that the inhibition of bacterial growth by berberine was more pronounced in a slightly alkaline region. This phenomenon seems undoubtedly to be related to the stronger competition between pyridoxal phosphate and berberine at this pH range and the irreversible nature of this inhibition.

It is not yet easy to explain the reasons for the dependence of tyrosine decarboxylase inhibition on pH of the reaction mixture. From the spectrophotometric observations, however, it appears quite unlikely that any marked changes are induced in the molecular structure of berberine by changing the pH. The spectral data also exclude the possibility of any interaction between pyridoxal phosphate and berberine in the pH region tested. Consequently, it seems highly probable that the behavior of apodecarboxylase towards pyridoxal phosphate and berberine is essentially varied depending on pH of the reaction media.

Furthermore, it should be pointed out that the action of berberine towards the apoenzyme at pH 8.0 is different from that at pH 7.0 or 7.4, since no protection is observable by the simultaneous presence of pyridoxal phosphate at pH 8.0, whereas the inhibition can be completely (at pH 7.0) or partially (at pH 7.4) protected by pyridoxal phosphate at more acidic reactions. It appears therefore that the susceptibility of the apoenzyme to berberine is remarkably augmented at pH 8.0. More experiments are required however, to draw any decisive conclusions concerning this problem.

Only a slight difference was detected in the inhibition by berberine between acetone-dried cells and cell-free enzyme preparations. The only differences observed were the facts that less inhibition was caused in the case of a cell-free preparation and that slightly higher pH value was required during preincubation with the cell-free preparation to detect the same degree of inhibition than in the case of acetone-dried cells. It is, however, premature to conclude that there is no essential difference between the mode of action of berberine towards the two enzyme preparations.

In order to attain maximal inhibition, the apoenzyme preparations should be incubated with berberine for a certain period of time prior to addition of the coenzyme. This is necessary because of the protective effect due to the coenzyme added simultaneously with the inhibitor. As mentioned earlier, the inhibition is irreversible in nature and the inhibition once introduced into the enzyme protein cannot be reversed by subsequent addition of the coenzyme.

The authors are grateful to Prof. K. Kimura of the University of Kyoto for his continued guidance and encouragement. They also acknowledge the helpful suggestions given during this investigation and measurement of absorption spectra by Prof. R. Sato of the Institute for Protein Research, Osaka University.

Summary

Berberine inhibits the tyrosine decarboxylase activity of *Streptococcus faecalis* if the enzyme preparation is preincubated with the alkaloid under slightly alkaline conditions prior to initiation of the enzyme reaction at its optimum pH (5.5). When the preincubation is carried out at acidic pH, berberine causes no inhibition. At the pH range from 7.0 to 7.4 the enzyme can be protected from the inhibitory action of berberine by conducting the preincubation in the presence of a large amount of pyridoxal phosphate. This fact suggests that berberine competes with pyridoxal phosphate for the apoenzyme at least at this pH range. At pH 8.0, however, no protection can be observed even if the preincubation mixture contains an excess of pyridoxal phosphate, the reason for which is as yet to be explored. The possibility that the competition between berberine and pyridoxal phosphate is, at least partly, responsible for the bacteriostatic action of the alkaloid is discussed.

(Received October 15, 1959)