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Biosynthesis of Thiamine. Inhibition of Thiamine Phosphatase Activity by Pyridoxal. (31661)

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Growth inhibition of *Neurospora crassa* cultures(1) grown in the presence of pyridoxal have been investigated and related to inhibitory effects on thiamine biosynthesis(2,3). The availability of cell-free preparations of yeast which biosynthesize readily measurable quantities of thiamine(4) from its precursors (hydroxymethylpyrimidine and thiazole)* has allowed several groups to establish the following sequence of reactions(5):

1) hydroxymethylpyrimidine —ATP; hydroxymethylpyrimidine kinase→ hydroxymethylpyrimidine-P

2) hydroxymethylpyrimidine-P —ATP; hydroxymethylpyrimidine phosphokinase→ hydroxymethylpyrimidine-PP

3) thiazole —ATP; thiazole kinase→ thiazole-P

4) hydroxymethylpyrimidine-PP + thiazole-P —thiamine-P synthase→ thiamine-P + PPi

5) thiamine-P —thiamine phosphatase→ thiamine + Pi

Using this yeast cell-free system it has been shown that pyridoxal-P and pyridoxal inhibit thiamine formation, although the other pyridoximers (pyridoxamine, pyridoxamine-P, and pyridoxine) do not(6). Evidence was presented to indicate that the inhibition caused by pyridoxal-P occurred at (or after) reaction 4 of the sequence(6). This paper continues the investigation of the inhibition caused by pyridoxal.

Materials and methods. Samples of hydroxymethylpyrimidine, the corresponding bromomethyl derivative, and thiazole were generously supplied by Merck and Co., Inc. Thiamine hydrochloride was obtained from Eastman Distillation Products Industries; thiamine phosphate from Calbiochem; pyridoxal·HCl and ATP (disodium salt) from Sigma Chemical Corp. Thiazole-P was prepared from thiamine-P by the method of Camiener and Brown(7). Hydroxymethyl-P and hydroxymethyl-PP were prepared from bromomethylpyrimidine by the method of Lewin and Brown(6).

Preparation of incubation mixtures. Cell-free extracts of baker's yeast (Fleischman's) were prepared and purified by ammonium sulfate fractionation procedures, as described by Camiener and Brown(7). These enzyme preparations were used to prepare thiamine-biosynthesizing incubation mixtures, which were incubated with or without pyridoxal at 38°C for specified lengths of time and then were heat inactivated at 100°C for 5 minutes (7). Further details are provided in connection with the appropriate Figures and Tables.

Microbiological determination of thiamine. Aliquots of incubation mixtures were diluted and thiamine content determined by microbiological assay using *Lactobacillus viridescens* A.T.C.C. 12706, according to the meth-

* The following abbreviations are used in this paper: hydroxymethylpyrimidine for 2-methyl-4-amino-5-hydroxymethylpyrimidine; bromoethylpyrimidine for 2-methyl-4-amino-5-bromomethylpyrimidine; thiazole for 4-methyl-5-(2-hydroxyethyl)thiazole; ATP for adenosine triphosphate; P and PP for phosphate and pyrophosphate, respectively.

od of Deibel *et al*(8). The resulting values were recorded as "thiamine equivalents," according to the convention of Camiener and Brown(7), who noted that the test organism responds to thiamine and its phosphate and pyrophosphate esters to somewhat different extents.

Fluorimetric measurement of thiamine and thiamine P. Aliquots of incubation mixtures (50 μl) were spotted on Whatman No. 3 MM paper, developed (ascending) with a solvent system consisting of n-propanol, H_2O, acetate buffer (1 M, pH 5.0), 7:2:1 v/v which separated thiamine from thiamine-P. The resulting zones of thiamine and thiamine-P were assayed by the thiochrome fluorimetric method of Lewin and Wei(9).

Bioautographic methods. Aerobacter aerogenes strain PD1, obtained from Dr. G. M. Brown, was used for detection of hydroxymethylpyrimidine and its derivatives (10); *Escherichia coli* thiazoleless strain (also obtained from the collection of Dr. G. M. Brown) for detection of thiazole and its derivatives(7); *Saccharomyces carlsbergensis* A.T.C.C. 9080 for detection of pyridoxal and its derivatives(11), on paper chromatograms using bioautographic procedures.

Results. Pyridoxal exerted a considerable degree of inhibition on thiamine biosynthesis as measured by the *L. viridescens* assay results. Inhibition was most dramatic at the high pyridoxal concentration (10^{-2} M), which was therefore used for later experiments (Table I). Pyridoxal, at the concentration present in the microbiological assay tubes, did not affect thiamine recovery values.

Since the inhibitory effect of pyridoxal on thiamine biosynthesis is less pronounced than that of pyridoxal-P(6) it was necessary to ascertain whether pyridoxal was phosphorylated during the incubation and exerted its effect in the form of pyridoxal-P. Bioautography of phosphatase-sprayed paper chromatograms of incubation mixtures, using *Saccharomyces carlsbergensis* A.T.C.C. 9080 as test organism, demonstrated the absence of pyridoxal-P in the mixtures.

Results of a time study (Fig. 1) indicated that thiamine synthesis (as measured by *L. viridescens* assay) from hydroxymethylpyrimidine and thiazole proceeded linearly for up to two hours, and that the degree of pyridoxal inhibition was constant throughout this time.

TABLE I. Inhibition of Thiamine Biosynthesis by Pyridoxal.

Concentrations of components in the reaction mixtures were: hydroxymethylpyrimidine, 58 μM; thiazole, 36 μM; ATP, 5 mM; $MgCl_2$, 0.01 M; pyridoxal as shown; phosphate buffer (pH 7.4), 0.1 M, and yeast autolysate containing approximately 3.4 mg protein per ml, prepared and charcoal treated according to the method of Camiener and Brown(7). Reaction mixtures (1 ml) were incubated for 3 hours at 38°C, inactivated by heating in a boiling water bath for 5 min, and analyzed for thiamine by microbiological assay with *Lactobacillus viridescens* A.T.C.C. 12706. Thiamine synthesis was calculated as the difference between thiamine contents of incubated and unincubated samples.

Pyridoxal concentration (M)	Thiamine* synthesis (nanograms)
0	572
10^{-5}	512
10^{-4}	408
10^{-3}	284
10^{-2}	82

* Thiamine expressed as thiamine HCl equivalents.

L. viridescens assay results (Table II) demonstrated pyridoxal inhibition of thiamine synthesis when either hydroxymethylpyrimidine, its phosphate or pyrophosphate esters supplied the pyrimidine moiety and whether thiazole or thiazole-P was the source of the thiazole moiety.

Since the data of Table II indicated that the inhibitory action of pyridoxal was not localized at steps 1, 2, or 3 of the reaction sequence it became necessary to utilize a thiamine assay which would distinguish between the products of steps 4 and 5. A paper chromatographic, fluorimetric method was developed to do this(9). The results of assays using this method (Table III) demonstrate little or no inhibition by pyridoxal of the total thiamine production, but demonstrate a definite increase in the ratio of thiamine-P to thiamine in the incubation mixtures. It would appear that thiamine-P is synthesized at approximately the normal rate in the presence of pyridoxal, but that its hydrolysis to thiamine is inhibited. To demonstrate this

TABLE II. Pyridoxal Inhibition of Thiamine Biosynthesis from Phosphorylated and Nonphosphorylated Moieties.

Concentrations of components in the reaction mixtures were: $MgCl_2$, 0.01 M; phosphate buffer (pH 7.4), 0.1 M; enzyme preparation (as described in Fig. 1, equivalent to 1.5 mg of protein per ml) and, where indicated, pyridoxal, 10 mM; ATP, 5 mM; hydroxymethylpyrimidine, 30 μM; hydroxymethylpyrimidine-P, 36 μM; hydroxymethylpyrimidine-PP, 30 μM; thiazole, 36 μM; and thiazole-P, 40 μM. Reaction mixtures (1 ml) were incubated at 38°C for 1 hr, then inactivated by heating to 100°C (water bath) for 5 min. Thiamine synthesized was calculated as the difference between thiamine contents of incubated and unincubated samples, as determined by assay with *L. viridescens* A.T.C.C. 12706.

Mixture No.	Components added to reaction mixture	Thiamine* synthesis (nanograms)
Exp I		
1.	Hydroxymethylpyrimidine, thiazole, ATP	2800
2.	1. + pyridoxal	1200
3.	Hydroxymethylpyrimidine-P, thiazole, ATP	3700
4.	3. + pyridoxal	1300
Exp II		
5.	Hydroxymethylpyrimidine, thiazole, ATP	2100
6.	5. + pyridoxal	930
7.	Hydroxymethylpyrimidine, thiazole-P, ATP	2300
8.	7. + pyridoxal	1400
9.	Hydroxymethylpyrimidine-PP, thiazole, ATP	2900
10.	9. + pyridoxal	1500
11.	Hydroxymethylpyrimidine-PP, thiazole-P	4200
12.	11. + pyridoxal	2200

* Thiamine expressed as thiamine HCl equivalent.

more clearly, thiamine phosphate was incubated with the enzyme preparation in the absence of pyrimidine or thiazole moieties. The results (Table IV) indicate that pyridoxal inhibits the thiamine phosphatase reaction.

Discussion. *L. viridescens* A.T.C.C. 12706, which has been used to measure thiamine biosynthesis in cell-free systems(6,7,10), has been shown to give a more vigorous growth response to thiamine than to its pyrophosphate and phosphate esters(7,8). Under conditions where the biosynthesizing systems produce different ratios of thiamine-P and thiamine the assay values will not be comparable. This is the case in reaction mixtures incubated with and without pyridoxal. The resulting values would therefore appear to indicate a decrease in total thiamine content when, actually, only the free thiamine content is decreased. The locus of the inhibitory action of pyridoxal on thiamine synthesis in these cell-free systems is best shown by use of the more specific paper chromatographic thiochrome fluorescence method.

The thiamine phosphatase step, which we have shown to be inhibited by pyridoxal, needs further investigation. Although previous workers have reported that thiamine

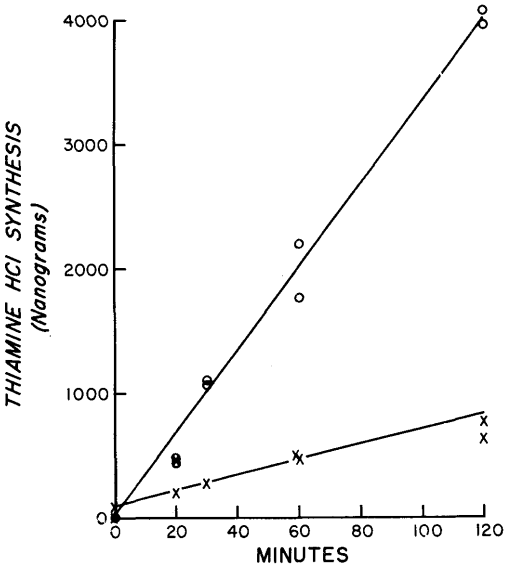

FIG. 1. Pyridoxal inhibition of thiamine synthesis as a function of incubation time. Reaction mixtures similar to those of Table I were prepared, containing 1.5 mg protein/ml of enzyme prepared by ammonium sulfate fractionation of yeast autolysate(8). After incubation at 37°C for specified times, biosynthesis was stopped by heating at 100°C (water bath) for 5 min, and thiamine assayed microbiologically with *L. viridescens* A.T.C.C. 12706. Pyridoxal (0.01 M) was present in samples marked x and was absent in those marked o.

TABLE III. Effect of Pyridoxal on Biosynthesis of Thiamine and Thiamine Phosphate.

Concentrations of components, in the reaction mixtures and the conditions of incubation and inactivation were similar to those described under Table II. Aliquots (0.05 ml) of the mixtures were chromatographed and assayed for thiamine and thiamine-P by the thiochrome fluorimetric method of Lewin and Wei(9). Amount of thiamine synthesized was calculated as the difference between incubated and unincubated samples.

Mixture	Components added to reaction mixture	Products synthesized: Thiamine (nanomoles)	Thiamine-P (nanomoles)	Thiamine-P thiamine
1.	Hydroxymethylpyrimidine, thiazole, ATP	3.0	3.8	1.3
2.	1. + pyridoxal	1.2	6.8	5.7
3.	Hydroxymethylpyrimidine-PP, thiazole-P	12.4	3.8	.3
4.	3. + pyridoxal	3.3	11.5	3.5

phosphate was converted to thiamine by enzyme preparations from yeast(5,12), no phosphatase specific for thiamine-P has been characterized. It has been shown by bioautography, however, that enzyme preparations of the type used in these experiments dephosphorylated thiamine-P while they did not cause appreciable hydrolysis of pyridoxal-P, hydroxymethylpyrimidine phosphate, or hydroxymethylpyrimidine pyrophosphate(6). This would indicate the presence of an enzyme with specificity for thiamine phosphate. Pyridoxal inhibition may provide a useful tool for investigation of this enzyme. It is interesting to note that there is no pyridoxal inhibition of thiamine phosphate hydrolysis when intestinal alkaline phosphatase is the enzyme used (Table IV). The mechanism by which pyridoxal exerts its inhibitory action on the yeast phosphatase remains to be elucidated.

TABLE IV. Effect of Pyridoxal on Thiamine Phosphatase Activity.

Concentrations of components in the initial reaction mixtures were: Thiamine-P, 58 μM; $MgCl_2$, 0.01 M; tris (hydroxymethyl) aminomethane buffer (pH 7.4), 0.1 M; pyridoxal and enzyme preparation as described below. Following incubation for 1 hr at 37°C, the mixtures (5 ml) were inactivated by the addition of HCl (1 N, 1 ml). Aliquots (0.05 ml) were chromatographed and assayed by the thiochrome fluorescence method of Lewin and Wei(9).

	Thiamine produced (nanomoles/ml)
Experiment I	
1. Complete system with yeast enzyme*	20.6
2. 1. + pyridoxal (10^{-4}M)	20.2
3. 1. + pyridoxal (10^{-3}M)	16.0
4. 1. + pyridoxal (10^{-2}M)	8.2
Experiment II	
5. Complete systems with intestinal phosphatase†	37.7
6. 5. + pyridoxal (10^{-2}M)	39.2
7. 5. without enzyme	0

* The yeast enzyme containing 1.5 mg of protein per incubation mixture, was prepared by ammonium sulfate precipitation of yeast autolysate, as described under Fig. 1.

† Commercial calf intestinal alkaline phosphatase (Calbiochem), 4 Bodansky units/mg, 2 mg of protein per incubation mixture (5 ml).

Summary. The biosynthesis of thiamine from 2-methyl-4-amino-5-hydroxymethylpyrimidine and 4-methyl-5-(hydroxyethyl) thiazole, or from their pyro- and monophosphate esters respectively, was inhibited by pyridoxal. Pyridoxal was shown to inhibit the conversion of thiamine monophosphate to thiamine by a cell-free enzyme system prepared from baker's yeast.

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Enumeration of VSV Particles and a Demonstration of Their Growth Kinetics by Electron Microscopy.* (31662)

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A knowledge of the physical particle count of a virus sample would be of great help in determining whether the variance in published results is due to host cell variation, virus adaptation, or amount of virus(1). Biological titer alone does not give sufficient information to properly quantitate and compare various results. The plaque forming unit (pfu) does not give the total number of virus particles (vp) in a given suspension nor does it give the number of infectious particles(2). Hackett(3) has expressed the need for meaningful particle counts of vesicular stomatitis virus (VSV) for a clearer understanding of homologous inhibition. In a study of the radiosensitivity of VSV, Pittman *et al* (4) have expressed the need for particle counts in understanding the effect of radiation on infected cells. It has been further suggested that the ratio of the actual number of virus particles to the number of biological units provides a more accurate estimation of the quantitative relationships between various virus samples(5). The present report is an attempt to answer the need for an accurate technique for particle counting of VSV and to demonstrate its application to certain growth properties of the virus.

Materials and methods. The cell line used throughout these studies was the Earle L strain originally obtained from Dr. W. Earle and kept in continuous passage. The cells were grown in Medium 199 containing 10% horse serum, 400 units/ml of penicillin and 40 μg streptomycin/ml.

The Indiana serotype of VSV was obtained from the American Type Culture Collection. It was adapted to growth in L cells and virus obtained from the 23-27th passage was used in these experiments.

The basic particle count technique is that described by Sharp(6) with minor modifications. Briefly, infected cell cultures were treated in a Raytheon sonic oscillator (Model S-102A) for 3 minutes in order to lyse the cells and disperse the virus to obtain homogenous samples. The crude cell lysates are diluted in phosphate buffered saline (PBS) and centrifuged onto an agar receiving surface at 17,600 $\times$ g in a SU Sorvall counting rotor for 20 minutes. The agar block is removed and exposed to 2% osmium tetroxide vapors for 10 minutes. Pseudoreplicas are made and the collodion film with the virus is placed on stainless steel grids, shadowcast and examined by electron microscopy (RCA EMU 3 H). Repeated stripping of the agar block revealed that more than 99% of the virus was removed with the first pseudoreplica. The particle count is calculated according to the formula described by Sharp(6).

Crude cell lysates with as little as 1 $\times$ 10^7 vp/ml could be counted with no purification procedures. When the particle count is less than 2 $\times$ 10^8 vp/ml, cellular debris tends to obscure the picture. This background level of debris may be satisfactorily reduced by enzymatic treatment with trypsin

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