The Conversion of Pyridoxine Phosphate into Pyridoxal Phosphate in *Escherichia coli*

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1. Evidence is presented for the presence of pyridoxine phosphate oxidase in aqueous extracts of *Escherichia coli*. Some comparison is made with pyridoxamine phosphate oxidase. 2. Isoniazid and iproniazid were found to combine with pyridoxal phosphate, but isoniazid did not combine with either pyridoxamine phosphate or pyridoxine phosphate. Both oxidase activities were somewhat inhibited by benzylamine and putrescine, but not by phenethylamine or cadaverine. 3. The significance of pyridoxine phosphate oxidase in cell metabolism is discussed.

The conversion of pyridoxine phosphate into pyridoxal phosphate was first demonstrated by Wada *et al.* (1959), who obtained a preparation from rabbit liver that catalysed this reaction. The enzyme pyridoxine phosphate oxidase was later purified from rabbit liver by Morisue, Morino, Sakamoto & Ichihara (1960). Wada & Snell (1961) and Snell (1961) presented evidence that the oxidations of both pyridoxine phosphate and of pyridoxamine phosphate to pyridoxal phosphate are catalysed by a single protein. These workers obtained a 66-fold purification of the enzyme activities from rabbit liver, and the two activities were found to run in parallel throughout the purification procedure.

The enzymic oxidation of pyridoxamine phosphate to pyridoxal phosphate was demonstrated by Pogell (1958, 1963) in mammalian liver preparations. Beechey & Happold (1957) demonstrated the conversion of pyridoxamine phosphate into pyridoxal phosphate by an enzyme present in crude extracts of apotryptophanase from *Escherichia coli*. The enzyme was later shown to be a pyridoxamine phosphate oxidase [pyridoxamine phosphate-oxygen oxidoreductase (deaminating), EC 1.4.3.5], which was extensively studied by Turner & Happold (1961).

It is the purpose of the present paper to report on the presence in *E. coli* of a system that will convert pyridoxine phosphate into pyridoxal phosphate.

EXPERIMENTAL

Enzyme preparations. The enzyme preparations used were obtained from acetone-ether-dried cells of *E. coli* N.C.T.C. 86 (kindly supplied by the Lister Institute of Preventive

Medicine, Elstree, Herts.). These cells had been grown for 18 hr. at 37° on the following medium solidified with agar: peptone, 10 g./l.; NaCl, 2.5 g./l.; pancreatic digest of casein, subjected to complete hydrolysis with HCl (half of the medium); tryptophan, 0.025 g./l.; medium adjusted to pH 7.4 (Beechey & Happold, 1957; Dawes, Dawson & Happold, 1947). Estimations of the pyridoxamine phosphate-oxidase and pyridoxine phosphate-oxidase activities have also been made (H. M. Henderson, unpublished work) on aqueous extracts of *E. coli* cells grown, under various conditions, in other culture media.

The cells were extracted overnight with water (20 mg. of cells/ml. of water) at 3–4° and then centrifuged at 12000g for 30 min. at 0° (Dawes, Dawson & Happold, 1947). The supernatant aqueous cell-free extract contained the enzyme activity.

Composition of standard reaction mixture. To estimate the pyridoxamine phosphate-oxidase and pyridoxine phosphate-oxidase activities, reactions were carried out in a total volume of 4.0 ml. The standard composition was: aqueous extract of *E. coli* (approx. 4.0 mg. of protein/ml.), 2.0 ml.; buffer, 1.0 ml.; 16 mm-MgSO₄, 0.5 ml.; pyridoxine phosphate or pyridoxamine phosphate (200 µg./ml.), 0.5 ml., i.e. approx. 0.4 µmole. Water replaced substrate in the blank. During the reaction the mixtures were incubated at 37°. Reactions were usually terminated by the addition of 1.0 ml. of 30% (w/v) trichloroacetic acid.

Measurement of enzyme activity. (a) Phenylhydrazine procedure. This method for the colorimetric estimation of pyridoxal phosphate was followed according to the procedure developed by Wada & Snell (1961).

(b) Spectrophotometric procedure. The progress of the conversion of pyridoxine phosphate into pyridoxal phosphate was followed in a Unicam SP. 500 spectrophotometer. Changes in extinction were estimated at wavelengths of maximum absorption characteristic of substrate and product, at the pH concerned, over a period of time (usually 1 hr.). The actual method used was a modification of that employed by Turner & Happold (1961) in their studies on the activity of pyridoxamine phosphate oxidase in *E. coli*. Preliminary experiments were carried out to obtain the absorption spectra of pyridoxine phosphate and of pyridoxal

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phosphate, each at pH 7.2, 8.0 and 10.1. From these spectra, wavelength values for maximum extinction and molar extinction coefficients were calculated.

Tryptophanase activity and indole assay. Tryptophanase activity was determined according to the method described by Beechey & Happold (1957). Indole estimation was carried out according to the method of Happold & Hoyle (1934), since modified by Scott (1961).

Estimation of protein. This was carried out either as a modification of the method of Lowry, Rosebrough, Farr & Randall (1951), or by the measurement of ultraviolet absorption.

Determination of inorganic phosphate. P was determined by the method of Gomori (1941-42).

RESULTS

Spectrophotometric evidence for the conversion of pyridoxine phosphate into pyridoxal phosphate. Enzyme activity was followed by spectrophotometric determination of the maximum extinction values, alternately, of the substrate pyridoxine phosphate and of the product pyridoxal phosphate at 37° over a period of 1 hr. This experiment was carried out at pH 7.2, 8.0 and 10.1, and a representative result at pH 10.1 is shown in Fig. 1. The absorption spectra of pyridoxine phosphate show maximum extinction at pH 10.1 at a wavelength of 312.5m μ , and at pH 7.2 and 8.0 at 325m μ . There was no absorption at wavelengths above 360m μ at any of these pH values.

Pyridoxal phosphate possesses maximal absorption at 390m μ at all these pH values, except in tris buffer where the maximal value is 415m μ owing to

Fig. 1. Conversion of pyridoxine phosphate into pyridoxal phosphate. Reaction mixtures were made up as follows: 2.0 ml. of aqueous extract of *E. coli* (4 mg. of protein/ml.); 1.0 ml. of 50 mM- Na_2CO_3 - NaHCO_3 buffer, pH 10.1; 0.5 ml. of 16 mM- MgSO_4 ; 0.5 ml. of pyridoxine phosphate (0.4 μ mole). In the blank mixtures 0.5 ml. of water replaced pyridoxine phosphate. The mixtures were incubated at 37°. Extinction readings were taken at 312.5m μ (○) and at 390m μ (●) at the indicated times.

Schiff-base formation (Matsuo, 1957), and absorbs comparatively weakly at 300-340m μ (Williams & Neilands, 1954).

Identification of the product as pyridoxal phosphate. The increase in extinction at 390m μ was taken to indicate an increase in the amount of pyridoxal phosphate formed from enzyme activity in the reaction mixture, this conclusion being based on absorption spectra data.

Confirmation of the product of the reaction as pyridoxal phosphate. The presence of pyridoxal phosphate was confirmed by two observations: (i) on incubation of pyridoxine phosphate with an aqueous extract of *E. coli*, the product of the reaction gave the characteristic stable yellow colour at room temperature 10 min. after the addition of phenylhydrazine reagent; (ii) the activation of apotryptophanase.

Effect of various compounds on enzyme activity. In view of the relationship between the reaction catalysed by pyridoxamine phosphate oxidase and those reactions catalysed by monoamine oxidase and diamine oxidase (Pogell, 1958; Zeller, 1951), and evidence that the oxidations of pyridoxamine phosphate and pyridoxine phosphate to pyridoxal phosphate are catalysed by a single protein (Wada & Snell, 1961), the effects of iproniazid, isoniazid and amines as indicated below on pyridoxine phosphate oxidase were investigated.

Isoniazid and iproniazid were found to combine with pyridoxal phosphate to form an isonicotinylhydrazone (Davison, 1956). Isoniazid was found, by spectrophotometric methods, not to combine with either pyridoxamine phosphate or pyridoxine phosphate.

The enzyme preparation was preincubated with 1.0 ml. of the amine at appropriate concentration for 30 min. before the addition of pyridoxamine phosphate or pyridoxine phosphate (Table 1). No inhibition was obtained in the presence of cadaverine.

For both oxidase activities, there was a significant degree of inhibition by benzylamine (1 mM) and by putrescine (0.1 mM). There was no significant inhibition in the presence of either of the respective homologues phenethylamine or cadaverine.

Control mixtures containing pyridoxal phosphate and the amines in different concentrations were made up, and a comparison was made with the standard pyridoxal phosphate calibration curve. There was no evidence to indicate any combination between pyridoxal phosphate and any of the amines concerned.

Effect of time on reaction velocity. Reaction mixtures were incubated at 37° for various times, after which the reactions were terminated by the addition of 1.0 ml. of 30% (w/v) trichloroacetic acid.

The curves obtained (Fig. 2) were very similar to

Table 1. Inhibition of pyridoxamine phosphate-oxidase and pyridoxine phosphate-oxidase activities after preincubation with amines

Reaction mixtures were made up as follows: 0.5 ml. of enzyme preparation; 1.0 ml. of 50 mM- Na_2CO_3 - NaHCO_3 buffer, pH 10.1; 0.5 ml. of 16 mM- MgSO_4 ; 1.0 ml. of amine at appropriate concentration. After 30 min. preincubation at 37°, 1.0 ml. of substrate was added (1.0 ml. of water to control mixtures). After incubation at 37° for 30 min., 1.0 ml. of 30% (w/v) trichloroacetic acid was added. Pyridoxal phosphate was determined by the phenylhydrazine procedure.

Amine	Final concn. (mm)	Percentage inhibition		Activities corresponding to 100% activity ($\mu\text{g.}$ of pyridoxal phosphate formed/ml.)	
		Pyridoxamine phosphate oxidase	Pyridoxine phosphate oxidase	Pyridoxamine phosphate oxidase	Pyridoxine phosphate oxidase
Benzylamine	10	38	37		
	1	32	31		
	0.1	5	6	5.05	4.15
	0.01	0	2		
Phenethylamine	10	0	10		
	1	5	10		
	0.1	4	6	3.85	3.15
	0.01	0	6		
Putrescine	10	54	44		
	1	55	43	4.90	3.50
	0.1	44	30		
	0.01	15	3		
Cadaverine		No inhibition		3.85	3.60

Fig. 2. Effect of time on reaction velocity. Reaction mixtures were set up as follows: 2.0 ml. of aqueous extract of *E. coli* (3.3 mg. of protein/ml.); 1.0 ml. of 50 mM-sodium phosphate buffer, pH 8.0; 0.5 ml. of 16 mM- MgSO_4 ; 0.5 ml. of pyridoxamine phosphate or pyridoxine phosphate (0.4 μmole). In the control mixtures, 0.5 ml. of water replaced the substrate. The mixtures were incubated at 37° for the indicated periods of time, after which 30% (w/v) trichloroacetic acid was added. Pyridoxal phosphate was determined by the phenylhydrazine procedure. Enzyme activities: ●, pyridoxamine phosphate oxidase; ○, pyridoxine phosphate oxidase.

Fig. 3. Effect of pH on enzyme activity. Reaction mixtures were set up as follows: 2.0 ml. of aqueous extract of *E. coli*; 1.0 ml. of buffer (0.2 M-tris-HCl, pH range 6.2-9.5, or 50 mM- Na_2CO_3 - NaHCO_3 , pH range 10.2-10.6); 0.5 ml. of 16 mM- MgSO_4 ; 0.5 ml. of pyridoxamine phosphate or pyridoxine phosphate (0.4 μmole). In the control mixtures, 0.5 ml. of water replaced the substrate. The mixtures were incubated at 37° for 1 hr., after which 1.0 ml. of 30% (w/v) trichloroacetic acid was added. Enzyme activities: ●, pyridoxamine phosphate oxidase; ○, pyridoxine phosphate oxidase.

the increases in extinction at 390m μ observed during the spectrophotometric studies. At pH 8.0, pyridoxine phosphate oxidase was more active than pyridoxamine phosphate oxidase. In both cases, the initial rate of reaction was fairly rapid, and a constant rate of activity was attained after 20 min.

Effect of pH on enzyme activity. Reaction mixtures were made up in the pH range 6.2–10.6, and incubated at 37° for 1 hr.

Pyridoxamine phosphate oxidase was the more sensitive of the two activities to changes in pH, the optimum value being pH 10.2 (Fig. 3). Pyridoxine phosphate oxidase was less sensitive to changes in pH, the optimum value being pH 9.6–9.8.

In the region pH 10.2–10.6, the two enzyme activities were approximately equal, whereas within the pH range 6.2–10.0 pyridoxine phosphate oxidase had a much greater activity than pyridoxamine phosphate oxidase.

Effect of protein concentration on enzyme activity. Reaction mixtures were made up within the final protein concentration range 0–7.3 mg./ml. and incubated at 37° for 1 hr., after which the reactions were terminated by the addition of 1.0 ml. of 30% (w/v) trichloroacetic acid.

With protein concentrations up to 1.6 mg./ml., the two enzyme activities increased linearly and

approximately equally. With further increases in protein concentration, there was a slight increase in pyridoxamine phosphate-oxidase activity, but a much greater increase in pyridoxine phosphate-oxidase activity. This suggests that pyridoxine phosphate oxidase is the more sensitive activity towards changes in enzyme concentration.

DISCUSSION

The key coenzymic role of pyridoxal phosphate in the activation of many enzymes associated with protein and amino acid metabolism has been extensively reviewed by a number of workers (Snell, 1958; Braunstein, 1960). Evidence was presented (Rabinowitz & Snell, 1947) for pyridoxamine phosphate as the predominant member of the vitamin B₆ group of compounds in the cell, and the consequent importance of the conversion of pyridoxamine phosphate into pyridoxal phosphate in cell metabolism was pointed out later (Beechey & Happold, 1957).

Pyridoxamine phosphate oxidase may now be thought to be responsible for the indirect activation by pyridoxamine phosphate of pig-heart glutamate-oxaloacetate transaminase. This idea emanates from the hypothesis of Evangelopoulos & Sizer (1963) that the only coenzyme for this transaminase is pyridoxal phosphate, and that in the mechanism of transamination a ternary complex is formed.

On the other hand, it seems that pyridoxine phosphate does not occur as a constituent of cells, although Snell (1963) has stated that it may occur naturally owing to interconversion with pyridoxal phosphate. Meister, Sober & Peterson (1954) found that pyridoxine phosphate markedly and competitively inhibited the activation of pig-heart glutamate-oxaloacetate apotransaminase by pyridoxal phosphate or pyridoxamine phosphate. Meister *et al.* (1954), however, postulated that pyridoxine phosphate could be converted into pyridoxal phosphate, and this has since been demonstrated.

As pyridoxine phosphate appears to be a potent inhibitor of pyridoxal phosphate-dependent enzymes, living organisms possess an enzymic mechanism whereby pyridoxine phosphate, instead of being allowed to accumulate in the cell, can be immediately converted into pyridoxal phosphate.

Some vitamin B₆ in plant material occurs in the form of pyridoxine. For example, an unidentified conjugate of pyridoxine occurs in cereal grains (Scudi, Buhs & Hood, 1942). If pyridoxine is available to bacterial cells, either by biosynthesis or from an exogenous source, or occurs in cereal diet of animals, it could be phosphorylated by pyridoxal kinase (ATP-pyridoxal 5-phosphotransferase, EC 2.7.1.35) to form pyridoxal phosphate, which would be immediately oxidized to pyridoxal phosphate.

Fig. 4. Effect of protein concentration on enzyme activity. Reaction mixtures were set up as follows: aqueous extract of *E. coli* (2.0 ml., giving final protein concentration 0–7.3 mg./ml.); 1.0 ml. of 50 mM- Na_2CO_3 – NaHCO_3 buffer, pH 10.1; 0.5 ml. of 16 mM- MgSO_4 ; 0.5 ml. of pyridoxamine phosphate or pyridoxine phosphate (0.4 μ mole). In the control mixtures, 0.5 ml. of water replaced the substrate. The mixtures were incubated, after which trichloroacetic acid was added. Enzyme activities: ●, pyridoxamine phosphate oxidase; ○, pyridoxine phosphate oxidase.

This may represent the reaction leading to pyridoxal phosphate production in micro-organisms, and in animals the conversion of a storage form of vitamin B₆ into the active coenzyme form.

Evidence is presented for the existence of the following metabolic pathway in *E. coli*:

(J. Hurwitz, unpublished work; see Hurwitz, 1953).

Pyridoxine phosphate oxidase may therefore play an important part in cell metabolism in assisting in the control of the concentration of pyridoxal phosphate, and hence of the rate of amino acid metabolism, within the cell.

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