Preliminary Studies for Fluorometric Determination of Pyridoxal and of Its 5'-Phosphate

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(Received October 24, 1969)

As fundamental studies for fluorometric determination of pyridoxal and of pyridoxal phosphate in biological materials, hydrolysis of pyridoxal phosphate, condition of the reaction and fluorometry, and interfering substances were examined.

Bonavita (1) found that the reaction of pyridoxal (PAL) and of pyridoxal 5'-phosphate (PAL-P) with cyanide in a slightly alkaline solution produced highly fluorescent compounds and this reaction has been applied to fluorometric determination of PAL and PAL-P as "cyanohydrin method". Recently it was found that PAL and PAL-P were oxidized with oxygen by a catalytic action of cyanide to 4-pyridoxolactone (PiAL) and 4-pyridoxic acid 5'-phosphate (PiA-P) respectively (2, 3).

The present studies deal with some fundamental works which were carried out in order to utilize the reaction to fluorometric determination of PAL and PAL-P in biological materials. The fluorescence intensity of the reaction product obtained from PAL was found to be much stronger than that from PAL-P and PAL could be more easily separated from interfering substances than PAL-P. For determination of PAL-P it is therefore preferable to determine it as PAL after hydrolysis. The hydrolysis of PAL-P, the reaction condition and interfering substances were studied.

EXPERIMENTAL

1. Materials

PAL, pyridoxine (PIN), pyridoxamine (PAM) and

their 5'-phosphate were purchased from E. Merck Aktiengesellschaft. 4-Pyridoxic acid (PiA) and PiAL were synthesized from PAL according to the method of Heyl (4). NAD was the preparation of Sigma Co.

Rat liver acid phosphatase (P-ase) was obtained by the method of Goodlad *et al.* (5). Potato acid P-ase was prepared according to the method of Katsunuma *et al.* (6) with some modifications. Potatoes (1 kg) were homogenized in 500 ml of 0.1 M acetate buffer (pH 3.4) and filtered with gauze into a beaker. Celite (40 g) was added to the filtrate, mixed well and filtered. The filtrate was cooled to 0°–4° and 34 g of (NH₄)₂SO₄ per 100 ml of the filtrate was added in portions under stirring. The mixture was allowed to stand for 1 hour at 0°–4° and centrifuged. Supernatant solution was discarded and the precipitate was dissolved in 0.05 M Tris-HCl buffer (pH 7.2). The solution was dialyzed twice against 5 liters of water for 12 hours. The dialysed solution was adjusted to pH 5 with 0.1 M acetate buffer (pH 3.4) and heated at 60° for 1 minute. The solution was cooled to 0°–4° and centrifuged. Supernatant solution was diluted with 0.2 M acetate buffer (pH 4.0) to obtain 0.4 unit/ml solution. The unit was defined as the amount of the enzyme which liberated 1 μ mole of inorganic phosphate (Pi) per minute from 20 μ moles (5×10^{-3} M) of β -glucerosphosphate at pH 4.0.

2. Apparatus

Excitation and emission spectra, and fluorescence intensity were measured in a Hitachi fluorescence spectrophotometer MPF-2.

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3. Reaction of PAL and of PAL-P with Cyanide

To 5 ml of PAL or of PAL-P solution (10^{-6} – 10^{-7} M) in 0.2 M phosphate buffer (pH 7.5) was added 0.1 ml of 0.1 M KCN solution. It was heated at 50°.

4. Hydrolysis of PAL-P

Acid hydrolysis — PAL-P (5×10^{-4} M, 5 μ moles) was heated in 2 N H₂SO₄ or in 0.2 N H₂SO₄ at 100° respectively, and liberated iP was assayed by the method of Fiske-SubbaRow (7). PAL was assayed by fluorometry after the cyanide reaction.

Enzymatic hydrolysis — A mixture of 1 ml of PAL-P (10^{-6} M), 2.5 ml of 0.2 M acetate buffer (pH 4.0) and 0.5 ml of acid P-ase was incubated at 37° for 1 hour. To the incubation mixture was added 1 ml of 10 % trichloroacetic acid (TCA) and the mixture was centrifuged. The supernatant solution was assayed for liberated iP and PAL.

RESULTS AND DISCUSSION

1. Fluorescence Intensities of the Reaction Product of PAL and of PAL-P with Cyanide

The reaction mixture of 10^{-7} M PAL and KCN was adjusted to pH 10 and the fluorescence intensity was measured at 356 m μ of excitation and 432 m μ of emission. In the case of PAL-P, 10^{-7} M solution was treated with KCN and adjusted to pH 3, and fluorescence intensity at 418 m μ , excited at 318 m μ , was measured. The fluorescence intensity of the former was about 5 times as strong as that of the latter. Therefore, it is favorable to measure PAL-P as PAL following hydrolysis in order to obtain higher sensitivity. Furthermore, the hydrolysis of PAL-P was advantageous for separation from interfering substances as described after.

2. Hydrolysis of PAL-P

As shown in Table 1, PAL-P failed to be hydrolysed completely either in 0.2 or in 2 N H₂SO₄. Furthermore, assayed values of PAL were lower than that of iP, suggesting that PAL might be decomposed during the hydrolysis.

On the other hand, almost quantitative hydrolysis was achieved by the enzymatic hydrolysis. As shown in Table 2, the hydrolysis rates by rat liver acid P-ase were about 90 % at most and the enzyme contained PAL

TABLE 1
Acid hydrolysis of PAL-P

	Time	Pi liberated	PAL liberated
	min.	μ moles	μ moles
PAL-P 5 μ moles 0.2 N H ₂ SO ₄ 100°	15	1.36	1.24
	30	2.76	2.66
	45	3.30	3.06
	60	3.58	3.41
PAL-P 5 μ moles 2 N H ₂ SO ₄ 100°	15	1.55	1.42
	30	2.74	2.44
	45	3.64	3.25
	60	4.32	3.56

TABLE 2
Enzymatic hydrolysis of PAL-P

	Amount of enzyme	Hydrolysis rate
	unit	per cent
PAL-P 1 m μ mole Rat liver acid phosphatase 37°, 60 min	0.07	78
	0.14	91
	0.28	90
	0.56	82
PAL-P 1 m μ mole Potato acid phosphatase 37°, 60 min	0.05	81
	0.10	95
	0.15	99
	0.20	98
	0.25	98
	0.40	94

FIG. 1 *Effect of pH and reaction time on the reaction*

or PAL-P in comparatively high concentration, while almost quantitative hydrolysis was effected by 0.15–0.2 unit of potato acid P-ase.

3. pH and Time Course of the Reaction

The reaction of 10^{-7} M PAL with KCN was carried out at each pH level as specified in Fig. 1. An aliquot of each reaction mixture

was taken at intervals of 30 minutes, adjusted to pH 10 with 0.4 M Na_2CO_3 and fluorescence intensities were measured. The maximum intensity was obtained by the reaction at pH 7.5 for 1.5-2 hours (Fig. 1).

4. Effect of pH on the Fluorescence Intensity

The fluorescence intensity of the reaction product of PAL, *i.e.*, PiAL, reached the maximum and was stable at pH 9-10, decreasing markedly as the pH approached an acidic region. PiAL was unstable above pH 10. Excitation and emission maxima of PiAL at pH 10 were 356 and 432 m μ respectively.

5. Interfering Substances

Metallic ions, such as Cu^{2+} , Zn^{2+} , Mg^{2+} and Fe^{3+} in 10^{-6} M, and 10^{-4} M EDTA showed no effect on the reaction. Glucose (10^{-3} M) also had little influence on the reaction. Borate inhibited the reaction markedly at the concentration of 0.1 M. Therefore, borate buffer should not be used for the reaction or pretreatment of the determination. PIN and PAM had little influence on the fluorometry even if they co-exist 10 times as much as PAL. Their fluorescence intensity was much lower than that of PiAL, and their excitation and emission maxima were 330 and 370-380 m μ , respectively, at pH 10. Since PAL itself showed only a weak fluorescence at 310 m μ of excitation and 370 m μ of emission at pH 10, the PAL in a blank test carried out in the absence of cyanide did not interfere with the fluorometry. PiA, a metabolite of vitamin B₆ group, interfered with the fluorometry because of its high fluorescence. When an equivalent amount of PiA co-existed with PAL, the fluorescence intensity was increased by about 5%. The reaction product of PAL-P, *i.e.*, PiA-P, showed a fluorescence spectrum similar to that of PiA, hence PAL-P is also an interfering substance for the determination of PAL. NAD was slightly fluorescent under the reaction condition. Excitation and emission maxima of KCN-treated NAD were 340

and 430 m μ , respectively, the values close to those of PiAL. When 100 times as much NAD as PAL co-existed, the fluorescence intensity was increased by about 15%.

From these findings the preliminary separation of PAL is indispensable for the fluorometric determination of PAL and PAL-P in biological materials.

6. Deproteinization

Deproteinization of human sera, plasma and rat liver homogenate was examined by using TCA, perchloric acid, metaphosphoric acid and heating. As reported by Yamada *et al.* (8), heating at 50° in the presence of TCA (final concentration, 4-5%) gave the best result.

7. Removal of the Interfering Substances

The main interfering substances are PiA, NAD and PAL-P for PAL determination. TCA used for the deproteinization of samples should be removed before determination. All these compounds are acidic and their removal was effected by anion exchange resins such as Amberlite IR 45 and Dowex 1 $\times$ 8. Detailed studies on separation of PAL shall appear in a subsequent paper. In PAL-P determination, preliminary hydrolysis makes it easier to separate from the interfering substances.

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