A REVISED METHOD FOR THE TRITIUM LABELING OF PYRIDOXAL-5'-PHOSPHATE.

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1. Introduction

In the course of studies on the binding of pyridoxal-5'-phosphate (PLP) to *E. Coli* K 12 tryptophanase, the need for radioactive PLP became apparent. Since Stock et al. [1] had already reported a very elegant and simple method for labeling this molecule with tritium, we tried to reproduce their procedure and, unexpectedly, were unable to recover radioactive (PLP).

This method involves three steps: reduction of PLP with ^{3}H-labeled sodium borohydride, oxidation of the pyridoxine-5'-phosphate (PLP) thus obtained with manganese dioxide, and separation of the products by chromatography on an ion-exchange resin. The work reported here deals with a further study of this separation step which leads to the unexpected finding that ^{3}H-labeled PLP can be readly separated from the unlabeled molecule by ion-exhange chromatography.

2. Materials and methods

2.1. *Reduction of PLP and oxidation of PNP*

Reduction of PLP (Sigma) with radioactive sodium borohydride (C.E.A.-France; 26.6 Ci/mmole) and the subsequent oxidation were performed as described by Stock et al. [1]. The solution obtained was freed from perchloric acid by precipitation of the potassium perchlorate obtained upon drop wise addition of 1 M potassium bicarbonate until the yellow color of PLP appeared. After centrifugation, the supernatant was diluted with 10 ml water, degassed, and layered on the ion-exchange column.

2.2. *Ion-exchange chromatography*

The resin used was Dowex AG 1X2 (200–400 mesh) purchased from Bio-Rad. The resin was thoroughly washed with 4.4 M acetic, then water, and stored as a suspension in water. A 1 X 30 cm packed column was washed as above just before chromatography. It is very important to carefully degas both the resin and solvents before use in order to avoid bubble formation in the column. After layering the sample, the column was washed with twice its volume of water, then eluted with a 140 ml linear gradient of acetic acid (0 to 4.4 M). The chromatography was performed at room temperature and in the dark since PLP is very photosensitive. The flow rate was adjusted so that elution lasted for 6 hr. Fractions of 3.7 ml were collected; their radioactivity was measured on 5$\mu\ell$ aliquots counted in Bray's mixture in an Intertechnique SL 30 scintillation counter. Their optical densities were measured in a Zeiss PMQ II spectrophotometer after a 20-fold dilution in 0.1 M potassium phosphate pH 7.8.

Fractions 21–24 (see fig. 1) were pooled and unlabeled PLP was added in order to minimize radiolysis of the labeled compound. The solution was lyophilized and the resulting material dissolved in 10 ml water. This solution, refered to as the stock solution, was stored frozen at -25° C in a brown glass vial. It contained a total of 1.6×10^{9} cpm and 17 μmoles of PLP.

2.3. *Thin-layer chromatography*

The thin-layer chromatography was performed according to Ahrens and Korytnyck [2]. The TLC

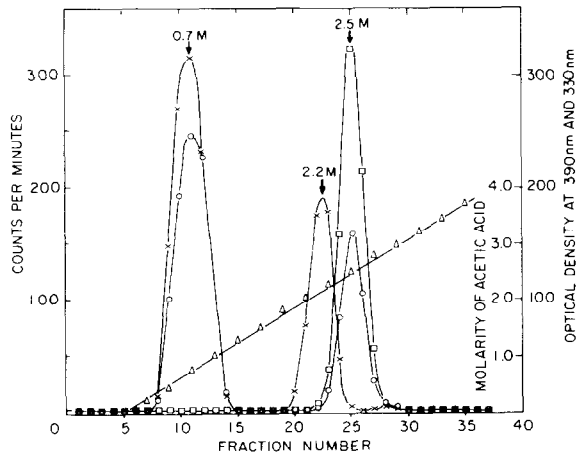

Fig. 1. Separation of the oxidation products on AG 1X2. The conditions for chromatography and analysis were as described under Materials and methods: (○-○-○) optical density at 330 nm; (□-□-□) optical density at 390 nm; (X-X-X) counts per minute per 5 μℓ; (Δ-Δ-Δ) acetic acid concentration determined by refractometry.

plates (10 X 20 cm; 0.25 mm thick silica gel F 254) were purchased from Merck. The solvent was methylethylketone–ethanol–concentrated ammonia–water (3/1/1/1 by vol.), and ascending chromatography was performed in a saturned chamber. PLP was detected with Gibbs' reagent according to Stahl [3]. Autoradiography was performed according to Rogers [4]. After spraying with a solution of 7% 2,5-diphenyloxazole (PPO) in diethylether, the plate was dried and left in contact for 24 hr at −70°C with a Kodak X-ray film.

2.4. *Apo-tryptophanase*

E. Coli K 12 apo-tryptophanase was prepared according to London and Goldberg [5]. The S-alkylcysteine lyase activity of tryptophanase was assayed as described earlier [6].

3. Results

3.1. *Chromatography of the oxydized mixture*

According to Stock et al. [1] the elution profile should consist in a first radioactive peak of PNP absorbing light at 330 nm but not at 390 nm) followed by a second radioactive peak of PLP (absorbing light at 330 and 390 nm [7]). Fig. 1 shows the profile obtained when O.D. and radioactivity were monitored; it can be seen that, while the first peak appears as expected, the second radioactive peak exhibits no detectable absorption between 250 and 450 nm. The last peak of absorbing material, which has an absorbing material, which has an absorption spectrum identical with that of PLP, contains practicaly no radioactivity.

The question was then to identify the material contained in the second radioactivity peak and to understand why radioactive PLP was not obtained. A simple, eventhough unlikely, explanation was that a PLP molecule carrying a tritium atom on its carbonyl group might be eluted from the column earlier than an unlabeled molecule; the second radioactive peak

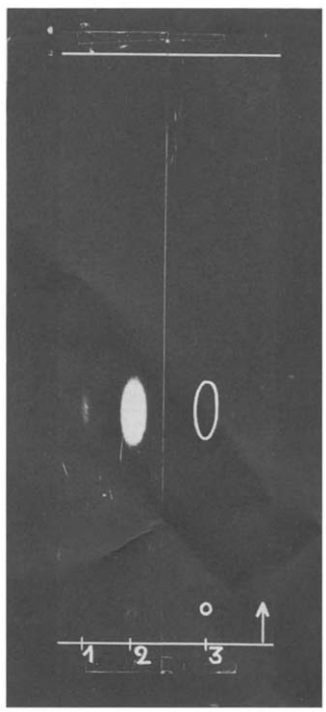

Fig. 2. Thin-layer chromatography of the radioactive material. One μℓ and 10 μℓ of the spock solution were deposited in 1 and 2 respectively. One μℓ of authentic PLP (2×10^{-2} M) was deposited in 3. After migration the radioactivity contained in samples 1 and 2 was revealed by autoradiography. The migration of sample 3 was analysed with Gibbs' reagent and the resulting spots were drawn on the plate.

would then contain radioactive PLP. Three types of experiments have been performed to test this hypothesis.

3.2. *Thin-layer chromatography*

Fig. 2 shows the result of a chromatography performed as described in Materials and methods. It can be seen that more than 90% of the radioactive material migrates, like PLP, with an R_F of 0.38. A slight contaminant, accounting for less than 10% of the material, migrates with an R_F of 0.1 and is detectable both by autoradiography and with Gibbs' reagent.

3.3. *Reduction of the radioactive material*

If this radioactive material were PLP, it should be easy to reduce it again with cold sodium borohydride and characterize the radioactive PNP thus obtained.

This experiment was performed as described in the legend to fig. 3. It can be seen that 93% of the radioactivity thus obtained was eluted from the column at the same acetic acid concentration as authentic PNP, while 7% of the material was not retained.

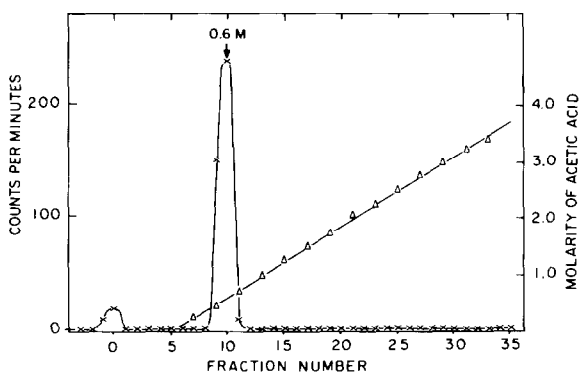

Fig. 3. Chromatography of the reduced radioactive material. 0.3 ml of the stock solution of radioactive material were diluted with 0.2 ml of 0.1 M potassium phosphate, pH 7.8, and reduced with no radioactive sodium borohydride until complete bleaching. Elution and analysis of the fractions were performed as described under Materials and methods. Symbols are as indicated in fig. 1.

3.4. *Interaction between the radioactive material and apo-tryptophanase*

The tryptophanase from *E. Coli* binds one molecule of PLP per subunit of 55 000 mol. wt. and the association between the apo-enzyme and the cofactor can be rendered irreversible by reduction with sodium borohydride [9]. The interaction between apo-tryptophanase and the radioactive material has therefore been investigated in order to further confirm that this material is PLP. For this purpose 0.15 ml of a 3.2×10^{-5} M apo-tryptophanase solution, previously dialysed against twice 200 ml of 0.1 M potassium phosphate, pH 7.8, and 10^{-2} M β-mercaptoethanol, were incubated 6 μl of the stock solution of radioactive material for 30 min at 37°C (final concentration of PLP: 7×10^{-5} M). 50 μl of this solution was then treated with 2 μl of solution borohydride (0.5 mg/ml in 0.2% sodium hydroxide); 50 μl were left untreated as a control. After 5 min at room temperature, the two samples were precipitated with 150 μl of 5% trichloroacetic acid and centrifuged. Whereas in the control all the radioactivity was recovered in the supernatant, the supernatant of the reduced sample contained only 8% of the initial radioactivity. When both pellets were washed twice 5% trichloroacetic acid then redissolved in 0.2 ml of 0.2% sodium hydroxide, 90% of the initial radioactivity was recovered in the reduced sample, while no radioactivity was detected in the non reduced control.

It therefore can be concluded that over 90% of the tritiated molecules are able to bind to apo-tryptophanase and yield an irreversible covalent bond with the enzyme after reduction with sodium borohydride.

4. Discussion

The experiments described above demonstrate that the reduction–oxidation procedure of Stock et al. [1] yields tritiated molecules which share with PLP the following properties:

1) Same migration by thin layer chromatography on silica plates.

2) Ability to be reduced by sodium borohydride to yield PNP as evidenced by chromatography on Dowex 1×2.

3) Ability to bind to apo-tryptophanase and form

an irreversible covalent bond by reduction with sodium borohydride. Since the dissociation constant of the apo-tryptophanase–PLP complex must be smaller than, or equal to, the K_M of this protein for PLP (2×10^{-6} M; reference [8]) incubation of 7×10^{-5} M of PLP with 3.2×10^{-5} M apo-tryptophanase (or 12.8×10^{-5} M binding sites) should result in the binding of more than 99% of the PLP molecules to the apo-enzyme. This binding can then be rendered irreversible by reduction with sodium borohydride. This behaviour has indeed been observed for 92% of the tritiated molecules obtained.

From these properties, it can be concluded with little ambiguity that more than 90% of the tritium atoms present in the second peak of radioactivity (see fig. 1) are incorporated in PLP molecules. It thus appears that the replacement of a hydrogen by a tritium atom on the carbonyl group of PLP modifies the migration of this molecule on an ion-exchange column. The observation that labeled and unlabeled PLP are separated on an ion-exchange column bears two essential advantages: the method of Stock et al. [1] can now be reproducibly applied with success; very hot ^{3}H-labelled PLP can now be obtained, regardless of the specific radioactivity of the sodium borohydride used.

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