PREPARATION OF DIHYDROPTERIDINEDIPHOSPHATE, AN INTERMEDIATE IN DIHYDROFOLATE SYNTHESIS

T. Shiota, M. N. Disraely and M. P. McCann

National Institute of Dental Research, National Institutes of Health, Public Health Service, U. S. Department of Health, Education and Welfare, Bethesda, Maryland.

Received February 20, 1962

The enzymatic synthesis of dihydrofolate involves the coupling of 2-amino-4-hydroxy-6-hydroxymethyldihydropteridine with either p-aminobenzoate (PABA) or p-aminobenzoylglutamate (PABG) (Shiota and Disraely, 1961; Brown et al., 1961). The demonstration of an adenosinetriphosphate (ATP) requirement for this reaction indicated the possibility that a phosphorylated intermediate may participate in the coupling reaction. The possible involvement of such an intermediate was further indicated by the observation that a mixture of phosphorylated pteridines was active in coupling with PABG or PABA in the absence of ATP. (Shiota and Disraely, 1960). Jaenicke and Chan, 1960, postulated that the active intermediate in the coupling reaction was a dihydropteridine derivative with pyrophosphate in the sixth position. This communication describes the preparation of a pteridinediphosphate which after reduction to the dihydro-derivative is active in the coupling reaction.

Dialyzed extracts of Lactobacillus plantarum 17-5 were prepared as described previously (Shiota, 1959) except that a French Pressure Cell (Aminco) was used to disrupt the cells. The assays performed were essentially those mentioned in a previous communication (Shiota and Disraely, 1961). Total nitrogen determinations were performed after a Kjeldhal digestion, according to the method described by Conway and Byrne, 1933. Total phosphorus (Chen, et al., 1956) after

digestion (Umbreit et al., 1949) and free phosphorus (Lowry, 1957) determinations were also carried out.

Fifty milligrams of 2-amino-4-hydroxy-6-hydroxymethylpteridine (Waller et al., 1950) and 4 g of pyrophosphoric acid were added to a 25 ml Erlenmeyer flask. The flask was stoppered and its contents were protected from light. The reaction mixture was stirred with a magnetic stirrer for 17 hr at 60°C. The flask was then chilled to 0°C and ice cold distilled water was added in small portions until 30 ml were added. Three milliliters of a 15% Norite A suspension (acid washed) were added and centrifuged in the cold. The charcoal was washed four times with 40 ml portions of cold water, and the pteridines eluted by centrifugation with 10 ml portions of a solution composed of equal volumes of 3N NH_4OH and ethyl alcohol. The ammonical Norite eluates were pooled, centrifuged again to remove residual charcoal and concentrated to about 2 ml in an evaporator (Rotovap) with the water bath temperature about 45°C. The concentrate was added quantitatively to the top of a 13 x 300 mm column containing Dowex 50 H^+ (100-200 mesh) and water pumped (Sigmamator) through (3 ml/min) until the effluent emerging from the column was free of fluorescent material (300-350 ml). The effluent was then added to the top of 13 x 340 mm DEAE column (jacketed for cooling) by a pump and was developed by gradient elution with the mixing chamber containing 400 ml of water and the reservoir containing 400 ml 0.3 M KCl in 01. M trishydroxymethylaminomethane, pH 7.2. Fractions were collected at a rate of 10 ml per tube per 4 min. The contents of the tubes were analyzed for absorption at 270 mμ (Optica).

The graphic illustration of the separation of the phosphorylated pteridines from a DEAE column is shown in Fig. 1. Four peaks were observed at 270 mμ. The contents of tubes 20-27, 29-39, 42-50, and 56-59 were combined and designated as fractions F1, F2, F3, and F4, respectively. The absorption spectrum of each fraction was identical

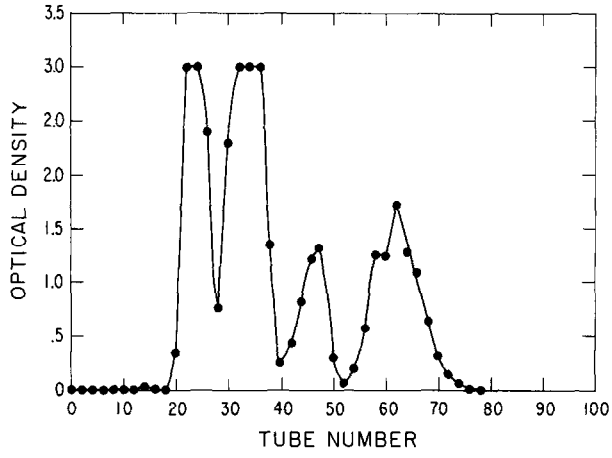

Figure 1

with that of 2-amino-4-hydroxy-6-hydroxymethylpteridine (Walker, et al., 1950).

The phosphorylated pteridines in fractions F1-F4, were precipitated as their lead salts. To each fraction was added 0.2 ml of 25% lead acetate and after 1 hr. in the cold the precipitates were collected by centrifugation. Each precipitate was then washed four times with 50 ml cold water, twice with ethanol and once with ether. The yields of F1 and F2 were 16.1 mg. and 14.8 mg. respectively. The lead was released by dissolving the lead salts of each fraction in cold 0.1N HCl. The lead was precipitated by flushing each solution with H_2S and the lead sulfide was removed by centrifugation.

Each of these fractions were tested for coupling with PABG or PABA. The results of such an experiment are shown in Table 1. It can be seen that only F2 was active. Results from bioautograms indicated the formation of dihydrofolate from PABG and pteroate from PABA.

Each of the combined fractions were analyzed for total nitrogen, total and free phosphorous. These results are shown in Table II. The ratios of P/N indicated that F1 was a pteridinemonophosphate; F2, pteridinediphosphate; and F3 and F4 possibly as pteridinetri

TABLE I

ENZYMATIC COUPLING ACTIVITIES OF DEAE FRACTIONS

Reaction mixture	*S. faecalis* activity, mμg/ml	
	ATP	W/O ATP
F1 + PABG	0	0
F2 + "	850	900
F3 + "	0	0
F4 + "	0	0
F1 + PABA	0	0
F2 + "	700	750
F3 + "	0	0
F4 + "	0	0

The complete system contained in 0.5 ml; 10 μmoles phosphate buffer, pH 8., 1 μmole $MgCl_2$, 1 mg potassium ascorbate, .05 ml dialyzed cell extract (450 μg. protein), 1 μmole PABG or PABA, 1 μmole ATP and each fraction diluted to contain 40 mμmoles (E = 23,500 at 253 mμ in 0.1 N NaOH) of phosphorylated pteridine after being reduced to dihydro-level. Dihydropteridine was prepared by adding .05 ml of 0.5 M $NaBH_4$ in .005 N NaOH (freshly prepared) .01 ml of 1N HCl and allowed to stand for 30 min. with occasional shaking. Residual $NaBH_4$ was decomposed by adding an additional .01 ml 1N HCl with shaking. The tubes containing the reaction mixture were gassed with argon, stoppered and incubated at 37°. Samples were assayed at zero time and at 1 hr.

and pteridinetetrapolyphosphates. The P/N values indicated further that the molar extinction coefficients of these phosphorylated pteridines are similar to that reported for the nonphosphorylated derivative (Waller *et al.*, 1950).

These results support the proposal by Jaenicke and Chan that the intermediate in the coupling reaction is 2-amino-4-hydroxydihydro-6-pteridinylmethylpyrophosphate. The failure of the pteridine-monophosphate derivative (F1) to participate in the coupling reaction, even in the presence of ATP, suggests the possible existence of a pteridine pyrophosphokinase.

TABLE II

NITROGEN AND PHOSPHORUS ANALYSES OF DEAE FRACTIONS

Fraction	Nitrogen	Phosphorous		N/P
		Total	Free	
	μmole/ml	μmole/ml		
F1	5.10	1.19	.05	4.5
F2	5.10	2.22	.06	2.4

REFERENCES

Brown, G. M. Weisman, R. A., and Molnar, D. A., J. Biol. Chem., 236, 2534 (1961).

Chen, P. S., Jr., Toribara, T. Y., and Warner, H., Anal. Chem. 28, 1758 (1956).

Conway, E. J., and Byrne, A., Biochem. J., 27, 419 (1933).

Jaenicke, L., and Chan, PH. C., Angew, Chem., 72, 752 (1960).

Lowry, O. H., Methods in Enzymol. Vol. IV, Academic Press, Inc., New York 1957, p. 371.

Shiota, T., Arch. Biochem. Biophys., 80, 155 (1959).

Shiota, T. and Disraely, M. N., Bacteriol. Proc. (Soc. Am Bacteriologists), 60, 174 (1960).

Shiota, T., and Disraely, M. N., Biochem. Biophys. Acta, 52, 467 (1961).

Waller, C. W., Goldman, A. A., Angier, R. B., Boothe, J. H., Hutchings, B. L., Mowat, J. H., and Semb. J., J. Am. Chem. Soc., 72, 4630 (1950).

Umbreit, W. W., Burris, R. H., and Stauffer, J. E., Manometric Techniques and Tissue Metabolism, Burgess Publishing Co., Minn., Minn., 1949, p. 190.