THE ENZYMATIC SYNTHESIS OF CRITHIDIA ACTIVE SUBSTANCE(S) AND A PHOSPHORYLATED D-ERYTHRONEOPTERIN FROM GTP OR GDP BY LIVER PREPARATIONS FROM SYRIAN GOLDEN HAMSTERS

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SUMMARY: Dialyzed liver homogenates from the Syrian golden hamster were found to be enzymatically active for the production of Crithidia active substance(s) from GTP or GDP. This activity was stimulated by a mixture of pyridine nucleotides. An Ultrogel AcA-34 fraction of a liver homogenate preparation produced a fluorescent substance from GTP which was not biopterin. This fluorescent substance was identified as a phosphorylated derivative of D-erythroneopterin.

INTRODUCTION: One of the reports by Kaufman (1), who extensively studied the phenylalanine hydroxylase system, dealt with the isolation of dihydrobiopterin from rat liver and the establishment of its structure (2). Although the identification of the natural cofactor for all the pterin-requiring hydroxylation reactions (3-11), as yet, has not been made, the variety of the compounds involved emphasize the relative importance of pterins.

Early studies on the biosynthesis of biopterin suggest that glucose, guanine or one of its derivatives was a precursor of biopterin (12-15). A more recent report (16), from studies with the Chinese hamster ovary cell cultures, indicated that guanosine or one of its phosphorylated derivatives is the precursor of biopterin and that the side chain of biopterin is of ribose origin. The results from another report (17), with neuroblastoma cell cultures, also support this conclusion. In the work reported here, the results from a study of the enzymatic synthesis of pterins from GTP or GDP by homogenate preparations of various organs from Syrian golden hamsters are presented.

MATERIALS AND METHODS: GTP and other purine derivatives, pyridine nucleotides and alkaline phosphatase were purchased from Sigma Chemical Company. ECTEOLA-Sephadex (18,19), biopterin (20), and D-erythroneopterin and L-threoneopterin (21) were prepared by published procedures.

Various organs from the Syrian golden hamster were homogenized in 4 volumes of 0.01 M Tris-HCl-0.04 M KCl buffer, pH 8.0 using the VirTis-45 homogenizer. The homogenates were centrifuged at 17,300 x g for 1 hour and dialyzed for 24 hours against 0.005 M Tris-HCl buffer, pH 8.0. These dialyzed supernatant fractions were used in some of the experiments. In other experiments, an Ultrogel AcA-34 preparation was used. To obtain this preparation, the supernatant fluid from a liver homogenate was concentrated against solid sucrose, dialyzed against 0.005 M Tris-HCl buffer, pH 8.0, and applied to an Ultrogel AcA-34 column (2.5 x 160 cm) (Fig. 1), and the column washed with the same buffer. The fractions which were collected were measured at 280 nm and assayed by the standard assay system for the production of fluorescent product(s). The peak fractions which produced fluorescence were pooled.

The standard assay system contained in a final volume of 0.15 ml:GTP or other purine derivatives, 0.3 µmole; Tris-HCl, pH 8.0, 7.5 µmoles and an enzyme preparation. The mixture was incubated at 37°C in the dark for 3 hours, after which the reaction was terminated by the addition of 2 ml of 0.1 N HCl. For purpose of converting any reduced pterins to their more stable oxidized form, the following solutions were added: 0.01 ml of 1% starch solution and 0.01 ml iodine solution (1 g iodine and 2 g KI in 100 ml H_2O). The mixture was allowed to stand for 15 min at room temperature and the excess iodine was reduced by the addition of ascorbic acid solution. The mixture was adjusted to pH 8 with 0.2 ml of 2 M Tris base and assayed for the production of Crithidia active (22) and fluorescent substance(s).

Thin-layer chromatography was carried out in the dark at room temperature by the ascending procedure. Eastman Chromagram sheets (13255 cellulose) were developed in the following solvent systems: Solvent A, 3% NH_4Cl; Solvent B, 1-propanol-ethylacetate-water (7:1:2); Solvent G, ethanol-ammonium borate (pH 8.2, 5% boric acid)-3% NH_4Cl (2:1:1); Solvent D, 1-propanol-1% NH_4OH (2:1); Solvent E, 1-butanol-acetic acid-water (4:1:2).

Fluorescence determinations were carried out on a Turner model 110 fluorometer, using a primary filter with peak transmission of 360 nm and a secondary filter allowing passage of light with wavelength greater than 415 nm. Protein was determined by the method of Lowry et al. (23).

RESULTS AND DISCUSSION: The ability of each dialyzed supernatant fluid from homogenate preparations of liver, kidney, brain, lung and uterus and ovary to produce Crithidia active substance(s) in the presence of GTP was determined These results are shown in Table I. Using the standard assay it was found that the preparation from liver was most active, the kidney slightly active and the other preparations inactive in the enzymatic synthesis of Crithidia active substance(s). In a separate experiment, a mixture of pyridine nucleotides were found to be stimulatory for the formation of Crithidia active substance(s). Accordingly, a mixture of pyridine nucleotides was added to the standard assay system. The addition of this mixture stimulated the production of Crithidia active substance(s) by all the organ preparations except the uterus-ovary preparation. These results indicate that with the exception of the uterus-ovary

TABLE I

Effect of GTP on the Synthesis of Crithidia Active Substance(s) by a Dialyzed Supernatant Fraction of Various Organs from the Syrian Golden Hamster

Organ	Condition	Biopterin equivalent ng
Liver	Complete	16.8
	Minus GTP	1.9
	Plus NAD(P)H and NAD(P)	28.2
Kidney	Complete	0.7
	Minus GTP	0.3
	Plus NAD(P)H and NAD(P)	0.9
Brain	Complete	0.1
	Minus GTP	0.1
	Plus NAD(P)H and NAD(P)	1.7
Lung	Complete	0.1
	Minus GTP	0.1
	Plus NAD(P)H and NAD(P)	0.9
Uterus and Ovary	Complete	0.1
	Minus GTP	0.1
	Plus NAD(P)H and NAD(P)	0.1

To the complete standard assay system, a mixture of 0.15 μmole each of NADH, NADPH, NAD and NADP was used as indicated. The reaction mixture contained dialyzed supernatant fluids from liver, kidney, brain, lung and uterus and ovary homogenates equivalent to 2.3, 1.7, 1.1, 1.8 and 1.0 mg protein, respectively.

preparation, the organ homogenates tested possess the enzyme system to utilize GTP for the enzymatic synthesis of biopterin-like substance(s). It is believed that an reaction, yet unknown, in the system is stimulated by the mixture of the pyridine nuceotides.

In Table II, the utilization of various purine derivatives in the formation of Crithidia active substance(s) by dialyzed supernatant fluid from a liver homogenate is shown. Of the compounds tested, either GTP or GDP was most active These results showing the enzymatic utilization of GTP or GDP as well as those earlier results, from studies using intact animals or growing cultures of mammalian cells (12-17) for biopterin biosynthesis, suggest that GTP is the precursor. This conclusion is also supported by results obtained in bacterial

TABLE II

Effect of Certain Purine Compounds on the Enzymatic Synthesis of Crithidia active substance(s) by Syrian golden Hamster Liver Homogenate Preparation

Compound	Biopterin Equivalent ng
GTP	37.5
GDP	25
GMP	2.5
Guanosine	2.5
ATP	3.6
None	2.5

The standard assay system contained a dialyzed supernatant fluid of liver homogenates equivalent to 2.1 mg protein.

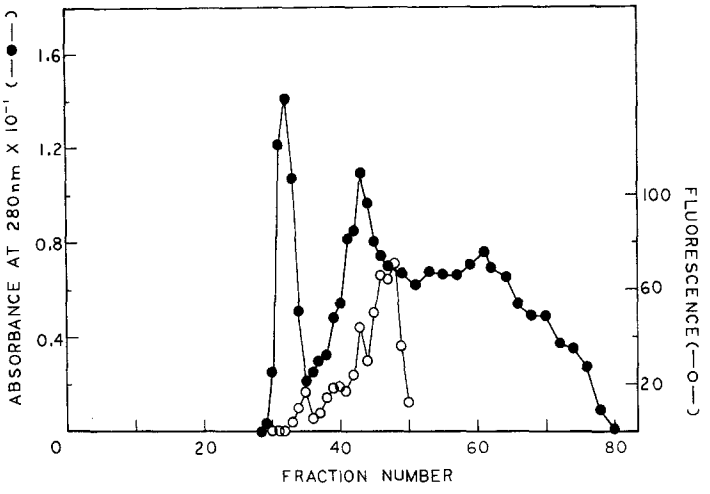

Fig. 1. Ultrogel AcA-34 column chromatography of the supernatant fluid from a liver homogenate.

A 10 ml portion (1.1 g of protein) of the concentrated supernatant fluid from a liver homogenate was applied to an Ultrogel AcA-34 column (2.5 x 160 cm) The fractions collected were 8 ml at a rate of 0.4 ml per min.

systems which specifically utilize GTP for pterin synthesis (24,25).

The subsequent experiments were designed for the purposes of determining the identity of the fluorescent compound produced from GTP by an Ultrogel AcA-34

treated liver homogenate preparation (Fig. 1). The pooled Ultrogel AcA-34 preparation was found to be free of phosphatase activity and furthermore does not produce biopterin from GTP. This preparation was incubated with GTP and the pterin produced was identified. The details of the procedure used is presented in Table III. The results obtained show that the product prior to phosphatase treatment, has the same mobility in five solvent systems as the iodine treated product of D-erythrodihydroneopterin triphosphate. On the other hand, the dephosphorylated substance (after alkaline phosphatase treatment) migrated identically to erythroneopterin.

In order to obtain information concerning the amount of the phosphorylated neopterin produced as well as the configuration of the side-chain, a portion

TABLE III

Thin-layer Chromatography of the Reaction Product

Compound	Solvents* Rf Values A	B	G	D	E
Biopterin	0.52	0.22	0.41	0.39	0.44
Erythro neopterin	0.50	0.09	0.28	0.25	0.28
Threo neopterin	0.46	0.08	0.20	0.21	0.24
Product from GTP with *L. p.* preparation**	0.85	0.0	0.12	0.03	0.18
Product from GTP with liver preparation					
Before alkaline phosphatase	0.85	0.0	0.12	0.03	0.17
After alkaline phosphatase	0.50	0.09	0.28	0.25	0.28

*Solvent systems are described in Materials and Methods.
**L. *plantarum* preparation

The incubation mixture contained: 24 µmoles of GTP, 0.6 mmole of Tris-HCl, pH 8.0; 12 µmoles EDTA and 8 ml of Ultrogel AcA-34 preparation (24 mg of protein) in a total volume of 12 ml and the mixture incubated at 37°C in the dark for 3 hours. The mixture was acidified to pH 1.0 and treated by the iodine procedure. After the treatment, the reaction mixture was concentrated and applied to a Sephadex G-25 column (1.2 x 33 cm) to remove protein and salts. The fluorescent peak fractions were pooled and a portion of the pooled fraction was treated with alkaline phosphatase. A phosphatase and a non-phosphatase treated sample were subjected to thin-layer chromatography using 5 solvent systems.

D-erythrodihydroneopterin triphosphate was prepared enzymatically by a preparation from Lactobacillus plantarum (26). This product was also treated by the iodine procedure and applied to TLC**.

of the fluorescent fraction obtained from the Sephadex G-25 column (Table III) was further purified. The purification procedure is described in Fig. 2. From this procedure, 8.6 nmoles of a phosphorylated neopterin was recovered which represents about 0.036% of the GTP used in the experiment. Furthermore, the product obtained after purifying the alkaline phosphatase-treated phosphorylated

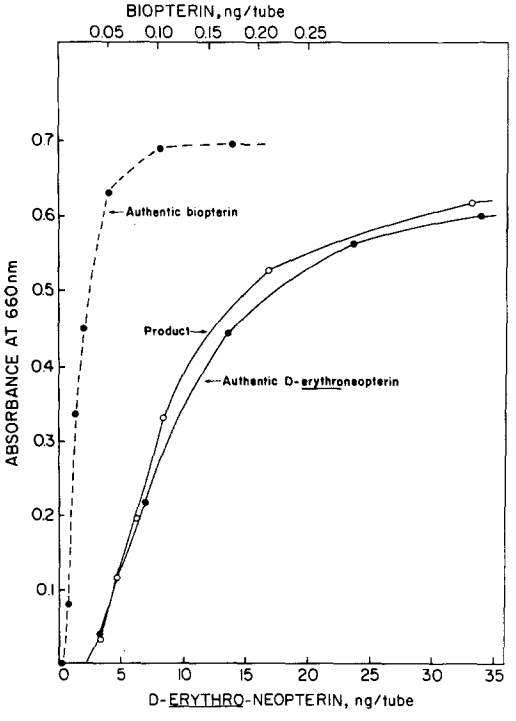

Fig. 2. C. fasciculata growth promoting activity of a dephosphorylated product from GTP.

The remaining fluorescent fraction obtained from the Sephadex G-25 column step (Table III) was further purified in order to determine the amount of the phosphorylated neopterin produced and to obtain information on the configuration of the side-chain by C. fasciculata assay. This fraction was concentrated, streaked on a cellulose TLC sheet and the sheet developed in solvent A. A single blue fluorescent band was removed, eluted with water and applied to an ECTEOLA-Sephadex (OH^-) column (1 x 2 cm). The column was washed with water and eluted with 1.0 M ammonium bicarbonate and the fluorescent fraction obtained was lyophilized. The amount of fluorescent material was determined by fluorometry using authentic neopterin as the standard. The isolated product was treated with alkaline phosphatase and purified by the Dowex-50-ECTEOLA-Sephadex column chromatography procedure (19) and assayed for C. fasciculata activity. The activity of authentic D-erythroneopterin and biopterin was also determined.

neopterin, exhibited similar growth promoting activity for C. fasciculata as that of authentic D-erythroneopterin. The results indicate that the side-chain of the phosphorylated neopterin has the D-erythro configuration.

The identification of the production of a phosphorylated derivative of D-erythroneopterin from GTP is incomplete. Additional work is required in order to establish the number of phosphate groups and the state of reduction of the pterin ring of the GTP product. However, from the results obtained, it is tempting to speculate that the immediate pterin product from GTP is D-erythro-dihydroneopterin triphosphate. If the additional data support such a conclusion, then, the initial set of reactions in mammalian systems which utilizes GTP for biopterin synthesis may be similar to that in bacterial systems for dihydrofolate synthesis (24,25).

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