NEW POSSIBILITIES OF ENZYMIC SYNTHESIS OF RADIOACTIVE NUCLEOTIDES.

IV. APPLICATION OF IN VIVO TRYPTOPHAN-STABILIZED ENZYME SYSTEM FROM REGENERATING RAT LIVER FOR THE SYNTHESIS OF RADIOACTIVE THYMIDINE 5'-PHOSPHATES IN VITRO.

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SUMMARY

The enzymatic extract ptepared from 48h. regenerating rat liver stabilized with dietary L-tryptophan possesses the enhanced thymidine and thymidylate kinase activity. The optimal conditions for the phosphorylation of thymidine in vitro *were found and the possibility for the preparative uses was considered. The amount of newly formed thymidine 5'-monophosphate and 5'-triphosphate depends on the level of adenosine 5'-triphosphate in the reaction mixture, on the temperature of incubation and on the concentration of* Mg^{2+} *-ions.*

INTRODUCTION

Among amino acids tryptophan has a special role on account of its effect on the synthesis of proteins and nucleic acids [1,2]. Its stabilizing effect on the activity of a number of

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amino acid metabolizing enzymes is well known[3,4], and it seems that this effect is of more general validity[5]. While studying the liver regeneration[6,7] we have observed that the dietary administration of tryptophan during the appropriate stages of regeneration results in the enhanced activity of thymidine and thymidylate kinase in the cell-free liver extract. At the same time tryptophan retarded the decrease of both enzymatic activities that occurred during the later phases of regeneration[8].

In this paper we report on some properties of the enzymatic system stabilized with tryptophan which catalyses the transformation of thymidine to corresponding 5′-phosphates in the liver. There is an indication that under suitable conditions of incubation this system may be applied for the preparative synthesis of radioactive thymidine 5 -phosphates in analogy to the synthesis of certain nucleoside 5′-phosphates as has been described in previous papers of this series[9,10].

MATERIALS

Thymidine-2-^{14}C (44 mCi/mmole) was a product of the Institute for Research, Production and Uses of Radioisotopes in Prague. Adenosine 5′- triphosphate (disodium, trihydrate), and thymidine 5′-triphosphate (disodium, hexahydrate) were products of Calbiochem, Luzerne. L-Tryptophan was purchased from Loba-Chemie, Wien. For the experiments female Wistar rats (175-180 g) kept under standard conditions were used.

METHODS

Preparation of cell-free liver extract.

Groups of rats including 4 animals were partially (66%) hepatectomized according to Higgins and Anderson[11] under ether narcosis. Thereafter, the animals were fed ad libitum, and 24 h following the hepatectomy they were intubated under ether anesthesia with the solution of L-tryptophan (100 mg per 100 g) dissolved in mildly alkaline physiologic saline (maximal volume 5 ml). After 24 h the animals were killed by decapitation, and the excised livers were homogenized under cooling in 3 volumes of ice-cold 0.025 M Tris-HCl buffer (pH 7.5) with 5 x 10^{-3}M Mg^{2+}-ions and 2.5 x 10^{-2}M KCl in a glass Potter-Elvehjem homogenizer with tight-fitting Teflon pestle driven by a motor. The homogenate was spun down (10 000 g, 20 min, 2°C), and the defatted supernatant fraction was kept prior to use in a water bath at the temperature of ice as a source of thymidine and thymidylate kinase. By freezing the preparation to -20°C the activity of enzymes decreases 10-20%. However, the sample may be stored at this temperature for 2 weeks or longer without any further loss of the activity.

Conditions of incubation and separation of the newly formed thymidine 5′-phosphates.

The stationary incubation was carried out usually at 40°C in 0.04M Tris-HCl buffer (pH 7.5) in a total volume of 0.5 ml. The reaction mixture contained thymidine-2-^{14}C (usually

5 x 10^{-5}M), adenosine 5 -triphosphate with Mg^{2+}-ions and 0.1 ml of liver cell-free extract. Aliquots of the reaction mixture were separated by chromatography without deproteinization using the Whatman paper No. 1 with corresponding standards in a solvent system composed of isobutyric acid - ammonium hydroxide - water (66:1,5:33). Radioactive zones were cut out and assayed with a Packard Liquid Scintillation Spectrometer.

RESULTS

The enhancement of thymidine and thymidylate kinases at early stages of liver regeneration is associated at later periods with the decrease of the activity of both enzymes[8,11] Table 1 indicates that the period of decreased activity is retarded by the dietary application of L-tryptophan at 24 h of regeneration, and that the amount of newly formed thymidine 5 -phosphates is increased in comparison to the controls approximately twofold. This phenomenon is described more in detail elsewhere[6,7].

The synthesis of thymidine 5′-phosphates from thymidine depends on the level of adenosine 5′-triphosphate (ATP) in the reaction mixture. At lower concentrations of ATP the synthesis of thymidine 5′-monophosphate occurs prevalently; at higher levels of ATP the reaction conditions are more favourable for the formation of thymidine 5′-triphosphate (Fig. 1). In certain cases the high concentration of ATP may be substituted with the ATP-regenerating system in analogy to the procedure for uridine 5′-phosphates synthesis[10]. Simultaneously with thymidine 5′-mono- and -triphosphate a small amount of thymidine 5′-dihposphate is formed together with an unidentified substance characterized as TDP-X.

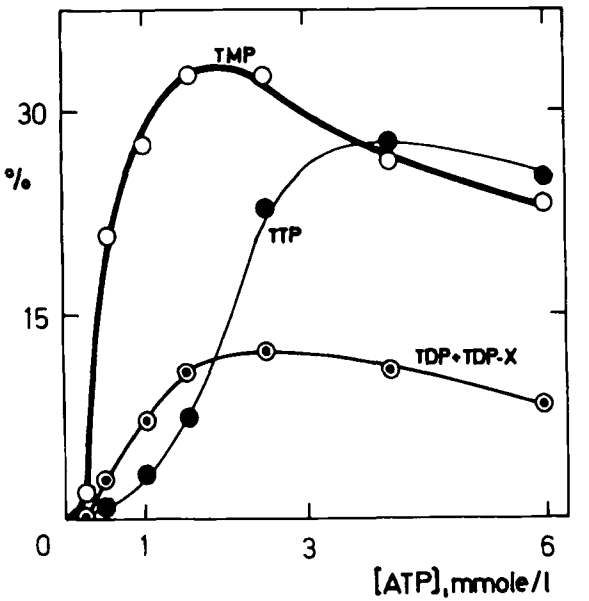

Fig. 1. Synthesis of thymidine-2-^{14}C 5'-phosphates in relation to the concentration of ATP in reaction mixture. Incubation of 5 x 10^{-5}M thymidine-2-^{14}C was for 20 min. at 37°C in 0.04M Tris-HCl buffer (pH 7.5). Equimolar ATP and Mg^{2+}-ions were used.

The time course of thymidine phosphorylation in the presence of an unpurified liver extract, and the formation of the respective 5'-mono- and -triphosphates is shown in Fig. 2. The synthesis of thymidine 5'-triphosphate is usually maximal at 30 - 60 min and at later periods it decreases, while the synthesis of thymidine 5'-monophosphate under appropriate conditions increases during 150 min of incubation with 80% yield.

Thymidine kinase and mainly thymidylate kinase are very sensitive towards higher temperature and are easily inactivated [7,8]. The stabilizing effect of thymidine and thymidine 5'-monophosphate on the activity of both enzymes is well known[8,14];

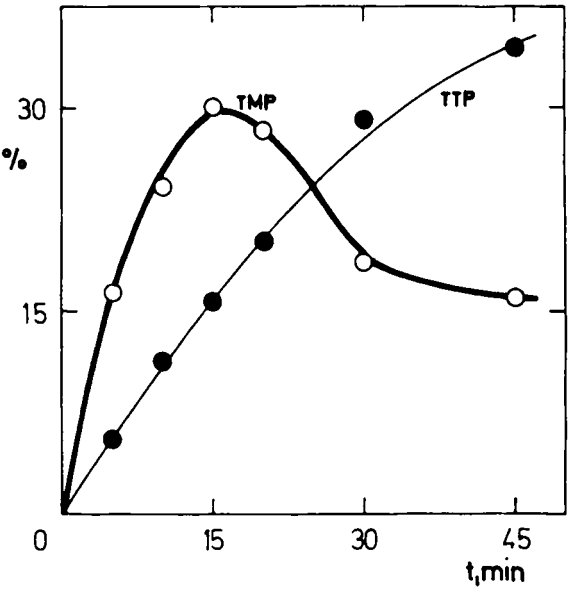

Fig. 2.

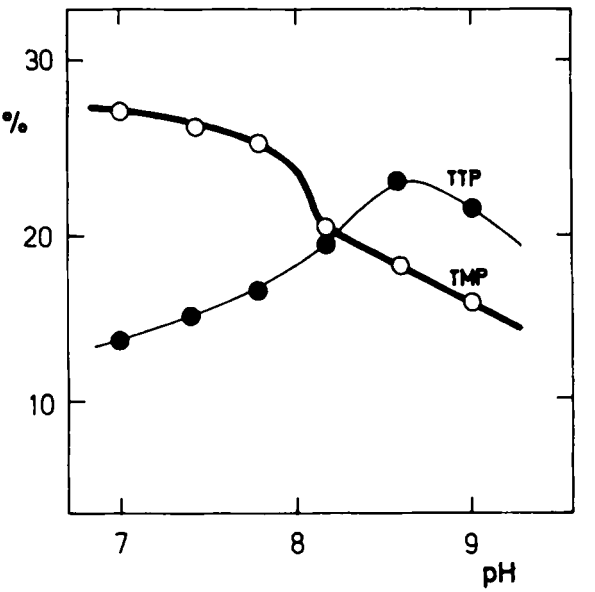

Fig. 3.

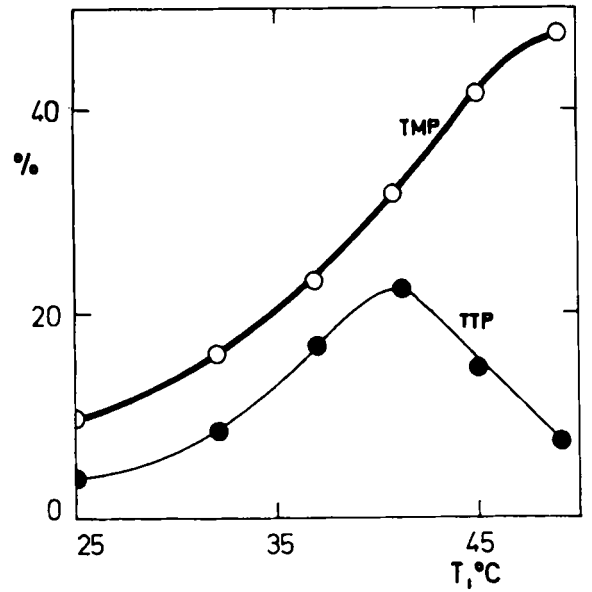

Fig. 4.

Fig. 2. The formation of thymidine-2-^{14}C 5′-monophosphate and 5′-triphosphate in the presence of enzyme fraction stabilized with tryptophan.

Incubation was done at 40°C as described in Fig. 1. ATP, 2.5 x 10^{-3}M and Mg^{2+}-ions, 1.25 x 10^{-3}M.

Fig. 3. Effect of pH on the level of newly formed thymidine-2-^{14}C 5′-phosphates.

Incubation was for 15 min at 37°C as described in Fig. 1 in 0.04M Tris-HCl buffer.

Fig. 4. Effect of temperature on the synthesis of thymidine-2-^{14}C 5′-monophosphate and 5′-triphosphate.

Incubation was for 15 min as described in Fig. 1 and 2.

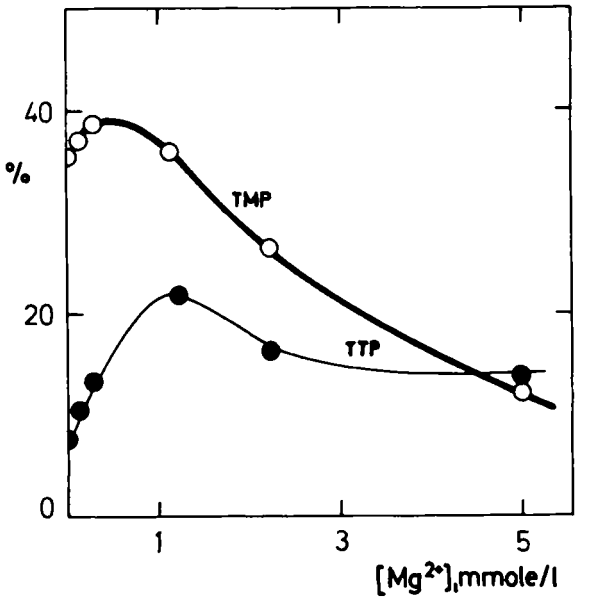

Fig. 5. Effect of Mg^{2+}-ions on the synthesis of thymidine-2-^{14}C 5'-monophosphate and 5'-triphosphate. Incubation was for 15 min. at 40°C at the constant concentration of ATP 2.5 x 10^{-3}M. Conditions of incubation are described in Fig. 1.

however, it is not convenient for the preparative synthesis of radioactive thymidine 5'- phosphates with high specific activity. The inactivation of thymidylate kinase depends on pH of the reaction mixture; pH 7,2-7,8 is optimal, although the synthesis of thymidine 5'-triphosphate is most efficient at mildly alkaline range (Fig. 3).

Considerable sensitivity of thymidylate kinase towards heat is obvious also from Fig. 4 which indicates the amount of newly formed thymidine 5'-monophosphate and 5'-triphosphate in relation to the incubation temperature. Thymidine kinase is more

stable enzyme since the level of thymidine 5′-monophosphate increases with increasing temperature up to 50°C. The temperature optimum for thymidine 5′-triphosphate under the conditions of a 15-min incubation period is 40-42°C.

Although the synthesis of thymidine 5′-phosphates was studied in the presence of an unpurified extract, it seemed desirable to follow the influence of Mg^{2+}-ions on the rate of their formation. The concentration of Mg^{2+}-ions at 0.5 - 1.0 x 10^{-3}M stimulates especially the synthesis of thymidine 5′-triphosphate. On increasing the concentration of Mg^{2+}-ions further to 5.0 x 10^{-3}M (Fig. 5), the activity of thymidine kinase is lower, and consequently the transformation of thymidine is decreased.

DISCUSSION

The activity of thymidine and thymidylate kinase in the adult mammalian liver is low, and it increases during the infiltration with leukemic cells[7] or following partial hepatectomy[15]. The enhanced activity of both enzymes is generally present in rapidly proliterating tissues, such as regenerating liver[8,13,15], different tumours[14,16] and embryonic tissue[17]. The most practical procedure seems to be to prepare the cell-free extract from regenerating rat liver.

It has been established that following partial hepatectomy thymidine kinase enhancement in the liver is observed prior to that of thymidylate kinase[8,18]. At 48-60 h of regeneration the activity of both enzymes sharply decreases and at 120-140 h. it does not differ from the activity of the intact liver. Dietary administration of L-tryptophan at 24 h of liver regenera-

Table 1. Effect of dietary tryptophan on the synthesis of thymidine-2-^{14}C 5′-phosphates in cell-free extract from 48 h regenerating rat liver [a].

Animals	5′-TTP %	5′-TDP %	5′-TMP %	Total %
Intact	0.4	0.5	3.8	4.7
48 h regeneration	6.8	3.2	17.8	27.8
48 h regeneration + tryptophan at 24 - 48 h	14.5	6.2	31.2	51.9

[a] The phosphorylation of thymidine-2-^{14}C, 5 x 10^{-5}M was done in 0.04M Tris-HCl buffer (pH 7.5) at 40°C, 15 min, in a total volume of 1 ml in the presence of 0.2 ml of cell-free liver extract. ATP, 2.5 x 10^{-3}M; Mg^{2+}-ions 1.25 x 10^{-3}M.

tion results in the enhanced activity of both enzymes, and furthermore it prevents the levelling off of their activity at later times[6,7]. Thus the animals may be killed at any time between 12-24 h following L-tryptophan administration when the activity of thymidine and thymidylate kinase is maximal.

The reported data may be used for preparative purposes with certain modifications. It is more convenient to apply the ATP regenerating system, and it is necessary to add the optimal amount of the enzyme to a given volume of reaction mixture. Higher temperature and lower levels of ATP lead to the enhancement of the synthesis of thymidine 5'-monophosphate. The conditions for the preparative synthesis of thymidine 5 -phosphates in high yield using the described enzyme system in combination with the extract from leukemic mouse cells are at present under investigation.

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