Biochemical Pharmacology, Vol. 24, pp. 1025–1027. Pergamon Press, 1975. Printed in Great Britain.

5-ETHYL-2′-DEOXYURIDINE-5′-MONOPHOSPHATE INHIBITION OF THE THYMIDYLATE SYNTHETASE FROM *ESCHERICHIA COLI*

ROLF D. WALTER and KAILASH K. GAURI*

Bernhard-Nocht-Institut für Schiffs- und Tropenkrankheiten, D-2000 Hamburg 4, Bernhard-Nocht-Str. 74 and Universitätskrankenhaus Eppendorf, Augenklinik, D-2000 Hamburg 20, Martinistr. 52, Federal Republic of Germany

(*Received* 6 *August* 1974; *accepted* 12 *November* 1974)

Abstract—The synthesis of 5-ethyl-2′-deoxyuridine-5-monophosphate (EtdUrdMP) and an improved method for the purification of deoxythymidylate synthetase (TdRMP synthetase) from *E. coli* are described. TdRMP synthetase was inhibited competitively by EtdUrdMP ($K_i = 2{\cdot}2 \times 10^{-5}$ M). Under similar conditions the K_i for deoxythymidine monophosphate (TdRMP) was $2{\cdot}0 \times 10^{-5}$ M. The possible role of EtdUrdMP in the virostatic activity of the nucleoside 5-ethyl-2′-deoxyuridine (EtdUrd) is considered.

5-Ethyl-2′-deoxyuridine (EtdUrd) inhibits the replication of DNA-viruses like herpes and vaccinia and serves as an antiviral drug [1, 2]. We have shown previously that this compound is phosphorylated by thymidine kinase (EC 2.7.1.21) to 5-ethyl-2′-deoxyuridine-5′-monophosphate (EtdUrdMP) and that it is incorporated into DNA [3–6]. The effect of EtdUrdMP on the activity of thymidylate synthetase (TdRMP synthetase, EC 2.1.1.b) is reported in this paper.

MATERIALS AND METHODS

^{14}C-Formaldehyde (10 mCi/mM) was purchased from New England Nuclear Corp. Deoxynucleosides and deoxynucleotides were obtained from Boehringer, Mannheim. Analytical grade reagents and frozen *E. coli* K 12 cells harvested in the late log phase were purchased from E. Merck, Darmstadt. DEAE-cellulose (DE 52) was obtained from Whatman, Kent, Sephadex G-150 from Pharmacia, Uppsala, hydroxypatite from Serva, Heidelberg and Ampholine from LKB-Instruments, Stockholm. Thin-layer chromatography was performed on PEI-cellulose from Machery-Nagel, Düren. Folic acid was reduced to the tetrahydro form according to the method of Lorenson *et al.* [7].

Synthesis of 5-ethyl-2′-deoxyuridine-5′-monophosphate (EtdUrdMP). 5-Ethyl-2′-deoxyuridine (EtdUrd) [5] was converted by a two-step reaction into its 3′-acetyl derivative according to the method of Michelson and Todd [8]. This derivative was ultimately phosphorylated to the monophosphate by the method of Tener using β-cyanoethyl phosphate [9].

2·6 g EtdUrd yielded 4·1 g of the trithyl derivative (yield 75%, m.p. 180°), which in turn gave 1·8 g of analytically pure 3′-acetyl derivative (yield 75%, m.p. 150°).

0·3 g of 3′-acetyl-5-ethyl-2′-deoxyuridine (0·001 mole), 2 ml of the standard β-cyanoethylphosphate solution (0·002 mole) and 1·2 g of dicyclohexylcarbodiimide yielded 200 mg (62%) of analytically pure EtdUrdMP: R_F 0·17 in ammonia–isopropanol–water (1:7:2).

pH 3·8 λ_{min} 234 e = 1745 pH 3 λ_{max} 267 e = 7755

Enzyme assay. Thymidylate synthetase activity was measured by a radio-assay as reported previously [10]. The complete reaction mixture contained in a final volume of 100 μl: 0·4 mM ^{14}C-formaldehyde, 0·5 mM *d*-uridine-5′-monophosphate, 0·4 mM tetrahydrofolic acid, 20 mM $MgCl_2$, 50 mM Tris–HCl pH 7·5, 20 mM 2-mercaptoethanol, and enzyme preparation. Protein was estimated by the methods of Warburg and Christian [11] or Lowry *et al.* [12] with crystalline serum albumin as a standard.

Purification of the thymidylate synthetase. 30 g packed *E. coli* cells were disrupted by sonication and a crude extract was obtained by centrifugation

* Dedicated to Mr. Ernst Mauz on his 75th birthday.

Table 1. Purification of thymidylate synthetase

Fraction	Volume (ml)	Protein (mg)	Activity (m-units)	Spec. act. (m-units/mg)	Recovery (%)	Purification
Extract	110	1240·0	112·0	0·090	100	—
Ammonium sulfate	30	685·0	96·5	0·141	86	1·5
Hydroxyapatite	38	78·0	82·0	1·051	73	12
Sephadex G-150	16	21·0	71·5	3·405	64	38
DEAE–cellulose	16	0·85	50·5	59·41	45	660
Isoelectric focusing	8	0·09	26·3	292·2	23	3246

at 40,000 *g* for 30 min. This was treated with streptomycin sulfate, final concentration 0·6%. The purification procedure summarized in Table 1 was carried out at 0–4° using buffers containing 10 mM 2-mercaptoethanol and 1 mM EDTA. The precipitate resulting from the streptomycin treatment was centrifuged off and the supernatant fractionated with ammonium sulfate (35–70%). The ammonium sulfate precipitate was dissolved in 70 mM potassium phosphate buffer pH 7 and dialysed against 6 l. of the same buffer. The protein solution was applied to a 2·4 × 10 cm hydroxyapatite column equilibrated with the 70 mM phosphate buffer. The unabsorbed enzyme was eluted with the same buffer at a flow rate of 30 ml/hr. The eluate was refractioned with ammonium sulphate. The precipitated protein dissolved in 3 ml of 50 mM Tris–HCl buffer were applied to a 2·4 × 40 cm Sephadex G-150 superfine column equilibriated with the Tris–HCl buffer. This was eluted with the same buffer at 4 ml/hr. Pooled fractions containing the enzyme activity was chromatographed on a 1·5 × 10 cm DEAE-cellulose column equilibriated as before. The column was eluted with 50 mM Tris–HCl buffer, and the same buffer containing 0·5 M ammonium sulphate. The pooled fractions which contained the enzyme activity were dialysed as stated above.

Isoelectric focusing, performed with an LKB 8100 column according to Vesterberg and Svensson [13], led to a single peak of thymidylate synthetase activity with its maximum at pH 4·1. This resulted in a specific activity of 0·292 μmole of thymidylate formed per min and per mg of protein, corresponding to a 3200-fold purification.

RESULTS AND DISCUSSION

EtdUrdMP (5-ethyl-2′-deoxyuridine-5′-monophosphate) inhibits TdRMP synthetase (deoxythymidylate synthetase) from *E. coli*. The apparent Michaelis constant for UdrdMP (deoxyuridine-5′-monophosphate) was calculated to be 5 μM (Fig. 1). This value agrees well with that observed previously (K_m 4 μM) [14]. The inhibitor constant for ErdUrdMP was found to be 22 μM, comparatively the K_i-value for TdRMP (2′-deoxythymidine-5′-monophosphate) was calculated to be 20 μM. Both the compounds compete with UdrdMP as shown in Fig. 1a–c. The inhibition by EtdUrdMP is competitive with respect to UdrdMP even if the enzyme is preincubated with the inhibitor for 10 min (Fig. 1c). Competitive product inhibition of TdRMP synthetase has also been observed in other organisms. K_i-values of 24 μM for *S. ficalis* [15] and *L. casei* [16], 59 μM for calf thymus [17] and 140 μM for chick embryo [7] have been reported.

The type of inhibition of TdRMP synthetase by EtdUrdMP and TdRMP is the same as that by 5-fluorodeoxyuridine monophosphate but differs from that by 5-trifluoromethyldeoxyuridine monophosphate. With trifluoromethyldeoxyuridine monophosphate the type of inhibition changes from competitive to non-competitive if a preincubation period is employed [18].

According to the inhibitor constants the exchange of the 5-methyl group in TdRMP with an ethyl group in the analogue EtdUrdMP does not impair

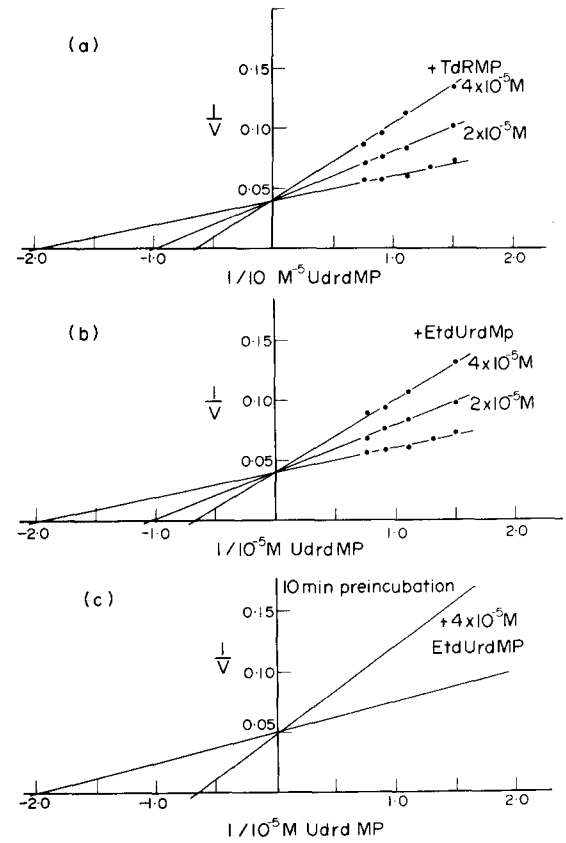

Fig. 1. Double-reciprocal plots of the rate of thymidylate synthetase activity as a function of 2′-deoxyuridine-5′-monophosphate (UdrdMP) concentration and its inhibition by 2′-deoxythymidine-5′-monophosphate (TdRMP) (a) and 5-ethyl-2′-deoxyuridine monophosphate (EtdUrdMP) (b). The velocity is expressed as pmoles of TdRMP formed per 30 min. The conditions are as described in Materials and Methods except that the reaction mixture contained varying amounts of UdrdMP. (c) UdrdMP was added after 10 min preincubation at 37° of the enzyme with all other components including the EtdUrdMP.

the "product" inhibition of thymidylate synthetase activity.

Since like TdR EtdUrd shows virostatic activity against *Herpes simplex* virus, it seems that EtdUrdMP and TdRMP are the possible rate limiting factors in virus multiplication, in that they compete with UdrdMP for the catalytic centre of TdRMP synthetase [19]. Application of ethyldeoxyuridine (EtdUrd) following its phosphorylation by deoxythymidine kinase might stop the *de novo* synthesis of the thymidylate.

Acknowledgements—The authors are grateful to Miss H. Meyer, Miss H. Jahn and Mr. W. Porzig for skilful assistance.

REFERENCES

1. K. K. Gauri and G. Malorny, *Naunyn-Schmiedebergs Arch. exp. Path. Pharmak.* **257**, 21 (1967).
2. K. K. Gauri, *Klin. Mbl. Augenheilk.* **153**, 837 (1968).
3. K. K. Gauri and R. D. Walter, *Chemotherapy* **18**, 269 (1973).
4. E. Riehm and K. K. Gauri, 69. *Tag. Dt Ophtal. Ges.*, Heidelberg (1968).

5. K. K. Gauri, K.-W. Pfughaupt and R. Müller, *Z. Naturforsch.* **24b,** 7 (1969).
6. H. A. Kunkel, K. K. Gauri and G. Malorny, *Biophysik* **5,** 88 (1968).
7. M. Y. Lorenson, G. F. Maley and F. Maley, *J. biol. Chem.* **242,** 3332 (1967).
8. A. M. Michelson and A. R. Todd, *J. chem. Soc.* 951 (1953).
9. C. M. Tener, *J. Am. chem. Soc.* **83,** 159 (1961).
10. R. D. Walter and E. Königk, *Z. Tropenmed. Parasitol.* **22,** 250 (1971).
11. O. Warburg and W. Christian, *Biochem. Z.* **310,** 384 (1941).
12. O. H. Lowry, N. J. Rosebrough, A. L. Farr and R. J. Randall, *J. biol. Chem.* **193,** 265 (1951).
13. O. Vesterberg and H. Svensson, *Acta chem. scand.* **20,** 820 (1966).
14. A. J. Wahba and M. Friedkin, *J. biol. Chem.* **237,** 3794 (1962).
15. R. L. Blakley, *J. biol. Chem.* **238,** 2113 (1963).
16. T. C. Crusberg, R. Leary and R. L. Kisliuk, *J. biol. Chem.* **245,** 5292 (1970).
17. H. Horinishi and D. M. Greenberg, *Biochim. biophys. Acta* **258,** 741 (1972).
18. P. Reyes and C. Heidelberger, *Molec. Pharmac.* **1,** 14 (1965).
19. K. K. Gauri, *Biochem. Pharmac.* **23,** 1153 (1974).