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THYMIDYLATE SYNTHETASE ACTIVITY IN NORMAL AND *BRUGIA PAHANGI*-INFECTED *AEDES AEGYPTI**

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Abstract—Thymidylate synthetase (5,10-methylenetetrahydrofolate:dUMP C-methyltransferase; EC 2.1.2.45; TMPS) activity was detected in crude extracts of 4 to 5 day-old adult male and uninfected female *Aedes aegypti*. Mosquito TMPS was strongly inhibited by Mg^{2+} and Ca^{2+} (ID_{50} values of 0.25 and 0.12 μM, respectively) but not by other divalent cations. The K_m values for dUMP and 5, 10-methylenetetrahydrofolate were 55 and 100 μM, respectively. It appeared that mosquito TMPS would not be subject to regulation by physiological concentrations of thymidine mono-, di- and triphosphates or di- and triphosphates of cytidine, uridine, adenosine or guanosine. When subjected to Sephadex G-200 gel filtration, TMPS activity occurred in three peaks, corresponding to molecular weights of 210,000, 310,000 and 450,000. Among a variety of compounds tested as inhibitors of the enzyme, FdUMP was the most potent, with an ID_{50} value of 0.5 nM; by contrast, FUdR was inactive at 1.0 mM. The polycyclic sulfonated anionic drug, suramin, was also a strong inhibitor of mosquito TMPS, with an ID_{50} value of 0.3 μM. Various 2,4-diaminoheterocyclic antifols, including methotrexate, either were inactive or were relatively weak inhibitors of this enzyme. The total activity of TMPS did not change in extracts of female mosquitoes prepared 7 and 12 days after they fed upon either normal or *Brugia pahangi*-infected jirds.

We reported previously that the total activity of dihydrofolate reductase (FH_2R), serine hydroxymethyltransferase (SHMT), and 5,10-methylenetetrahydrofolate reductase (MFH_4R) increased in crude extracts of adult *Aedes aegypti* females with advanced *Brugia pagangi* infections [1–3]. On the other hand, no change in the total activity of 5,10-methylenetetrahydrofolate dehydrogenase (MFH_4D) was detected [3], and that of 10-formyltetrahydrofolate synthetase (10-FFH_4S) decreased under identical circumstances (unpublished observations). There is experimental evidence suggesting that this pattern of change in the folate metabolism of filaria-infected mosquitoes reflects a specific biochemical aspect of the host–parasite relationship and is not merely a host response to parasite-induced mechanical injury of flight muscles [3]. To gain further insight into the involvement of folate-related enzymes in filaria–mosquito interactions, we turned to a comparative study of thymidylate synthetase (TMPS) in normal and *B. pahangi*-infected adult *A. aegypti* females. TMPS (5,10-methylenetetrahydrofolate:dUMP C-methyltransferase; EC 2.1.2.45) catalyzes the methylation of the pyrimidine ring of dUMP at position 5 to form dTMP. Methylenetetrahydrofolate donates its methylene substituent and a pair of hydrogen atoms in the transmethylation reaction and thus is reoxidized to dihydrofolate. The latter must then be reduced by FH_2R to FH_4 before it can again participate in 1-carbon donating reactions.

This paper reports on the occurrence of TMPS in crude extracts of 4 to 5 day-old *A. aegypti* adults of both sexes and the partial characterization of its properties. The total activity of this enzyme in crude extracts of female mosquitoes 7 and 12 days after they fed upon either normal or *B. pahangi*-infected jirds (*Meriones unguiculatus*) is compared.

MATERIALS AND METHODS

The Liverpool blackeye strain of *A. aegypti* used in this study was maintained as described previously [1].

Batches of 1000 adult mosquitoes of the following descriptions were quick-frozen at −75° before processing: (1) 4 to 5 day-old males and uninfected females that fed on raisins, segregated by sex; (2) identically aged females 7 days after they fed upon normal jirds or upon *B. pahangi*-infected jirds (100–150 microfilariae/20 mm^3 of blood); and (3) identically aged females 12 days after they fed upon normal or *B. pahangi*-infected jirds. The filaria-infected jirds were obtained from Dr. J. W. McCall, University of Georgia, Athens, GA. It was determined that approximately 75 per cent of the mosquitoes that fed upon the filaria-infected jirds became infected and that the mean third stage larval burden in the infected cohort was 14/mosquito.

Crude extracts were prepared by homogenizing 1000 females or 2000 males in 3.0 ml of 40 mM Tris–HCl buffer (pH 7.4) at 0°. The homogenates were centrifuged at 10^5 *g* for 30 min at 4°. The particle-free supernatant fractions were dialyzed against 100 vol. of the above-mentioned buffer for 18–20 hr at 4°, after which they were assayed for TMPS activity. The protein content of these extracts was determined by the method of Lowry *et al.* [4], using Fraction V bovine albumin (Sigma, St. Louis, MO) as a standard.

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TMPS activity was estimated by a modified radioactive assay adapted from both the method of Roberts [5] with regard to most of the components of the system and that of Lorenson *et al.* [6] with regard to the use of $^{14}CH_2O$ and the chromatographic separation of formed product.

Mixture 1. A stock solution of 40 mM Tris-HCl buffer (pH 7.4), 100 mM 2-mercaptoethanol, and 100 mM KF was freshly prepared each week and was stored at 4° when not in use.

The components of the assay system were placed in a thick-walled 7-ml capacity polycarbonate ultracentrifuge tube in the following order: 0.09 ml of Mixture 1, 0.1 ml *dl*-L-FH_4 (0.1 μmole) dissolved in Mixture 1, and 0.01 ml of $^{14}CH_2O$ (1.0 μCi in 0.5 μmole) dissolved in water. These ingredients were allowed to stand at room temperature for 5 min to permit the nonenzymatic chemical synthesis of 5,10-methylenetetrahydrofolate [7]. Thereafter, 0.1 ml dUMP (0.2 μmole dissolved in Mixture 1) was added, and the reaction was started by the addition of the crude extract, usually in a volume of 0.2 ml. If smaller volumes of extract were used, the reaction mixture was brought to a final volume of 0.5 ml by addition of the appropriate amount of 40 mM Tris–HCl (pH 7.4) buffer, and it was then incubated at 30° for 2 hr. A control without dUMP was incubated simultaneously. Under these conditions the assay was found to be linear with respect to time for up to 3 hr. The reaction was terminated by heating at 100° for 2 min. After cooling, the denatured protein was removed by centrifugation. The unreacted $^{14}CH_2O$ in the supernatant fraction was removed by dimedon trapping and sequestration of the dimedon-CH_2O adduct into toluene [8]. After aspiration of the upper toluene layer, a 0.01-ml aliquot of the aqueous phase was spotted on an Eastman Chromogram sheet (cellulose adsorbent with fluorescent indicator). An aqueous solution of nonradioactive dTMP, 2.4 μg in 0.002 ml, was applied as an overlay and served as a carrier; the same amount of dTMP applied separately served as a marker. Ascending chromatographic separation of ingredients in the aqueous phase of the reaction mixture was accomplished by the use of a solvent system composed of 1.0 M ammonium acetate (pH 7.5) and 95% ethanol (7:3, v/v) and development for a distance of 15 cm. After visualization under a u.v. lamp (254 nm), the dTMP-containing spot in a 1-cm square was cut out and the radioactivity in it was measured by liquid scintillation spectrometry. The amount of quenching was determined by means of an internal standard. Identical processing of five 0.01-ml aliquots yielded results that were very reproducible. No radioactivity could be detected in spots in which thymidine or thymine served as carriers. TMPS activity was expressed as nmoles dTMP formed/hr/mg of protein.

Estimation of molecular weight by Sephadex G-200 gel filtration was accomplished by a method described method previously [9].

To test the potency of various chemically heterogeneous compounds as inhibitors of TMPS, each was tested in the standard reaction system initially at concentrations of 0.01 to 0.1 mM, and 1 mM, after 5 min preincubation with the enzyme. Any compound showing marked inhibitory activity at these concentrations was diluted serially to determine the concentration that would inhibit enzyme activity by 50 per cent (ID_{50}).

Table 1. Requirements for thymidylate synthetase assay

Conditions	dTMP formed (nmoles/hr/mg protein)
Complete system *	0.54
Omit extract	0
Omit FH_4	0
Omit dUMP	0
Omit FH_4; add $MeFH_4$	0
Omit dUMP; add UMP	0.02
Omit dUMP; add CMP	0.02
Omit dUMP; add dCMP	0

* See Materials and Methods. Where indicated, equimolar amounts of 5-methyltetrahydrofolate ($MeFH_4$) were substituted for tetrahydrofolate (FH_4), and equimolar amounts of uridylate (UMP), cytidylate (CMP) and deoxycytidylate (dCMP) were substituted for deoxyuridylate (dUMP).

dl-L-FH_4 and dUMP were purchased from the Sigma Chemical Co., St. Louis, MO; $^{14}CH_2O$ was from New England Nuclear, Boston, MA; dimedon was from the Aldrich Chemical Co., Inc., Milwaukee, WI; methotrexate and diethylcarbamazine were from Lederle Laboratories, Pearl River, NY; pyrimethamine, trimethoprim, and 2,6-diaminopurine were from Burroughs Wellcome Co., Research Triangle Park, NC; suramin was kindly provided by Dr. G. Lämmler, Justus Liebig University, Giessen, Federal Republic of Germany; clofazimine by Dr. W. A. Vischer, CIBA-Geigy AG, Basel, Switzerland; and 2,4-diamino-6([(4-chloro-1-naphthyl)nitrosamino]-methyl) quinazoline (JJM-70) by Dr. J. J. McCormack, University of Vermont, Burlington, VT. All reagents were of the highest available grade.

RESULTS AND DISCUSSION

As indicated previously, mosquito TMPS activity under specified assay conditions was linear with time for up to 3 hr; activity was also linear with respect to enzyme concentration. Values obtained in repeated experiments were satisfactorily reproducible.

Table 1 indicates the requirements for the reaction. Omission of the source of enzyme, FH_4 or dUMP prevented formation of radiolabeled dTMP. When an equimolar amount of N^5-methyl-FH_4 was substituted for FH_4, no enzyme activity could be detected. When equimolar amounts of UMP or CMP were substituted for dUMP, roughly 4 per cent of the original activity was detected, indicating that either uridylate and cytidylate could by methylated directly in this system to a small extent or small amounts of these ribonucleoside monophosphates were converted to dUMP by enzymes present in the crude mosquito extracts. It is noteworthy in this connection that Lorenson *et al.* [6] reported that the methylation of UMP by extracts of T_2-infected *Escherichia coli* was 5 per cent that of dUMP, whereas TMPS from chick embryo methylated UMP at 40 per cent the rate for dUMP. No radiolabeled dTMP was formed when dCMP replaced dUMP, suggesting a lack of dCMP deaminase activity in the dialyzed crude mosquito extracts. Mosquito TMPS activity was strongly inhibited by both Mg^{2+} and Ca^{2+}, ID_{50} values being 0.25 and 0.12 μM, respectively; concentrations of these two cations above 2 μM abolished enzyme

Table 2. Comparative activity of structurally heterogeneous compounds as inhibitors of thymidylate synthetase from *Aedes aegypti*

Compound	Concentration ($M \times 10^9$) for 50 per cent inhibition*
FdUMP	0.5
$CaCl_2$	120
$MgCl_2$	250
Suramin	3000
Pyrimethamine	50,000
Trimethoprim	50,000
Clofazimine	50,000
A 2,4-diaminoquinazoline derivative (JJM-70)	80,000
Methotrexate	Inactive at 100,000
Diethylcarbamazine	Inactive at 100,000
dTMP	1,000,000
dTDP	1,000,000
dTTP	1,000,000
FUdR	Inactive at 1,000,000

* Each compound was tested in the standard reaction system (see Materials and Methods) after preincubation with the enzyme for 5 min.

activity (Table 2). Two-thirds of the original activity could be restored following complete inhibition of TMPS by 2 μM Mg^{2+} when the reaction system was supplemented with 100 μM EDTA. The following divalent cations were not inhibitory at 2 μM: Mn, Fe, Zn, Co and Cu. Inhibition by Mg^{2+} of TMPS from L1210 mouse ascites leukemic cells [5] and from trypanosomatids [10] has been reported, but the concentrations of this cations required to inhibit TMPS from these sources were 2–4 orders of magnitude higher than those required to inhibit mosquito TMPS. By contrast, Mg^{2+} is an activator of bacterial TMPS [11–13] and was found to have no influence on TMPS activity in extracts of Ehrlich mouse ascites tumor cells [14], calf thymus [15], or chick embryo [6].

The elution pattern of *A. aegypti* TMPS subjected to Sephadex G-200 gel filtration was variable. In two separate experiments, activity occurred in three peaks, corresponding to molecular weights of approximately 210,000, 310,000 and 450,000; on another occasion,

Table 3. Comparative activities of thymidylate synthetase in crude extracts of *Aedes aegypti* and other organisms or tissues

Source	Enzyme activity*	Reference
A. aegypti adults†		
Females (4 to 5 day-old)	0.54 (0.45–0.76)	
Males (4 to 5 day-old)	0.56 (0.28–0.86)	
Females 7 days after normal blood meal	0.31 (0.23–0.41)	
Females 7 days after infected blood meal	0.33 (0.26–0.38)	
Females 12 days after normal blood meal	0.22 (0.13–0.33)	
Females 12 days after infected blood meal	0.21 (0.19–0.23)	
Silkworm (*Bombyx mori*) adults	0.02	[21]
Mouse L1210 leukemia cells	2.0	[5]
Rat Novikoff hepatoma cells	2.4	[22]
Human RPMI 4265 cultured leukocytes	2.5	[23]
Chick embryo	5.6	[6]
Brucei-group trypanosomes	7.8	[10]
T. cruzi (bloodstream form)	71.4	[10]
E. coli	90.0	[11]
T. cruzi (culture form)	93.6	[10]
D. pneumoniae	120.0	[13]

* Expressed as nmoles dTMP formed/hr/mg of protein; range of values is given in parentheses.

† Average values of three or more experiments.

all activity was confined to a single peak corresponding to 450,000. This pattern of response to gel filtration suggests that either mosquito TMPS is a protein aggregate composed of an undetermined number of subunits or that it forms a high molecular weight complex with other proteins; this uncertainty has not yet been resolved. TMPS from protozoal sources [10, 16] also has a relatively high molecular weight, ranging between 100,000 and 200,000. In this respect, TMPS from protozoal sources and *A. aegypti* appears to differ markedly from the isofunctional enzymes from bacterial and vertebrate sources, the molecular weights of which range between 36,000 and 71,000 [13, 14].

The apparent affinity (K_m) of *A. aegypti* TMPS for dUMP and 5,10-methylenetetrahydrofolate was 55 and 100 μM respectively. These values are reasonably close to those reported for TMPS from trypanosomatids: K_m for dUMP, 25 μM and approximately 50 μM [16, 10]; K_m for methylenetetrahydrofolate, approximately 500 μM [10]. These K_m values are significantly higher than those reported for TMPS from most bacterial and vertebrate sources; generally in these cases the K_m for dUMP is around 6 μM while that for 5,10-methylenetetrahydrofolate is around 30–40 μm [14, 17]. Notable exceptions are the K_m values of TMPS from *Diplococcus pneumoniae* for dUMP and 5,10-methylenetetrahydrofolate, 31 and 266 μM [13] and the K_m value of TMPS from three rodent neoplastic cell lines for 5,10-methylenetetrahydrofolate, around 100 μM [18].

The potencies of various structurally heterogeneous compounds as inhibitors of mosquito TMPS are compared in Table 2. 5-Fluorodeoxyuridylate (FdUMP) was the strongest inhibitor, with an ID_{50} of 0.5 nM; by contrast, 5-fluorodeoxyuridine (FUdR) was inactive at 1 mM. The highly selective sensitivity to inhibition by the deoxyribonucleoside monophosphate form of 5-FU and FUdR is characteristic of TMPS from all known sources [17]. The polycyclic sulfonated anionic compound, suramin, a clinically useful trypanocidal and filaricidal drug, was also a strong inhibitor of *A. aegypti* TMPS, with an ID_{50} of 3 μM. Suramin was also found to be a strong inhibitor of TMPS from trypanosomatids (ID_{50} = 2 μM) [10]; it was a much weaker inhibitor of TMPS from fetal rat liver (ID_{50} = 1 mM) [10]. We previously reported [3] that suramin was a strong inhibitor of other folate-related enzymes from *A. aegypti*, with the following ID_{50} values: FH_2R (5 μM); MFH_4R (2 μM); and 10-FFH_4S (1 μM). On the other hand, suramin did not inhibit mosquito SHMT at a concentration of 20 μM or MFH_4D at a concentration of 100 μM. Various 2,4-diaminoheterocyclic compounds, reported to be inhibitors of FH_2R, either were inactive or were relatively weak inhibitors of mosquito TMPS: the ID_{50} values of the 2,4-diaminoquinazoline derivative, JJM-70, was 80 μM; pyrimethamine and trimethoprim had an identical ID_{50} value of 50 μM. Methotrexate was inactive at 0.1 mM. Methotrexate was also inactive against trypanosomatid TMPS at a relatively high concentration [10], and 2,4-diaminoquinazoline derivatives, although relatively weak, were found to be better inhibitors of TMPS from *D. pneumoniae*, by at least one order of magnitude, than the corresponding 2,4-diaminopteridines [13]. By contrast, methotrexate and related folate analogs significantly inhibited TMPS from proliferating neoplastic mammalian cell lines [18] and regenerating rat liver cells [19]. Two other drugs with antifilarial activity, clofazimine [20] and diethylcarbamazine, were tested as inhibitors of mosquito TMPS; the ID_{50} of the former was 50 μM, and the latter was inactive at 0.1 mM.

Thymidine nucleotides (dTMP, dTDP, dTTP) inhibited mosquito TMPS by approximately 50 per cent at a concentration of 1.0 mM, but their inhibitory activity fell off sharply at lower concentrations. Moreover, when present at the concentration at which dUMP was present (0.4 mM) in the reaction system, the 5′-di- and triphosphates of adenosine, guanosine, cytidine and uridine did not inhibit mosquito TMPS significantly. These findings suggest that TMPS from this source is not subject to allosteric regulation by nucleotides. TMPS from chick embryo [6] and from trypanosomatids [10] were also considered to be independent of feedback-regulatory mechanism.

The ranges of TMPS activity in crude extracts of cells and organisms from diverse sources, including *A. aegypti*, are compared in Table 3. The total activity of TMPS in extracts of 4- to 5-day-old adult mosquitoes of both sexes was roughly the same, namely, an amount capable of producing approximately 0.55 nmole dTMP/hr/mg of protein under specified assay conditions. This level of TMPS activity in crude extracts of young *A. aegypti* adults falls within the lower range of a broad activity spectrum for this enzyme from bacterial [11–13], invertebrate [10, 21], and vertebrate [5, 6, 22, 23] sources and probably reflects significant rates of cell division in a limited number of mosquito tissues. The levels of TMPS activity in extracts of female mosquitoes prepared 7 and 12 days after they had fed upon either normal or *B. pahangi*-infected jirds were roughly the same at each time interval (approximately 0.32 nmole dTMP and 0.22 nmole dTMP formed/hr/mg of protein respectively). It is noteworthy that the total activity of TMPS seemed to fall progressively in aging mosquitoes.

The finding that TMPS activity did not change in mosquitoes with advanced *B. pahangi* infections, considered together with our previous findings, provides further evidence that folate metabolism in the filaria-infected mosquito is modified in a particular way. The nature of the biochemical interactions between the developing parasites and their arthropod host that induces this pattern of change in the latter's folate metabolism remains to be elucidated. The increase in the activity of FH_2R, SHMT and MFH_4R and the concomitant decrease in the activity of 10-FFH_4S suggest at this time that the ultimate response of the mosquito host to filarial infection might be to increase the availability of 5-methyl-FH_4, either for its direct assimilation by the parasites (which, however, possess the enzymatic capacity to produce this FH_4 cofactor) or for its participation directly or indirectly in transmethylation reactions. The latter function could conceivably serve to replace losses of such vital methylated metabolites as methionine or choline. However, there is considerable evidence to indicate that methionine cannot be synthesized by insects, including *Aedes* [24], and that Diptera require preformed choline for growth and reproduction [25]. It is clear that much more work is required to elucidate the role of 5-methyl-FH_4 in the physiology of normal as well as *B. pahangi*-infected mosquitoes.

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