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# PRESENCE AND PROPERTIES OF THYMIDYLATE SYNTHASE IN TRYPANOSOMATIDS

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## Summary

High speed centrifugal supernatant fractions of homogenates of a number of trypanosomatids were assayed for thymidylate synthase (5,10-methylenetetrahydrofolate: dUMP *C*-methyltransferase, EC 2.1.1.45) activity using the method of Lomax and Greenberg (1967) J. Biol. Chem. 242, 109—113). Similar activities were detected in *Crithidia fasciculata*, *Crithidia oncopelti*, the blood forms of *Trypanosoma brucei*, *Trypanosoma congolense* and *Trypanosoma lewisi* and the blood, intracellular and culture forms of *Trypanosoma cruzi*, suggesting that all species synthesize at least some thymidylate de novo. The properties of the activities in *C. fasciculata* and the three forms of *T. cruzi* were compared with those of the isofunctional bacterial and mammalian enzymes. The trypanosotamid enzyme was inhibited by $Mg^{2+}$, was much more sensitive to mercaptoethanol, had higher apparent $K_m$ values for substrate (dUMP) and cofactor (tetrahydrofolate), had a higher apparent molecular weight and was markedly more sensitive to inhibition by suramin. It is, therefore a possible target for chemotherapeutic attack, either on its own or in combination with a dihydrofolate reductase inhibitor. No evidence was obtained for the regulation of the trypanosomatid enzyme, either by its product, dTMP, or by dTDP or dTTp. This result agrees with previous studies which suggested that in trypanosomatids, the level of dTMP was regulated, at least in part, by a catabolic pathway consisting of a thymidylate phosphatase and a thymidine phosphorylase which degraded the excess of dTMP to thymine.

## Introduction

Thymidylate synthase (5,10-methylenetetrahydrofolate: dUMP *C*-methyltransferase, EC 2.1.2.45) catalyses the methylation of the pyrimidine ring of

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dUMP to form dTMP. The source of the methyl group is in part the methylene residue of $N^5$,$N^{10}$-tetrahydrofolate and in part a pair of electrons from the pteridine moiety of tetrahydrofolate itself [1]. Thus during the reaction, the tetrahydrofolate becomes oxidised to dihydrofolate and must be reduced by dihydrofolate reductase if the thymidylate synthase cycle is to continue [1]. Dihydrofolate reductase has been studied in many organisms, including trypanosomatids and has been shown to be sensitive to selective inhibition by a range of 2,4-diaminoheterocycles and especially by 2,4-diaminopyrimidines [2]. Depletion of the cellular pool of tetrahydrofolate by the thymidylate synthase reaction is believed to be an integral part of the growth inhibitory action of dihydrofolate reductase inhibitors, since in all the other active one-carbon transfer reactions known, tetrahydrofolate is left unaltered at the end of the reaction [2]. It was of interest, therefore, to determine if thymidylate synthase activity was present in trypanosomatids in significant amounts. Since some antiviral and antitumour drugs might act by inhibition of thymidylate synthase [1], it was also of interest to compare the detailed properties of the trypanosomal enzyme with those of the isofunctional bacterial and mammalian equivalents in order to determine if there are differences which might be exploitable chemotherapeutically. There is some evidence from experiments with intact cells that in trypanosomatids, dTMP synthesis is not regulated at the level of synthesis but rather by the catabolism of the excess dTMP to thymine which is excreted [3]. Thus, finally, it was of interest to determine whether there is any regulation of the trypanosomatid thymidylate synthase by thymine nucleotides. A preliminary report of this work has been presented [4].

The presence of thymidylate synthase activity has in fact already been reported in two species of trypanosome (*Trypanosoma gambiense* and *Trypanosoma rhodesiense*), but few details of its properties were included [5].

## Materials and Methods

*Materials.* Deoxy[5-$^3$H]uridylate was obtained from the Radiochemical Centre, Amersham, Bucks., U.K. The sources and chemical structures of the drugs used can be obtained elsewhere (Gutteridge, W.E., Cover, B. and Gaborak, M., unpublished).

*Organisms. C. fasciculata* (*Anopheles* strain ATCC 11,745) was grown at 25°C in Kidder and Dutta's defined medium [6]. *Crithidia oncopelti*, obtained originally from Dr. B.A. Newton, Molteno Institute, University of Cambridge, was grown at 25°C in peptone medium [7]. Cultures were harvested in the mid log phase of growth.

The Sonya strain of *Trypanosoma cruzi* was maintained in male and female ASH/XP mice (≈20 g) by syringe passage of blood (subcutaneous inoculation) every 14—21 days. Blood from these animals (containing $10^7$ trypomastigotes) was used to infect subcutaneously batches of rats (90—110 g weight range) which had been immunodepressed with 580 R of whole-body γ-irradiation not more than 24 h previously. Blood stages (mainly broad forms) were isolated from the rats 8—20 days after infection, using a technique involving differential centrifugation to remove most of the erythrocytes and DEAE-cellulose

chromatography (with phosphate/saline/glucose buffer, pH 7.5, $I$ = 0.206) to remove the remaining blood cells. Intracellular stages (mainly amastigote forms) were isolated from the rats 10—15 days after infection. Hind limb muscle tissue was disrupted in an M.S.E. tissue homogeniser and the homogenate incubated with DNAase, collagenase and trypsin. Parasites, contaminated only by a few blood cells, were obtained by differential centrifugation. They were further purified in a linear sucrose gradient (0.25—0.70 M sucrose; 225 × $g$ for 5 min) (Gutteridge, W.E., Cover, G. and Gaborak, M., unpublished). Culture epimastigote forms of the organism were grown at 28°C in a modified LIT-medium [8].

Blood forms of *Trypanosoma brucei* (London School of Hygiene and Tropical Medicine No. 14/2/164) were obtained from infected rat blood using DEAE-cellulose chromatography (phosphate/saline/glucose buffer, pH 8.0, $I$ = 0.217) [9,10]. Blood forms of *Trypanosoma congolense* (Pfizer strain) and *Trypanosoma lewisi* (obtained originally from Dr. J.F. Ryley, I.C.I. Ltd.) were obtained from infected rat blood using a technique involving defribrination [11] and centrifugation in a linear sucrose gradient [12].

*Thymidylate synthase assay.* Organisms, once prepared and washed, were resuspended in 50 mM Tris · HCl buffer, pH 7.8, at $10^9$ organisms/ml. They were then disrupted by three cycles of freezing (liquid nitrogen) and thawing (to 37°C) and the homogenate centrifuged at 105 000 × $g$ for 60 min. The supernatant fraction was used as the source of enzyme activity.

Thymidylate synthase activity was estimated in these crude extracts by assay of the release of $^3H_2O$ from [5-$^3$H]dUMP, using the method of Lomax and Greenberg [13]. Assays were carried out in a total volume of 0.2 ml. The complete system contained (μmol): formaldehyde, 1.0; (±)-L-tetrahydrofolate (dissolved in 1 M mercaptoethanol), 0.2; Tris · HCl buffer, pH 7.8, 10.0; mercaptoethanol, 8.0; [5-$^3$H]dUMP, 0.04 ($4 \cdot 10^6 - 6 \cdot 10^6$ cpm/μmol); enzyme (200 μg protein). Incubation was at 37°C (30°C for *C. fasciculata* and *C. oncopelti*) for 30 min. The reaction was terminated by the addition of 0.5 ml of a slurry of Norit A (100 mg/ml in 1 mM phosphate, 1 mM pyrophosphate buffer, pH 7.0). The $^3H_2O$ released during the reaction is not adsorbed by the Norit A, whereas the [5-$^3$H]dUMP is [14]. Thus, after mixing, the entire suspension was filtered through a membrane filter (Millipore, pore size 0.45 μm). Portions of the filtrate (0.25 ml) were mixed with 3 ml of methyl cellosolve (2-methoxyethanol) and 6 ml of a toluene-based scintillation fluid and estimated for radioactivity in a scintillation counter (Packard Tricarb, liquid scintillation spectrometer). Control samples containing distilled water in place of the enzyme, were incubated in each experiment so that an allowance could be made, where necessary, for natural tritium exchange between the substrate and water.

*Protein estimations.* For the determination of enzyme specific activities, protein was estimated by the Biuret method [15], using bovine serum albumin as standard.

*Molecular weight determinations.* These were made by the gel filtration method, using Sephadex G-200, according to the general procedures of Andrews [16].

## Results

*Initial experiments*

Initially, crude homogenates of *C. fasciculata* were assayed directly for thymidylate synthase activity, using the assay system described for bacteria [14]. Activity was detected and shown to be present in the high speed centrifuged supernatant fraction of the cell. Thus, this cell fraction was used as the source of enzyme in all subsequent experiments.

The optimum conditions detailed in Materials and Methods for the trypanosomatid enzyme were then determined by a systematic examination of the effects of varying each of the components of the reaction system, using *C. fasciculata* as the source of the activity. The changes made from the original bacterial assay involved deletion of $Mg^{2+}$ (see below), reduction of the mercaptoethanol concentration from 100 to 40 mM (see below) and the doubling of the substrate (dUMP) and cofactor (tetrahydrofolate) concentrations to 0.2 and 1 mM, respectively. Under these optimum conditions, the rate of the reaction was linear with respect both to time (for at least 30 min) and to the amount of enzyme added. In most of the experiments with *C. fasciculata*, 2000—4000 cpm of tritium were released during the 30 min incubation period.

*Determination of specific activity in trypanosomatids*

Once the optimal conditions had been identified, the distribution of the activity among key members of the Trypanosomatidae was determined (Table I). Activity was found to be present in all species investigated, including two species of *Crithidia* often used as model trypanosomes, two salivarian trypanosomes, *T. brucei*, which is closely related to *T. rhodesiense*, which causes human sleeping sickness, and *T. congolense*, which causes nagana, the equivalent disease of cattle, and two stercorarian trypanosomes, *T. lewisi*, a natural pathogen of rats and *T. cruzi*, which causes Chagas' disease in man. The specific activities varied over the range of an order of magnitude and the differences are statistically significant, but it is not clear whether they have any

TABLE I

THYMIDYLATE SYNTHASE ACTIVITY IN TRYPANOSOMATIDS

The standard assay, described in Materials and Methods, was used. Results indicate the mean, standard deviation and the number of determinations made, expressed as nmol of tritium released/min per mg protein.

| Organism | | Specific activity |
|---|---|---|
| *C. fasciculata* | | 2.18 ± 0.24 (6) |
| *C. oncopelti* | | 0.54 ± 0.07 (6) |
| *T. brucei* (blood forms) | | 0.12 ± 0.02 (3) |
| *T. congolense* (blood forms) | | 0.14 ± 0.01 (2) |
| *T. lewisi* (blood forms) | | 0.38 ± 0.03 (3) |
| *T. cruzi* | (blood forms) | 1.19 ± 0.04 (4) |
| | (intracellular forms) | 0.35 ± 0.05 (4) |
| | (culture forms) | 1.56 ± 0.16 (6) |

practical significance in the metabolism of the organisms. Similar values have been recorded for *T. gambiense* and *T. lewisi* [5].

*Properties of the trypanosomatid activity*

Highest thymidylate synthase activities were recorded with *C. fasciculata* and *T. cruzi*. Since we were also particularly interested in the question of regulation of thymidylate synthase activity in these two species, they were used as the source of enzyme in all remaining experiments.

The optimum temperature for the assay of the activity in *C. fasciculata* (grown at 25°C) was 30°C, whereas that for *T. cruzi* (a mammalian parasite) was 37°C. The optimum pH of 7.8 was the same for both species. A value of 6.5—6.7 has been reported for the enzyme in *T. gambiense* and *T. lewisi* [5]. The molarities of the Tris · HCl buffer and the sulphydryl-reducing agent, mercaptoethanol, markedly affected activities, the optima being in the range 50—70 and 40 mM, respectively.

The effect of the omission of various components from the assay system are detailed in Table II. Omission of enzyme or tetrahydrofolate abolished activity completely, indicating a lack of significant non-enzymatic exchange between [5-$^3$H]dUMP and water. A small amount of activity remained, however, if formaldehyde was not added, suggesting the presence of a small amount of an alternative source of one carbon group, such as serine, together with the appropriate enzyme, in the crude extracts. The apparent stimulation of activity caused by the omission of $Mg^{2+}$ led to its deletion from the assay system.

Reciprocal plots of reaction velocity versus substrate or cofactor concentrations were linear for the activities in both species. The apparent $K_m$ values derived from these plots ($\approx 5 \cdot 10^{-5}$ M for dUMP and $\approx 5 \cdot 10^{-4}$ M for L-tetrahydrofolate) are somewhat higher than those reported for bacterial and mammalian thymidylate synthases. Values of $2.5 \cdot 10^{-5}$ M have been reported for dUMP for the enzymes from *T. gambiense* and *T. lewisi* [5].

The apparent molecular weights of the enzymes were determined on Sephadex G-200 to be in the range 175 000—200 000, values about three times higher than those of the isofunctional bacterial and mammalian enzymes but similar to that reported for the malaria enzyme (Table III).

TABLE II

REQUIREMENTS FOR THYMIDYLATE SYNTHASE ACTIVITY

The standard assay, described in Materials and Methods, was used except that $MgCl_2$ (4 μmol) was also present.

| Omission | Percent of activity with no omissions | | | |
|---|---|---|---|---|
| | *T. cruzi* | | | *C. fasciculata* |
| | Culture | blood | intracellular | |
| Tetrahydrofolate | 0 | 0 | 0 | 0 |
| Formaldehyde | 10 | 12 | 8 | 4 |
| $MgCl_2$ | 136 | 125 | 119 | 113 |
| Enzyme | 0 | 0 | 0 | 0 |

TABLE III

APPARENT MOLECULAR WEIGHTS OF TRYPANOSOMATID THYMIDYLATE SYNTHASES

The molecular weights were determined on a Sephadex G-200 column which was equilibrated and eluted with 50 mM Tris · HCl buffer (pH 7.8). Fractions were assayed as described in Materials and Methods. Results indicate the mean, standard deviation and the number of determinations made. The data for *Plasmodium berghei* and bacteria and mammalian cells are from refs. 28 and 29.

| Organism | Molecular weight |
|---|---|
| *C. fasciculata* | 175 000 ± 15 000 (2) |
| *T. cruzi* (culture forms) | 200 000 ± 14 000 (3) |
| *P. berghei* | >100 000 |
| Bacteria and mammalian cells | 60 000—70 000 |

The trypanosomal activities were sensitive to inhibition by fluorinated pyrimidines and folate analogues, though not 2,4-diaminopyrimidines (Table IV), a property they share with thymidylate synthases from all sources [1]. The trypanosomatid enzymes were also sensitive to inhibition by a number of antitrypanosomal drugs at high concentration (1 mM), including pentamidine, antrycide, ethidium, dimidium, prothidium and suramin, but not berenil, Bayer 7602 Ac, nitrofurazone, lampit and SQ 18,506. However, only with suramin was significant inhibition noted below this concentration and, therefore, with that possible exception, the effects are unlikely to be related to the biochemical mode of action of these drugs. The trypanosomatid enzyme was much more sensitive to inhibition by suramin than was the isofunctional foetal rat liver enzyme (concentration for 50% inhibition $\approx 1.8 \cdot 10^{-6}$ and $1.1 \cdot 10^{-3}$ M, respectively).

Thymine nucleotides at high concentration ($10^{-2}$ and $10^{-3}$ M) altered the activity of the trypanosomatid thymidylate synthase activity, (Table V), even in the presence of NaF (10 mM) which blocks their degradation by phosphatases [3]. They had no effect, however, at the concentration at which substrate was present ($2 \cdot 10^{-4}$ M) and thus it is unlikely that the trypanosomatid enzyme is regulated by these compounds.

TABLE IV

EFFECT OF STANDARD THYMIDYLATE SYNTHASE INHIBITORS

The standard assay, described in Materials and Methods, was used.

| Inhibitor | Concentration (· M) for 50% inhibition of activity | | | |
|---|---|---|---|---|
| | *T. cruzi* | | | *C. fasciculata* |
| | culture | blood | intracellular | |
| 5-Fluorodeoxy-pyrimidine | $8 \cdot 10^{-5}$ | $2 \cdot 10^{-4}$ | $8 \cdot 10^{-5}$ | $9 \cdot 10^{-5}$ |
| 5-Fluorouracil | $8 \cdot 10^{-4}$ | $7 \cdot 10^{-4}$ | $7 \cdot 10^{-4}$ | $5 \cdot 10^{-4}$ |
| Aminopterin | $3 \cdot 10^{-4}$ | $2 \cdot 10^{-4}$ | $2 \cdot 10^{-4}$ | $8 \cdot 10^{-5}$ |
| Trimethoprim | $>1 \cdot 10^{-2}$ | — | — | $>1 \cdot 10^{-2}$ |

TABLE V

EFFECT OF THYMINE DEOXYRIBONUCLEOTIDES ON TRYPANOSOMATID THYMIDYLATE SYNTHASE ACTIVITY

The standard assay, described in Materials and Methods, was used except that NaF ($10^{-2}$ M) was added to prevent breakdown of the deoxyribonucleotides by phosphatases in the crude enzyme extract used. No inhibitions were detected at the substrate concentration used ($2 \cdot 10^{-4}$ M).

| Nucleotide | Concentration (M) | % inhibition | | | |
|---|---|---|---|---|---|
| | | *T. cruzi* | | | *C. fasciculata* |
| | | culture | blood | intracellular | |
| dTMP | $10^{-2}$ | 67 | 70 | 70 | 72 |
| | $10^{-3}$ | 26 | 29 | 27 | 21 |
| dTDP | $10^{-2}$ | 65 | 75 | 61 | 73 |
| | $10^{-3}$ | 23 | 27 | 28 | 21 |
| dTTP | $10^{-2}$ | 61 | 60 | 65 | 73 |
| | $10^{-3}$ | 17 | 18 | 27 | 20 |

## Discussion

Thymidylate synthase activity was detected in all species of trypanosomatid tested. The specific activities measured are similar to those recorded with crude homogenates of bacteria [17] but higher than those in mammalian tissue cells [18], indicating that all the species have a potential to synthesise dTMP de novo. Many of those tested have been reported also to be able to salvage thymidine, though not thymine, from culture media [19] and thymidine kinase has been isolated from *T. rhodesiense* [20]. Thus, it seems likely that in most trypanosomatids, dTMP can be made both by synthesis and salvage. This situation is in direct contrast to that in two other groups of protozoa, the malaria parasites and the coccidia, where there is no salvage on any scale and thus dTMP is obtained only by direct synthesis [19,21].

All the components of the standard bacterial and mammalian assay systems were required for maximum activity, with the exception of $Mg^{2+}$ which were found to be inhibitory. In bacteria, $Mg^{2+}$ are markedly stimulatory [17,22]; in mammalian assays they have little effect [23]. However, the substrate (dUMP) and cofactor (tetrahydrofolate) concentrations needed to be doubled to ensure maximum rates of activity, suggesting, as was later shown experimentally, that the $K_m$ values for the trypanosomatid enzymes are somewhat higher than those for the most of the isofunctional mammalian and bacterial enzymes [17,24–27]. Furthermore, the trypanosomatid enzyme showed a sharp optimum for mercaptoethanol concentration at less than half that used in the bacterial and mammalian assays [14,18], an apparent molecular weight three times higher [28,29] and a greater sensitivity to inhibition by suramin. Apart from these differences, the basic properties of the trypanosomatid thymidylate synthases were the same as those of the isofunctional bacterial and mammalian enzymes.

All the properties of the enzymes in *C. fasciculata* and *T. cruzi* were very similar and those of the different forms of *T. cruzi*, were, within experimental

error, the same. Such similarities were previously noted in studies with the dihydrofolate reductases of these two species [30].

The trypanosomatid activity was apparently not regulated by thymine nucleotides, a feature which it shares with most but not all thymidylate synthases [1]. Thus if there is any enzyme regulation, in addition to catabolism of excess dTMP by the combined functioning of a thymidylate phosphatase and a thymidine phosphorylase to thymine [3], it is likely to be at the ribonucleotide diphosphate reductase step [1].

Suramin was a very sensitive inhibitor of the trypanosomatid thymidylate synthase and also discriminated between it and the isofunctional mammalian enzyme. It has been shown that this drug also inhibits, at similar concentrations, the dihydrofolate reductases of *Onchocerea volvulus* and *T. rhodesiense* [31] and the L-α-glycerophosphate oxidase of *T. brucei* [32]. Clearly in the absence of other evidence, it is not possible to decide which effect, if any, is significant as far as the mode of action of the drug is concerned.

Thymidylate synthase activity has been detected in a number of species of *Plasmodium* [5,29,33,34]. Its properties were found to be similar to those of the isofunctional bacterial and mammalian enzymes, though some distinctions were observed, notably that the activity was excluded from Sephadex G-200, indicating that the molecular weight was >100 000 compared with 60 000 for the host enzyme [29]. Thus, it is possible that protozoal thymidylate synthases have higher molecular weights than their bacterial and mammalian equivalents, a property which they share, curiously, with protozoal dihydrofolate reductases [2,35,36].

The presence of thymidylate synthase activities in trypanosomatids indicates that they have the potential to deplete their tetrahydrofolate pools themselves, in the presence of dihydrofolate reductase inhibitors and thus inhibitors of dihydrofolate reductase should rapidly block all active one-carbon transfer reactions. The trypanosomatid dihydrofolate reductase has a different drug-sensitivity profile to that of the mammalian enzyme and is thus a potential target for selective drug action [30]. The differences noted between the trypanosomatid and mammalian and bacterial thymidylate synthases suggest that this enzyme too must be a potential target, especially as the possibility of bypassing the metabolic lesion by thymidine salvage is unlikely in vivo [37]. A mix of an inhibitor of dihydrofolate reductase and an inhibitor of thymidylate synthase might even produce a synergistic drug combination.

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