Pyrimidine metabolism in *Trichomonas vaginalis*

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Pyrimidine metabolism in *Trichomonas vaginalis* was investigated using washed cell suspensions of the organism with radiolabelled pyrimidine ring precursors and preformed pyrimidines. The precursors [^{14}C]orotate, [^{14}C]bicarbonate and [^{14}C]aspartate were not incorporated into the pyrimidine bases of trichomonal nucleic acids, indicating that the protozoan is unable to synthesise the pyrimidine ring and is dependent on the salvage of exogenous pyrimidines. [^{3}H]uracil, [^{3}H]uridine, [^{3}H]cytidine, deoxy[^{3}H]cytidine and [^{3}H]thymidine were all efficiently salvaged, and interconversion between cytosine and uracil nucleotides was detected. Thymidylate synthase activity was not detected, suggesting that *T. vaginalis* is dependent upon an exogenous supply of thymidine for TMP synthesis.

Pyrimidine synthesis *Pyrimidine salvage* Trichomonas vaginalis *Parasitic protozoa*

1. INTRODUCTION

Whilst the purine metabolism of several general of parasitic protozoa has been intensively studied, frequently with a view to establishing possible chemotherapeutic targets [1–4], our knowledge of pyrimidine metabolism in this group of organisms is less well advanced. *Trypanosoma, Leishmania, Toxoplasma* and *Plasmodium* species are known to synthesise at least part of their pyrimidine requirements de novo from aspartate, glutamine and carbon dioxide [5–8] and are therefore presumably relatively insensitive to inhibitors of pyrimidine salvage pathways. We recently reported on the ability of the protozoan *Trichomonas vaginalis,* the causative agent of trichomonal vaginitis, to salvage the purines adenine and guanine and their nucleosides, and on the apparent inability of the organism to interconvert adenine and guanine nucleotides and to synthesise the purine ring de novo [9]. We describe here similar experiments, using both preformed pyrimidines and precursors of the pyrimidine ring, which indicate that the organism is able to salvage bases and nucleosides and to interconvert uracil and cytosine compounds, but, in contrast to the majority of the parasitic protozoa, is unable to synthesise the pyrimidine ring. In addition, enzymic studies failed to demonstrate thymidylate synthase cycle activities in *T. vaginalis,* suggesting that this protozoan is dependent upon an exogenous source of thymidine for TMP synthesis.

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2. MATERIALS AND METHODS

2.1. *Growth and harvesting of organisms*

Trichomonas vaginalis (Bushby strain) from the Wellcome Research Laboratories was grown, harvested and washed as described in [9].

2.2. *Pyrimidine incorporation experiments*

Cells were incubated aseptically at 37°C at an initial density of 10^6 cells/ml in 10 ml of a defined medium, based on that described in [5]. The medium contained either preformed, radiolabelled pyrimidines at a final concentration of 80 μM and 5 μCi/ml, or potential precursors of the pyrimidine ring. The precursors used were Na[^{14}C]bicarbonate (180 μM, 5 μCi/ml), [6-^{14}C]orotate (80 μM,

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1 μCi/ml) and [U-^{14}C]aspartate (80 μM, 5 μCi/ml). As *T. vaginalis* is neither able to synthesise purines nor to interconvert adenine and guanine nucleotides [9], adenine and guanine were included in all incubations at 80 μM. Samples (1 ml) were removed at intervals, rapidly mixed with cold 5% (w/v) trichloroacetic acid, collected on Millipore filters (1.2 μm pore size) and treated as in [9]. The radioactivity in the acid-insoluble material (protein, nucleic acid and glycogen) was measured in a toluene-based liquid scintillation fluid.

2.3. *Chemical fractionation of cellular material*

After incubation for 4 h the remaining organisms were washed twice with phosphate-buffered saline. The resulting pellet of cells was extracted twice with 5% (w/v) trichloroacetic acid at 4°C for 30 min, twice with an ethanol/ether mixture (50:50 by volume) at 45°C for 15 min to remove lipids and finally twice with 5% (w/v) trichloroacetic acid at 95°C for 30 min to give a fraction containing solubilised nucleic acids. Details of the subsequent hydrolysis of the nucleic acids, chromatography of the resulting bases and the determination of radioactivity profiles are given in [9].

In some experiments with the deoxyribonucleosides thymidine and deoxycytidine, DNA and RNA were extracted separately after the ethanol/ether treatment, using the method of [10] as modified in [11].

2.4. *Enzyme assays*

Cell-free extracts of *T. vaginalis*, prepared as described in [9], were used in the following enzyme assays, all of which are based on previously published methods.

Thymidylate synthase was measured by following the release of ^3H_2O from [5-^{3}H]dUMP [12]. Centrifugation was used rather than filtration, to remove the Norit A charcoal added to stop the reaction, as this resulted in lower and more stable levels of background radioactivity. The assay mixture for thymidine kinase, based on [13], contained 50 mM Hepes (pH 7.5) 5 mM ATP, 5 mM $MgCl_2$, 15 mM NaF, 0.2 mM [2-^{14}C]thymidine (specific activity 2.5 mCi/mmol) and cell extract in a total volume of 100 μl. Reactions were stopped by heating at 95°C for 4 min, and 20-μl portions spotted on to DEAE discs (Whatman DE81). To remove unreacted thymidine, these were rapidly washed 5 times in 1 mM ammonium formate (~10 ml/disc) and once in methanol, and the remaining radioactivity measured by scintillation counting after the addition of 5 ml PCS (Amersham). Dihydrofolate reductase and serine hydroxymethyltransferase were assayed using the methods in [14] and [15], respectively.

Carbamoyl-phosphase synthase was assayed by following the formation of [^{14}C]citrulline from [^{14}C]bicarbonate in the presence of excess ornithine and ornithine carbamoyltransferase, as described in [16]. Aspartate carbamoyltransferase and dihydroorotase were measured as in [17] and [18], respectively. Dihydroorotate dehydrogenase was assayed spectrophotometrically as described in [19]. The final two enzymes in the pyrimidine biosynthetic pathway, orotate phosphoribosyltransferase and orotidine 5-phosphate decarboxylase, were assayed simultaneously using [6-^{14}C]orotate (5 mCi/mmol) (and in the presence of excess commercial orotate phosphoribosyltransferase in the case of the decarboxylase), and the substrate and products separated by thin-layer chromatography on PEI-cellulose with 0.2 M LiCl [20].

Protein was measured by the method of [21] using bovine serum albumin as a standard.

3. RESULTS

Comparative figures for the incorporation of pyrimidines into the cold trichloroacetic acid-insoluble fraction of *T. vaginalis* are shown in table 1. Incorporation remained linear with time

Table 1

Incorporation of pyrimidines into the nucleic acid of *T. vaginalis*

Pyrimidine	(nmol · 10^6 cells^{-1} · 4 h^{-1})[a]
[6-^{3}H]Uracil	1.026 ± 0.086 (5)
[6-^{3}H]Thymine	0.011 ± 0.001 (3)
[5-^{3}H]Cytidine	1.629 ± 0.164 (5)
Deoxy[5-^{3}H]cytidine	1.126 ± 0.153 (3)
[G-^{3}H]Uridine	1.741 ± 0.378 (5)
[6-^{3}H]Thymidine	0.039 ± 0.007 (4)
[5-^{3}H]UMP	0.246 (2)
[5-^{3}H]UMP	0.325[b] (2)

[a] Mean ± SD (no. determinations)
[b] Obtained in the presence of 10 mM NaF

for more than 6 h. The value for uracil compares well with those given for adenine and guanine (1.09 and 1.05 nmol·10^6 cells^{-1}·4 h^{-1}, respectively) in [9]. There appears to be some preference for the pyrimidine nucleosides compared to the base. The higher values for incorporation of pyrimidine nucleosides compared with purine bases and nucleosides were the first indication obtained that, unlike purines, they are interconvertible. The rate of UMP incorporation was not decreased in the presence of NaF, added to inhibit extracellular phosphatase activity, suggesting that this nucleotide is taken up intact, albeit slowly.

As found for other parasitic protozoa [1] there was negligible uptake of thymine; thymidine salvage was also low, but the ratio of thymidine incorporation to that of adenine (none of which is converted to guanine [9]) is approximately 1:28, which corresponds closely to the ratio of DNA to RNA in the trichomonal cell (1:27, unpublished). Deoxycytidine was salvaged to a much greater extent than thymidine, suggesting that this deoxyriboside is incorporated into both RNA and DNA. This was substantiated by chemical separation of the nucleic acids which revealed that deoxycytidine labelled DNA and RNA in the ratio 1:25, after allowing for ~5% alkaline digestion of the DNA seen in control experiments using thymidine.

Evidence for active interconversion between cytosine and uracil nucleosides/nucleotides is shown in fig.1. The nucleic acid of cells incubated with either [^{3}H]cytidine or [^{3}H]uridine contained both labelled uracil and cytosine bases. Incubation with labelled thymidine resulted solely in the labelling of the thymine base.

Fig.1 also shows the total absence of incorporation of ^{14}C from [^{14}C]bicarbonate into the pyrimidine bases of trichomonal nucleic acid; similarly there was no trace of incorporation from [U-^{14}C]aspartate and [6-^{14}C]orotate. Aspartate and a small amount of bicarbonate were incorporated into the protein of the cell, indicating the continuation of macromolecular synthesis during incubation.

Attempts to detect thymidylate synthase in cell-free extracts of *T. vaginalis* proved negative. Extracts of *Trypanosoma cruzi* were assayed under identical conditions as a positive control. A specific activity of 2.40 ± 0.52 nmol·min^{-1}·mg protein^{-1} was obtained for the trypanosomal enzyme

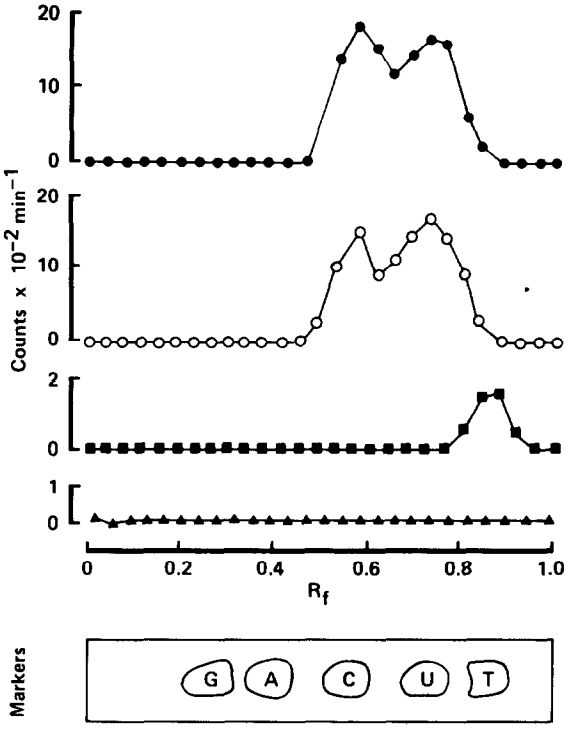

Fig.1. Radioactivity profiles of chromatographed nucleic acid (DNA + RNA) hydrolysates after incubation of *T. vaginalis* with radiolabelled cytidine (●), uridine (○), thymidine (■) and bicarbonate (▲). Markers: G, guanine; A, adenine; C, cytosine; U, uracil; T, thymine.

and this value was unaffected by the addition of *T. vaginalis* extracts. An attempt was also made to detect activity of dihydrofolate reductase and serine hydroxymethyltransferase, both of which are intimately connected in a cycle with thymidylate synthase in many organisms, especially in trypanosomes, where the former occurs with thymidylate synthase as a bifunctional protein [22]. Using the standard, relatively sensitive, assay methods, neither enzyme was detected in *T. vaginalis*. In this context it is interesting to note that when suspensions of *T. vaginalis* were incubated with radiolabelled formate or methyl-labelled [^{14}C]serine, either of which could theoretically supply the 5-methyl carbon of TMP, neither labelled the thymine residues of trichomonal DNA [9]. The limits of our detection of thymidylate synthase were 0.002 nmol·min^{-1}·mg protein^{-1}; this would not be high enough to support the organism's requirement for thymidine (≤0.008 nmol·

$min^{-1} \cdot mg$ $protein^{-1}$), calculated on the basis of known DNA content and doubling time ($28\,\mu g \cdot 10^8$ $cells^{-1}$ and 6.5 h, respectively; unpublished).

The absence in *T. vaginalis* of TMP synthesis from dUMP implies a dependence on the uptake of preformed thymidine. Metabolism to the nucleoside monophosphate is presumably achieved by thymidine kinase, which was detected in cell-free extracts at a specific activity of 2.10 ± 0.60 ($n = 3$) $nmol \cdot min^{-1} \cdot mg$ $protein^{-1}$.

Preliminary reports [23] that, in spite of the apparent absence of de novo pyrimidine synthesis from radiolabelled precursors in *T. vaginalis,* activity of all 6 pyrimidine biosynthetic enzymes could be detected have not been confirmed. A stable, ammonia-dependent carbamoyl-phosphate synthase (CPS) was found with a specific activity of 2.14 ± 0.53 ($n = 4$) $nmol \cdot min^{-1} \cdot mg$ $protein^{-1}$ but its inactivity with glutamine and lack of inhibition by pyrimidine nucleotides suggests that it is similar to the arginine-specific mammalian CPS I rather than the pyrimidine-specific CPS II [24]. Aspartate carbamoyltransferase activity at a specific activity of 5.70 ± 1.31 ($n = 4$) $nmol \cdot min^{-1} \cdot mg$ $protein^{-1}$ was also detected in *T. vaginalis,* as were low levels of dihydroorotase and dihydroorotate dehydrogenase (<0.2 $nmol \cdot min^{-1} \cdot mg$ $protein^{-1}$), but further attempts to detect the last two enzymes of the pathway, orotate phosphoribosyltransferase and orotidylate decarboxylase, proved negative. Thus, overall, our experiments on whole cell utilisation of pyrimidine precursors and further enzymatic studies both point to the same conclusion: there is no pyrimidine biosynthesis de novo in *T. vaginalis.*

4. DISCUSSION

The non-incorporation of bicarbonate, orotate and aspartate into the pyrimidine ring and the apparent absence of the final two enzymes of the de novo synthetic pathway is evidence that *T. vaginalis* (Bushby strain) is unable to synthesise the pyrimidine ring, but must rely upon the salvage of exogenous, preformed pyrimidines. This is an unusual situation in the parasitic protozoa which are generally considered to derive at least some of their pyrimidine requirement from biosynthesis [5–8]. However, recent reports have shown that *Giardia lamblia* [25] and *Tritrichomonas foetus* [26,27] also lack the de novo pathway, and *T. foetus* was shown to incorporate uracil, uridine, cytidine and thymidine into pyrimidine nucleotides [26,27]. *T. vaginalis* (ATCC 30001) has also recently been shown to lack de novo pyrimidine biosynthesis, and to salvage pyrimidine bases and nucleosides [28].

The presence in *T. vaginalis* of several pyrimidine salvage enzymes, including nucleoside phosphorylases and kinases, has been demonstrated [29], and the results presented here confirm that these pathways are actively involved in the essential salvage of a range of pyrimidine bases and nucleosides. Current knowledge of pyrimidine salvage pathways in *T. vaginalis* is summarised in fig.2.

Cytosine was not used in this study, but the absence of cytidine synthesis from cytosine by a phosphorylase in *T. vaginalis* [29], and the absence of cytosine phosphoribosyltransferase from *T. foetus* [26], suggest that *Trichomonas* cannot

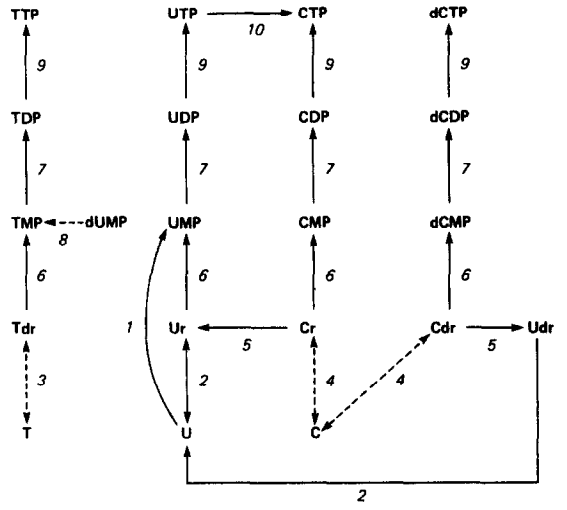

Fig.2. Pathways of pyrimidine in *T. vaginalis:* (⟶) pathways shown by enzymic or metabolic studies to be present; (⇢) pathways apparently absent. Enzymes are: 1, uracil phosphoribosyltransferase; 2, (deoxy)uridine phosphorylase; 3, thymidine phosphorylase; 4, (deoxy)cytidine phosphorylase; 5, (deoxy)cytidine deaminase; 6, pyrimidine nucleoside kinase; 7, nucleoside monophosphate kinase; 8, thymidylate synthase; 9, nucleoside diphosphate kinase; 10, CTP synthetase. U, uracil; C, cytosine; Ur, uridine; Udr, deoxyuridine; Cr, cytidine; Cdr, deoxycytidine; T, thymine; Tdr, thymidine.

utilise this base. Prior to the incorporation of the labelled cytosine ring of deoxy[5-^{3}H]cytidine into RNA it must be converted from a deoxyribose to a ribose derivative. Of the two possible routes for this conversion, via deoxyuridine and uracil (involving cytidine deaminase and phosphorylase enzymes) and via cytosine (involving a nucleoside phosphorylase), the first seems the most likely in view of the absence or very low activity of cytidine phosphorylase in *T. vaginalis* and the presence of (deoxy)cytidine deaminase and (deoxy)uridine phosphorylase [29]. These two enzymes were also detected in *T. foetus* [26].

Deamination at the nucleoside level is the normal pathway of conversion from cytosine to uracil based compounds, whilst conversion in the opposite direction principally occurs by amination at the triphosphate level. *T. vaginalis* freely interconverts cytidine and uridine (fig.1), and possesses cytidine deaminase [29] for the cytidine to uridine conversion; it seems reasonable to assume that CTP synthetase, converting UTP to CTP, is present in the Bushby strain of *T. vaginalis* used in this study. In contrast to these results, no incorporation of uracil or uridine into cytosine nucleotides was seen in *T. vaginalis* (ATCC 30001), although cytidine incorporation into uracil nucleotides was detected [28]. There appear to be distinct enzymatic differences between this strain of the organism and the Bushby strain, in that pyrimidine nucleoside phosphotransferases predominate in the former, whereas uridine kinase and thymidine kinase are present in the latter [28,29].

The absence of thymidine synthase and dihydrofolate reductase from *T. vaginalis* is in striking contrast to all other parasitic protozoa studied, with the exception of *T. foetus* [26], and the majority of all other cells. Thymidine salvage in *T. vaginalis* appears to depend upon thymidine kinase, which has previously been detected in the organism [29], although at a lower specific activity ($0.37\ nmol \cdot min^{-1} \cdot mg\ protein^{-1}$) than the value reported here. In *T. foetus,* thymidine is phosphorylated not by a kinase but by a phosphotransferase, for which a range of nucleoside monophosphates were effective phosphate donors [26]. There is one report that a similar enzyme occurs in *T. vaginalis* [28].

The results presented here, together with those in [28] and [29], show that the pyrimidine metabolism of *T. vaginalis* is dissimilar to that in most other genera of parasitic protozoa and the majority of mammalian tissues. These metabolic studies substantiate the suggestion made after experiments with a defined growth medium, that both purines and pyrimidines are obligate growth factors for *T. vaginalis* [30], and highlight the apparent simplicity of the pyrimidine salvage network, with thymidine metabolism quite independent of the other pathways. From a chemotherapeutic point of view, the inability of *T. vaginalis* to synthesise the pyrimidine ring and its consequent dependence on the salvage of preformed pyrimidines, is as potentially interesting as the organism's dependence upon preformed purines.

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