

THE DEOXYRIBONUCLEOSIDE PHOSPHOTRANSFERASE OF *TRICHOMONAS VAGINALIS*

A Potential Target for Anti-trichomonal Chemotherapy

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Trichomonas vaginalis is a human protozoan parasite adhering to the epithelial layer of the infected urogenital tract and causing trichomonal vaginitis (1). It is incapable of de novo synthesis of either purine (2) or pyrimidine nucleotides (3). The lysed epithelial cells from the infected host may provide an abundant source of nucleic acids, which are likely taken up in the parasite food vacuoles and digested by the nucleases (4) and phosphatases (5) of *T. vaginalis*. Adenosine kinase and guanosine kinase activities were identified in the cytoplasm of *T. vaginalis* (2), and are presumably responsible for salvaging the purine ribonucleosides (5). We have detected uridine and cytidine phosphotransferases in the *T. vaginalis* cytoplasm, and postulated that they may be essential for the salvage of pyrimidine ribonucleosides.

Another unique enzyme activity, the thymidine phosphotransferase, was detected in the membranous fraction of *T. vaginalis* (3). This enzyme appears to be essential for supplying the parasite with TMP because no dihydrofolate reductase, thymidylate synthetase, or thymidine kinase activity can be detected in *T. vaginalis* and no radiolabel from uridine can be incorporated into the parasite DNA (3). It has been assumed, however, that the other three deoxyribonucleotides, dAMP, dGMP, and dCMP, also needed for *T. vaginalis* DNA synthesis, can be derived from the corresponding ribonucleotides in the diphosphate form by the action of a ribonucleotide reductase in the organism. This assumption has been based on our previous discovery of a ribonucleoside diphosphate reductase in another closely related cattle parasite *Tritrichomonas foetus* (6), which is also deficient in the de novo synthesis of purine (7) and pyrimidine nucleotides (8). The assumption is also based on the fact that ribonucleotide reductase is a ubiquitous enzyme detectable in all living organisms.

In later studies it became apparent to us that *T. vaginalis* differs from *T. foetus* as well as all other known organisms in possessing no detectable ribonucleotide reductase activity. No detectable radiolabel from adenosine, guanosine, and cytidine can be incorporated into *T. vaginalis* DNA even after prolonged in vitro incubations with log-phase *T. vaginalis* cells. The origins of dAMP, dGMP, and dCMP for DNA synthesis in the parasite thus turned into a mystery, because of its unusual metabolic deficiencies. One simple explanation could be that *T.*

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vaginalis has, in addition to thymidine phosphotransferase, also deoxyadenosine, deoxyguanosine, and deoxycytidine phosphotransferase activities. The purpose of the present study is to examine this possibility and to identify, isolate, and characterize the crucial *T. vaginalis* enzyme(s) responsible for providing the basic building blocks for *T. vaginalis* DNA.

Materials and Methods

***T. vaginalis* Cultures.** The *T. vaginalis* ATCC 30001 strain was cultivated at 37°C in Diamond's TYM medium (9), pH 6.2 with 10% heat-inactivated bovine serum. The cells grow consistently to the mid-log phase of 4×10^6 cells per ml in 17 h, and have been used for all the subsequent studies. Cell countings were routinely performed in a Coulter ZF counter (Coulter Electronics Inc., Hialeah, FL). Periodic examinations of the cultures for possible mycoplasma contamination were also carried out routinely; there have been no such contamination.

Chemicals. Radiolabeled ribonucleosides and deoxyribonucleosides were purchased from New England Nuclear, Boston, MA or ICN, Irving, CA. ^3H -TMP were obtained from Amersham, Arlington Heights, IL. All other chemicals used in the studies were of the highest purities commercially available.

Enzyme Assays. Phosphotransferases were ordinarily assayed by using *p*-nitrophenylphosphate (100 mM) as the phosphate donor in an assay mixture also consisting of 100 mM Na-acetate, pH 5.4 and 1.0 mM of the radiolabeled nucleoside, which was usually [^3H -methyl]-thymidine ($50 \text{ mCi} \cdot \text{mmole}^{-1}$) unless otherwise stated. The incubation at 37°C, initiated by adding the enzyme sample, was terminated after various time intervals by a 10-fold dilution with an ice-cold solution of 2 mM of the unlabeled nucleoside. The mixture was further diluted 10-fold in 5 mM ammonium acetate, pH 5 and filtered through glass fiber filters loaded with polyethyleneimine (PEI)-cellulose in the same buffer as previously described (2). Levels of radioactivity trapped in PEI-cellulose, representing nucleotides formed from the radiolabeled nucleosides during the incubation, were determined with a liquid scintillation spectrometer.

The radiolabeled nucleotides formed in the phosphotransferase assays were separated, identified, and quantitated in an ion-exchange high pressure liquid chromatography system (HPLC), whenever a new phosphate donor or a radiolabeled nucleoside was tested in the assay for the first time. Samples were injected into an Ultrasil AX (10 μm) 4.6 \times 250 mm column and eluted with a programmed gradient from 7 mM phosphate buffer, pH 3.8 to 250 mM phosphate buffer, pH 4.5 plus 500 mM KCl at a flow rate of 1.0 ml/min. The effluent was monitored at 254 nm, mixed with Aquasol 2 (2), and the radioactivity was recorded in a Flo-one radioactive flow detector (Radiomatic Instruments & Chemical Co., Inc., Tampa, FL). For the analysis of bases and nucleosides, a reverse-phase HPLC system with an ODS (octadecanysilane) (5 μm) reverse-phase column was used and eluted with a programmed gradient from 7 mM KH_2PO_4 , pH 6.0, to 50% acetonitrile at a flow rate of 0.75 ml/min; the effluent was monitored as previously described.

The ribonucleoside diphosphate reductase activity was assayed by the procedure of Waddell and Ullman (10) using 100 μM [U^{14}C]CDP (7,000 cpm/nmole), 3 mM ATP, 6.6 mM MgCl_2 , 5 mM NaF, 10 mM dithiothreitol, 50 mM Tris-HCl, pH 7.4 and enzyme. The reaction was carried out at 37°C for 60 min and stopped by boiling. Radioactive product formed was measured on borate-Dowex 1 columns as described by Steeper and Steuart (11). For assay of ribonucleoside triphosphate reductase activity, [U^{14}C]-UTP was the substrate and adenosyl cobalamin (20 μM), a kind gift from Dr. H. A. Barker of the University of California, Berkeley, was added as a cofactor. The rest of the assay was the same as that for ribonucleoside diphosphate reductase.

The acid phosphatase activity was assayed in 100 mM Na-acetate, pH 5.4 with 4.58

¹ Abbreviations used in this paper: HPLC, high pressure liquid chromatography; PEI, polyethyleneimine; TD, 25 mM Tris-HCl, pH 7.2 plus 1 mM dithiothreitol.

mM of *p*-nitrophenylphosphate as the substrate. The reaction, proceeding at 37°C, was stopped by a twofold dilution with an ice-cold solution of 0.1 N NaOH and the quantity of newly formed *p*-nitrophenol was estimated at 405 nm with a Beckman DU7 spectrophotometer and an extinction coefficient of 18.5 cm²/μmol (12).

Assays of Precursor Incorporation. Assays of precursor incorporation into nucleic acids of *T. vaginalis* were performed according to a previously described procedure (2) with radiolabeled ribonucleosides or deoxyribonucleosides each present at 10 μM in the incubation mixture.

Preparation of *T. vaginalis* Crude Extracts. *T. vaginalis* mid-log phase cells were washed and resuspended in one volume of 25 mM Tris-HCl, pH 7.2 and 1 mM dithiothreitol (TD), and then homogenized in an ice-bath using a Brinkman polytron for three 15-s periods at a setting of 10. The homogenate was centrifuged at 10⁴ g for 30 min to remove the cell debris, and then at 10⁵ g for 1 h to separate the soluble and pelletable fraction for enzyme assays. Protein concentrations were determined by the method of Bradford (12), with bovine serum albumin solution as the standard.

Results

Lack of Conversion of Ribonucleotides to Deoxyribonucleotides in *T. vaginalis*. When log-phase *T. vaginalis* was incubated at 37°C for 2 h with a mixture of the four radiolabeled ribonucleosides, adenosine, guanosine, uridine, and cytidine, each present at a concentration of 10 μM and a specific radioactivity of ~50 mCi·mmol⁻¹, there were 7.80 ± 1.35 pmol of the ribonucleosides incorporated into the RNA fraction of 10⁶ cells but no detectable incorporation into the DNA fraction (<0.05 pmol/10⁶ cells). When similar experiments were performed with the four radiolabeled deoxyribonucleosides as precursors, there were 2.43 ± 0.56 pmol of deoxyribonucleosides incorporated into the DNA of 10⁶ cells, but there was no detectable incorporation into RNA. These results suggest that there may be very little conversion of ribonucleotides to deoxyribonucleotides in *T. vaginalis*.

This suggestion has been further supported by the observation that no activities of ribonucleoside diphosphate reductase or ribonucleoside triphosphate reductase could be detected in freshly prepared crude extracts of *T. vaginalis* (<0.01 nmol/h/mg protein). The same assay was, however, able to detect in *T. foetus* crude extracts significant ribonucleoside diphosphate reductase activity of ~15 nmol/h/mg protein. The absence of ribonucleotide reductase in *T. vaginalis* thus further confirms the lack of conversion of ribonucleotides to deoxyribonucleotides in the parasite.

Identification of Deoxyribonucleoside Phosphotransferase in *T. vaginalis*. The negative findings described above raised the question of possible sources of dATP, dGTP, and dCTP needed for DNA synthesis of *T. vaginalis*. Our previous assays of deoxyribonucleoside kinase activities in the crude extracts of *T. vaginalis* have turned out negative results in both supernatant and pelletable fractions (2) (also see Discussion). Assays of nucleoside phosphotransferase activities in the supernatant fraction found only uridine phosphotransferase and cytidine phosphotransferase activities (2). Thus the 10⁵ g pellet fraction was re-examined in the present study and was found to consist of, in addition to thymidine phosphotransferase (2), deoxyadenosine, deoxyguanosine, and deoxycytidine phosphotransferase activities, also. These four enzyme activities were present at a similar specific activity of ~1.0 nmol/min/mg protein, which represented an activity

level capable of converting 1.7 pmol of each of the four deoxyribonucleosides to the corresponding 5'-monophosphates by 10^6 *T. vaginalis* cells in 1 min. This capacity of salvaging deoxyribonucleosides is more than adequate in coping with the requirement for DNA synthesis in log-phase *T. vaginalis* (see Discussion).

Solubilization and Partial Purification of Deoxyribonucleoside Phosphotransferase from *T. vaginalis*. The fraction of *T. vaginalis* homogenates pelletable between 10^4 and 10^5 g centrifugal force was resuspended in the TD buffer and solubilized with 1% Triton X-100 after a 30-min incubation at 0–4°C. The supernatant fraction harvested after an hour of centrifugation at 10^5 g consisted of ~56% of the original total protein and ~20% of the total enzyme activity. There was thus a reduction in the specific enzyme activity as a result of the solubilization. But the solubilized enzyme activity remained stable for several weeks at 0–4°C; suitable for further purifications.

The solubilized enzyme sample was applied to a DEAE-Sepharose Cl-6B column pre-equilibrated in TD buffer + 1% Triton X-100 and washed with the same solution. The column was then eluted with a salt gradient from 0 to 200 mM KCl in the buffer-detergent mixture at a flow rate of 4.0 ml per hour, and the effluent was collected in 0.6-ml fractions (see Fig. 1). This was a crucial step in the purification that separated the phosphotransferase activity into two apparent peaks. The front activity peak was clearly separated from the acid phosphatase activity (see Discussion). It was collected, combined, named the deoxyribonucleoside phosphotransferase I, and used for all the subsequent purification and characterization studies. Since Enzyme I constituted only about half of the total activities of deoxyribonucleoside phosphotransferase in the solubilized enzyme sample, the DEAE-Sepharose Cl-6B column chromatography resulted in only 50% recovery of activity in Enzyme I (see Table I). The other half was in the second peak, which overlapped with the acid phosphatase activity but did not

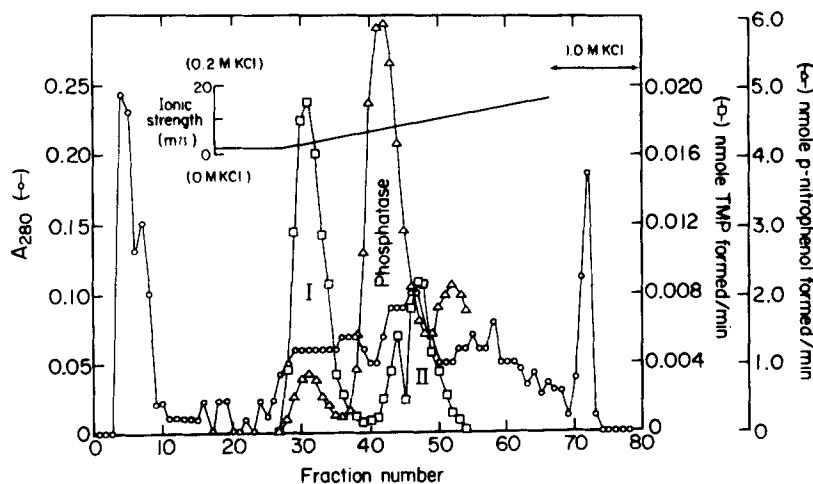


FIGURE 1. Separation of deoxyribonucleoside phosphotransferase from acid phosphatase by DEAE-Sepharose Cl-6B column chromatography. A linear salt gradient made in TD-Triton X-100 buffer was used to elute and separate the enzymes from a crude extract of *T. vaginalis*. Deoxyribonucleoside phosphotransferase activity, (□); acid phosphatase activity, (Δ); I, deoxyribonucleoside phosphotransferase I; II, deoxyribonucleoside phosphotransferase II.

TABLE I
Purification Scheme of *T. vaginalis* Deoxyribonucleoside Phosphotransferase*

Fraction	Total volume (ml)	Protein		Activity (nmol/min)		Purification	Percent yield
		mg/ml	Total (mg)	Specific	Total		
Membranes	8.0	5.0	40.0	1.0	40.00	—	—
Solubilized fraction	8.0	2.8	22.4	0.37	8.29	1	100
DEAE-Sepharose C1-B6 I	9.2	0.25	2.3	1.61	3.70	4.4	44.6
II	16.0	0.14	2.2	1.18	2.60	3.2	31.4
Sephacryl S-200 I	14.4	0.07	1.0	3.62	3.62	10.0	43.7

* The material was from a six-liter batch of *T. vaginalis* in vitro culture that consisted of about 2.4×10^{10} cells.

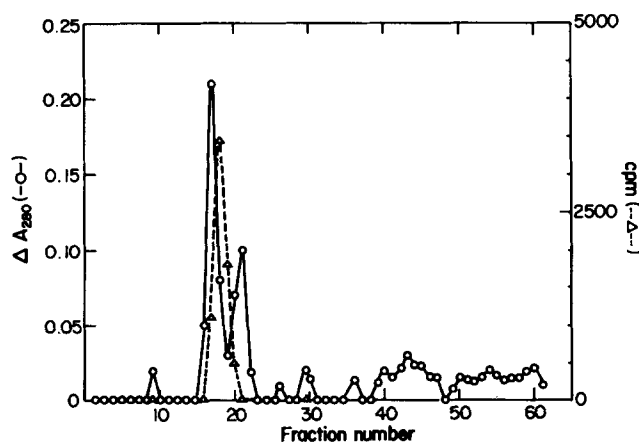


FIGURE 2. Sephacryl S-200 gel filtration of *T. vaginalis* deoxyribonucleoside phosphotransferase I. The column (1.5×46 cm) was equilibrated and eluted with TD-Triton X-100 buffer and was calibrated with the Bio-Rad gel filtration protein standards. The Enzyme I activity, (Δ --- Δ).

coincide completely with it (see Fig. 1). It thus must be a separate enzyme distinctive from the acid phosphatase, and we termed it the deoxyribonucleoside phosphotransferase II. We have not yet had an opportunity to further purify or characterize Enzyme II. But preliminary indications on Enzyme II suggested substrate specificities and molecular weight similar to those of Enzyme I. It thus could be an isozyme of Enzyme I.

Enzyme I was filtered through a 50-fold volume of Sephacryl-200 gel column with 25 mM potassium phosphate buffer, pH 7.2 plus 1% Triton X-100 at a flow rate of 5 ml per hour. The enzyme activity was recovered in a single, well-defined peak with slightly higher than twofold purification and nearly quantitative yield (see Fig. 2 and Table I). Calibrations of the gel column with standard protein samples (Bio-Rad, Richmond, CA) of thyroglobulin (mol wt 670,000), γ -globulin (mol wt 158,000), ovalbumin (mol wt 44,000), and myoglobin (mol wt 17,000) enabled an estimation of the molecular weight of Enzyme I as 200,000 after several independent repetitions of the experiment.

This partially purified sample of Enzyme I represented a 10-fold purification

of the originally solubilized enzyme but counted for only 10% of the total activity originally identified in the membranous pellet fraction of *T. vaginalis* (Table I). This relatively poor yield resulted in ~1.0 mg of the partially purified Enzyme I from six liters of cell cultures that consisted of $\sim 2.4 \times 10^{10}$ *T. vaginalis* cells. The enzyme sample was relatively stable and could be stored at -80°C for several months without any detectable loss of activity.

Properties of Deoxyribonucleoside Phosphotransferase I. The relatively low yield of enzyme I has made its further purification difficult, but the effective removal of acid phosphatase from the preparation has made it suitable for further characterization of the enzyme property. We examined the pH profile of Enzyme I by assaying the enzyme activity in 100 mM of Na-acetate buffer from pH 4.0 to pH 6.5 and in the 100 mM Tris-HCl buffer from pH 7.0 to pH 9.0 with 100 mM *p*-nitrophenyl phosphate as the phosphate donor (see Materials and Methods). The results, presented in Fig. 3, indicated that the pH optimum for thymidine phosphotransferase is from 5.0 to 6.0. Further tests suggested that the pH optima for deoxyadenosine, deoxyguanosine, and deoxycytidine phosphotransferase activities in the same enzyme preparation are also between pH 5.0 and pH 6.0 (data not shown). These four enzyme activities, present in the crude membrane fraction of *T. vaginalis* at about the same level, were co-purified by our procedure and resulted in a similar level of activities in the partially purified Enzyme I preparation (data not shown). These indications suggest that the four activities may all reside in the same enzyme.

All the radiolabeled nucleosides commercially available have been tested at

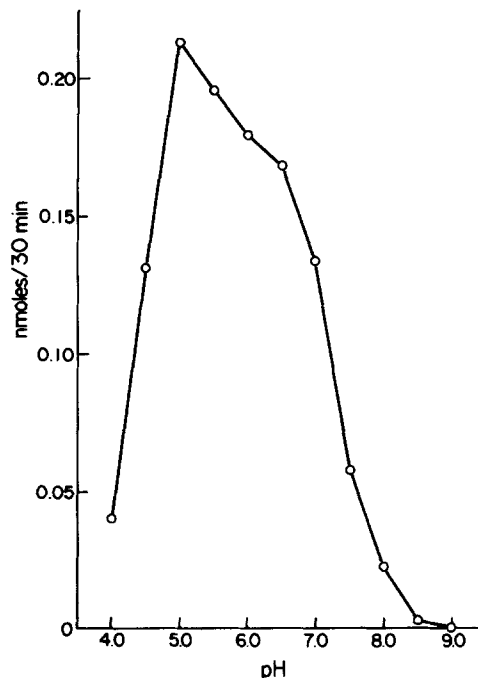


FIGURE 3. The pH optimum profile of *T. vaginalis* deoxyribonucleoside phosphotransferase I.

1.0 mM in our phosphotransferase assay of Enzyme I, and the PEI-cellulose adsorbable radioactivity was monitored after a 60-min incubation at 37°C (see Materials and Methods). Adenosine, guanosine, cytidine, uridine, and deoxyuridine all turned out to be totally incapable of serving as the phosphate recipient. The only phosphate recipients identified so far have been thymidine, deoxyadenosine, deoxyguanosine, and deoxycytidine. Reaction mixtures containing each of the four substrates were analyzed in ion-exchange HPLC, and the radiolabeled products identified by retention times were TMP, dAMP, dGMP, and dCMP, respectively (data not shown). Detailed kinetic analysis of these four enzymic reactions, by following the linear, initial rates of radiolabeled nucleotide formation within the first 30 min of incubation, has enabled us to obtain reasonably accurate estimations of the apparent K_m and V_{max} values of each substrate by Lineweaver-Burke analysis (13). The values listed in Table II indicate that all four deoxyribonucleosides have a similar K_m of 2–3 mM, but the V_{max} values of thymidine and deoxycytidine appear to be twice as high as those of deoxyadenosine and deoxyguanosine.

In an effort to verify once again whether the four substrates all bind to the same active site of the same enzyme, kinetic analysis of Enzyme I preparation was performed with radiolabeled thymidine as substrate and each of the other three unlabeled deoxyribonucleosides as the potential inhibitor. The data, analyzed by Lineweaver-Burke plots (13) and presented in Figs. 4–6, demonstrated that all the three unlabeled deoxyribonucleosides are competitive inhibitors with thymidine, and the K_i values are estimated to be 4.19 mM, 2.53 mM, and 0.63 mM for deoxyadenosine, deoxyguanosine, and deoxycytidine, respectively. It is thus likely that we are studying the same active site in Enzyme I. The lower K_i value of deoxycytidine suggests, however, that the two pyrimidine deoxyribonucleosides may compete more directly with each other.

Many potential phosphate donors other than *p*-nitrophenylphosphate were tested in the enzyme assay. At a concentration of 100 mM, inorganic phosphate, glucose-6-phosphate, fructose-6-phosphate, ribose-1-phosphate, glycerol-3-phosphate, and phosphoenolpyruvate were totally inactive. AMP, GMP, CMP, UMP, IMP, and XMP had only ~10% of the activity of *p*-nitrophenyl phosphate, and their diphosphate and triphosphate derivatives were without detectable activity.

TABLE II
*Kinetic Constants of Deoxyribonucleosides in the Reactions Catalyzed by Deoxyribonucleoside Phosphotransferase I of Trichomonas vaginalis**

Substrate	Apparent K_m (mM)	Apparent V_{max} (nmol/30 min)
Thymidine	3.57 ± 0.42	2.40 ± 0.23
Deoxyadenosine	2.98 ± 0.45	1.33 ± 0.17
Deoxyguanosine	2.25 ± 0.22	1.12 ± 0.27
Deoxycytidine	2.35 ± 0.38	2.30 ± 0.56

* *p*-Nitrophenyl phosphate was present at 100 mM as the phosphate donor in all experiments. Each deoxyribonucleoside was tested in the concentration range of 0.5 to 15 mM.

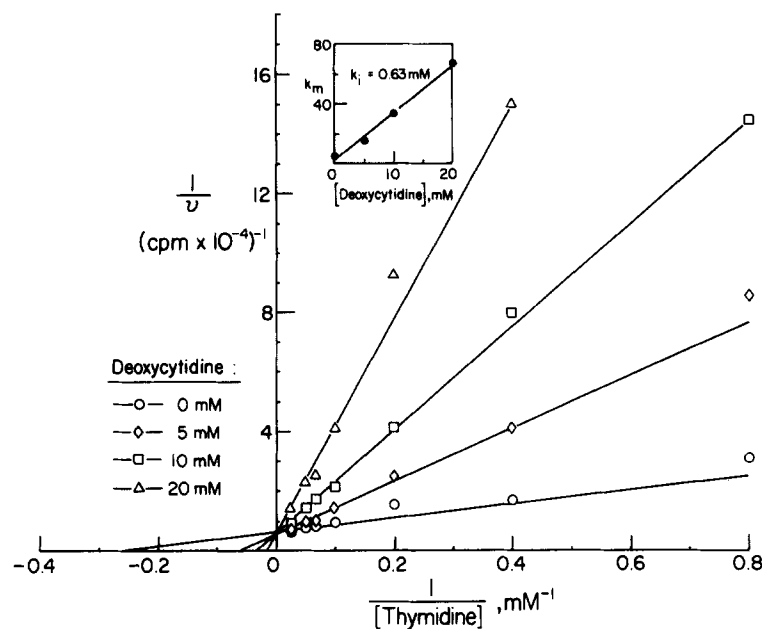


FIGURE 4. Double reciprocal plot of the initial rates of converting thymidine to TMP by *T. vaginalis* deoxyribonucleoside phosphotransferase I. Radiolabeled thymidine was assayed at various concentrations with 100 mM of *p*-nitrophenylphosphate and different fixed concentrations of unlabeled deoxycytidine.

The most potent phosphate donors turned out to be TMP, dAMP, dGMP, and dCMP (Fig. 7A); dAMP and dGMP at 5 mM appeared to be nearly twice as efficient as TMP and dCMP of the same concentration. dUMP and 5-FdUMP were also active phosphate donors with activities higher than that of *p*-nitrophenyl phosphate (Fig. 7B). The enzyme specificity for the phosphate donors appears thus less stringent than that for the phosphate recipients. But the real physiological phosphate donor in *T. vaginalis* may yet to be identified.

Phosphatase Activity of Deoxyribonucleoside Phosphotransferase I. On a closer examination of the Enzyme I peak in Fig. 1, one found in each fraction of the peak both deoxyribonucleoside phosphotransferase activity and phosphatase activity at a relatively constant ratio of 1:40. Enzyme I thus could be an enzyme with two activities. This was verified by further kinetic studies of the phosphatase activities of Enzyme I with *p*-nitrophenylphosphate as the only substrate. The results, obtained after a Lineweaver-Burke analysis of the data, showed an apparent K_m value of 0.74 mM and a V_{max} of 10.6 nmol/30 min (Fig. 8). The phosphatase activity of Enzyme I had also a pH optimum of 5.5. When thymidine was added to the phosphatase assay, there was indication of slightly enhanced rates of hydrolysis of *p*-nitrophenylphosphate by Enzyme I. This thymidine effect was further analyzed and the results presented in Fig. 8 show that the initial velocities for hydrolysis of various concentrations of *p*-nitrophenylphosphate at different fixed concentrations of thymidine result in a series of parallel lines in a double reciprocal plot. The V_{max} of *p*-nitrophenylphosphate hydrolysis is in-

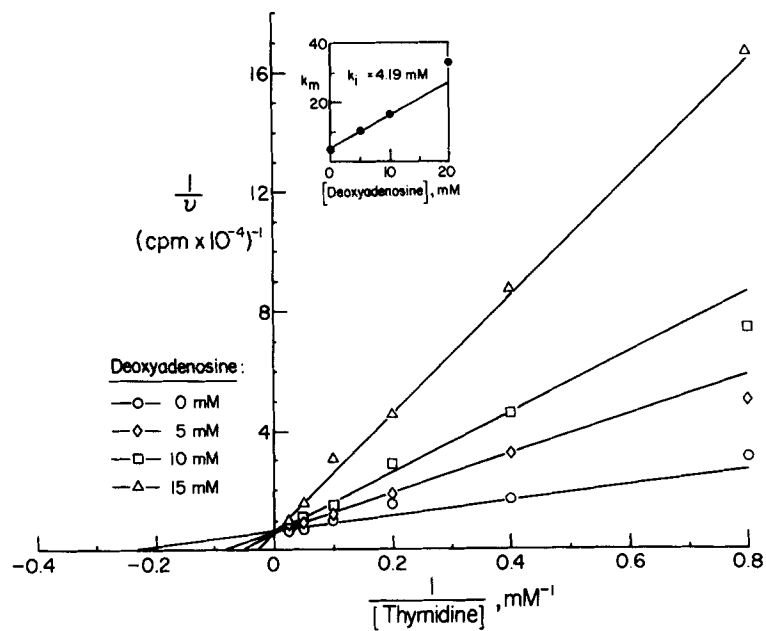


FIGURE 5. Double reciprocal plot of the initial rates of TMP formation from radiolabeled thymidine catalyzed by *T. vaginalis* deoxyribonucleoside phosphotransferase I. Unlabeled deoxyadenosine was present at different fixed concentrations.

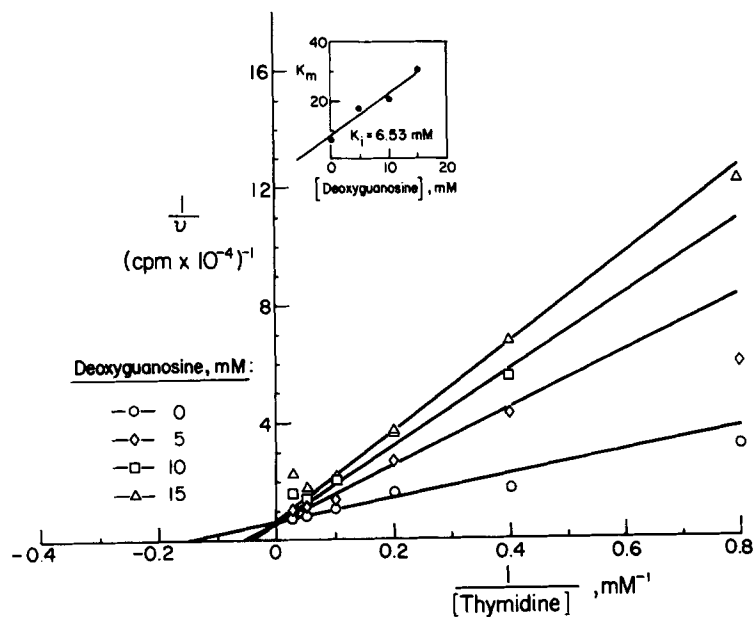


FIGURE 6. Double reciprocal plot of the initial rates of *T. vaginalis* deoxyribonucleoside phosphotransferase I with radiolabeled thymidine and 100 mM *p*-nitrophenylphosphate as the substrates and unlabeled deoxyguanosine as the inhibitor present at different fixed concentrations.

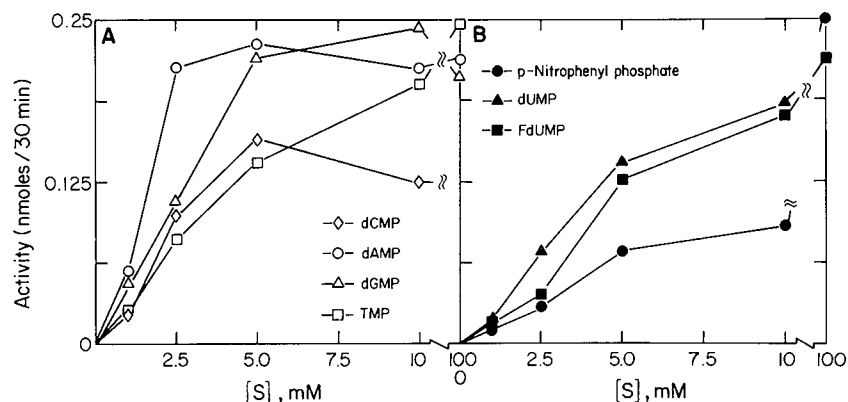


FIGURE 7. The phosphate donors [S] recognized by *T. vaginalis* deoxyribonucleoside phosphotransferase I.

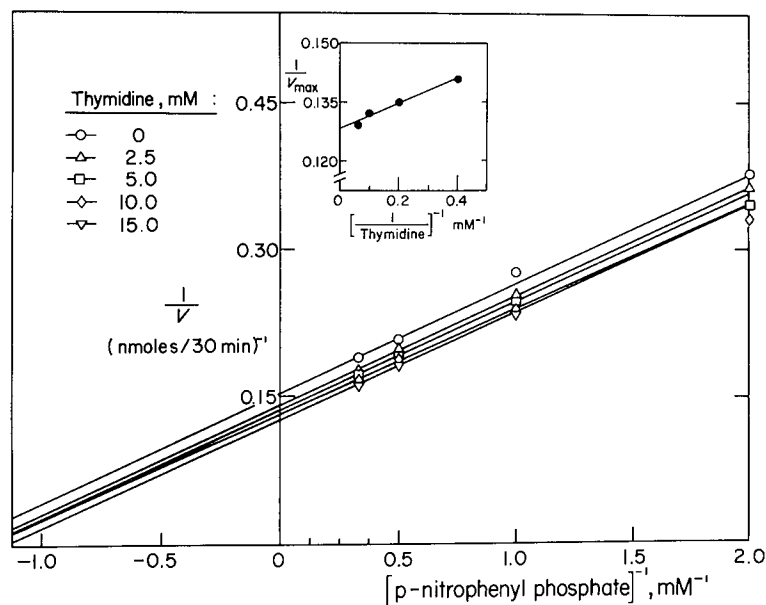


FIGURE 8. Double reciprocal plot of the initial rates of *p*-nitrophenylphosphate hydrolysis by *T. vaginalis* deoxyribonucleoside phosphotransferase I at different fixed concentrations of thymidine.

creased to an estimated value of 14.3 nmol/30 min by the presence of 10 mM thymidine.

Radiolabeled TMP, dAMP, dGMP, and dCMP were also hydrolyzed by Enzyme I in the absence of a phosphate recipient. The hydrolytic products were analyzed in reverse-phase HPLC and were identified as thymidine, deoxyadenosine, deoxyguanosine, and deoxycytidine, respectively (data not shown). The enzyme was, however, incapable of hydrolyzing any of the tested purine or pyrimidine ribonucleotides.

The acid phosphatase peaks observed in fractions 38–41 of Fig. 1 were pooled

together and the kinetics of its hydrolysis of *p*-nitrophenylphosphate was studied. It had a similar K_m (0.91 mM) and a 10-fold higher V_{max} value (54 nmol/30 min) as Enzyme I. It had a pH profile very much like that in Fig. 3, and a very similar molecular weight as Enzyme I in gel filtration experiments (data not shown). The major differences between these two enzymes other than the higher V_{max} values of the phosphatase were that: (a) the phosphatase does not transfer the phosphate group to any nucleoside to form nucleotide; (b) the phosphatase hydrolyzes all nucleotides; (c) the phosphatase activity was not affected by the presence of any nucleosides.

Discussion

Our present investigations have further revealed several unique features of purine and pyrimidine metabolism in *T. vaginalis*. We have learned that there are no apparent ways of providing the parasite with any of the four deoxyribonucleotides needed for DNA synthesis other than via the action of a membrane enzyme of *T. vaginalis*, deoxyribonucleoside phosphotransferase, which only recognizes thymidine, deoxyadenosine, deoxyguanosine, and deoxycytidine as substrates. From the data presented in Table I, this enzyme activity in 2.4×10^{10} *T. vaginalis* cells was capable of converting 40 nmol of deoxyribonucleosides to the corresponding monophosphates in 1 min. Our previous data have indicated that there are ~1.3 mg of bulk DNA in this number of parasites, which is equivalent to ~4.0 μ mol of deoxyribonucleotides (14). At an estimated in vitro generation time of 5–6 h (2), a maximal level of 14.4 μ mol of deoxyribonucleosides could be converted to the nucleotides by this deoxyribonucleoside phosphotransferase activity within one generation time, which far exceeds that required for DNA synthesis. Although the presence of high levels of purine nucleoside phosphorylase (1) and uridine phosphorylase (2) in *T. vaginalis* could, in theory, generate deoxyribonucleosides from ribonucleosides, the existence of this pathway is probably insignificant because no appreciable radiolabel from ribonucleosides was incorporated into *T. vaginalis* DNA. It is thus likely that this deoxyribonucleoside phosphotransferase may provide the main mechanism for supplying *T. vaginalis* deoxyribonucleotides needed for DNA synthesis, and the enzyme thus could be a potential target for anti-trichomonal chemotherapy.

The possible presence of deoxynucleoside kinase activities in *T. vaginalis* extracts, suggested by Miller and Linstead (5) in another strain of *T. vaginalis* (Bushby), was repeatedly examined by us (2) in *T. vaginalis* strain ATCC 30001. In no case were we able to detect any kinase activity above the margin of error of 0.05 nmol per 60 min. In order to verify the possibility that the substrates may have been rapidly destroyed by the high deaminase and phosphorylase activities of *T. vaginalis* in the kinase assays at pH 7.5 (2), we examined the kinase assay mixtures after 60 min incubation in the reverse-phase HPLC analysis. The results indicated that after the incubation with supernatant and pelletable fractions of *T. vaginalis* crude extracts in the kinase assay mixtures, 61.5% of the deoxyadenosine, 82.1% of the deoxyguanosine, 97.7% of the deoxycytidine, and 103.7% of the thymidine remained intact. There is thus no evidence supporting the presence of deoxyribonucleoside kinase in *T. vaginalis* ATCC 30001 under the present assaying conditions.

The relatively low pH optimum (5.0–6.0) of the deoxyribonucleoside phosphotransferase I suggests that the enzyme may be in the lysosomal membrane of *T. vaginalis* (15). This agrees with our working hypothesis that host DNA is digested in *T. vaginalis* intracellular vacuoles and salvaged from the vacuoles in the form of deoxyribonucleosides. This mechanism of salvage may also explain the relatively high substrate K_m values of Enzyme I: the enzyme may be facing relatively high concentrations of the four substrates in the vacuoles following inclusion and digestion of host DNA.

There are also many similarities in the properties of our Enzyme I and the nucleoside phosphotransferases isolated from carrot (16, 17) and barley seedlings (18, 19). All three enzymes have optimal pH values between 5.0 and 6.0, and all exhibited inherent phosphatase activities. The carrot enzyme utilized both ribonucleosides and deoxyribonucleosides as phosphate acceptors with an estimated K_m value of 3.5 mM for uridine (17). Phenylphosphate, UMP, and AMP were all effective phosphate donors, whereas ribose-5-phosphate and β -glycerophosphate acted with somewhat lower efficiencies. These phosphate donors were hydrolyzable by the carrot enzyme in the absence of phosphate acceptors, with two K_m values of 0.7 and 3 mM for phenylphosphate and 2 and 60 mM for ribose-5-phosphate, suggesting two hydrolytic centers in the enzyme (17). The hydrolytic activities were always much higher than the transferase activities. Uridine acted as a noncompetitive inhibitor of the hydrolase function, with $K_i = 4.3$ mM. The barley seedling phosphotransferase also recognized all nucleosides as phosphate acceptors, but had a decided preference for purine deoxyribonucleosides, e.g., deoxyadenosine was the best phosphate acceptor with a K_m value of 0.087 mM, whereas cytidine was the poorest of all ($K_m = 6.39$ mM) (18). All the 5'-nucleotides, ribose-5-phosphate, and *p*-nitrophenylphosphate could serve as phosphate donors for the barley enzyme (19). The enzyme exhibited much higher nucleotidase activity than the transferase activity. In its transferase reaction, retention of the stereochemical configuration of the transferred phosphorothioate was demonstrated (19), and the isolation of a covalently bonded [^{32}P] phosphoenzyme was accomplished by incubating α - ^{32}P -AMP with the enzyme for 2–3 s (18). The evidence thus clearly suggested a ping-pong mechanism of the barley phosphotransferase catalyzed reaction (18). AMP had a Michaelis constant of 5.1 μM in the transferase assay, but showed two apparent K_m values of 5.4 μM and 12 mM in the phosphatase assay which suggested again two hydrolytic centers in the enzyme (18). AMP hydrolysis was also inhibited by nucleosides such as thymidine (18).

Our Enzyme I is similar to the carrot and barley enzymes in also possessing low transferase and high phosphatase activities, but it differed from the two plant enzymes by its strict specificities in choosing phosphate donors and phosphate acceptors. It also demonstrated a much higher molecular weight (200,000) than those of the other enzymes, which are in the 50,000 range (16, 18). Enzyme I was a membranous enzyme that remained soluble only in the presence of Triton X-100, whereas the plant enzymes were isolated from the soluble extracts (16, 18). These facts made Enzyme I more difficult to purify, and thus relatively insufficient kinetic information on this enzyme is available at this time. However, the moderate stimulatory effect of thymidine on the rate of Enzyme I hydrolysis

of *p*-nitrophenylphosphate suggests a somewhat different mechanism of catalytic action from those of the carrot and barley enzymes. Assuming a ping-pong mechanism for the Enzyme I action, the phosphoenzyme intermediate may have binding site(s) for both thymidine and H₂O, but thymidine may have a higher affinity of binding. This suggestion implies that Enzyme I is primarily a transferase with a reaction mechanism similar to that of kidney γ -glutamyl transpeptidase, i.e., a ping-pong mechanism modified by a hydrolytic shunt (20). Verification of this mechanism will have to wait for further purifications of the enzyme.

Summary

Trichomonas vaginalis, a human protozoan parasite known to lack the capability of synthesizing purine and pyrimidine nucleotides de novo, was found also incapable of converting its ribonucleotides to deoxyribonucleotides. The only apparent means of providing deoxyribonucleotides for DNA synthesis relies on salvaging exogenous deoxyribonucleosides by a deoxyribonucleoside phosphotransferase activity in the *T. vaginalis* 10⁵ g pelletable fraction. The activity, constituted by at least two isozymes I and II, can be solubilized by Triton X-100, has a pH optimum of 5.0–6.0, and recognizes only thymidine, deoxyadenosine, deoxyguanosine, and deoxycytidine as the phosphate acceptor. TMP, dAMP, dGMP, dCMP, dUMP, FdUMP, and *p*-nitrophenylphosphate can serve as phosphate donors. Enzyme I has been purified 10-fold by DEAE-Sepharose chromatography and Sephacryl 200 filtration, and is totally freed of the acid phosphatase of *T. vaginalis*. It has an estimated molecular weight of 200,000 and *K_m* values of 2–3 mM for the four deoxyribonucleosides, which act on each other as competitive inhibitors. It also possesses phosphatase activity capable of hydrolyzing *p*-nitrophenylphosphate with a Michaelis constant of 0.74 mM. The rates of hydrolysis are enhanced by thymidine, which suggests that the latter may be the preferred phosphate acceptor, and Enzyme I may be, thus, more a transferase than a phosphatase. This enzyme could be a potential target for antitrichomonal chemotherapy.

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