Cytosolic thymidine kinase (EC 2.7.1.21), an enzyme of the pyrimidine salvage pathway, catalyzes the phosphorylation of thymidine to thymidine 5'-monophosphate (1, 2). In mammalian tissue, two distinct activities occur with cytosolic or mitochondrial localization and it is the cytosolic activity which increases with cellular DNA synthesis, whereas the mitochondrial activity remains relatively constant during various phases of the cell cycle (3-7). Interest in the human enzymes has been recently stimulated by the finding that the levels of the mitochondrial or cytosolic thymidine kinase activities of involved neoplastic tissue of patients with a variety of lymphoproliferative disorders are useful indicators for predicting either tumor behavior or the clinical course of these patients (8-10). Mitochondrial thymidine kinase has been purified to near homogeneity from human liver (11), but our attempts to develop a radioimmunoassay for cytosolic thymidine kinase has necessitated isolation and definition of its properties. In this report, we describe a purification procedure for cytosolic thymidine kinase from human placenta and some characteristics of this highly purified enzyme not previously reported are described.

**EXPERIMENTAL PROCEDURES**

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‡ Portions of this paper (including "Experimental Procedures," Figs. 1 to 4, and Tables I to III) are presented in miniprint at the end of this paper. Miniprint is easily read with the aid of a standard magnifying glass. Full size photocopies are available from the Journal of Biological Chemistry, 9650 Rockville Pike, Bethesda, MD 20814. Request Document No. 82M-3014, cite the authors, and include a check or money order for $5.20 per set of photocopies. Full size photocopies are also included in the microfilm edition of the Journal that is available from Waverly Press.

‡ The abbreviations used are: dThd, thymidine; dTMP, thymidine monophosphate; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; Mes, 2-(N-morpholino)ethanesulfonic acid.

![Image](https://via.placeholder.com/150)

**RESULTS**

Subcellular Fractionation and Purification of Thymidine Kinase—Subcellular fractionation studies performed on two separate occasions showed that 70 to 85% of the total activity in placental crude homogenate occurred in the 160,000 g supernatant with the remainder of activity sedimenting with the 15,000 g pellet. The results of the purification procedure are summarized in Table I. The enzyme preparation obtained after Step 1 when stored at -70 °C in the indicated buffer showed no appreciable loss of activity for up to two weeks. Optimum condition for enzyme binding to blue-Sepharose (Step 3) was found to be within a pH range of 7.5 to 8.9 with a 0.02 M Tris-HCl buffer. As shown in Fig. 1 for its dissociation from the blue-Sepharose complex, thymidine kinase was eluted as a single peak of activity with 0.5 M Tris-HCl, pH 8.0, containing 5 mM ATP and 10 mM MgCl2. Enzyme preparations obtained after Steps 2 and 3 when stored at -70 °C in the respective buffers outlined in the purification procedure were stable for up to 72 h. The enzyme preparation obtained after Step 4 was labile with loss of more than 70% activity in 24 h when stored at either 4, -20, or -70 °C in 0.2 M Tris-HCl and 200 μM dThd. Efforts to stabilize this activity with EDTA (5 mM), ATP (5 mM), MgCl2 (10 mM), bovine serum albumin (10 mg/ml), and protease inhibitors including soybean trypsin inhibitor (0.01 mg/ml), sodium heparin (10 units/ml), and aprotinin (16 units/ml) were unsuccessful. The final enzyme preparation (Step 4) when subjected to electrophoresis showed a single Coomassie blue staining band with identical Rf of 0.32 (Table II) to the single peak of thymidine kinase activity, and with the outlined purification procedure the overall yield was about 16%.

Physical Properties and Subunit Molecular Weight—When the purified enzyme was denatured with sodium dodecyl sulfate, dithiothreitol, and β-mercaptoethanol and electrophoresed on 7.5% polyacrylamide gels, a single Coomassie blue stained band was observed which corresponded to the single band of the autoradiograph of 1125 radiolabeled purified enzyme (Step 4) preparation processed in the same fashion (Fig. 2). By comparison of the migration of these bands to proteins of known subunit molecular weight, the subunit molecular weight of thymidine kinase appears to be 44,000 ± 1,500 (mean ± S.D. of 4 determinations). Because of the relative lability of the final enzyme preparation, some of the physical properties of thymidine kinase were determined with 1125 radiolabeled protein of Step 4 enzyme preparations. With sucrose density ultracentrifugation, the final enzyme preparations yielded a single peak of catalytic activity with an s20,w value of 5.2 and a molecular weight of 92,000 (Table II). Gel
filtration of the radiolabeled Step 4 preparations with Bio-Gel A-0.5M yielded a single peak of radioactivity with a Stokes radius of 41 Å. The isoelectric point of the purified enzyme is 5.1 (Fig. 3). Rabbit immune serum was prepared with preparations of human placental thymidine kinase obtained after Step 4 of the purification procedure and absorbed against human liver mitochondrial extracts. This antisera showed precipitation lines of identity with crude placental extract, partially purified placental thymidine kinase (Step 2 and 3 of the purification procedure) but no immunological reactivity with either crude human liver extract or purified liver thymidine kinase or placental mitochondrial extract (Fig. 4).

Properties of the Reaction—The effect of pH range of 4.4 to 10.0 Mes buffers (50 mM) were used for the pH range of 4.4 to 6.5, Hepes buffers (50 mM) for the pH range 7.0 to 8.0, and Tris-HCl (50 mM) buffers for pH range 7.4 to 10.0. Thymidine kinase exhibited a sharp pH optimum of 7.4. Preliminary substrate specificity studies showed the following. (a) Mg²⁺ is the preferred divalent cation although Ca²⁺ and Mn²⁺ showed greater than 20% of the activity with Mg²⁺. (b) Compounds that showed ability to donate a phosphate group include GTP, CTP, dATP, dGTP, and dCTP. No compounds tested had greater activity than ATP, with dATP showing 60% and the other compounds less than 30% of the activity with ATP. (c) Nucleoside substrate specificity studies showed that amongst the compounds tested only 5 iododeoxyuridine, 5 diazodeoxyuridine, and 5 bromodeoxyuridine produced significant degree of inhibition of activity (Table III). The apparent Ki for thymidine was determined to be 3 μM.

**DISCUSSION**

Mammalian cytosolic thymidine kinase has been partially purified and characterized from a few tissue sources (27–33). We have purified thymidine kinase 5000-fold to apparent homogeneity from human placenta and the critical aspect of our preparation was the sequential elution of the enzyme from blue-Sepharose and a thymidine-Sepharose column. The final purified enzyme was presumed to be the cytosolic form. That this is indeed the case is suggested by the findings that the bulk of human placental thymidine kinase occurs in the cytosol fraction; the behavior of the purified enzyme with polycrylamide electrophoresis, isoelectric focusing, or sucrose density ultracentrifugation is quite distinct from human liver mitochondrial thymidine kinase (11) and these properties compare favorably with those reported for the cytosolic thymidine kinase(s) from a variety of mammalian sources (28–32). In addition, rabbit immune serum elaborated with purified human placenta thymidine kinase preparations does not cross-react with purified mitochondrial thymidine kinase activity. In concert, these findings are compatible with the suggestion that human mitochondrial and cytosol thymidine kinase are products of distinct gene loci on human chromosome 16 and 17, respectively (34, 35).

The final enzyme preparation appears to be homogeneous based on the findings that: it yields a single protein band corresponding to enzyme activity with polycrylamide electrophoresis at pH 8.3; with isoelectric focusing, it migrates as a single activity peak with a pI of 9.1 corresponding to a single protein band; and the final enzyme preparations yield single protein and 11S enzyme preparations a single autoradiographic band with sodium dodecyl sulfate electrophoresis.

The native molecular weight of placental cytosolic thymidine kinase of 25,000, determined either with sucrose density ultracentrifugation or gel filtration of the radiolabeled enzyme, are in good agreement with that obtained with the enzyme either from HeLa cells (29, 29) or from human myelocytic blasts (32), but is distinct to the molecular heterogeneity of highly purified human liver mitochondrial enzyme (11) or the molecular weight of 177,000 reported for the cytosolic form of the human breast carcinoma cell line MCF-7 (33). The subunit molecular weight of 44,000 for the human placental enzyme, determined with sodium dodecyl sulfate electrophoresis, would suggest that this activity in its native state exists as a dimer.

The catalytic lability of the final enzyme preparations has precluded extensive characterization of its kinetic characteristic and attempts to stabilize this activity were unsuccessful. Despite this, the pH optimum of 7.4 for human placental thymidine kinase is similar to that reported for the cytosolic enzyme from a number of mammalian sources (5, 28, 31). The substrate specificity, including the compounds which could donate a phosphate in the reaction and potential nucleosides, are in accordance with previous observations (28, 29, 31, 36).

The present purification procedure is adaptable for the isolation of cytosolic thymidine kinase from other tissue sources as with this method the enzyme from human CEM lymphoblasts has been purified to homogeneity. In addition, our previous findings have suggested that human mitochondrial thymidine kinase and deoxycytidine kinase activity reside with the same protein (11) whereas nucleoside specificity reporting for the highly purified cytosolic activity is entirely compatible with the suggestion that human cytosolic deoxycytidine kinase and thymidine kinase are different enzyme entities (37).

**Acknowledgments**—We would like to thank the labor ward nursing staffs of Margaret Coles Hospital and Queen Victoria Hospital, Melbourne for the supply of fresh human placentas. We would also like to thank Dr. P. H. Ellims for his help and advice, and M. Brown and A. Devlin for the typing of the manuscript.

**REFERENCES**

Human Placental Thymidine Kinase

Supplemental Material to Human Thymidine Kinase. Purification and Properties of the Cytosolic Enzyme of Placenta,
by T. Cigler, D. S. Bradley and M. B. Van Der Meyden.

EXPERIMENTAL PROCEDURES

Materials

- [3H]dThd (specific activity 150 Ci/mmol), [3H]dThd-5'-(5-C14) (Specific activity 11 Ci/mmol) and [14C]dThd (specific activity 40 Ci/mmol) were purchased from New England Nuclear, 1,1-dithio-2-propanol, dithiothreitol, acrylamide, N,N',N'-tetraacrylamidobisacrylarnide, N,N,N',N'-tetramethylenediamine, bovine serum albumin and human transferrin were purchased from Sigma, Bioblock or Calbiochem.

Polyacrylamide gel electrophoresis

Cytochemistry of human placenta obtained immediately after delivery were homogenized gently in a blender at the fastest speed for 10 seconds in one volume (w/v) of 50 mm phosphate buffer, pH 7.2 containing 10 mm KCl, 1 mm EDTA and 0.15 m NaCl. Mitochondria were prepared by subsequent centrifugation at 15,000 g for 10 minutes. After the mitochondria have been removed, the soluble supernatant fraction was obtained by centrifugation at 105,000 g for 30 minutes. These fractions were collected in 50 mm phosphate buffer, pH 7.4 containing 10% glycerol and 0.5 M DTT and lyzed by rapid freezing to liquid nitrogen. The cytosol was centrifuged at 15,000 g for 20 minutes, the precipitate was suspended in approximately 45 ml of 20 mm Tris-HCl buffer, pH 8.0 containing 10% glycerol and 0.5 M DTT (buffer B). Aliquots (50 ml) of this suspension were sequentially applied to a Sephadex G-50 column (12.5 x 70 mm) equilibrated and eluted with buffer B. Ten 0.7 ml samples were collected and fractions containing thymidine kinase activity pooled. The figures were performed on a Bio-Rad model 162 instrument with minor modification.

TABLE I

<table>
<thead>
<tr>
<th>Step</th>
<th>Protein</th>
<th>Units</th>
<th>Specific Activity</th>
<th>Purification Factor</th>
<th>Fold Change</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Placental supernatant</td>
<td>940</td>
<td>97.3</td>
<td>0.015</td>
<td>100</td>
</tr>
<tr>
<td>2</td>
<td>Ammonium sulfate (0.5M)</td>
<td>1555</td>
<td>61.5</td>
<td>0.052</td>
<td>3</td>
</tr>
<tr>
<td>3</td>
<td>Blue agarose affinity chromatography</td>
<td>31.04</td>
<td>55.7</td>
<td>1.7</td>
<td>59</td>
</tr>
<tr>
<td>4</td>
<td>Thymidine affinity column</td>
<td>0.167</td>
<td>25.7</td>
<td>0.833</td>
<td>50</td>
</tr>
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</table>

**TABLE II.**

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sedimentation coefficient</td>
<td>5.7 ± 0.06 (4)</td>
</tr>
<tr>
<td>Stokes radius (nm)</td>
<td>41 (2)</td>
</tr>
</tbody>
</table>

**Molecular weight**

- Subunit density ultracentrifugation 92,000 ± 1500 (4)
- Gel filtration 92,000 (2)
- PAGE 94,000 ± 1500 (4)
- PAGE (4) 30 ± 0.02 (4)

**Isoelectric point**

9.1 (2)

**Notes:**

1. The value given is the mean ± 1 SD.
2. The figure in parentheses indicates the number of determinations.

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HUMAN PLACENTAL THYMIDINE KINASE

Purification of Human Liver Thymidine Kinase

Human liver thymidine kinase was purified from the liver of a 23 week pregnant woman by the method of Cahn and Naarden (24). The standard used were human transferrin and bovine albumin, the migration of which were determined by absorption at 280 nm.

Purification of liver thymidine kinase

Aliquots (70-100) of pooled Step 4 enzyme preparations were mixed with an equal volume of a mixture complete albumin and destilled water. Aliquots (50 ml) of the mixture were injected intravenously into brains of the back and proximal limbs of New Zealand white rabbits (24). Four to eight weeks after primary immunization, antibody production was assessed by the Ouchterlony technique (26). The rabbits were immunized at each time of 1 ml antisera was injected. The immunized rabbits were bled weekly and ant serum pooled. The immune rabbit serum was absorbed against human liver mitochondriomes extract. Human liver was obtained at autopsy and homogenized at the specified speed in a blender in 10 mm Tris-HCl buffer, pH 7.4 containing 10% glycerol. The homogenate was centrifuged at 1000 g for 10 min at 4°C and the supernatant obtained at 15,000 g for 30 min at 4°C. The mitochondrial pellet so obtained was washed five fold in 0.15 M sodium chloride solution. The pellet was then lysed by rapid freezing in liquid nitrogen. One volume of the homogenate was taken and a rabbit rabbit serum at 75°C for 50 min and then centrifuged at 100,000 g at 4°C for 1 hour.

**Molecular weight and Stokes radius determination**

The purified dThd kinase preparations (Step 4) were pooled and 0.2 mg of protein was radioactivity with 1122 using the chromogenic method (18). This preparation was applied to a Sephadex G-50 column (1.5 x 40 cm) which was equilibrated and eluted with 50 mm phosphate buffer, pH 7.4 containing 0.15 m sodium chloride. The column was calibrated with catalase, bovine transferrin, bovine serum albumin, ovalbumin and myoglobin. Fractions of 1.7 ml were collected. The elution volume of the standard proteins was determined by following the absorbance at 280 nm for all standards except for ovalbumin which was determined at 210 nm and the void volume was determined by elution of blue dextran. The molecular weight of dThd kinase was determined by the method of Andrews (23). To determine the molecular weight and Stokes radius of dThd kinase, the enzyme was calculated by the method of Kinner (21) and the molecular weight of each standard was obtained from the literature (27). The molecular weight of dThd kinase was determined by the method of Andrews (23).
Table III

<table>
<thead>
<tr>
<th>Additional Nucleoside</th>
<th>Percent Activity</th>
<th>Phosphate Donor</th>
<th>Percent Activity</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>100</td>
<td>ATP</td>
<td>100</td>
</tr>
<tr>
<td>Adenosine</td>
<td>100</td>
<td>CTP</td>
<td>29</td>
</tr>
<tr>
<td>Guanosine</td>
<td>20</td>
<td>GTP</td>
<td>24</td>
</tr>
<tr>
<td>Cytidine</td>
<td>12</td>
<td>UTP</td>
<td>10</td>
</tr>
<tr>
<td>Thymidine</td>
<td>100</td>
<td>TTP</td>
<td>17</td>
</tr>
<tr>
<td>Deoxyadenosine*</td>
<td>100</td>
<td>dCTP</td>
<td>62</td>
</tr>
<tr>
<td>Deoxyguanosine</td>
<td>100</td>
<td>dGTP</td>
<td>36</td>
</tr>
<tr>
<td>Deoxyxylidine</td>
<td>100</td>
<td>dXTP</td>
<td>36</td>
</tr>
<tr>
<td>5-Fluoro-deoxyuridine</td>
<td>100</td>
<td>dfTP</td>
<td>1</td>
</tr>
<tr>
<td>5-iodo-deoxyuridine</td>
<td>44</td>
<td></td>
<td></td>
</tr>
<tr>
<td>5-diazido-deoxyuridine</td>
<td>64</td>
<td></td>
<td></td>
</tr>
<tr>
<td>5-Fluoro-deoxyuridine</td>
<td>80</td>
<td></td>
<td></td>
</tr>
<tr>
<td>5-bromodeoxyuridine</td>
<td>38</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

For nucleoside specificity studies, assays were performed in duplicate with 50 μL [3H]-thymidine, 5 μL ATP, 10 μL MgCl2, and 5 μL of the indicated compound. Results are expressed as percent TK performed in the absence of the additional purine or pyrimidine compound.

For phosphate donor studies, the concentration of nucleotides used was 5 μM and 10 μM MgCl2. Results are expressed as percent TK performed with ATP as the phosphate donor. Each assay contained 2-5 μg of the purified enzyme.

Fig. 1. Elution pattern of human placental cytosolic thymidine kinase with Blue Sepharose affinity chromatography. An aliquot (10 μL) of step 2 preparation of the purification procedure was applied to the column which was eluted with 0.5 M Tris-HCl pH 8.0 containing 5 μM ATP and 30 mM MgCl2. Fractions of 10 μL were collected and activity occurring as shown by fractions 20-26 were pooled. Activity with ATP (a→g).

Fig. 2. Polyacrylamide electrophoresis of human placental cytosolic thymidine kinase obtained after step 4 of the purification procedure. Panel A. Coomassie blue-stained gel. Panel B. Same preparation stained with 0.1% Coomassie blue R-250. Panel C shows SDS-PAGE of the [3H] radio-labelled step 4 enzyme preparation as described under Experimental Procedures. Panel D. Standards applied to SDS-polyacrylamide gels: phosphorylase, molecular weight 97,400; bovine serum albumin (66,000), ovalbumin (43,000), carbonic anhydrase (30,000), trypsin inhibitor (20,100) and lactic dehydrogenase (14,400). The Rf of the single band in Panel B is identical to that of the autoradiograph band in Panel C corresponding to a molecular weight of 44,000. The arrows indicate standards.

Fig. 3. Isoelectric focusing of human placental cytosolic thymidine kinase activity obtained after step 4 of the purification procedure. Isoelectric focusing with 7% polyacrylamide gels were performed as described under "Experimental Procedures". Activity with ATP (a→g) and with CTP (a→e).

Fig. 4. Double immunodiffusion precipitin reaction of anti-human placental thymidine kinase rabbit serum with various antigens. Immune serum (25 μL) was used in the centre well and various other preparations (25-100 μL) in the peripheral wells of the micro-immunodiffusion plate. The wells are designated 1 at the top and sequentially numbered in the clockwise direction. Well 1 containing crude placenta extract; 2 purified placental thymidine kinase (Step 2); 3 purified placental thymidine kinase (Step 4); 4 crude liver extract; 5 purified human liver thymidine kinase (Step 4); 6 placental mitochondrial extract.