Rat liver dihydrothymine dehydrogenase, the rate-limiting enzyme of thymidine and uridine degradation, was purified to homogeneity as judged by polyacrylamide disc gel electrophoresis, sedimentation velocity, and Ultrogel ACA-34 elution profile. The enzyme has a molecular weight of 220,000 ± 5,000 as determined by Ultrogel ACA-34 and sedimentation equilibrium. The $s_{20,w}$ value of the enzyme was 9.2 S. The isoelectric point was at pH 5.25. The enzyme is composed of two identical subunits of an approximate molecular weight of 110,000 ± 3,000 as determined by sodium dodecyl sulfate disc gel electrophoresis. The enzyme contains 4 mol of FAD and 3 mol of iron per mol of enzyme. Flavin released from the enzyme by boiling was identified as FAD by absorption spectra and thin layer chromatography, indicating that the enzyme is a flavometal protein. During dialysis, the enzyme was stabilized by 2-mercaptoethanol, but neither NADPH nor thymine was effective. The relative rates of reduction of pyrimidine analogues substituted at position 5 were 5-fluorouracil > 5-bromouracil > 5-diazauracil > 5-iodouracil > 5-nitroauracil, with 5-fluorouracil and 5-diazauracil 70% faster than thymine. Uracil was reduced 25% faster than thymine. The pH optimum for the forward and reverse reactions was 7.4. In the presence of NADPH, the apparent $K_m$ was 2.6 mM for thymine and 1.8 mM for uracil. Apparent $K_m$ for NADPH was 15 μM with thymine as substrate and 11 μM with uracil. In the reverse reaction, apparent $K_m$ values were 43 μM for dihydrothymine and 193 μM for dihydrouracil; apparent $K_m$ for NADPH was 3.8 μM with dihydrothymine as substrate and 2.9 μM with dihydrouracil.

Dihydrothymine dehydrogenase (4,5-dihydrothymine: oxidoreductase, EC 1.3.1.12) catalyzes the reaction

Thymine + NADPH $\rightarrow$ dihydrothymine + NADP$^+$ + H$^+$

and was identified as the rate-limiting enzyme in thymidine and uridine degradation in rat liver (1-6). The forward reaction requires NADPH as a hydrogen donor. This enzyme was active with both thymine and uracil (7-13). The biological significance of this enzyme in the degradative pathways of thymidine and uridine was demonstrated by inhibition of the activity of the catabolic pathway and of this enzyme by pyrimidine analogues substituted at position 5 (8, 10, 12, 20, 21). The enzyme activity decreased in all examined rat hepatomas in parallel with the increase in tumor growth rate (13-15). In regenerating (13-16) and differentiating (13-18) liver, enzyme activities were lower than in normal liver. Decreased activity was also reported in mouse colon tumor (19). These results suggest a linking of this enzyme activity with cell proliferation. Dihydrothymine dehydrogenase was purified to varying degrees from liver of beef (7), rat (5), mouse (22), pig (23), from human and pig leukocytes (24), and from bacteria (25). High purification was not achieved in any of these preparations because of the instability of this enzyme, and kinetic characteristics have not been examined in detail. This paper describes the purification of the rat liver enzyme to homogeneity and the evidence that the enzyme is a flavometal protein. The methods for stabilization and the kinetic properties of both forward and reverse reactions are reported.

MATERIALS AND METHODS

The miniprint supplement contains all details under "Materials and Methods."

RESULTS

Enzyme Purification—In a typical purification scheme, a 1400-fold purification was achieved (Table I, miniprint). During purification it was especially important to remove other NADPH-oxidizing enzymes. These enzyme activities were not found after Fraction 6. When the column chromatography of DEAE-cellulose, 2'5'-ADP-Sepharose 4B, or Ultrogel ACA-34 was run in 35 mM potassium phosphate buffer, pH 7.4, containing 2.5 mM MgCl$_2$, 70 to 80% of the activity was lost. Addition of 1 mM NADPH or 20 μM thymine did not prevent the loss of activity, but by addition of 5 mM 2-mercaptoethanol, 80 to 90% of activity was recovered. These results suggest that thiols such as 2-mercaptoethanol are essential for enzyme stabilization. The specific activity of the final fraction was 25 units per mg of protein with thymine as substrate, and with uracil, 30 units per mg of protein were obtained.

Parity—Purified dihydrothymine dehydrogenase was homogeneous by the criteria of polyacrylamide disc gel electrophoresis (Fig. 1A), gel electrophoresis in the presence of SDS$^1$

$^1$ Portions of this paper (including "Materials and Methods," Figs. 2, 4, 5, and 6, and Tables I to IV) 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. 20014. Request Document 80M-710, cite author(s), and include a check or money order for $4.80 per set of photocopies. Full size photocopies are also included in the microfilm edition of the Journal that is available from Waverly Press.

$^2$ The abbreviations used are: SDS, sodium dodecyl sulfate; DHT, dihydrothymine; DHU, dihydrouracil; MTB, 3-(4,5-dimethylthiazolyl)-2,5-diphenyl.
Purification of Rat Liver Dihydrothymine Dehydrogenase

FIG. 1. Polyacrylamide disc gel electrophoresis of purified dihydrothymine dehydrogenase. A, 20 µg of enzyme subjected to electrophoresis using 5% polyacrylamide disc gel; B, 10 µg of enzyme subjected to electrophoresis using 5% polyacrylamide disc gel in the presence of SDS. Electrophoresis was from top (cathode) to bottom (anode) (see under "Materials and Methods," miniprint).

Dye

(Dye)

(Dye)

FIG. 2. Ultrogel ACA-34 elution profile (Fig. 2, miniprint), and sedimentation velocity (Fig. 3). The purified enzyme gave a single, sharp protein band on polyacrylamide disc gel electrophoresis in the absence and presence of SDS. The activity band had an identical mobility with that of the protein band (data not shown). Dihydrothymine dehydrogenase was eluted from Ultrogel ACA-34 column in a symmetrical peak where the specific activity over the peak was nearly constant (Fig. 2, miniprint). The schlieren pattern obtained in sedimentation velocity experiments indicated the presence of a single component, with an $s_{20,w}$ value of 9.2 S (Fig. 3). All studies reported in this paper were conducted with the homogeneous enzyme, except for heat inactivation experiments.

Molecular Weight Determination—The molecular weight of native dihydrothymine dehydrogenase was 220,000 ± 2,000 as determined with calibrated gel filtration. The same activity peak was observed when thymine was replaced by uracil, suggesting that the molecular weights of dihydrothymine dehydrogenase and dihydouracil dehydrogenase were the same. A value of 220,000 ± 3,000 was found in sedimentation equilibrium experiments. A subunit molecular weight of 110,000 was estimated by gel electrophoresis in the presence of SDS (Fig. 4, miniprint). A single band on SDS gels indicated that the subunits were of the same molecular weights. Based on the weight of the native enzyme and enzyme subunit, it was concluded that the molecular weight of the enzyme was 220,000, and it was a dimer consisting of two identical subunits.

Isoelectric Point—The isoelectric point of the enzyme was at pH 5.25 by isoelectric focusing. The elution pattern was symmetrical, suggesting that the enzyme was homogeneous.

Amino Acid Composition—The amino acid composition of dihydrothymine dehydrogenase indicates that the enzyme contained residues of all the amino acids (Table II, miniprint) and showed the usual composition of acidic protein judged by the isoelectric point.

Flavometal Protein Nature of the Enzyme—The purified enzyme in Buffer A was amber in color and showed the characteristic absorption spectrum of a reduced flavoprotein (Fig. 5, miniprint). The absorption spectrum of purified enzyme had maxima at 272 and 375 nm and a shoulder at 450 nm. The absorption ratios were $A_{272}/A_{375}/A_{450} = 4.28:1.19:1$. To examine the nature of the flavin cofactor in the enzyme molecule, the purified enzyme (3 mg in 2 ml of Buffer A) was boiled in a water bath for 10 min in the dark to liberate bound flavin. The $R_F$ values of thin layer chromatography of flavin extracted from the enzyme were compared with authentic riboflavin, FMN, and FAD. The spectrum of the flavin extracted from the enzyme was the same as that of FAD with absorption maxima at 262, 373, and 447 nm (Fig. 5, miniprint). These data suggest that FAD binds to the enzyme. Assuming the enzyme-bound FAD extinction coefficient to be equal to that of free FAD at 450 nm ($11.3 \text{ m}^{-1} \text{ cm}^{-1}$), the FAD content was 3.75 mol per mol of enzyme, suggesting that 2 mol of FAD were bound to the subunit.

Analysis of metals known to be involved in the oxidative-reductive processes, e.g. iron or copper (34), was carried out spectrophotometrically with the purified enzyme, and we found that 3 mol of iron were contained per mol of enzyme. In the purification procedure, the enzyme was eluted from 2'5'-ADP-Sepharose 4B column with the linear gradient of NADPH (0 to 0.1 mM). As shown by Rosenberg and Goswami (35), NADPH causes the reduction of the enzyme. Preparation of the half-reduced form may be due to elution of the enzyme.

FIG. 3. Sedimentation pattern of purified dihydrothymine dehydrogenase. The photograph was taken after 40 min at 55,000 rpm. Protein concentration was 8 mg/ml in Buffer A.
with NADPH. When the enzyme was passed through Ultragel ACA-34, the spectrum remained unchanged, suggesting that NADPH bound tightly to the enzyme molecule. Addition of 20 and 200 μM thymine to the purified enzyme did not cause a change of spectra. When sodium hydrosulfite was added to the purified enzyme, the 375-nm peak and the shoulder at 445 nm extending to longer wavelength were completely eliminated (Fig. 5, miniprint). Attempts to obtain the oxidized form were unsuccessful.

**Stabilization of Dihydrothymine Dehydrogenase**—The purified enzyme dissolved in Buffer A was stable when stored at -20°C; an enzyme solution of 2 mg/ml retained 90% of the activity for 2 weeks. Dilute solutions of the enzyme (below 1 mg/ml), however, were irreversibly denatured; only 10% of activity remained at -20°C. Storage of a sample solution (2 mg/ml) at 4°C resulted in 30% loss of activity in 1 week. Addition of glycerol, NADP+, and thymine failed to prevent loss of activity. Therefore, the purified enzyme was stored at -20°C and dissolved before use.

When the enzyme solution was dialyzed for 24 h against 35 mM potassium phosphate buffer, pH 7.4, 54% of the activity was lost. Addition of 1 mM NADP+ or 20 mM uracil or thymine did not prevent the decrease of activity. During dialysis for 24 h enzyme activity was completely retained by the addition of 5 mM 2-mercaptoethanol or dithiothreitol.

When the enzyme solution in 35 mM potassium phosphate buffer, pH 7.4, and 25 mM MgCl₂ was heated at 45°C, 50% of activity was lost at 2 min (t₁/₂), and 100% was lost by 4 min. Addition of 1 mM NADP+ or 20 mM thymine did not protect, but 20% of enzyme activity was still retained in the presence of 5 mM 2-mercaptoethanol and with a t₁/₂ of 2½ min. The same protection was observed on addition of 5 mM 2-mercaptoethanol and 1 mM NADP+. Stabilization by 2-mercaptoethanol suggests that sulfhydryl groups are essential for the enzyme activity or to maintain the conformation of the enzyme.

**Optimum pH**—With thymine and uracil as substrates, the pH optimum was 7.4 (Fig. 6, miniprint). Enzyme activity in Tris-HCl buffer was about 30 to 40% lower than in phosphate buffer, although Fritson (5) reported no difference in activity when phosphate buffer was replaced by Tris buffer. The optimum pH of the reverse reaction was also 7.4 for the substrates dihydrothymine or dihydrouracil. The same difference in activity was observed when potassium phosphate buffer and Tris-HCl buffer were used.

**Substrate Specificity**—With 20 μM thymine as a standard (100%), the relative rate of uracil reduction was 25% higher (Table III, miniprint). Among pyrimidine analogues substituted at position 5, the relative rates of reduction were 70% and 68% higher with 5-fluorouracil and 5-bromouracil; by contrast, 75%, 77%, and 17% rates of oxidation were found with 5-iodouracil, 5-diazauracil, and 5-nitroauracil, respectively. Similar results were found with 5 μM substrate concentrations.

**Kinetic Properties**—With standard assay conditions at 37°C, the amount of reduction was proportional to the incubation time at a constant enzyme amount of 5 μg of protein for up to 15 min. The rate of reduction was also proportional to incubation time for 5 min and when the protein amount was 10 μg or less. Hyperbolic kinetics were obtained for thymine and uracil with an NADPH concentration of 240 μM. Full saturation of enzyme activity was at 20 μM of substrate. The apparent Kᵣₚ for thymine and uracil was 2.6 and 1.8 μM, respectively. The optimum rate of reduction of thymine or uracil was observed at NADPH concentration of 150 μM, and the apparent Kᵣₚ for NADPH was estimated at 15 and 11 μM with thymine or uracil as substrates. Reversibility of the reduction of thymine or uracil was observed. There was an increase in optical density at 340 nm on addition of enzyme to dihydrothymine or dihydrouracil in presence of NADP+. Proportionality between the rate of oxidation and enzyme amount or time of incubation at 37°C was obtained within the same parameters observed in the forward reaction. The apparent Kᵣₚ values for dihydrothymine and dihydrouracil were 43 and 193 μM with an NADP+ concentration of 0.5 mM. No “blank” reduction of NADP+ occurred in the presence of the enzyme. The apparent Kᵣₚ values for NADP+ were 3.8 and 2.9 μM at 1 mM dihydrothymine and dihydrouracil concentration. Enzyme activity was saturated with 20 μM NADP+. In the reverse reaction, dihydrothymine was oxidized about 4 times more rapidly than dihydrouracil. A summary of kinetic parameters of both reactions is given in Table IV (miniprint).

**DISCUSSION**

We purified dihydrothymine dehydrogenase from rat liver to homogeneity as judged by electrophoretic criteria, elution profile of gel filtration, and sedimentation velocity. It was concluded that the enzyme has a molecular weight of 220,000 and consists of two identical subunits with a molecular weight of 110,000 each. The purified enzyme was free from NADPH and NADP+ converting nonspecific activities. The requirement for NADPH and NADP+ for the forward and reverse reactions is strict; NADH and NAD+ were inactive. Enzyme from pig liver was purified 61-fold, and it was shown that the partially purified enzyme was unstable, since 50% of activity was lost after 24 h. Conventional methods of purification, especially those involving column chromatography with DEAE-cellulose, CM-Sephadex, and CM-cellulose, failed to give satisfactory enrichment of the protein yield (23). In all purifications of the dehydrogenase, including from beef liver (7) and Clostridium uracilicum (25), instability of the enzyme was reported. We found that 5 mM 2-mercaptoethanol and dithiothreitol, 4 to 6 μM, protected the activity, suggesting that sulfhydryl reduction is necessary for retaining the enzyme activity. Indeed, it was demonstrated that addition of dithiothreitol resulted in a significant stimulation of uracil catalysis in mouse liver (22). Goode et al. (24), using Sephadex G-200, suggested the existence of aggregate forms of the enzyme in human leukocytes, but we could not find any other activity peak in the same experiment. However, disc gel electrophoresis of Fraction 7, stored for 2 weeks at 4°C with a saturating level of ammonium sulfate, showed the appearance of several bands with mobilities slower than the enzyme band. This suggests that the enzyme may aggregate at a high protein concentration.

The purified enzyme has an amber color and shows a typical half-reduced flavoprotein spectrum which has maxima at 272 and 375 nm and a shoulder at 445 nm (Fig. 5, miniprint). The oxidized enzyme was not obtained in these purification procedures. This may be due to the use of 2′-ADP-Sepharose 4B column chromatography, in which the enzyme was eluted with a linear gradient of NADPH (0 to 0.1 mM). As indicated by Rosenberg and Goswami in iodotyrosine deiodinase (35), NADPH will bind and reduce the enzyme, resulting in production of a half-reduced spectrum of the enzyme. The 375-nm peak and a shoulder at 445 nm extending to longer wavelengths were eliminated with further reduction of the purified enzyme by sodium hydrosulfite. Flavin removal experiments from the enzyme by boiling and atomic absorption spectrophotometric studies showed that the enzyme contained 4 mol of FAD and 3 mol of iron per mol of enzyme. It is known that FAD-containing enzymes have one or two FAD moieties per mol of enzyme. The significance of high content of FAD in dihydrothymine dehydrogenase is not clear at present.
When compared to the rate of reduction with thymine as a substrate, the 5-halogenated analogues reacted significantly (Table III, miniprint). Of these, the greatest rate of reduction was obtained with 5-fluorouracil, and the lowest with 5-nitroacil; thymine reacted less well than did uracil. In general, our findings on substrate specificity with enzyme purified from rat liver agree with previous reports (5, 7, 20). Newmark et al. (36) and Dorsett et al. (9) described somewhat larger differences for the 5-bromouracil and 5-iodouracil, but in their work 5-bromouracil was also reduced more rapidly and 5-iodouracil less rapidly than uracil. Barrett et al. found an inhibition of pyrimidine degradation by 5-substituted uracils which were more effective inhibitors of uracil than of thymine degradation. They concluded that inhibition occurred only during the initial reductive step in pyrimidine degradation and suggested that the inhibition resulted from substrate competition for the active site on dihydrothymine dehydrogenase (8). Optimum pH of dihydrothymine and dihydrouracil dehydrogenase was 7.4 and that of reverse reaction was also 7.4. No difference in enzyme activity was reported (5) when potassium phosphate buffer was replaced by Tris buffer in the crude extract from rat liver, but lower activity was observed in our purified enzyme when phosphate buffer was replaced by Tris buffer (Fig. 6, miniprint). Similar pH optimum was reported for the enzyme from several tissues (8, 10, 12, 13, 20, 21). Apparent $K_m$ values obtained for our purified enzyme were 2.6 and 1.8 $\mu M$ for thymine and uracil, respectively. Using fixed substrate concentrations of thymine and uracil, apparent $K_m$ values of 15 and 11 $\mu M$ were observed for NADPH, which agree with those of Fritzson (5). Michaelis constants for the reverse reaction were 43 and 193 $\mu M$ for dihydrothymine and dihydrouracil, respectively, in the presence of NADP$.^+$ For NADP$^+$ the constants were 3.8 $\mu M$ with dihydrothymine and 2.9 $\mu M$ with dihydrouracil. Higher $K_m$ values of 1.5 and 2.5 $\mu M$ for dihydrothymine were reported by Fritzson (5) and Smith and Yamada (11), but their enzyme preparation still contained considerable "blank" reduction of NADP$^+$, and enzyme activities with substrate concentrations below $K_m$ values were not assayed. They felt that the actual rate of dihydrouracil oxidation might be much greater. Fritzson (5) postulated that the reverse reaction may be without physiological significance because of the relatively low affinity of the dehydrogenase for dihydrouracil and the slow rate of conversion of dihydrouracil to uracil. In our experiments, the apparent $K_m$ values for dihydrothymine or dihydrouracil were 20 to 100 times higher than those of thymine or uracil. The specific activities of the forward reaction were 30 to 100 times higher than that of the reverse reaction. These results argue for the dominance of the forward reaction over the reverse one. By studying degradation of thymine or uracil to CO$_2$, it was assumed that reduction of both uracil and thymine was catalyzed by the same enzyme, dihydrothymine dehydrogenase (8). Our results establish that dihydrothymine and dihydrouracil dehydrogenases from rat liver cytoplasm are the same enzyme, since the same mobility on polyacrylamide disc gel electrophoresis in activity staining and the same elution pattern on the Ultrogel ACA-34 column chromatography were observed when thymine was replaced by uracil.

Several new characteristics of dihydrothymine dehydrogenase were observed in our studies. First was the purification to homogeneity made possible by the discovery of the stabilizing effect of sulfhydryl reagent on the enzyme. This observation led to the use of column chromatography, and finally we achieved about 1,400-fold purification to homogeneity and estimated the molecular weight as 220,000. Second was the finding that the enzyme is a flavometal protein. Third was the precise comparison of the kinetic properties of this enzyme using the purified enzyme in the forward and reverse reactions and in both substrates. These results established that dihydrothymine and dihydrouracil dehydrogenase are the same enzyme. As the activity of this enzyme was decreased in all examined rat hepatomas, in colon tumor in mouse, and in regenerating and differentiating rat liver (13-19), it should be of interest to purify and compare the differences in the properties of the enzyme from normal and proliferating tissues.

REFERENCES

2. Canellakis, E. S. (1957) J. Biol. Chem. 227, 701-709
Purification of Rat Liver Dihydrothymine Dehydrogenase

Supplemental Materials

Purification and Properties of Dihydrothymine Dehydrogenase from Rat Liver

by Talal Shibly and George Weber

Materials and Methods

Cells
d - MRP, NRMP (27), (thymine, uracil, dihydrothymine) dihydrofuran, DHTC, DACT, and DACTH. The fresh cell fraction and the supernatant were incubated with 50 μM NADPH and 10 μg/ml of DACTH for 2 h at 37°C. The reaction was stopped by the addition of 0.5 ml of 10% trichloracetic acid. The mixture was then centrifuged at 10,000 g for 10 min, and the supernatant was used for the determination of the enzyme activity.

Enzyme Assay

The enzyme activity was determined by measuring the decrease in absorbance at 340 nm due to the formation of dihydrothymine. The reaction mixture contained 50 mM potassium phosphate buffer, pH 7.4, 1 mM DACTH, and 0.1 mg/ml of liver homogenate in a final volume of 1 ml. The decrease in absorbance was measured at 340 nm using a Beckman DU-800 spectrophotometer.

Reactions were conducted at 37°C for 10 min. The reaction was then stopped by adding 0.5 ml of 0.5 M perchloric acid. The mixture was boiled for 10 min, and the supernatant was used for the determination of enzyme activity.

All results are expressed as means ± SD of triplicate determinations. The significance of the differences between the means was assessed by the Student's t-test.

The enzyme activity was expressed as nmoles of dihydrothymine formed per mg of protein.

One unit of enzyme activity was defined as the liberation of 1 nmol of dihydrothymine per min at 37°C.

Enzyme Preparation

The liver homogenate was prepared by homogenizing the liver tissue in 50 mM potassium phosphate buffer, pH 7.4, containing 0.1 M sucrose. The homogenate was centrifuged at 10,000 g for 10 min, and the supernatant was used as the enzyme source.

RESULTS

The purification of dihydrothymine dehydrogenase from rat liver was carried out as described in Materials and Methods. The enzyme was purified to homogeneity by a combination of affinity chromatography and gel filtration. The enzyme was eluted from the columns with a column of DACTH-Sepharose 4B and a column of DACTH-Sepharose 6B. The enzyme was then dialyzed against 0.1 M Tris-HCl buffer, pH 7.4, and stored at -20°C.

Purification of dihydrothymine dehydrogenase from rat liver

The purification procedures are described in Materials and Methods.

Fraction 1: Ammonium sulfate fractionation

The ammonium sulfate fraction was obtained by dissolving 1.2 g of ammonium sulfate in 10 ml of water and then adjusting the pH to 7.4 with 0.1 M NaOH. The solution was then dialyzed against 0.1 M Tris-HCl buffer, pH 7.4, and stored at -20°C. The enzyme solution was then centrifuged at 10,000 g for 10 min, and the supernatant was used as the enzyme source.

Fraction 2: Sepharose 4B chromatography

The enzyme solution was applied to a column of DACTH-Sepharose 4B (45 ml) and was eluted with 0.1 M Tris-HCl buffer, pH 7.4, containing 0.5 M NaCl. The enzyme was eluted at a flow rate of 5 ml/min, and the eluate was collected in 5-ml fractions.

Fraction 3: Sepharose 6B chromatography

The enzyme fractions from Fraction 2 were applied to a column of DACTH-Sepharose 6B (30 ml) and were eluted with 0.1 M Tris-HCl buffer, pH 7.4, containing 0.5 M NaCl. The enzyme was eluted at a flow rate of 5 ml/min, and the eluate was collected in 5-ml fractions.

Fraction 4: Gel filtration

The enzyme fractions from Fraction 3 were loaded onto a column of Sephadex G-200 (2.5 cm x 100 cm) and were eluted with 0.1 M Tris-HCl buffer, pH 7.4, containing 0.5 M NaCl. The enzyme was eluted at a flow rate of 5 ml/min, and the eluate was collected in 5-ml fractions.

Electrophoresis

The enzyme solution was analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and by isoelectric focusing. The enzyme was found to be a single band on both gels.

Stability

The enzyme was stable for at least 6 months at -20°C. The enzyme activity was measured at various pH values and at different temperatures. The enzyme was found to be stable at pH 7.4 and at temperatures ranging from 4°C to 37°C.

DISCUSSION

The purification of dihydrothymine dehydrogenase from rat liver has been achieved by a combination of ammonium sulfate fractionation, affinity chromatography, and gel filtration. The enzyme was purified to homogeneity and was found to be a single band on SDS-PAGE and on isoelectric focusing gels. The enzyme was stable at pH 7.4 and at temperatures ranging from 4°C to 37°C. These results indicate that the purification procedure described here is effective and reproducible.

The enzyme was purified to homogeneity and was found to be a single band on SDS-PAGE and on isoelectric focusing gels. The enzyme was stable at pH 7.4 and at temperatures ranging from 4°C to 37°C. These results indicate that the purification procedure described here is effective and reproducible.
Purification of Rat Liver Dihydrothymine Dehydrogenase

**TABLE III**

Substrate specificity for dihydrothymine dehydrogenase

The specific activity expressed as units/mg is listed for each substrate at 5 and 10 μM.

<table>
<thead>
<tr>
<th>Substrate concentration</th>
<th>5 μM</th>
<th>10 μM</th>
</tr>
</thead>
<tbody>
<tr>
<td>Specific activity</td>
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<td>27.9</td>
</tr>
<tr>
<td>Relative activity</td>
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<td>100</td>
</tr>
</tbody>
</table>

**TABLE IV**

Comparison of kinetic parameters of purified enzyme

Apparent K_m and V_max values were calculated from the double reciprocal plots. Assay conditions are described under "Materials and Methods." The forward reaction means the oxidation of NADPH and the reverse reaction the reduction of NADP⁺. Apparent K_m values were expressed as μM and V_max as units/mg protein.

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Thymine</th>
<th>Uracil</th>
<th>NADPH</th>
<th>Dihydrothymine</th>
<th>Dihydro-uracil</th>
<th>NADP⁺</th>
</tr>
</thead>
<tbody>
<tr>
<td>Apparent K_m</td>
<td>2.6</td>
<td>1.6</td>
<td>15</td>
<td>43</td>
<td>15</td>
<td>0.8</td>
</tr>
<tr>
<td>V_max</td>
<td>25.4</td>
<td>47.3</td>
<td>24.1</td>
<td>46.6</td>
<td>2.5</td>
<td>0.4</td>
</tr>
</tbody>
</table>


Fig. 3: The absorption spectrum of purified enzyme and its redox enzyme and NADP⁺ released from it by boiling. Curve 1, purified enzyme; 2, enzyme reduced by sodium hydrosulfite; 3, NADP⁺ released from purified enzyme by boiling and 1, standard NADP⁺.

Fig. 4: Molecular weight determination of purified dihydrothymine dehydrogenase by SDS polyacrylamide gel electrophoresis. Two kinds of molecular weight markers were used: molecular weight range 16,000 to 86,000 (marker, 12.5 kDa, heavier 95,000). The molecular weight of rat liver dihydrothymine dehydrogenase is approximately 22 kDa. A, Marker, 12.5 kDa; heavier, 95,000. B, Rat liver dihydrothymine dehydrogenase, 22 kDa.