BBA 65739

PYRIMIDINE DEOXYRIBONUCLEOSIDE PHOSPHORYLASE

I. SUBSTRATE INHIBITION BY THYMINE

GEORGE M LYON, JR
Department of Pediatrics, Duke University Medical Center, Durham, N C (U S A)

(Received December 27th, 1967)

SUMMARY

Thymidine phosphorylase (thymidine orthophosphate deoxyribosyltransferase, EC 2.4 2 4) has been approximately 500-fold purified from adult rabbit liver and the kinetics of the conversion of thymine to thymidine, with deoxyribose-1-phosphate as deoxyribosyl donor, are presented The reaction is subject to inhibition by high concentrations of thymine and this inhibition is modified by urea or low pH The inhibition persists when the reaction is carried out in 0 5 M NaCl The effects of both thymine and urea are immediately reversible, and the inhibition by thymine is non-competitive with respect to deoxyribose-1-phosphate

INTRODUCTION

Thymidine phosphorylase (thymidine:orthophosphate deoxyribosyltransferase, EC 2 4 2 4) catalyzes the reversible conversion of thymine to thymidine when deoxyribose-1-phosphate is the deoxyribosyl donor[1] Associated with this phosphorylase activity is a transferase function which uses a pyrimidine deoxyribonucleoside as the deoxyribosyl donor[2] These reactions are shown below

"Phosphorylase"

$$\text{thymine} + \text{deoxyribose-1-phosphate} \underset{\text{degradative}}{\overset{\text{synthetic}}{\rightleftharpoons}} \text{thymidine} + \text{inorganic phosphate} \qquad (1)$$

"Transferase"

$$\text{thymine} + \text{pyrimidine deoxyribonucleoside} \underset{\text{degradative}}{\overset{\text{synthetic}}{\rightleftharpoons}} \text{thymidine} + \text{pyrimidine base} \qquad (2)$$

Neither the relative importance of these reactions, nor their mechanistic relationship is known

Recent studies of the enzyme from *Tetrahymena pyriformis*[3,4] and human leucocytes[5] have demonstrated complex kinetics for these reactions Various substrates

and products of the reactions act as inhibitors, and certain of these inhibitions are modified if the reactions are carried out in the presence of urea or low pH (pH 5.5) (ref 3). This paper describes the characteristics of the synthetic phosphorylase reaction (Reaction 1) as catalyzed by enzyme partially purified from rabbit liver Inhibition by thymine has been observed, and it is modified by urea and pH. Furthermore, sensitivity to thymine is apparently stabilized in the presence of 0.5 M NaCl. The characteristics of the rabbit liver enzyme are similar to those of enzymes from other sources. We have also found that thymidine phosphorylase is precipitated by Zn^{2+}; the enzyme being recovered intact from the precipitate by treatment with EDTA

MATERIALS AND METHODS

Thymine, thymidine, $(NH_4)_2SO_4$, sucrose, and urea were obtained from Mann Research Corp. 2-Deoxy-α-D-ribose-1-phosphate, as the dicyclohexyl ammonium salt, was from the California Corp for Biochemical Research. Imidazole was a product of Eastman Organic Chemicals. Sephadex G-25 (coarse) and G-200 were obtained from Pharmacia and DEAE-cellulose was from Schleicher and Schuell Protamine sulfate was from Lilly, and [2-^{14}C]thymine (20 μC/μmole) was from Schwarz BioResearch, Inc

Enzyme assay

We have used a spectrophotometric assay, which takes advantage of the differential absorption characteristics of thymine, and thymidine, in an alkaline medium[6] The reaction was followed by observing the change in absorbance of the reaction samples with respect to time at $\lambda = 300$ mμ The enzymatic activity was proportional to $\Delta A_{300\ m\mu}$ when $\Delta A_{300\ m\mu} = A_{(sample)} - A_{(zero\ time)}$ The thymidine formed was calculated from the ΔA at 300 mμ $= -3.60$, when one μmole of thymidine was formed The isosbestic point of the pyrimidine base and deoxyribonucleoside spectra, $\lambda = 279$ mμ, was also monitored to assure specificity to the assay. A similar assay has been previously used to quantitate the conversion of uracil to deoxyuridine[4]. The accuracy of the spectrophotometric assay was validated by comparison with a radioactive assay Using [2-^{14}C]thymine as a radioactive marker, incubation samples were obtained simultaneously for each assay. The samples for analysis of radioactivity were deproteinized by the addition of one-third volume of 4 M $HClO_4$, following addition of non-radioactive carrier thymine and thymidine After chilling, the precipitate was removed by centrifugation, and the supernatant was neutralized with 4 M KOH. The precipitated $KClO_4$ was removed by centrifugation; the supernatant was applied to Whatman No. 1 paper, and the chromatograms were developed by descent of the organic phase of equilibrated ethyl acetate–water–formic acid[7] (12:7.1, v/v/v) for 8 h. The chromatograms were air dried and the ultraviolet-absorbing spots were localized using an ultraviolet source (Mineralight) The spots were cut out; pyrimidine was eluted from the paper with water, dried on copper planchets, and counted as described previously[8]. Excellent agreement was obtained between the spectrophotometric and radioactive assays.

The standard reaction mixture contained 0.0025 M thymine, 0.01 M deoxyribose-1-phosphate; 0.05 M imidazole, pH 6.5, and enzyme protein in a final volume of 0.1 to 0.4 ml The reactions were carried out at 37° in stoppered glass tubes containing air. Duplicate 0.015- to 0.04-ml samples were withdrawn immediately following initiation

of the reaction and at various times thereafter, usually at 5 and 10 min The samples were pipetted directly into enough 0 3 M NaOH to give a final volume of 1.0 ml Spectrophotometric readings were taken at $\lambda = 279$ mμ and 300 mμ, using matched silica cuvettes, with a light path of 1 cm, at room temperature, in a Gilford Model 220 spectrophotometer, immediately following completion of the incubation No changes in the readings were noted if the samples were allowed to stand for several hours Sample size was adjusted, when possible, so that the $\Delta A_{300\ m\mu}$ in the first 10 min of the reaction was at least 10% of the starting $A_{300\ m\mu}$ of the reaction sample

Enzyme purification

Livers were obtained from New Zealand White rabbits (2–3 kg) killed by air injection into the marginal vein of the ear. The livers were trimmed of excess fat and connective tissue, the gall bladders were removed, and the livers were stored at −10° until use No loss of enzymatic activity was noted following 6 weeks storage at −10°, although livers which had been stored for longer were discarded

For each purification, 5 to 15 livers were thawed and added to two volumes of 0 05 M imidazole, pH 6 5; 0 001 M EDTA, 0 06 M sucrose The data reported in this paper were obtained from 5 separate purifications. Table I illustrates the results of a typical purification The livers were homogenized in a Waring blendor for 3 min at 4°,

TABLE I

PURIFICATION OF THYMIDINE PHOSPHORYLASE

Enzymatic activity was analyzed by the spectrophotometric method described in METHODS The reaction mixtures contained 0 05 M imidazole, pH 6 5, 0 0025 M thymine, 0 01 M deoxyribose-1-phosphate, and enzyme protein

Fraction	*Volume (ml)*	*Protein concentration (mg/ml)*	*Enzyme units*		*Specific activity (units/mg)*	*Yield (%)*	*Purification*
			total	*per ml*			
I Crude extract	1200	64	847	0 70	0 011	100	—
II 15 000 × *g* supernatant	850	33	653	0 77	0 023	77	2 1
III Protamine sulfate supernatant	650	27	603	0 93	0 034	71	2 6
IV 25–50% saturated precipitate	142	29	552	3 9	0 13	65	11 8
V Zinc sulfate precipitate, dialyzed	17	19	339	19 9	1 05	40	95 5
VI Sephadex G-25	35	4 8	317	9 6	1 9	37	173
VII DEAE-cellulose	5	11	298	59 6	5 4	35	491

and the crude extract was centrifuged for 30 min at 15 000 × *g* in a refrigerated centrifuge The supernatant was decanted, brought to a final concentration of 5 g/l with respect to protamine sulfate, and allowed to chill in an ice bath for 30 min with constant stirring The precipitate was removed by centrifugation and solid $(NH_4)_2SO_4$ was added to 25% of saturation calculated at 4° (ref 9) After chilling for 60 min in an ice bath, the precipitate was sedimented by centrifugation The supernatant was brought to 50% saturation with respect to $(NH_4)_2SO_4$, allowed to chill in an ice bath for 4 h, and the precipitate was recovered by centrifugation at 15 000 × *g* for 30 min

The precipitate was dissolved in a minimal amount of the homogenizing buffer; the solution was made 0 03 M with respect to zinc sulfate (1 M), allowed to chill in an ice bath for 1 h, and the precipitate was recovered following centrifugation at 4° The precipitate was dissolved in 0 05 M imidazole, pH 6 5, 0.05 M EDTA; 0.06 M sucrose and dialyzed overnight at 4° against 100 volumes of the same buffer, containing 0 001 M EDTA The precipitate which formed during the dialysis was removed by centrifugation, and the dialysate was applied to a 1 0 cm × 25 cm Sephadex G-25 (coarse) column which had previously been equilibrated with 0.01 M imidazole, pH 6 5, 0 001 M EDTA; and 0 06 M sucrose. The active fractions were eluted using the same buffer. Enzymatic activity was found in the initial protein-containing fractions eluting from the column The pooled, active fractions were briefly centrifuged to remove the precipitate which formed during the fractionation step, and were applied to a 2 5 cm × 10 cm DEAE-cellulose column which had been equilibrated with the same buffer used in the Sephadex G-25 step The column was washed with 50 ml of the starting buffer. A bright yellow band formed in the top 2 to 3 cm of the column The DEAE-cellulose, which contained this material, was removed from the top of the column and placed in conical centrifuge tubes The material was suspended in a minimal amount of 0 5 M NaCl, 0.05 M imidazole, pH 6 5, 0 001 M EDTA, 0 06 M sucrose, and shaken briefly. The DEAE-cellulose was sedimented by centrifugation, the bright yellow supernatant was decanted and dialyzed overnight against the same buffer without NaCl at 4° Both the Sephadex and the DEAE-cellulose steps were carried out at toom temperature. Recovery of active protein fractions from the DEAE-cellulose, by the method described, has the advantage of yielding enzyme of high specific activity in a relatively small volume The rabbit liver enzyme, like thymidine phosphorylase from other species[2,4], is difficult to concentrate from dilute solutions The adsorption of the enzyme upon the DEAE-cellulose is highly reproducible and is strictly dependent upon the ionic strength of the solution applied to the column Although the yellow color appears to be an excellent indicator for the localization of thymidine phosphorylase activity upon the column, it is probably not associated with the phosphorylase When a sample from this fraction was passed through a Sephadex G-200 column, there is a peak of material absorbing at $\lambda = 420$ mμ which eluted before the peak of enzymatic activity

Protein was determined by the method of LOWRY *et al* [10] using bovine albumin as the protein standard. DEAE-cellulose was prepared by the method of PETERSON AND SOBER[11]. Enzyme activity is routinely expressed as μmoles of thymidine formed per h per mg protein, based upon the initial velocity of the reaction observed

RESULTS

Characteristics of the enzyme and the reaction

The reaction was linear for 20–30 min under the conditions described. At higher concentrations of thymine, in the range of 5 to 10 mM, the reaction was linear up to 1 h. To ensure that only initial, linear velocity rates were obtained, at least two time points, besides the initial time point, were always analyzed Fig 1A shows the results of such an assay Duplicate 0 015-ml samples were taken at the times indicated, and the absorbances at 279 mμ and 300 mμ were determined as described in METHODS The spectrophotometric assay consistently agreed to within 10% of the conversions calculated by the radioactive assay As expected, the spectrophotometric assay be-

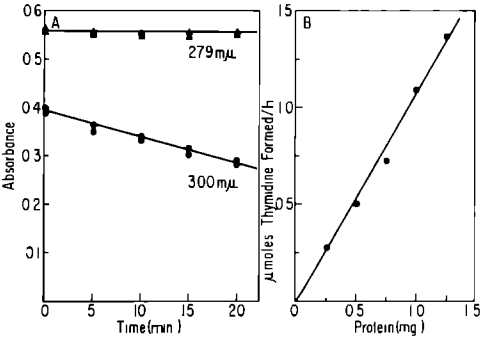


Fig 1A Time course of the conversion of thymine to thymidine The incubation mixture contained 0 05 M imidazole, pH 6 5, 0 0025 M thymine, 0 01 M deoxyribose-1-phosphate, and enzyme protein (zinc precipitate) in a final volume of 0 3 ml 0 015-ml samples were withdrawn, in duplicate, at the indicated times, pipetted directly into 0 3 M NaOH, and the absorbance determined at 279 mμ and 300 mμ, as described in METHODS (▲—▲) describe the readings obtained at 279 mμ, (●—●) the readings at 300 mμ Note the lack of absorbance change at 279 mμ, as compared to the decrease observed at 300 mμ

Fig. 1B Dependence of the thymidine phosphorylase reaction upon the amount of enzyme added Enzyme from the zinc precipitate was used for these determinations Linear results were obtained at all levels of purification For these determinations 0 0025 M thymine and 0 01 M deoxyribose-1-phosphate were used

comes less reliable at very high or low concentrations of thymine, where the ratio of $\Delta A/A_{(\text{zero time})}$ is less than 0.02.

Fig 1B shows the proportionality of added protein to the reaction velocity observed All experiments reported in this paper were carried out within the linear range of protein concentration with respect to velocity

The pH optimum of the reaction is more complex. There is an apparent peak of activity in the range of pH 5 5, as shown in Fig 2. There is consistently a shoulder, or asymmetry, to the curve in the region of pH 6 5 Identical results have been described for thymidine phosphorylase from *T pyriformis*[3] and human leucocytes[5] Experiments elucidating the possible mechanism for this complex pH optimum curve are described below.

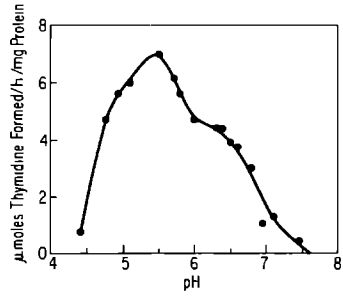
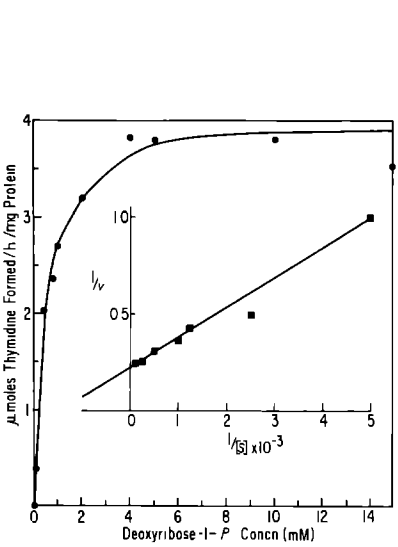


Fig 2 Dependence of the thymidine phosphorylase reaction upon pH The buffer mixture consisted of 1 M Tris, 1 M imidazole; 1 M succinate, adjusted to the desired pH by the addition of 10 M HCl or 10 M NaOH The final concentration of each buffer, in the reaction mixture, was 0 05 M, and the pH of each tube was checked following incubation 0 0025 M thymine and 0 01 M deoxyribose-1-phosphate were used and the enzyme was from the DEAE-cellulose fraction



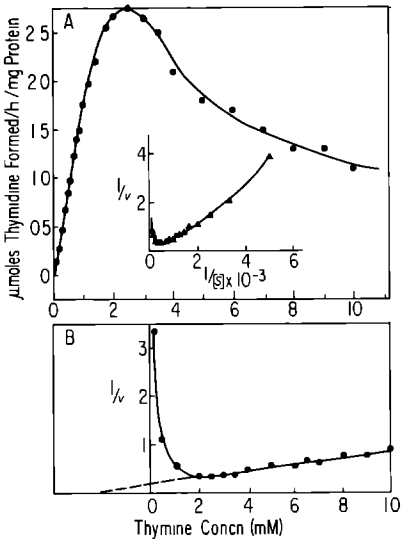


Fig 3 Substrate concentration *versus* velocity curve for deoxyribose-1-phosphate, in the thymidine phosphorylase reaction with a thymine concentration of $2\ 5\ 10^{-3}$ M The observed K_m for deoxyribose-1-phosphate is $7\ 10^{-4}$ M Enzyme was from the DEAE-cellulose fraction

Fig 4A Velocity *versus* substrate concentration curve for thymine in the thymidine phosphorylase reaction at a deoxyribose-1-phosphate concentration of 0 01 M and 0 05 M imidazole, pH 6 5 Enzyme was from the Sephadex G-25 fraction Adequate absorbance changes were obtained from the samples at very low thymine concentrations by using larger sample volumes (0 04 ml) and incubation times of 2 5 and 5 min

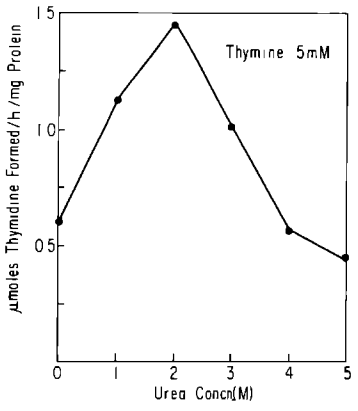
Fig 4B A reciprocal of the velocity *versus* substrate concentration plot of the data presented in Fig 4A Here, the linear portion of the curve can be extrapolated to the abscissa and an apparent K_s' for thymine of $2\ 5\ 10^{-3}$ M is obtained All of the experimental points obtained at low thymine concentrations are not plotted on this graph

Kinetics of the reaction

Hyperbolic kinetics are obtained for deoxyribose-1-phosphate with a thymine concentration of $2.5 \cdot 10^{-3}$ M (Fig 3) The apparent K_m for deoxyribose-1-phosphate is $7 \cdot 10^{-4}$ M Fig 4A shows excess substrate inhibition by high concentrations of the pyrimidine base, thymine Maximal activity was found at a thymine concentration of $2.5 \cdot 10^{-3}$ M The inhibition was also observed if uracil was substituted for thymine. A plot of the data on reciprocal coordinates yields a line which is again typical of excess substrate inhibition We are attempting to further characterize the reaction at very low concentrations of thymine using both spectrophotometric and radioactive assays Fig. 4B shows the data presented in Fig. 4A plotted as the reciprocal of the velocity *versus* substrate concentration Here, the expected linearity of the plot at high thymine concentrations is observed, and an apparent $K_s' = 2.5 \cdot 10^{-3}$ M is calculated for thymine[12]. All of the thymine concentration curves were carried out in 0.01 M deoxyribose-1-phosphate

Modifications of the substrate inhibitions

Urea has been found to stimulate the synthetic thymidine phosphorylase reaction at all concentrations of thymine when assayed at pH 6 5. The stimulation is



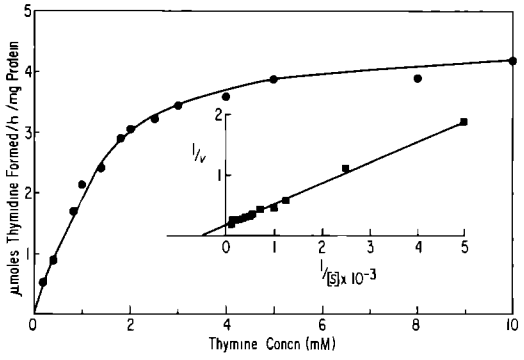


Fig 5 Effect of urea on thymidine phosphorylase activity at an initial thymine concentration of $5 \cdot 10^{-3}$ M The incubations were carried out in the indicated concentrations of urea and the activity was determined spectrophotometrically, as described in the text The reactions were initiated by the addition of enzyme protein, and were incubated for 10 min at 37° Zinc-precipitated enzyme was used

Fig 6 Velocity *versus* substrate concentration curve for thymine in the thymidine phosphorylase reaction in the presence of 2 M urea, at a deoxyribose-1-phosphate concentration of 0 01 M Enzyme protein was from the Sephadex G-25 fraction The K_m for thymine was calculated to be 0 002 M

maximal at a final concentration of 2 M urea, and there is inhibition of enzymatic activity at higher concentrations of urea Fig 5 shows the effect of different concentrations of urea found with an initial concentration of $5 \cdot 10^{-3}$ M thymine. There is progressive inactivation of the enzyme in the presence of 2 M urea, although the apparent half-life of the enzyme is approximately 2–3 h at 37° (G LYON, unpublished observation). ZIMMERMAN[2], studying the effect of urea upon the conversion of thymidine to thymine by enzyme from human spleen, also found inactivation of the enzyme in urea.

As in *T. pyriformis*[3] urea affects the thymine-induced substrate inhibition of rabbit liver enzyme (Fig 6). In the presence of 2 M urea there is an apparent conversion of the reaction kinetics to the hyperbolic form. Essentially normal kinetics are observed over the range of thymine concentrations which produce marked substrate inhibition in the absence of urea We are currently investigating the possibility that there may be substrate inhibition at very high concentrations of thymine (0 02 M) even in the presence of urea Because of the difficulty of making spectrophotometric determinations at these high levels of thymine substrate inhibition cannot as yet be ruled out Although the non-linearity of the LINEWEAVER–BURK[13] plot makes a definite assignment of K_m for thymine difficult, under standard conditions the K_m can be estimated to be between $1 \cdot 10^{-3}$ and $3 \cdot 10^{-3}$ M thymine This is not significantly different from the K_m obtained either in the presence of urea ($2 \cdot 10^{-3}$ M) or at pH 5.5 ($2\ 3 \cdot 10^{-3}$ M). Therefore, changes in the velocity of the reaction observed at different pH values and under the various conditions are not due to alteration in the affinity of enzyme for substrate but most likely are the result of modifications of the inhibitory properties of thymine

We next examined the effect of NaCl upon the enzyme activity We had noted that in the presence of 0.5 M NaCl there was stimulation of enzymatic activity at

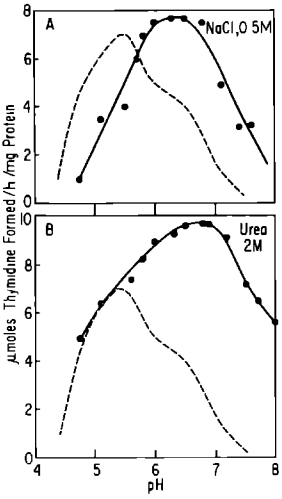


Fig 7A Effect of 0 05 M NaCl upon the pH optimum curve The assays were identical to those described in the legend of Fig 2, except that NaCl was added to each reaction mixture to a final concentration of 0 5 M The dashed line represents the activities found when the reactions are carried out in the absence of NaCl The same enzyme fraction was used for the experiments represented in Fig 4 as well as for Figs 7A and 7B, and maintained constant activity over the period required for completion of the studies

Fig 7B Effect of 2 M urea upon the pH optimum curve The assays were identical to those described in the legend of Fig 2, except that urea was added to each reaction mixture to a final concentration of 2 M Again, the same enzyme fraction and assay conditions were employed, and the dashed line represents enzymatic activity observed under standard conditions Thymine concentration was 0 0025 M and deoxyribose-1-phosphate was 0 01 M for all of the pH optimum studies

pH 6 5 and inhibition at pH 5.5 when compared to reactions carried out under the standard conditions (see METHODS) Fig 7A shows the pH curve obtained in the presence of 0.5 M NaCl In the presence of NaCl the pH optimum is shifted up to 6 5

A similar analysis of enzyme activity as a function of pH was carried out in the presence of 2 M urea, as shown in Fig 7B There is stimulation of enzyme activity over

TABLE II

EFFECT OF 0 5 M NaCl ON SUBSTRATE INHIBITION

The reaction mixtures contained 0 05 M imidazole, pH 6 5, 0 01 M deoxyribose-1-phosphate, and thymine at the concentration indicated The concentration of NaCl is indicated and enzyme was from the Sephadex G-25 fraction

Assay conditions	*μmoles thymidine formed per h per mg protein*	*Percent inhibition*
Thymine, 0 0025 M	1 82	—
Thymine, 0 008 M	1 04	43
Thymine, 0 0025 M + 0 5 M NaCl	2 28	—
Thymine, 0 008 M + 0.5 M NaCl	1 51	34

the pH range observed, when compared to the normal assay, except in the region of pH less than 5 5. A broad pH optimum peak between pH 6.5 and 6 8 is seen, and, again, the curve has become essentially symmetrical

The effect of 0 5 M NaCl upon the substrate inhibition was examined Table II presents data indicating that, in the presence of NaCl, substrate inhibition is observed, and, although there is stimulation, the relative amount of substrate inhibition is essentially unchanged Table III shows experiments examining the effect of pH 5 5 upon the substrate inhibition At pH 5 5 there is relatively little substrate inhibition, when compared to the inhibition seen at pH 6 5 Other studies have shown that in the presence of 0 5 M NaCl there is substrate inhibition at pH 5 5 to approximately the same degree as seen at pH 6 5 Thus, the complex pH optimum curves observed under standard assay conditions may be due to transition of enzyme from a substrate-inhibited to a non-substrate-inhibited form at lower pH

TABLE III

EFFECT OF pH 5 5 ON THE SUBSTRATE INHIBITION

The reaction mixtures contained 0 05 M each of Tris–imidazole–succinate, adjusted to pH 6 5 or 5 5, deoxyribose-1-phosphate, 0 01 M, enzyme protein from the zinc precipitation fraction, and thymine at the concentration indicated

Assay conditions	*μmoles thymidine formed per h per mg protein*	*Percent inhibition*
Thymine, 0 0025 M, pH 6 5	1 18	—
Thymine, 0 006 M, pH 6 5	0 53	55
Thymine, 0 0025 M, pH 5 5	1 71	—
Thymine, 0 006 M, pH 5 5	1 55	9

Experiments designed to demonstrate physical alteration of the enzyme under conditions of high ionic strength or urea have not been successful. When identical samples of enzyme were passed through the same Sephadex G-200 column which had previously been equilibrated with homogenizing buffer, containing 0 5 M NaCl, 2 M urea, or no additions, no significant alterations in the elution patterns of the enzymatic activity were noted Because of the inactivation of the enzyme by urea, prolonged centrifugations or elutions are not feasible Currently, we are attempting to take advantage of the absence of substrate inhibition at low pH and, using sucrose density gradient centrifugations, to demonstrate possible alterations in the sedimentation characteristics of the enzyme at different pH

The effects of both urea and thymine appear to be immediately reversible. If enzyme protein was preincubated for 10 min at 37° in 2.0 M urea or $6 \cdot 10^{-3}$ M thymine, in the absence of deoxyribose-1-phosphate, subsequent enzyme activity was dependent upon the final concentration of urea or thymine present in the complete reaction mixture, when assayed immediately following completion of the preincubations. Thus, enzyme which was preincubated in $6 \cdot 10^{-3}$ M thymine, then assayed in 2 5 10^{-3} M thymine or 6 10^{-3} M thymine, was inhibited only at $6 \cdot 10^{-3}$ M thymine. Similarly, enzyme preincubated in 2 M urea was inhibited by thymine when the concentration

of urea was diluted to 0.5 M for the assay Preincubation at 37° for 10 min did not alter the activity of the enzyme, even in the presence of 2 M urea. Also, enzyme inhibition by thymine does not appear to be dependent on the concentration of deoxyribose-1-phosphate in the reaction mixture, as shown by the data presented in Fig 8 Thus, the excess substrate inhibition by thymine appears to be non-competitive with respect to deoxyribose-1-phosphate

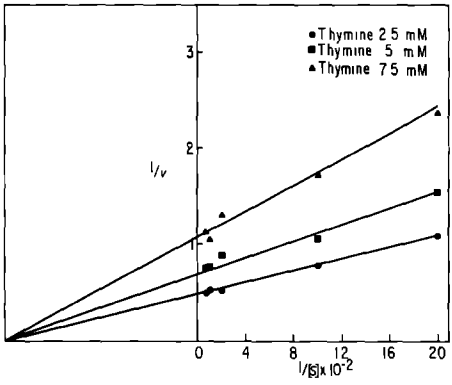


Fig 8 Inhibition of the thymidine phosphorylase reaction by thymine at varying concentrations of deoxyribose-1-phosphate Enzyme was from the Sephadex G-25 fraction, and the assays were carried out in 0 05 M imidazole, pH 6 5, at different concentrations of thymine Thymine concentration was 0 0025 M (●—●), 0 005 M (■—■) and 0.0075 M (▲—▲) The data suggest that the inhibition by thymine is non-competitive with respect to deoxyribose-1-phosphate

DISCUSSION

The data presented in this paper suggest that the enzymatic characteristics of the synthetic thymidine phosphorylase reaction (Reaction 1), catalyzed by rabbit liver enzyme, are essentially the same as those described in *T. pyriformis*[3] The finding that the enzyme is precipitated by Zn^{2+}, and is recovered apparently intact following treatment with EDTA, provides a method of obtaining high yields of the enzyme from relatively dilute solutions.

These investigations further indicate that the complex pH optimum curves, which are characteristic of thymidine phosphorylase from rabbit liver, as well as enzyme from *T pyriformis*[3] and human leucocytes[5], can be resolved into two components by addition of either NaCl (final concentration, 0.5 M) or urea (final concentration, 2 M) to the reaction mixture In the presence of NaCl there is substrate inhibition by thymine throughout the pH range observed, whereas in the presence of urea, no substrate inhibition is observed. It is suggested that the pH optimum curve obtained under standard assay conditions, that is, in the absence of NaCl and urea, is a composite. The shoulder of activity in the range of pH 6.5 may be the true pH optimum, and the rise in activity at lower pH may be due to escape of the enzyme from substrate inhibition, the curve assuming the configuration of the enzyme's pH *versus* activity curve when studied in the presence of urea. Although preliminary attempts to demonstrate structural alterations in the enzyme under these different conditions have not been

successful, a more critical analysis of the physical characteristics under these modifying conditions has been initiated

Analysis of the kinetic data by the Hill equation[14] reveals that for thymine concentrations of less than 2.5 10^{-3} M the slope is 1.22 In the region corresponding to 2.5 10^{-3} M thymine there is a break in the curve and at higher concentrations of thymine the line has a negative slope equal to -1.04 When carried out in the presence of 2 M urea, the slope of the curve at low levels of thymine is equal to 1.14 and at $2.5 \cdot 10^{-3}$ M thymine, there is also a break in the curve and a positive slope of less than unity is found. The finding of a Hill coefficient of less than one has previously been reported[15] In the case of thymidine phosphorylase it may suggest that urea only partially modifies the substrate inhibition and the resultant slope is due to both catalytic (positive) and inhibitory (negative) functions being observed These findings suggest that both the catalytic and the inhibitory functions occur at single distinct loci Whether or not there are separate sites cannot be definitely determined The finding that urea and low pH affect the substrate inhibition without appreciably altering the K_m for thymine may suggest separate sites for these phenomena.

The functional significance of these findings cannot be assessed until the kinetic properties of the reaction are fully understood Preliminary experiments do suggest, however, that when excess substrate inhibition is modified, there is a concomitant inhibition of the transferase reaction (Reaction 2) ZIMMERMAN[2] has reported that in the presence of 2 M urea there is significant inhibition of transferase activity, with relatively less inhibition of degradative phosphorylase activity (Reaction 1). Conceivably, the inhibitions may function to control the rate of activity of the phosphorylase and transferase reactions, depending upon the amounts of substrates and products which are available to the enzyme. Control of enzymatic activity by the relative concentrations of the substrates and products of a reaction may be an important intracellular regulatory mechanism[16] Experimental attempts to further document the enzymatic and regulatory characteristics of thymidine phosphorylase are currently in progress in this laboratory

ACKNOWLEDGEMENTS

The author wishes to thank Dr. O. W JONES for his aid and encouragement during the course of these experiments, and Drs J S. HARRIS, I FRIDOVICH, and J B SIDBURY for their helpful evaluations of the manuscript Part of this project was supported by an institutional research grant to Duke University by the American Cancer Society.

REFERENCES

1 M FRIEDKIN AND D ROBERTS, *J Biol Chem*, 207 (1954) 245
2 M ZIMMERMAN, *J Biol Chem*, 239 (1964) 2622
3 G LYON AND E. ANDERSON, *Federation Proc*, 26 (1967) 843.
4 G LYON AND E ANDERSON, in preparation
5 R GALLO, S PERRY AND T BREITMAN, *J Biol Chem*, 242 (1967) 5059.
6 R HOTCHKISS, *J. Biol Chem*, 175 (1948) 315
7 K FINK, R CLINE, R HENDERSON AND R FINK, *J Biol Chem*, 221 (1956) 425
8 E ANDERSON AND W BROCKMAN, *Biochim Biophys Acta*, 91 (1964) 380

9 A GREEN AND W HUGHES, in S. P COLOWICK AND N O KAPLAN, *Methods in Enzymology*, Vol 1, Academic Press, New York, 1955, p 76
10 O H LOWRY, N J ROSEBROUGH, A. L. FARR AND R J RANDALL, *J. Biol Chem*, 193 (1951) 265
11 E PETERSON AND H SOBER, in S P COLOWICK AND N O KAPLAN, *Methods in Enzymology*, Vol 5, Academic Press, New York, 1962, p 3
12 M DIXON AND E C WEBB, *Enzymes*, 2nd ed , Academic Press, New York, 1964, p 78
13 H LINEWEAVER AND D BURK, *J Am. Chem Soc*, 56 (1934) 658
14 D ATKINSON, J HATHAWAY AND E SMITH, *J Biol Chem*, 240 (1965) 2682
15 C WOOLFOLK AND E STADTMAN, *Arch Biochem Biophys*, 118 (1967) 736
16 A WILSON AND A B PARDEE, in M. FLORKIN AND H MASON, *Comparative Biochemistry*, Vol 6, Academic Press, New York, 1963, p 73