Uridine Phosphorylase, Molecular Properties and Mechanism of Catalysis†

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ABSTRACT: Uridine phosphorylase of the cytosol fraction of rat liver was purified to electrophoretic homogeneity. It was found to be a tetrameric protein with subunits of 26,000 molecular weight. The enzyme contained 24 half-cystine residues and 12 histidine residues. The enzyme was much less stable in Tris buffer than in phosphate buffer and new electrophoretic bands appeared after storage at −40°. Isotope exchange studies under steady-state conditions confirmed the ordered bi-bi mechanism proposed earlier (Kraut, A., and Yamada, E. W. (1971), *J. Biol. Chem. 246*, 2021) in which phosphate binds to the enzyme before uridine and uracil leaves the enzyme before ribose 1-phosphate. The rate equation for the exchange reaction was derived. The equilibrium constant for the phosphorolysis of uridine was calculated to be 0.031 at pH 7.4 and 0.078 at pH 8.2. From log V_m and pK_m plots *vs.* pH, histidyl and sulfhydryl residues were implicated in the enzyme–substrate complex. A model outlining the catalysis mechanism was formulated.

Uridine phosphorylase, because of its role in the degradation of pyrimidine nucleosides as well as in the "salvage" pathway of nucleic acid synthesis (Kornberg, 1957), occupies an important amphibolic position in metabolism. That it is an enzyme distinct from thymidine phosphorylase was shown for several mammalian tissues (Krenitsky *et al.*, 1964; Yamada, 1968) as well as for bacteria (Razzell and Khorana, 1958).

Uridine phosphorylase resides mainly in the cytosol fraction of rat liver (Yamada, 1968) and has been extensively purified and characterized by initial velocity and product inhibition studies as to the ordered bi-bi mechanism[1] it catalyzes (Kraut and Yamada, 1971). The reverse order of addition of substrates and release of products was proposed for uridine phosphorylase of guinea pig intestine (Krenitsky, 1968) on the basis of pentosyl transferase activity; the interpretation of these data was not, however, based on rigorous mathematical analysis. With this discrepancy in mind the rat liver enzyme was investigated further in present work. The molecular and kinetic data obtained were then integrated into a model for the catalysis mechanism. At the present time the most detailed model on the mechanism of action of this enzyme revolves about an explanation for the inversion of configuration of C′-1 after cleavage of the β-*N*-glycosidic linkage (Koshland, 1959).

Experimental Section

Animals. Frozen livers (50–100) from young rats (Sprague-Dawley, fasted 24 hr prior to death, Pel-Freeze, Inc.) were used for each of six enzyme purifications. The livers weighed between 5 and 7 g each.

Enzyme Assays. Kinetic constants were determined by the computer program described by Cleland (1967a).

PHOSPHORYLASE. Uridine and thymidine phosphorylase activities were determined by the spectrophotometric procedures of Yamada (1968). One unit of activity is defined as that amount of enzyme that is required to form 1 μmol of free base/hr. Specific activity is defined as the number of enzyme units per milligram of protein. Protein was determined usually by the method of Lowry *et al.* (1951) with crystallized bovine serum albumin (Mann) as reference standard; however, the method of Warburg and Christian (1942) was used for fractions separated by column chromatography.

PENTOSYL TRANSFERASE. The methods of Gallo *et al.* (1967) and De Verdier and Potter (1962) were modified as described before (Kraut and Yamada, 1971). The reaction was initiated by the addition of labeled substrate; incubation was for 30 min at 37°. The tubes were then placed in a boiling-water bath for 4 min. The reaction mixture (10–20 μl) was applied to strips of Whatman No. 1 filter paper (1.8 × 35 cm). The strips were equilibrated for 2.5 hr in the aqueous phase of the solvent (ethyl acetate–water–formic acid (12:7:1)) used by Fink *et al.* (1965) and then developed for 3 hr. In this solvent uracil (R_F 0.24) was separated from uridine (R_F 0.07), deoxyuridine (R_F 0.12), and thymine (R_F 0.37) but not from thymidine (R_F 0.25). These compounds were eluted with water and counted in a Beckman LS-250 scintillation spectrometer. Activity is expressed as the micromoles of substrate transferred per hour per milliliter of incubation medium.

Disc Gel Electrophoresis. AT pH 8.3. The method of Davis (1964) was followed. Routinely, 7% acrylamide was used; sample gel was omitted and enzyme preparations (0.02–0.4 mg of protein/ml of 4% sucrose) were applied in volumes of 50–100 μl. Electrophoresis was performed in Tris–glycine buffer (pH 8.3) in a cold room at 2–4° with a current of 2.5 mA/gel for 2–3 hr or until the tracking dye migrated to the end of the gel.

Protein bands in the gels were stained with Coomassie Blue (Chrambach *et al.*, 1967). A Joyce–Lobel Chromoscan was used for densitometer tracings of the developed gels.

AT pH 7.0. The method of Williams and Reisfeld (1964) was used. Spacer gel was prepared in Tris-HCl buffer (pH 5.5) and separation gel (7% acrylamide) in Tris-HCl buffer (pH 7.5). Electrophoresis was performed in Tris–barbiturate buffer (pH 7.0) as described for the pH 8.3 system.

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‡ Submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy.

[1] The nomenclature of Cleland (1963) is used throughout.

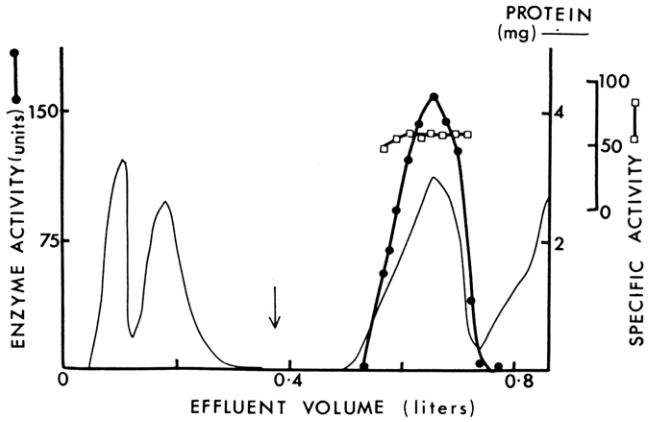


FIGURE 1: Hydroxylapatite chromatography at pH 7.3, the final step of purification. The sample contained 236.5 mg of protein and 2609 units of uridine-cleaving activity of which 87% was recovered. Bed volume was 137 ml and flow rate, 22 ml/hr. The vertical arrow indicates the place at which a linear gradient of P_i buffer (0.02–0.15 M) was started. Enzyme activity was assayed at pH 7.4 with uridine as substrate by the standard assay.

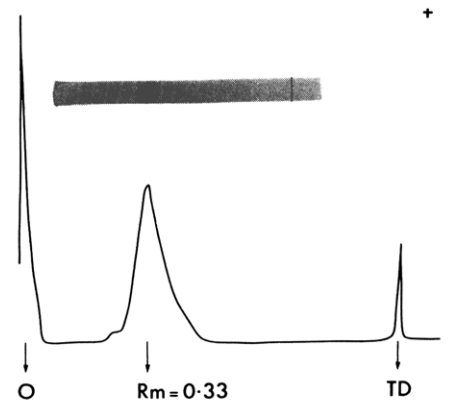


FIGURE 2: Densitometer tracing of gel after electrophoresis of purified uridine phosphorylase (15.6 μg of protein, 0.77 unit[2]) in 7% acrylamide in the pH 8.3 system. Gel stained with Coomassie Blue; the vertical arrows indicate the origin (O), position of the protein and relative mobility (R_m), tracking dye (TD), anode (+), and cathode (−). Above the tracing is a photograph of the developed gel.

DETECTION OF ENZYME ACTIVITY. The procedure was an adaptation of that developed for purine nucleoside phosphorylase (Gardner and Kornberg, 1967). After electrophoresis the gels were incubated for 30 min at 37° in 3 ml of the standard incubation mixture (Yamada, 1968) in which the P_i buffer was replaced by potassium arsenate buffer. After treatment with alkali the gels were stained with triphenyltetrazolium chloride. At least 0.5 unit of uridine-cleaving activity was required to give significant staining. Appropriate controls lacking uridine were prepared with each test.

MOLECULAR WEIGHT DETERMINATION. The method of Hedrick and Smith (1968) was used for purified enzyme fractions. Marker proteins included apoferritin, catalase, γ-globulin, alcohol dehydrogenase, and serum albumin. Gel concentrations were from 4 to 9%.

SUBUNIT COMPOSITION. Electrophoresis was performed in 0.1% sodium dodecyl sulfate–4 M urea–sodium phosphate buffer (pH 7.2) and 10% acrylamide according to the method of Weber and Osborn (1969). Current was maintained at 7 mA/tube. Enzyme fractions (500 μg of protein/ml) were pretreated with 0.5% β-mercaptoethanol (v/v)–4 M urea–0.5% sodium dodecyl sulfate in 0.05 M sodium phosphate buffer (pH 7.2) at 37° for 4 hr prior to electrophoresis according to the method of Dunker and Reuckert (1969). The developed gels were placed in 12% acetic acid overnight to leach out sodium dodecyl sulfate prior to staining with Coomassie Blue. Marker proteins used for a standard plot of migration relative to molecular weight in 10% acrylamide included γ-globulin, serum albumin, ovalbumin, chymotrypsinogen, myoglobin, hemoglobin, and cytochrome *c*.

Amino Acid Composition. Samples containing 1.5 mg of protein were hydrolyzed in 6 N HCl at 110° in sealed, evacuated tubes for 22 hr. Amino acid analysis was carried out on a Spinco Model 120C amino acid analyzer by the method of Spackman *et al.* (1958) as outlined in the Spinco manual. Additional samples were oxidized with performic acid as described by Hirs (1956) prior to hydrolysis in 6 N HCl for the determination of cysteine and cystine residues.

Results

Enzyme Purification. The procedures of Kraut and Yamada (1971) were followed except that routinely enzyme fractions were stored in P_i buffer (0.05 M potassium phosphate (pH 7.0)–1 mM EDTA (pH 7.0)–10 mM β-mercaptoethanol) and storage in Tris buffer was avoided. As before, approximately 1500-fold purification was achieved. A representative elution profile during the last step of purification is shown in Figure 1. It is apparent that specific activity was quite constant over the peak of enzyme activity indicating that a high degree of homogeneity had been achieved. The specific activity at pH 7.4 of the purified preparations was in the ratio of 10:6.3:1 for the substrates, uridine, deoxyuridine, and thymidine, respectively.

In one preparation activity was eluted from DEAE-Sephadex (pH 7.0) (step 6) by a shallower salt gradient (linear from 0 to 0.2 M KCl) than that used previously. Under these conditions and because elution was prolonged over several days completely distinct peaks of uridine phosphorylase activity appeared. These peaks were not, however, distinguishable upon electrophoresis and were thought to be artefacts of the experimental procedure.

Disc Gel Electrophoresis. HOMOGENEITY OF PURIFIED ENZYME. The active fractions from the final step of purification were concentrated, dialyzed and subjected to electrophoresis. A densitometer tracing of a developed gel is shown in Figure 2. A single band of protein was discernible at either pH 8.3 or 7.0 which also stained for activity with either uridine or deoxyuridine as substrate. The R_m (relative mobility) of the active band was 0.33 (range 0.33–0.36) in the pH 8.3 system and 0.30 (range 0.28–0.31) in the pH 7.0.

Unless specified otherwise enzyme fractions of this degree of purity were used in all subsequent experiments.

MOLECULAR WEIGHT DETERMINATION. The relationship between acrylamide concentration and R_m for uridine phosphorylase was determined according to the method of Hedrich and Smith (1968). The slope (±SE) of the resulting linear plot, calculated by unweighted linear regression analysis, was 6.5 (±0.3). The molecular weight of the enzyme was calculated to be 102,500 (±8500).

SUBUNIT COMPOSITION. After incubation of the purified enzyme for 4 hr in buffer containing sodium dodecyl sulfate, urea, and β-mercaptoethanol, four protein bands were separated upon electrophoresis in 10% acrylamide. The molecular weights of these proteins were estimated from a standard plot

[2] In the legend to figures, enzyme units are given in terms of uridine-cleaving activity.

to be 104,500, 75,500, 51,500, and 25,750. Incubation of the purified enzyme in 6 M urea + 0.5% sodium dodecyl sulfate in the absence of β-mercaptoethanol, resulted in no detectable dissociation of the enzyme into subunits. These data indicate that uridine phosphorylase is a tetrameric protein with four subunits of a molecular weight close to 26,000; disruption of disulfide linkages facilitated subunit dissociation. The enzyme resembles purine nucleoside phosphorylase of *Bacillus cereus* (Gilpin and Sadoff, 1971) but not purine nucleoside phosphorylase of man which was suggested to be a trimeric protein (Edwards *et al.*, 1971).

EFFECTS OF STORAGE. Earlier, enzyme fractions, purified as much as 1900-fold, still contained two proteins bands upon electrophoresis (Kraut and Yamada, 1971). These enzyme fractions had been dialyzed and stored in Tris buffer (0.05 M Tris-HCl (pH 7.0)–5 mM β-mercaptoethanol). In present work all enzyme activity was found to be lost after storage for 4 weeks at −40° in Tris buffer in concentrations of 0.2–2.3 mg of protein/ml. On the other hand, comparable samples stored in P_i buffer (P_i replacing Tris) still retained 90% of their activity after 6 weeks. Further, fractions suspended in Tris buffer and heated at 50° for 20 min lost all activity whereas comparable fractions heated in P_i buffer lost only 10% of their activity.

This instability in Tris buffer was found to be reflected in the appearance of the new more rapidly migrating protein species observed earlier (Kraut and Yamada, 1971). Both bands remained after reactivation of the enzyme (for 90 min at 25° in 0.05 M potassium phosphate buffer (pH 7.0)–0.075 M β-mercaptoethanol) to 25% of its original activity; enzyme activity was now discernible in a single peak associated with the original protein band. The active band retained its original molecular weight but the molecular weight of the new protein species determined by electrophoresis at different acrylamide concentrations (Hedrick and Smith, 1968) was almost double and this species was resistant to reactivation.

Additional small protein bands also appeared upon electrophoresis after storage for 7 weeks at −40° in P_i buffer despite the fact that the enzyme still retained 70–80% of its original activity. Slight changes in electrophoretic patterns were discernible after about 1 week and became more prominent as the aging process was prolonged.

The new protein bands which formed upon storage were mainly isomeric proteins of similar charge but of different size (*i.e.*, enzyme aggregates) (Hedrick and Smith, 1968) although there was also some dissociation into lower molecular weight species. Activity was associated with the major protein band. The electrophoretic patterns were somewhat variable depending on protein concentration and the aging period. Routinely then, enzyme fractions were stored in P_i buffer at −87° and dialyzed just prior to electrophoresis.

Amino Acid Analysis. The amino acid composition of uridine phosphorylase was determined after hydrolysis of duplicate samples. There appeared to be no unusual amino acid ratios and the composition resembled that of purine nucleoside phosphorylase of *Bacillus cereus* (Engelbrecht and Sadoff, 1969). Noteworthy was the high percentage of half-cystine residues (24 per mole) and histidine residues (12 per mole).

Kinetic Studies. LOG V_m, pK_m, AND pH. Dixon plots (1953) relating changes in kinetic parameters with pH are shown for uridine phosphorylase (Figure 3). In the direction of phosphorolysis with uridine as substrate a histidyl residue (pK_{es} = 7.0) as well as a sulfhydryl group (pK_{es} = 8.0) are implicated in the enzyme–substrate complex (Figure 3A). That a sulfhydryl group (pK_e = 7.4) dissociates on the free enzyme is inferred from the plot of pK_m *vs.* pH (Dixon and Webb (1964)).

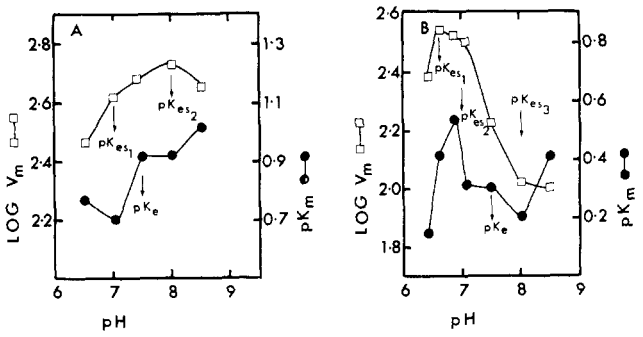


FIGURE 3: Log V_m and pK_m plots of uridine phosphorylase *vs.* pH. Kinetic parameters were calculated from double-reciprocal plots of initial velocity (nmol of uracil/ml per hr) against nucleoside concentration (mM) at saturating levels of P_i (0.110 M). The standard assay was adjusted to the required pH by either 150 μl of 1 M acetate buffer (pH 6–6.5) or 150 μl of 1 M Tris-HCl (pH 6.8–9). Fifty microliters of 1.0 M glycylglycine buffer were also present at pH values over 8.0. Each tube contained 34.5 μg of enzyme protein (1.69 units). The vertical arrows indicate the values of pK_{es} and pK_e, the pH values at which an enzyme–substrate complex or free enzyme is half-ionized, respectively: (A) uridine was varied over the range of 0.016–3.33 mM; (B) deoxyuridine was varied over the range of 0.33–0.66 mM.

With deoxyuridine as substrate the participation of P_i (pK_{es} = 6.5), a histidyl (pK_{es} = 7.0) and a sulfhydryl group (pK_{es} = 8.0) in the enzyme–substrate complex can be seen (Figure 3B). As well, from the pK_m *vs.* pH plot a sulfhydryl group dissociating in the free enzyme (pK_e = 7.4) is apparent. The apparent K_m values for uridine and deoxyuridine are in line with those reported for uridine phosphorylase of Ehrlich ascites tumor cells (Krenitsky *et al.*, 1964).

In the direction of synthesis with saturating levels of ribose-1-P (0.533 mM) and varying uracil concentrations (0.333–0.5 mM) pK_{es} values of 6.5, 7.0, and 8.0 and pK_e values of 7.6 and 8.4 were detected.

EQUILIBRIUM CONSTANTS. In three experiments in which uridine and P_i concentrations were both 0.667, 0.333, or 0.133 mM, equilibrium was obtained after 190–240 min. Constants (K_{eq}) were calculated to be 0.031 at pH 7.4 and 0.078 at pH 8.2. For comparison, K_{eq} for the phosphorolysis of thymidine catalyzed by thymidine phosphorylase was 0.102 at pH 7.4. The K_{eq} values at pH 7.4 are similar for the reactions catalyzed by uridine phosphorylase of rat liver and tumor cells (Pontis *et al.*, 1961) and purine nucleoside phosphorylase of calf or rat liver (Kalckar, 1947; Friedkin, 1950). The synthetic direction is favoured much more in the reactions catalyzed by the mammalian enzymes than in that catalyzed by uridine phosphorylase of bacteria (Paege and Schlenk, 1952).

MIXED SUBSTRATES. The addition to the incubation medium of uridine and deoxyuridine together gave an activity intermediate between that for uridine and deoxyuridine alone indicating that there is a common active site for both substrates (Dixon and Webb, 1964).

PENTOSYL TRANSFERASE ACTIVITY. a. Exchange between 2-[^{14}C]Uracil and Uridine. Transferase activity was maximal at uridine concentrations greater than 1.0 mM, 0.9 mM 2-[^{14}C]-uracil (at constant specific radioactivity) and occurred only when P_i was present. Exchange was a linear function of time up to 45 min and enzyme concentration up to 30 μg of enzyme protein/tube with optimal P_i concentrations of 0.4 mM or greater.

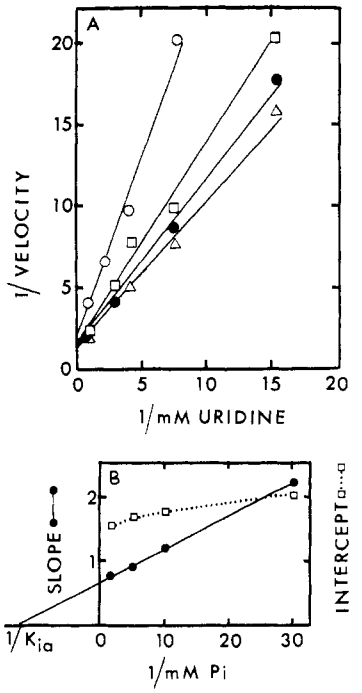


FIGURE 4: Isotope exchange between 2-[^{14}C]uracil and uridine catalyzed by uridine phosphorylase. (A) Double-reciprocal plot of velocity (μmol of uracil transferred/ml per hr) *vs.* uridine concentration. The standard assay medium contained per milliliter: 1.346 μmol of 2-[^{14}C]uracil (0.67 μCi), 30.7 μg of uridine phosphorylase protein (1.5 units), uridine (0.066–2.0 mM) at different levels of P_i: 0.033 mM (○), 0.1 mM (□), 0.2 mM (●), and 1.0 mM (△). Incubation was at 37° for 30 min. (B) Replot of slopes and intercepts of the lines from A *vs.* the reciprocal of P_i concentration.

Transferase activity was determined with P_i as nonvariable substrate, uridine as variable substrate at a saturating concentration of labeled uracil. A pattern of competitive inhibition by P_i was found (Figure 4A); such a pattern is predicted by the rate equation which was derived by a modification[3] of the method of Cleland (1967b, 1970). This rate equation is given below in the reciprocal form

$$\frac{1}{v} = \left[\frac{K_b}{V_1}\left(1 + \frac{K_{ia}}{[A]}\right)\left(\frac{K_p K_{iq}}{K_q}\frac{1}{[P]} + 1\right)\right] \times \frac{1}{[B]} + \frac{K_p K_{iq}}{V_1 K_{ip} K_q}\left[\left(\frac{K_a}{[A]} + 1\right)\frac{K_{ip}}{[P]} + 1\right] \quad (1)$$

where $A + B \rightleftharpoons P^*$, Q is absent, and v is the initial velocity of formation of labeled uridine (B) from labeled uracil (P*) in the presence of P_i (A) with ribose-1-P (Q) absent.

The K_{ia} value determined from the slope replot (Figure 4B) was 0.083 mM which is approximately $^1/_{10}$th the K_{ia} value obtained by initial velocity studies of the phosphorolytic reaction for this as well as previous preparations (Kraut and Yamada, 1971). This result was not unexpected; in isotope exchange there is a low rate of dissociation of the EA complex; bound A is reused without being released from EA. Further, as noted by Cleland (1963), inhibition constants may be identical with dissociation constants under appropriate conditions; in the case in hand P_i acts as a competitive inhibitor.

b. Effect of Arsenate. Arsenate stimulated transferase activity much less than did P_i (Table I) at both pH 7.4 and 8.2. When equal amounts of arsenate and P_i were present together an activity intermediate between that of each alone was obtained indicating that the two compounds bind at the same enzyme site.

[3] E. W. Yamada and J. T. Yamada, manuscript in preparation.

TABLE I: Pentosyl Transferase Activity of Uridine Phosphorylase in the Presence of Arsenate.[a]

Expt	pH	Act. (μmol of 2-[^{14}C]Uracil Transferred/ml per hr)		
		P_i	Arsenate	P_i + Arsenate
1	7.4	0.686	0.199	0.346
2	7.4	0.280	0.098	0.168
	8.2	0.265	0.150	0.229

[a] The standard reaction mixture contained per ml: uridine (1.66 μmol), 2-[^{14}C]uracil (1.68 μmol, 0.833 μCi), and either 1.0 mM P_i or potassium arsenate (expt 1) or 3.33 mM of each (expt 2). Each tube contained 76 μg of enzyme protein but different enzyme preparations were used in the two experiments.

It is known that free ribose 1-arsenate esters are very unstable but it is apparent from Table I that they are stabilized sufficiently in the enzyme-bound form to allow them to act as pentosyl donors.

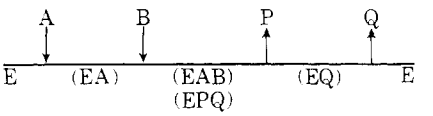
c. Isotope Exchange between [U-^{3}H]Uridine and Uracil. Activity was dependent upon the addition of ribose-1-P and was maximal at concentrations greater than 0.1 mM in the presence of 1.99 mM uracil and 1.333 mM labeled uridine (1.67 μCi).

Ribose-1-P at concentrations greater than 0.6 mM inhibited activity; there was 50% inhibition with 3.0 mM ribose-1-P. This result is also predicted by the rate equation (eq 1 with V_1 replaced by V_2 and B and P and A and Q interchanged). Ribose-1-P at concentrations greater than 16 mM completely inhibited the transferase activity; such complete inhibition indicates a sequential mechanism without randomness (Cleland, 1970).

d. Comparison with Thymidine Phosphorylase. Transferase activity of thymidine phosphorylase, purified from rat liver as described previously (Kraut and Yamada, 1971), with [U-^{3}H]-deoxyuridine as pentosyl donor and thymine as acceptor was increased when deoxyribose-1-P was added to the reaction mixture (Figure 5). In confirmation of the findings of Gallo and Breitman (1968) for thymidine phosphorylase of human leukocytes, the reaction is not inhibited by deoxyribose-1-P even at concentrations as high as 16 mM. In contrast, as was also the case for ribose-1-P, as little as 4 mM deoxyribose-1-P significantly inhibited the activity of uridine phosphorylase (Figure 5) even though with this enzyme, deoxyribosyl exchange was very low. This lack of inhibition of thymidine phosphorylase has been taken to indicate that the transferase and phosphorylase reactions occur at separate sites on the enzyme and through different enzyme complexes (Gallo and Breitman, 1968).

Discussion

Present studies confirm the ordered bi-bi mechanism for uridine phosphorylase represented as



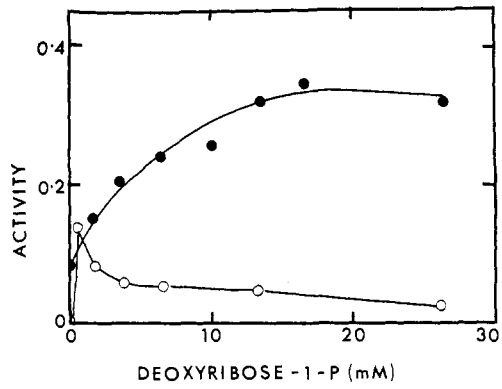


FIGURE 5: Effect of deoxyribose-1-P on pentosyl transferase activity of uridine phosphorylase (○) and thymidine phosphorylase (●). Velocity is expressed as the μmol of [U-^{3}H]deoxyuridine transferred/ml per hr. The standard reaction mixture contained per ml: [U-^{3}H]deoxyuridine (3.333 μmol, 1.67 μCi), thymine (2.0 μmol) and enzyme (127 μg of protein). Incubation was at 37° for 30 min.

where A, B, P, Q, and E represent P_i, uridine, uracil, ribose-1-P, and enzyme, respectively. They cannot be reconciled with the reverse order of addition of substrates and release of products (Krenitsky, 1968) for then uracil and uridine would be the outer pair of reactants (*viz.*, A and Q) and there would be no exchange in the absence of either P_i or ribose-1-P under steady-state nonequilibrium conditions such as used in present work (Cleland, 1970). This conclusion is clearly in accordance with the rate equation for this exchange.[4]

There was a significant difference between uridine and thymidine phosphorylases in the effect of deoxyribose-1-P on isotope exchange between thymine and deoxyuridine. This is surely a consequence of the fact that thymidine phosphorylase catalyzes direct as well as indirect pentosyl transfer (Krenitsky, 1968; Gallo *et al.*, 1967; Gallo and Breitman, 1968) whereas uridine phosphorylase catalyzes indirect transfer only (Krenitsky, 1968; Kraut and Yamada, 1971). It may well be that isomeric forms of thymidine phosphorylase are involved in the two reactions. In this regard it is of interest that isomeric forms of purine nucleoside phosphorylase, an enzyme which also catalyzes direct and indirect pentosyl transfer (Abrams *et al.*, 1965; Kim *et al.*, 1968), have been postulated (Krenitsky, 1967).

The study of the effect of P_i on electrophoretic patterns of uridine phosphorylase was prompted by the suggestion that the enzyme is unstable in Tris buffer (Kraut and Yamada, 1971). As well it was decided to ascertain whether P_i caused interconversion of enzyme forms such as was reported for bacterial uridine phosphorylase (Gilpin and Sadoff, 1971; Engelbrecht and Sadoff, 1969). No evidence for such an ordered interconversion was found.

Finally, a model for the mechanism of catalysis by uridine phosphorylase is proposed (Figure 6). The essential features of this model as illustrated in Figure 6A are as follows. (a) The active site of the enzyme has three SH groups which are essential for full activity. Uridine protects the enzyme from inhibition by *o*-iodosobenzoate (which reacts with vicinial

$$[4]\quad \frac{1}{v_{A^*\rightarrow Q}} = \frac{1}{V_1}\frac{K_a}{[A]}\left[1 + \frac{K_{ia}K_b}{K_a[B]}\left(1 + \frac{K_q}{K_{iq}}\frac{[P]}{K_p}\right) + \frac{[Q]}{K_{iq}}\left(1 + \frac{K_{ia}K_b}{K_a[B]} + \left(1 + \frac{K_{ib}}{[B]}\right)\frac{K_{ia}'}{K_a'}\frac{K_b}{K_{ib}}\frac{[P]}{K_p}\right)\right] + \frac{1}{V_1}\left[1 + \frac{K_b}{[B]} + \left(1 + \frac{K_{ip}K_qK_b}{K_pK_{iq}[B]}\right)\frac{[P]}{K_{ip}}\right] \quad (2)$$

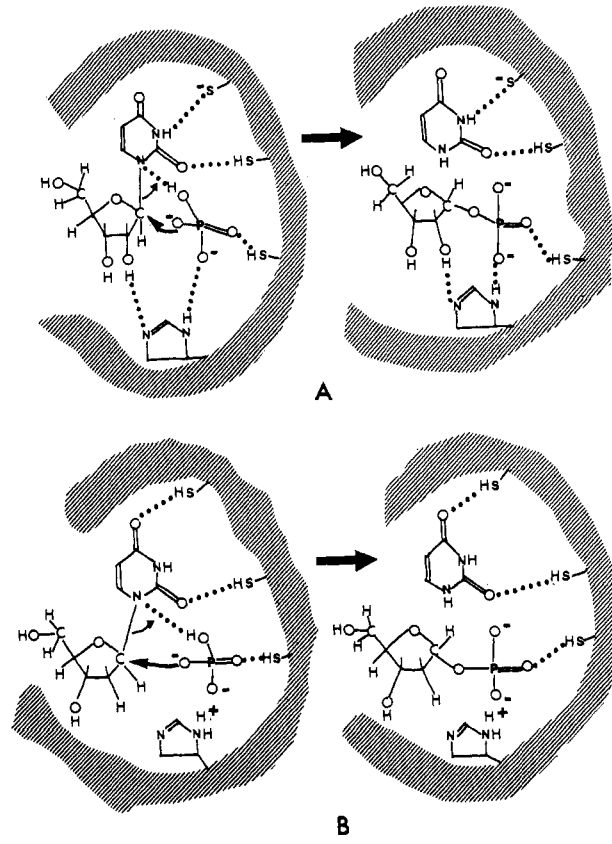


FIGURE 6: Catalysis mechanism for uridine phosphorylase: (A) phosphorolysis of uridine at pH 8.0; (B) phosphorolysis of deoxyuridine at pH 6.5.

SH groups), P_i does not but both protect against sulfhydryl inhibitors that react with single SH groups (Kraut and Yamada, 1971). On this basis it is proposed that uridine binds to two SH groups, one of which is implicated in the enzyme–substrate complex (pK_{es} = 8.0), while P_i binds to the third. (b) A histidyl residue in the unprotonated form (pK_{es} = 7.0) forms a hydrogen bond with the pentose moiety of uridine and one with P_i. (c) The three-point attachment of uridine results in the withdrawal of electrons from N-1 of the β-*N*-glycosidic bond and orients this bond for "back-side" (Koshland, 1954, 1959) nucleophilic attack by P_i. There is facilitated proton transfer (Wang, 1968) and replacement of the more weakly nucleophilic N-1 of uridine by P_i, possibly by a SN2 mechanism. The "back-side" single displacement reaction results in a product of inverted configuration on the asymmetric carbon under attack.

In Figure 6B, the pH is optimum for deoxyuridine cleavage and now the following hold true. (a) The SH group of the enzyme is not ionized; deoxyuridine protects the enzyme only partially against inhibition by *o*-iodosobenzoate (Kraut and Yamada, 1971) and is apparently bound more loosely to the enzyme than is uridine at pH 8.0 (*viz.*, by hydrogen bonds alone). P_i binds to one SH group as before. (b) The protonated form of the histidyl residue forms a salt bridge with P_i (pK_{es} = 6.5) and of course there is no interaction between C′-2 of the pentose and the histidyl residue. (c) At this pH, uridine is bound less tightly not only to the SH groups but also to the histidyl residue for in the protonated form the opposing N of this group is more weakly nucleophilic and there is less tendency for H bonding to occur with the pentose moiety.

Certainly, chemical modification studies should be done to support and add to this model.

Acknowledgments

The skillful technical assistance of Mr. Alec Leung is gratefully acknowledged. The authors also extend thanks to Dr. Frits C. Stevens for his help with the amino acid analyzer.

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