in Me_2CO–Et_2O–NH_4OH (2:20:0.02). The band corresponding to cotinine[6] (R_f 0.15) was removed and eluted with ~10 mL of MeOH. The MeOH was removed in vacuo and the clear oil was dissolved in 2 mL of THF. This (4) was added to 17 mg (0.5 mmol) of $LiAlH_4$ in 15 mL of THF (by syringe) under an atmosphere of Ar. The mixture was refluxed for 20 min and cooled to 25 °C. Et_2O (20 mL) was added and then (cautiously) $Na_2SO_4 \cdot 10H_2O$ to destroy excess $LiAlH_4$. The precipitate was filtered and THF–Et_2O was removed in vacuo. The residue **5** (oil, 0.17 Ci, 3.6 mmol) was dissolved in MeOH containing 0.025% *l*-tartaric acid.

Determination of Specific Activity of 5. The specific activity of **5** was determined by liquid-scintillation counting and GS/MS. A Finnigan 4000 GC/MS was equipped with a 1.83 m × 2 mm glass column packed with 3% SP 2250 on Supelcoport 80–100 mesh. Mass spectra were obtained under the following conditions: column temperature, 120 °C; carrier gas (He) flow, 30 mL/min; electron energy, 70 eV. Single-ion monitoring was used to construct a standard calibration curve for 1 (0.1–1000 ng). The ions monitored for both the standard curve and samples were *m/e* 84, 86, and 88, which corresponded to the base peaks of nicotine, nicotine-*t* and nicotine-t_2, respectively. The nicotine standards did not contain detectable levels of *m/e* 86 and 88. A known quantity of radiolabeled 1 (**5**) (1 μCi as detemined by liquid-scintillation counting) was injected into the GC/MS, and the quantity of total nicotine (labeled and unlabeled) was determined from the calibration curve. The specific activity was found to be 4.7 Ci/mmol. Since the base peak of 1 corresponds to the pyrrolidine ring fragment,[7] the presence of *m/e* 86 and 88 established that the tritium atoms were in this ring.

Binding of 5 to Brain Tissue. Male, Sprague–Dawley rats (200–250 g) were decapitated and their brains removed for homogenization in 4 volumes of 0.32 M sucrose. The homogenates were centrifuged at 1000*g* for 10 min at 0 °C. The resulting supernatants were recentrifuged at 10000*g* for 20 min at 0 °C to provide crude mitochondrial (CM) pellets. The pellets were suspended in Krebs–Henseleit (K–H) buffer (2–4 mg of protein/mL) and pipetted into microfuge tubes. Compound **5**, in 50 μL of K–H buffer, was added to the tubes and optimum incubation conditions were established. For displacement experiments, unlabeled drugs were incubated 5 min prior to the addition of **5**. After incubation, the tubes were spun on a Beckman Microfuge, Model B, for 3 min and the supernatants were pipetted into scintillation vials containing 10 mL of scintillation fluid [2 parts of PhMe containing 0.4% of diphenyloxazole, 0.01% of 1,4-bis[2-(4-methyl-5-phenyloxazolyl)]benzene, and 1 part of Triton X-100 (TPP-TX)]. The tips of the centrifuge tubes containing the bound **5** were cut off and counted in 10 mL of TPP-TX. Radioactivity was quantitited by liquid-scintillation spectrometry and quench corrected by external standardization.

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Thymidine Phosphorylase. Substrate Specificity for 5-Substituted 2′-Deoxyuridines

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A continuous spectrophotometric assay for thymidine phosphorylase has been developed and used to analyze the substrate properties of a number of 5-substituted 2′-deoxyuridines. A QSAR was found which relates the catalytic efficiency for various substrates to a single variable, the inductive field constant $\mathcal{F}$.

Thymidine (dThd) phosphorylase (EC 2.4.2.4) catalyzes the reversible conversion of dThd and phosphate to thymine (Thy) and 2-deoxyribose 1-phosphate. The enzyme also catalyzes transfer of the deoxyribosyl moiety of dThd to appropriate pyrimidine bases. In addition to its role as a salvage enzyme in pyrimidine metabolism, the enzyme is responsible for the catabolism of a number of 5-substituted pyrimidine deoxyribonucleoside analogues with real or potential chemotherapeutic usefulness. In this report we describe the behavior of a number of 5-substituted pyrimidine nucleosides as substrates of mammalian dThd phosphorylase, as well as an improved continuous spectrophotometric assay for phosphorolysis and transferase activities of this enzyme.

Results and Discussion

Continuous Spectrophotometric Assay. dThd phosphorylase activity is usually monitored by use of radiolabeled substrates and separation and quantitation of reactants and products,[3,4] or by quenching reaction mixtures and observing differences in the UV spectra of nucleoside and pyrimidine base in strongly alkaline solutions.[4-6] Both methods suffer from the tedium and inaccuracies of single point assays. A continuous spectrophotometric assay has been reported for Urd phosphorylase which monitors the decrease in absorbance at the 282 nm which accompanies conversion of Urd to uracil;[7] while this assay is convenient, the change in absorbance at 282 nm is small ($\Delta\epsilon$ = 1370), and it is less sensitive than either of the aforementioned single point assays. The assay described here is a modification of the reported continuous assay in which the change in absorbance is monitored at the wavelength ($\Delta\lambda_{max}$) where the *maximal* difference in absorbance ($\Delta\epsilon_{max}$) exists between the nucleoside and base

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Table I. Phosphorolysis of 5-Substituted 2'-Deoxyuridines by Horse Liver Thymidine Phosphorylase

5 substituent	$\Delta\epsilon_{max}$, nm	K_m, mM	V_{max}^{rel} [a]	$\mathcal{F}$ [b]	π [c]	MR [b]	log (V_{max}^{rel}/K_m) obsd	log (V_{max}^{rel}/K_m) calcd [d]	log V_{max}^{rel} obsd	log V_{max}^{rel} calcd [e]
H	2840 (270)	0.559	0.88	0	0	0.103	0.198	0.250	−0.054	0.050
CH_3	2420 (275)	0.187	1.0	−0.04	0.45	0.565	0.728	0.162	0	−0.005
F	2310 (278)	0.631	3.42	0.43	0.22	0.092	0.734	1.20	0.534	0.598
Cl	2790 (285)	0.186	4.20	0.41	0.72	0.603	1.35	1.15	0.623	0.563
Br	2690 (288)	0.121	3.51	0.44	0.86	0.888	1.46	1.22	0.545	0.534
I	2140 (300)	0.157	3.85	0.40	1.11	1.394	1.39	1.13	0.586	0.383
CF_3	3640 (270)	0.798	8.53	0.38	1.11	0.502	1.03	1.09	0.931	0.724
CHO	2760 (286)	0.125	1.46	0.31	0.04	0.688	1.07	0.932	0.163	0.158
CN	3300 (280)	0.049	0.74	0.51	−0.57 [b]	0.633	1.18	1.37	−0.134	0.155
CH_2OH	2150 (273)	0.577	0.60	0	−0.85	0.719	0.019	0.250	−0.220	−0.531
C_2H_5	1880 (275)	0.719	0.79	−0.05	1.02 [b]	1.03	0.043	0.140	−0.100	0.017
$CH_2CH{=}CH_2$	2050 (275)	0.943	0.76	−0.08 [f]	1.1 [b]	1.449	−0.091	0.074	−0.117	−0.154
$CH{=}CHCH_3$	1510 (305)	0.314	0.57	0.07 [g]	1.2 [g]	1.50	0.262	0.404	−0.241	0.024

[a] Expressed as the ratio of the V_{max} of the analogue to that of dThd for that particular assay. The V_{max} of dThd was 0.14 μmol min^{-1} (mg of protein)$^{-1}$. [b] Taken from ref 11. MR values are scaled by 0.1. [c] Unless otherwise noted, π values for 5-substituted uracils are taken from ref 19. [d] Calculated from eq 2. [e] Calculated from eq 1. [f] Estimated, using $\mathcal{F}$ for benzyl. [g] Estimated, using values for $-C_2H_3$.

(Table I). As shown in Table I, $\Delta\lambda_{max}$ and $\Delta\epsilon_{max}$ values depend upon the 5-substituent of the uracil heterocycle and must be determined for each individual substrate. In general, $\Delta\lambda_{max}$ is ca. 10 to 12 nm higher than λ_{max} of substrates or products, and $\Delta\epsilon_{max}$ is sufficiently large to permit quantitation of conversion of ca. 10^{-5} M nucleoside to base or vice versa; as such, the assay is about twice as sensitive as the reported continuous assay of Urd phosphorylase. It is apparent that this assay is applicable to all enzymes which catalyze the cleavage and/or formation of glycosidic bonds of nucleosides/nucleotides possessing the uracil heterocycle.

Further utility of this assay arises from the fact that $\Delta\lambda_{max}$ varies with the 5-substituent of the uracil heterocycle. First, use of a synthetic substrate with a high $\Delta\lambda_{max}$ (e.g., IdUrd, $\Delta\lambda_{max}$ = 300 nm) provides additional sensitivity to assays of crude enzyme preparations containing interfering absorbance at the lower wavelength necessary to monitor phosphorolysis of naturally occurring substrates (e.g., dThd, $\Delta\lambda_{max}$ = 275 nm). Second, in addition to the use of this assay for initial velocity experiments, dThd phosphorylase catalyzed trans-deoxyribosylation[3] can be directly monitored without product isolation. For this purpose, a nucleoside deoxyribosyl donor and pyrimidine base acceptor are chosen which have large differences in their $\Delta\lambda_{max}$ values, and the phosphate concentration is kept at a sufficiently low concentration to minimize phosphorylalysis of nucleosides. For example, the dThd phosphorylase catalyzed conversion of dThd ($\Delta\lambda_{max}$ = 275 nm) and IUra to Thy and IdUrd is conveniently monitored by the increase in absorbance at 300 nm ($\Delta\lambda_{max}$ of IdUrd) which accompanies this reaction (see Experimental Section).

Substrate Specificity. While Urd phosphorylase will catalyze phosphorolysis of 5-substituted uracil ribosides or 2'-deoxyribosides, dThd phosphorylase appears to be highly specific for 5-substituted 2'-deoxyribofuranosyluracils.[5,8-10] Kinetic properties of 13 5-substituted 2'-deoxyuridines which were substrates for dThd phosphorylase are given in Table I. In general, compounds having electron-withdrawing 5-substituents at least the size of a methyl group showed lower K_m values and, in most cases, higher V_{max} values.

In an attempt to develop a clearer picture of the effect of the 5-substituent on the ability of the substituted 2'-deoxyuridines to serve as substrates of thymidine phosphorylase, the enzymatic data in Table I was subjected to multiple regression analysis. The independent variables examined in the fit were π, MR (scaled by 0.1), σ_p, σ_m, $\mathcal{F}$, and $\mathcal{R}$. Substituent constants used were from Hansch et al.[11] No significant correlation could be found for log ($1/K_m$) using any combination of the above substituent constants. The most significant fit for log V_{max} is given in eq 1. The parenthesisized values are the standard errors

$$\log V_{max}^{rel} = 1.06(\pm 0.26)\mathcal{F} + 0.39(\pm 0.10)\pi - 0.41(\pm 0.16)\mathrm{MR} + 0.15 \quad (1)$$

$$n = 13,\ s = 0.202,\ r = 0.896,\ F_{3,9} = 12.2$$

of the coefficients; n represents the number of data points employed, r is the correlation coefficient, s is the standard deviation, and F is the stepwise F statistic.[12] Only π and MR had a significant cross-correlation (r^2 = 0.55) in this equation. In toto, this equation should be considered only marginally significant, since it uses 3 independent variables for only 13 data points. It does, however, indicate the intuitively apparent importance of electron-withdrawing effects of the 5-substituent on the velocity of the phosphorolysis. The equation also suggests contribution of hydrophobic interactions of the substituent on V_{max}, along with possible steric interference as indicated by the negative coefficient of the MR term. By far the most significant equation generated from fitting any of the experimental data to the independent variables listed above is eq 2. Here, all 13 experimental values of log (V_{max}/K_m)

$$\log (V_{max}^{rel}/K_m) = 2.20(\pm 0.36)\mathcal{F} - 0.30 \quad (2)$$

$$n = 13,\ s = 0.282,\ r = 0.881,\ F_{1,11} = 38.25$$

have been correlated with the single variable $\mathcal{F}$, the inductive field constant, which indicates the most important effect of the 5-substituent is its pure electron-withdrawing ability. The data used in this fit and the calculated log (V_{max}^{rel}/K_m) values are given in Table I.

The parameter V_{max}^{rel}/K_m, in effect equivalent to k_{cat}/K_m, is a measure of the second-order rate constant of an enzyme-catalyzed reaction at low substrate concentra-

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tion and represents the "efficiency of catalysis" of the reaction of a given substrate.[13] Thus, since the intracellular concentrations of deoxynucleoside substrates are far below their K_m values for dThd phosphorylase, those with higher V_{max}^{rel}/K_m values will be more rapidly catabolized and the QSAR described for this parameter should be of predictive utility. Important drugs which are degraded by dThd phosphorylase are also converted to their active form via initial phosphorylation at the 5'-hydroxyl by dThd kinase. It follows that the efficacy of such drugs in a given cell will largely depend on the relative rates of these divergent pathways. Thus, at low substrate concentrations, the fraction of a 5-substituted 2'-deoxyuridine which will be converted to its 5'-phosphate rather than be catabolized by dThd phosphorylase is described by eq 3,

$$\frac{v_{TK}}{v_{TK} + v_{TP}} = \frac{(k_{cat}/K_m)_{TK}[TK]}{(k_{cat}/K_m)_{TK}[TK] + (k_{cat}/K_m)_{TK}[TP]} \quad (3)$$

where v_{TK} and v_{TP} are the velocities of reaction with dThd kinase (TK) and dThd phosphorylase (TP), respectively. If, as described here for dThd phosphorylase, a QSAR could be established for the catalytic efficiencies (k_{cat}/K_m) for phosphorylation of 5-substituted 2'-deoxyuridines by dThd kinase, it should be possible to predict their metabolism in cells once the relative activities of these two enzymes are ascertained. As these activities vary in differing cell lines,[14] it may be possible to predict the sensitivity of a given cell to these analogues. Studies to test this hypothesis are in progress.

Experimental Section

General. dThd phosphorylase from horse liver was purchased from Gipep Co., Ltd. (Paris, France). Using conditions described below, the phosphorolysis of Urd proceeded at less than 2% the rate of dThd; the absence of Urd phosphorylase in this preparation was further verified by lack of inhibition of dThd phosphorolysis by 0.45 mM 5-benzyluridine, a potent inhibitor of Urd phosphorylase.[15] dUrd, dThd, BrdUrd, CldUrd, CF_3dUrd, FdUrd, and IdUrd were obtained from P-L Biochemicals. CHOdUrd[16] and CNdUrd[17,18] were prepared by the cited procedures. 5-Ethyl-, 5-allyl- and 5-propenyl-2'-deoxyuridines were gifts from Dr. Donald Bergstrom. Other materials were of the highest commercial grade available. All nucleosides used had proper UV spectra and were homogeneous as determined by high-performance LC analysis using a Lichrosorb RP18 column (4.6 × 250 mm) and 2% (v/v) CH_3CH/water as eluant; flow rate was 1 mL/min and compounds were detected at 254 and/or 280 nm with an ISCO UA-5 detector. Ultraviolet spectra and kinetic analysis were obtained on a Cary 118 recording spectrophotometer equipped with a repetitive scan accessory.

Enzyme Assays. With substrates of dThd phosphorylase, the wavelength of maximal difference ($\Delta\lambda_{max}$) between each nucleoside and its corresponding base and the magnitude of absorbance change ($\Delta\epsilon_{max}$) were determined as follows: The UV spectrum of a solution of the nucleoside (ca. 0.1 mM) in 0.1 M potassium phosphate (pH 6.0) was obtained and, from reported ϵ_{max} values, the concentration was determined. Identical amounts of the dThd phosphorylase preparation in 1% of the total volume were added to both reference and sample cuvettes to give a final concentration of ca. 0.08 mg/mL. The UV spectrum was scanned repetitively until no further changes were observed. The final spectrum was scanned repetitively until no further changes were observed. The final spectrum of the pyrimidine base was subtracted from the initial spectrum of the nucleoside to obtain the difference spectrum from which $\Delta\lambda_{max}$ and $\Delta\epsilon_{max}$ were obtained. Completion of reaction was verified by high-performance LC analysis using the systems described above.

For initial velocity experiments of *phosphorolysis*, the change in absorbance was continuously monitored at $\Delta\lambda_{max}$ for each nucleoside, and $\Delta\epsilon_{max}$ was used to quantitate the extent of reaction. Reaction mixtures contained variable amounts of nucleoside (60–320 μM) in 0.1 M potassium phosphate (pH 6.0) and limiting enzyme; assays were performed at 25.0 °C. *Pentosyl transfer* (trans-deoxyribosylation) was assayed by monitoring the increase in absorbance at 300 nm which accompanies the formation of IdUrd upon transfer of the deoxyribose moiety from a nucleoside donor which does not absorb at 300 nm (e.g., dThd) to IUra. A typical reaction mixture contained, in 1 mL, 75 mM morpholinoethyl sulfonate (pH 6.2), 0.1 mM IUra, 0.1 mM potassium phosphate, 90 μg of enzyme, and 0.1 to 1 mM of dThd as the deoxyribosyl donor. The validity of this assay was confirmed by high-performance LC separation of reactants and products, which were then quantitated by UV spectroscopy.

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Antiallergy Activity of 10-Oxo-10*H*-pyridazino[6,1-*b*]quinazoline-2-carboxylic Acids

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A series of substituted 10-oxo-10*H*-pyridazino[6,1-*b*]quinazoline-2-carboxylic acids was prepared and evaluated as antiallergy agents. The 8-chloro and unsubstituted analogues were more potent than cromolyn sodium and doxantrazole intravenously in the rat PCA test. None of the analogues possessed significant oral activity.

A new series of 11-oxo-11*H*-pyrido[2,1-*b*]quinazoline-8-carboxylic acids was recently reported to possess significant oral antiallergy activity. 2-Methyl-11-oxo-pyrido[2,1-*b*]-quinazoline-8-carboxylic acid (11) was 30 times more potent than cromolyn sodium in the rat PCA test intravenously and 4–6.7 times better than doxantrazole orally.[1]

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