pramol. Struct. 1, 8.
Tanford, C., & Reynolds, J. A. (1976) *Biochim. Biophys. Acta 457,* 133.
Tomita, M., & Marchesi, V. T. (1975) *Proc. Natl. Acad. Sci. U.S.A. 72,* 2964.
Webster, R. E., & Cashman, J. S. (1973) *Virology 55,* 20.
Williams, R., & Dunker, A. K. (1977) *Biophys. J. 17,* 71a.
Wong, K. P., Roxby, R., & Tanford, C. (1971) *Anal. Biochem. 40,* 459.
Wyman, J. (1964) *Adv. Protein Chem. 19,* 224.

O^2- and O^4-Alkyl Pyrimidine Nucleosides: Stability of the Glycosyl Bond and of the Alkyl Group as a Function of pH†

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ABSTRACT: Alkylation of the O^2 of deoxypyrimidine nucleosides greatly destabilizes the glycosyl linkage. Within the pH range studied, cleavage of the glycosyl bond of O^2-alkyl thymidine and O^2-alkyl deoxyuridine is fastest in acid pH but also occurs with little or no pH dependence over the range pH 4.0–13.0. The glycosyl bond of O^2-alkyl deoxycytidine is the least stable of any O^2-alkyl derivative at neutrality and is cleaved without significant pH dependence. The glycosyl bond of O^2-alkylated ribopyrimidine nucleosides is cleaved at a much slower rate than that of the deoxy ribo derivatives. The relative stability, at pH 7.0, of the glycosyl bond of the ethyl derivatives studied is O^2-Et-dCyd $\ll$ O^2-Et-dUrd < O^2-Et-Thd < O^2-Et-Cyd $\ll$ O^2-Et-Urd. Those methylated derivatives investigated were found to behave similarly. The stability of the glycosyl bond of O^2-Et-dCyd and O^2-Et-Thd was also compared with that of 7-Et-dGuo at pH 7.0, 37 °C. In contrast, all of the O^4-alkylated deoxyribo- and ribopyrimidine nucleosides resemble the unmodified nucleosides in the stability of the glycosyl bond. All *O*-alkyl groups are labile, particularly in strong acid and base, and the rate of their hydrolysis was studied at various pHs. pH dependence was similar to that for glycosyl bond cleavage; dealkylation being much faster in acid than in alkali for all except O^2-alkyl deoxycytidine. The relative stability, at pH 1.5, of the ethyl groups is O^4-Et-Urd < O^4-Et-Thd < O^4-Et-dUrd < O^4-Et-Ura < O^2-Et-Urd < (O^2-Et-dUrd) O^2-Et-Ura < (O^2-Et-Thd) O^2-Et-Thy $\ll$ O^2-Et-Cyd < O^2-Et-Cyt. pK_a values were determined for the new derivative, O^2-Et-Thy, and for O^2-Et-Thd, O^2-Et-dUrd, O^4-Et-Thd, O^4-Et-dUrd, and O^2-Et-Ura.

Depyriminidation of deoxyribonucleotides has been found to occur in neutral solution, although very much slower than depurination (Shapiro & Kang, 1969; Lindahl & Karlstrom, 1973). Both purine and pyrimidine base release proceeds by the same mechanism, namely, hydrolytic cleavage of the glycosyl bond without opening of the sugar ring. Although the rate of depurination of dAdo[1] and dGuo is a linear function of the hydrogen ion concentration, the hydrolysis rate of dCyd and Thd is virtually pH independent over the range pH 3.0–7.0 (Shapiro & Kang, 1969; Shapiro & Danzig, 1972).

The glycosyl bond of the pyrimidine ribonucleosides is more stable than that of the corresponding deoxyribonucleosides (reviewed by Kochetkov & Budowsky, 1972), and at pH 7.0, 37 °C, the rate constants for hydrolysis of Cyd and Urd are two orders of magnitude less than for dCyd and dUrd. Various substituents on the C-5 of dUrd (but not dCyd) increase the rate of glycosyl cleavage to a slight but measurable extent (Kochetkov & Budowsky, 1972). No comparable data appear to exist for other positions of ring substitution or for any ribopyrimidine nucleoside modified on the ring.

Recent studies on the reaction of nucleic acids with carcinogens of the *N*-nitroso type showed that *O*-alkyl pyrimidines were major products (Singer, 1976a). There were clear indications that O^2-alkyl thymidine and O^2-alkyl deoxycytidine were converted to the corresponding alkyl base under mild conditions (Singer, 1976a). It thus appeared important for the study of the biological role of O-alkylation to systematically study the effect of the position of substitution on the stability of the glycosyl linkage and of the alkyl group for both ribo- and deoxyribopyrimidine nucleosides.

We now report that alkylation of the O^2 of all pyrimidines greatly destabilizes the glycosyl bond at all pHs investigated, while alkylation of the O^4 of Urd, dUrd, or Thd has no measurable effect on glycosyl bond cleavage.

Experimental Section

Preparation of Alkylated Nucleosides and Bases. Nucleosides and bases were alkylated using several methods. Thd, dUrd, Urd, dGuo, and Cyt were reacted in anhydrous solution with both radioactive and unlabeled diazoalkanes according to Kuśmierek & Singer (1976). The following derivatives used in this work were isolated from reaction mixtures using paper chromatography (Whatman 3MM) or thin-layer chromatography on silica gel plates (Eastman No. 6060): O^2-alkyl-Thd, O^4-alkyl-Thd, O^2-Et-dUrd, O^4-Et-dUrd, O^2-alkyl-Urd, O^4-alkyl-Urd, 7-Et-dGuo, 1,7-diEt-Guo, and O^2-alkyl-Cyt. The paper chromatographic systems were 80 1-butanol, 10 ethanol, 25 water (solvent A); or 75 ethanol, 30 1 M pH 7.5 ammonium acetate (solvent B). Silica gel plates were devel-

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[1] Abbreviations used: Me, methyl; Et, ethyl; Ado, Guo, Urd, and Cyd refer to ribonucleosides; dAdo, dGuo, dUrd, dCyd, and Thd refer to deoxyribonucleosides; Ade, Gua, Ura, Cyt, and Thy refer to bases. A single letter abbreviation refers to the base moiety in a polynucleotide.

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TABLE I: Approximate pK_a of Alkyl Pyrimidine Nucleosides and Bases.

Compound	pK_a
Nucleoside	
O^2-Ethylthymidine	0.5
O^2-Ethyldeoxyuridine	0.92
O^4-Ethylthymidine	−0.32
O^4-Ethyldeoxyuridine	0.66
O^2-Ethyldeoxycytidine	>9.5[a]
O^2-Ethylcytidine	>9.2[b]
Base	
O^2-Ethylthymine	0.8
O^2-Ethyluracil	0.7
O^4-Ethyluracil	1.0[c]
O^2-Ethylcytosine	5.4[d]

[a] Singer (1976b). [b] Singer (1967a). [c] Katritzky & Waring (1962). [d] Sukhorukov et al. (1972).

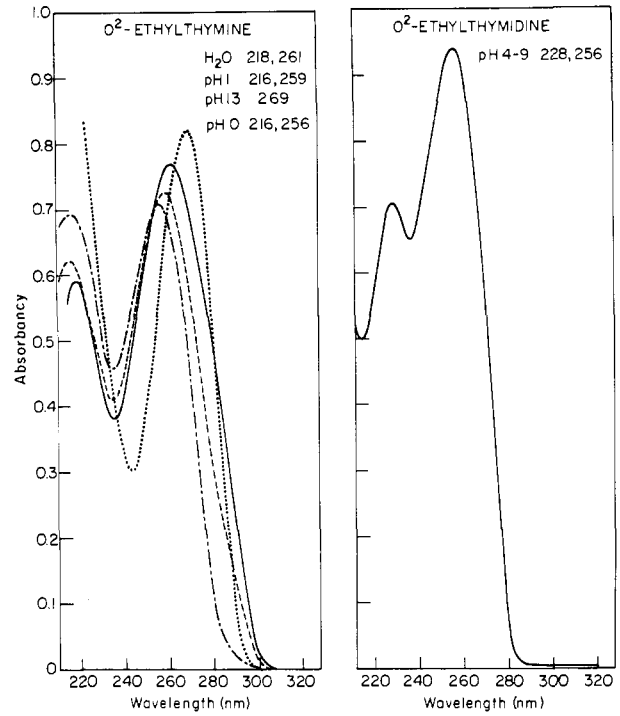

FIGURE 1: UV absorption spectra of O^2-ethylthymidine and O^2-ethylthymine in H_2O (—), 0.1 N KOH (- - - -), and 0.1 N HCl (— — —). The spectrum of O^2-ethylthymine was also plotted in 1 N HCl (· · · ·) inasmuch as the pK_a of this compound is 0.8 (Table I). The difference in spectra between pH 0 and 1 illustrates the use of the spectrophotometric method of determining dissociation constants. The λ_{max} at the various pHs are shown in the figure.

oped in 20 acetone, 10 benzene (solvent C). R_f values for the majority of these compounds have been previously published (Kuśmierek & Singer, 1976; Singer, 1972, 1976a). ^{14}C-labeled and unlabeled O^2-Et-Cyd and O^2-Et-dCyd were prepared as described by Singer (1976a,b).

Unlabeled O^4-Me-Urd and O^4-Et-Urd were also prepared using the method of Robins & Naik (1971). It was found that O^4-Et-Urd, which was not described by Robins & Naik, could also be made in high yield by using sodium ethoxide instead of sodium methoxide.

The pyrimidine bases O^2-Et-Ura, O^4-Et-Ura, and O^2-Et-Thy were obtained by base treatment of the corresponding 2,4-dialkoxypyrimidines (Hilbert et al., 1935). 2,4-Diethoxypyrimidine was a commercial product from Sigma, while 2,4-diethoxy-5-methylpyrimidine was prepared according to Schmidt-Nickels & Johnson (1930). Separation of the starting material, O^2-Et-Ura and O^4-Et-Ura was performed on silica gel thin-layer plates (Merck 60, no. 5766) using 50 benzene, 10 acetone. Only O^2-Et-Thy could be isolated by this method since the alkyl group on the O^4 was labile (Wong & Fuchs, 1970). The UV spectra of the new derivative, O^2-Et-Thy, and of the corresponding nucleoside, O^2-Et-Thd, are shown in Figure 1.

One additional series of derivatives was prepared using [*deoxyribose*-5-^{3}H]thymidine (Amersham). This compound was alkylated with unlabeled diazoethane as described above.

For double-label experiments, the ^{3}H-labeled derivative (labeled deoxyribose) was mixed with the comparable ^{14}C-labeled derivative (labeled ethyl group) in order to detect and characterize derivatives from both the sugar and the base.

Acidic Dissociation Constants. The approximate pK_as of O^2-Et-Thd, O^2-Et-dUrd, O^4-Et-Thd, O^4-dUrd, O^2-Et-Thy, and O^2-Et-Ura were determined using a spectrophotometric method as described by Singer (1972). These data as well as previously determined pK_as for other derivatives used in this paper are shown in Table I.

Hydrolysis of Alkyl Nucleosides and Determination of Products. The treatment of alkyl nucleosides and bases was carried out by the same procedure for both radioactive labeled and unlabeled compounds. However, the method for the determination of the products of hydrolysis differed. The first part of this section, a, is the hydrolysis procedure while section b is the analytical method for radioactive nucleoside samples, section c is the analytical method for unlabeled nucleoside samples, and d is the method for determination of dealkylation of alkyl bases.

(a) Fifty-microliter samples containing 2000–10 000 cpm (0.1–1 OD) or 5–20 A_{260} OD units were sealed into ampules after adding 25 μL of buffer, 0.1 M HCl or 0.1 M KOH, depending on the pH desired. The buffers used to bring nucleosides to the various pHs were: pH 3.0, Sorensen's citrate I; pH 4.15, 0.1 M dihydrogen sodium phosphate; pH 5.0, Sorenson's citrate II; pH 7.0, Sorensen's phosphate; and pH 9.0, Sorensen's glycine II.

Although the pH of the buffers was not measured by us at 100 °C, it has been shown that phosphate buffers at pH 4.0 or 7.0 exhibit the same pH ± 0.01 unit, at temperatures from 0 to 95 °C (Sober, 1973). The pH of the citrate and glycine buffers was measured at 37 °C, the only temperature where they were used. All temperatures were maintained with water baths, including a boiling water bath for 100 °C. The times of heating varied with the stability of the derivative tested but for each experiment two to four time periods were used.

(b) After hydrolysis, the radioactive samples were applied to silica gel thin-layer plates and chromatographed in solvent C which separates pyrimidine bases from nucleosides. UV markers of each nucleoside and base were separately applied to the same plate. However, in most experiments there was enough UV absorbancy in the test samples to detect whether an alkyl base was formed as well as the starting nucleoside. When double-label experiments were performed with alkylated thymidine, the chromatographic system used to separate deoxyribose, nucleoside, alkyl nucleoside, and alkyl base was generally by thin-layer chromatography (cellulose plates, Eastman 6065) developed in solvent A. The relative movement of derivatives is, relative to thymine: deoxyribose, 0.7; thymidine, 1.1; O^2-ethylthymidine, 1.4; O^2-ethylthymine, 1.7. The ribose moiety on chromatograms was detected using a spray

TABLE II: Half-Life of Depyrimidination and Dealkylation of O^2- and O^4-Alkyl Pyrimidine Nucleosides and Bases as a Function of pH.[a]

		Approximate half-life[b]											
		Deoxyribonucleosides					Ribonucleosides			Bases			
pH	Temp. (°C)	O^2-Et-Thd	O^2-Et-dUrd	O^2-Et-dCyd	O^4-Et-Thd	O^4-Et-dUrd	O^2-Et-Urd	O^2-Et-Cyd	O^4-Et-Urd	O^2-Et-Thy	O^2-Et-Ura	O^2-Et-Cyt	O^4-Et-Ura
						Depyrimidination							
1.5	50	<5 min	<5 min	2 h	[c]	[c]	[c, d]	>200 h	[c]				
	100			<5 min				3 h					
4.15	100			<5 min		nd (5 h)	100 h	6 h	nd (5 h)				
7.0	37	~220 days		26 h[e]									
	100	2.7 h	1 h	<5 min	nd (5 h)	nd (5 h)	100 h	5 h	nd (5 h)				
12.5	100	2.7 h	1 h	[c]	nd (5 h)	[c]	20 h[f]	[c]	[c]				
						Dealkylation							
1.5	50	[c]	[c]	nd (5 h)	20 min	20 min	40 min[g]	nd (2 h)	4 min	3 h	1 h	nd (10 h)	30 min
	100							8 h				18 h	
4.15	100	nd (15 h)		[c]	70 h	20 h	nd (24 h)	9 h	20 h			nd (2 h)	
7.0	100	nd (5 h)	nd (5 h)	[c]	125 h	50 h	nd (24 h)	2 h	50 h			nd (10 h)	
12.5	100[h]	13 h	10 h	<5 min	30 h	<10 min	4 h	[i]	<10 min	90 h	110 h	nd (10 h)	2 h

[a] The method for determination of half-life is described in the Experimental Section. [b] The majority of results are averages of two or more separate experiments, each with two to four time periods. Actual times of hydrolysis ranged from 5 min to 48 h at 100 °C, or 10 days at 37 °C, depending on the time necessary to obtain significant data. In the case of half-lives of <5 min, it was not possible to be more exact and the actual half-lives may be as little as 1 min. ND means that no detectable reaction (<1%) occurred during the time period studied. The maximum time is shown in parentheses. [c] Not measurable due to the speed of the competing reaction. [d] O^2-Ethyluracil was detected in 1 h. [e] Exactly the same half-life was observed at pH 3, 5, and 9. [f] Not measurable with accuracy due to the speed of the competing reaction. [g] The half-life of O^2-Me-Urd under the same conditions is 30 min. [h] Sugar decomposition and other products are also observed with long treatment at this temperature. [i] Dealkylation occurs at 24 °C at a rate too fast to measure by these techniques (<5 min). Reaction at 100 °C results in decomposition products.

containing $AgNO_3$ (Trevelyan et al., 1950). After marking the position of the UV markers, the chromatograms were cut into 1.0- or 1.5-cm strips and placed in scintillation vials with 0.5 mL of H_2O for 30 min. Five milliliters of Aquasol 2 scintillation fluid was added and the radioactivity determined using a Beckman liquid scintillation counter.

(c) After hydrolysis, samples without radioactivity were chromatographed on Whatman 3MM in solvent A. The UV-absorbing areas were cut out and eluted in water. The total absorption of each product was measured. Half-lives of depyrimidination and dealkylation were calculated from these data. There was no significant difference in the half-lives as determined using methods b and c.

(d) After hydrolysis, alkyl base samples were placed in a 37 °C water bath and dried in an airstream in order to evaporate any liberated alcohol and then redissolved in 0.5 mL of H_2O. An unheated control sample was treated in the same manner, then the radioactivity in each sample was determined. The difference between the radioactivity of the control and heated sample was taken as a measure of dealkylation. In some experiments, 5–30 A_{260} units of unlabeled alkyl base were heated in the same way as in (a) and then the entire sample was applied to Whatman 3MM paper and chromatographed using solvent B which separates alkyl bases from unmodified bases. Optical density was determined for each UV-absorbing area, and the proportion of products were used to calculate the rate of dealkylation. No significant difference in half-life was found between the two methods of detecting dealkylation.

Under all conditions of heating at pH 1.5–9.0, the products of hydrolysis included and were identical with alkyl bases, nucleosides, and deoxyribose or ribose. No other products could be detected by either UV absorbancy or radioactivity. In addition, no losses, other than by handling, occurred.

Determination of Rates of Depyrimidination and Dealkylation. When reaction conditions or times indicated that there was a single product, either as a result of dealkylation or depyrimidination, the log of the percent of starting material remaining, plotted as a function of time, was linear indicating a pseudo-first-order reaction. The half-life was then calculated from the slope of each plot.

When both dealkylation and depyrimidination occurred at similar rates as judged by the proportion of products, the rate of each was calculated independently using the slope of the log of the difference between 100% and the percent product formed as plotted against increasing time of reaction.

Results

Hydrolysis of O^2-Ethyl Deoxyribonucleosides. O^2-Et-Thd and O^2-Et-dUrd are depyrimidinated so rapidly in acid solution that it was not possible to measure this rate accurately at pH 1.5, 50 °C. Although this reaction is slower at pH 7.0 and 12.5, it is still extremely fast compared with that of the unmodified nucleoside. O^2-Et-dCyd glycosyl bond cleavage showed much less pH dependence and this compound is rapidly converted to O^2-Et-Cyt at pHs 1.5, 4.0, and 7.0, while the rate at pH 12.5 was not measurable due to almost instantaneous dealkylation (Table II).

Since O^2-Et-dCyd was converted to the alkyl base with such rapidity at pH 7.0, 100 °C, it seemed important to compare the rate of such cleavage with that of 7-ethyldeoxyguanosine; Table III presents comparative data on the rate of glycosyl bond cleavage at pH 7.0, 37 °C, of several alkylated nucleosides.

Dealkylation of O^2-Et-Thd and O^2-Et-dUrd is not measurable at pH 1.5 due to rapid depyrimidination. At pH 4.15 and 7.0 the alkyl group of both derivatives is very stable, but at pH 12.5, 100 °C, dealkylation occurs with half-lives of 13 and 10 h, respectively (Table II). The rate of depyrimidination of O^2-Et-dCyd differs from the other two O^2-alkyl compounds and it is rapidly depyrimidinated at pH 4.15 and 7.0. Therefore dealkylation can only be examined at pH 1.5 where it is undetectable after 5 h at 50 °C and at pH 12.5 where it occurs in less than 5 min at 100 °C (Table II).

The bases resulting from depyrimidination (O^2-Et-Thy,

O^2-Et-Ura, and O^2-Et-Cyt) were also heated at various pHs and it was found that they were all relatively acid stable (Table II).

Thus, for all three O^2-ethyl deoxynucleosides, when the pH favors rapid depyrimidination (pH 1.5 for O^2-Et-Thd, O^2-Et-dUrd; pH 1.5, 4.0, and 7.0 for O^2-Et-dCyd), dealkylation is not of importance (Figure 2, reaction I), the resulting alkyl bases being stable during the period of heating necessary to determine half-lives.

Hydrolysis of O^2-Ethyl Ribonucleosides. The glycosyl bonds of ribopyrimidine nucleosides are much more acid stable than those of the corresponding deoxyribonucleosides (Venner, 1964, 1966). We also find that the glycosyl bond of O^2-Et-Cyd and O^2-Et-Urd is more stable than those of O^2-Et-dCyd and O^2-Et-dUrd, while the alkyl groups are slightly less stable (Table II).

Both dealkylation and depyrimidination of O^2-Et-Cyd occur with similar rates, though depyrimidination is at pH 1.5–7.0 slightly faster than dealkylation. At pH 13.0, 24 °C, dealkylation to cytidine is instantaneous. O^2-Et-Urd is primarily dealkylated at both pH 1.5 and 12.5 but the rate is considerably faster in acid. Although a half-life cannot be calculated, it was possible to identify O^2-Et-Ura as a minor product after heating O^2-Et-Urd at pH 1.5, 4.15, or 7.0.

Both O^2-Et-Urd and O^2-Et-Cyd can be shown to undergo the same pH-independent cleavage of the glycosyl bond as found for the O^2-ethyl deoxyribonucleosides, although at a greatly reduced rate. However, in terms of the stabilities of the parent nucleosides, our data indicate that the glycosyl bond of O^2-alkyl ribopyrimidine nucleosides is greatly weakened.

Hydrolysis of O^4-Ethyl Deoxyribo- and Ribonucleosides and O-Ethyl Bases. The only reaction detected in acid pHs was dealkylation which occurred most readily at pH 1.5 (Table II; Figure 2, reaction II). In general, the *O*-alkyl groups on Thd were more stable than on Urd or dUrd. This was also the case for the *O*-alkyl bases.

When O^4-alkyl-Urd or O^4-alkyl-dUrd were heated in alkali it was found that the treatment of the sample prior to heating had a major effect. When the alkaline samples were kept at 0–25 °C before being heated at 100 °C, dealkylation was rapid. When the same samples were first kept in dry ice, a new product was formed upon heating, which had the spectrum and radioactivity of the original alkyl nucleoside but was chromatographically distinct. These products are still under investigation and appear to result from rearrangements. Table II presents results for the dealkylation reaction without dry ice pretreatment.

Discussion

Cleavage of glycosyl bonds of nucleosides is now generally believed to occur by direct hydrolysis of the C–N bond (Garrett & Mehta, 1972; Shapiro & Danzig, 1972, 1973) rather than by hydrolysis of an intermediate Schiff's base. The data of Shapiro & Kang (1969) indicated that water was involved in the mechanism of hydrolysis since they found that heating deoxyuridine in ethanol rather than water greatly reduced glycosyl bond hydrolysis. In line with this we find that 5 h heating at 100 °C under anhydrous conditions[2] did not cause any detectable depyrimidination of O^2-Et-dCyd, while the reaction is complete in less than 5 min in neutral aqueous solution.

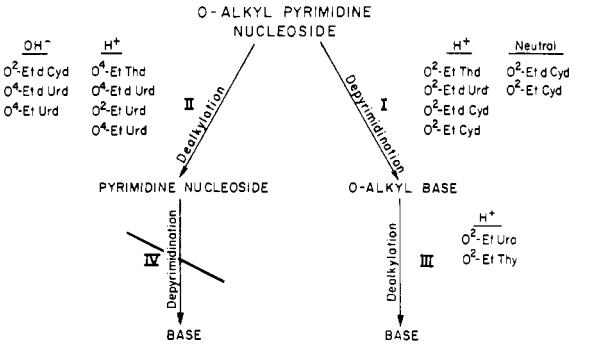

FIGURE 2: Diagram of the pathways for loss of the sugar or alkyl group from *O*-alkyl pyrimidines. For each reaction, a list is given of the derivatives and the pH favoring a given reaction (see Table II).

TABLE III: Rate of Hydrolysis of Glycosyl Bond of Alkyl Nucleosides at pH 7.0, 37 °C.[a]

Nucleoside	Half-life of glycosyl bond
7-Ethyldeoxyguanosine	5 h[b]
1,7-Diethyldeoxyguanosine	7 h
O^2-Ethyldeoxycytidine	26 h
O^2-Ethylthymidine	~220 days[c]

[a] The method for determination of half-lives is described in the Experimental Section. [b] Calculation of the half-life of this compound, based on data in Figure 5 from Lawley & Brookes (1963), gives a value of 6.3 h. [c] This figure was obtained by extrapolation from the amount of O^2-ethylthymine found after a maximum incubation of 10 days.

The pH at which glycosyl bond cleavage occurs seems to be correlated with the pK_a of the alkyl pyrimidine, protonation enhancing depyrimidation (Table I). The apparent pH independence of glycosyl bond cleavage in some instances is in line with the mechanism proposed by Shapiro & Kang (1969).

Both deoxyribo- and ribopyrimidines which are alkylated on the O^2 position are more labile than the parent compounds. Michelson & Todd (1955) report that O^2-5′-*O*-cyclothymidine and O^2-3′-*O*-cyclothymidine undergo hydrolysis in acid (pH 1, 100 °C, 10 min) with thymine as the final product. The rate of hydrolysis appears to be greater than that for thymidine under the same conditions. However, the influence of the attached sugar intermediate is different for the two cyclothymidines. Acid lability of the glycosyl bond of isocytidine has also been observed in 0.66 N H_2SO_4 (reflux, 1 h) (Doerr & Fox, 1967) but no data are given for the pH range used in this study. Another example of the influence of a substituent at the C-2 of pyrimidines labilizing the glycosyl bond was found by Kröger & Cramer (1977) who studied *S*-alkyl 2-thiocytidine. On the basis of these earlier suggestions the great labilization of the glycosidic linkage observed by us upon alkylation of the O^2 of the deoxypyrimidines was not expected. Thus the glycosyl bond of O^2-alkyl dCyd is four orders of magnitude more labile than that of a deoxypurine, and O^2-alkylation of dUrd or Thd weakens the glycosyl linkage so that these compounds are much less stable than deoxypurines. In contrast, O^4-alkylation does not appear to labilize the glycosidic bond of pyrimidine nucleosides.

The glycosyl bond of the deoxyribo derivatives is, as expected, cleaved much more rapidly than that of the ribo derivatives. The half-life of depyrimidation was 100 times faster for O^2-Et-dCyd than O^2-Et-Cyd. This 100-fold difference is

[2] The authors thank Dr. Carl Carrano of the Chemistry Department, University of California, Berkeley, for preparing the anhydrous samples which were heated in pyridine. The pyridine was predried over KOH and then distilled over BaO. All procedures followed normal Schlenk techniques under oxygen-free nitrogen (see Schriver, 1969).

also observed for the acid-catalyzed depyrimidination of dCyd as compared with Cyd (Kochetkov & Budowsky, 1972). Thus while O^2 alkylation increases the velocity of depyrimidination strikingly, it does not affect the ratio of rate constants influenced only by the nature of the sugar.

One other rate comparison is that of the influence of the 5-methylation of the ring on glycosyl bond cleavage. When one compares the depyrimidination rates of O^2-Et-dUrd and O^2-Et-Thd at pH 7.0, 100 °C, the half-life of the latter is 2.7 h while that of the former is 1 h. Under similar hydrolysis conditions, Shapiro & Kang (1969) report that the rate constant of deoxyuridine is 2.5 times that of thymidine. Therefore the influence of the methyl substituent in decreasing the hydrolysis rate appears to be independent of the *O*-alkyl modification.

At pH 1.5 given enough time and/or temperature, all of the *O*-alkyl pyrimidine nucleosides and bases are dealkylated (Table II). As shown in Figure 2, the O^2 derivatives follow reactions I and III, while the O^4 derivatives follow reaction II. At pH 12.5 the decomposition of the O^2-alkyl derivatives follows both paths, reaction I followed by III, and also directly II. Under the same conditions the O^4 derivatives are simply dealkylated (reaction II). Reaction IV does not occur under our experimental conditions.

In considering the roles of these degradative reactions in the effects of in vivo alkylation, only the data relating to neutral conditions are pertinent. In discussing glycosyl bond cleavage under physiological conditions it should be pointed out that nonenzymatic hydrolysis of alkyl bases from DNA is very much slower than cleavage of this bond in mononucleotides, which in turn have a more stable glycosyl bond than nucleosides. There exist several examples to illustrate this fact, but no accepted hypothesis. One example is a comparison of half-lives at pH 7.0, 37 °C, of the glycosyl bond of 7-Et-dGuo, 5 h (Table III); 7-Et-dGMP, 19 h (Lawley & Brookes, 1963); and 7-EtG in DNA, 225 h (Rajewsky et al., 1976).

The half-life of 26 h for the glycosyl bond of O^2-Et-dCyd at neutrality, 37 °C, can thus be compared with about 5 h for 7-Et-dGuo (Table III). One of the next most labile glycosyl bonds now studied, O^2-Et-Thd, has a half-life at pH 7.0, 37 °C, of about 220 days (Table III). It seems clear that, of the pyrimidines, only O^2 alkylation of deoxycytidine would lead to significant nonenzymatic removal from DNA under physiological conditions. This does not mean, however, that this and the other *O*-alkyl pyrimidines may not be released from DNA in vivo at a much more rapid rate than in vitro, as has been demonstrated for 3-alkyl-A, O^6-alkyl-G, 3-alkyl-G, and possibly other alkyl derivatives (Craddock, 1973; Walker & Ewart, 1973; Goth & Rajewsky, 1974; Margison et al., 1976). Besides the potential role of loss of an O-alkylated base, or excision of that nucleotide, the possibility of its mispairing during DNA replication or transcription must be considered. Experiments on the template properties of copolymers of U or C with O^2 or O^4-alkyl U indicate that anomalous base pairing results from these alkylations (Singer et al., 1978).

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References

Craddock, V. M. (1973) *Biochim. Biophys. Acta 312,* 202.
Doerr, I. L., & Fox, J. J. (1967) *J. Org. Chem. 32,* 1462.
Garrett, E. R., & Mehta, P. J. (1972) *J. Am. Chem. Soc. 94,* 8532.
Goth, R., & Rajewsky, M. F. (1974) *Z. Krebsforsch. 82,* 37.
Hilbert, G. E., Jansen, E. F., & Hendricks, S. B. (1935) *J. Am. Chem. Soc. 57,* 552.
Katritzky, A. R., & Waring, A. J. (1962) *J. Chem. Soc.,* 1540.
Kochetkov, N. K., & Budowsky, E. I. (1972) in *Organic Chemistry of Nucleic Acids,* Part B, Chapter 8, p 425, Plenum Press, London.
Kröger, M., & Cramer, F. (1977) *Chem. Ber. 110,* 361.
Kuśmierek, J. T., & Singer, B. (1976) *Nucleic Acids Res. 3,* 989.
Lawley, P. D., & Brookes, P. (1963) *Biochem. J. 89,* 127.
Lawley, P. D., & Warren, W. (1976) *Chem.-Biol. Interact. 12,* 211.
Lindahl, T., & Karlstrom, O. (1973) *Biochemistry 12,* 5151.
Margison, G. P., Margison, J. M., & Montesano, R. (1976) *Biochem. J. 157,* 627.
Michelson, A. M., & Todd, A. R. (1955) *J. Chem. Soc.,* 816.
Rajewsky, M. F., Goth, R., Laerum, O. D., Biessmann, H., & Hülser, D. F. (1976) in *Fundamentals in Cancer Prevention* (Magee, P. N., Ed.) p 313, University Park Press, Baltimore, Md.
Robins, M. J., & Naik, S. R. (1971) *Biochemistry 10,* 3591.
Schmidt-Nichels, W., & Johnson, T. B. (1930) *J. Am. Chem. Soc. 52,* 4511.
Schriver, D. F. (1969) *The Manipulation of Air Sensitive Compounds,* McGraw-Hill, New York, N.Y.
Shapiro, R., & Danzig, M. (1972) *Biochemistry 11,* 23.
Shapiro, R., & Danzig, M. (1973) *Biochim. Biophys. Acta 319,* 5.
Shapiro, R., & Kang, S. (1969) *Biochemistry 8,* 1806.
Shugar, D., & Fox, J. J. (1952) *Biochim. Biophys. Acta 9,* 199.
Singer, B. (1972) *Biochemistry 11,* 3939.
Singer, B. (1976a) *Nature (London) 264,* 333.
Singer, B. (1976b) *FEBS Lett. 63,* 85.
Singer, B., Fraenkel-Conrat, H., and Kuśmierek, J. T. (1978) *Proc. Natl. Acad. Sci. U.S.A.* (in press).
Sober, H., Ed. (1973) *Handbook of Biochemistry,* 2nd ed, p J227, CRC Press, Cleveland, Ohio.
Sukhorukov, B. I., Gukovskaya, A. S., Sukhoruchkina, L. V., & Lavrrenova, G. I. (1972) *Biophysik 17,* 5.
Trevelyan, W., Procter, D. P., & Harrison, J. S. (1950) *Nature (London) 166,* 444.
Venner, H. (1964) *Hoppe-Seyler's Z. Physiol. Chem. 339,* 14.
Venner, H. (1966) *Hoppe-Seyler's Z. Physiol. Chem. 344,* 189.
Walker, I. G., & Ewart, D. F. (1973) *Mutat. Res. 19,* 331.
Wong, J. L., & Fuchs, D. S. (1970) *J. Org. Chem. 35,* 3786.