inhibitor are reasonably high as early as 12 hr after birth and increase dramatically as the animal becomes an adult. The data do not eliminate the birth process itself as being the triggering mechanism, however, studies carried out with fasted animals show that it is possible to delay the appearance of inhibitor activity by withholding food from the newborn animal for at least 48 hr.

Recently, Sharma and Borek (1970) have shown a tRNA methylase inhibitor to be under estradiol regulation in both rat and pig uteri. In pigs, growth hormone levels are high at birth and remain elevated in animals that are starved for 60 hr after birth. In contrast, growth hormone levels decrease in animals fed immediately after birth to those levels observed in adult pigs (Swiatek *et al.*, 1968). It would be interesting to determine whether the differences in levels of inhibitor in fed and fasted newborn pig brain are hormonally regulated or dependent on some other dietary factors as yet unexplained. Experiments are currently in progress to answer this question.

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Glutathione-Catalyzed Hydrogen Isotope Exchange at Position 5 of Uridine. A Model for Enzymic Carbon Alkylation Reactions of Pyrimidines*

Thomas I. Kalman

ABSTRACT: The effect of glutathione (GSH) on the exchange of hydrogen to deuterium at position 5 of uridine (Urd) was studied by using proton magnetic resonance spectroscopy. It was found that in D_2O solutions, at 80°, the rate of H-isotope exchange was enhanced in the presence of GSH and that the enhancement of the pseudo-first-order rate of exchange was proportional to the GSH concentration. The results obtained with GSH derivatives indicated the requirement of a free SH group for catalysis. The GSH-catalyzed H-isotope exchange showed a bell-shaped dependence on the OD^- ion concentration, suggesting that in the rate-determining step the ionized SH group of GSH reacts with the nonionized species of Urd. Ionization of Urd causes a substantial shielding of the proton at position 6, indicating the increased electron density of the 5,6-double bond, which may account for the lack of reactivity observed at high pD values. The results are consistent with a catalytic mechanism of H-isotope exchange involving the reversible addition elimination of the SH group of GSH across the 5,6-double bond of Urd.

The relevance of these findings to the mechanism of enzyme-catalyzed C-alkylation reactions of pyrimidine nucleotides is discussed.

Lomax and Greenberg (1967) and Yeh and Greenberg (1967) reported that thymidylate synthetase and deoxycytidylate hydroxymethylase, respectively, catalyze the exchange of hydrogen to tritium at position 5 of the substrates, deoxyuridylate and deoxycytidylate, and suggested that the labilization of H-5 may be the initial step in the overall substitution reaction. Both of these enzymic reactions belong to a group of metabolic transformations of pyrimidine nucleotides, which involve C–C-bond formation at position 5 of the pyrimidine ring. C-alkylation reactions of this type also include hydroxymethylation of deoxyuridylate (Roscoe and Tucker, 1966), the formation of the thymine, hydroxymethyluracil, and 5-carboxyuracil moieties of the nucleoside antibiotics, polyoxins (Isono *et al.*, 1969), and certain modifications of pyrimi-

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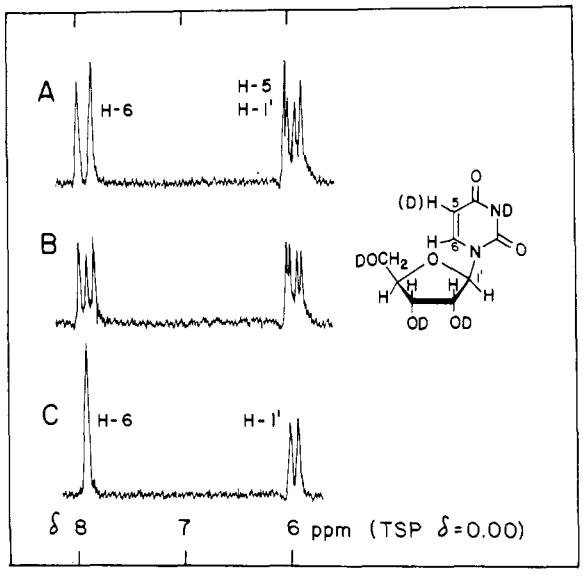


FIGURE 1: The change of the proton magnetic resonance spectrum of Urd during incubation with GSH. Spectra of 0.5 M GSH-d_7 and Urd-d_4 in D_2O (pD 8.0) were recorded at the beginning of the reaction (A) and after 1-day (B) and 2-weeks (C) incubation at 80°.

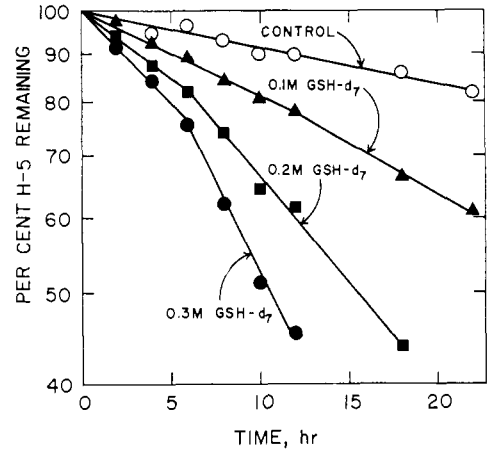


FIGURE 2: Semilog plots of the time course of the H-isotope-exchange reaction of Urd in the presence of various concentrations of GSH. Solutions of Urd-d_4 in D_2O (0.2 M, pD 9.6) were incubated in the absence and presence of the indicated concentrations of GSH-d_7 at 80° for various length of time and the H-isotope ratio at C-5 was calculated from the recorded nuclear magnetic resonance spectra (see Experimental Section).

dine bases at the polynucleotide level, such as the methylation (Borek and Srinivasan, 1966) of uracil in RNA and cytosine in RNA and DNA, and also the synthesis of the pseudouridylate (Goldwasser and Heinrichson, 1966), 2-thiouracil-5-acetic acid methyl ester (Baczynskyj *et al.*, 1968), 5-methylaminomethyl-2-thiouridine (Carbon *et al.*, 1968), and 5-carboxymethyluridine (Gray and Lane, 1968) residues of tRNA. The catalytic mechanisms of these reactions are of considerable interest, since C alkylations, in general, require larger activation energies than the analogous alkylations of the more electronegative S, N, or O atoms.

It was recently proposed (Kalman, 1970) that certain biological C-methylation reactions may have a common catalytic mechanism involving the nucleophilic addition of an enzymic SH group to the double bond of the methyl acceptor, forming a thioether intermediate. This proposal provides a working hypothesis for the elucidation of the mechanism of C-alkylation reactions of pyrimidine nucleotides. The present work was undertaken to obtain evidence for the catalytic potential of cysteinyl residues in these reactions by demonstrating the catalytic effects of glutathione on the H-isotope exchange at position 5 of uridine.

Experimental Section

Materials. Uridine was purchased from Aldrich, Milwaukee, Wis.; glutathione (reduced and oxidized) and *S*-methylglutathione (lot 98B-2070) were from Calbiochem, Los Angeles, Calif., and Sigma, St. Louis, Mo., respectively. Silanor–D_2O–TSP[1] (D_2O containing 0.75% TSP) was supplied by Merck, Rahway, N. J., D_2O and NaOD in D_2O were obtained from Diaprep, Atlanta, Ga. 3,3′-Dithiobis(6-nitrobenzoic acid) was a product of Eastman, Rochester, N. Y.

Measurement of pD. Solutions were made up with Silanor–D_2O–TSP, flushed with N_2, and then adjusted with 2 N NaOD at 25° to the desired pD, which was obtained according to the method of Glasoe and Long (1960) by adding 0.40 to the apparent pH value measured using a Radiometer M26 pH meter, standardized with aqueous buffers.

H-Isotope-Exchange Measurements. The hydrogens attached to N, O, or S of Urd, GSH, oxidized glutathione, and *S*-methylglutathione were exchanged to deuterium by dissolving the materials in D_2O, followed by freeze-drying. Solutions (0.6-ml total volume) adjusted to the desired pD were put in nuclear magnetic resonance tubes and flushed with N_2. The tubes were closed with plastic caps and incubated at 80° for various length of time. The proton magnetic resonance spectra were recorded at ambient temperature on a Varian A-60 nuclear magnetic resonance spectrometer, at 60 MHz. The extent of isotope exchange was calculated from the relative intensities of the singlet and doublet signals of the H-6 proton of Urd-d_5 and Urd-d_4, respectively, according to the method of Santi *et al.* (1970). The value of the sum of the doublet intensities of H-6 divided by the sum of the singlet and doublet intensities of H-6 was taken as a measure of the remaining fraction of H-5. The k_{obsd} values were calculated from the slopes of the initial linear portions of the log per cent H-5 remaining *vs.* time plots and they represent apparent pseudo-first-order rate constants.

Measurements of Chemical Shifts. All chemical shifts were measured as signal positions from the internal standard TSP (δ 0.00) in either hertz or parts per million units, as indicated.

Results

Labilization of the Hydrogen at Position 5 of Urd by GSH. Incubation of Urd-d_4 in slightly alkaline D_2O solutions of GSH-d_7 (pH 8.0) at 80° was accompanied by changes in the proton magnetic resonance spectrum of the pyrimidine moiety of the nucleoside, while no significant change was observed in the absence of GSH-d_7 under the same conditions. Figure 1 shows that during the course of a typical experiment the doublet signal of the H-5 proton (5.95) disappears and the H-6 doublet is replaced by a singlet of the same chemical shift (7.93). These spectral changes are characteristic for the substitution of the hydrogen by deuterium at C-5 of Urd

[1] Abbreviations used are: TSP, sodium 3-trimethylsilylpropionate-2,2,3,3-d_4; GSH-d_7 and Urd-d_4 represent glutathione and uridine, respectively, with their labile hydrogens replaced by deuterium.

TABLE I: Apparent Pseudo-First-Order Rate Constants Obtained at Various Concentrations of GSH.[a]

Additions	Concn (M)	$pD_{25°}$	k_{obsd} (hr^{-1})[b]
None	0	9.62	0.0090
GSH-d_7	0.1	9.59	0.0206
GSH-d_7	0.2	9.57	0.0327
GSH-d_7	0.3	9.58	0.0447

[a] Experimental conditions were those described in the legend of Figure 2. [b] Pseudo-first-order rate constants, calculated from the initial linear portions of the plots of Figure 2.

TABLE II: Apparent Pseudo-First-Order Rate Constants Obtained in the Presence and Absence of GSH Derivatives.[a]

Addns[b]	Concn (M)	$pD_{25°}$	k_{obsd} (hr^{-1})[c]	Δk_{obsd} (hr^{-1})[d]
None	0	9.62	0.0087	
GSSG-d_{12}	0.1[e]	9.61	0.0095	0.0008
GSMe-d_6	0.2	9.55	0.0118	0.0031

[a] Experimental conditions were those described in the legend of Figure 4. [b] Abbreviations are: GSSG, oxidized glutathione; GSMe, *S*-methylglutathione. Pseudo-first-order rate constants, calculated from the initial linear portions of the plots of Figure 4. [d] Pseudo-first-order rate constants corrected for the blank reaction. [e] Equivalent with 0.2 M GSH-d_7.

(Heller, 1968). Thus it is apparent that complete H-D exchange is achieved after 2-weeks incubation (Figure 1C).

It was of interest to determine the effect of the concentration of GSH on the rate of the H-isotope-exchange reaction. Figure 2 shows the time course of the exchange reaction in the absence and presence of various amounts of GSH-d_7 (pD 9.6). The H-isotope-exchange reaction follows pseudo-first-order kinetics during the initial 6–12-hr period, as is indicated by the linearity of the plots. Deviations from simple kinetics were observed during prolonged incubation and could be attributed to the degradation of GSH[2] under the reaction conditions. It is shown that there is a slow exchange even in the absence of GSH in the control experiment, however the rate is significantly enhanced by the addition of increasing amounts of GSH-d_7. The k_{obsd} values calculated from the initial linear portions of the plots are given in Table I. By subtracting the k_{obsd} value of the control experiment from those of the others, Δk_{obsd} values are obtained, which represent the rate enhancement due to the presence of GSH-d_7. A plot of Δk_{obsd} *vs.* the concentration of GSH-d_7 is shown in Figure 3. It is apparent that the rate enhancement of the H-isotope exchange in Urd is proportional to the GSH concentration.

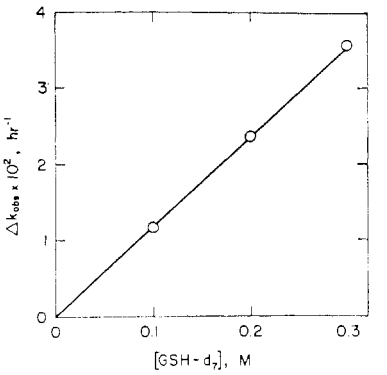


FIGURE 3: The dependence of the rate enhancement of the H-isotope-exchange reaction on the concentration of GSH. The Δk_{obsd} values represent the difference between the pseudo-first-order rate constants obtained in the presence and absence of GSH-d_7, and were calculated from the data presented in Table I.

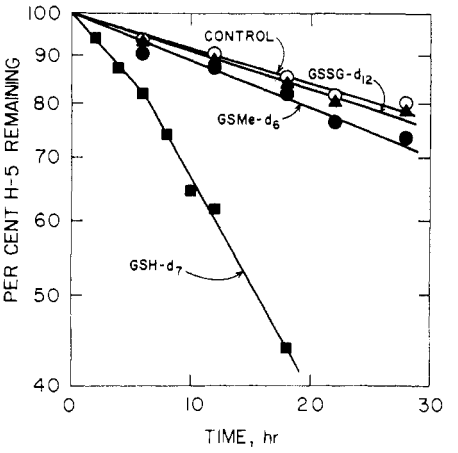


FIGURE 4: Semilog plots of the time course of the H-isotope-exchange reaction in the presence and absence of various GSH derivatives. Abbreviations are: GSSG, oxidized glutathione; GSMe, *S*-methylglutathione. D_2O solutions of Urd-d_4 were incubated under identical conditions with those described in the legend of Figure 2, except that GSH-d_7 was replaced by 0.1 M GSSG-d_{12} and 0.2 M GSMe-d_6, where indicated. The GSH-d_7 (0.2 M) plot is reproduced from Figure 2 for comparison.

Complete exchange of H-5 to deuterium was observed even at GSH-d_7 concentrations much smaller than that of Urd, which is consistent with a catalytic participation of the tripeptide in the reaction.

Requirement of a Free SH Group for Catalysis. In order to demonstrate that the functional group participating in the exchange reaction is the SH group of GSH, the rate-enhancing effects of two S-substituted GSH derivatives, oxidized glutathione and *S*-methylglutathione, were compared to that of GSH itself. Figure 4 shows the results of this experiment. Oxidized glutathione did not increase the rate significantly over that of the control, while a slight rate enhancement was observed in the presence of *S*-methylglutathione. The numerical rate constants are presented in Table II. The rate enhancement caused by oxidized glutathione and *S*-methylglutathione was 3.4% and 13.1%, respectively, of that produced by an equivalent amount of GSH-d_7. In order to explain the small, but

[2] Paper chromatographic analysis (Gutcho and Laufer, 1954) of a 24-hr reaction mixture, using 80% aqueous phenol as solvent, revealed in addition to GSH (R_F 0.34) the presence of three ninhydrin- (Toennies and Kolb, 1951) positive components (R_F 0.09, 0.14, and 0.23) and a major ninhydrin-negative component (R_F 0.57), which was detected by the 2,2′-dithiobis(5-nitropyridine) spray (Grassetti and Murray, 1969), indicating the presence of a free SH group. Since small molecular thiols are more efficient catalysts than GSH (T. I. Kalman, unpublished results) the accumulation of a SH-bearing degradation product is expected to result in an increase of the exchange rate, when its concentration reaches a kinetically significant level.

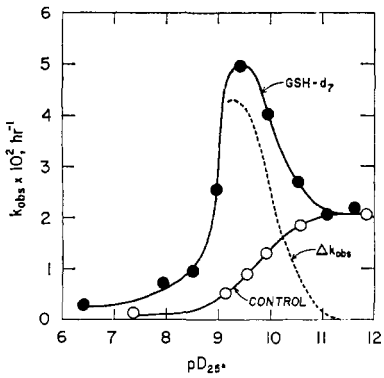


FIGURE 5: The pD dependence of the H-isotope-exchange reaction. The pD of the solutions of Urd-d_4 (0.2 M) and GSH-d_7 (0.25 M) in D_2O were adjusted with 2 N NaOD to the indicated values (see Experimental Section). Nuclear magnetic resonance spectra were recorded at regular intervals during incubation of the solutions at 80°. The k_{obsd} values were calculated from the measured H-isotope-exchange rates (see Experimental Section). Broken line represents the difference between the plots obtained in the presence and absence of GSH-d_7 (Δk_{obsd}).

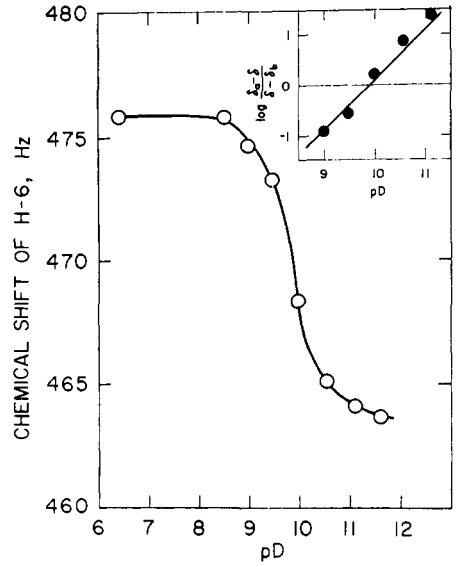
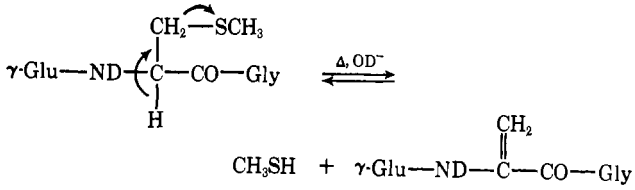


FIGURE 6: The pD dependence of the chemical shift of the proton at C-6 of Urd. The chemical shifts refer to the position of the midpoint of the H-6 doublet signal from the internal standard TSP. Insert: δ_a and δ_b are the chemical shifts of H-6 of the acidic and basic (ionized) forms of Urd-d_4, respectively, and are approximated by the maximum and minimum δ values measured. Dots represent experimental points, solid line is a theoretical ionization plot calculated for an acid with a pK_a of 9.9 (in pD units).

significant rate increase in the latter case, the possibility of the degradation of *S*-methylglutathione, yielding methylmercaptan, was considered. This may occur at high temperatures by base-catalyzed elimination of CH_3SH as shown



Since the SH group of methylmercaptan could participate in the H-isotope-exchange reaction *in lieu* of that of GSH, a degradation reaction of this type could account for the apparent discrepancy. Indeed, the odor of methylmercaptan could be noticed after opening the nmr tube. Furthermore, the SH content of the 24-hr reaction mixture was determined by the method of Ellman (1959) and found to be 5×10^{-4} M, which also supports the above conclusions.

The results of these experiments clearly indicate that the catalytic effect of GSH is dependent upon the availability of a free SH group.

Dependence of the Rate of H-Isotope Exchange on the pD. It was of interest to examine the effects of pD on the GSH-catalyzed H-isotope-exchange reaction. The rates of H-5 exchange were determined in 0.2 M Urd-d_4 solutions of varying pD's, in the presence and absence of 0.25 M GSH-d_7. The pseudo-first-order rate constants plotted against the pD are shown in Figure 5. The rate of H-isotope exchange in the presence of GSH first becomes faster by increasing the pD, than declines above pD 9.3–9.5. The rate of the control reaction increases beyond pD 9.5, but reaches a plateau at pD 11. It is important to note that the rates of exchange in the presence and absence of GSH become equal above pD 11. Thus, the rate enhancement due to the presence of GSH, which is represented by the broken line in Figure 5, approaches $\Delta k_{obsd} = 0$ at pD 11.3.

The bell-shaped dependence of the GSH-catalyzed H-isotope exchange on the pD indicates the opposing effects of two ionization reactions. It is reasonable to assume that these are the ionizations of the SH group of GSH and the NH-3 of Urd. The former seems to be required for catalytic activity. The finding that Δk_{obsd} becomes 0 at pD >11 indicates that GSH reacts *only* with the nonionized form of Urd.

According to these considerations, the pseudo-first-order rate constants can be expressed by eq 1, where k_0 and k_G are

$$k_{obsd} = k_0[OD^-]\frac{[D^+]}{K_U + [D^+]} + k_G[G]\frac{K_G[D^+]}{(K_G + [D^+])(K_U + [D^+])} \quad (1)$$

the rate constants of the control and the GSH-d_7- (G) catalyzed reactions, respectively, K_U and K_G are the respective acidic ionization constants of Urd-d_4 and GSH-d_7 in D_2O. At low pD, eq 1 becomes 0 and at high pD, k_{obsd} approaches a constant value (taken from Figure 5), according to eq 2, where

$$k_{obsd} = k_0[D_2O]\frac{K_{D_2O}}{K_U} = 0.02\ hr^{-1} \quad (2)$$

K_{D_2O} is the dissociation constant of D_2O.

The pD optimum of 9.3 obtained from the Δk_{obsd} curve in Figure 5 is in agreement with the pK_a values[3] of 9.20 and 9.25 reported for GSH (Benesch and Benesch, 1955) and Urd (Fox and Shugar, 1952), respectively, which supports the above conclusions.

[3] All values in eq 1 and 2 refer to 80°. Although in the experiments described in Figure 5 the pD was measured at 25°, a similar k_{obsd} *vs.* pD pattern, but somewhat different numerical values would presumably be obtained by using pD values measured at 80°. The pK_a's are also expected to have different values in D_2O, at 80°. No attempts were made to determine these values. It should be noted, however, that kinetic data obtained at 95° in D_2O and pD values measured at room temperature could also be correlated with regular pK_a values in the study of the H-5 exchange of cytidine (Shapiro and Klein, 1967).

Dependence of the Chemical Shift of the H-6 Proton of Urd on the pD. It was observed that by increasing the pD of the reaction mixture of 0.2 M Urd-d_4 and 0.25 M GSH-d_7, the signal of the H-6 proton of Urd was shifted upfield. In Figure 6, the chemical shift of the H-6 proton is plotted as a function of pD. The resulting spectrometric titration curve yields an apparent pK_a of 9.9. The same pK_a value was obtained by the titration of 0.2 M Urd-d_4 solutions in D_2O with 2 N NaOD.

The total shielding effect associated with the ionization of Urd was $\Delta\delta$ = 0.20–0.21 ppm (12.2–12.4 Hz),[4] indicating a significant increase in the electron density of the 5,6-double bond of the ionized Urd.

Discussion

H-isotope exchange at position 5 of Urd has been studied previously (Fink, 1964; Santi and Brewer, 1968; Heller, 1968; Cushley *et al.*, 1968; Wechter, 1970). It was suggested that the mechanism of the exchange observed in basic D_2O solutions may involve the 1,4 addition of a nucleophile to the 5,6-double bond of Urd, followed by a tautomeric shift and the elimination of the H-5 proton and the base (Heller, 1968; Cushley *et al.*, 1968; Wechter, 1970). A similar mechanism was proposed for the analogous hydroxide ion catalyzed deuterium exchange reaction of 1-methyluracil-*5-d* (Santi *et al.*, 1970). Such a mechanism is also consistent with the results presented in this communication. Thus, it is likely that the GSH-catalyzed isotope exchange involves the nucleophilic addition elimination of the SH group of GSH across the 5,6-double bond of Urd. Sulfhydryl addition to the 5,6-double bond has been demonstrated by the formation of 6,5′-cyclonucleosides from 5′-thiouridine and its acetonide (Bannister and Kagan, 1960; Chambers and Kurkov, 1963; Reist *et al.*, 1964) and has also been implicated in the mechanism of certain substitution reactions of 5-bromouracils (Szabo *et al.*, 1970). Similar adduct formation between bisulfite and Urd was recently reported to occur readily in aqueous solutions (Shapiro *et al.*, 1970; Hayatsu *et al.*, 1970). It was also established that the position of attack by these sulfur nucleophiles is C-6 of the pyrimidine ring. That GSH may similarly add to position 6 and *not* 5 of Urd is indicated by the complete lack of exchange of the H-6 proton, since C-5 attack would result in the labilization of the H-6 proton (Cushley *et al.*, 1968).

Based on the above considerations, a mechanism outlined in Figure 7 may be suggested for the GSH-catalyzed isotope-exchange reaction. According to this hypothetical mechanism, the reaction starts with the 1,4 addition of the ionized SH group of GSH to the pyrimidine ring. Tautomerization leads to an unstable adduct intermediate, which quickly decomposes. The 1,2-trans elimination of GSH (route c) or GSD

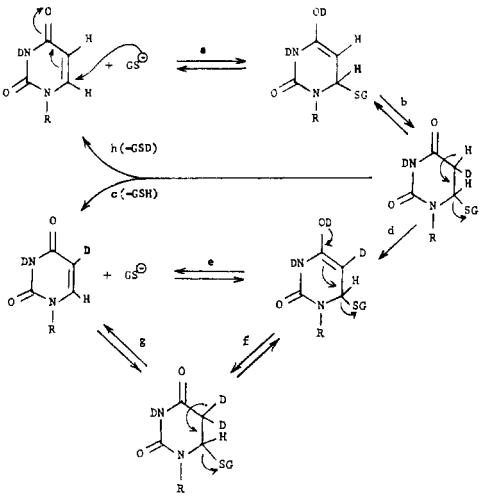


FIGURE 7: Mechanistic scheme for the GSH-catalyzed H-isotope exchange at position 5 of Urd. Abbreviations: GS^-, glutathione-d_6 with ionized SH group; GSD, glutathione-d_7; GSH, glutathione-d_6.

(route h) from the intermediate yields either the deuterated product or the starting material, respectively, depending on the configuration at position 5. An alternative path to the deuterated species is a reverse of the 1,4 addition, which is preceded by a tautomeric shift (route a-b-d-e). Formation of the 5-dideuterated adduct also leads to the product (route a-b-d-f-g). Since the rate-determining step is the nucleophilic attack of Urd by GS^- (step a), as is indicated by the dependence of Δk_{obsd} on the GSH concentration (Figure 3) and also by the pD profile (Figure 5), the kinetic data do not allow us to determine the relative contributions of these alternative pathways to the formation of the deuterated product.[5]

It is conceivable that the SH group of an enzyme[6] can undergo a reaction similar to that of the tripeptide, GSH, described in Figure 7. The formation of the saturated intermediate in this case, however, should be more favorable due to the proper positioning of the enzyme-bound substrate and the functional SH group. This argument is supported by the finding that at equilibrium, in neutral solution, 20% of 5′-thiouridine is in the adduct form (Reist *et al.*, 1964), as a consequence of the intramolecular nature of the reaction. Furthermore, the equilibrium of the addition of the 5′-SH group of the 2′,3′-isopropylidene derivative of 5′-thiouridine to the 5,6-double bond is shifted further toward the formation of the adduct, yielding a stable, crystallizable compound (Chambers and Kurkov, 1963), showing the strong influence of the molecular conformation on this equilibrium. Thus, it can be concluded that the nucleophilic addition of an enzymic SH group to the 5,6-double bond of pyrimidine nucleotides could be

[4] The dependence of the chemical shift of the H-6 proton on the ionization of N-3 of Urd explains the apparent shielding effects observed by Heller (1968) in the presence of various bases, since an increase in the pD of such solutions can be anticipated. It can be estimated from the reported chemical shift data of Heller (1968), using the titration curve of Figure 6 of this study, that the pD of the solution of Urd and 2-mercaptoethylamine used in the isotope-exchange studies could have a value of 9.4–9.7. Since the pK_a of 2-mercaptoethylamine is 8.35 (Benesch and Benesch, 1955) the SH group must have been almost completely ionized under the experimental conditions described. The lack of isotope exchange reported in the presence of mercaptoethanol, could be the consequence of the low pD of the solution, rather than the inability of the SH group, as opposed to the amino group to catalyze the H-isotope exchange. In fact, mercaptoethanol effectively catalyzes the exchange of the H-5 proton of Urd if the pD of the solution is adjusted to 8–10 (T. I. Kalman, unpublished results).

[5] A direct 1,2-trans addition followed by exchange through ionization of H-5 and subsequent elimination is also compatible with the results, as was pointed out by one of the reviewers. That tautomerization may play a role in both the 1,4- and the 1,2-addition mechanisms of isotope exchange is shown by the fact that exchange occurs inspite of the known *trans* stereospecificity of addition eliminations across the 5,6-double bond of pyrimidines. Tautomerization may also explain the finding that the two hydrogens at position 5 of the photohydrate of uridylate are exchanged relatively rapidly in neutral solutions (Chambers, 1968).

[6] Other basic amino acid side chains may similarly participate in such an addition reaction. However, it is well established that *N*-ethylmaleimide, which has a somewhat similar, but much more reactive α,β-unsaturated carbonyl system, reacts preferentially with cysteinyl residues of proteins, with a high degree of specificity (Webb, 1966).

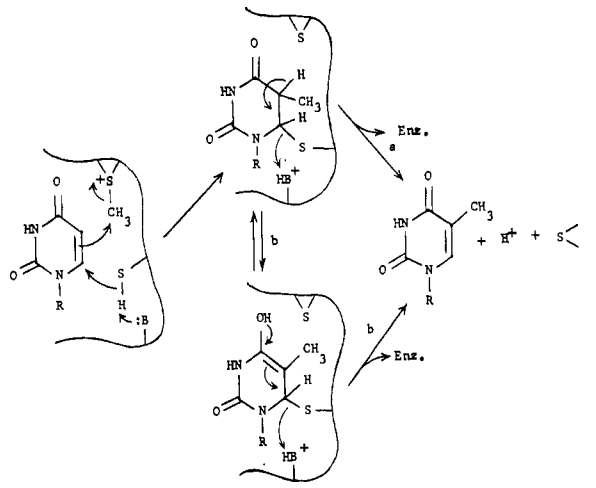


FIGURE 8: Hypothetical mechanism for the methylation of uridylic acid in tRNA. Abbreviations: $CH_3S^+ <$, *S*-adenosylmethionine; $S<$, *S*-adenosylhomocysteine; B, basic group of the enzyme, R, polynucleotide chain of tRNA.

responsible for the observed H-isotope exchange at position 5 (Lomax and Greenberg, 1967; Yeh and Greenberg, 1967). In the case of cytosine nucleotides, protonation of the pyrimidine ring by the enzyme may be a prerequisite for substitution to occur, as is suggested by the results of Shapiro and Klein (1967).

In the H-isotope-exchange reaction, the negative charge of the ionized SH group is directed toward the carbonyl oxygen at position 4, as a consequence of the Michael-addition mechanism discussed above. It is conceivable that the electron flow may also be attracted by a strong and properly positioned alkylating electrophile, in the proximity of position 5, resulting in C–C-bond formation.[7] Thus, a common mechanism of catalysis can be proposed for the C-alkylation reaction of pyrimidines, exemplified by the *S*-adenosylmethionine-mediated methylation of uridine residues of tRNA (Figure 8). According to this hypothesis, the electrophilic substitution by the methylsulfonium ion of *S*-adenosylmethionine at C-5 of the pyrimidine ring is initiated by the nucleophilic addition of an ionized SH group of the enzyme to C-6 of the substrate. The ionization of the SH group is likely to be facilitated by enzymic base catalysis, as indicated. The saturated intermediate thus formed can subsequently regenerate the double bond yielding the product either by a direct 1,2 elimination of the elements of the SH group (*via* route a) or by a 1,4 elimination, preceded by a tautomeric shift (*via* route b). If we assume that the sulfur atom and the methyl group are added to the double bond in a trans manner, then route b is the more likely course of the reaction, since it does not involve the energetically less favorable cis-elimination step. Thus, the potential tautomerizability of the α,β-unsaturated carbonyl systems may have functional significance in these reactions. Nucleophilic addition of SH groups to carbon–carbon double bonds of substrates bearing analogous α,β-unsaturated carbonyl systems have been implicated in the mechanism of the C methylation of demethylmenaquinones (Lederer, 1969) and the enzyme-catalyzed cis–trans isomerization of maleylacetoacetate (Edwards and Knox, 1956) and maleylpyruvate (Lack, 1961).

In previously proposed mechanisms of the tRNA–Urd methylation reaction, the activation of the 5,6-double bond was attributed to the electron-donating effect of the neighboring N-1 of the pyrimidine ring (Tropp *et al.*, 1964; Borek and Srinivasan, 1965; Lederer, 1965). There is no direct experimental evidence available in favor of either the SH addition or the neighboring group participation mechanisms. A recent report, however, which provides evidence for the requirement of reduced SH groups for the activity of tRNA–Urd methylase, but not for that of a tRNA–guanine *N*-methylase isolated from the same source (Svensson *et al.*, 1969), is consistent with the idea of SH participation in C-methylation reactions.

The proposed hypothetical mechanism can be applied to other C-alkylation reactions, by replacing tRNA–Urd and *S*-adenosylmethionine in Figure 8 with the proper substrate and alkylating electrophile. For instance, in the case of thymidylate synthetase, the alkylation of deoxyuridylate by the methylene group of the quarternary imine, $CH_2{=}N^+<$, proposed by Kallen and Jencks (1966), may proceed by a similar mechanism. Indeed, it was suggested by Santi and Brewer (1968) that the activation of position 5 of deoxyuridylate toward electrophilic attack may involve the addition and subsequent elimination of a nucleophilic group of this enzyme, at position 6. The above considerations suggest that the SH group of a cysteine residue of the enzyme is a likely candidate for such a nucleophile, especially in the light of recent results which indicated the existence of essential SH groups in highly purified preparations of thymidylate synthetase (Crusberg *et al.*, 1970; Fridland and Heidelberger, 1970). The operation of a sulfhydryl addition–elimination mechanism in this enzymic reaction would also have a bearing upon the mode of action of certain 5-substituted pyrimidine analogs (Kalman and Bardos, 1970).

Further work is in progress to investigate the actual participation of enzymic SH groups in certain representative C-alkylation reactions of pyrimidine nucleotides.

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Added in Proof

Recent studies of Dunlap *et al.* (1971) demonstrated that the catalytic activity of *L. casei* thymidylate synthetase is lost when one of its four accessible SH groups has reacted with *p*-mercuribenzoate.

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[7] An excellent example of a successful competition of the alkylation reaction with the simple 1,4 addition is the reaction of thiols with the double bond of an α,β-unsaturated carbonyl system of jatrophone, which results in the formation of an energetically more stable transannular C–C bond (Kupchan, 1970; S. M. Kupchan, personal communication).

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