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# Methylation and Ethylation of Uridylic Acid and Thymidylic Acid. Reactivity of the Ring and Phosphate as a Function of pH and Alkyl Group†

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ABSTRACT: At pH 6.8 in aqueous solution (4 hr, 22°), all methylating agents tested, i.e., dimethyl sulfate, methyl methanesulfonate, and methylnitrosourea, react with both the N-3 of the ring and the phosphate of UMP and dTMP. Although the extent of reaction varies from 17 to 76%, the ratio of phosphate/ring methylation is ~4. Both the 3-methyl nucleotides and the methyl ester of 3-methyl nucleotides are identified, as well as the methyl esters of unmodified UMP and dTMP. At pH 8.2 the extent of total methylation is similar but reactivity of the N-3 is increased and that of the phosphate decreased so that the phosphate/ring ratio is ~1. At pH 6 almost all reaction is with the phosphate group. Uridine, under the same conditions, is methylated at pH 6.8 to form 15% 3-methyluridine and, at pH 8.2, the N-3 of uridine and thymidine is methylated to about 50%. Neither uridine nor UMP forms detectable ribose methyl products at any of these pH's. The comparable ethylating agents (diethyl sulfate, ethyl methanesulfonate, and ethylnitrosourea) are less reactive and the total ethylation of UMP or dTMP is about 1/5 that of methylation. There is little ethylation of the N-3 but the phosphate is alkylated to a relatively high extent so that the phosphate/base ratio at pH 6.8 is 10–23, and at pH 8.2 the ratio is 5–8. The fact that ethylating agents have a greater affinity than methylating agents for alkylating phosphates is proposed as the basis for the previously reported analytical data in which ethylating agents, acting on DNA or RNA at neutrality, form more phosphotriesters than the analogous methylating agents.

It has been believed that the nucleosides lacking amino groups, uridine and thymidine, as monomer or polymer, do not react with alkylating agents at neutrality, but only with diazomethane or in highly alkaline solution (Brown, 1974). Thus, the reaction of poly(U) with dimethyl sulfate was performed in tri-*n*-butylamine (pH ~12) to yield, quantitatively, 3-methyl poly(U) (Pochon and Michelson, 1967). Similarly, 3-methyluridine was found in high yield when an aqueous solution of poly(U) was treated with ethereal diazomethane (Brimacombe et al., 1965). Considerable methylation of the N-3 of U was observed in yeast RNA also treated in aqueous solution with diazomethane (Kriek and Emmelot, 1963).

Since this laboratory is studying phosphate alkylation in neutral aqueous solution, UMP and dTMP appeared to be useful models as it was not expected that the ring would react and, indeed, Rhaese and Freese (1969) reported that thymine and thymidine did not react with methyl methanesulfonate at pH 7.4.

We now report that uridine, 5′-UMP, 2′(3′)-UMP, thymidine, and dTMP all react significantly at the N-3 of the ring at pH 6.8 when treated with dimethyl sulfate and other methylating agents. We further discuss the reactivity of the ring and the phosphate groups when UMP and dTMP are alkylated by methylating and ethylating agents at various pH's, and the relationship between these findings and the formation of phosphotriesters in RNA and DNA.

## Experimental Section

*Materials.* All unmodified nucleotides and nucleosides were commerical products of the highest purity obtainable. Chromatography of 100–250 absorbancy units indicated that no impurities with uv absorbancy were present in amounts exceeding 0.5%, with the exception of thymidine which contained about 1–2% uridine. Even at this level of contamination the possible reaction products of the uridine in thymidine would be below the level of detection. Dimethyl sulfate ($Me_2SO_4$) and diethyl sulfate ($Et_2SO_4$) were from Matheson Coleman and Bell, methyl methanesulfo-

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Table I: $R_f$ Values of Alkylated Uracil and Thymine Nucleotides and Nucleosides.[a]

| | Solvent A | Solvent B |
|---|---|---|
| 5′-UMP | 0.36 | 0 |
| 3-Methyl-5′-UMP | 0.52 | 0.04 |
| Methyl ester of 5′-UMP | 0.70 | 0.04 |
| Methyl ester of 3-methyl-5′-UMP | 0.81 | 0.09 |
| 2′,3′-UMP | 0.41 | 0 |
| 3-Methyl-2′,3′-UMP | 0.57 | 0.06 |
| Methyl ester of 2′,3′-UMP | 0.75 | 0.06 |
| Methyl ester of 3-methyl-2′,3′-UMP | 0.85 | 0.11 |
| 3-Ethyl-5′-UMP | 0.55 | 0.04 |
| Ethyl ester of 5′-UMP | 0.78 | 0.04 |
| 2′,3′-cUMP | 0.70 | 0 |
| 5′-dTMP | 0.46 | 0.02 |
| 3-Methyl-5′-dTMP | 0.62 | 0.04 |
| Methyl ester of 5′-dTMP | 0.77 | 0.09 |
| Methyl ester of 3-methyl-5′-dTMP | 0.86 | 0.21 |
| 3-Ethyl-5′-dTMP | 0.69 | |
| Ethyl ester of 5′-dTMP | 0.82 | 0.13 |
| Ethyl ester of 3-ethyl-5′-dTMP | 0.91 | |
| Uridine | 0.80 | 0.28 |
| 3-Methyluridine | 0.88 | 0.49 |
| 3-Ethyluridine | 0.89 | 0.62 |
| 2′-*O*-Methyluridine | 0.87 | 0.50 |
| $O^2$-Methyluridine | 0.88 | 0.36 |
| Thymidine | 0.85 | 0.52 |
| 3-Methylthymidine | 0.91 | 0.74 |
| 3-Ethylthymidine | 0.92 | 0.81 |

[a] Solvent A is 75 ml of ethanol and 30 ml of 1 *M* pH 7.5 ammonium acetate run descending on Whatman 3MM for 18–24 hr. Solvent B, run similarly, is 80 ml of butanol, 10 ml of ethanol, and 25 ml of $H_2O$. The $R_f$ values are averages of 3–20 separate values. Similar data for $R_f$ values in a solvent equivalent to solvent A are reported by Szer and Shugar (1961) for uridine, 5′-UMP, and their methyl derivatives.

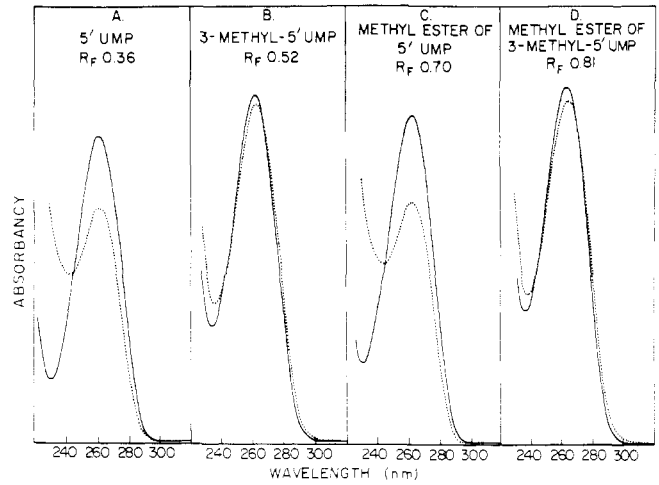


FIGURE 1: Uv absorption spectra of 5′-UMP and its methyl derivatives in $H_2O$ or 0.1 *N* HCl (—) and 0.1 *N* KOH (- - -). The $R_f$ values in solvent A are given in the figures to show that while 1A and 1C have the same spectra, as do 1B and 1D, they are each completely separated by chromatography. The spectra of the ethyl derivatives differ only in a slight increase (1–2 nm) in the $\lambda_{max}$ of the basic form. The spectra of 3-alkyluridine is identical with that of 3-alkyluridylic acid. dTMP and its alkyl products exhibit the same general spectral characteristics as UMP and its products except that the $\lambda_{max}$ of dTMP is 267 whereas the $\lambda_{max}$ of UMP is 262.

nate (MeMes)[1] and ethyl methanesulfonate (EtMes) were from Eastman, and methylnitrosourea and ethylnitrosourea were from K & K Laboratories. [$^{14}$C]$Me_2SO_4$ from Schwarz/Mann had a corrected specific activity of 4.4 Ci/mol and [$^{14}$C]$Et_2SO_4$ from ICN had a corrected specific activity of 2 Ci/mol. 2′-*O*-Methyluridine, $O^2$-methyluridine, and $O^4$-methyluridine were gifts from Dr. J. T. Kuśmierek. All other alkyl nucleosides and bases were prepared in this laboratory (Singer and Fraenkel-Conrat, 1975; Sun and Singer, 1974, 1975).

*Reaction of Nucleotides and Nucleosides with Alkylating Agents.* (A) Twenty-five milligrams of 5′-UMP, 2′(3′)-UMP, 5′-AMP, dTMP, Urd, or dThd was dissolved in 1.5 ml of $H_2O$. Using a Radiometer pH-Stat, the pH was adjusted and maintained at the desired value (±0.1 pH unit). The alkylating agent was added in four aliquots over a 2-hr period at room temperature and the reaction continued for a total of 4 hr. The total amount of reagent used was as follows: 80 μl of $Me_2SO_4$; 0.12 ml of $Et_2SO_4$, MeMes, or EtMes; 0.4 ml of a 250 mg/ml of ethanol solution of methylnitrosourea or ethylnitrosourea. In all cases, the reagent was in 10–15-fold excess, an amount sufficient to compensate for decomposition of the reagent and maintain an excess throughout the reaction (Singer et al., 1975). When 5′-UMP, 2′(3′)-UMP, or 5′-AMP were reacted with [$^{14}$C]$Me_2SO_4$(0.09 Ci/mol) or [$^{14}$C]$Et_2SO_4$(0.02 Ci/mol), the major portion of unbound radioactivity was removed by repeated ether extraction. Similar ether extraction of samples treated with unlabeled reagents indicated that no alkyl nucleotide was detectably soluble in the ether phase.

(B) Ten milligrams of 5′-UMP or 2′(3′)-UMP in 1 ml of $H_2O$ was methylated almost to completion (95%) with 0.6 m*M* diazomethane (Haines et al., 1964) in order to prepare authentic samples of 3-methyluridylic acid, the methyl ester of uridylic acid and the methyl ester of 3-methyluridylic acid.

*Methods for Separation and Identification of Alkyl Derivatives.* Two paper chromatographic systems were used to separate the products of alkylation of nucleotides and nucleosides. The $R_f$ values of the various derivatives are given in Table I. Solvent A is ethanol–1 *M* pH 7.5 ammonium acetate (75:30) and solvent B is butanol–ethanol–$H_2O$ (80:10:25). Nucleotides which were either unmodified or alkylated only on the ring were also identified by their $R_f$ after dephosphorylation to nucleosides with alkaline phosphatase (1–10 absorbancy units, 25 μg of enzyme, 0.01 *M* pH 9 borate, and 0.005 *M* $MgCl_2$, 37°, 4 hr). Similarly, 5′-alkyl esters of nucleotides were treated with snake venom phosphodiesterase to obtain nucleotides (1–10 absorbancy units, 50 μg of enzyme, 0.01 *M* pH 9 borate, and 0.005 *M* $MgCl_2$, 37°, 18 hr), while the 3′ isomer of 2′(3′)-alkyl esters of nucleotides was hydrolyzed with pancreatic ribonuclease (1–10 absorbancy units, 25 μg of enzyme, 0.02 *M* pH 7.4 acetate, and 0.005 *M* $MgCl_2$, 37° 18 hr), or in 1 N NaOH (100°, 30 min) to yield nucleotides. Chromatography in a borate-containing solvent (Al-Arif and Sporn, 1972) was used as a method of identifying possible ribose alkylation products. Since $O^4$-methyluridine did not separate from 3-methyluridine in either solvent used, possible $O^4$ alkylation of uridine was indirectly measured as [$^{14}$C]methanol or [$^{14}$C]ethanol released upon 1 *N* HCl hydrolysis (100°, 20 min).

The spectra of phosphate- and/or ribose-alkylated nu-

[1] Abbreviations used are: $Me_2SO_4$, dimethyl sulfate; $Et_2SO_4$, diethyl sulfate; MeMes, methyl methanesulfonate; EtMes, ethyl methanesulfonate; Urd, uridine; Thd, thymidine; U, T, A stand for the 3 bases, regardless of the nature of their pentose substituent; poly(U), poly(uridylic acid).

Table II: Extent and Site of Reaction of UMP and TMP with Methylating and Ethylating Reagents at Various pH's.[a]

| Nucleotide | Reagent[b] | pH | Extent of Reaction (%) | Alkylation Products % of Starting Material | | | Distribution of Alkyl Products | | |
|---|---|---|---|---|---|---|---|---|---|
| | | | | 3-Alkyl | Alkyl Ester of 3-Alkyl- | Alkyl Ester | Ring | $PO_4$ | Ratio $PO_4$/Ring |
| 5′-UMP | $Me_2SO_4$ | 6.0 | 61 | 1 | n.d. | 60 | 1 | 60 | 60 |
| 5′-UMP | $Me_2SO_4$[c] | 6.8 | 52 | 4 | 7 | 41 | 11 | 48 | 4.3 |
| 5′-UMP | MeMes | 6.8 | 17 | 2.7 | 0.7 | 14 | 3.4 | 14.7 | 4.3 |
| 5′-UMP | MeNU[d] | 6.8 | 56 | 8 | 4 | 44 | 12 | 48 | 4 |
| 2′(3′)-UMP | $Me_2SO_4$[c] | 6.8 | 55 | 7 | 8 | 40 | 15 | 48 | 3.2 |
| dTMP | $Me_2SO_4$ | 6.8 | 76 | 1.3 | 10 | 65 | 11.3 | 75 | 5.8 |
| 5′-UMP | $Me_2SO_4$[c] | 8.2 | 44 | 17 | 11 | 16 | 27 | 26 | 1 |
| 5′-UMP | MeMes | 8.2 | 31 | 17 | 2.8 | 11 | 20 | 14 | 0.8 |
| dTMP | $Me_2SO_4$[c] | 8.2 | 86 | 11 | 43 | 29 | 54 | 72 | 1.3 |
| 5′-UMP | $Et_2SO_4$[c] | 6.8 | 11 | 1 | n.d. | 10 | 1 | 10 | 10 |
| 5′-UMP | EtMes[c] | 6.8 | 4 | <0.2 | n.d. | 3.5 | <0.2 | 3.5 | >17 |
| 5′-UMP | EtNU[d] | 6.8 | 7 | 0.3 | n.d. | 7 | 0.3 | 7 | 23 |
| dTMP | $Et_2SO_4$[c] | 6.8 | 14 | 0.1 | 0.6 | 13 | 0.7 | 14 | 20 |
| 5′-UMP | $Et_2SO_4$[c] | 8.2 | 11 | 1.5 | n.d. | 9 | 1.5 | 9 | 6 |
| 5′-UMP | EtMes | 8.2 | 5 | 0.8 | n.d. | 4 | 0.8 | 4 | 5 |
| 2′(3′)-UMP | $Et_2SO_4$ | 8.2 | 9 | 1 | n.d. | 8 | 1 | 8 | 8 |
| dTMP | $Et_2SO_4$[c] | 8.2 | 17 | 0.9 | 0.5 | 15 | 1.4 | 16 | 11 |

[a] Reaction conditions and the method of separating and identifying products are given in the Experimental Section. 100–250 OD units of a reaction mixture was chromatographed in a band 3–6 cm wide for the primary separation. After elution and determination of the absorbancy, derivatives were rechromatographed (0.5–5 absorbancy units) to obtain $R_f$ values and spectra of the purified compounds. Since the alkyl ester of 3-alkyl-UMP or -dTMP is alkylated on both the ring and phosphate, it is counted twice when calculating the distribution of alkyl products; e.g., in the case of 5′-UMP + $Me_2SO_4$ at pH 6.8, 7% of the reaction is dialkylated and thus 7% is added to 4% 3-alkyl-5′-UMP (total 11%) and 7% is also added to 41% alkyl ester (total 48%). [b] Includes data obtained with both radioactive and unlabeled reagents. [c] Average of 2–3 separate alkylations. All others are single alkylations, analyzed twice. [d] MeNU, methylnitrosourea; EtNU, ethylnitrosourea.

cleotides are identical with those of the unmodified nucleotides while alkylation of the $O^2$, $O^4$, or N-3 of uridylic acid or the N-3 or $O^4$ of thymidylic acid causes distinctive changes in the spectra. Figure 1 illustrates this with the anionic and cationic spectra of 5′-UMP (Figure 1A), 3-methyl-5′-UMP (Figure 1B), the methyl ester of 5′-UMP (Figure 1C), and the methyl ester of 3-methyl-5′-UMP (Figure 1D). Note that while the $\lambda_{max}$ are not different, the $\lambda_{min}$ and the absorbancy of the 3-methyl derivatives are different from UMP and its ester in alkaline solution. The spectra of uridine and of 3-methyluridine are virtually identical with those in Figure 1A and B, respectively. $O^2$-Methyluridine has a $\lambda_{max}$ (pH 1) 253 and $\lambda_{min}$ 238 (J. T. Kuśmierek, unpublished) while $O^4$-methyluridine has a $\lambda_{max}$ (pH 1) 275 and $\lambda_{min}$ 235 (J. T. Kuśmierek, unpublished), clearly distinguishing them from 3-methyluridine which has a $\lambda_{max}$ (pH 1) 263, $\lambda_{min}$ 233. Similar spectral characteristics are also found for thymidine, thymidylic acid, and their alkyl products (Singer, 1975b).

## Results

*Reaction of UMP, dTMP, and AMP with Methylating Agents.* The three methylating agents used ($Me_2SO_4$, MeMes, and methylnitrosourea) are known to react with the rings of AMP, as well as GMP and CMP, over a wide pH range. In the present experiments, at pH 6.8, AMP reacted with $Me_2SO_4$ to form 25% 1-methyladenylic acid, 13% methyl ester of adenylic acid, and 50% methyl ester of 1-methyladenylic acid. These results are in accord with those of Griffin and Reese (1963) and indicate that, under the experimental conditions used, the ring and the phosphate group of AMP react equally well.

The same reaction conditions (pH 6.8) were then used for the methylation of UMP and dTMP and the results are shown in Table II. While the extent of reaction varies with the reagent and the nucleotide, in all cases the 3-methyl derivative is found, as well as the methyl ester and the methyl ester of 3-methyl-UMP and -dTMP. The relative reactivity of phosphate/ring is about 4, or about 25% of the methylation is on the N-3 of the ring. As the pH of the methylation is increased, ring alkylation increases and at pH 8.2 the ring and phosphate are equally reactive. Lowering the pH to 6 leads to reaction of almost only the phosphate (although a very small amount of ring alkylation occurs).

A competitive experiment was also performed in which a mixture of equal amounts of 5′-UMP and 5′-AMP was reacted with $Me_2SO_4$ at pH 6.8. In this experiment the N-3 of UMP was methylated as well as the phosphate since the products included the methyl ester of 3-methyl-5′-UMP and 3-methyl-5′-UMP. Methylation of AMP was, however, greater than that of UMP (ca. sevenfold) as would be predicted from the relative extent of reaction of the ring nitrogens of the separate nucleotides.

It can be noted in Table II that much of the ring alkylation occurring at pH 6.8 and 8.2 is in the form of the methyl ester, particularly in the case of dTMP. It might appear that the ester is more readily ring methylated than the original nucleotide. This proved to be the case when comparing methylation of the methyl ester of 5′-UMP with that of 3-methyluridylic acid, both reacted with $Me_2SO_4$ at pH 8.2. The final product in both reactions is the methyl ester of 3-methyluridylic acid which is formed about three times as readily from the ester.

*Reaction of UMP and AMP with Ethylating Agents.* The experimental procedures were first used with AMP which was reacted with both $^{14}C$-labeled and unlabeled $Et_2SO_4$ at pH 6.8. All products were identified on the basis of spectra and specific activity. The extent of ethylation was

Table III: Alkylation of the N-3 of Uridine and Thymidine at Various pH's.[a]

| Nucleoside | Reagent | pH | % 3-Alkyl Derivative |
|---|---|---|---|
| Uridine | $Me_2SO_4$ | 6.8 | 14 |
| | $Me_2SO_4$ | 8.2 | 41 |
| Thymidine | $Me_2SO_4$ | 6.8 | 7 |
| | MeMes | 7.4 | 4 |
| | $Me_2SO_4$ | 8.2 | 42 |
| Uridine | $Et_2SO_4$ | 6.8 | <1 |
| | $Et_2SO_4$ | 8.2 | 3 |
| Thymidine | $Et_2SO_4$ | 8.2 | 2 |

[a] Reaction conditions are given in the Experimental Section. Solvent B was used to separate unreacted nucleosides from the 3-alkyl derivative.

about 15% of which 2% was 1-ethyladenylic acid, 0.4% was the ethyl ester of 1-ethyladenylic acid, and 13% was the ethyl ester of adenylic acid. In contrast to methylation of AMP, the predominant site of ethylation was the phosphate group; the ratio of phosphate/ring ethylation being about 6.

Table II shows the comparable data obtained when UMP or dTMP is reacted with $Et_2SO_4$, EtMes, or ethylnitrosourea. The same general observations can be made as were found for ethylation of AMP. The total extent of reaction is much lower than with methylation, and the relative alkylation of the phosphate group is greatly increased. At pH 6.8 the 3-alkyl derivatives are about 5–10% of the total and even at pH 8.2 they represent only 10–20% of the total.

*Reaction of Uridine and Thymidine with Methylating and Ethylating Agents.* Further proof that the N-3 of the ring of U and T reacts with alkylating agents at neutrality came from experiments in which phosphate alkylation was not a factor. The nucleosides reacted at both pH 6.8 and 8.2 to a similar extent as did the N-3 of the nucleotides (Tables II and III). Uridine, reacted at pH 6.8 with $Me_2SO_4$, formed 14% 3-methyluridine, while UMP was methylated on the N-3 11–15%. Similarly thymidine formed 7% 3-methylthymidine while the nucleotide was 11% modified at the N-3. Although the extent of ethylation was very low, it could be shown to occur by isolation of uv absorbing derivatives which had the same spectral characteristics and chromatographic behavior as authentic 3-ethyluridine or 3-ethylthymidine (Singer, 1975b). Moreover when the alkylating agent was labeled the specific activity of such derivatives indicated that one ethyl group was bound.

At pH 8.2, 4 hr reaction at room temperature with an excess of $Me_2SO_4$ increased the extent of reaction of the N-3 to 40–50%, without concomitant alkylation of the ribose, in the case of uridine.

The reaction of thymidine with MeMes was also performed at pH 7.4 since Rhaese and Freese (1969) reported that they did not find any 3-alkylthymidine with MeMes or EtMes reaction. Although their reaction was begun at pH 7.4 they note that the pH dropped 0.8–1.2 units. Even with a constant pH of 7.4 only 4% 3-methylthymidine was found and at a lower pH it can be presumed that little or no reaction would occur. MeMes and EtMes also are much less effective alkylating agents for nucleotides than are $Me_2SO_4$ and $Et_2SO_4$ (Table II).

[2] In work to be published separately, Kuśmierek and Singer find that the extent of alkylation of the $O^2$ and for $O^4$ of the uracil ring in poly(U) is a function of the alkylating agent used. The alkyl sulfates are the least efficient in this respect.

Besides the N-3 of U or T, the exocyclic oxygens are possible sites of alkylation. In experiments to be published separately, J. T. Kuśmierek and B. Singer find that $O^2$-methyluridine is chromatographically separable from 3-methyluridine (Table I) while $O^4$-methyluridine cochromatographs with 3-methyluridine in solvents A and B. Both $O^2$- and $O^4$-methyluridine are dealkylated in 1 *N* HCl at 100° and this characteristic was utilized as a means of detecting O-methylation (or O-ethylation) in $[^{14}C]Me_2SO_4$ and $[^{14}C]Et_2SO_4$ treated uridine. At pH 6.8, the maximum acid labile material was 6–8% of the total reaction, representing a maximum of 1% O-alkylation.[2]

## Discussion

Alkylation of the three nucleosides or deoxynucleosides, adenosine, guanosine, and cytidine, by methylating agents in neutral aqueous solution, which occurs readily, has been studied extensively and has recently been reviewed by Singer (1975a). It has, however, been assumed for uridine and thymidine, on the basis of their lack of amino groups and weak basicity, that these nucleosides (as monomers or in polymers), would not become alkylated except in highly alkaline solution (Lawley, 1961). This belief has been reiterated (Haines et al., 1964; Friedman et al., 1965; Brimacombe et al., 1965; Rhaese and Freese, 1969; Brown, 1974) even though there exists some experimental evidence (not necessarily believed by the authors) that both U and T residues in polymers are methylated extensively near neutrality (Loveless and Hampton, 1969; Lawley and Shah, 1972). On the other hand, there is also experimental evidence that these reactions do not occur, but in these experiments the pH is known to be approximately 6 or less (Rhaese and Freese, 1969; Loveless and Hampton, 1969) or the methylating agent chosen (Ludlum, 1966) was one which, in the present paper, is found to be the least efficient of those used. Nevertheless, even under these conditions Ludlum found measurable amounts of 3-methyluridine in methyl methanesulfonate treated poly(U) (0.5% compared to 4.5% 1-methyladenosine from poly(A)). Another school of thought exists which denies the existence of 3-alkyluridine in neutral alkylated nucleic acids on the basis that it results from deamination of 3-alkylcytidine during hydrolysis.

In the experiments reported here, we are dealing only with the simple model system of nucleosides and nucleotides reacted at constant pH with common alkylating agents. The problems of alkylating homo- or heteropolymers with their attendant complications such as secondary structure, competing reactions, and hydrolysis conditions do not exist. Under the conditions used, uridine and thymidine react to form the N-3 derivative (Table III). The extent varies with the pH and the alkyl group. When UMP or dTMP are similarly alkylated, the secondary phosphate is esterified as well as the N-3, also to varying extents, again depending on pH and alkyl group (Table II). The alkylation of the N-3 of U is also independent from the alkylation of competing nucleotides. This differs from the observations of Brimacombe et al. (1965) who did not find any 3-methyluridine in ApU treated with $Me_2SO_4$ under similar conditions to those described in this work. It is likely that Brimacombe et al. (1965) did not hydrolyze enough modified ApU to detect 3-methyluridine since this reaction occurs to a much lesser extent than that of the N-1 of A.

It is apparent, and in accord with expectation, that small differences in pH, even near neutrality, i.e., when less than 1% of the bases are dissociated, can noticeably affect the

proportion of alkylation on the ring. This fact, as well as other differences in reaction conditions, may account for the great variation of values for N-3 methylation of U and T reported by different investigators (Singer, 1975a).

While methylation is, by a factor of 10–30, more efficient than ethylation in alkylating the ring of nucleotides at pH 6.8, phosphate alkylation is less affected by the nature of the alkyl group, and the formation of ethyl esters is thus the by far predominant event. This is in line with our earlier finding that ethylation of DNA or RNA leads to increased alkylation of phosphodiesters (Singer and Fraenkel-Conrat, 1975; Sun and Singer, 1975). We thus propose that ethylating agents have a similar affinity for tertiary phosphates as they are now shown to have for the secondary phosphates of nucleotides. Preliminary experiments on the alkylation of poly(U) (Kuśmierek and Singer, unpublished) also support this, since phosphotriesters are formed in poly(U) when treated at pH 6.8 with $Me_2SO_4$ and more with $Et_2SO_4$, and the relative amounts of ethyl and methyl triesters are similar to the ethyl and methyl diesters in Table II.

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# DNA-Dependent Protein Methylase Activity in Bull Seminal Plasma†

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ABSTRACT: The existence of a DNA-dependent protein methylase activity without any concomitant DNA methylase activity was demonstrated in bull seminal plasma. The enzyme utilized *S*-adenosyl-L-methionine as a methyl donor, and endogenous seminal plasma protein as the substrate. There was no demonstrable enzyme activity when the seminal plasma was preheated at 100° for 10 min, or when the enzyme reaction mixture was incubated at 4°. The protein methylase required a heterologous DNA source, had optimal activity at pH 8.1, and was enhanced in the presence of $Mg^{2+}$, $NH_4^+$, and reduced glutathione. After the methylated protein product was separated from DNA by extraction with 0.2 *M* HCl, the incorporated radioactivity was shown to be totally solubilized by incubating the protein either with Pronase or 1 *M* NaOH, while RNase and DNase had no effect. Approximately 70% of the enzymatically synthesized amino acids in the protein product were tentatively identified as O-methylated amino acid ethers by virtue of their elution from a Dowex 50 $H^+$ column with 0.2 *M* pyridine, and their stability to acid and base hydrolysis. The partially purified methylated product was shown by Sephadex G-50 chromatography to consist of three distinct radioactive proteins with molecular weights of approximately 21,000, 15,000, and 10,000.

The enzymatic methylation of specific amino acid residues in preformed protein macromolecules with *S*-adenosyl-L-methionine as the active biological methyl donor is a well-recognized biological phenomena (Paik and Kim, 1971). However, the physiological consequences of this type of enzymatic restructuring of proteins after their initial synthesis remains an enigma.

Burdon et al. (1967) and Burdon (1971) demonstrated

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