Identification of the modified nucleotides produced by covalent photoaddition of hydroxymethyltrimethylpsoralen to RNA

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Received 30 January 1981

ABSTRACT

The reaction between RNA and 4'-hydroxymethyl-4,5',8-trimethylpsoralen has been studied. Both natural RNA and synthetic RNAs were used. The base specificity of the reaction was found to be the same in natural RNA, homopolymers, and mononucleotides. Uridine was found to be the most reactive base in all cases. The kinetics of formation and reversal of monoadducts and crosslinks has been examined. Paper electrophoretic conditions are described which provide a separation of the monoaddition and crosslinked photoproducts. The relative and absolute amounts of monoadducts and crosslinks can be determined very accurately with this system. Paper electrophoresis provides good separations of the different photoproducts. The mobilities of the products are a simple function of their molecular weights and charges.

INTRODUCTION

Psoralen and its derivatives intercalate between base pairs in the nucleic acid double helix and photoreact when excited with 320 to 380 nm light (1,2). The 5,6 double bond of a pyrimidine can react with either the 4',5' double bond or the 3,4 double bond of the psoralen. When there is a pyrimidine in an adjacent base pair on the opposite strand of the double helix, both ends of the psoralen can react to form a crosslink covalently linking the two strands. Such a stabilization of a nucleic acid structure is a valuable tool for understanding the conformation of DNA and RNA (3-6).

In order to improve the solubility and reactivity of psoralen, new derivatives of psoralen have been synthesized and shown to be effective at photoaddition (7). Several electron microscopy studies have been done with 4'-hydroxymethyl-4,5',8-trimethylpsoralen and its ability to crosslink single stranded RNA has been proven (8,9). The mapping of sites of monoaddition and crosslinking by psoralen derivatives allows the elucidation of secondary and tertiary interactions (10-12). The potential also exists for studying the effect of specific monoadducts or crosslinks on biological activity.

In order to exploit this potential, we have studied the specificity of reaction of HMT with model systems of RNA. Ribomononucleotides and ribohomopolymers were reacted and the products compared to those obtained with natural RNA.

MATERIALS AND METHODS

Materials. Homopolymers were obtained from P.L. Biochemicals. Nucleotide monophosphates and T2 RNase (E.C. 3.1.4.23) were purchased from Sigma. Calf intestine alkaline phosphatase was (E.C. 3.1.3.1) a Boehringer-Mannheim product. ^3H -HMT (3.7×10^7 cpm/ μg) was synthesized by Steve Isaacs of this lab according to Isaacs et al. (13). Uniformly labeled ^{32}P -tRNA from cultured *Drosophila* cells was prepared as indicated by Thompson et al. (11).

Irradiation of Samples. The samples to be irradiated were put in Eppendorf tubes which were placed in a water bath. The bath was surrounded by a double-walled glass vessel containing a circulating 40% (w/w) cobaltous nitrate solution. This solution served as a temperature regulator and an ultraviolet and visible light filter with a maximum transmittance of 365 nm light (window: 320-380 nm). The samples were irradiated by two GE 400 W mercury lamps, one on each side of the sample. The light intensity at the surface of the sample was approximately 100 mW/cm². After irradiation, polymers were extracted twice with phenol and then ethanol precipitated two or more times to remove all traces of unreacted HMT. The RNA was redissolved in 50 mM sodium acetate, pH 5, containing 2000 units/ml T2 RNase. For up to 50 μg of RNA, 10 μl was used. Digestions were done at 37° for 18 hours. After photoreaction, mononucleotides were extracted two or more times with chloroform/isoamyl alcohol (24:1, v/v) and then electrophoresed directly.

Electrophoresis. Electrophoresis was done on Whatman #1 chromatography paper in the form of 46 x 57 cm sheets (VWR Scientific) or 27 cm wide rolls (Savant Instruments). The running buffer was 5% acetic acid, 0.5% pyridine (v/v/v), pH 3.5. Runs were done in a Savant LT 48 tank, normally for 50 minutes at 3000 V. Longer runs at 4000 V for 3 or more hours were also done. An aliquot of a mixture of (^{32}P)3'-phosphate mononucleotides was added to each sample to serve as mobility markers. After electrophoresis, the paper was dried and cut into 0.5 or 1 cm slices. In order to obtain high counting efficiencies, 0.5 ml of water was added to each slice for one hour to elute the nucleotides from the paper. Each fraction was counted after addition of a 5 ml scintillation mixture containing 2 parts toluene, 1 part Triton X100, 4 g/l omnifluor (98% PPO, 2% Bis MSB).

When it was necessary to recover the electrophoresed samples, the paper strips were counted directly at low efficiency in toluene with 6 g/l PPO and .075 g/l POPOP. After counting, the paper strips were rinsed with toluene and dried. They were then each placed in a 200 μ l pipet tip and rinsed with 200 μ l of H_2O in order to elute the nucleotides from the paper.

Photoreversal. Photoreversal of crosslinks and monoadducts was done with a hand-held Rayonet 6 W short wave UV lamp. The lamp was positioned 5 cm above the sample to be reversed. The optical density of the samples was low enough that the rate of photoreversal was independent of concentration.

RESULTS

The photoproducts of HMT with different types of natural and synthetic RNAs have been studied, taking advantage of a highly radiolabeled psoralen derivative synthesized in this laboratory (13). After the photoreacted RNA has been completely hydrolyzed by extensive digestion with T2 RNase, the products can be separated by electrophoresis on neutral paper at pH 3.5.

Reaction of (3H) HMT with all the natural RNAs we have tested so far (*E. coli* tRNAs, *Drosophila* 5S and 18S rRNAs) yields only two major labeled peaks separable by paper electrophoresis. Typical radioactivity profiles of RNA hydrolysates are shown in Figure 1, for a mixture of *E. coli* tRNAs and for synthetic poly(U). The relative mobilities of each peak (taking m^7G as a reference, $R_F(m^7G) = -1$) are .62 and .99 for peak 1 and peak 2, respectively. In every case, we observed that the (3H) cpm ratio between peaks 1 and 2 was highly dependent upon the light dose. The proportion of peak 2 photoadducts increases with irradiation time (as shown here in Figure 1b for poly(U)). Moreover, "pulse-chase" experiments, in which a short irradiation of RNA in the presence of (3H)HMT is followed by removal of unreacted psoralen and further irradiation, have indicated that a fraction of the pulse-labeled peak 1 component can be converted into peak 2 product by 340-380 nm light during the chase period (not shown). In order to aid in the identification of the composition of these two major peaks, the reaction was examined in simple systems such as homopolymers and mononucleotides.

When HMT was photoreacted with the four homoribopolymers in standard conditions, the reactivity of poly(U) was found to be 10-20 times higher than that of other synthetic homopolymers (see Table I). The paper electrophoresis analysis of T2 RNase digests showed that only poly(U) yields a profile similar to that observed with natural RNAs. The much less efficient reaction of HMT with poly(C) gives rise to a labeled component with the same mobility ($R_F =$

Figure 1. Paper electrophoresis analysis of T2 RNase digests of HMT-photo-reacted *E. coli* tRNAs and poly(U). After photoreaction with (^3H) HMT followed by phenol extraction and ethanol precipitation, RNA samples were hydrolyzed by an overnight treatment with T2 RNase and analyzed by electrophoresis on Whatman #1 paper at pH 3.5. An aliquot of a mixture of the four (^{32}P) labeled unreacted 3'ribonucleotides monophosphates was comigrated as markers. Radioactivity profiles were determined by liquid scintillation counting of 0.5 cm paper slices (see MATERIALS AND METHODS). (a) A mixture of *E. coli* tRNAs (200 $\mu\text{g}/\text{ml}$) was photoreacted with 1 $\mu\text{g}/\text{ml}$ (^3H) HMT (10 min, 5°C in 5 mM NaCl, 1 mM Tris-HCl, pH 7.5, 0.1 mM EDTA). (b) Poly(U) (660 $\mu\text{g}/\text{ml}$) was photoreacted with 1.3 $\mu\text{g}/\text{ml}$ (^3H) HMT (16 $^\circ\text{C}$ in 1 mM Tris-HCl, pH 7.6, 0.1 mM EDTA) for 15 seconds ($\bullet\text{---}\bullet$) or 2 minutes ($\Delta\text{---}\Delta$). R_F values were determined using $m^7\text{G}$ as a reference ($R_F(m^7\text{G}) = -1$).

0.14) as a minor component seen in natural RNA, as well as a component ($R_F = 0.62$) identical to the one observed with poly(U). These are detected in varying proportions, depending upon how the photoreacted polymer is handled (see DISCUSSION). A more complex electrophoretic pattern of labeled products was obtained for poly(G) (not shown). Its complete identification is still in progress.

The reaction of HMT with the different mononucleotides was also studied.

Polynucleotide	³ H incorporated Radioactivity (cpm/sample)	Nucleotide Content (nmole/sample)	Specific Photoreactivity 10 ⁵ x (mol. HMT/mol. nucleo.)
Poly(U)	101,320	22	46.1 (100%)
Poly(C)	8,935	30.3	2.95 (6.4%)
Poly(G)	19,260	37.3	5.16 (11.2%)
Poly(A)	5,600	21	2.67 (5.8%)

Table I. Photoreactivity of homoribopolymers with HMT. Each homopolyribonucleotide solution (600 μ g/ml) was photoreacted with 1.3 μ g/ml (³H) HMT (2 min at 16°C in 1 mM Tris-HCl, pH 7.4, 0.1 mM EDTA). Samples were extracted twice with phenol before ethanol precipitation (2x) and redissolution in 10 mM Tris-HCl, pH 7.4. UV absorption spectra were determined and aliquots were assayed for (³H) radioactivity.

The products detected with the mononucleotides have mobilities identical to those observed in the respective homopolymers. The reactions were found not to vary with temperature in the range 4–50°C. Ionic concentration did have an effect on the reactivity. Raising the concentration of NaCl from 0 mM to 100 mM increased the amount of HMT incorporated into UMP by 25%. In the case of the mononucleotide, the photoreactivity of uridine was higher than that of the other major bases. CMP was about one third as reactive while no reactivity above background could be detected for AMP and GMP. We also examined the HMT photoreactions with different uridine analogs, in the mononucleotide monophosphate form. No reaction could be detected with dihydrouridine, even though it would have been possible to observe a reaction which is 20 times less efficient than the reaction with uridine. Pseudouridine and 4-thio-uridine were approximately 10 times less reactive than uridine.

The electrophoretic mobilities of the observed HMT-monomonucleotides products are listed in Table II. These mobilities have been compared to those calculated from an empirical equation (14). Based on this and on the nature of the reactive nucleotides used in the simple systems, a tentative assignment can be made as to the identity of these products. The major peak (Peak 1, $R_F = 0.62$) in Figure 1 has the same mobility as predicted for an HMT monoadduct of uridine monophosphate, while the other major product peak (Peak 2, $R_F = 0.99$) has the mobility expected for an HMT crosslink between two uridine monophosphates. (For this reason, as well as additional evidence shown below, we shall refer to these products as U monoadduct and U-U crosslink, respectively.) When the two components are dephosphorylated by alkaline phosphatase, they

Compound	Predicted R_F	Observed R_F
CMP	.21	.19
AMP	.33	.37
GMP	.76	.74
UMP	.93	.93
UMP+HMT	.64	.57
		.61
		.66
2UMP+HMT	.95	.99
		1.04
UMP+UMP+HMT	.58	.57
CMP+HMT	.14	.14

Table II. Electrophoretic mobilities of photoproduct at pH 3.5. The predicted mobilities were calculated from the equation $R_F = 45.4 \frac{Q}{M^{2/3}}$ where Q is the net charge and M is the molecular weight (14).

both remain at the origin during electrophoresis at pH 3.5. This is exactly what is predicted by their assigned structures.

More resolution can be obtained in the separation of the U monoadduct by carrying out the electrophoresis for a longer time. A typical profile is shown in Figure 2a. The broad monoadduct peak is actually resolved into three sub-species with $R_F = .57$, $.61$, and $.66$. The relative proportions of the two faster migrating components is highly dependent upon the ratio of the concentration of monoadduct to the concentration of T2 RNase present during the hydrolysis of the photoreacted RNA. When the ratio is high (low enzyme concentration), the peak migrating with $R_F = .66$ is the major species. When the T2 RNase concentration is raised sufficiently, this peak disappears with a concomitant increase in the size of the peak with $R_F = .61$. The faster migrating peak can also be quantitatively converted into the component migrating with $R_F = .61$ by a one hour treatment at pH = 1 (Figure 2b). The $R_F = .66$ component is resistant to dephosphorylation with calf alkaline phosphatase, unlike the $R_F = .61$ component (Figure 2c). The acid treatment of the $R_F = .66$ component allows its subsequent dephosphorylation by alkaline phosphatase (not shown). It should also be noted here that the experiment reported in Figure 1 was carried out with a low substrate to enzyme ratio. This results in almost complete absence of the $R_F = .66$ component.

The stability and reactivity of the presumptive U monoadduct and U-U

Figure 2. High resolution separation of UMP-HMT monoadducts. A T2 RNase digest of poly(U,A) previously photoreacted with (^3H) HMT (15 sec in 0.1 M NaCl, 1 mM Tris-HCl, pH 7.4, 0.1 mM EDTA) has been analyzed on 90 cm-long sheets of Whatman 1 paper by electrophoresis at pH 3.5. (a) Control. (b) The T2 RNase digest was pretreated by 0.1 N HCl for 1 hr at 37°C before electrophoresis. (c) The T2 RNase digest was treated by calf intestine alkaline phosphatase (10 mUnits, 75 min, 37°C in 50 mM Tris-HCl, pH 8.3) before electrophoresis. Each sample contained 6 pico-moles of HMT-UMP monoadduct. R_F values of HMT-UMP monoadducts are given with m^7G as a reference ($R_F(m^7G) = -1$).

crosslink were also examined. Both products were purified by paper electrophoresis and assayed in various conditions.

A further irradiation at 360 nm resulted in no detectable change in the electrophoretic profiles. When irradiation of the U-U crosslink at 360 nm was carried out in the presence of additional nucleotides, the U-U crosslink was again unchanged. The U monoadduct, on the other hand, could be converted to a U-U crosslink if the irradiation was carried out in the presence of additional UMP. In the presence of additional CMP, the irradiation of U monoadduct gave rise to a new product migrating as would be predicted for a U-C crosslink using the empirical equation mentioned above (Figure 3). However, even with careful handling, this new product slowly converts to a compound

Figure 3. Formation of Crosslink from Monoadduct. U monoadduct from *E. coli* tRNAs was purified and reacted further. A 10 minute irradiation was performed in 1 mM Tris, pH 7.4, 0.1 mM EDTA at 20°C. The concentration of monoadduct was 2.4×10^{-7} M and no additional nucleotides (O—O), 50 mg/ml UMP (□—□), or 50 mg/ml CMP (X—X) was added.

migrating like the U-U crosslink (see Discussion). No new product was detected when the further irradiation of U monoadduct was performed with additional AMP or GMP. Both of these crosslinks could also be photoreversed (Figure 4). After a 260 nm irradiation of (^3H) HMT-uridine monoadduct, the fraction of tritium counts remaining at the origin when electrophoresed on paper increased proportionally with the light dose (not shown). When irradiated with short UV light, the uridine-HMT-uridine crosslink was progressively converted into a labeled compound migrating like the uridine monoadduct (Figure 4b). This was, in turn, broken down to a labeled product which remains at the origin after further UV exposure (not shown). The 260 nm irradiation of the presumptive U-C crosslink resulted in the transient detection of U monoadduct and a small amount of C monoadduct (Figure 4a).

The kinetics of formation of these products was studied. In order to get

Figure 4. Photoreversal of crosslinks. The U-C crosslinks (A) and the U-U crosslink (B) formed as shown in Figure 3 were purified and then reversed with 20 minutes of short wave ultraviolet light. The crosslinks are shown before ($\circ$ — $\circ$) and after ($\bullet$ — $\bullet$) reversal.

efficient reaction, large excesses of nucleotides have to be added. A plot of these data is shown in Figure 5. The straight lines obtained indicate a reaction which is first order in monoadduct. In order to determine the order of reaction in which monoadduct is formed, the change in the initial rate of reaction was observed with varying concentrations of UMP. This is shown in Figure 6.

In order to confirm the presumptive nature of the HMT-uridine photoproducts, further experiments were done using ^{32}P labeled RNA. *In vivo* uniformly labeled ^{32}P -tRNA was photoreacted with ^3H -HMT and then hydrolyzed by T2 RNase before paper electrophoresis. All of the tritium labeled peaks are listed in Table 3. As previously shown (Figure 2), the U monoadduct could actually be resolved into three components, while the U-U crosslink was separated into two species.

Figure 5. Kinetics of formation of crosslink from monoadduct. The formation of the U-U crosslink (○---○) and the U-C crosslink (●—●) as described in Figure 4 is shown in a first order plot. The initial concentration of monoadduct is $[MA]_0$ and the concentration of crosslink at any time is $[XL]$. The initial concentration of monoadduct was 2.4×10^{-7} M and the initial concentration of mononucleotides was 50 mg/ml.

The tritium to ^{32}P ratios also support the assignments. The crosslink has twice as much ^{32}P per HMT when compared to the monoadduct. All of these species were also reversed with short UV light. When the U monoadducts were reversed (Table III), the slower two yielded UMP while the faster one yielded a product migrating like cyclic UMP. When additional UMP was added to the two faster migrating monoadducts (and then irradiated at 360 nm), U-U crosslink was formed. This was not performed on the slowest moving component ($R_F = 0.57$). When the crosslinks were reversed, monoadducts, UMP, and HMT were formed.

DISCUSSION

Identification of photoproducts (monoadducts vs. crosslink). The process of identifying the photoproducts of the reaction between HMT and natural RNA is simplified greatly by the use of model compounds such as homopolymers and mononucleotides. In the case of both polymers and nucleotides, uracil is the most reactive of the four bases. For uridine, there is no difference in reactivity

Figure 6. Kinetics of formation of monoadduct. Varying concentrations of UMP were reacted with 15 $\mu\text{g/ml}$ HMT, 1 mM Tris, pH 7.4, and 0.1 mM EDTA at 20° for 30 seconds and analyzed by paper electrophoresis. The curved lines were generated from a best fit of the data to an exponent in the equation listed in the DISCUSSION. The dashed line is the best fit for the four lowest concentration, while the solid line is for all concentrations.

between the nucleotide and the polymer. This is in agreement with previous work (15,16) and indicates that polymerization has no effect on the reaction.

The products obtained from poly(U) and UMP are identical in electrophoretic mobility to the major products obtained in natural RNAs. This suggests that the reaction in natural RNA involves principally uridine.

R_F	Percent of Total ^3H	$^3\text{H}/^{32}\text{P}$	Products of UV Photoreversal
.57	4.48	28	UMP, HMT
.61	24.22	33	UMP, HMT
.66	56.88	35	cUMP, HMT
.98	6.61	18	UMP, cUMP, U monoadducts (R_F = .57, .66), HMT
1.04	2.61	—	cUMP, U monoadduct (R_F = .66), HMT

Table III. Photoproducts of reaction between ^3H -HMT and ^{32}P tRNA. The remaining 5.2% of the tritium counts was present in HMT breakdown products and incompletely hydrolyzed material. The ^{32}P tRNA was irradiated for 10 min at 4°C in a solution which contained 1 $\mu\text{g/ml}$ HMT, 5 mM NaCl, 1 mM Tris pH 7.4, and 0.1 mM EDTA.

This assignment is supported by the use of an empirical equation which can be used to predict electrophoretic mobilities on neutral paper. This equation assumes that only the charge and molecular weight of a compound determine its mobility. While other factors are certainly involved, the equation provides a good first approximation of the mobility of the common and modified nucleotides that have molecular weights from 200 to 2000 D. We have used the mobility of UMP as our standard and equated its R_F with that observed by Sommer (14), who measured mobilities relative to m^7G . Using the assumption that, at pH 3.5, addition of HMT does not affect the net charge of the nucleotide to which it is attached, the mobilities were calculated and compared to those observed. The close agreement is strong evidence for the assignment of the major peak I (R_F around 0.61) to the HMT monoadduct of uridine and the faster peak II (R_F around 0.99) to a crosslink between two uridines. That the global charge of the uridine adducts does remain identical to that of unreacted uridine (i.e., $R_F \approx 0$ at pH 3.5) was experimentally confirmed by the absence of mobility of the dephosphorylated photoproducts.

The possibility that the peak II product results from an incomplete hydrolysis of the phosphodiester chain by T2 RNase was examined. The nucleolytic activity of various RNases can actually be altered adjacent to the photoadduct (17) and resistant dinucleotide diphosphates containing a U-monoadduct can be detected for shorter periods of T2 RNase digestion; they also have been characterized by paper electrophoresis. An HMT monoadduct of UpUp migrates only marginally slower than the crosslink between two uridine monophosphates ($R_F = 0.93$ instead of 0.99). However, these two components can be easily distinguished after dephosphorylation by alkaline phosphatase ($R_F = 0.50$ for U_pU monoadduct, $R_F \approx 0$ for U-U crosslink), thus providing a good assay for monitoring the extent of T2 RNase digestion. In the experiments described here, no detectable fraction of resistant phosphodiester linkages was observed. Another possibility, pUp-HMT, migrates significantly faster than peak II.

Photoreversal (first observed by Musajo et al. (17)) of peak II yields additional evidence for this assignment. Peak II can be completely converted to monoadduct which is in turn reversed. When ^{32}P crosslink is reversed, the ^{32}P is ultimately found as UMP and cUMP. If peak II contained partially hydrolyzed monoadduct, reversal would lead immediately to HMT and other ^{32}P products.

Kinetics of peak I and peak II appearance were also performed during the initial period of 360 nm irradiation, when changes in HMT concentration due to photoreaction and breakdown can be neglected. While the rate of peak I appearance is linear with light-dose, the rate of peak II production is a quadratic

function of light-dose (not shown), in line with previous observations that the HMT crosslinking formation involves a two photon reaction (18).

Comparison of the products obtained in the reaction of HMT and model compounds with the products of HMT and natural RNA shows that there is a preferential reactivity with uridine. This is in line with the observations of Ou and Song (19), who found that the major psoralen photoproducts in fluorouracil substituted tRNA involved fluorouracil. Also, the absence of reactivity observed for dihydrouridine agrees well with the hypothesis that the 5,6 double bond of the pyrimidine is involved in the psoralen addition. This process is also clearly inhibited when this double bond is sterically hindered by a C₆-C₁ ribose linkage as in the case of pseudouridine.

Besides uridine photoadducts, some minor products have been detected. Their mobilities agree well with predicted values (Table II): a CMP-monoadduct was detected by irradiating CMP, poly(C), or natural RNA and a U-C crosslink was detected by irradiating U monoadduct in the presence of CMP. Identification of the C-photoproducts was complicated by the fact that cytidine deaminates readily when the 5,6 double bond is saturated by the addition of HMT. This process is accelerated by heat but it occurs even at low temperature and neutral pH. CMP monoadduct is then converted to UMP monoadduct. Deamination has been observed previously with psoralen adducts (17), cytidine dimers (20), and bisulfite adducts (21). This process clearly interferes with determining the accurate extent of cytidine photoaddition in natural RNA. In DNA, this problem can be solved because uridine adducts arising from cytidine deamination can be separated from thymidine adducts (22).

Assignment of isomers of U-monoadduct and U-U crosslinks. The number of possible isomers from the reaction of HMT with any of the bases is very large. Clearly, most of these would not be separable by paper electrophoresis. Nevertheless, high resolution migration allowed the detection of three subspecies in the peak I (U monoadduct) component obtained either from natural RNAs or U-containing synthetic polymers. However, only two of these subspecies can be detected after photoreacting with UMP. The reasons for an additional fast-migrating ($R_F = 0.66$) U monoadduct in the case of polynucleotides can be understood upon considering the RNA hydrolysis process itself. T2 RNase digestion involves the formation of a 2'-3' cyclic phosphate intermediate. The decyclization of this into the final 3' phosphate nucleotide is also catalyzed by the enzyme. Cyclic phosphate mononucleotides migrate slightly but significantly faster than the noncyclic form (not shown). Our results are consistent with the fastest moving U monoadduct ($R_F = 0.66$) being a 2'-3' cyclic phosphate

nucleotide, the conversion of which is strongly inhibited by the presence of HMT. As expected, increased amounts of T2 RNase promotes the conversion of $R_F = 0.66$ species into $R_F = 0.61$ species. Also, the classical procedure of using acid to decyclize the phosphate (23) allows us to perform the same conversion. This assignment is in line with the inhibition of dephosphorylation of the fast moving U monoadduct ($R_F = 0.66$). The dephosphorylation becomes possible once the decyclizing acid-treatment has been carried out. The short-UV light reversal experiment reconfirms this identification, since the $R_F = .61$ U-monoadduct yields (^{32}P) labeled noncyclic UMP and the $R_F = .66$ monoadduct yields (^{32}P) labeled cyclic UMP (Table III).

Kinetics. The order of the reaction between UMP and HMT was determined by varying the concentration of UMP and determining the initial rate of reaction. If the amount of reacted material is very small compared to the total amount present, the following relation can be proven:

$$v_0 = k[X]^n$$

The order of reaction, n , can be found by simply varying the concentration of the reactant in question while keeping all other factors constant and then measuring the initial rate. Figure 6 demonstrates that the reaction is first order in UMP. The deviation from linearity at higher concentrations is caused by the breakdown of the above relationship because of the large amount of HMT reacted. It could also be caused by the presence of more than one type of mechanism for the reaction. The two lines shown in Figure 6 were obtained from a least squares fit of $\ln v_0 = n \ln[X] + C$. The slope of this line yields the best value of the exponent (order of reaction). For the four lowest concentrations only, a value of $n = .99$ was obtained. For all concentrations, a value of $n = .90$ fit the data best.

A first order reaction was to be expected based on the similarity of reaction rates of UMP and poly(U). If the reaction was higher order, the high local concentrations of UMP in poly(U) would lead to increased reactivity. This rules out the possibility of an intercalation complex for the simple systems studied here.

The kinetics of formation of crosslink from monoadduct is shown in Figure 5. The tremendous excess of UMP and CMP used causes the reaction to be pseudo first order in these components. The standard method of graphing first order reactions is to plot the logarithm of the initial concentration of the starting material divided by the difference between the starting material and product versus time. The fact that straight lines are produced for both reactions indicates that they are first order in monoadduct..

The deviation from linearity observed is caused by the fact that not all of the starting material can be driven to crosslink. The monoadduct used in this experiment is derived from a mixture of E. coli tRNAs. Only 80% can form crosslinks in these conditions. This could be the result of the presence of unreactive isomers of the monoadduct (those with the 3,4 bond reacted) or photobreakdown products of the monoadduct which cannot react further but have the same mobility (e.g., photohydrates).

Advantages of electrophoresis. In previous studies of the reactions of psoralens with RNA and DNA, paper (19) and thin layer chromatography (24) have been used to separate the photoproducts. These methods as well as changes in the absorption or fluorescence have been used to monitor the progress of reaction (25,26). Although these techniques have been very useful, we have found paper electrophoresis to be more versatile in studying the complex products found in natural RNA as well as in monitoring the production of small amounts of products.

When natural RNAs are hydrolyzed in an effort to study the products formed, the inhibitory effects of the attached psoralen can lead to a large amount of partially digested material. This problem was encountered in a previous study of the specificity of reaction in tRNA (19). Paper chromatography yielded a spot at the origin which was attributed to the various photoadducts. Most of this was assigned to fluorouracil present in the tRNA, but some was also attributed to adenosine. Based on our findings that AMP and poly A are relatively unreactive, it is more likely that some adenosine was adjacent to the reacted fluorouracil and the tRNA was not completely hydrolyzed. Since electrophoresis can distinguish these possibilities, it is the method of choice.

Electrophoresis can also separate the major photoproducts easily. This technique is well suited for studies in which quantifying the absolute or relative amounts of monoadduct and crosslink is desirable. Reproducible and accurate results can be obtained more readily than with current techniques.

The use of paper electrophoresis has enabled us to examine the reaction of HMT and RNA in great detail. The ability to detect extremely small amounts of product (as low as 1 picogram under ideal circumstances) has made it possible to examine the reactions under a wide variety of conditions. Reactions with model compounds have allowed us to understand the kinetics and specificity of reaction. This knowledge has led to a better understanding of the way in which HMT reacts with natural RNAs in vivo.

ACKNOWLEDGMENTS

This work was supported in part by the Assistant Secretary for Environment,

Office of Environmental Research and Development, Biomedical and Environmental Research Division of the U.S. Department of Energy under Contract No. W-7405-ENG-48. JPB was supported by an International Research Fellowship from the Fogarty International Center, Department of Health, Education and Welfare, while in this laboratory.

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