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Reactivity and selectivity in light-induced free radical reactions of 2-propanol with purine and pyrimidine mononucleotides and dinucleoside monophosphates

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ABSTRACT

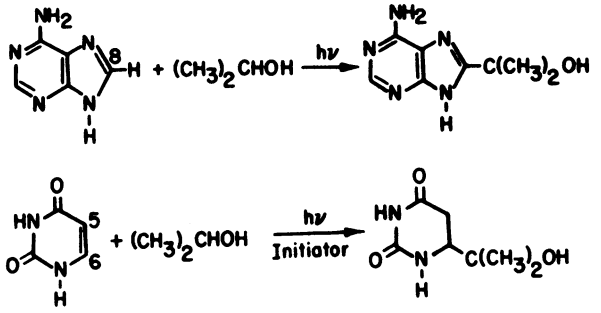
Photoalkylation reactions with 2-propanol, initiated with di-*tert*-butyl peroxide, of a variety of purine and pyrimidine mononucleotides and dinucleoside monophosphates lead to the substitution of an α-hydroxy*iso*propyl group for the H-8 atom of adenosine and the addition of the alcohol across the 5,6-double bond of the pyrimidines. Adenosine moieties blocked at their 3'-hydroxyl group are alkylated faster than those blocked at their 5'-hydroxyl. The reactivity of the uridine moieties of 3'-UMP, 5'-UMP, and uridylyl-(3',5')-uridine is not affected by the location of the phosphate group. However, the uridine moiety of uridylyl-(3',5')-adenosine is modified faster than that of adenylyl-(3',5')-uridine. It is suggested that steric hindrance imposed by the phosphate group determines the reactivity of adenosine moieties, while base stacking involving adenosine determines the reactivity of uridine moieties. These two effects play a major role in controlling the nature and degree of the selectivity of these photoalkylation reactions for either adenosine or uridine. Cytidine has been found to be inert in these reactions.

INTRODUCTION

The pyrimidine bases in nucleic acids have been regarded as the sensitive sites for ultraviolet radiation damage. Accordingly, the study of the photochemistry of nucleic acid constituents concentrated mainly on the reactions of these bases, in which the major photoproducts characterized were cyclobutane-type dimers (1). It has been shown recently that purines also undergo photochemical reactions in which the purine skeleton is preserved (2-9). Thus, with nucleic acid occurring purines, these reactions resulted in the substitution of the appropriate moiety for the H-8 atom of the purine system, e.g. the reaction of adenine with 2-propanol to form 8-α-hydroxy*iso*propyladenine (6-9). Uracil and thymine and their nucleosides also form photoadducts with alcohols under similar reaction conditions (10-12). These light induced reactions of adenine and uracil with 2-propanol are described in Scheme I. High yields (>95%) of purine-alcohol photoproducts were obtained when di-*tert*-butyl peroxide [$(Bu^tO)_2$] was used as a photoinitiator in these reactions which were shown to involve free radical intermediates. The use of the same photoinitiation system in the reactions of uracil or thymine led to the exclusive

formation of the pyrimidine-alcohol adducts, without any formation of cyclobutane dimers (10).

Scheme I



The application of these reactions to native DNA resulted in the substitution of an α-hydroxyalkyl group for the H-8 atom of both adenine and guanine moieties. When the reactions were initiated directly with UV light of λ>260 nm or photosensitized with acetone and light of λ>290 nm, pyrimidine dimers were formed in the irradiated DNA together with the 8-alkyl derivatives of adenine and guanine (13). However, with $(Bu^tO)_2$ as a photoinitiator alkylation of the purines occurred exclusively (14).

The fact that several types of sites sensitive to light or free radicals are present in nucleic acids, increases the number and types of expected photoproducts. It has been necessary, therefore, to sort out selective photochemical reactions for the appropriate bases in a given nucleic acid. The photoalkylation with 2-propanol, initiated with $(Bu^tO)_2$, of purines and pyrimidines leads to a single type of photoproduct of each base (10). It presents, therefore, a suitable system for the study of the selectivity of light-induced and free radical reactions of nucleic acid constituents.

We have shown previously that when reacted separatedly, under the above conditions, the pyrimidines exhibited a comparable or even higher reactivity than the purines. In pyrimidine-purine mixtures, however, the reactivity of the pyrimidine was reduced considerably, while that of the purine remained virtually unchanged. It has been proposed that the selectivity observed in these systems resulted from the heteroassociation of the bases, which under the conditions of the reaction was of a base stacking type (11).

In this paper we report the application of the photoalkylation reactions with 2-propanol to a variety of purine and pyrimidine mononucleotides and dinucleoside monophosphates. It has been found that (a) the reactivity of the adenosine moieties depends strongly on the site of binding of the phosphate

to the sugar ring; thus, adenosine blocked at its 3'-position is more reactive than that blocked at the 5'-position; (b) the degree of selectivity of the reaction for adenine or uracil in the heterodinucleoside monophosphates depends on the location of these moieties in the molecule; and (c) cytidine is unreactive in this reaction.

MATERIALS AND METHODS

Materials. Mononucleotides and dinucleoside monophosphates were purchased from Sigma. Nucleosides were purchased from Fluka, Bucks. 2-Propanol and di-*tert*-butyl peroxide [$(Bu^tO)_2$] (Merck-Schuchardt, Munich) were of analytical grade.

Authentic samples of 8-α-hydroxy*iso*propyladenine and 8-α-hydroxy*iso*propyl-AMP were prepared according to Salomon and Elad (7). 6-α-Hydroxy*iso*propyl-5,6-dihydrouridine, 6-α-hydroxy*iso*propyl-5,6-dihydro-UMP, and 6-α-hydroxy*iso*propyl-5,6-dihydro-TMP were prepared according to procedures described by Leonov *et al.* (10).

Irradiation procedure. Irradiations were carried out with a 450 W Hanovia high pressure mercury lamp inserted in a water-cooled Pyrex jacket. Solutions of mononucleotides or dinucleoside monophosphates (0.5 mM) in 2-propanol:water mixture (3:2 v/v) containing $(Bu^tO)_2$ (100 mM), were flushed with nitrogen for 5 min and irradiated at room temperature in 3 ml Pyrex spectrophotometric cells at a distance of 2 cm from the light source. In experiments carried out in the presence of salts, ammonium sulfate (0.2 M) or sodium perchlorate (0.2 M) was included in the irradiated reaction mixture.

Separation, identification, and quantitative determination of products. Samples (0.5 ml) of the irradiated solutions were withdrawn periodically, freeze-dried, and analyzed as follows:

Mononucleotides. The freeze-dried irradiated samples of the mononucleotides and the corresponding non-irradiated controls, were dissolved in water (300 µl) and samples (100 µl, in duplicates) were loaded onto Whatman No. 3 MM paper. Each sample was chromatographed in two solvent systems: A) ascending in ethanol: 1M ammonium acetate (7:3 v/v); B) descending in 2-propanol:water: concentrated ammonium hydroxide (3:1:1 v/v). The paper was then thoroughly dried in a stream of air, and fluorescent areas corresponding to markers of authentic samples were located by mineralight lamp. These were cut out and extracted with ammonium bicarbonate (0.25 M, 3x1 ml) by centrifugation. The yields of purine photoproducts were determined directly by measuring the optical density of the extracts at 260 nm. The fidelity of this method was

demonstrated by showing that the sum of each purine starting material and its photoproduct was equal to the amount of starting material extracted from the non-irradiated controls. The yields of pyrimidine photoproducts were calculated from the decrease in the amount of starting materials which was determined spectrophotometrically at 260 nm. Identical results were obtained by using both chromatographic solvent systems.

Ribodinucleoside monophosphates. Freeze-dried samples of irradiated ribodinucleoside monophosphates were hydrolyzed in NaOH (0.5 M, 300 µl) at 37° for 18 h to yield mixtures of nucleosides, nucleoside 3'-monophosphates, and their corresponding photoproducts. These were analyzed chromatographically as described above, using hydrolyzed non-irradiated dinucleoside monophosphates as controls.

Deoxyribodinucleoside monophosphates. Deoxyadenylyl-(3',5')-thymidine (dA-dT), thymidylyl-(3',5')-deoxyadenosine (dT-dA) and their photoproducts were hydrolyzed according to Burton (15), by adding their aqueous solution (0.2 ml) to 2 volumes of a solution of diphenylamine in 98% formic acid (3% w/v) and incubating the mixtures for 18 h at 30°. After ether extraction the aqueous phase was freeze-dried, dissolved in 300 µl of water, and subjected to chromatography as described above.

The R_f values of the various photoproducts are given in Table 1.

TABLE 1. R_f Values of Purine and Pyrimidine Photoproducts

Photoproduct	Solvent System* A	B
8-α-Hydroxy*iso*propyladenine	0.66	0.69
8-α-Hydroxy*iso*propyladenosine	0.66	0.78
8-α-Hydroxy*iso*propyl-3'(5')-AMP	0.30	0.45
6-α-Hydroxy*iso*propyl-5,6-dihydrouridine	0.52	0.58
6-α-Hydroxy*iso*propyl-5,6-dihydro-3'(5')-UMP	0.20	0.32
6-α-Hydroxy*iso*propyl-5,6-dihydro-3'(5')-TMP	0.26	0.38

*Solvent system A: ethanol:1M ammonium acetate pH 7.5 (7:3 v/v); solvent system B: 2-propanol:water:concentrated ammonium hydroxide (3:1:1 v/v).

RESULTS

The mononucleotides were irradiated with ultraviolet light of λ>300 nm in the presence of 2-propanol and $(Bu^tO)_2$, the latter serving as a photoinitiator. The reactions of adenine derivatives led to the substitution of the α-hydroxy-

*iso*propyl group for the H-8 atom of the purine, while with uracil and thymine derivatives addition of the alcohol across the 5,6-double bond of the pyrimidine took place. Products were determined quantitatively as described under Materials and Methods, and the results are described in Figure 1.

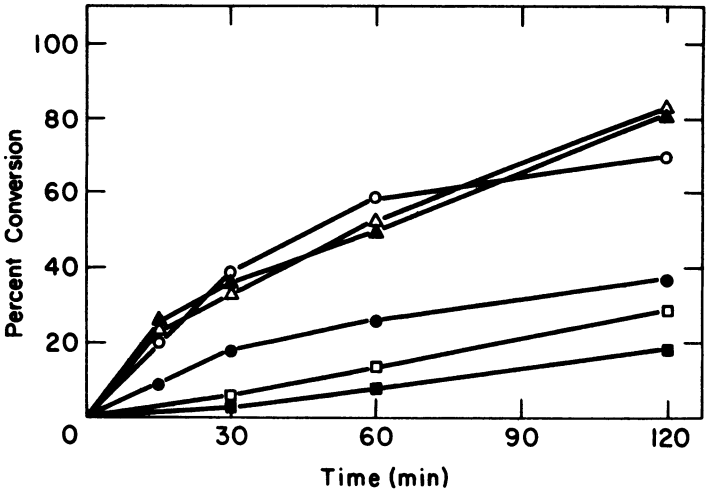


FIGURE 1: Photoalkylation of mononucleotides. Reaction conditions and analytical procedures are described under Materials and Methods. —○— , 3'-AMP; —●— , 5'-AMP; —△— , 3'-UMP, and 3'-UMP in the presence of 0.2M $(NH_4)_2SO_4$; —▲— , 5'-UMP; —□— , 3'-TMP; —■— ,5'-TMP.

Cytidine and cytidine monophosphates were unreactive under these conditions, and were recovered unchanged from the irradiated mixtures. Additional proof for the quantitative recovery of cytidine and its monophosphates was achieved by treatment of the irradiated nucleoside or nucleotide with aqueous NaOH (0.5N) at 37° for 18 h; this led to their quantitative conversion to uridine or to the appropriate uridine derivatives, as determined by paper chromatography.

It is seen from Figure 1 that 3'-AMP is modified nearly twice as fast as 5'-AMP. On the other hand, 3'-UMP and 5'-UMP are alkylated with nearly equal rates, and the presence of $(NH_4)_2SO_4$ does not affect the rate of modification of 3'-UMP. It is also seen from Figure 1 that 3'-TMP and 5'-TMP react rather slowly.

The dinucleoside monophosphates were irradiated in the presence of 2-propanol and $(Bu^tO)_2$ as described previously. Alkaline treatment of the irradiated ribonucleotides, or apurination of the deoxyribonucleotides, yielded mixtures of the appropriate nucleosides, mononucleotides, adenine, and their

respective photoproducts. These were analyzed by paper chromatography and the results are described in Figures 2 and 3.

We have found that the cytidine moieties of adenylyl-(3',5')-cytidine (A-C) and cytidylyl-(3',5')-adenosine (C-A) are unreactive under our irradiation conditions, as evidenced by their quantitive conversion to the appropriate uridine moieties following alkaline treatment of the irradiated dinucleoside monophosphates. In adenylyl-(3',5')-adenosine (A-A), as indicated in Figure 2, the adenosine which is blocked at its 3'-hydroxyl is modified two fold faster than that blocked at its 5'-hydroxyl. Figure 2 shows, in addition, that the adenosine moieties of adenylyl-(3',5')-uridine (A-U) and A-C react faster than those of uridylyl-(3',5')-adenosine (U-A) and C-A, respectively. Concerning the reactivity of the uracil moieties, it is seen from Figure 3 that both uracil moieties of uridylyl-(3',5')-uridine (U-U), as well as that of U-A, react with nearly the same rates; however, the uracil moiety of A-U is modified much slower. It should be noted that the presence of 0.2M $(NH_4)_2SO_4$ in the reaction of U-A reduces considerably the reactivity of the uracil moiety. It is further seen from Figure 3 that the thymine moieties of dA-dT and dT-dA react sluggishly.

DISCUSSION

It has been shown previously that the described photoalkylation reactions involve free radical intermediates resulting from the photolysis of $(Bu^tO)_2$, which absorbs most of the incident light (8). The excited peroxide fragments to oxy radicals which abstract a hydrogen atom from C-2 in 2-propanol to yield the ketyl radicals $C(CH_3)_2OH$; these are subsequently scavenged by the purine or the pyrimidine to yield the appropriate photoproduct (6,8). The addition of the free radical to the 7,8-double bond of the purine or the 5,6-double bond of the pyrimidine involves an initial attack at C-8 of the purine (6) or at C-6 of the pyrimidine. Our results indicate, therefore, that the 8-position of the adenine moiety of 3'-AMP is more exposed to an attack by ketyl radicals than that of 5'-AMP (see Figure 1). A similar effect consistently operates in adenine-containing dinucleoside monophosphates, where an adenosine moiety blocked at its 3'-hydroxyl is more exposed to the attack by the free radicals than a 5'-blocked adenosine. However, the 5,6-double bond of the uracil moiety in 3'-UMP is nearly as equally exposed to an attack by ketyl radicals as that of 5'-UMP. The same effect is observed in U-U, where both uracil moieties are exposed to the same extent to an attack by the free radicals. It should be noted, however, that the uracil moiety of U-A is more exposed than that of A-U.

We have shown previously that, when reacted separatedly, uracil and thymine

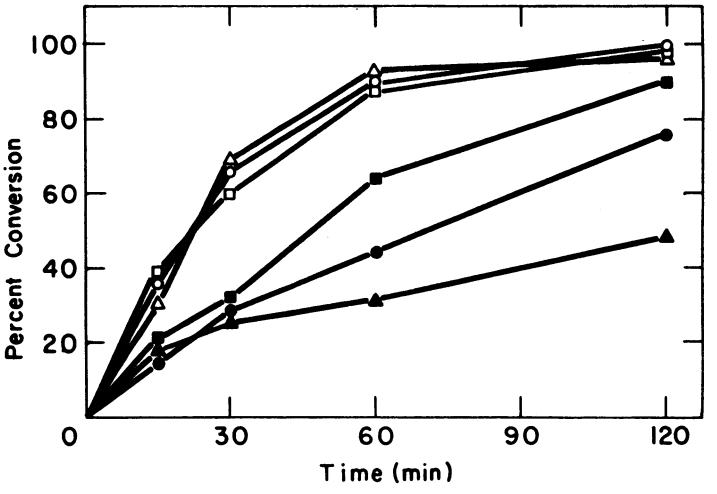


FIGURE 2: Photoalkylation of adenosine moieties of dinucleoside monophosphates. Reaction conditions and analytical procedures are described under Materials and Methods. —o— ,*A*-A; —●— , A-*A* (*A* indicates the analyzed adenine moiety); —△— , A-C; —▲— , C-A; —□— ,A-U; —■— ,U-A.

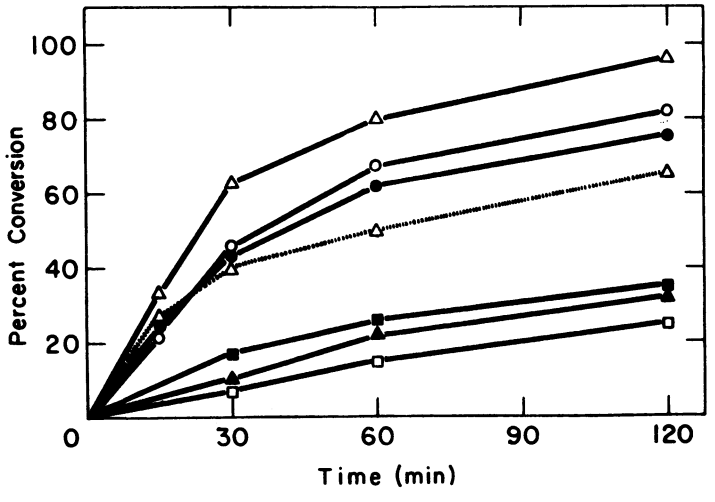


FIGURE 3: Photoalkylation of pyrimidine moieties of dinucleoside monophosphates. Reaction conditions and analytical procedures are described under Materials and Methods. —o— , *U*-U; —●— ,U-*U*(*U* indicates the analyzed uridine moiety); —△— ,U-A;--△-- , U-A in the presence of 0.2M $(NH_4)_2SO_4$; —▲— , A-U; —□— , T-A; —■— , A-T.

are modified with 2-propanol at least as fast as adenine or guanine; however, in purine-pyrimidine mixtures the reactivity of the pyrimidine is reduced considrably resulting in the selective modification of the purines. It has been suggested that this suppression of the reactivity of the pyrimidines results from the heteroassociation of the bases (11). The presently observed selectivity in nucleotides, and presumably in polynucleotides, may result from additional factors which are dictated by the conformation of the sugar-phosphate backbone.

In the present work reactions were carried out in very dilute solutions of the nucleotides (10^{-3} - 10^{-4}M), thus eliminating the possibility of intermolecular base stacking; furthermore, the presence of the alcohol facilitates destacking (16). Therefore, the reactivities of the adenine or uracil moieties in the mononucleotides must be evaluated in terms other than base stacking. Since the addition of free radicals to multiple bonds is sensitive to steric effects (17) , our results imply that the approach to the 8-position of the adenine moiety of 5'-AMP is more hindered than the approach to the 8-position of the adenine of 3'-AMP. Our conclusion is in agreement with that derived from physicochemical data, like nmr and CD or ORD (16), which asigned the *anti* conformation to 5'-AMP in solution. In this conformation the phosphate group is in close proximity to the 8-position of the adenosine, as detected by the deshielding effect of the phosphate on the H-8 proton of the purine (16). Nmr data further indicated that such an interaction of the phosphate group and the 8-position of the adenosine does not exist in 3'-AMP.

Concerning A-A, it is noteworthy that the adenosine linked through the 3'-hydroxyl is modified faster than that linked through the 5'-hydroxyl. Nmr data have indicated that A-A in solution assumes the *anti* conformation (16); thus, the phosphate group is in close proximity to the 8-position of the adenosine moiety which is linked through the 5'-hydroxyl. Unlike the case of AMP, base stacking may exist in A-A under the condition of the reaction (18, 19), and thus contribute to the observed difference in the reactivity of the two adenine moieties. Our results indicate, however, that base stacking does not account for this difference since the relative reactivities of the bases are not affected by the presence of salts which are known either to enhance or to diminish base stacking (16, 20). The same conclusion can be reached from the observation that the ratio of the rates of modification of adenosine moieties of 3'-AMP ($R_{3'\text{-AMP}}$) and 5'-AMP ($R_{5'\text{-AMP}}$) is nearly equal to that of the respective moieties of A-A.

$$\frac{R_{3'\text{-AMP}}}{R_{5'\text{-AMP}}} \Big/ \frac{R_{A\text{-}A}}{R_{A\text{-}A}} = 1.1$$

A similar effect has been observed in all the adenine-containing ribodinucleoside monophosphates. We, therefore, conclude that the reactivity of adenine in the reported photoalkylation reactions depends primarily on steric hindrance imposed by the phosphate group.

Regarding the selectivity of the photoalkylation reaction for the various heterocyclic bases, it is noteworthy that cytidine itself and cytosine moieties of nucleotides are inert to these reactions. In addition, the reactivity of 3'-TMP and 5'-TMP, as well as that of thymine moieties of thymine-containing dinucleoside monophosphate, is relatively low. Thus, the reaction of adenine-cytosine or adenine-thymine systems are highly selective for the adenine moieties, irrespective of their location in the dinucleoside monophosphate.

The adenine-uracil system presents, however, a different case since uracil exhibits a higher reactivity in the reported reactions as compared with other pyrimidines, and in some instances it is even higher than that of adenine (11). Furthermore, our results indicate that the reactivities of both bases strongly depend on their location in the nucleotide. Thus, while 3'-AMP and 5'-UMP are modified with comparable rates, the rate of modification of adenine of A-U is about 8-fold faster than that of the uracil moiety. On the other hand, 3'-UMP reacts nearly twice as fast as 5'-AMP, and this preference of the reaction for the uracil moiety is preserved in U-A. Unlike the reactions of adenosine moieties, the reactions of uridine moieties are not sensitive to steric hindrance imposed by the phosphate group,since 3'-UMP and 5'-UMP are modified with equal rates, as is the case for both uracil moieties of U-U. We, therefore, assume that the difference in the reactivity of the uracil moiety of A-U, as compared with that of U-A, results from intramolecular stacking of the bases which appears to be stronger in A-U. This assumption is supported by previously reported nmr data which indicate that the 5,6-double bond of uracil of A-U is in close proximity to the adenine moiety, while in U-A only partial overlap of the uracil and adenine is possible (21, 22). Thus, in the latter the 5,6-double bond of the uracil is well away from the adenine. Our photoalkylation reaction can detect this difference, which is manifested in a faster rate of modification of the uridine moiety of U-A as compared with that of A-U. We have further shown that the presence of $(NH_4)_2SO_4$, which is known to enhance stacking interactions in nucleotides (20), suppresses considerably the reactivity of uracil of U-A, thus reversing the selectivity of the reaction in favor of adenine. This observation can be interpreted by assuming that the addition of salt forces the uracil closer to the adenine, resulting in an increased hindrance for the approach to its 5,6-double bond. The possibility

that the presence of $(NH_4)_2SO_4$ quenches the reactivity of the uracil moiety of U-A is ruled out by showing (Figure 1) that the reactivity of 3'-UMP is not affected by the presence of $(NH_4)_2SO_4$.

CONCLUSION

Our present and previous results have led us to the tentative conclusion that the reactivity of adenosine moieties in light-induced and free radical reactions, involving the 8-position of the purine, is affected by the location of the sugar-phosphate linkage. This structural feature and intramolecular base stacking determine the degree of selectivity of these reactions for the appropriate base in systems containing adenine and uracil. Cytidine moieties may be unreactive in free radical reactions.

Our results verify some predictions concerning the chemical reactivity of various nucleotides, which could have been made on the basis of conformational features derived from physicochemical data. This work further implies that a free 5' end in polynucleotides will be the initial target for photochemical reactions of this nature. These points are currently under investigation.

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