Volume 12 Number 20 1984 Nucleic Acids Research

The photoreactivity of T-A sequences in oligodeoxyribonucleotides and DNA

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Received 30 July 1984; Accepted 24 September 1984

ABSTRACT

Photoaddition between adjacent adenine and thymine bases occurs, with a quantum yield of $\sim 5 \times 10^{-4}$ mol einstein^{-1}, when d(T-A), dT-A, d(pT-A), d(T-A-T), d(T-A-T-A) and poly(dA-dT) are irradiated, at 254 nm, in aqueous solution. The photoadduct thus formed is specifically degraded by acid to the fluorescent heterocyclic base 6-methylimidazo[4,5-*b*]pyridin-5-one (6-MIP) with retention of C(8) of adenine and the methyl group of thymine. This reaction, coupled with either spectrofluorimetric or radiochemical assay of 6-MIP isolated by high voltage paper electrophoresis, has been used to demonstrate formation of the adenine-thymine photoadduct on UV irradiation of poly(dA-dT). poly(dA-dT) and both native and denatured DNA from calf thymus and *E.coli*. Estimated quantum yields for this new type of photoreaction in DNA show that it is substantially quenched by base pairing. Possible biological implications of the photoreaction are discussed.

INTRODUCTION

The photochemical lesions produced in DNA by ultraviolet (UV) radiation have been associated with many diverse biological effects including the mutation and killing of microorganisms and carcinogenesis in man and other species (1). In DNA irradiated under physiological conditions, the primary photoproducts hitherto identified are derived exclusively from the pyrimidine bases and there is no convincing evidence for the occurrence of photochemical modification to the purines adenine and guanine (1,2). We have recently observed (3,4) that when the deoxydinucleoside monophosphate d(T-A) is irradiated at 254 nm, in aqueous solution, it undergoes a sequence - specific intramolecular photoaddition reaction between its constituent adenine and thymine bases. Formation of the resultant photoadduct, designated TA*, can be detected by virtue of its acid catalysed degradation to the fluorescent heterocyclic base 6-methylimidazo[4,5-*b*]pyridin-5-one (6-MIP). In this paper, we describe experiments showing that the photochemically induced formation of TA* is a common property exhibited by T-A sequences in simple oligodeoxyribonucleotides, the alternating copolymer poly(dA-dT), and DNA

from calf thymus and *E.coli*; estimates of the quantum yield of the photoreaction have been made in each case. Although the feasibility of photoaddition reactions between thymine and a purine nucleus has previously been demonstrated in trimethylene-bridged model systems (5,6), TA* constitutes the first example of a purine-pyrimidine photoadduct to be characterised in UV-irradiated nucleic acids.

EXPERIMENTAL

Materials

1,3 Dimethyluracil, d(T-A) sodium salt and calf thymus DNA (Type I) were supplied by Sigma, and the ammonium salts of d(T-A-T) and d(T-A-T-A) by Collaborative Research. The following were obtained from P-L Biochemicals: d(pT-A) ammonium salt, poly(dA-dT), poly([8-^{14}C]dA-dT), poly(dA-[2-^{14}C]dT) and poly(dA-dU). Poly(dA-[*methyl*-^{3}H]dT) and [*methyl*-^{3}H]thymine were from Miles and Amersham International respectively. Radiolabelled *E.coli* DNA was prepared according to Marmur (7) from a thymine - requiring strain of *E.coli* (NCIB 10246) grown in the presence of [*methyl*-^{3}H]thymine. dT-A was a gift from Dr. G.M. Blackburn, Sheffield University.

General Methods

UV spectra were recorded with Cary 118 and Unicam SP 800 instruments; spectrofluorimetric measurements were made with a Perkin-Elmer model 204 fluorescence spectrophotometer . High voltage paper electrophoresis at pH 2.5 and thin layer chromatography (TLC) were carried out as described elsewhere (4). An LKB-Wallac Rackbeta II instrument was used for liquid scintillation counting.

Irradiation Procedures

UV irradiation was carried out at room temperature, with either two 8W germicidal strip lamps (Sylvania G8T5) in parallel, or with one or more of the circular elements comprising a PCQ-X1 Photochemical Lamp (Ultra-Violet Products, San Gabriel, California). Both sources emit almost exclusively at 254 nm in the ultraviolet. In the former case, solutions were irradiated in sealed quartz UV cells positioned with their transparent faces parallel to the strip lamps; the incident fluence was measured with a J-225 short wave UV meter (Ultra-Violet Products). Samples irradiated with the PCQ-X1 Lamp were contained in quartz fluorimeter cells or quartz tubes positioned vertically along its central axis; ambient temperature was maintained by directing a jet of cool air over the apparatus. For this lamp, the incident fluence was determined by actinometry with 1,3-dimethyluracil, assuming a quantum yield

for photohydration of 0.0138 mol einstein^{-1} (8). Effectively opaque solutions of 1,3-dimethyluracil were irradiated in the same configuration as experimental samples and the rate of photohydration was established from the decrease in absorbance at 266 nm with time.

The oligonucleotides, single stranded poly(dA-dT) and denatured DNA were irradiated as solutions in distilled water adjusted to pH 7 with dilute ammonia. Double stranded poly(dA-dT) and native DNA were irradiated in pH 7.0, 0.10 M Na^+ buffer (0.025 M Na_2HPO_4, 0.05 M NaCl adjusted to pH 7.0 with HCl). The concentrations of species in solution were estimated spectrophotometrically using the following extinction coefficients ($M^{-1}cm^{-1}$): 1,3-dimethyluracil, ε_{266} 9130; d(T-A), dT-A and d(pT-A), ε_{260} 23400 (9); d(T-A-T) and d(T-A-T-A), ε_{260} 33900 and 49200 respectively (supplier's specifications); poly(dA-dT), ε(P) 9600 at 260 nm when single stranded, ε(P) 6650 at 262 nm when double stranded (10); DNA, ε(P) at 260 nm 9200 (denatured), 6800 (native). For the purpose of calculating quantum yields, the extinction coefficient of the photoproduct TA* was taken as 3500 $M^{-1}cm^{-1}$ at 260 nm (3).

Photoproduct Analysis. Following UV irradiation, samples in aqueous solution were lyophilised directly while those in pH 7.0 buffer were first desalted by dialysis against distilled water. The lyophilised material was dissolved in 1M HCl (1 ml) and heated at 100°C for 4h in a sealed tube. The resultant hydrolysate was evaporated to dryness on a rotary evaporator and then re-evaporated several times from a small quantity of water to remove all traces of HCl. Finally the residue was subjected to high voltage electrophoresis on Whatman 3MM paper strips at pH 2.5. This efficiently separates 6-MIP, produced by acid degradation of the photoproduct TA*, from adenine, guanine, cytosine, thymine and uracil; it is readily detected on the electrophoretogram by its characteristic fluorescence on illumination with UV light. To quantify the amount of 6-MIP produced it was extracted from the paper with water and assayed by UV spectrophotometry or spectrofluorimetry assuming ε_{322} 14800 $M^{-1}cm^{-1}$ at pH 7 (4).

In the case of UV-irradiated [*methyl*-^{3}H]thymine - labelled *E.coli* DNA, the distribution of tritium on the final electrophoretogram was determined by cutting the paper into strips 1.5 cm wide and measuring their associated radioactivity by liquid scintillation counting.

RESULTS

On irradiation at 254 nm, in neutral aqueous solution, single stranded poly(dA-dT) and the oligonucleotides d(T-A), dT-A, d(pT-A), d(T-A-T) and d(T-A-T-A) all showed very similar changes in their UV spectra. As

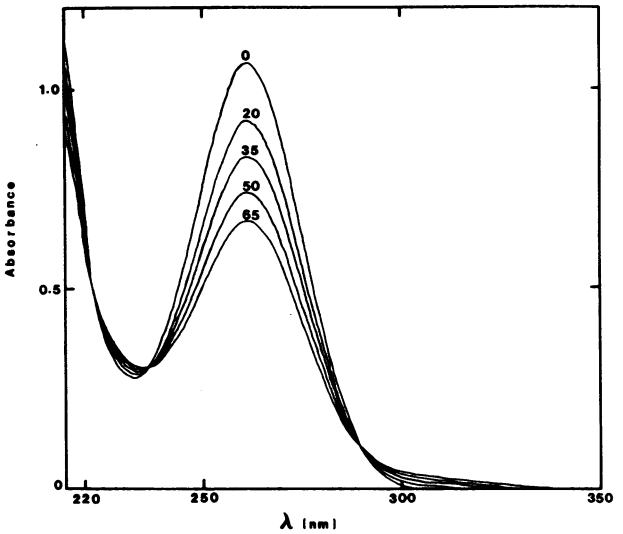

Fig. 1. Changes in the UV spectrum of d(T-A) induced by 254 nm radiation. A 0.045 mM solution of d(T-A) in neutral aqueous solution (3.1 ml) was irradiated in a 1 cm pathlength quartz cell with an incident fluence of ∿14 mW for the times (in min) indicated on the spectra.

illustrated for d(T-A) in Fig. 1, consecutive spectra pass through isosbestic points in the region of 222, 236 and 290 nm, consistent with the occurrence of a specific photochemical reaction. Analysis by high voltage paper electrophoresis and TLC revealed that all of the oligonucleotides gave a single major photoproduct except for d(T-A-T-A) which gave two (arising, presumably, from one or both T-A doublets being present as TA*). When the irradiated samples were treated with 1M HCl, at 100°C for 4h, the fluorescent base 6-MIP, whose formation is diagnostic of the photoadduct TA*, was produced (together with adenine and thymine) in every case. Its identity was confirmed, after recovery by paper electrophoresis, by its distinctive spectroscopic properties and its mobility on TLC (4).

The approximate quantum yields for the photoreaction in the oligonucleotides and single stranded poly(dA-dT), that are given in Table 1, have been calculated from the decrease in absorbance of the irradiated samples at 260 nm as a function of UV fluence at 254 nm. It has been assumed that the formation of TA* was solely responsible for the observed decrease in absorbance.

With native and denatured calf thymus DNA and double stranded poly(dA-dT).poly(dA-dT) the quantum yield of the photoreaction could not be

Table 1. Approximate quantum yields for formation of the photoproduct TA* in oligo- and polydeoxyribonucleotides on irradiation at 254 nm

Material	Quantum Yield (μmol einstein^{-1})
d(T-A)	700
dT-A	700
d(pT-A)	500
d(T-A-T)	400
d(T-A-T-A)	500
poly(dA-dT)	500
poly(dA-dT).poly(dA-dT)	100
Denatured DNA (calf thymus)	60
Native DNA (calf thymus)	14

correlated with the absorbance changes occurring during irradiation. Instead, the values given in Table 1 are based on the transformation of TA* into 6-MIP which occurs with a yield of ∿50% (4). In this procedure, essentially opaque solutions ($A_{254} \geqslant 2$) of the relevant materials were exposed to a known incident fluence of 254 nm radiation. The amount of TA* thus produced was estimated from the amount of 6-MIP isolated after acid treatment of the irradiated samples as detailed in Experimental. To allow for recovery losses, as well as the conversion yield of ∿50%, the amount of TA* has been calculated as three times that of the isolated 6-MIP. This factor is obviously somewhat approximate but is unlikely to seriously over-estimate the yield of TA*. Within experimental error, the respective quantum yields for native and denatured calf thymus DNA remained constant as the absorbed dose varied over the range 20 to 200 J/mg of DNA.

Experiments were carried out with radioactively labelled poly(dA-dT) to establish which atoms of TA* are retained during its degradation to 6-MIP. In addition to providing some insight concerning the mechanism of the process (4), these studies defined which radiolabelled bases are appropriate for detecting and assaying the formation of TA* in DNA by radiochemical procedures. The 6-MIP produced from irradiated samples of poly(dA-dT) was quantified by spectrophotometry and then its specific activity was determined by liquid scintillation counting. When the poly(dA-dT) was labelled with ^{14}C at C(8) of adenine or with ^{3}H in the methyl group of thymine the specific activity of the recovered 6-MIP was the same, within ±15%, as that of the corresponding

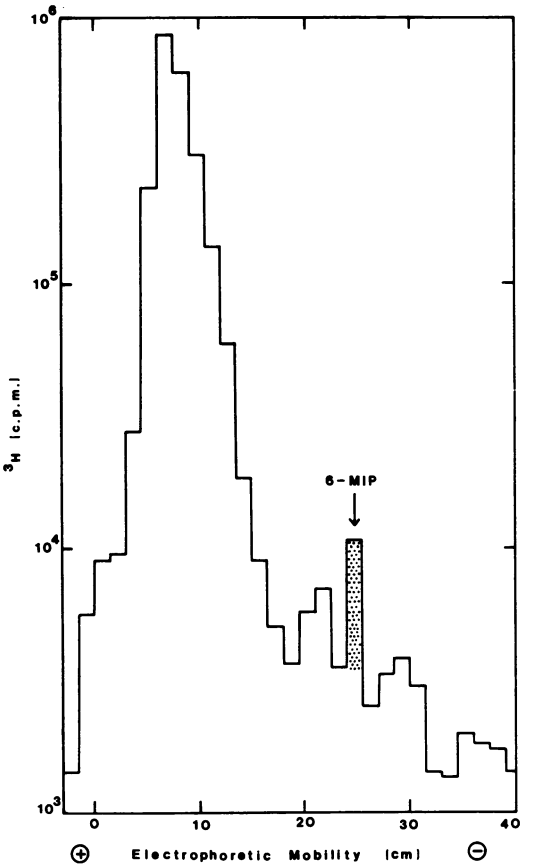

Fig. 2. Profile of ^3H radioactivity on high voltage paper electrophoresis (pH 2.5, 3 kV, 90 min) of the acid hydrolysate of native [*methyl*-^3H]thymine - labelled *E. coli* DNA irradiated with a fluence of 58 kJ m^{-2} at 254 nm. The position of 6-MIP, produced by degradation of the photoadduct TA*, is indicated.

labelled base. However, when a ^{14}C label was located at C(2) of thymine it was not retained.

Based on these results, *E.coli* DNA labelled with [*methyl*-^3H]thymine was used to examine the feasibility of detecting small amounts of TA* in UV-irradiated DNA by radiochemical assay. Residues from the acid hydrolysis of irradiated DNA were fractionated by electrophoresis and the distribution of tritium on the paper determined as detailed in Experimental. As illustrated in Fig. 2, a distinct peak of activity could be observed at the position expected for 6-MIP. Its identity was confirmed by eluting the radioactive

material from the paper and mixing it with unlabelled 6-MIP. The 6-MIP was then subjected to paper chromatography in three different solvent systems in succession. After each run the 6-MIP extracted from the paper was assayed for radioactivity. Tritium counts co-migrated with 6-MIP in all three solvent systems and the specific activity of the recovered 6-MIP remained constant within experimental error.

When double stranded *E.coli* DNA was irradiated at 254 nm with an average fluence of 14 kJ m^{-2} (calculated by applying the appropriate Morowitz (11) correction factor) the radioactivity associated with 6-MIP represented $\sim$0.1% of the total tritium in the acid hydrolysate. For an average fluence of 58 kJ m^{-2} the corresponding figures were $\sim$0.6% for double stranded and $\sim$2.2% for single stranded DNA. Allowing for 50% conversion of TA* into 6-MIP, the estimated quantum yields for formation of TA* in *E.coli* DNA are $\sim 1 \times 10^{-5}$ and $\sim 4 \times 10^{-5}$ mol einstein^{-1} for the native and denatured forms respectively, in good agreement with the data (Table 1) for calf thymus DNA.

Finally, an attempt was made to establish whether the adjacent adenine and uracil bases in poly(dA-dU) undergo a similar photoreaction to the adenine and thymine bases in poly(dA-dT). In this case, acid degradation of the resultant adenine-uracil photoadduct should, by analogy with TA*, give imidazo[4,5-*b*]pyridin-5-one which, like 6-MIP, is strongly fluorescent (4). To inhibit photohydration of the uracil bases the single stranded polymer was irradiated in frozen aqueous solution whereas base paired poly(dA-dU). poly(dA-dU) was irradiated in pH 7.0, 0.10 M Na^+ buffer as for poly(dA-dT). However, after treating the irradiated samples with 1M HCl, at 100°C for 4h, no imidazo[4,5-*b*]pyridin-5-one could be detected by paper electrophoresis or TLC.

DISCUSSION

The photoreactivity of the individual bases in oligo- and polynucleotides is very sensitive to their physical and chemical environment and can vary markedly with the molecular conformation of the irradiated species (2). To establish how these factors affect formation of the photoadduct, TA*, which is specific to T-A sequences, the occurrence and yield of the photoreaction were investigated in a series of simple oligodeoxyribonucleotides, as well as poly(dA-dT) and DNA.

It is clear from the data in Table 1 that the quantum yield for formation of TA* is highest for the deoxydinucleoside monophosphate d(T-A) and is unaltered by replacement of the deoxyadenosine moiety by adenosine in

dT-A. The yield is reduced slightly on phosphorylation to d(pT-A) but thereafter remains effectively constant at higher degrees of polymerization in d(T-A-T), d(T-A-T-A) and poly(dA-dT). A marked (approximately 5-fold) decrease in the quantum yield is observed, however, with double stranded poly(dA-dT).poly(dA-dT) and a similar difference exists between denatured and native DNA.

The quenching of the photoreaction associated with base pairing may be due to the restricted conformational freedom of the double helix, compared to the single stranded state, making it more difficult to attain optimal alignment of adjacent adenine and thymine bases for the photoaddition process. In this context, it should be noted that, at pH 7.0, 0.10 M Na^+, the secondary structure of poly(dA-dT).poly(dA-dT) differs somewhat from that of DNA. Although it has been shown (12) by 2-dimensional NMR measurements (utilising the nuclear Overhauser effect) that poly(dA-dT).poly(dA-dT), like DNA, assumes a right handed B conformation in solution, the presence of two discrete resonance signals in its ^{31}P-NMR spectrum (13) indicates that the alternate phosphodiester linkages of the copolymer are conformationally distinct.

The progress of the photoreaction in the oligodeoxyribonucleotides and poly(dA-dT) could be conveniently monitored from the decrease in absorbance at 260 nm as a function of UV fluence; TA* absorbs only very weakly above 240 nm (3). As significant amounts of other photoproducts did not appear to be formed on irradiating these compounds, the observed absorbance changes were used to estimate the relevant quantum yields given in Table 1. This approach was not applicable to poly(dA-dT).poly(dA-dT) where prolonged irradiation caused almost no change in absorbance at 260 nm; presumably, the reduction in extinction associated with photoadduct formation is compensated by hyperchromic effects arising from localised denaturation of the double helix. Obviously, direct quantitation of TA* on the basis of absorbance changes is impossible in UV-irradiated DNA where a variety of more abundant pyrimidine photoproducts are formed. In this case, and with poly(dA-dT).poly(dA-dT), it was necessary to assay for the formation of TA* by taking advantage of its specific conversion into 6-MIP on treatment with 1M HCl, at 100°C for 4h. Were it not for this remarkable transformation of the photoadduct into a chemically stable heterocylic base with distinctive spectroscopic properties, the detection of TA* in DNA would pose very severe technical problems. Fortunately, however, the weak basicity of 6-MIP and its intense fluorescence emission permit very small amounts of it to be isolated from hydrolysates of UV-irradiated DNA by paper electrophoresis and then

quantified by spectrofluorimetry.

On electrophoresis at **pH 2.5**, 6-MIP (whose pK for protonation is 2.9) migrates as a cation and is clearly separated from the normal nucleic acid bases and from the species arising by acid hydrolysis of the major thymine-derived photoproducts *viz* cyclobutyl dimers and 6-(2'-oxopyrimidin-4'-yl)-thymine. It can therefore be selectively excised from the electrophoretogram, eluted from the paper with water and assayed by spectrofluorimetry. Less than 1 nmol of 6-MIP can be recovered and measured by this procedure. As mentioned under **Results**, quantum yields estimated from such measurements of 6-MIP have been adjusted to allow for recovery losses and for the non-quantitative conversion of TA* into 6-MIP.

It is noteworthy that fractionation of acid hydrolysates of UV-irradiated DNA has hitherto relied heavily on paper chromatography (2) rather than electrophoresis. The latter technique, however, is not only more rapid but provides greater scope for resolving charged photoproducts. It has the advantage that, because the major DNA photoproducts derived from thymine are effectively uncharged in the pH range 2.5 to 7.0, they remain close to the origin and do not interfere with the detection of minor photoproducts that are electrophoretically mobile under these conditions.

Radiochemical detection of 6-MIP provides greater sensitivity than can be achieved by spectrofluorimetry. This is necessary for measuring the formation of TA* in DNA exposed to low UV fluences. As the conversion of TA* into 6-MIP involves substantial rearrangement and elimination of the component atoms of the original adenine and thymine bases, it is essential to establish which radiolabelling patterns are compatible with retention of radioactivity in 6-MIP. Results obtained with specifically labelled samples of poly(dA-dT) showed that 6-MIP incorporates the C(8) atom from adenine and the methyl group of thymine but not the C(2) atom of thymine. The use of [2-^{14}C]thymine - labelled DNA, which has been widely employed in photochemical studies, is therefore not appropriate for monitoring the production of TA*. In an early study with [2-^{14}C]thymine - labelled poly(dA-dT), Smith (14) detected several radioactive photoproducts after hydrolysis of the UV-irradiated copolymer with trifluoroacetic acid. However, the presence of 6-MIP, expected as the main photoproduct-derived species in this experiment, would have gone unnoticed owing to non-incorporation of the ^{14}C label.

As illustrated in Fig. 2, paper electrophoresis at pH 2.5 separates 6-MIP as a discrete peak of radioactivity from acid hydrolysates of UV-irradiated *E.coli* DNA labelled with [*methyl*-^{3}H]thymine. Its position on the

electrophoretogram is readily established by spiking the hydrolysate with unlabelled 6-MIP and detecting the associated fluorescence. Other small peaks of radioactivity are evident which possibly arise from minor, but as yet uncharacterised, photoproducts of thymine.

The quantum yield for formation of TA* in *E.coli* DNA ($\sim 1 \times 10^{-5}$ mol einstein^{-1}), calculated on the basis of radioactivity in the 6-MIP peak, corresponded closely with the value based on spectrofluorimetric estimation for calf thymus DNA (Table 1). It must be emphasised that these quantum yields define the efficiency of TA* formation in terms of quanta absorbed by total DNA. From nearest neighbour analysis (15) it is known that in both *E.coli* and calf thymus DNA the dinucleotide frequency of T-A sequences is 0.05. Hence, to a first approximation, the quantum yields expressed in terms of the quanta specifically absorbed by the thymine and adenine bases in T-A doublets would be $\sim$10 times greater and comparable with the value for poly(dA-dT).poly(dA-dT) i.e. $\sim 1 \times 10^{-4}$ mol einstein^{-1}. As this is less than one hundredth of the quantum yields quoted for pyrimidine dimerisation in DNA (2,16) it is evident that TA* must constitute a relatively minor photoproduct. Nonetheless, exposure of wild type *E.coli* bacteria to one mean lethal fluence (F_{37} = 50 J m^{-2}) of 254 nm radiation (17) should induce the formation of TA* at $\sim$10 sites per genome. Presumably, it will be formed preferentially at Pu-T-A rather than Py-T-A sequences as dimerisation of thymine with the adjacent pyrimidine base should be favoured in the latter case (18). The presence of TA* probably contributes to the decrease in ellipticity at 275 nm that is observed (19) when calf thymus DNA is exposed to high UV fluences because this wavelength coincides with a minimum in the circular dichroism spectrum of TA* itself (3).

The formation of TA* is the first example of an intramolecular photoreaction involving a purine base to be characterised in native DNA. Although the adenine bases in poly(dA) undergo photodimerisation with a relatively high quantum yield (20) attempts to demonstrate the occurrence of this reaction in DNA have been inconclusive (21). At present, the photochemistry and photobiology of nucleic acids are interpreted almost exclusively in terms of pyrimidine photoproducts (1,2). However, evidence for photochemical modification at purine bases in DNA has very recently emerged from the DNA sequencing results obtained with a novel photofootprinting technique (22). The nature of the relevant purine photoproducts is still obscure but their chemical characterisation is clearly of great importance.

A key factor influencing the biological significance of any photoreaction in DNA is the efficiency with which the resultant lesion can be corrected by cellular DNA repair systems. In this regard it is noteworthy that, after irradiation at 254 nm, poly(dA-dT).poly(dA-dT) acts as a substrate for a damage-specific DNA-binding protein from human placenta that is reported to recognise a singlet state-derived thymine lesion (23). The latter most probably corresponds to TA* which is known (4) to arise via a singlet excited state in d(T-A), and would be formed under the irradiation conditions. The greatly reduced affinity of the protein for similarly irradiated poly(dA-dU).poly(dA-dU) is consistent with our failure to detect the formation of an adenine-uracil photoadduct in this copolymer.

In principle, the mutagenic potential of TA* might be assessed by directly correlating UV-induced mutations with nucleotide changes at T-A doublets within a gene of known DNA sequence. This approach has been used very effectively in defining the respective roles of pyrimidine dimers and bipyrimidine (6-4) photoadducts as mutagenic lesions (24,25). However, the expected low abundance of TA* compared to the latter photoproducts implies that any mutations arising from it will be relatively rare events. Although most mutations caused by UV radiation can be attributed to a single base change, a small proportion involve tandem double base changes. It is interesting, and possibly significant, that of 40 UV-induced nucleotide changes determined in the *lac* promoter of M13*lac* hybrid phage DNA the only two tandem base changes both occurred at T-A sites (26). As this DNA is single stranded the relative yield of TA* would have been higher (Table 1) than for a double stranded substrate. By comparison, none of the 17 tandem double base changes characterised in two other studies with double stranded DNA (25,27) involved T-A sequences. Clearly, far more data are needed to definitively evaluate the status of TA* as a mutagenic lesion.

Finally, it should be recognised that as T-A is a self-complementary sequence, the photoadduct TA* has the unique characteristic that it could be formed, albeit with very low probability, at the same site on both strands of a DNA duplex. Unless the formation of TA* can be reversed *in vivo*, as occurs with pyrimidine dimers during photoreactivation, this type of event would almost inevitably have mutagenic or lethal consequences because the original DNA sequence could not be restored by template-directed repair enzymes.

ACKNOWLEDGEMENTS

This work was supported by grants from the Science and Engineering Research Council and the Nuffield Foundation.

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