Specific truncated variants would result from strong transcription initiation or termination sites internal to the repeat element. In this context, it is interesting to compare the internal structure and pattern of transcription of the long repeat element *Copia* in the *Drosophila* genome (Fouts & Manning, 1981) with the repeated family described here. There are two principal RNA transcripts from *Copia*, one full length and the other terminating near the center of the element within a tandemly repeating region strikingly similar to the one described here; it too is composed of a few repetitions of a 35–37-bp element. Thus, the truncated variants described here might derive not from recombination at the DNA level but by specific termination of RNA transcripts followed by reinsertion of the corresponding DNA copies.

In some measure, the data presented here serve to explain the limited number of structural variants detected in this, and perhaps in other, long repeated sequence family. There are apparently internal structural features that facilitate the appearance of certain variants. Like "hot spots" for mutation or recombination, these special regions may cause certain specific variants to predominate against a background of random variants generated by other mechanisms.

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Formation of Radiation-Induced Cross-Links between Thymine and Tyrosine: Possible Model for Cross-Linking of DNA and Proteins by Ionizing Radiation[†]

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ABSTRACT: A model for radiation-induced cross-linking of DNA and proteins has been developed. It is based on initial formation of free radicals on a DNA base, i.e., thymine, and on an amino acid, i.e., tyrosine. It was demonstrated that interaction of these radicals is highly favored as measured by their kinetics and the cross-linked products. The gas chromatography–mass spectrometry methodology used for the identification of the thymine–tyrosine cross-links is suggested as an experimental approach in the measurements of biological cross-links.

Cross-links within DNA and between DNA and proteins induced by different agents and processes, such as drugs (Kohn, 1977; Kohn & Ewig, 1979), autoxidation (Riess & Tappel, 1973), and ionizing radiations (Fornace & Little, 1977; Mee & Adelstein, 1981; Bowden et al., 1982), and intrastrand links caused by UV light (Smith, 1976) are becoming recognized as a common occurrence in vitro and in vivo. However, the cross-linking has not received a deserved attention in the overall consideration of DNA damage and its consequences, although cross-linking is by no means a minor process. In most cases, the kinetics and mechanisms of cross-linking are poorly understood, whereas UV light-induced intrastrand links are well-known to take place mainly via condensation of two double bonds, i.e., the formation of cyclobutane-type dimers (Setlow, 1966). Similarly, UV light causes interstrand

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cross-linking by agents such as psoralen (Song & Tapleg, 1979). In autoxidation, interstrand cross-links are induced by malondialdehyde, and the nature of those cross-links may be derived from the studies in model systems (Summerfield & Tappel, 1983).

Ionizing radiation-induced cross-links have been shown to occur between DNA and the accompanying histone molecules (Mee & Adelstein, 1981). The observed DNA–protein cross-links should be a consequence of the interaction of two vicinal free radicals, as we perceive it—one on the DNA strand and one in close proximity on the adjacent protein. Vicinal free radicals can be formed in spurs (Mozumder & Magee, 1966), which contain two to three radical pairs, generated either by direct ionization of DNA and the protein or by the reactions of OH radicals with DNA and the protein components. Free radicals on DNA and the protein that are formed randomly would have a minimal chance of interacting because of impaired mobilities associated with macromolecules.

The most likely components to be involved in radiation-induced cross-linking would be DNA bases and the aromatic or positively charged amino acids of histones. Those particular amino acids have been singled out because they participate in the formation of the DNA–protein complex (Takeda et al., 1983). Negatively charged amino acids, e.g., glutamic and aspartic acids, would be located away from the negatively charged DNA strand in the DNA–histone complex and would have minimal probabilities for a cross-link formation. 2-Deoxyribose (dR)[1] cannot be ruled out as a participant in cross-linking. However, because of rapid consecutive intramolecular reactions in the dR radical (Schulte-Frohlinde, 1983) and in the absence of observed dR–dR cross-links, participation of dR in DNA–protein cross-link formation has not been considered so far.

In order to assess the feasibility of radiation-induced cross-links between various DNA and protein components, we chose to investigate the mixture of thymine (T) and tyrosine (Tyr) as an appropriate model system. A methodology using capillary gas chromatography–mass spectrometry (GC–MS) was developed for separation and characterization of possible cross-links between T and Tyr. We report here on a highly efficient OH radical induced cross-linking between T and Tyr that indicates a possibility of T–Tyr linkage formation in the radiation-induced cross-linking between DNA and proteins.

MATERIALS AND METHODS[2]

Materials. Thymine (T), thymidine (dT), thymidine 5′-monophosphate (pdT), tyrosine, and uracil were purchased from Sigma. Bis(trimethylsilyl)trifluoroacetamide (BSTFA) and acetonitrile were from Pierce. Water purified through a Millipore system was used for all purposes.

Irradiations. Aqueous solutions of the mixtures of T, dT, or pdT with Tyr (each 0.5 mM in the mixture) were saturated with oxygen-free N_2O for 30 min and irradiated in a ^{60}Co-γ-source [dose range 110–440 Gray (Gy); dose rate 110 Gy/min]. The samples were then lyophilized. The dose rate of the source was determined by using a Fricke dosimeter (Fricke & Hart, 1966).

Hydrolysis with Hydrochloric Acid. To remove the sugar moiety, lyophilized samples of irradiated mixtures of dT or pdT with Tyr were hydrolyzed with 1 n HCl in evacuated and sealed tubes at 100 °C for 4 h prior to GC–MS analysis. For comparison, a sample of irradiated T was also treated with HCl in the same manner. After hydrolysis, samples were dried in a desiccator under vacuum prior to trimethylsilylation.

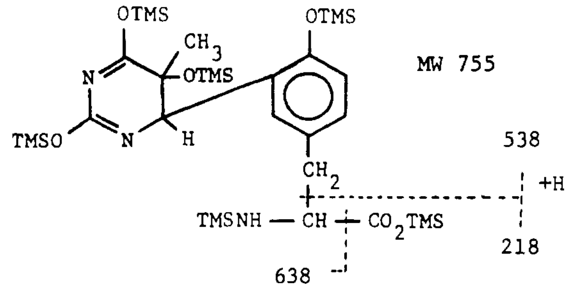
Trimethylsilylation. Samples (ca. 2 mg) were trimethylsilylated in Teflon-capped Hypovials (Pierce) with 0.2 mL of a mixture of BSTFA and acetonitrile (1:1) by heating for 15 min at 140 °C.

Gas Chromatography (GC). A Hewlett-Packard Model 5880A microprocessor-controlled gas chromatograph equipped with a flame ionization detector was used. The injection port and detector were both maintained at 250 °C. Separations were performed in a fused silica capillary column (12 m, 0.2-mm i.d.) coated with SE-54 (5% phenyl methyl silicone gum; cross-linked; siloxane deactivated) (Hewlett-Packard). Helium was used as the carrier gas at an inlet pressure of 100 kPa. The split ratio was 20:1.

Gas Chromatography–Mass Spectrometry (GC–MS). Mass spectra were taken at 70 eV with a Hewlett-Packard Model 5970A mass selective detector interfaced to the above gas chromatograph.

RESULTS

Irradiated mixtures of T and Tyr (~25% conversion) were lyophilized and analyzed by GC–MS after trimethylsilylation. Figure 1 shows a typical gas chromatogram. The assignment of the peaks was done by MS and is listed in Table I. Mass spectra were interpreted on the basis of typical fragmentation patterns of trimethylsilyl (Me_3Si) derivatives of DNA pyrimidine bases (White et al., 1972) and of amino acids (Leimer et al., 1977). Peaks 1 and 10 correspond to Me_3Si derivatives of T and Tyr, respectively. Peaks 2 to 9 represent the Me_3Si derivatives of radiation-induced monomeric products of T as reported previously (Dizdaroglu & Simic, 1984). Peaks 11 and 12 correspond to 2- and 3-hydroxytyrosines, which are the radiation-induced products of tyrosine (Karam et al., 1984). Peaks designated by 13 and those designated by 20 represent Me_3Si derivatives of radiation-induced dimers of T (Dizdaroglu & Simic, 1984) and Tyr (Karam et al., 1984), respectively. Peaks 14–19 were attributed to Me_3Si derivatives of radiation-induced cross-links between T and Tyr. Essentially identical mass spectra were obtained from peaks 14, 15, 17, and 19 with characteristic fragment ions at *m/z* 755 ($M^{+\cdot}$), 740 ($M^{+\cdot} - CH_3$), 638 ($M^{+\cdot} - CO_2Me_3Si$), 538 ($M^{+\cdot} - 218 + H$), 218, 147, and 73. The assigned structure representing several isomers along with fragmentation patterns is



The mass spectrum taken from peak 16 had characteristic ions at *m/z* 665 ($M^{+\cdot}$), 650 ($M^{+\cdot} - CH_3$), 622 ($M^{+\cdot} - CH_3 - CO$), 548 ($M^{+\cdot} - CO_2Me_3Si$), 448 ($M^{+\cdot} - 218 + H$; base peak), 218, 147, and 73. This compound was apparently formed by loss of a H_2O molecule from some isomers of the compound, whose Me_3Si derivative is shown above, because

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[1] Abbreviations: dR, 2-deoxyribose; T, thymine; Tyr, tyrosine; GC–MS, gas chromatography–mass spectrometry; Me_3Si, trimethylsilyl; M^+, molecular ion; M_r, molecular weight; dT, thymidine; pdT, thymidine 5′-monophosphate; *k*, reaction rate constant; kPa, kilopascal; BSTFA, bis(trimethylsilyl)trifluoroacetamide; Gy, Gray.

[2] Certain commercial equipment or materials are identified in this paper in order to adequately specify the experimental procedure. Such identification does not imply recommendation or endorsement by the National Bureau of Standards, nor does it imply that the materials or equipment identified is necessarily the best available for the purpose.

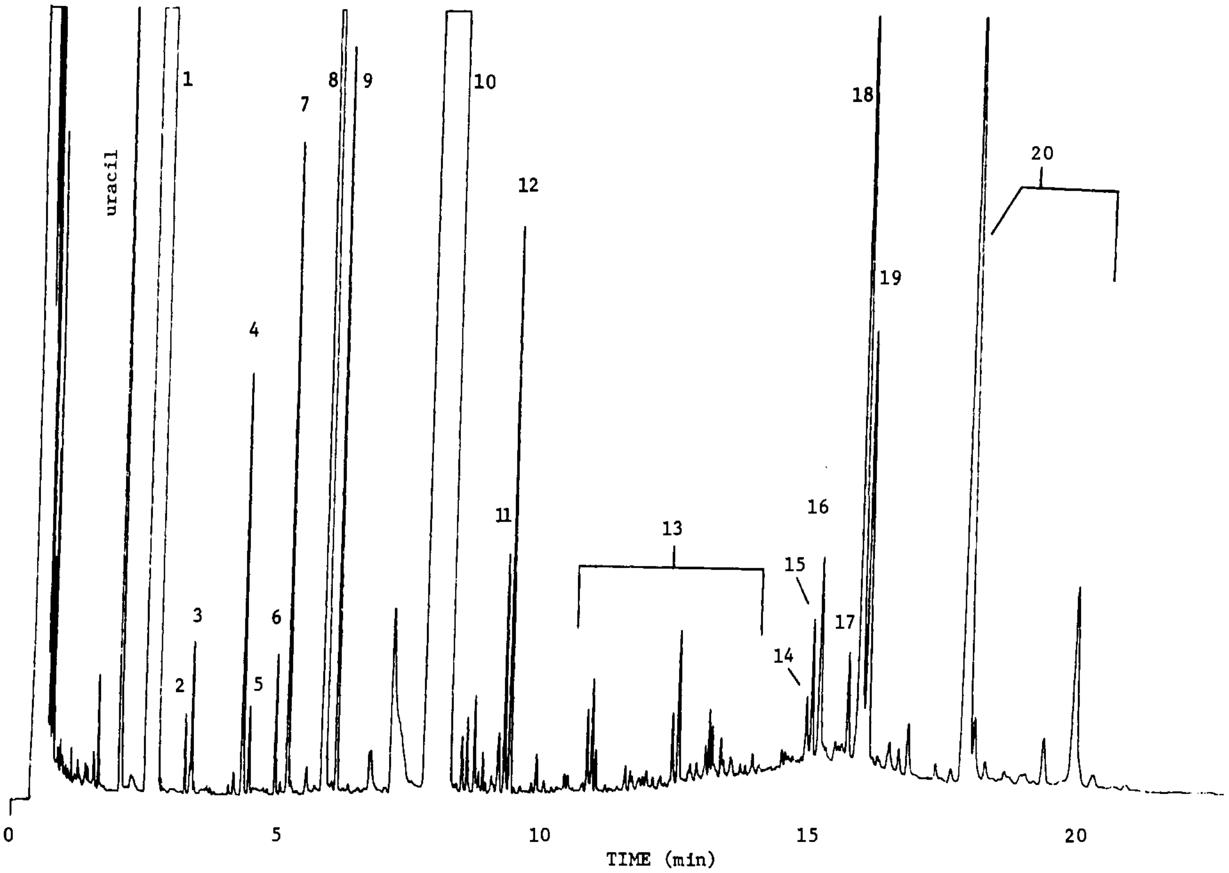
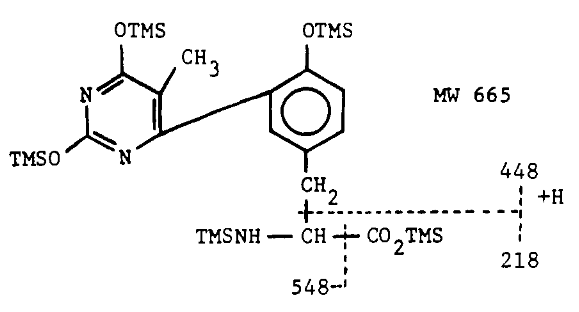


FIGURE 1: Gas chromatogram obtained from a γ-irradiated mixture of thymine and tyrosine after trimethylsilylation. Column, fused silica capillary coated with cross-linked SE-54 (12 m × 0.2-mm i.d.), was programmed at 10 °C/min from 100 to 250 °C. Column head pressure was 100 kPa. Split ratio was 20:1. Uracil was added to the sample as an internal standard before derivatization.

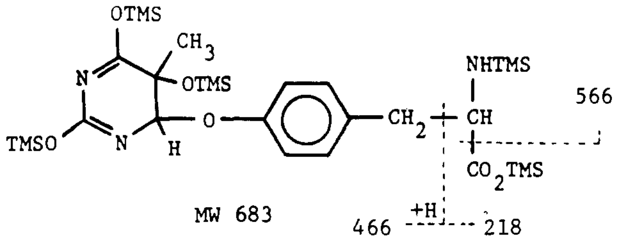
Table I

peak in Figure 1	product assignment	*G* value
1	thymine	
2	5,6-dihydrothymine	0.01
3	not detected	
4–6	5- or 6-hydroxy-5,6-dihydrothymine	0.1
7	5-(hydroxymethyl)uracil	0.1
8, 9	thymine glycol	1.1
10	tyrosine	
11	2-hydroxytyrosine	0.05
12	3-hydroxytyrosine	0.1
13	thymine dimers	0.2
14–19	T–Tyr cross-links	1.0
20	tyrosine dimers	0.8

a difference of 90 amu ($HOMe_3Si$) exists between the ions $M^{+\cdot}$, $M^{+\cdot} - CH_3$, $M^{+\cdot} - CO_2Me_3Si$, and $M^{+\cdot} - 218 + H$ in both mass spectra. It is not known at what stage the H_2O elimination takes place or whether it is induced by trimethylsilylation. The assigned structure representing a number of isomers is



Peak 18 in Figure 1 gave a mass spectrum with characteristic ions at *m/z* 668 ($M^{+\cdot} - CH_3$), 640 ($M^{+\cdot} - CH_3 - CO$), 566 ($M^{+\cdot} - CO_2Me_3Si$), 466 ($M^{+\cdot} - 218 + H$), 218 (base peak), 147, and 73. It was attributed to the following compound (other isomers are also possible):



dT and pdT were also used instead of T to find out whether radiation-induced cross-linking between these compounds and Tyr also takes place. Samples of irradiated mixtures of Tyr with dT or pdT were hydrolyzed with HCl to remove the sugar or sugar–phosphate moieties and then analyzed by GC–MS after trimethylsilylation. As a control, a sample of irradiated mixture of tyrosine and thymine was also treated with HCl and then analyzed by GC–MS. In all instances, similar gas chromatograms were obtained. All the products described above were observed in these systems as well.

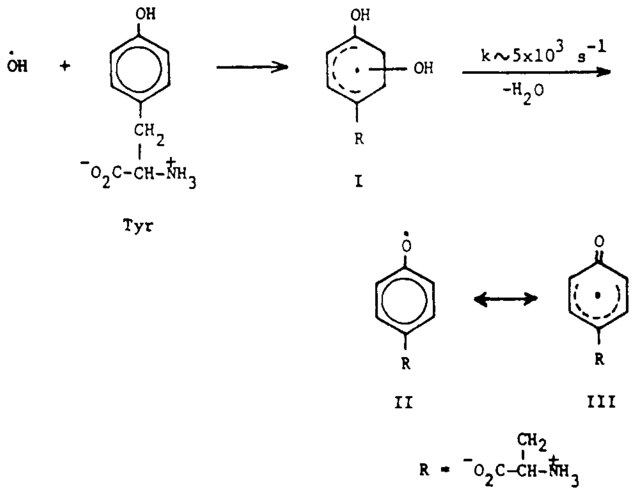
Quantitative determination of the radiation-induced products was also undertaken by GC with uracil as an internal standard. Their *G* values (number of molecules formed per 100 eV of radiation energy) are listed in Table I. The yields of the products of cross-linking in dT–Tyr or pdT–Tyr systems were comparable with the yields of these products in the T–Tyr system within a limit of ±10%.

DISCUSSION

OH radical reacts with T predominantly by addition to the C(5)=C(6) double bond, with a preference for addition at

C(5), giving rise to ·T–OH radicals (Fujita & Steenken, 1981). For practical reasons, the small fraction (a few percent) of T radical on the methyl group will be neglected. When T is the only reactant, a variety of dimeric products are observed (Dizdaroglu & Simic, 1984) in addition to a small number of monomeric products (Teoule & Cadet, 1978), where about half of OH radicals lead to formation of dimeric products.

Similarly, OH radical reacts with Tyr predominantly by addition to the benzene ring to give dihydroxycyclohexadienyl radicals (I) (Dorfman et al., 1962; Land & Ebert, 1967); these radicals are not stable and rapidly lose water to give a phenoxy radical (II), which is in resonance with radical III (Land & Ebert, 1967):



Radical I is responsible for the formation of 2- and 3-hydroxytyrosines via disproportionation (Karam et al., 1984). Radical I does not appear in the observed cross-linked products, and it apparently either does not dimerize or loses water after cross-linking occurs (Karam et al., 1984). Radicals II and III disproportionate and dimerize. The cross-linking is of two types. It takes place both through C–C bonds, (i.e., reaction of III + III) and also through C–O–C bonds, (i.e., reaction of II + III) (Karam et al., 1984). When both T and Tyr radicals are present simultaneously, the following cross-reactions between T and Tyr radicals take place as inferred from the isolated products:[3]

$$\cdot\text{T-OH} + \text{II} \rightarrow \text{HO-T-O-Phe}$$

$$\cdot\text{T-OH} + \text{III} \rightarrow \text{HO-T-Phe-OH}$$

The above reactions are evidently favored over the T + T and Tyr + Tyr radical reactions, since the products via hetero-cross-links are predominant. This, although unexpected, is corroborated by the reaction rate constant measured by pulse radiolysis. For the cross-reaction, the rate constant, $k = 4.8 \times 10^{8}\ \mathrm{M}^{-1}\ \mathrm{s}^{-1}$, is higher than the reaction rate constants for the homogeneous reactions of T radicals ($k = 3.2 \times 10^{8}\ \mathrm{M}^{-1}\ \mathrm{s}^{-1}$) and Tyr radicals ($k = 2.4 \times 10^{8}\ \mathrm{M}^{-1}\ \mathrm{s}^{-1}$) (E. L. P. Hunter and M. G. Simic, unpublished results).

Although radiation-induced cross-linking mechanisms in cells are not understood at present and the actual DNA and protein components that cross-link are not known, the facility with which the cross-links take place for some components, as demonstrated here, suggests a possibility of occurrence. It is possible that this type of radiation damage to DNA is more difficult to repair and that the slow component of repair (Blakely et al., 1982) is in fact associated with the elimination of the cross-linked protein by proteolytic enzymes in the first step, before DNA repair enzymes can act.

This and many other questions such as the role of cross-links in radiation-induced lethality, mutagenicity, and carcinogenicity have yet to be answered. It is hoped that the approach presented in this paper will lead toward solution of these problems.

Registry No. dT, 50-89-5; pdT, 365-07-1; thymine, 65-71-4; tyrosine, 60-18-4.

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[3] Tyrosine is depicted as hydroxyphenylalanine (Phe-OH).