# THE EXCITED-STATE PRECURSOR OF THE THYMINE DIMER

by

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We wish to describe two experiments designed to help identify the excited electronic state which leads to the thymine dimer ($\widehat{TT}$) in DNA.

The first experiment is an attempt to quench dimerization in thymidylyl-(3′-5′)-thymidine (TpT) solutions by the use of triplet state quenchers. Since no quenching is observed, one may conclude that either (1) the dimerization proceed via the excited singlet state, or (2) the dimerization proceeds via a triplet state with minimum reaction rate of $4 \times 10^9$ sec$^{-1}$. The second experiment is a comparison of the fluorescence emission from dimethyl thymine (DMT) under two circumstances In one the DMT molecules are isolated from each other and in the other they are in the correct relative orientation to form DMT dimers efficiently. The results support a model in which dimerization is a fast process compared to fluorescence emission when an excited pyrimidine molecule has a pyrimidine neighbor in a position favorable for dimer formation.

Thymine dimers are the most abundant photoproducts produced in DNA and in thymine containing polynucleotides and dinucleotides and as such have been studied extensively (Setlow, 1966 and Johns, et al., 1964). Their role in the biological consequences of ultraviolet light damage to DNA has been demonstrated in certain systems (Setlow, 1966). Thymine dimers of several different structures have been isolated (Weinblum and Johns, 1966). It has been shown that the dimer formed in frozen aqueous solutions of thymine, the so-called "ice dimer", has the *cis*-head-to-head structure (Weinblum and Johns, 1966 and Blackburn and Davies, 1965, 1966A and 1966B) and that this isomer is the most abundant one formed upon irradiation of room temperature solutions of TpT (Johns, et al., 1964) and DNA (Blackburn and Davies, 1965, 1966A and 1966B and Weinblum, 1967).

It has been shown (Greenstock, et al., 1967) that the dimers formed during irradiation of aqueous solutions of thymine are quenched by oxygen which implicates a triplet precursor. Lamola and Mittal (1966) have demonstrated a triplet state intermediate for thymine dimerization in acetonitrile solutions. These results are not unexpected for dimerization of monomers in dilute solutions since, even if the rate of the dimer-forming step is diffusion-controlled, a long lived ($10^{-6}$ sec) excited state is needed to permit two molecules, one of which is excited, to approach each other. The very short ($10^{-11}$ sec) lifetime of the monomer singlets at room temperature makes them therefore unlikely precursor states for the dimer formed in monomeric solutions.

Recently, Lamola and Yamane (1967) have shown that thymine dimers can be created in DNA by triplet state sensitization. This result must be carefully distinguished from one implicating the thymine triplet state as the precursor to the dimer following the direct excitation of thymine in its first absorption band. Eisinger and Shulman (1967) have in fact argued that in frozen aqueous thymine solutions the singlet state is the most likely precursor of the dimer and that this conclusion may be valid for other systems where thymine molecules are in close proximity to each other, such as dinucleotides and polynucleotides.

## METHODS AND MATERIALS

The TpT was kindly given to us by Professor Khorana. The cis-head-to-head dimer of dimethylthymine was prepared from the thymine dimer by the method described by Wulff and Fraenkel (1961). Isoprene and 2,4-hexadienol were purchased from Matheson, Coleman, and Bell and were distilled prior to use. Unbuffered mixtures of water and reagent ethanol were used for solvents in the TpT experiments. The irradiations were carried out with the samples in thin-walled synthetic quartz tubes (1.5 mm I.D.) which were filled to a height of 2 cm and sealed off in air. Irradiations and fluorescence measurements were accomplished in a variable temperature fluorospectroscope which has been described (Eisinger, et al., 1967). Absorption measurements used a Bausch and Lomb 500 spectrophotometer which had been provided with special sample holders so that the sealed sample tubes could be centered in the beam. The reproducibility of these measurements was better than 1 percent.

## RESULTS AND DISCUSSION

### 1. Quenching of Thymine Dimerization in TpT

We measured the photoreaction rate of TpT at room temperature in solution in the presence of two triplet state quenchers by monitoring the decrease in the 266 nm absorption peak of thymine. The quenchers used were 2,4-hexadienol (HDE) and isoprene (ISO) whose spectroscopic triplet states lie at 20,800 $cm^{-1}$ and 21,000 $cm^{-1}$ respectively (Lamola and Mittal, 1966). The triplet state of thymine lies much higher, at 26,300 $cm^{-1}$ (Lamola, et al., 1967). On the other hand, these dimers absorb to the blue of thymine.

It has been shown (Johns, et al., 1964 and Weinblum and Johns, 1966) that four products are obtained upon irradiation of TpT in water and that if the irradiation wavelength is 280 nm the cis-head-to-head dimer accounts for 80% of the product at conversions up to 40%. They report a dimerization quantum yield of 0.02. Our solvents were chosen to accommodate the limited water solubility of the quenchers. We used 5% ethanol and 50% ethanol solutions for the HDE and ISO respectively. The rate of loss of TpT in the first solvent was virtually identical to that found in water but was reduced to 45% in the latter. We have no reason to doubt that dimerization within the TpT is the main photochemical event in our solvents but we will perform product analyses in the near future to ensure this point.

The TpT samples were irradiated with and without quenchers at a wavelength of 280 nm. HDE was used at 0.005 M, the highest concentration which did not compete for 280 nm light. ISO was used at 0.07 M, about half the solubility in 50% ethanol. The results for ISO (see Fig. 1) show that ISO has a quenching efficiency of less than 10% and that of HDE was found to be no greater.

The results show that the dimerization of TpT occurs either (1) via the singlet level or (2) via the triplet level in a time short compared to that required to quench the triplet

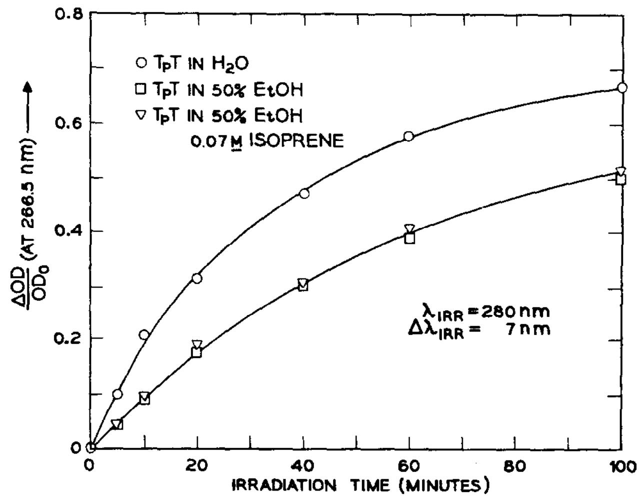

Figure 1 - The fractional decrease in absorptivity at 266 nm of TpT solutions irradiated in the presence and absence of ISO which is capable of quenching the thymine triplet state. No quenching of the thymine dimerization is observed.

level by means of quenchers. If this quenching rate is $k_Q$ and the dimerization via the triplet level occurs at a rate $^3k_{T\widehat{T}}$ in the presence of quenchers at a concentration Q, $^3k_{T\widehat{T}} \geq 10\ k_Q\ [Q]$. The bimolecular quenching rate is determined by the diffusion rate so that $k_Q$ is about $6\times10^9\ \underline{M}^{-1}\text{sec}^{-1}$ for 50% ethanol from the Debye equation. Since $[Q] = 0.07\ \underline{M}$, we conclude that $^3k_{T\widehat{T}} \gtrsim 4\times10^9\ \text{sec}^{-1}$.

This lower limit for the dimerization rate via the triplet level is not large enough to eliminate the triplet route but does lead us to favor a mechanism involving the singlet level. The upper limit for the lifetime of the thymine singlet state in water at room temperature is $10^{-11}$ sec since no fluorescence is detected under these conditions. Thus for a quantum yield of 0.02 the dimerization rate via the singlet level $^1k_{T\widehat{T}} \geq 2\times10^9\ \text{sec}^{-1}$ This is also very fast. However, singlet excimers have been observed in many dinucleotides at low temperature (Eisinger, et al., 1966). Such species could serve as intermediates between the excited singlet state of thymine and the dimer since the excimer is well along the way to the dimer, and excimer formation can compete successfully with monomer fluorescence. Triplet excimers, on the other hand, have not been observed.

How can this model be proved or disproved conclusively? One approach is to look for other more soluble triplet quenchers in order to further raise the lower limit of $^3k_{T\widehat{T}}$ . But since it is difficult to give a firm theoretical estimate for values of $^3k_{T\widehat{T}}$ which could be considered unreasonably high, conclusive evidence for a singlet state precursor should be sought in a different manner. One other possibility which we are pursuing is to repeat the foregoing experiment with radioactive TpT which will make it possible to measure chromatographically the effect of the triplet quencher on all four products rather than the ice dimer only, which is responsible for nearly all the absorption changes. If it should turn out that one of the "minor" products is quenched by a triplet quencher, one may conclude that all the products which are not, have an excited singlet state as a precursor.

### 2. Splitting and Reformation of a DMT Dimer at Low Temperature

We suggested above that when thymine molecules are in a suitable relative orientation with respect to each other to form the cis-head-to-head dimer this photoreaction will occur in a time which is short compared to the singlet lifetime. In support of this model we offer the following experiment (c.f. Chandross et al, 1966)

The cis-head-to-head dimer of DMT was dissolved in 50% ethylene glycol-water solutions and the samples were cooled to 80°K. The fluorescence spectrum was then recorded with the sample excited at 265 nm. The observed emission was very weak and might be due to small amounts of DMT monomers or impurities in the sample (see Fig. 2b). The sample was then irradiated for 15 minutes in situ with 248 nm light to break the dimers after which its weak fluorescence spectrum was found to be unchanged. The sample was then melted and its absorption spectrum was measured at room temperature (Fig. 2c). This confirmed that some 50% of the DMT dimers had indeed been broken by the short wavelength irradiation (Setlow, 1966). The sample was then cooled to 80°K in the fluorimeter, this time it showed a strong fluorescence spectrum typical of DMT (Fig. 2d).

If the 248 nm irradiation was followed by a 15 minute irradiation at 280 nm, before the sample was allowed to melt, most of the dimers were remade and many fewer DMT monomers were found by absorption (following melting) or by emission (following melting and refreezing at 80°K).

These results are readily understood in terms of a

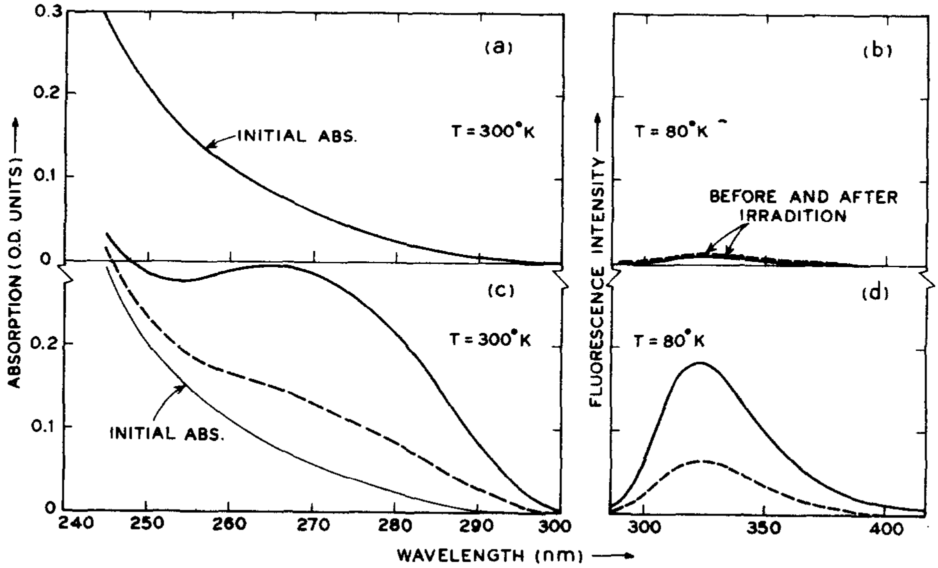

Figure 2 - Absorption and fluorescence results for DMT dimers dissolved in 50% ethylene glycol. (a) is the initial dimer absorption spectrum at R.T., (b) is the fluorescence at 80°K before and after irradiation at 248 nm. Between (b) and (c) the sample is melted and between (c) and (d) it is refrozen. The solid curves in (c) and (d) show the absorption and emission of DMT molecules. The dashed curves refer to a sample in which the 248 nm irradiation was followed by irradiation at 280 nm. This remade some 70% of the broken DMT dimers.

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model which makes dimerization the most likely photoreaction between DMT molecules which are correctly positioned to form dimers. This accounts for the absence of emission from excited DMT molecules which have DMT neighbors from which they had previously been separated. After melting, these DMT monomers diffuse apart and the isolated DMT molecules fluoresce with high quantum yield (about 10 percent). Irradiation with 280 nm light before allowing the monomers to diffuse apart reforms the previously broken dimers. This shows that the absence of fluorescence emission and the ability to form dimers are indeed connected. An excimer is a likely precursor to the dimer but its existence can only be conjectured since no emission from it is observable.

We are extending this study to all four dimers of thymine.

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