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Ultraviolet Irradiation

of

Nucleic Acids and Related Compounds

Final Progress Report

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Baltimore, Maryland

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A. Introduction

On November 1, 1974, Dr. Roy A. Jensen informed me of the decision to terminate my contract on the study of UV irradiation of nucleic acids and related compounds. Some twenty years ago, I undertook this study at Tufts Medical School in Boston. In 1961, I transferred to Baltimore to join the faculty of the Johns Hopkins University. With the help of Dr. James Liverman, I was able to continue this project without interruption despite the move. During this period, the U.S. Atomic Energy Commission, now ERDA, provided the financial support. Although I received a Career Development Award from NIH between 1961 to 1970, I did not seek any additional grant support from NIH despite a grant usually accompanied with the award during that time. Naturally, I am most appreciative for the confidence shown me by the present and past members of DBER. On the other hand, I trust that my accomplishments justify your past and possibly future support. We have witnessed the explosive development in this area from the interest of a few investigators to the involvement of multidisciplinary laboratories. I have been much involved during the entire span. Undoubtedly, the study of photochemistry and photobiology of nucleic acids has achieved its popularity in recent years. However, I believe that further research is essential to reach the acme. Inevitably, I hope our laboratory can get the proper financial support and can continue to bring about the necessary advancement. For this purpose, I wish to make a comment at the end of Section B.

B. Summary of Research Accomplishments

When a research project is progressing well, few enjoy being terminated and preparing a Final Progress Report. Nevertheless, it provides an opportunity to reflect on one's past achievements and to plan future experiments. Especially, these thoughts have passed through my mind

frequently in the last two or three years because I was the Editor of the 21-chapter, two-volume monograph on Photochemistry and Photobiology of Nucleic Acids (Appendix B).

It is generally assented that the isolation and identification of photoproducts provided the major breakthrough in the study of photochemistry and photobiology of nucleic acids. Until now, there are five types of photoproducts that have been characterized when nucleic acids are exposed to UV light at doses comparable to biological studies. A sixth type, which was first characterized by K.C. Smith and required much higher doses, is also of current interest in UV-radiation produced protein - nucleic acid cross-linkings. Because it was generally believed that products formed with high doses may have no biological significance, I did not engage in this phase of study. Otherwise, our laboratory is responsible for the characterizations or discoveries of all the five types of photoproducts of nucleic acids. The following are brief summaries of each. Two additional summaries will deal with the hypothesis of "Molecular aggregates — puddle formation" and the study of "topochemistry".

B.1. Pyrimidine Photohydrates

In the area of photochemistry and photobiology of nucleic acids my first publication was concerned with photohydration of pyrimidine derivatives and appeared in 1956. This publication together with a second one in this area drew the comments of Dr. Michael Kasha (a member of the National Academy of Science, U.S.) in his appraisal of the status of molecular photochemistry in that era. He wrote: "Photochemical kinetic studies constitue a major part of the research in photochemistry undertaken by physical chemists. Much of this research is on a stoichiometric level and may give little information

on the intramolecular electronic processes occurring, although the advent of flash spectroscopic techniques is rapidly changing the picture (e.g., Bridge and Porter, '58; Porter and Strachan, '58). On the other hand, the powerful and systematic methods of organic chemistry certainly have much to offer in the unravelling of the intricate molecular mechanisms of photochemical reactions (e.g. the studies on a uracil by Wang et al., '56; Wang, '58) --------". (Appendix B.1.a.). This event predated or may have led the tremendous expansion of photochemial studies among organic chemists. In these articles, I also proposed a zwitterion species for the intermediate and a "hot" ground state for such an intermediate based on our findings. Both aspects turned out to be controversial issues; however, most arguments rested on general principles of photochemistry rather than on our experimental data. After long and close examinations of this reaction, the opponents eventually became advocates. For instance, Summers, Enwall, Burr, and Letsinger in 1973 (Appendix B.1.b.) indicated that they supported our suggestion of a zwitterion intermediate. In addition, Dr. H.E. Johns is now in favor of a "hot" ground state intermediate leading to photohydration (Appendix B.1.c.). The acceptance of these new suppositions are of consequence in photochemistry in general and also in the biological importance of pyrimidine addition products in nucleic acids.

B.2. Pyrimidine Dimers

In the study of photochemistry and photobiology of nucleic acids, it is generally agreed that the major breakthrough is the discovery of thymine dimerization. Along with a Holland group, I was credited with this discovery as evidenced from the earliest review that dealt with thymine dimer by Deering in 1963 to the two most recent reviews by Patrick and by Harm (Appendix B.2.).

B.3. Thyminyl Radicals (Thy^{α})

The formation of pyrimidinyl radicals by UV irradiation of nucleic acids has interested us for quite some time. Recently, the structural identification study of the so-called "spore product" indicates that such radicals are indeed occurring in in vivo systems (Appendix B.3.).

B.4. Pyrimidine Coupled Product

Also in the mid-sixties, the formation of a coupled product by the irradiation of 5-bromouracil derivatives was discovered. This coupled product has now been identified as a photoproduct from polynucleotides containing bromouracil (submitted for publication). This work was done in collaboration with Drs. Ehrlich and Riley who showed that the nature of the UV photoproduct produced in these polynucleotides has properties in common with our coupled products (Appendix. B.4.). Hopefully, this finding provides the molecular mechanism responsible for the increased radiation sensitivity of DNA containing bromouracil.

B.5. Pyrimidine Adducts

In the mid-sixties, we discovered a new class of pyrimidine photoadducts that attracted great attention. Our work has been used as a model for other laboratories as seen from the remarks made by Dr. Nelson J. Leonard (a member of the Academy). Another member of the Academy, Dr. Bernhard Witkop designated these compounds as "Wang adducts" (Appendix B.5.).

B.6. "Molecular Aggregates — Puddle Formation" Hypothesis

The original discovery of pyrimidine photodimerization was made by the irradiation of pyrimidine in frozen aqueous solutions. In this connection, I proposed a "molecular aggregates — puddle formation" hypothesis relating reactions in frozen states. This hypothesis is in direct contrast to two

others proposed by two Nobel laureates, Szent Györgyi and Manfred Eigen. My hypothesis is now generally accepted and has been credited with leading to the development of a new area of research in chemistry as cited in a review (Appendix B.6.).

B.7. Topochemistry

In the course of our study of the mechanism of UV irradiation of frozen aqueous solutions, we discovered that upon freezing, the solute forms microcrystals interspersed among the ice crystals. Thus, the irradiation of compounds in frozen aqueous solutions should be similar to the irradiation in solid films. From the study of solid films, we observed some unexpected results that led us to suggest that the stereoarrangements of molecules in the crystals are the determining factor for the stereoconfigurations of the resultant photodimers. Conversely, the stereoconfiguration of a dimer could be used to predict the packings of the crystalline structures. This correlation allowed a Caltech group to predict the correct stereoisomer of a dimer (Appendix B.7.). This concept has been developed into topochemical studies of the structures of the dimers and of the crystalline arrangements by several laboratories.

B.8. Miscellaneous

Although our research objective has been directed toward understanding the photochemistry and photobiology of nucleic acids, at times the uncovering of other broader underlying principles of fundamental importance to chemistry in general and new methods of interest to chemists and biologists resulted as by-products. For instance, our proposed mechanisms of halogenation of pyrimidines and the dehalogenation of halogenated pyrimidines are two of current interest in reaction mechanisms. Similarly, the determinations of

base compositions of DNA by bromination and the enrichment of ^{18}O in the nucleic acid bases are techniques used by many. In addition, our studies led to the recognition of a novel electronic transition, i.e. $C\pi \rightarrow \pi^*$ transition, which should be of fundamental importance.

B.9. Comments

To submit a Final Progress Report under the present circumstances is indeed anticlimatic. At present, our activity and interest in this area remain high and our group consists of visiting scientists who were trained in this area from many laboratories. This kind of teamwork is most productive and should be encouraged by the Administration. Therefore, I hope that the members of DBER will fund our project that was approved several months ago.

C. Training of Personnel

C.1. Doctoral Students

	Present Position
John C. Nnadi	Manager - Special Products Mobil Oil Co., stationed in Nigeria

C.2. Postdoctoral Students

	Ph.D. Source	Present Position
G. Sherman	Boston U.	Director of Laboratories Mt. Auburn Hospital, Cambr., Mass.
A.J. Varghese	U. of Maryland	Faculty University of Toronto
M.H. Patrick	U. of Chicago	Associate Professor University of Texas at Dallas
A. Rafi	U. of Newcastle	Scientific Staff Baltimore Gas & Electric Co.
K.C. Padmanabhan	Eidgenössische Tech Hochschule	Member Hopkins Hospital Laboratories
D.F. Rhoades	U. of Washington	Faculty University of Washington
O.C. Zafiriou	Johns Hopkins	Senior Chemist Woods Hole Laboratory
W.H. Huang	Colorado State	Associate Cornell University
W. Hauswirth	Oregon State U.	Fellow Johns Hopkins Medical School
R. Drisko	Johns Hopkins	Assistant Professor Essex Community College
B.S. Hahn	University of Saskatchewan	Assistant Professor Johns Hopkins University
B.R. Toth	Johns Hopkins	Associate North Dakota University
S. Sasson	Weizmann Inst.	Faculty Weizmann Institute
H. Taguchi	Australian Nat.U.	Associate Harvard University
J. McKee	U. of Maryland	Associate Johns Hopkins University
K. Acholonu	U. of Pacific	Lecturer Howard University
A. Gupta	New York U.	Fellow Johns Hopkins University
H.S. Ryang	Osaka U.	Fellow Johns Hopkins University

C.3. Senior Scientists on Leave

R. Alcantara	Assistant Professor	Instituto Poletecnico Nacional (currently Professor)
H. Ishihara	Assistant Professor	Nagoya City University (currently Professor)
M.N. Khattak	Staff Scientist	Pakistan Atomic Energy Commission (currently Staff Scientist, REAS, Baltimore)
B. Czochralska	Lecturer	University of Warsaw (currently Lecturer)
J. Cadet	Staff Scientist	Centre D'etudes Nucleaires de Grenoble

D. List of Publications (meeting abstracts excluded)*

S. Sasson, S.Y. Wang, and M. Ehrlich, "5,5'-Diuridinyl, A Major Photoproduct from UV- Irradiation of Polynucleotides containing Bromouracil" submitted for publication (1976).

B. Czochralska, B.S. Hahn, and S.Y. Wang, "Electroreduction of Biologically Important Pyrimidine Photoadducts" submitted for publication (1976).

B.S. Hahn, and S.Y. Wang, "The Preparation of trans-Pyrimidine Glycols by near UV Irradiation" submitted for publication (1976).

W.W. Hauswirth, and S.Y. Wang, "Cytidine-C(5)-Photoexchange: A Photokinetic Analysis" submitted for publication (1976).

W.W. Hauswirth, and S.Y. Wang, "Excited State Processes and Solution Conformation of Dipyrimidine Adducts" submitted for publication (1976).

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*This list contains only those publications pertaining to photochemistry and photobiology of nucleic acids.

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Photochemistry and Photobiology of Nucleic Acids

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Michael Kasha in "Comparative Effects of Radiation", eds. M. Burton, J.S. Kirby-Smith and J.L. Magee (1960).

chemists. Much of this research is on a stoichiometric level and may give little information on the intramolecular electronic processes occurring, although the advent of flash spectroscopic techniques is rapidly changing the picture (e.g., Bridge and Porter '58; Porter and Strachan '58). On the other hand, the powerful and systematic methods of organic chemistry certainly have much to offer in the unraveling of the intricate molecular mechanism of photochemical reactions (e.g. the studies on a uracil by Wang et al, '56; Wang '58). Unfortunately, however, spectroscopic theory has not been applied often in the elucidation of such mechanisms, partly because its own nomenclature details have served to make it somewhat inscrutable, but largely because of actual limitation in the development of the theory. However, the last decade has brought great extensions in the understanding of molecular electronic spectra, so it is worthwhile to inquire if any connections between theory and experiment can now be made.

5.1. THE PHOTOCHEMICAL LAWS

5.1.1. Zeroth Law:* The Activation Principle. - Grotthus and Draper, over a century ago, established the principle which may be called the photochemical activation principle: Only the light that is absorbed by a molecule is effective in producing a photochemical change. In earlier times, when the distinction between scattering phenomena and quantum transitions was unclear, this principle had a central role in the interpretation of photochemical reactions. In the perspective of the quantum age, the utility of this principle has diminished. It seems appropriate to recognize the activation principle as a zeroth law -- a now self-evident starting point for any photochemical interpretation.

* Editors' note: This law has been sometimes called the first law of photochemistry (cf. G. K. Rollefson and M. Burton, Photochemistry and the Mechanism of Chemical Reactions, Prentice-Hall, Inc., New York 1939).

Photochemistry and Photobiology, 1973, Vol. 17, pp. 295–301. Pergamon Press. Printed in Great Britain

THE PHOTOADDITION OF NUCLEOPHILES TO URACIL

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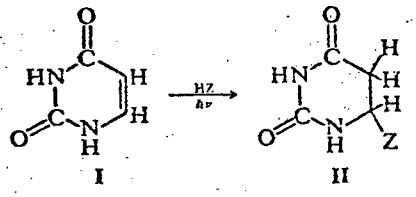
(*Received* 31 *July* 1972; *accepted* 8 *December* 1972)

Abstract—Quantum yields for 254 nm ultraviolet photoaddition of the nucleophiles hydrazine, HCN, HSO_3^-, methyl amine, and BH_4^- to uracil have been measured; the quantum yields for hydrazine, HCN, and HSO_3^- additions are pH-dependent. The nucleophiles sulfide, azide, chloride, bromide, iodide, nitrite and thiocyanate failed to photo-add under similar conditions. These reactions are interpreted as 1,4-additions to the conjugated enone system of the anti-aromatic compound, uracil; as suggested by S. Y. Wang (Wang and Nnadi, 1968). The nuclear magnetic resonance (NMR) spectrum of the photohydrate of uracil-5-d showed that the proton was added to the 5-position in a stereochemically random manner. The photoaddition of HSO_3^- takes place at much lower concentrations than required for the thermal addition of this anion and is also stereochemically random.

INTRODUCTION

THE photoinduced addition of water to the 5,6-double bond of uracil has been considered to involve the nucleophilic attack of water on the relatively positive 6-carbon of an excited-state uracil molecule, I. (Burr, 1968; Smith and Hanawalt, 1969; Burr *et al.*, 1968; Burr and Park, 1968a; Wang and Nnadi, 1968; Burr *et al.*, 1972; Summers and Burr, 1973). Since a test of this hypothesis lies in the generality of the reaction, we now report the photochemical reactions of a number of other nucleophiles with the 5,6-double bond of uracil, producing compounds of type II. In addition, evidence about the stereochemistry of water and bisulfite addition could be obtained by examination



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of the nuclear magnetic resonance (NMR) spectra of the products. Accordingly, we now report the results of such a study on the photohydrate of 1-ethyluracil-5-d and on the product of photoadditions of HSO_3^- to uracil.

EXPERIMENTAL

Uracil and 1-ethyluracil were supplied by Cyclo Chemical Co. and used without further purification. The water was doubly distilled from a glass apparatus.

Irradiations of uracil with the nucleophile in a series of buffered aqueous solutions

*Present address: Ash Stevens, Inc., Detroit Research Park, 5861 John C. Lodge Freeway, Detroit, Michigan 48202.

Photochemistry and Photobiology of Nucleic Acids, Vol. 1,
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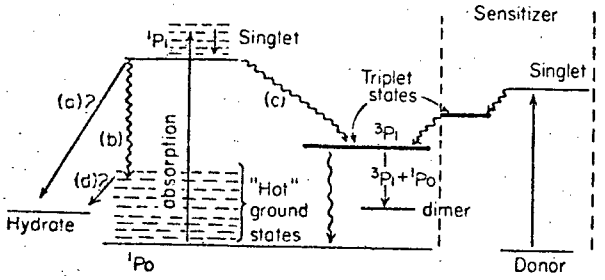


Fig. 7. *Energy level scheme for the pyrimidines indicating the precursor states responsible for dimers and hydrates.*

excited to its triplet. When this is done triplet states are produced without any involvement of the Pyr singlet. Under these circumstances, Greenstock and Johns (1968) showed that no hydrates result. *Hence, hydrates do not arise from the triplet state.* [The 6-azapyrimidines are exceptions to this rule (Kittler and Löber, 1969; Kittler, 1972); however, they are not true pyrimidines.] This leaves paths a and d as possible precursors for the hydrate, along with such suggestions as an alternate singlet-state or an excited-state tautomer (for a discussion, see Summers and Burr, 1972).

EVIDENCE FOR AND AGAINST THE HOT GROUND STATE. We first consider the dependence of hydration on pH, illustrated in Fig. 5 which shows that hydrates are formed by interactions of water with a neutral Pyr. For example, with Cyt and its derivatives, the hydrate yield increases markedly above pH 4 when the base becomes uncharged in its ground state. The simplest explanation is that hydration occurs from a hot uncharged ground state.

For Ura which is neutral up to pH 9.3 in the ground state (Shugar and Fox, 1952), a sudden reduction in hydrate yield occurs at about pH 4. Fluorescence data, summarized by Burr *et al.* (1972), indicate that this is the pH at which the excited state becomes negatively charged. The simplest explanation in this case is that hydration of Ura takes place mainly from an uncharged excited singlet state. However, the involvement of an uncharged hot ground state cannot be ruled out. For example, if a neutral Ura molecule at pH 7 were excited, it would quickly deprotonate to yield a negatively charged singlet. If this molecule underwent internal conversion in less time than that required to regain a proton by diffusion, it would then be uncharged in a hot ground state and so available for hydration. For Ura the pH evidence alone cannot allow us to choose between the two models, pathways a and d of Fig. 7. Therefore, other lines of evidence must be considered.

Table 2 Molecular Orbital Calculations and Photohydration

Compound	ϕ_H	Polarization of 5,6-bond	
		$^1P_0$	$^1P_1$
Thy	10^{-5}	0.052	0.158
Ura	10^{-2}	−0.122	−0.193
Cyt	10^{-2}	−0.189	0.083

Molecular orbital calculations have been carried out by Danilov (1967) and Malrieu (1967), and some of their results are reproduced in Table 2. The polarization of the 5,6-bond is defined as the difference between the calculated charge densities on C(5) and C(6). A positive value indicates that C(6) is relatively negative and, therefore, that it has a lower probability for nucleophilic addition of a water molecule. Hence a positive polarization would be expected to lead to a low hydrate yield. The values for ϕ_H are approximate values for the neutral molecules taken from Table 1. The yield of hydrates correlates with the polarization of the 5,6-bond for the electronic ground state much better than with the values for the first excited singlet, $^1P_1$.

Whitten *et al.* (1970) reported that the quenching of fluorescence, and thus of the electronic singlet, of Me_2Ura by a variety of nucleophiles, including water, does not correlate well with the rate of photochemical addition to the Pyr. This suggests that the addition is not to the fluorescent singlet state but rather to a vibrationally excited ground state. Wang and Nnadi (1968) and Wang *et al.* (1968, 1970) also interpreted substituent, pH, and isotope effects in terms of a hot ground state intermediate.

The nucleophilic attack of bisulfite, HSO_3^-, on Ura and Cyt derivatives to give the 6-sulfite product analogous to the hydrate has been reported (Shapiro *et al.*, 1970; Hayatsu *et al.*, 1970). Since this reaction is a thermal addition which cannot involve the electronically excited singlet state, it provides yet another suggestion that hydration may occur via a hot ground state.

The thermal deamination of Cyd at 95°C may also involve the formation of a hydrate intermediate with a saturated 5,6-bond which deaminates and then rapidly eliminates water (Wechter and Kelly, 1970). Such a thermal hydrate could be formed only from a vibrationally excited ground state, since the electronic singlet is not produced by heating.

Although some evidence can be interpreted as showing that Pyr photohydrates are formed from the electronically excited singlet state,

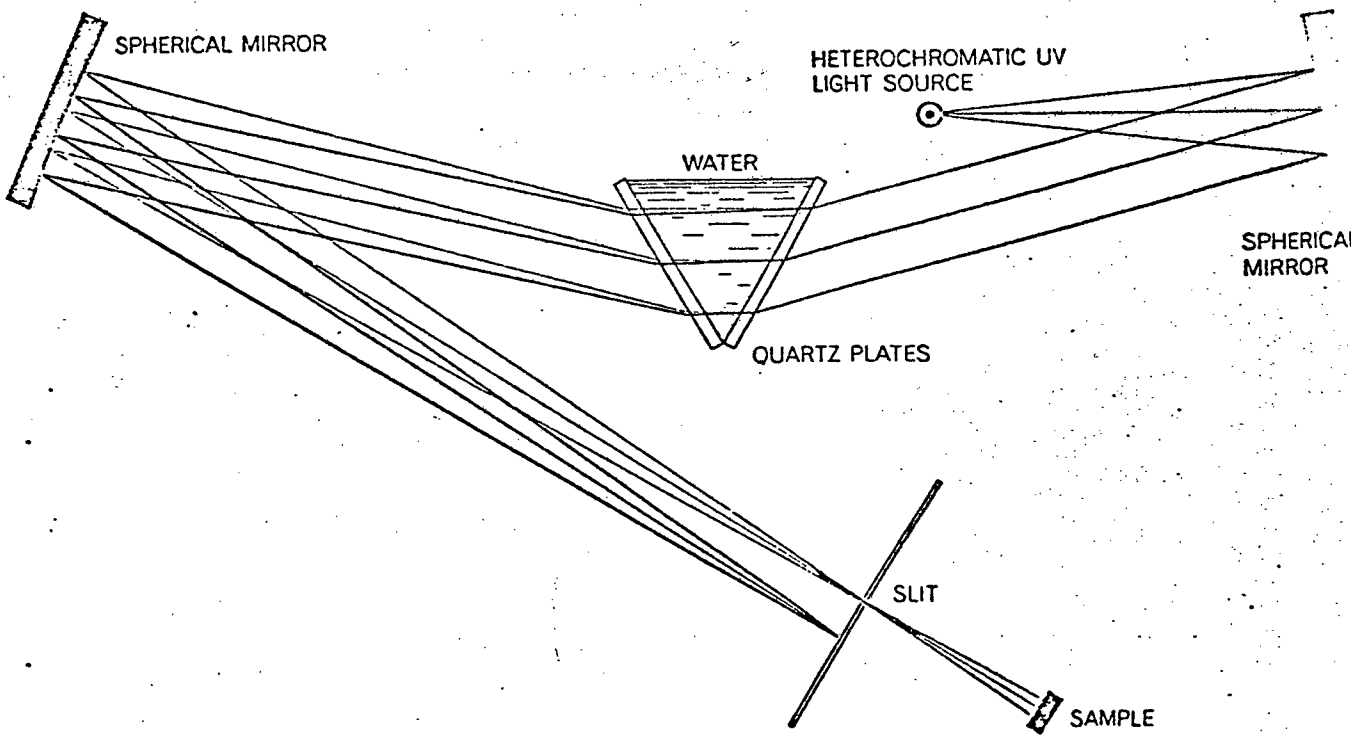
of substances called pyrimidines, are far more sensitive to ultraviolet than are adenine and guanine, which are purines. About one in every 100 quanta of ultraviolet energy absorbed by pyrimidines alters the molecules; for purines the ratio is one in 10,000. (In general only a few of the quanta absorbed by a molecule will be effective in producing permanent changes.) The search was therefore narrowed to the pyrimidines.

The first effect to be discovered was that ultraviolet acts on cytosine molecules or the cytosine units of DNA in water solution, adding a water molecule across a double bond [*see middle illustration on page 6*]. Heating the altered cytosine, even to the temperatures required for biological growth, or acidifying it, partly reverses the reaction. Therefore the hydration of cytosine did not seem likely to be of major biological importance.

For some years, however, this hydration was the only sensitive, ultraviolet-induced change in the bases that could be detected. Heavy doses of radiation did produce complex rearrangements, but these doses were far in excess of the smallest ones known to have biological effects. About three years ago a breakthrough in the photochemistry of DNA came when R. Beukers, J. Ijlstra and W. Berends of the Technological University of Delft in Holland and Shih Yi Wang, now of Johns Hopkins University, discovered that although in a liquid solution thymine is not particularly sensitive to ultraviolet, in a concentrated, frozen aqueous solution it is extremely sensitive. It developed that irradiation of the frozen solution causes thymine molecules to combine and form two-molecule chains, or dimers. As in the case of the cytosine conversion, a double bond changes to a single, and new bonds between carbon atoms link the two thymines [*see bottom illustration on page 6*]. Unlike the altered cytosine, the thymine dimer is stable to heat and acid. But when the solution is melted, irradiation can convert the dimer back into the two original thymine molecules. What the freezing does is to hold the thymines close together in a crystalline or semicrystalline configuration, making it possible for the dimer bonds to form between two neighboring thymines when they absorb ultraviolet. It seemed likely that such a conversion would also occur in DNA, where thymine units are sometimes adjacent to each other on a helical strand and are held in relatively fixed positions. In 1960 Adolf Wacker and his associates at the University of Frankfort found thymine dimers in DNA extracted from irradiated bacteria.

In order to get more complete information on the formation and splitting of thymine dimers in polymer chains such as DNA, Richard B. Setlow and I carried out experiments on some model polymers at the Oak Ridge National Laboratory. Similar experiments were performed independently at the California Institute of Technology by Harold Johns and his collaborators. The compounds we used were short polymers—in effect short single strands of DNA in which all the bases were thymine. Some of our test molecules contained only two backbone units and two thymines; others had 12 or more. Since the sugar-phosphate backbone holds the thymines in fairly close proximity, we anticipated that ultraviolet radiation should form dimers between adjacent thymines in a chain even in a liquid solution. And we expected that once the dimers had formed they would be subject to breakage by ultraviolet, as were the isolated thymine dimers. When thymine loses a double bond in changing to a dimer, it also loses its ability to absorb light at 2,600 angstroms. Therefore measuring the change in 2,600-angstrom absorption gives an indication of the ratio between thymine monomers and thymine dimers in the solution.

When we irradiated our polymers, dimers were in fact produced. Since the rate of formation did not vary with thymine concentration, we concluded



MONOCHROMATOR provides ultraviolet light of a single wavelength for experiments. Light of mixed wavelengths is rendered parallel by a spherical mirror and passes through a quartz-and-water prism. (Glass would not transmit the desired wavelengths.) The prism splits the light into many components of different wavelengths, only two of which are indicated here, and the beams are refocused by a second mirror. The sample to be irradiated is positioned behind a slit that excludes all but the desired wavelength.

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D. Properties of Photoproducts Formed in DNA

1. Cyclobutyl Dipyrimidines (Pyr<>Pyr)

a. Isolation and Purification

i. THY<>THY. It is generally agreed that the most significant breakthrough in DNA photochemistry was the isolation and identification of Thy<>Thy from irradiated frozen solutions of Thy (Beukers and Berends, 1960, 1961; Wang, 1960, 1961). Evidence for Thy<>Thy in irradiated DNA was initially obtained by Beukers *et al.* (1960) and substantiated by Wacker *et al.* (1960). In these, and in subsequent studies by others, identity of the photoproduct rested on chromatographic evidence and its similarity to the chemical and spectroscopic properties of the product obtained from irradiated frozen solutions of Thy. The first chemical evidence for the structure of Thy<>Thy derived from DNA was reported by Blackburn and Davies (1966, 1967). These authors showed that the major product obtained from chromatograms of acid hydrolyzed, irradiated DNA ([^{3}H]Thy from *E. coli*), when mixed with Thy<>Thy(c,s) from irradiated frozen solutions of Thy, co-chromatographs with the latter and undergoes no significant change in specific activity after repeated recrystallizations. The identity of the two photoproducts was confirmed by treatment with sodium hydroxide and bromine; under these conditions the Thy<>Thy(c,s) rearranges to a triazatricyclodecane derivative, a reaction sterically unfavorable for the other three geometrical isomers. The only drawback to this proof is that both Thy(6-5)Pyo and Thy<>Thy(c,s) are formed in irradiated DNA, and these photoproducts are chromatographically indistinguishable and co-crystallize under the conditions employed. Shortly thereafter, however, pure Thy<>Thy was isolated in milligram quantities from irradiated DNA and was shown to give the UV, IR, and NMR spectra identical to those of the Thy<>Thy(c,s) made from irradiated frozen solutions of Thy (Varghese and Wang, 1967a; Weinblum, 1967).

ii. CYT-CONTAINING DIMERS. Cyclobutyl dimerization was shown by Setlow and Carrier (1966) to extend to pyrimidines in general. As noted earlier, these dimers undergo easy deamination to form Ura<>Ura and Ura<>Thy from Cyt<>Cyt and Cyt<>Thy, respectively. The crystal structure of these dimers isolated from DNA has yet to be determined. However, Weinblum (1967) showed that Ura<>Thy isolated from DNA and Ura<>Thy obtained from irradiated frozen solutions of a Thy and Ura mixture have identical infrared spectra. On the basis of

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DNA or any other DNA) is added to a reaction mixture consisting of enzyme extract and irradiated transforming DNA, the PR reaction rate decreases. However, no such slowdown is observed when the irradiated unspecific DNA has been previously photoreactivated. Evidently the nonspecific UV-irradiated DNA molecules compete with the irradiated transforming DNA for PRE. Increasing concentration and UV exposure of the nonspecific DNA enhances its competing power by increasing the probability for any given PRE molecule to react with that DNA. If the ratio [amount of competing substrate] : [amount of substrate in the biologically tested DNA] is r, one would expect that the PR rate in the studied substrate would be reduced to $1/(1 + r)$ times the rate obtained without competition. This was indeed found by Rupert (1962a) when he varied the concentration of competing DNA or its UV exposure.

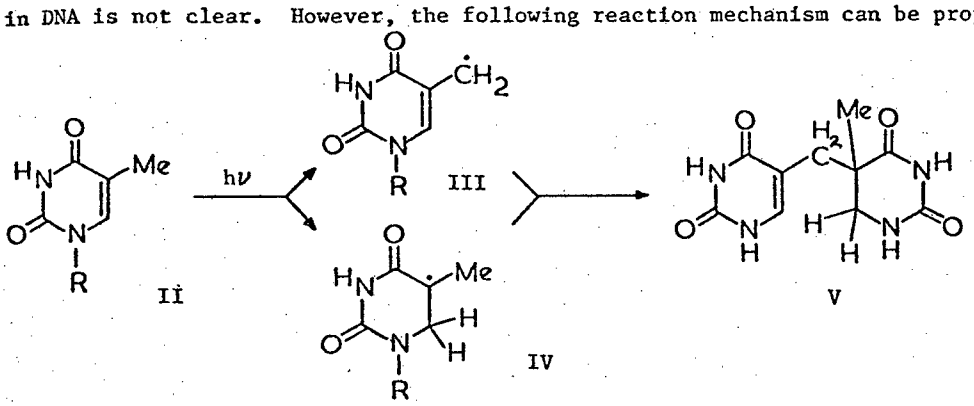
The competitive inhibition assay not only lends strong support to the overall concept of photoenzymatic repair, but it has also become a useful tool for demonstrating the presence of photoreparable UV lesions in any kind of DNA, or even in synthetic oligo- or polynucleotides. This is particularly relevant in cases in which DNA lesions cannot be tested directly for photoreactivability, it may be that the cells do not contain PRE or that PR is masked by other processes.

5. The Photoreparable UV Lesion and Its Alteration

When it was established that UV-irradiated, but not unirradiated DNA serves as substrate for the PRE, an obvious question arose; which kinds of photoproducts or structural alterations in the DNA are recognized by the enzyme? Fortunately a major breakthrough in the UV photochemistry of DNA was accomplished by Wang (1960, 1961) and by Beukers and Berends (1960, 1961): the discovery of a major group of photoproducts, the cyclobutanedipyrimidines (or "Pyr dimers" from here on symbolized by Pyr<>Pyr) formed between adjacent Pyr bases within the same strand. Thus, first evidence for the biological significance of these photoproducts was indeed obtained by PR experiments. In 1962, Wulff and Rupert showed that these dimers disappear from UV-irradiated transforming DNA under PR conditions *in vitro*, but that they remain in the DNA under comparable conditions not causing PR. Since ~90% of the inactivating UV lesions in *Haemophilus* DNA are photoreparable, these results suggest that Pyr<>Pyr are the major, if not the only, cause of UV lethality.

Further evidence for Pyr<>Pyr being the predominant lethal, and by inference photoreparable, UV lesions was obtained in quite a different way. As is discussed in Section E,1, a large fraction of the Pyr<>Pyr

While the evidence presented above suggests that 5-thyminyl-5,6-dihydrothymine is the most probable structure of P_3, the mechanism for its formation in DNA is not clear. However, the following reaction mechanism can be proposed:



There is some evidence for the formation of radicals III and IV as a result of UV irradiation. Thus the oxidation of the 5-methyl group of thymine to give 5-hydroxymethyluracil, 5-formyluracil, and 5-carboxyuracil, has been observed when thymine is irradiated with UV (λ~254 nm) in solution (17), the first step for such a reaction sequence is probably the formation of the thyminyl radical III. The formation of the thymyl radical IV is suggested from electron-spin-resonance studies of DNA and thymine irradiated with UV or ionizing radiations (18-20). It is therefore not unreasonable to assume that UV can induce radicals III and IV under suitable conditions. To explain the differences in the E.S.R. spectra of wet and dry DNA, Pershan et al (18) have postulated the necessity of two sources of hydrogen, one of which predominates in dry DNA and the other in moist DNA. Recently Rahn and Hosszu (8) have shown that the absence of water is necessary for the formation of spore photoproducts (mainly P_3) in dry DNA. From the above considerations it can be postulated that the nature of the hydrogen source determines the type of thymine-derived product in UV-irradiated DNA. In the presence of water, hydrate formation and dimerization may take place concurrently and, the hydration product of thymine being very unstable, the isolable product will be mainly the dimer. In the dry state the methyl group of one thymine residue may be the hydrogen source for the other, thus leading to III and IV, and finally the product V.

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EFFECT OF BASE SEQUENCE ON THE ULTRAVIOLET IRRADIATION PRODUCTS OF DOUBLE-STRANDED POLYNUCLEOTIDES CONTAINING BROMOURACIL AND ADENINE

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Abstract—The effects of ultraviolet irradiation of double-stranded synthetic polynucleotides containing BrU and A have been investigated. Homopolymer pairs and alternating copolymers composed of either ribo- or deoxyribo-nucleotides were prepared and were irradiated with either 313 nm or ~285 nm light. Strand separation and a modest amount of strand breakage followed irradiation of the homopolymer pairs. Changes in the ultraviolet absorption spectra of the polymers during irradiation reflected the sum of hyperchromic increases caused by progressive strand separation and loss of absorbance caused by photoproduct formation. Extensive debromination occurred. An RNase digest of irradiated poly(rA)·poly^{14}C(rBrU), analysed by column chromatography, showed components similar to those found previously upon irradiation of single-stranded poly(rBrU). Little photoproduct was released by RNase digestion as mononucleotides. The major photoproduct was in the dinucleotide fraction, and may be 5,5′-diuracil. Base sequence had a profound effect on the sensitivity of the polynucleotides. Irradiation of alternating copolymers with doses of light comparable to those that produced major photochemical changes in the homopolymer pairs brought about little if any change in the copolymers of alternating base sequence.

INTRODUCTION

Work on the nature of the UV photoproducts produced in the synthetic polynucleotide poly(rBrU) has been reported ((Ehrlich and Riley, 1972a,b). Irradiation of this polynucleotide led to changes in absorption spectrum, debromination, formation of photoproducts, and strand breakage. The major photoproduct was released by enzymatic digestion as a dinucleotide and was found to have properties in common with 5,5′-diuracil (Ishihara and Wang, 1966).

Many biological studies have made use of the increased radiation sensitivity of organisms containing BrU-DNA (i.e. Djordjevic and Szybalski, 1960; Setlow and Boyce, 1963; Mennigman, 1967; Cleaver, 1968). To learn more about the molecular consequences of irradiation of double-stranded poly-deoxyribonucleotides containing BrU, we have extended our earlier studies to double-stranded synthetic polynucleotides containing adenine and bromouracil. Four double-stranded polymers were used, which differ from one another only in base sequence or in the nature of the sugar moiety: homopolymer pairs and double-stranded copolymers of alternating base sequence were both used, composed of either ribo- or deoxyribonucleotides. Irradiation of the polynucleotide complexes poly(rA)·poly(rBrU), poly(dA)·poly(dBrU), poly[r(A—BrU)·r(A—BrU)], and poly[d(A—BrU)·d(A—BrU)] resulted in photochemical changes in the homopolymer pairs similar to those observed previously for single-stranded poly(rBrU), but had little effect on the alternating copolymers.

MATERIALS AND METHODS

Preparation of synthetic polynucleotides

(a) *Single-stranded polymers.* Poly^{14}C(rBrU): ^{14}C-BrUDP was prepared by bromination of ^{14}C-UDP (New England Nuclear Co. radioactive salt diluted with nonradioactive compound). The ^{14}C—BrUDP was purified by column chromatography then polymerized with polynucleotide phosphorylase as previously described (Riley and Paul, 1970). The preparation of poly^{14}C(rBrU) used in these experiments had a specific activity of $2{\cdot}0 \times 10^5$ counts min^{-1} μmol^{-1}.

Poly(dBrU): both ^{14}C-labelled and non-radioactive poly(dBrU) were prepared. Non-radioactive dBrUTP was

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photoproduct [5-(4'-pyrimidin-2'-one)uracil] obtained from uracil and 4-thiouracil under the same conditions.

5-(1'-β-D-Ribofuranosyl-4'-pyrimidin-2'-one)uridine (Ib) (Cyd⌒Srd_1 or C⌒$^4S_1$). The conditions for the photoreaction at 335 nm for cytidine and 4-thiouridine were the same as those described above. The product was dissolved in 2 N HCOOH, filtered, and the acid was removed in vacuo. Purification of the solid residue was effected by solution in 1 N HCl, precipitation with NaOH, washing with water, and drying, dp 270°; nmr (CF_3COOH) δ9.61 (s, 1); 8.70-8.77 (d and s, 2); 7.45 (d, J = 7 Hz, 1); 6.16-6.20 (ss, 2, anomeric H's). Anal. Calcd for $C_{18}H_{23}N_5O_{10}\cdot H_2O$: C, 44.35; H, 5.17. Found: C, 44.64; H, 4.99.

RESULTS AND DISCUSSION

The photoreaction of 4-thiouracil and cytosine yielded only one product with ultraviolet absorption and fluorescence emission (on borohydride reduction) spectral characteristics in agreement with those of the photochemically crosslinked products resulting from the irradiation of E. coli $tRNA_1^{Val}$, $tRNA_2^{Val}$, $tRNA_f^{Met}$, $tRNA_m^{Met}$, $tRNA^{Phe}$ (Yaniv et al., 1969) and $tRNA^{Arg}$ (Chaffin et al., 1971) at 335 nm. The cytosine — 4-thiouracil photoproduct, Cyt⌒Sur_1, exhibiting $\lambda_{max}^{H_2O}$ 323 nm and $\lambda_{sh}^{H_2O}$ 334 nm had a molecular ion peak in the mass spectrum and microanalysis, as the monohydrate, correct for $C_8H_7N_5O_2$, corresponding to combination of cytosine and 4-thiouracil with the loss of H_2S.

For the next stage in the structure determination, we were guided by theoretical considerations resulting from (a) the direction of photoaddition of thiobenzophenone to the electron-deficient olefin acrylonitrile at 366 nm ($\pi \rightarrow \pi^*$) to give a substituted β-cyanothietane (Ohno et al., 1969), which was opposite to the direction of the photoaddition of acetone ($n \rightarrow \pi^*$) to acrylonitrile to give a subtituted α-cyanooxetane (Barltrop and Carless, 1968), and (b) the structures of the photoproducts from uracil (Khattack and Wang, 1969), thymine (Varghese and Wang, 1968), and a mixture of uracil and thymine (Rhoades and Wang, 1970), which are postulated to have arisen via

Table I. Nmr Spectra of Thymine Dimers and Their Products of Hydrolysis, Hydrogenolyis, and Oxidation[a]

	>CCH$_3$		>C–CH$_2$		–C(H)–C(H)–		
Compd	DMSO-d_6	D_2O	DMSO-d_6	D_2O	DMSO-d_6	D_2O	Remarks
1	8.67 (6 H, s)				6.24 (2 H, s)		
5		8.71 (3 H, s) 8.68 (3 H, s)		5.93 (1 H, s) J = 10.0 Hz 5.52 (1 H, s) J = 10.0 Hz		5.50 (1 H, d) J = 8.5 Hz 5.78 (1 H, d) J = 8.5 Hz	AB pattern for >CCH$_2$– and cyclobutane protons
6	8.97 (3 H, s) 8.93 (3 H, s)	8.91 (3 H, s) 8.79 (3 H, s)			6.23 (2 H, broad multiplet)	5.98 (2 H, s)	Carbinol protons, 4.97 and 4.87 (1 H, s, D_2O) 5.30 and 5.15 (1 H, d, J = 5.0 Hz, DMSO-d_6)
7	8.97 (6 H, s)	8.84 (6 H, s)	6.57 (4 H, broad singlet)	6.25 (4 H, s)	6.17 (2 H, d, J = 4.5 Hz)	5.85 (2 H, s)	Addition of D_2O changes cyclobutane doublet to singlet at 6.11
10		8.71 (6 H, s)				5.90 (2 H, s)	
12		8.66 (3 H, s) 8.60 (3 H, s)				6.16 (1 H, d) 5.90 (1 H, d) J = 6.0 Hz	AB pattern for cyclobutane protons
13		9.11 (6 H, s)		6.53 (4 H, s)		6.13 (2 H, s)	Solvent, D_2O and trifluoroacetic acid (1:1)
14	8.77 (6 H, s)				5.55 (2 H, d) J = 10 Hz		–NH$_2$, 4.20 (4 H, s) >NH, 3.15 (2 H, d) J = 10.0 Hz
15		8.74 (6 H, s)		6.23 (4 H, s)		6.33 (2 H, s)	

[a] The spectra were measured on a Varian A-60 spectrometer. Chemical shifts are given in τ values (tetramethylsilane as internal or external (D_2O) standard).

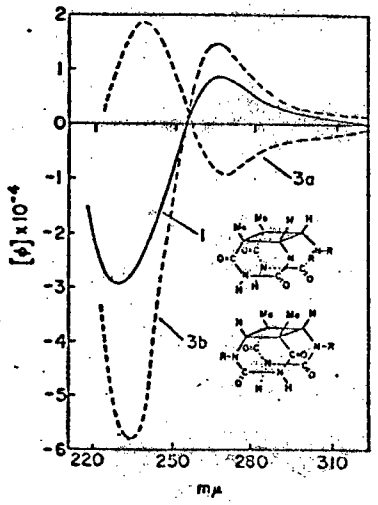


Figure 2. ORD curves of thymidine dimers 1 (—) and 3a and 3b (- - -) (R = 2-deoxyribofuranosyl) in MeOH.

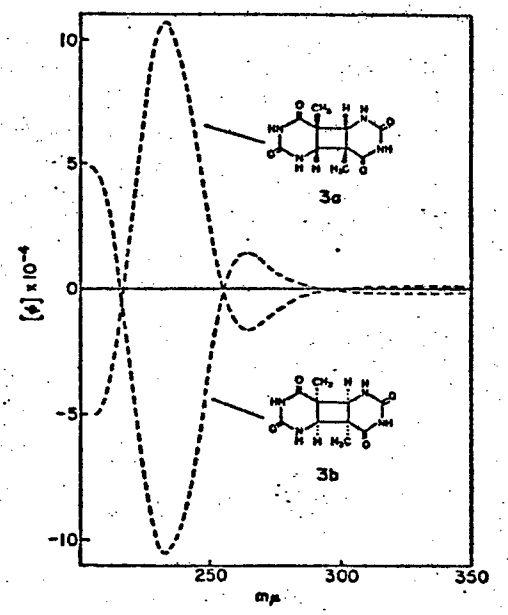
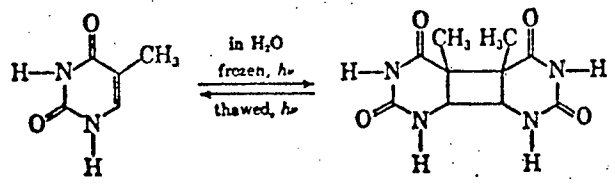


Figure 3. ORD curves of the optically active photodimers 3a and 3b. The assignments are tentative.

furanosyl), were isolated from the irradiated frozen solution of thymidine[12] and purified by chromatography on silica gel. Their ORD spectra are shown in Figure 2. Mild acid hydrolysis of these dimers gave optically inactive dimer 1 (R = H), (+)-dimer 3a, $[\alpha]^{25}D$ +94° (H_2O), and (−)-dimer 3b, $[\alpha]^{25}D$ −92° (H_2O). In addition we observed the formation of a new fluorescent product, λ_{max}^{MeOH} 318 nm, which presumably corresponds to Wang's adduct ($\lambda_{max}^{H_2O}$ 316 nm).[15,16]

The ORD spectra of (+)-3a (R = H) and (−)-3b (R = H) exhibited Cotton effects with a strong intensity, *i.e.*, $|[\phi]_P - [\phi]_T|/100$ ($a = 1.2 \times 10^3$), around 250 nm (Figure 3), probably due to the intensive n–π^* transitions of the ureidocarbonyl group in analogy to dihydrothymine[27] and cyclothymine nucleosides.[26] Al-



to photodeactivation and reactivation of microorganisms,[10] together with speculation on whether the reaction occurs in the solid state or in "puddles" of liquid in the frozen system, has resulted in a great deal of interest in this type of frozen-state reaction. Dimerization of thymine in ice has recently[11] been quite easily established as a true solid-state reaction (see below).

Several kinetic studies of chemical reactions in frozen systems have been prompted by initial observations that some reactions appear to be catalyzed by the frozen conditions. The increase in rate is often so great that it makes possible the quenching of the reaction simply by thawing a sample; a leisurely analysis of reaction progress can then be ordinarily carried out at room temperature. Such accelerations and other unusual kinetic features of frozen reactions have been interpreted in a great variety of ways.

Grant, Clark, and Alburn[12] described the base-catalyzed hydrolysis of penicillin in frozen systems at −5 to −30°. Here reaction occurred in frozen samples, but not in identical nonfrozen samples held long periods at 38°. As has been generally found, the reaction was not influenced by the manner in which samples were frozen, but the presence of various solutes (glycerol, ethanol) stopped the reaction in the frozen samples. The authors considered a possible explanation involving concentration of reactants on freezing, but suggested that a favorable substrate–catalyst positional constraint, and possibly the exceptionally high proton mobility in ice, might be factors.[12] They have also suggested that reactant and product diffusion, crystal imperfections, as well as the dielectric properties of ice may play some role in certain reactions in frozen systems.[13] In the hydroxylaminolysis of some amino acid esters in frozen solutions they showed that reactions were often inhibited by addition of compounds structurally analogous to the reactants; the kinetic relationship found was that of a Lineweaver–Burk plot for competitive inhibition. This seemed to suggest the existence of catalytically active sites on the ice surface.[14]

Butler and Bruice[15] compared the kinetics of catalyzed hydrolysis of acetic anhydride, β-propriolactone, and *p*-nitrophenyl acetate in water and in ice and pointed out that a concentration effect was responsible for increased rates of these reactions in ice. The crystallization of water resulted in high concentrations of reactant in liquid regions of the frozen systems, and the rate of any bimolecular reaction was simply increased by this change in concentration. In a later investigation of the reaction of morpholine with two thiolactones[16] they noted that the over-all observed reaction order changes from three in nonfrozen solution to two in frozen solutions. This seemed opposite to results anticipated for a concentration phenomenon, and they suggested that the ice structure itself takes part in a proton transfer reaction in place of one of the morpholine molecules.

Some inorganic reactions in frozen aqueous solutions have also given interesting results and explanations. The autoxidation of iodide ion occurs with enhanced rates in frozen solutions[17] and the oxidation of iodide by arsenic acid proceeds very rapidly even at −70° in frozen solutions.[18] Perhaps most interesting is the electron exchange between ferrous and ferric ions in $HClO_4$–H_2O solutions frozen to −78°.[19] Under the conditions used it was estimated that the reactants were separated in the solid medium by roughly 100 Å, yet, except for an ordinary decrease due to temperature, the reaction rate was not different from that in liquid solutions at higher temperatures. A facile electron transfer by a water bridging mechanism was suggested for reaction in both liquid and solid systems.[19]

Such interesting possibilities, combined with the fortuitous discovery of a particularly simple reaction in frozen organic solvents,[20] indicated that a systematic investigation of some reactions in frozen solutions would clarify or establish some of the many suggested explanations.[21] Although the projected study did not initially require it, it now seems best to begin a description of the work with definitions of the terms "frozen" and "rate."

"Frozen State." Often, in subconscious but mistaken analogy to one-component systems, there is a tendency to think of two- (or more) component systems below their freezing points as solids. The frequent existence of a liquid in equilibrium with a solid at temperatures below the freezing point is often neglected.[18] It is important, therefore, to mention that only below its eutectic point is a system completely solid. It seems best to consider that a "frozen state" exists in the range of temperatures below the freezing point and above the eutectic point, *i.e.*, where solid is

(10) S. Y. Wang, *Fed. Proc.*, 24, Suppl. 15, S-71 (1965), and references therein.
(11) (a) W. Fuchtbauer and P. Mazur, *Photochem. Photobiol.*, 5, 323 (1966); (b) S. Y. Wang, *ibid.*, 3, 395 (1964).
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Short Communications

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On 1-methylthymine photoproduct*

Ultraviolet irradiation of a frozen solution of thymine produces thymine photoproduct[1]. When the solution is thawed the latter product reverts to thymine upon further ultraviolet irradiation[2]. The photoproduct has been shown to be a dimer with a cyclobutane ring formed by addition of 5,6 double bonds of the two thymines[3–6]. Uracil and N-substituted uracils and thymines behave similarly[1,5–10]. It is probable that formation of thymine photodimer is favored when thymine residues are close together in a suitable configuration. Thus the dinucleotide TpT readily forms the photoproduct in dilute solution[4,9–12]. The dimer is also formed, presumably mainly from adjacent thymines in the polynucleotide chain, upon irradiation of DNA in solution[10,13–16]. WANG proposes that microcrystalline aggregates of thymines form when thymine solutions are frozen and that in these crystals the thymines are favorably oriented for photodimerization. He was also able to prepare thymine dimers by irradiating thin films of thymine[5].

The photostationary state depends on wavelength. Formation of the dimer is favored by long wavelengths where only thymine absorbs; dissociation is favored at around 230 mμ where the absorption coefficients of the dimer are comparable to those of the monomer.

I wish to present evidence which supports the hypothesis that 2 thymine molecules held in a favorable configuration readily undergo the photochemical formation of the dimer. These fragmentary observations were made in the course of a study of the single crystal absorption spectra of purines and pyrimidines[17]. While measuring transmissions with light polarized perpendicular to the *b*-axis in the (102)-plane (the plane of the molecular layers[18]) of a 1-methylthymine single crystal, which was 0.1 μ thick, considerable bleaching took place. (The absorption coefficient for the first absorption band of the crystal perpendicular to the *b*-axis in (102) is about 10 times the absorption coefficient parallel to the *b*-axis in (102).) At the end of the experiment the birefringence of the crystal had fallen by about 30 % and its light transmission at 280 mμ had increased by 75 %.

An experiment to demonstrate that this bleaching was due to the formation of thymine photodimer was done as follows. A preparation of thin plates of 1-methylthymine was obtained by rapid evaporation of a hot solution spread out on the bottom of a Petri dish. The predominant form is (102), parallel to the molecular layers, and the thickness of the crystals ranged from 0.7–20 μ. The absorption maximum for the crystal is 273 mμ, the same as for solution. By irradiating the crystals mostly to the red of 270 mμ, a region where the dimer absorption coefficient is small relative to the monomer, a favorable photosteady state with major production of the photoproduct should be established. By employing a continuous source one is assured

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