References

Arnott, S., Fuller, W., Hodgson, A., and Prutton I. (1968), *Nature (London) 220*, 561.
Blake, R. D., Massoulié, J., and Fresco, J. R. (1967), *J. Mol. Biol. 30*, 291.
Brahms, J. (1965), *J. Mol. Biol. 11*, 785.
Brahms, J., and Mommaerts, W. F. H. M. (1964), *J. Mol. Biol. 10*, 73.
Cohen, P., and Kidson, C. (1968), *J. Mol. Biol. 35*, 241.
Davies, H. G., and Small, J. V. (1968), *Nature (London) 217*, 1122.
De Vries, H. (1951), *Acta Cryst. 4*, 219.
Dubin, D. T. (1960), *J. Biol. Chem. 235*, 783.
Eigner, J., and Doty, P. (1965), *J. Mol. Biol. 12*, 549.
Elliott, A. (1965), *J. Sci. Inst. 42*, 312.
Franks, A. (1955), *Proc. Roy. Soc., Ser. A 68*, 1054.
Fuller, W. (1961), Ph.D. Thesis, London University, London.
Inoue, S., and Ando, T. (1960), *Biochim. Biophys. Acta 129*, 649.
Ji, T. H., and Urry, D. W. (1969), *Biochem. Biophys. Res. Commun. 34*, 404.
Katchalsky, A. (1964), *Biophys. J. 4*, 9.
Langridge, R., Wilson, H. R., Hooper, C. W., Wilkins, M. H. F., and Hamilton, L. D. (1960), *J. Mol. Biol. 2*, 19.
Leng, M., and Felsenfeld, G. (1966), *Proc. Nat. Acad. Sci. U. S. 56*, 1325.
Maestre, M. F., and Schneider, M. J. T. (1969), *Fed. Proc., Fed. Amer. Soc. Exp. Biol. 28*, 875.
Matsuo, K., Fuke, M., Tsuboi, M., and Wada, A. (1969), *Biochim. Biophys. Acta 179*, 39.
Miller, I. R., and Inbar, L. (1969), *Biopolymers 7*, 619.
Nirenberg, M. W., and Matthaei, H. (1961), *Proc. Nat. Acad. Sci. U. S. 47*, 1588.
Pardon, J. F., Wilkins, M. H. F., and Richards, B. M. (1967), *Nature (London) 215*, 508.
Robinson, C. (1961), *Tetrahedron 13*, 219.
Robinson, C., Ward, J. C., and Beevers, R. B. (1958), *Disc. Faraday Soc. 25*, 29.
Shapiro, J. T., Leng, M., and Felsenfeld, G. (1969), *Biochemistry 8*, 3219.
Spencer, M., Fuller, W., Wilkins, M. H. F., and Brown, G. L. (1962), *Nature (London) 194*, 1014.
Spirin, A. S. (1963), *Progr. Nucleic Acid Res. 1*, 301.
Stevens, C. L., and Felsenfeld, G. (1964), *Biopolymers 2*, 293.
Tinoco, I. (1968), *J. Chim. Phys. 65*, 91.
Urry, D. W., and Ji, T. H. (1968), *Arch. Biochem. 128*, 802.
White, M. J. D. (1948), Animal Cytology and Evolution, Cambridge, Cambridge University Press.
Wilkins, M. H. F. (1956), *Cold Spring Harbor Symp. Quant. Biol. 21*, 75.
Zubay, G., Wilkins, M. H. F., and Blout, E. R. (1962), *J. Mol. Biol. 4*, 69.

Uracil–Thymine Adduct from a Mixture of Uracil and Thymine Irradiated with Ultraviolet Light*

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ABSTRACT: A uracil–thymine adduct isolated from a uracil and thymine mixture irradiated with ultraviolet (254 nm) light in frozen aqueous solution is characterized as 5-hydroxy-6-(4′-pyrimidin-2′-one)dihydrothymine. This assignment was made on the basis of mass, ultraviolet, infrared, and nuclear magnetic resonance spectra. The compound readily undergoes dehydration to yield P_2B (6-(4′-pyrimidin-2′-one)thymine) which has been identified as a product from the acid hydrolysates of DNA irradiated with ultraviolet light. A mechanism for its formation is presented and its possible importance in the photochemistry and photobiology of nucleic acids is discussed.

A new product has recently been isolated from acid hydrolysates of DNA irradiated with ultraviolet light (254 nm) both *in vivo* and *in vitro* (Varghese and Wang, 1967). This product, P_2B, which has been characterized as 6-(4′-pyrimidin-2′-one)thymine (I) (Wang and Varghese, 1967), is derived from a cytosine–thymine adduct (II) or (III). It can also be isolated from acid hydrolysates of thymidine–cytidine mixture irradiated in frozen aqueous solution (Varghese and Patrick, 1969) and from thymine–uracil mixture irradiated in frozen aqueous solution (M. N Khattak and S. Y. Wang, unpublished results).

We now wish to report the isolation and characterization of a uracil–thymine adduct (U–T adduct, III), a precursor of P_2B (I). This compound may be analogous to, indeed may even be identical with, the pyrimidine moiety of the ultraviolet-induced lesions in DNA responsible for the isolation of P_2B in acid hydrolysates. In addition, this U–T adduct readily undergoes dehydration to give P_2B.

* From the Department of Biochemistry, The Johns Hopkins University, School of Hygiene and Public Health, Baltimore, Maryland. *Received May 20, 1970.* This work was supported in part by AEC Contract AT-(30-1)-2798 and by a Public Health Service Research Career Development award (K3-GM-4134). This publication is identified as NYO-2798-53.

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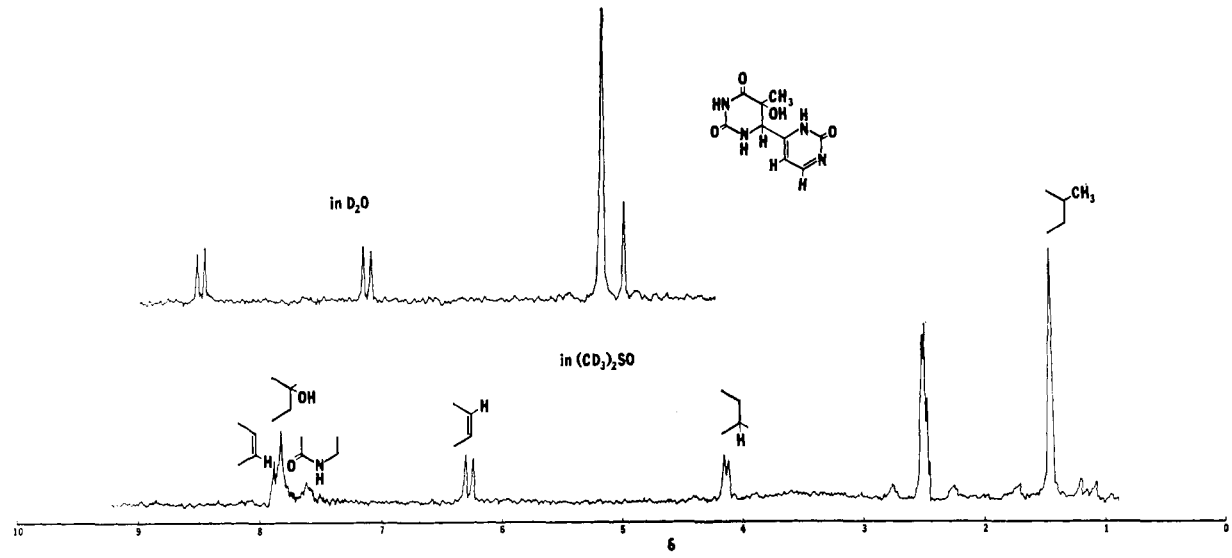


FIGURE 1: Nuclear magnetic resonance spectra of U–T adduct in D_2O and $(CD_3)_2SO$ at 100 MHz with internal standard tetramethylsilane.

Experimental Procedures

An aqueous solution containing thymine (1.67 mmoles/l.) and uracil (3.33 mmoles/l.) was irradiated for 60 min in the frozen state over crushed Dry Ice at a distance of 7 cm from the G. E. germicidal lamps (254 nm) of an irradiator (Wang, 1961). After thawing, the solution (4 l.) was evaporated to a volume of 2 ml. This was accomplished in stages with periodic filtration to remove solid precipitates of cyclobutyl dimers. The concentrated solution was applied on Whatman No. 3MM paper and was developed with eluent A [80% ethanol]. Sections were cut from the dried chromatograms and the material was eluted with water. Ultraviolet spectral examination of the eluents revealed the presence of a band, R_F 0.47, exhibiting an ultraviolet absorbancy maximum at 303 nm. However, irradiation of this eluent with 254-nm light at room temperature showed continued increase in the absorbancy at 265 nm, indicating the presence of considerable quantities of cyclobutyl dimers. Further purification of this mixture of adduct and dimers was attempted by rechromatography on paper with eluents B [1-butanol–water (84:16)], C [2-propanol–ammonia–water (70:20:10)], and D [2-propanol–water (75:25)]. However, no separation was achieved and bands with R_F 0.05 (eluent B), R_F 0.30 (C), and R_F 0.40 (D), respectively, were observed. Thus, in order to eliminate dimers from the mixture, it was necessary to resort to the irradiation of the eluent with 254-nm light. This was accomplished by adjusting the eluent with water to give an absorbancy at 303 nm of 0.9 optical density and then irradiating the solution at room temperature in quartz tubes with a bank of germicidal lamps (Wang, 1958) until little further increase in optical density at 265 nm was observed. The process took about 5 min. After irradiation, the solution was concentrated, applied on Whatman No. 3MM paper and developed with eluent A. Three bands were detected on the chromatograms: the 303-nm absorbing band at R_F 0.47 (U–T adduct) and two 265-nm absorbing bands at R_F 0.66 (uracil) and R_F 0.77 (thymine). The 303-nm absorbing band was eluted with cold water, followed by concentration to ~1 ml and addition of 3 ml of absolute methanol. It was allowed to stand overnight at 5°, producing 11 mg of U–T adduct as colorless needles. Recrystallized from water at 5°, it gave purified U–T adduct.

Results and Discussion

Structure. The nuclear magnetic resonance spectrum at 100 MHz of U–T adduct in D_2O (Figure 1) exhibits: a sharp singlet (δ 2.10, 3 H) indicating a single methyl group; a singlet (δ 4.99, 1 H) indicating C (6) methine; and two doublets (δ 7.12, 1 H; δ 8.50, 1 H; J = 6 cps) indicating C (5′) and C(6′) vinylic protons, respectively. In $(CD_3)_2SO$, the C(6) methine proton exhibits a doublet (δ 4.48, 1 H; J = 3 cps) coupling with N(1)-H, assigned to a broad signal (δ 7.99). C(5)-OH appears as a singlet (δ 8.22) and coincides with the C(6′) vinylic doublet (δ 6.6. 1 H; J = 6 cps). The C(5)-OH singlet of the U–T adduct occurs 2.22 ppm downfield from that observed in the thymine–thymine adduct (T–T adduct) (Varghese and Wang, 1968) in the same solvent. This shift indicates that the proton of C(5)-OH is more acidic and could be attributed to the fact that intramolecular hydrogen bonding occurs between C(5)-O and N(3′)-H in U–T adduct. Such hydrogen bonding is unlikely to occur in the T–T adduct as the angle of inclination between the two rings is approximately 96° (Karle *et al.*, 1969; Karle, 1969) due to the presence of C(5′)-CH_3. Intramolecular hydrogen bonding would also explain the absence of a well-defined N(3′)-H resonance although a very broad signal in the δ 10.3 region can be discerned.

The infrared spectrum of U–T adduct (Figure 2) in a potassium bromide pellet lacks an OH band characteristic of C(5)-OH (~2.95 μ) or C(6)-OH (~2.99 μ) of saturated pyrimidines (Wang *et al.*, 1956; Wang, 1958) or the strong OH band at 2.83 μ observed in T–T adduct (Varghese and Wang, 1968). This again may be due to the presence of strong intramolecular hydrogen bonding which shifts the OH band to the 3.0- to 3.5-μ region. Otherwise, the gross spectral features of T–T and U–T adducts are quite similar.

The ultraviolet spectrum of U–T adduct (Figure 3) exhibits

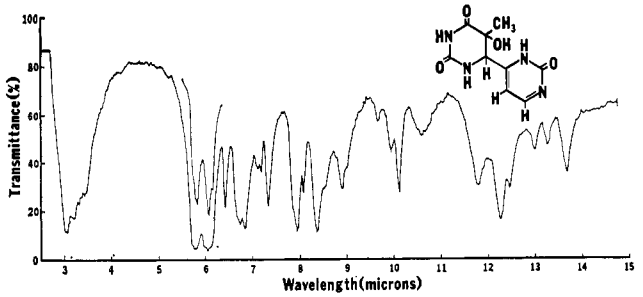


FIGURE 2: Infrared spectrum of U–T adduct in a KBr pellet.

absorbancy maxima at 303 nm (ϵ 4.68 $\times$ 10^3) in aqueous solution at neutral pH and pH 2, and 294 nm (ϵ 5.30 $\times$ 10^3) at pH 12. This corresponds well with the spectrum of T–T adduct, which has maxima at 316 nm and 306 nm, respectively. This bathochromic shift must be due to the presence of C(5′)-CH_3 in T–T adduct.

Attempts to determine the mass spectrum of U–T adduct failed to give the molecular ion peak and the spectrum obtained is identical with that of P_2B (Fenselau and Wang, 1969; Wang and Varghese, 1967). This dehydration process is to be expected at the high temperatures used for such determinations. However, a silyl derivative of U–T adduct obtained by treatment with Regsil No. 270002 in pyridine gave a mass spectrum with molecular ion peak *m/e* 526 (Figure 4). This corresponds to the molecular weight of the expected tetratrimethylsilyl U–T adduct, *i.e.*, 5-hydroxy-6-(4′-pyrimidin-2′-one)dihydrothymine (IIIa or IIIb).

Reactivity. No spectral change was detected after storage of a neutral aqueous solution of U–T adduct for 1 week at room temperature. At pH 2, however, complete conversion into P_2B occurred in this time span. Dehydration of U–T adduct (5 mg) was also achieved in 10 min by refluxing with 2 N HCl (25 ml). The reaction solution was concentrated, applied on Whatman No. 3MM paper and developed with eluent C. The major band (R_F 0.29) was eluted with water; the eluent was concentrated and cooled at 5° to give crystals of P_2B (4 mg), which were identified by ultraviolet and infrared spectra. Interestingly, quantitative dehydration of U–T adduct occurred simply by means of passage through a column of Dowex 50W-X12 (H^+ form). [In contrast, T–T adduct is stable upon this treatment. On the other hand, U–U adduct is even more unstable than U–T and can only be isolated as the dehydrated product (Khattak and Wang, 1969).] At pH 11, dehydration also takes place; however, a product(s) with an absorbancy maximum in the 265-nm region is formed in addition to P_2B.

On irradiation with 313-nm or 360-nm light in neutral aqueous solutions, U–T adduct showed little spectral change and therefore appeared to be stable under these conditions. On the other hand, U–T adduct undergoes a ~50% absorbancy loss at 303 nm after irradiation for 30 min with 254-nm light under the same conditions described above for its isolation. This change is accompanied by a slight initial rise in absorbancy in the 265-nm region, although this decays on continued irradiation to produce essentially a spectrum with only end absorption.

Mechanism of Formation and Biological Importance. A mechanism for the formation of P_2B from the acid hydrolysates of ultraviolet-irradiated DNA has been proposed (Wang and Varghese, 1967). Photoinduced intrastrand addition of the 5,6-double bond of thymine to the exocyclic imino group of cytosine yielding an azetidine derivative IV was considered probable. Intermediate IV would be unstable and undergo rearrangement with ring opening. As shown in Scheme I, there are three conceivable pathways for this to occur: (1) acid-catalyzed ring opening yields the pyrimidinone derivative (II) directly; (2) nucleophilic attack by HOH at C(4′) gives the hydroxyamino derivative V and with subsequent dehydration also yields II; and (3) nucleophilic attack by HOH at C(5) forms an isomeric hydroxyamino derivative VI which eliminates NH_3 to produce a U–T adduct (IIIb). Since the first two modes do not involve the optically active centers, the relative configuration at C(5) and C(6) of II thus formed should be the same as the azetidine derivative IV, *i.e.*, trans C(5)-OH and C(6)-H. On the other hand, the third pathway leading to IIIb, necessitates the approach of HOH from the side opposite to the oxetane ring and must result in the inversion of configuration at C(5).

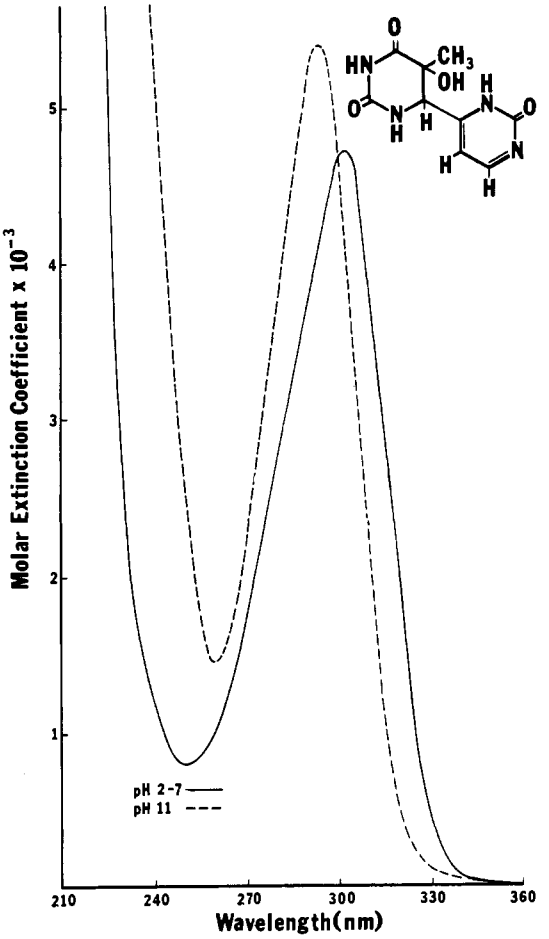


FIGURE 3: Ultraviolet absorption spectra of U–T adduct.

Similarly, the U–T adduct III obtained from the irradiation of a mixture of uracil and thymine is thought to be formed through the ring opening of an oxetane derivative (VII) *via* a similar mechanism.

In short, it may be said that if ring opening of both azetidine in DNA and the U–T oxetane occurs *via* the third pathway

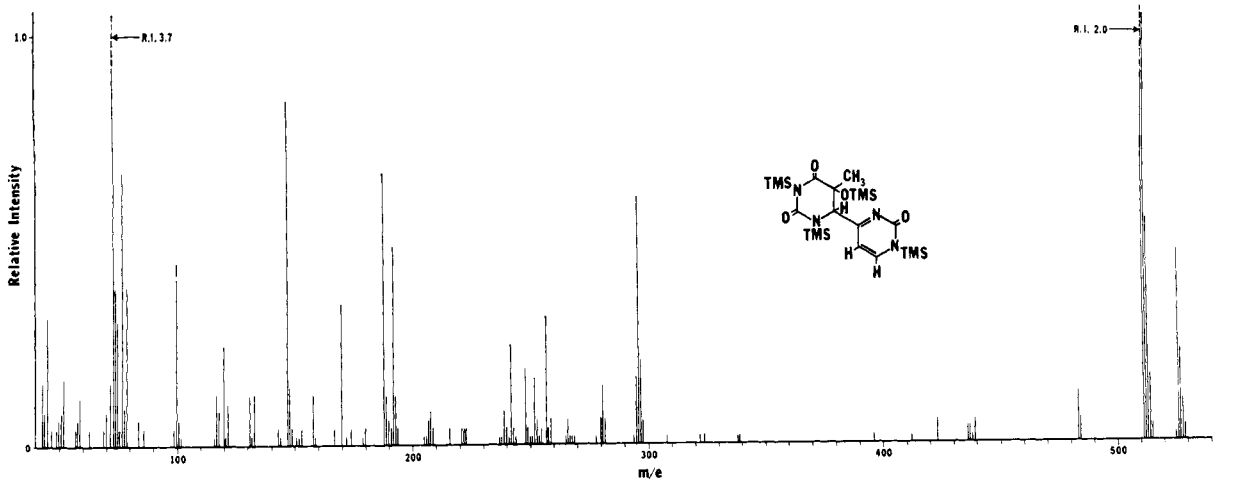


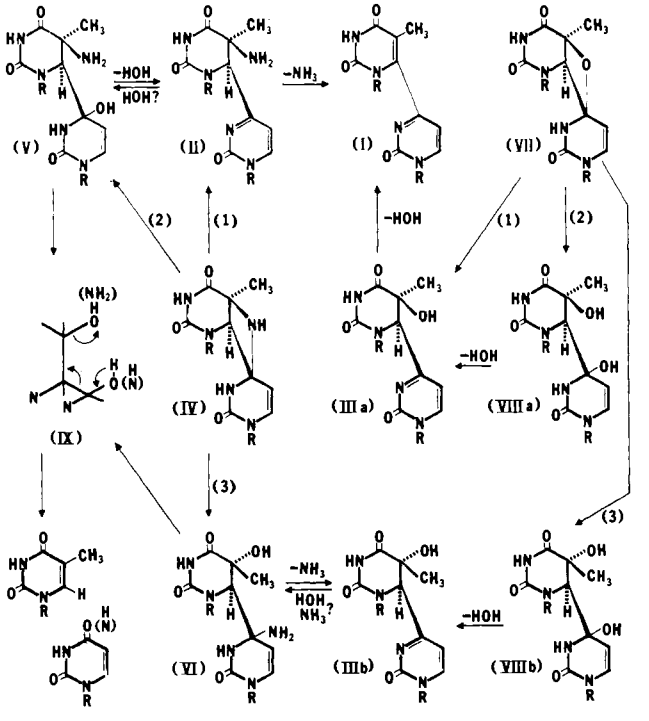
FIGURE 4: Mass spectrum of tetratrimethylsilyl U-T adduct.

then the resulting adducts should be identical, as shown in IIIb. If ring opening in both occurs by either of the other modes, then the U-T adduct IIIa and the DNA lesion II will be heteroanalogous.

Studies are presently under way to determine the configuration at C(5) and C(6) of U-T adduct in order to select the most probable mechanism. On the other hand, the structure of T-T adduct has been established by X-ray diffraction (Karle *et al.*, 1969; Karle, 1969). In this case, the trans configuration is assigned for C(5)-OH and C(6)-H giving a structure analogous to II and indicating that oxetane opens either directly by acid catalysis or by the attack of HOH at C(4′). Furthermore, a diol structure like VIII for a thymine phototrimer has been established recently (Wang, 1971; Flippen *et al.*, 1970), and is analogous to V, suggesting that oxetane opening by HOH attack at C(4′) is the favored mechanism. However, one must be cautious in ruling out the possibility that hydration (II) → (V) may occur under certain conditions.

Earlier observation (Varghese and Wang, 1967) showed that when native DNA solution (40 μg/ml of 0.15 M NaCl) was irradiated at a dose rate of 110 ergs/mm² sec for 30, 60, 90 sec, etc., there was a gradual decrease in the absorbancy at 260 nm with a simultaneous increase in the absorbancy in the 300- to 340-nm region. Therefore, an investigation was begun on the nature of this absorbancy increase and its possible biological roles. Subsequently it was found that the so-called "thymine dimer" was in fact a mixture of cis-syn T=T (P_2A) and the deaminated cytosine-thymine adduct (P_2B) having an absorbancy maximum at 316 nm (Varghese and Wang, 1967). The ratio of P_2A:P_2B was found to be about 9:1 at all doses (4800–28,800 ergs/mm²) of irradiation as assayed from the acid hydrolysates of ultraviolet-irradiated *Escherichia coli* 15 T⁻ cells. From the P_2B characterization study (Wang and Varghese, 1967), it was realized that P_2B actually results from vigorous conditions of acid hydrolysis and is not the product formed directly on irradiation. (This further strengthens our earlier belief that the usual conditions used to analyze DNA photoproducts are only applicable to those products which are stable under these conditions.) Therefore, it would be of interest to isolate a precursor of P_2B from the irradiation of a mixture of uracil and thymine in the frozen state. The present paper reports the successful isolation and elucidation of such a precursor. In fact, it is one of the major products formed, but it has eluded detection of its facile dehydration to P_2B on a Dowex column as discussed above. [A similar product (D. F. Rhoades and S. Y. Wang, unpublished results).] has now been isolated from deoxycytidine and thymidine. It is important to note that the various adducts have absorbancy maxima of 300–340 nm which is in the range of the most effective light wavelengths for direct photoreactivation (Jagger, 1967). (In contrast, the cyclobutyl dimers have virtually no absorption above 240 nm.) Although the functional importance of pyrimidine adducts in ultraviolet inactivation of

SCHEME I



biological systems is yet to be established (Small *et al.*, 1968; Jagger *et al.*, 1969), their possible significance in biological mutation and reactivation processes may not be trivial. In such processes, the adducts (II or III) may add water across N–(3′)–C(4′) to give derivatives such as V and VI. This type of linkage is known to convert adducts into individual bases, as shown (IX), under various conditions (Wang, 1971; Flippen *et al.*, 1970). Since such rearrangement will occur under rather mild conditions, these derivatives (V and VI) could not be detected by assay of the acid hydrolysates of ultraviolet DNA. On the other hand, their presence may be manifested by certain biological effects in *in vivo* systems.

Acknowledgments

We thank Dr. Kai N. Fang for the nuclear magnetic resonance spectra, Dr. Catherine Fensealu for the mass spectra, and Mrs. Linda Roth for technical assistance.

References

Fenselau, C., and Wang, S. Y. (1969), *Tetrahedron 25*, 2853.

Flippen, J. L., Karle, I. L., and Wang, S. Y. (1970), *Science* 169, 1084.

Jagger, J. (1967), Introduction to Research in Ultraviole Photobiology, Englewood Cliffs, N. J., Prentice-Hall Inc.

Jagger, J., Stafford, R. S., and Snow, J. M. (1969), *Photochem Photobiol. 10*, 383.

Karle, I. L. (1969), *Acta Crystallogr. B 25*, 2119.

Karle, I. L., Wang, S. Y., and Varghese, A. J. (1969), *Science 164*, 183.

Khattak, M. N., and Wang, S. Y. (1969), *Science 163*, 1341.

Small, G. D., Tao, M., and Gordon, M. P. (1968), *J. Mol Biol. 38*, 75.

Varghese, A. J., and Patrick, M. H. (1969), *Nature (London 223*, 299.

Varghese, A. J., and Wang, S. Y. (1967), *Science 156*, 955.

Varghese, A. J., and Wang, S. Y. (1968), *Science 160*, 186.

Wang, S. Y. (1958), *J. Amer. Chem. Soc. 80*, 6196.

Wang, S. Y. (1961), *Nature (London) 190*, 690.

Wang, S. Y. (1971), *J. Amer. Chem. Soc.* (in press).

Wang, S. Y., Apicella, M., and Stone, B. R. (1956), *J. Amer Chem. Soc. 78*, 4180.

Wang, S. Y., and Varghese, A. J. (1967), *Biochem. Biophys Res. Commun. 29*, 543.