Acetone-Sensitized Photochemistry of Some Pyrimidine Dinucleoside Phosphates†

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ABSTRACT: The kinetics of acetone-sensitized photochemistry of UpU, UpC, CpU, and CpC have been measured. At an exciting wavelength of 312 nm, no significant difference in the efficiency of triplet energy transfer from acetone to CpU, UpC, or CpC was found (sensitization constant, $K_s = 1.7 \times 10^4$ M^{-1}), and transfer to UpU was only slightly less efficient ($K_s = 1.2 \times 10^4$ M^{-1}). The efficiency of converting the excited dinucleoside phosphate to photoproducts (presumably cyclobutane-type dimers) ranged from 0.011 to 0.20 and increased in the order, CpC < CpU < UpC < UpU. Preliminary results show that pseudouridine 3′-phosphate also undergoes sensitized photochemistry that resembles closely the previously reported reactions induced by direct irradiation with 254-nm light (Tomasz, M., and Chambers, R. W. (1964), *J. Amer. Chem. Soc. 86*, 4216; (1966), *Biochemistry 5*, 773). These results provide a quantitative base line for detailed studies on the acetone-sensitized photochemistry of tRNA without the complication of photohydration. They indicate that, in the absence of ordered structure effects, formation of UpU dimers and chain cleavage at pseudouridine residues in the tRNA should be major photoreactions. CpC targets should be rather unreactive with a rate $^1/_{18}$ of UpU, while CpU and UpC sequences should fall in between with rates of $^1/_7$ and $^1/_5$ that of UpU, respectively.

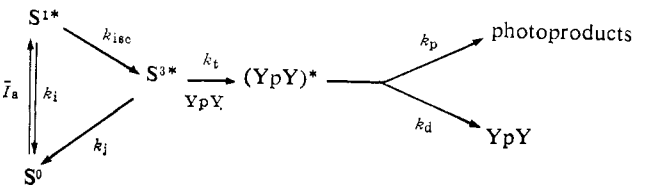
It is well established that photochemical dimerization of pyrimidines can be achieved by ketone-sensitized energy transfer without concomitant photohydration (Lamola and Yamone, 1967; Elad *et al.*, 1967; von Wilucki *et al.*, 1967; Krauch *et al.*, 1967; Greenstock and Johns, 1968; Ben-Ishai *et al.*, 1968). In view of this, it seems likely that dimer formation between adjacent pyrimidines in tRNA is at least partially responsible for the loss in aminoacyl acceptor activities that accompanies acetone-sensitized photochemistry of yeast $tRNA^{Tyr}$ and $tRNA^{Ala}$ (Chambers *et al.*, 1969). In order to establish a basis for further studies of this inactivation process, we have exmained several model compounds which represent potential dimer-forming targets in these tRNAs.

This paper describes kinetic studies which demonstrate that significant rate differences exist in the acetone-sensitized photochemistry of UpU, UpC, CpU, and CpC.[1] Results indicate that transfer of energy from sensitizer to acceptor is close to the diffusion controlled rate in all four cases and that differences in the partitioning of the excited dinucleoside phosphate to photoproducts and starting material, as shown in Scheme I, are a major source of the observed rate differences.

Results

Photoproducts. Although we have not examined the photoducts derived from acetone-sensitized photochemistry of the pyrimidine dinucleoside phosphate in any great detail, data presented in the Experimental Section indicate that irradiation of UpU at 312 nm in the presence of acetone (0.03–0.2 M) produced material with chromatographic properties similar to those described for isomeric dimers (Brown *et al.*, 1966). We were unable to detect any photohydrates. Acetone addition products, measured with [^{14}C]acetone, were found in small amounts (approximately 10%) when [acetone] = 0.2 M. The other pyrimidine dinucleoside phosphates, UpC, CpU, and CpC, also reacted under these conditions, presumably giving dimers, although the reaction products were not examined in detail. There was no evidence of any ultraviolet-absorbing photoproduct that might interfere with the spectrophotometric measurements described in the following section.

SCHEME I



Kinetics. According to the above scheme, disappearance of YpY is given by

$$\frac{-\mathrm{d[YpY]}}{dt} = k_t \phi_p [S^{3*}][\mathrm{YpY}] \qquad (1)$$

where $\phi_p = k_p/(k_d + k_p)$. If the usual steady-state assumption for [S^{3*}] is applicable, then the disappearance of YpY should be first order and proportional to $1 - 10^{-\epsilon l[S]_0}$, where $[S]_0$ = concentration of acetone added, ϵ = molar extinction coefficient of acetone at the exciting wavelength, and l = light path. As shown in Figure 1, first-order kinetics are obtained when a solution of UpU (2.5×10^{-5} M) and acetone (0.03–0.21 M) are irradiated under conditions where acetone is the only absorbing species (312 nm). This not only justifies the basic assump-

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[1] We shall use the abbreviation YpY to represent these compounds, as recommended by the IUPAC–IUB Commission on Biochemical Nomenclature.

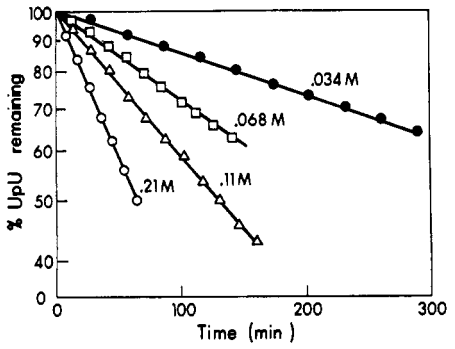

FIGURE 1: First-order kinetics of acetone-sensitized photochemistry of UpU at various concentrations of acetone. Two milliliters of UpU (2.5×10^{-5} M) in a cell with a 1-cm light path was irradiated in the presence of various concentrations of acetone, as indicated for each line. Change in [UpU], measured spectrophotometrically, was followed as a function of time. I_0 in this experiment was 0.05 μE min^{-1} cm^{-2}.

tion, but demonstrates that the photochemistry is indeed dependent upon acetone.

At steady state, $\bar{I}_a \cdot \phi_{isc} = k_t[S^{3*}][YpY] + k_j[S^{3*}]$, where $\bar{I}_a$ = light absorbed by sensitizer[2] and ϕ_{isc} = quantum yield for intersystem crossing of sensitizer from excited singlet to excited triplet, $k_{isc}/(k_{isc} + k_i)$. Solving for $[S^{3*}]$ and substituting into eq 1 gives

$$\frac{-d[YpY]}{dt} = v = \frac{\bar{I}_a \phi_{isc} \phi_p [YpY]}{K + [YpY]} \quad (2)$$

where $K = k_j/k_t$.

This equation predicts that at a low [YpY], where $[YpY] \ll K$, the rate will be first order with respect of [YpY] at a given acetone concentration and constant I_0. Conditions satisfying this requirement are shown in Figure 1. The equation also predicts that, at high [YpY], where $[YpY] \gg K$, the rate will approach a maximum, and the kinetics will approach zero order. In this case, $v = V_{max} = \bar{I}_a \phi_{isc} \phi_p$. Substituting this into eq 2 gives a familiar equation for saturation kinetics

$$v = \frac{V_{max}[YpY]}{K + [YpY]} \quad (3)$$

Thus, if the kinetic scheme is correct, v will approach V_{max} when [YpY] is high enough so that essentially every acetone triplet formed transfers its energy to [YpY]. However, both [acetone] and I_0 vary slightly from experiment to experiment, so it is necessary to normalize the rate data with respect to $\bar{I}_a$ (the amount of light absorbed by acetone). Since $v/\bar{I}_a$ = apparent quantum yield, ϕ, and $V_{max}/\bar{I}_a$ = maximum quantum yield, Φ, eq 3 can be written as

$$\phi = \frac{\Phi[YpY]}{K + [YpY]} \quad (4)$$

Experimentally, eq 4 was examined by measuring initial velocities normalized for the absorption of light by acetone, at various initial concentrations of YpY. Typical data for UpU are shown in Figure 2. The apparent quantum yields, ϕ, are calculated from the initial velocities. The data for UpU are plotted as a function of initial concentration in Figure 3. A typical saturation curve predicted by eq 4 is obtained, as required by the kinetic scheme.

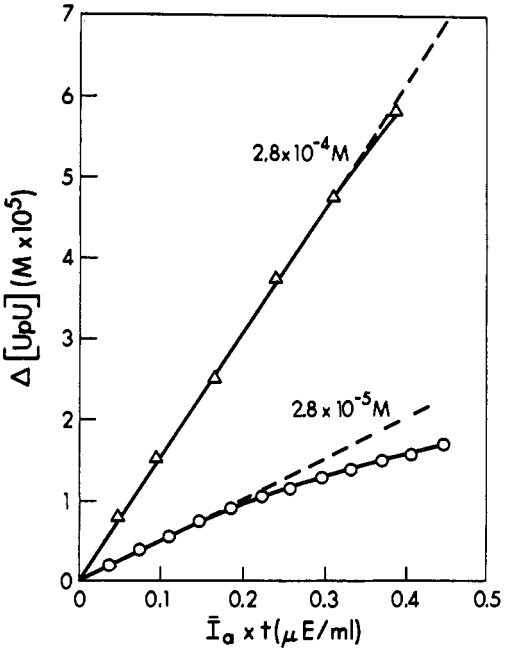

FIGURE 2: Kinetics of acetone-sensitized photochemistry of UpU at different initial concentrations of UpU, as indicated by the numbers above the curves. The dashed lines represent the initial rates of reaction. $\bar{I}_a$ corrects for slightly different I_0 and [acetone] in different experiments.

The maximum quantum yield, Φ, must be evaluated in order to compare the reaction rates of the different dinucleoside phosphates. Rearranging eq 4 gives

$$\frac{1}{\phi} = \frac{K}{\Phi} \frac{1}{[YpY]_0} + \frac{1}{\Phi} \quad (5)$$

Double-reciprocal plots of normalized initial velocity data for UpU, UpC, CpU, and CpC are shown in Figure 4. The straight lines obtained with all four compounds are consistent with the general kinetic scheme outlined above. The or-

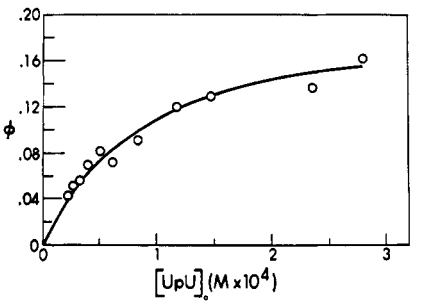

FIGURE 3: Saturation kinetics for acetone-sensitized photochemistry of UpU. ϕ is the apparent quantum yield, as defined in the text. This plot represents eq 4. The data were obtained from normalized initial velocity measurements similar to those shown in Figure 2.

[2] More precisely, $\bar{I}_a = I_0(1 - e^{-\epsilon l[S]_0})/l.$, which is the absorbed intensity averaged over the entire photolysis cell of path length, l. This assumes efficient stirring. Its units are Einsteins min^{-1} cm^{-3}. I_0 is expressed in Einsteins min^{-1} cm^{-2} (Shaw and Toby, 1966).

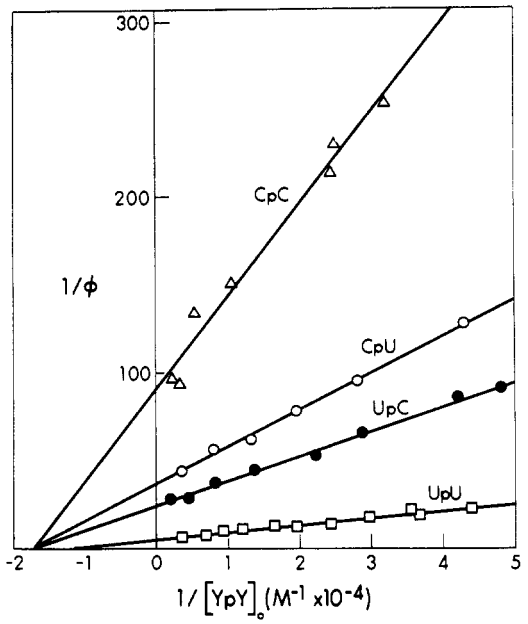

FIGURE 4: Evaluation of the sensitization constants and the maximum quantum yields for acetone-sensitized photochemistry of UpU, UpC, CpU, and CpC.

dinate intercept gives $1/\Phi$, and maximum quantum yields (Φ) for the four dinucleoside phosphates are summarized in Table I. Comparison of Φ values gives the relative rates for photoproduct formation from these compounds, normalized for the light absorbed by acetone.

The sensitization constant,[3] $K_s = k_t/k_j = 1/K$, can be evaluated from the slopes or the abscissa intercepts in Figure 4. These values are shown in Table I. Since k_j is a property of acetone, the comparison of the K_s values provide a measure of the relative efficiency of the energy-transfer process from sensitizer to different acceptors. The data shown in Figure 4 are not sufficiently precise to decide whether or not the small difference in k_t for UpU and the other dinucleoside phosphates is significant. It should be noted that the presence of an impurity in the UpU reaction mixture which can quench the acetone triplet would give a low value for K_s and could be responsible for the difference observed between UpU and the other dinucleoside phosphates.[4]

Since $\Phi = \phi_{isc}\phi_p$, and ϕ_{isc} is a property of acetone, comparison of the values for Φ shown in Table I provides a measure of the relative efficiency for converting the various excited dinucleoside phosphates to photoproducts. It is clear from the data in Table I that fundamental differences exist in the efficiency with which this process occurs with different dinucleoside phosphates, and this is the major cause of the observed rate differences. These findings are of considerable interest since similar inefficiencies have been noted in photochemical dimerization of uracil by direct irradiation (Wagner and Bucheck, 1968, 1970; Jellinek and Johns, 1970). Unfortunately, these three sets of data cannot be compared directly because of differences in experimental conditions. However, triplet sensitization provides an important technique for exploring the fundamental reasons for the observed inefficiencies without some of the complications that accompany direct irradiation experiments. The fact that the efficiency is dependent not only upon the composition of YpY, but also upon its sequence, is most intriguing.

TABLE I: Constants for Acetone-Sensitized Photochemistry of Some Pyrimidine Dinucleoside Phosphates.[a]

Compound	No. of Points Analyzed	Φ[b]	K_s[c]
UpU	19	0.20 ± 0.06	1.2 ± 0.3 × 10^4
UpC	8	0.041 ± 0.004	1.7 ± 0.2 × 10^4
CpU	6	0.027 ± 0.003	1.7 ± 0.2 × 10^4
CpC	7	0.011 ± 0.002	1.7 ± 0.5 × 10^4

[a] Data from Figure 4 were used to calculate the constants. Additional points are included for UpU. The lines were fitted by the method of least squares. The variation represents a 95% confidence level. [b] Maximum quantum yield, $\Phi = \phi_{isc}\phi_p$ (as defined in the text). [c] $K_s = k_t/k_j$ = sensitization constant as defined in the text.[3]

It should be noted that the absolute efficiency for conversion of (YpY)* to photoproducts is given by $\phi_p = \Phi/\phi_{isc}$. If ϕ_{isc} for acetone is 1.0 ± 0.1 in water as it is in methanol (Borkman and Kearns, 1966a), then $\Phi = \phi_p$, and the data in Table I also represent absolute efficiencies. The low value for dimerization of UpU (0.20) by acetone sensitization is particularly interesting, compared to the value of 0.56 obtained for dimerization of uracil and 0.52 for UpU by direct irradiation at 254 nm in water (Brown and Johns, 1968; Jellinek and Johns, 1970). However, ϕ_p is a composite that reflects the nature of the photoproducts as well as the nature of the precursor. We have found all three of the UpU dimers that Brown and Johns predicted should be formed by triplet sensitization (1968), but a detailed product analysis using radioactive substrates, though not part of this kinetic study, is clearly in order. It would be particularly interesting to know if the ratios of the three UpU dimers change since $\widehat{UpU}^1$ apparently can arise from both the singlet and triplet states, while $\widehat{UpU}^2$ and $\widehat{UpU}^3$ arise exclusively from the triplet state (Brown and Johns, 1968).

Regardless of the fundamental causes for the variations in Φ, it is clear that differences in the quantum yields exist for different dinucleoside phosphates. Therefore, the probability of altering these dinucleoside phosphate targets in tRNA will depend not only on the frequency of their occurrence, but also on their structure. A UpU sequence, for ex-

[3] The sensitization constant is usually defined as $K_s = k_t\tau_D^T$, where k_t is the bimolecular rate constant for triplet energy transfer from sensitizer to the acceptor and τ_D^T is the actual mean lifetime of the triplet donor. In the absence of the acceptor, this lifetime is $\tau_D^T = 1/k_j$, where k_j is the rate constant for first-order decay of the triplet (Wilkinson and Dubois, 1963; Borkman and Kearns, 1966a). Thus, $K_s = k_t/k_j$.

[4] The limits for k_t can be calculated from K_s and the values reported for the triplet lifetimes of acetone. From these, k_j has been estimated to be $\leq 0.33 \times 10^5$ sec^{-1} from triplet–triplet absorption (Porter and Windsor, 1958) and from phosphorescence emission studies (Borkman and Kearns, 1966b) in solution at room temperature; and 0.25×10^7 sec^{-1} from a quenching study (Wilkinson and Dubois, 1963). Using $K_s = 1.7 \times 10^4$ M^{-1} for CpC, CpU, and UpC (Table I), the limits, $4 \times 10^{10} > k_t > 6 \times 10^8$ M^{-1} sec^{-1}, are obtained for these compounds. For UpU, $K_s = 1.2 \times 10^4$ M^{-1} (Table I), and the limits, $3 \times 10^{10} > k_t > 4 \times 10^8$ M^{-1} sec^{-1}, are obtained. All of these values approach that expected for a diffusion controlled, bimolecular reaction in water at 20°, $k = 6.5 \times 10^9$ M^{-1} sec^{-1}, calculated from the Debye equation (1942), and summarized in convenient form by Lamola (1969).

ample, should be 18 times more susceptible than a CpC. However, ordered structure can play a very important role by lowering k_t. In fact, some potentially reactive targets may not react at all because they are inaccessible to excited acetone (Ben-Ishai *et al.*, 1968). In addition, base interactions may alter ϕ_p for a particular target. Therefore, considerable caution should be exercised in using these results for the interpretation of inactivation kinetics in tRNA.

It should also be noted that the minor nucleotide, pseudouridine 3′-phosphate, which occurs in all tRNAs, also undergoes two acetone-sensitized photoreactions, giving products which appear to be identical with those observed by direct irradiation of pseudouridine 3′-phosphate at 254 nm. One of these reactions involves cleavage of a phosphodiester bond and leads to fragmentation of the tRNA (Tomasz and Chambers, 1964, 1966). The second involves modification without release of inorganic phosphate to gave a photoproduct whose structure is still unknown (L. H. Schulman and R. W. Chambers, unpublished data). The preliminary kinetic data shown in Figure 5 indicate that the release of inorganic phosphate from pseudouridine 3′-phosphate proceeds at a rate similar to that of UpU photochemistry. Unpublished results also indicate that cleavage of yeast $tRNA^{Tyr}$ at the pseudouridine residue in its anticodon is a major photoreaction in this tRNA. Thus, pseudouridine photochemistry must also be considered as a possible inactivation event.

Because of the multiplicity of sites which might be inactivation targets and because of possible ordered structure effects, it is clear that kinetic studies alone cannot locate the cause of photochemical inactivation of tRNA. For this, detailed structural studies of the changes occurring in both active and inactive molecules after irradiation (Schulman and Chambers, 1969; Kucan *et al.*, 1970) will have to be pursued. The kinetic data presented here do, however, provide valuable guidelines for further studies at the macromolecular level.

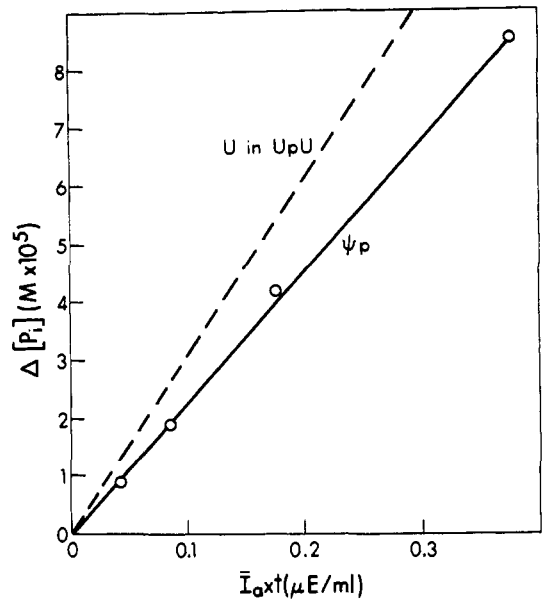

FIGURE 5: Release of inorganic phosphate by acetone-sensitized photolysis of pseudouridine 3′-phosphate. Initial concentration of Ψp was 5×10^{-4} M. The dashed line represents the photoreaction of UpU at the same pyrimidine residue concentration (*i.e.*, 2.5×10^{-4} M UpU). For the purpose of comparing the two rates, the photoreaction of UpU is represented here as moles of U residues reacted, and not moles of UpU, as in Figure 3.

Experimental Section

Compounds. UpU (Miles), CpC (Schwarz), CpU, and UpC (Gallard Schlesinger) were freed of contaminating nucleotides and nucleosides by paper chromatography on Whatman No. 3MM paper using solvent I (2-propanol–H_2O–concentrated NH_3, 7:2:1). Purified compounds gave single spots on thin-layer chromatography using MN-polygram cellulose 300 sheets (Macherey-Nagel and Co.) and solvents I, II (1-butanol–CH_3COOH–H_2O, 5:2:3), and III (*tert*-butyl alcohol–HCOOH–H_2O, 70:13:17). Pancreatic ribonuclease A (Worthington) digestion of each compound (2.5–5 A_{max} units[5] of YpY were incubated for 17 hr at 37° in 0.01 M phosphate buffer (pH 7) with 0.08 mg of enzyme, in which phosphatase activity was previously inactivated by heating for 20 min at 85°) gave only the expected nucleoside 3′-phosphate and nucleoside. Less than 2.5% UpC and CpU and less than 5% CpC and UpU remained undigested.

Irradiation Procedure. Radiation at 312 nm was obtained from a Bausch and Lomb high-intensity grating monochromator with a 200-W superpressure Osram mercury lamp (HB0200W) and a uv-visible grating (1200 grooves/mm). Entrance and exit slits were 2.4 and 1.35 mm, respectively. To reduce stray light, the beam leaving the exit slit was filtered through Corning 0-54 and 7-54 glass filters, and then focused by means of a quartz, planoconvex lens (38-mm diameter, 50-mm focal length). The sample cell, placed in a convenient holder, was positioned so that the maximum amount of light reached the solution. The intensity of the incident light just behind the front face of the photolysis cell (I_0^i) was determined for each experiment by ferrioxalate actinometry (Calvert and Pitts, 1966), using the same geometry and a quartz cell of identical optical properties as the sample cell. I_0^i varied from 0.2 to 0.06 $\mu E\ min^{-1}$ depending upon the individual lamp and the hours of use. During any single experiment, however, I_0^i was essentially constant as determined by actinometry at the beginning and the end of the irradiation. $\bar{I}_a$ was calculated from I_0^i the volume of the solution being irradiated and the absorbance of the acetone at the wavelength used for irradiation, *i.e.*, $\bar{I}_a = (I_0^i/l)(1. - 10^{-\epsilon l[S]_0})$. Note that I_0^i and I_0 have different units. I_0^i = total photons striking the solution per unit time. This quantity is obtained directly by ferrioxalate actinometry. $I_0 = (I_0^i/V) \times l$, where V = volume of the solution and l = light path.

Neutral, aqueous solutions of the compound to be irradiated were placed into a 10-mm light-path cylindrical or 2-mm light-path rectangular-necked quartz cell provided with a small stirring bar and stoppered with a rubber serum cap. Two no. 22 hypodermic needles were introduced through the stopper and prepurified nitrogen (AirCo, N. Y.) was bubbled gently through one into the stirred solution using the other as an outlet. After at least 30 min, the needles were withdrawn, and the absorbance of the solution at 290 nm (A_{290}) and at λ_{max} (A_{max}) was determined with a Cary 14 recording spectrophotometer. A small volume of previously nitrogenated acetone sufficient to make a final concentration of 0.1–0.2 M was injected into the reaction solution using a Hamilton microsyringe. The solution was stirred and A_{290} was determined again. The acetone concentration was calculated from

[5] One A_{max} unit is the amount of material in 1 ml of solution which gives an absorbance of 1.0 measured at λ_{max} in a cell with a 1-cm light path.

TABLE II: Distribution of Radioactivity from Acetone-Sensitized Photochemistry of UpU.

R_{UpU}[a]	Compound	Cpm	% Yield[b]
0	?	10,000[c]	
0.44	$\widehat{UpU}^3$		
0.56	$\widehat{UpU}^1$ [d]	50	1.6
0.68	$\widehat{UpU}^2$		
1.00	UpU	350	11.5
2.00	?	100	3.3
2.35	?	100	3.3

[a] Mobility relative to UpU on Whatman No. 50MM paper (descending; 2-propanol–95% ethanol–0.05 M potassium tartrate, pH 4.5; 30:40:40, v/v) (Moscarello *et al.*, 1961). [b] Calculated from a counting efficiency of 12.4% and based on a 1:1 acetone–UpU adduct where 1% product = 246 dpm. [c] This is a nonvolatile impurity present in the acetone. [d] These spots did not resolve completely. The assignment of structure is based on the R_{UpU} values given by Brown *et al.* (1966). The entire area was counted.

the difference in A_{290} before and after adding acetone to the YpY solution using ϵ_{290} 6.59 (determined for dilute aqueous solutions of acetone.[6] The same amount of acetone was added now to the aqueous solution in the reference cell so that the A_{max} reading of the sample was very close to the value it had before the addition of acetone. The sample cell was placed in the cell holder of the monochromator and stirred continuously during the irradiation. At suitable time intervals, the sample cell was returned to the spectrophotometer and A_{max} was read against the acetone-containing reference solution. Changes in [YpY] were calculated from these data using the molar extinction coefficients: UpU, ϵ 20,000; UpC, and CpU, ϵ 17,400; CpC, ϵ 16,800. These values, in turn, were obtained from absorption spectra (measured on a Cary Model 14 spectrophotometer at pH 7) and phosphate analysis as described previously (Reeves *et al.*, 1970). In spite of all precautions, some quenching by oxygen or impurities was occasionally noticed, and such experiments were rejected.

Identification of Photoproducts from UpU. A sealed ampoule containing 1.4 mg of [2-^{14}C]acetone (New England Nuclear Corp.) was cooled in a Dry Ice–acetone bath. Water was added to 1 cm above the mark, and the break seal was broken. The tube was opened, and the acetone–water solution was diluted to 2.0 ml. The solution was 1.21×10^{-2} M in acetone and gave 4.63×10^6 dps/ml measured with a Nuclear-Chicago Mark I scintillation spectrometer calibrated for counting efficiency with standard [^{14}C]toluene.

A reaction mixture was prepared as follows: UpU, 0.3 ml of a solution containing 31.8 A_{260} units/ml; 0.3 ml of [^{14}C]acetone solution described above; 2.40 ml of 0.250 M [^{12}C]acetone. This solution was 1.59×10^{-4} M in UpU, 0.2 M in acetone, and had a specific activity of 0.06 Ci/mole. Irradiation was carried out in the usual manner until 80% of the UpU had reacted, based on the disappearance of absorbance at 260 nm.

[6] The ϵ_{290} value for acetone varied slightly with concentration from 6.35 at 0.1 M to 6.59 at 0.3 M.

The reaction mixture was evaporated under reduced pressure at 35° to dryness. The residue was taken up in 100 μl of H_2O and 50 μl was spotted for chromatography. Inspection of the chromatogram under a short-wavelength ultraviolet lamp initially revealed only one spot corresponding to UpU. On additional exposure of the paper to ultraviolet light, spots corresponding to dimers of UpU appeared at the mobilities indicated in Table II. The chromatogram was also inspected for hydrates by saturating the paper with ammonia vapor. No additional ultraviolet-absorbing spots appeared during this procedure. The chromatogram was then scanned with a Nuclear-Chicago Actigraph III gas-flow counter for radioactivity. The results are summarized in Table II. The material at the origin is nonvolatile material apparently derived from acetone. The combined dimer area showed less than 2% activity, and this may, in fact, have been due to overlap with the large amount of activity at the origin. Approximately 11% activity was associated with the area corresponding to UpU, and this, in turn, may correspond to an acetone–UpU adduct. The nature of the nonultraviolet-absorbing, fast-moving materials with approximately 3% activity each is unknown. The identity of the dimers was confirmed by eluting the spot after irradiation with short-wavelength ultraviolet light and rechromatographing. Only a light spot of R_{UpU} = 0.48 and corresponding to $\widehat{UpU}^3$ was observed in the dimer region. All of the remaining material had been converted to UpU.

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Oxidation of Sulfhydryl Groups of Bovine Liver 2-Keto-4-hydroxyglutarate Aldolase by Tetranitromethane†

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ABSTRACT: Treatment of bovine liver 2-keto-4-hydroxyglutarate aldolase with a 42-fold molar excess of tetranitromethane at pH 8.0 and 20° rapidly and completely destroys both the aldolase and oxaloacetate β-decarboxylase activities of the enzyme. Various substrates and competitive inhibitors partially protect both enzymatic activities. Loss of aldolase and β-decarboxylase activities occurs concomitant with the modification of free sulfhydryl groups; four such groups are oxidized in the completely inactivated enzyme. Spectral and amino acid analyses demonstrate that the reaction is limited solely to cysteinyl residues. Partial recovery of enzymatic activity is obtained when the inactivated aldolase is incubated with an excess of several thiols. Cysteic acid is present in acid hydrolysates of the tetranitromethane-treated protein indicating that oxidized forms of cysteine other than disulfides are produced. The apparent Michaelis constants for substrates of the enzyme are not altered by modification of the protein with tetranitromethane. In contrast, tetranitromethane-inactivated aldolase does not bind either pyruvate or glyoxylate when aldolase is incubated with these compounds in the presence of sodium borohydride nor is glyoxylate stably bound by cyanide addition to the aldimine. Loss of both aldolase and β-decarboxylase activities, therefore, is most likely due to impaired Schiff-base binding of substrates. Since the native and oxidized enzymes show no significant differences in molecular weight or polyacrylamide gel electrophoretic mobility, the formation of more than one molecular species of the aldolase does not seem to occur.

Bovine liver 2-keto-4-hydroxyglutarate aldolase is a bifunctional enzyme catalyzing the β-decarboxylation of oxaloacetate as well as the reversible dealdolization of KHG.[1] The mechanism of aldol cleavage or condensation involves Schiff-base formation between the carbonyl group of KHG or pyruvate and the ε-amino group of a reactive lysyl residue in the protein molecule (Kobes and Dekker, 1966, 1971). The demonstration (Kobes and Dekker, 1967) that aldolase and β-decarboxylase activities are both lost by treatment of KHG-aldolase with substrates in the presence of $NaBH_4$ implicates a common functional role for the active site lysyl residue in the two catalytic activities of this enzyme. No information is presently available, however, on the nature of other aminoacyl residues in KHG-aldolase which may be essential for enzymatic activity.

Since its initial description as a mild and selective nitrating agent for tyrosine (Riordan *et al.*, 1966; Sokolovsky *et al.*, 1966), tetranitromethane has proved to be a convenient reagent to investigate the role of tyrosyl residues in protein structure–function relationships (Riordan *et al.*, 1967b; Cuatrecasas *et al.*, 1968; Atassi and Habeeb, 1969; Kassab *et al.*, 1970; Vincent *et al.*, 1970). It has been used successfully to delineate the chemical factors contributing to the unusual reactivity of phenolic side chains (Riordan *et al.*, 1967a; Cuatrecasas *et al.*, 1968), to demonstrate the occurrence of carbanion intermediates in enzymatic reaction mechanisms (Christen and Riordan, 1968; Riordan and Christen, 1969; Shlyapnikov and Karpiesky, 1969), and also to detect localized conformational changes that appear synchronous with enzymatic catalysis (Christen and Riordan, 1970). Amino acid residues other than tyrosine have been shown to react with tetranitromethane, notably sulfhydryl groups of rabbit muscle fructose diphosphate aldolase (Riordan and Christen, 1968) and a tryptophanyl residue in the extracellular nuclease of *Staphylococcus aureus* (Cuatrecasas *et al.*, 1968).

In an earlier brief report (Lane and Dekker, 1969b), we noted the rapid and concomitant loss of both aldolase and β-decarboxylase activities of KHG-aldolase when the enzyme is treated with low molar quantities of tetranitromethane under mild conditions. The data presented here indicate that this loss is associated with the preferential oxidation of four cysteinyl residues; disulfides and other oxidation products

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[1] The abbreviations used are: KHG, 2-keto-4-hydroxyglutarate; KHB, 2-keto-4-hydroxybutyrate; DTNB, 5,5′-dithiobis(2-nitrobenzoic acid); FDP, fructose diphosphate.