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PHOTOCHEMICAL REACTIONS OF THYMINE, URACIL, URIDINE, CYTOSINE AND BROMOURACIL IN FROZEN SOLUTION AND IN DRIED FILMS*

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Abstract—The photochemistry of uracil, uridine, cytosine, thymine and bromouracil has been investigated in frozen aqueous solution and in dried films. Essentially the same photoproducts were obtained in the two conditions; however, the yield of photoproducts was considerably greater in frozen solution. Uracil forms a dimer which can exist in two forms. Some kinetic data are presented for the interconversion of these two forms. The mixed dimer of thymine and uracil can also exist in two forms. Uridine forms only one acid stable photoproduct and does not appear to form mixed photoproducts under the conditions used. Two new photoproducts of thymine other than the dimer are described. Cytosine was at first considered to be completely inert but using more sensitive detecting equipment it has recently been found to form uracil dimer as a result of dimerization and deamination. The most remarkable response was shown by bromouracil. When irradiated by itself it formed no photoproducts but when irradiated in the presence of uracil, uridine, cytosine or NaOH it formed many photoproducts. Most of these products were devoid of bromide, but two still contained bromine. One of these has been identified as the mixed dimer of uracil and bromouracil while the other has been tentatively identified as the dimer of bromouracil. Dimers of thymine or bromouracil were not formed by X-rays.

IN 1928 GATES[10] pointed out the probable relation between the bactericidal effectiveness of the various wave lengths of ultraviolet light (u.v.) and the absorption of u.v. by deoxyribonucleic acid (DNA), but it was not until some twenty-one years later that a photochemical reaction of the pyrimidines was described that appeared to be of biological importance. In 1949 Sinsheimer and Hastings[20] reported the reversible photochemical alteration of uracil and cytidylic acid. It was ultimately shown by Moore[16] that this reaction, which involves the reversible hydration of the 5-6 double bond of uracil, yields 6-hydroxy, 5-hydro uracil. In 1960, Beukers and Berends[2] reported that the irradiation of thymine in frozen solution resulted in the formation of a thymine dimer. This dimer was also found in DNA when irradiated *in vitro*[6] and in the DNA of bacteria when irradiated *in vivo*[32]. We had been studying the radiation sensitizing effect of the incorporation of base analogs, such as 5-bromouracil[13, 14], into DNA. It seemed of interest to determine whether bromouracil were more sensitive than thymine to photochemical dimerization; such a finding would provide a chemical basis for the radiation sensitizing effect that incorporated bromouracil imparts to irradiated bacteria. A systematic search was therefore undertaken for u.v. irradiation products of C-14 labelled pyrimidines,

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irradiated singly or in pairs in frozen aqueous solution and in dried films. It was expected that these data would be helpful in evaluating the results obtained *in vivo*. The possible production of dimers by X-irradiation was also investigated. The present report deals with our findings *in vitro* and relates the identification of these photoproducts and some of their properties. Certain of these data have appeared in a preliminary communication[23] and in a review article[27]. A subsequent paper[28] will discuss the photoproducts, especially of thymine and bromouracil, obtained *in vivo*.

METHODS

The u.v. light source used was the Mineralight Model SL2537 (Ultra-Violet Products, Inc., San Gabriel, California). The intensity of the lamp at 2537 Å was about 685 ergs/mm²/min at 5 cm (at —20°C) and was monitored by uranyl oxalylate actinometry by the method of Bowen[7]. Thymine-2-C-14, bromouracil-2-C-14 and adenine (unlabelled) were obtained from Calbiochem (Los Angeles); uracil-2-C-14 from New England Nuclear (Boston, Mass.); uridine-C-14 (uniformly labelled) and cystosine-2-C-14 were from Schwarz BioResearch, Inc. (Orangeburg, N.Y.); Br-82-bromouracil was kindly supplied by Dr. Joseph P. Kriss[15]. The solutions of purines or pyrimidines were 0·5 mg/ml (unless otherwise stated) in water. About 200 μl of a solution or 100 μl of each of two solutions were mixed in a Pyrex spot plate and frozen in a freezer (—20°C) and irradiated (in a freezer, usually for 60 min) with the Mineralight so placed that the filter was 5 cm from the top of the sample being irradiated. The solutions were then thawed and aliquots (approximately 10^5 dpm) spotted on strips of Whatman #1 paper and chromatographed for about 18 hr (descending) in butanol/acetic acid/water (80/12/30). Table 1 lists representative Rf values in this solvent. The chromatograms were then photographed in ultraviolet light[22] and the radioactive areas were determined using a commercial strip scanner. For quantitation the radioactive areas were eluted in 0·1 *N* HCl and an aliquot counted in a liquid scintillation counter, using a counting solution previously described[25].

TABLE 1. R_f VALUES FOR SAMPLES CHROMATOGRAPHED IN BUTANOL/ACETIC ACID/WATER (80/12/30) ON STRIPS OF WHATMAN NO. 1 (DESCENDING)

	Spotted in 1 N HCl	Spotted in H_2O
Thymine	0·61	0·59
Uracil	0·47	0·47
Guanine	0·23	0·30
Cytosine	0·22	0·36
Bromouracil	0·61	0·60
Adenine	0·28	0·50
Thymine Dimer	0·26	0·24
Uridine	0·31	0·32
Uridylic Acid	0·09	0·09
Cytidine	0·15	0·23
Cytidylic Acid	0·04	0·07
Bromodeoxyuridine	0·62	0·60
Thymidine	0·58	0·57
Thymidylic Acid	—	0·09

RESULTS AND DISCUSSION

Formation and Identification of Photoproducts

The results are presented in Table 2 and Figs. 1 and 2. Many of the compounds or mixtures of compounds exhibited no photochemical response (Table 2). Those exhibiting a photochemical response are separately described below.

TABLE 2. PERCENT PHOTOCHEIMCAL CONVERSION IN FROZEN SOLUTION.

U*	69%	BU*	0%
BU*+U	64%	BU*+T	0%
U*+BU	44%	T*+BU	0%
T*	85%	T*+A	0%
T*+U	78%	BU*+A	0%
U*+T	68%	C*	0%
BU*+C	32%	C*+U	0%
T*+C	77%	C*+T	0%
U*+C	55%	C*+BU	0%
U*+A	36%	C*+A	0%
US*	22%		
US*+T	28%		
T*+US	63%		
US*+BU	21%		
BU*+US	21%		
BU*+NaOH (0·02*N*)	58%		

Experimental conditions are described in the section on Methods. Uracil is designated by U; bromouracil by BU; thymine by T; cytosine by C; adenine by A; and uridine by US. An asterisk (*) indicates that the compound is C-14 labeled.

Thymine. Thymine forms no mixed photoproducts with bromouracil in frozen solution; in fact, the presence of bromouracil completely inhibits the formation of photoproducts by thymine (Table 2). Adenine also inhibits the formation of thymine photoproducts (see addendum). Thymine does not react with cytosine (see addendum) but the presence of cytosine only slightly inhibits the photochemical reactivity of thymine. The presence of uridine or barbituric acid is also only slightly inhibitory to the photochemical reactivity of thymine. Attempts to dimerize thymine and the several aromatic amino acids in frozen solution were also unsuccessful. This was attempted in view of the observation of the *in vivo* crosslinking of DNA and protein by small doses of u.v.[24, 26, 27]. In the presence of uracil the total reactivity of thymine was somewhat reduced but mixed photoproducts were formed (see below).

Beukers *et al*[5] found that 5 per cent ethanol added to thymine at $10^{-4}M$ completely inhibited the formation of thymine dimer in frozen solution At $10^{-4}M$ thymine forms the same types and amounts of photoproducts as at 0·5 mg/ml (0·04 *M*). At 0·5 mg/ml the presence of 5 per cent ethanol had no effect upon the photochemical reactivity of the thymine. At $10^{-4}M$ no thymine dimer was produced in the presence of 5 per cent ethanol, as reported by Beukers *et al*[5]; however, we did find that about 6 per cent of the thymine was changed to a material with an R_f of 0·33 and 2 per cent to an R_f of 0·88. In an experiment to test the effect of quick and slow freezing on the formation of thymine photoproducts, only about 3 per cent more dimer was produced when the samples were slowly frozen at —20° than when they were quickly frozen at —78°.

The per cent photochemical conversion of thymine to photoproducts in frozen solution agrees with that reported by Wang[37] and Smietanowska and Shugar[21]. The extent of conversion was about 85 per cent. There was about 9 per cent conversion to a photoproduct having an R_f in our solvent of 0·13 and 76 per cent conversion to a product whose R_f was 0·24 (Fig. 1). This latter compound is the thymine dimer based upon equivalence in R_f values in isopropanol-HCl as described by Beukers *et al*[6] and Wacker *et al.*[32]. The minor photoproduct has the same R_f as the dimer in this solvent and probably explains why it was not detected by the above authors. If it is formed in irradiated DNA it would be difficult to demonstrate since it is largely destroyed by hydrolysis in trifluoroacetic acid or formic acid. As a consequence of acid hydrolysis some of the material with an R_f of 0·13 was converted to material having an R_f similar to that of the dimer, some to thymine, and some to unidentified products. Reirradiation of this R_f 0·13 material in distilled water (at 2537 Å) led to the formation of one mole of free thymine and a second product that contained two moles of thymine and chromatographed as did the dimer. The ordinary thymine dimer however, should have been completely converted to thymine under the

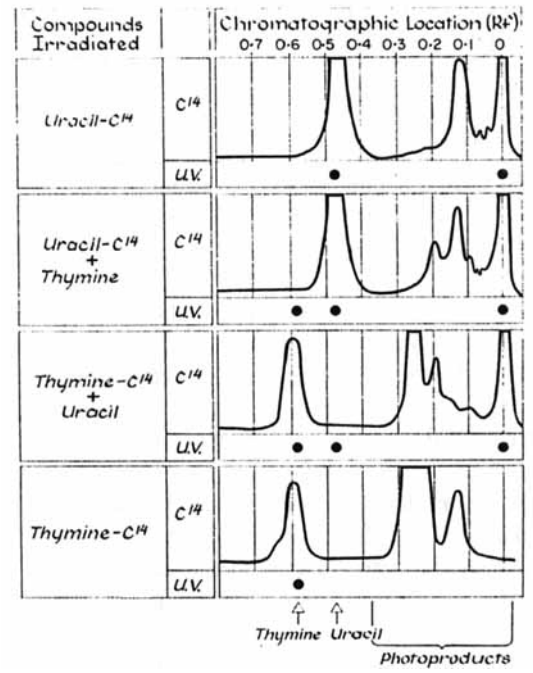

FIG. 1. Photoproducts or uracil and thymine. See section on Methods for experimental conditions and legend to Table 2 for explanation of symbols.

U*: R_f 0·01 and 0·12; different physical forms of uracil dimer; the two are interconvertible.

U*+T: R_f 0·0 and 0·19; mixed dimers or uracil and thymine (two forms are interconvertible, see U*)
R_f 0·12 uracil dimer

T*+U: R_f 0·0 and 0·19, see U*+T
R_f 0·24 thymine dimer

T*: R_f 0·24 thymine dimer
R_f 0·13 unknown photoproduct of thymine

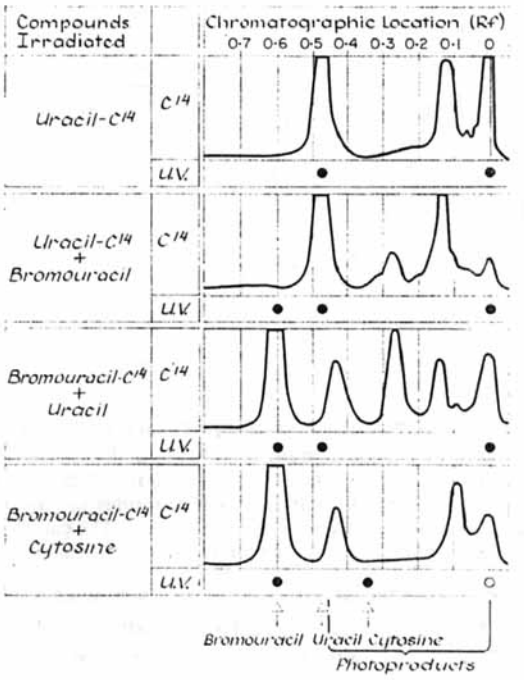

FIG. 2. Photoproducts of bromouracil and uracil. See section on Methods for experimental conditions and legend to Table 2 for explanation of symbols.

U*: R_f 0·01 and 0·12; different physical forms of uracil dimer; the two are interconvertible

U*+BU: R_f 0·00 probably uracil dimer, see U*
R_f 0·13 mixture of uracil dimer and unknown photoproduct of BU
R_f 0·26 a mixed dimer of uracil and bromouracil (still contains bromine)

BU*+U: R_f 0·00 photoproduct of bromouracil; has no bromine but absorbs in the u.v.; probably a dimer by a single carbon-carbon bond
R_f 0·13, see U*+BU
R_f 0·26, see U*+BU
R_f 0·44 probably a dimer of bromouracil (still contains bromine)

BU*+C: R_f 0·00, see BU*+U
R_f 0·09 unknown photoproduct of BU
R_f 0·44, see BU*+U

conditions of the experiment. This new thymine photoproduct is alkali labile. 1 hr in 0·1*N* NH_4OH converts it to two new products with R_f values of 0·17 (75 per cent) and 0·30 (25 per cent). Sixty minute irradiation ($4{\cdot}3\times10^3$ ergs/mm^2) in 0·1*N* NH_4OH gave rise to five new products [R_f: 0·01 (3 per cent); 0·09 (2 per cent); 0·17 (17 per cent); 0·28 (7 per cent); 0·38 (11 per cent); 0·46 (22 per cent); 0·62 (thymine; 38 per cent)].

The spectrum of this material (R_f 0·13) is quite different from that of the dimer (Fig. 3) in that there is very little absorption at wave lengths below 2600 Å but both have a peak at about 2820 Å. Per mole of thymine (measured on the basis of radioactivity content) the extinction for the dimer at this wave length is about one-fifth that of the R_f 0·13 material.

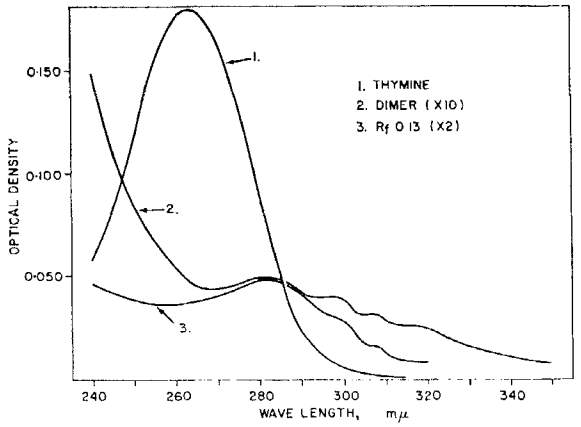

FIG. 3. Absorption spectra of thymine and its photoproducts

The radioactive areas on a chromatogram of irradiated thymine such as shown in Fig. 1 were eluted in 0·1 *N* HCl (as was a blank chromatogram) and their spectra determined. The amount of radioactivity was determined in each of the samples and the optical density readings were then normalized to an equal concentration of thymine. The curves therefore represent the [absorption spectra per unit amount of thymine

The thawed solution of irradiated thymine exhibits a peak at 3200 Å (Fig. 4)*. Unlike the thymine dimer, this peak is not destroyed by reirradiation at 2390 Å, but it is destroyed by reirradiation at 3100 Å (Setlow[18]). Recrystallized thymine dimer does not exhibit this peak yet the dimer area eluted from a chromatogram does show this (Fig. 3). Wang[38] finds a similar peak in irradiated DNA (proportional to A-T content) and reports that after treatment with photoreactivating enzyme it disappears. The importance of the enzyme is uncertain since this new thymine photoproduct is reversed by long wave light alone. The 3200 Å peak in irradiated DNA is not reversed by short wave light[19].

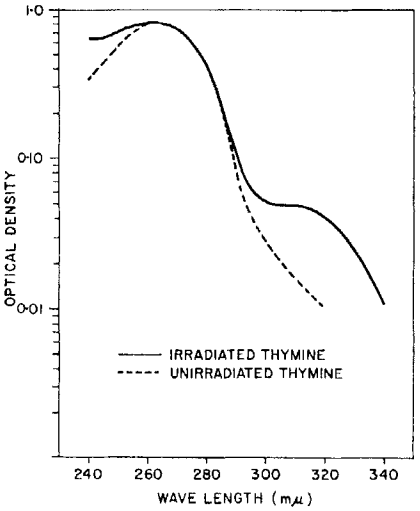

FIG. 4. The absorption spectra of irradiated and non-irradiated thymine

Thymine was frozen and irradiated (at 2537 Å) with $6{\cdot}7\times10^4$ ergs/mm^2, thawed, made to 0·1 *N* HCl and its spectrum determined. The spectrum of unirradiated thymine, normalized to give the same reading at 262 mμ as the irradiated sample, is also included for comparison.

* This phenomenon was called to the author's attention by Dr. R. B. Setlow (unpublished observations).

Other photoproducts of thymine are produced *in vivo* and will be described in a subsequent publication[28].

Uracil. When uracil was irradiated under our standard conditions about 69 per cent of it was converted to photoproducts (Table 2, Fig. 1). The number and relative amount of the photoproducts was a function of the conditions used both before and after irradiation. If the irradiated sample was spotted on a chromatogram immediately after thawing and developed in the chromatographic solvent only one photoproduct was observed. This material had an R_f of about 0·12 in our solvent and is presumably a photodimer of the type found for thymine[3]. If instead of spotting the irradiated material immediately after thawing, it was either allowed to stand at room temperature for up to 24 hr or at 4° for up to 6 hr the amount of the first photoproduct diminished and a second product appeared with an R_f of 0·01 (Fig. 5). The process can be considerably accelerated by simply refreezing the solution for a few minutes.

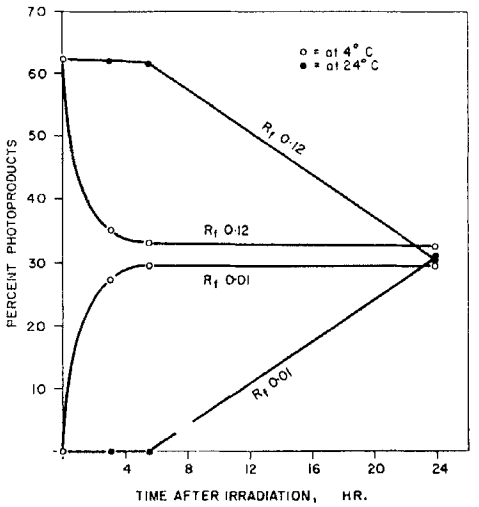

FIG. . he interconversion of the two forms of uracil dimer

Experimental conditions are described in the text. After thawing, the u.v. irradiated samples of uracil-2-C-14 were allowed to stand either at 4° or 24° and aliquots were removed at various times for chromatography to determine the relative amounts of the two forms of the uracil dimer present in each aliquot. The form with R_f 0·01 can be reconverted to the form with R_f 0·12 by acid, alkali or heat.

The relative formation of these two photoproducts of uracil was also very much dependent upon the concentration of the uracil solution. In very dilute solutions the total amount of uracil photoproduct formed was maximal and the formation of material with an R_f of 0·01 was essentially zero (when chromotographed immediately after thawing). As the concentration of uracil increased the total amount of photoproducts formed decreased for the same dose of u.v. (probably due to shielding of the uracil molecules lower in the frozen solution). As the uracil concentration increased the material with an R_f of 0·01 appeared and its concentration continued to increase while the amount of material with an R_f of 0·12 decreased (Fig. 6).

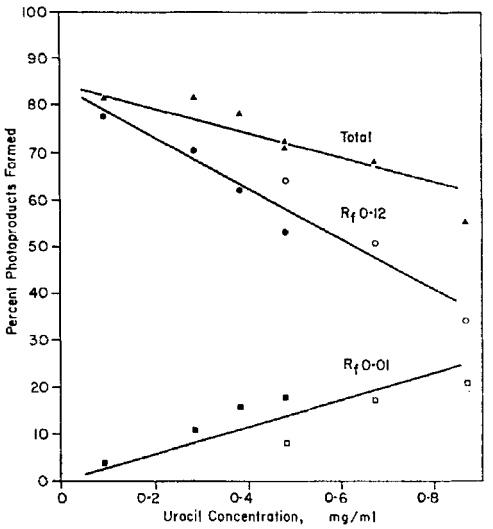

FIG. 6. The formation of uracil dimer at 2537 Å as a function of the concentration of uracil. Solutions of the several concentrations of uracil were frozen and irradiated as described in the section on Methods. The samples were then thawed and immediately spotted on chromatograms. The curve labeled TOTAL gives the total amount of uracil dimer formed. The R_f numbers refer to the two forms of uracil dimer (see Fig. 1). The closed squares and circles refer to those samples which took about 35 min to freeze while the open squares and circles took about 20 min to freeze in the freezer.

The isolation of a photoproduct of uracil has been previously described by severa authors. Wacker *et al*[36] irradiated frozen uracil at 0·06 mg/ml and obtained only one photoproduct. We have determined this material to be identical to the photoproduct having an R_f of 0·12 in our solvent. The results in Fig. 6 would predict the formation of only one photoproduct at this concentration. Wang[37] irradiated uracil at 0·11 mg/ml and presumably also would have obtained the material with R_f 0·12. Smietanowska and Shugar[21] irradiated at 0·9 mg/ml and would have obtained a mixture of both photoproducts. We have performed a bulk isolation of photoproducts when uracil was irradiated at 1·0 mg/ml. The material subsequently isolated after several recrystallizations was exclusively the material with an R_f of 0·01. (Identified by chromatography and reirradiation of the two areas (R_f 0·01 and 0·12) eluted from the chromatogram. The appearance of u.v. absorbing material (free uracil) occurred only after reirradiation of the solution containing the extract of R_f 0·01. The photoproduct having an R_f of 0·12, however, was present in the mother liquor of the first crystallization.)

The photoproduct with an R_f of 0·12 is probably the simple dimer of uracil. A product formed when thymine and uracil are irradiated together (mixed dimer of thymine and uracil) falls in the central position (R_f 0·19) between the thymine dimer (R_f 0·24) and the material with R_f 0·12 (see below). The R_f 0·12 material also responds to reirradiation in alkaline solution in a manner similar to the thymine dimer, yielding, however, uracil as the product. As we have seen the R_f 0·12 photoproduct can be easily changed to the photoproduct with an R_f of 0·01 by standing at room temperature or at 4° and it can be accelerated by freezing and thawing of the solution. The change from R_f 0·12 to R_f 0·01 is apparently not an all-or-nothing change. If the sample is allowed to freeze slowly, irradiated, thawed and then not refrozen before chromatography numerous small peaks of radioactivity appear between

the main peaks at R_f 0·12 and R_f 0·01. We have assumed these products to be in transition between R_f 0·12 material and R_f 0·01 material. The material with an R_f of 0·01 can be reconverted to the R_f 0·12 material by treatment with acid, alkali or heat. Since these two products are readily interconvertible it would appear that the two are related but differ in physical form. Both compounds have the same spectrum, which resembles that of the thymine dimer[12, 17], that is, they only show "end absorption". On reirradiation in solution at 2537 Å both compounds yield uracil at about the same rate. In Figs. 1 and 2 it is indicated that the uracil photoproduct at the origin on the chromatogram is u.v. absorbing, as judged by photography using light at 2537 Å, yet when eluted from the chromatogram it showed very little absorption at this wave length. The material at the origin is probably in crystalline form and is blocking the transmission of the u.v. light passively rather than by true absorption. A similar explanation is probably also applicable to the more insoluble form of the mixed dimer of thymine and uracil (see below).

Uracil does not form a mixed photoproduct when irradiated in frozen solution with cytosine (see addendum). The presence of cytosine is only slightly inhibitory to the photochemical response of the uracil. The presence of adenine markedly reduces the yield of uracil photoproducts. Uracil forms a mixed photoproduct with thymine (see below). Uracil not only enhances the photochemical reactivity of bromouracil but also forms a mixed dimer with bromouracil (see section on bromouracil).

Quick freezing (dry ice) results in about 16 per cent less photoproducts than slow freezing in the freezer at minus 20° and the loss is in the material with R_f 0·01. (Experimental sequence: fast or slow freezing, irradiation, thawing, refreezing and chromatography)

The problem of the chemical lability of uracil dimer has been raised. Beukers and Berends[1] found that reconversion of the uracil photoproduct to uracil by reirradiation was not accomplished "if the time between the two irradiations was too long. It is assumed that the reaction product of uracil is rather unstable". We have found that the more insoluble form of the uracil dimer (at the origin on chromatogram) is progressively formed from the more soluble form by standing at room temperature after irradiation (Fig. 3). It is possible that the more insoluble form of the dimer precipitated out of solution on standing (as it does in our hands) and could explain the results of Beukers and Berends just cited.

A more serious claim for the instability of uracil dimer has been made by Wacker *et al*[29, 30, 36] who reported that it is not very stable in acid or alkili or even in water at 100°C for 15 min. It is difficult to explain the observations of these authors on the lability of uracil dimer when Smietanowska and Shugar[21] found it "will withstand heating for 1 hr in 10 *N* HCl 0⁴. We have also found it to be quite stable to a variety of conditions as shown in Table 3. Failure to isolate uracil dimer from irradiated polymers would therefore *not* seem to be due to its chemical lability (unless its presence in a polynucleotide chain confers lability) but is more probably related to the frequency of neighbouring uracil residues and to the inefficiency of dimer formation because of competing hydration reactions[27].

Mixed dimers of thymine and uracil. Mixed dimers of thymine and uracil are formed (see also Beukers and Berends[2]; Wacker *et al.*[31, 36].) Analogous to the situation with uracil, two forms of the dimer are produced. The one at R_f 0·19 (Fig. 1) predominates immediately after irradiation but after standing in the cold, it was largely converted to the form having an R_f of 0·0. The origin material can be reconverted to the material at R_f 0·19 by heating at 75° for 5 min.

TABLE 3. THE CHEMICAL STABILITY OF URACIL DIMER

Experiment No.	% Uracil Dimer				% Destruction of Dimer
	1	2	3	Average	
Control	69·9	71·5	66·6	69·3±1·8	0·0%
10 min at 100°C	67·2	70·1	69·0	68·8±1·0	0·7%
1 *N* NH_4OH	69·9	70·5	67·4	69·2±1·2	0·1%
Dry: in HCl	69·4	69·4	68·1	69·0±0·6	0·4%
TFA at room T	67·7	67·5	66·9	67·4±0·3	2·8%
TFA at 155°C	59·4	65·2	59·8	61·5±2·5	11·3%

Uracil-2-C^{14} (1·5 μc/ml; 0·5 mg/ml) was frozen and irradiated at —20°C with 4×10^4 ergs/mm^2 (2537Å). The solution was thawed and an aliquot (20 μl) spotted on a chromatogram. Another aliquot was heated at 100° for 10 minutes and then spotted. A third aliquot was made to 1 N NH_4OH (with 2 N NH_4OH) and allowed to stand at room temperature for 60 minutes and then was spotted on a chromatogram. A fourth aliquot was taken to dryness in a vacuum desiccator and then dissolved in 1 N HCl for spotting. A fifth and sixth aliquot were taken to dryness and then dissolved in trifluoroacetic acid (TFA) and sealed in tubes. One tube stood at room temperature while the other was heated at 155°C for 60 minutes[9]. The two samples were than taken to dryness over NaOH and dissolved in 1 N HCl for spotting. The chromatograms were run in butanol/acetic acid/water (80/12/30; v/v/v) and the distribution of the radioactivity determined and quantitated using a Vanguard strip scanner and Automatic Data System.

For purposes of identification an experiment analogous to that described in Fig. 1 was performed except that both the uracil and the thymine were C-14-labeled. The resulting four photoproducts were isolated from the chromatograms and then resubmitted to chromatography so that contaminating photoproducts from adjacent peaks could be removed. The purified photoproducts were then reirradiated in alkaline solution (0·1*M* NH_4OH) at 2537 Å and the increase in optical density at 2600 Å was followed to a maximum value. This required about 30–60 min at 5480 ergs/mm^2/min to reach the plateau. The solutions were again chromatographed and quantitated. Knowing the specific activity of the uracil and thymine, the molar amounts of monomers liberated could be determined. The peak at R_f 0·24 yielded only thymine and was therefore the thymine dimer. The peak at R_f 0·12 yielded only uracil and was therefore the uracil dimer. The origin peak yielded $12\cdot7\times10^{-2}$ μM uracil and $6\cdot9\times10^{-2}$ μM of thymine indicating that this material was a mixture of uracil dimer and uracil-thymine mixed dimer. The material with an R_f of 0·19 yielded $3\cdot7\times10^{-2}$ μM uracil and $3\cdot3\times10^{-2}$ μM of thymine indicating that this was in fact a mixed dimer of thymine and uracil. The absorption spectrum for the thymine-uracil dimer was quite similar to that described for the thymine dimer[12, 17]. The thymine-uracil dimer was much more insoluble in water than either the thymine dimer or the uracil dimer. If the crystals were still wet with the mother liquor they would dissolve in 1*M* NH_4OH at room temperature. If the crystals were dried then the only solvent found that would dissolve them at room temperature was 1*N* NaOH.

Uridine. The per cent photochemical conversion of uridine (22 per cent) agrees with that reported by Wang[37]. Only one photoproduct was detected and this had an R_f of 0·05 in our acidic solvent system. In similar experiments, Wacker *et al.*[36] used a neutral chromatographic solvent and were also able to demonstrate the presence of the hydrated photoproduct of uridine (which reverts to uridine in acid). Uridine did not appear to interact photochemically with any of the compounds with which it was mixed (Table 2). The presence of thymine or bromouracil did not effect the photochemistry of uridine and the presence of uridine did not grossly suppress the photochemical reactivity of thymine.

The presence of uridine, however, made bromouracil photochemically labile. Three debrominated photoproducts of bromouracil were formed which appeared to be identical with the three debrominated photoproducts produced when bromouracil was irradiated in the presence of uracil. Unlike uridine, however, uracil elicited the formation of two additional photoproducts of bromouracil that still contained the bromine group (see section on bromouracil).

Cytosine. Under the conditions used in the present experiments cytosine was completely inert to photochemical alteration (see addendum) either by itself or in combination with any other compound that we have tested (Table 2) (see also Beukers *et al*[4]). Its effect upon the photochemical response of bromouracil, however, was quite dramatic (see section on bromouracil), although it appeared to have little effect upon the photochemistry of thymine or of uracil.

However, at about one-fifth the concentration of cytosine used here and at about ten times the dose of u.v., there was a 7 per cent conversion of cytosine to a photoproduct behaving like uracil dimer (Wacker[30]). It has been shown that cytosine is very susceptible to deamination when its 5-6 double bond is saturated[11]. We have confirmed this response and also find that when this product is reirradiated in solution uracil is formed. It has also been reported that uracil was isolated from irradiated DNA labeled with cytosine-2-C-14[8].

Bromouracil. Under the conditions used, bromouracil was completely resistant to photochemical alteration in frozen solution (see also Wacker *et al*[29, 33, 35]). When mixed with thymine it completely inhibited the photochemical reactivity of thymine (Table 2). The presence of adenine had no effect upon the photochemistry of bromouracil (see addendum). Bromouracil had no effect upon the photochemistry of cytosine; however, the presence of the cytosine had a marked effect upon the photochemistry of the bromouracil. In the presence of cytosine, which itself undergoes no photochemical alteration under these conditions, bromouracil was converted to the extent of 32 per cent to three photoproducts. Uracil caused the conversion of 64 per cent of the bromouracil to photoproducts. At least one of these photoproducts was different from those produced in the presence of cytosine (see below). In the presence of uridine bromouracil formed most of the photoproducts formed in the presence of uracil except that those photoproducts with R_f values above 0·2 were missing. In the presence of 0·01 *N* NaOH, bromouracil was quite photochemically reactive when irradiated in frozen solution. At this pH the absorption maximum of bromouracil is shifted from 276 mμ to 298·5 mμ. Photoproducts similar in R_f to those obtained in the presence of uridine were obtained plus another one that had an R_f similar to that for uracil. All the photoproducts produced in the presence of the NaOH were debrominated as judged by parallel experiments with C-14 labeled bromouracil and Br-82 labeled bromouracil. The following compounds added to bromouracil did not stimulate the production of photoproducts: an equal volume of NaCl or benzoic acid at 0·5 mg/ml; glycerine or ethanol at a final concentration of 5 per cent; formamide at a final concentration of 10 per cent; HCl at a final concentration of 0·01 *N* or 0·1 *N*; magnesium, ferric, ferrous, uranyl or cobalt salts at $5\times10^{-4}M$.

When bromouracil and uracil were irradiated together in frozen solution, one of the new compounds had an R_f of 0·26 in our butanol-acetic acid solvent (Fig. 2). Since this material was labeled regardless of whether the bromouracil or the uracil was labeled, it seemed to be a mixed photoproduct of bromouracil and uracil. Experiments using Br-82 bromouracil and unlabeled uracil demonstrated that this photoproduct still contained

bromine. One way of identifying this new compound as the bromouracil-uracil mixed dimer would be to label it both with Br-82 bromouracil and uarcil-2-C-14. The radioactivity content of this compound could be determined at zero time and again after the Br-82 had decayed (36 hr half life). Knowing the specific activity of the Br-82 bromouracil and the uracil-2-C-14 one could then calculate the relative content of the two compounds present in the material whose R_f is 0·26. Such an experiment indicated that it contained $0{\cdot}343 \times 10^{-8}$ moles of uracil and $0{\cdot}375 \times 10^{-8}$ moles of bromouracil. The ratio of U/BU was 0·92 indicating that this compound is a mixed photoproduct containing equal moles of uracil and bromouracil, and is presumably a mixed dimer analogous to the thymine-uracil mixed dimer.

Reirradiation of this material (2537 Å) in 0·1 *N* HCl led to the disappearance of material at R_f 0·26 and the appearance of material at R_f 0·47 (uracil) and material at R_f 0·0. The sample was lost before it could be determined whether or not Br-82 was still present after this reirradiation. Resubmitting the R_f 0·26 material to chromatography after the Br-82 had decayed gave only a trace of uracil and one large peak at R_f 0·30. If bromouracil had been transformed to uracil as a consequence of the decay of the Br-82 one would have expected an R_f of 0·12 or 0·01, the R_f values for the uracil dimers.

In the above experiment the material with an R_f of 0·26 (uracil–bromouracil dimer) was eluted from the chromatograms in 0·1 *N* HCl, evaporated to dryness in a vacuum desiccator, and redissolved in 1 *N* HCl for spotting on the chromatograms. Under these conditions very little of the material was degraded. If, however, the uracil-bromouracil dimer (labelled with bromouracil-2-C-14) was eluted from the chromatograms in water at room temperature, more than 70 per cent of the material was converted to five or six other products. A few minutes treatment with 1 *N* NH_4OH will also destroy this material. The uracil–bromouracil dimer is therefore only stable in acid.

When bromouracil was irradiated in the presence of uracil the photoproducts having an R_f below 0·2 were devoid of bromine. The photoproduct with an R_f of 0·43 still contained bromine and appeared to be identical with a photoproduct of bromouracil produced in the presence of cytosine and having the same R_f.

Of the photoproducts of bromouracil produced in the presence of cytosine only the one with an R_f of 0·43 still contained bromine, as judged by repeating the experiment with Br-82 labeled bromouracil (Fig. 2). Since uracil dimer has an R_f of 0·12 and the mixed dimer of uracil and bromouracil an R_f of 0·26, one would expect to find the bromouracil dimer at an R_f of about 0·40. We did find a bromouracil photoproduct at an R_f of 0·43 that still contained bromine. The assumption that this material is the bromouracil dimer seems therefore not unreasonable. Experiments to test this conclusion directly, however, have thus far failed. The planned experiment was to isolate the material whose R_f was 0·43 from a series of chromatograms and then reirradiate it in solution, in the manner used to split the thymine dimer to thymine, and thereby recover bromouracil. Unfortunately, the process of eluting this material from the chromatogram with water at room temperature and then taking it to dryness in a vacuum desiccator led to its destruction. Rechromatography indicated the presence of nine radioactive peaks. One of these peaks (40 per cent of the total radioactivity) has been identified as uracil and a second (3·4 per cent) as bromouracil. Judging from the results obtained with the mixed dimer of bromouracil and uracil, this experiment should be repeated with this compound kept at all times in strong acid. Positive proof that this material is the bromouracil dimer will have to await these results, although the evidence cited strongly suggests this identity.

The presence of bromouracil almost completely inhibits the formation of the uracil dimer that has an R_f of 0·0 but the presence of the uracil causes the formation of a photoproduct of bromouracil with this same R_f (Fig. 2). Ultraviolet photographs of the chromatograms indicated that this material at the origin still possessed ultraviolet absorption at around 2537 Å (the wavelength of the light used to prepare the photographs). When this material was eluted from the chromatogram it was found to have a sharp symmetrical spectrum with a maximum at 2920 Å and a minimum at 2600 Å. Clearly this cannot be uracil, bromouracil or a dimer of the type described for thymine. Moreover, this material is devoid of bromine. Since the position of this material on the chromatogram is similar to that found for several dimers (Fig. 1), it is postulated that this material may be a dimer by a single carbon-carbon bridge analogous to the formation of biphenyl by the u.v. irradiation of iodobenzene in benzene as described by Wolf and Kharasch[39]. Final proof, however, must await the isolation of sufficient material for chemical analysis.

Effect of X-Rays on Dimer Formation

X-irradiation was carried out at 250 kV, 15 mA, 30 cm distance, 0·25 mm Cu + 1·0 mm Al filter, 1·10 mm Cu HVL, dose rate 360–390 r/min. 10kr and 20kr had no effect on the u.v. absorption or chromatographic behaviour of thymine-2-C-14, bromouracil-2-C-14, thymine-2-C-14 plus uracil or bromouracil-2-C-14 plus uracil at approximately 0·5 mg/ml (in water) either irradiated frozen (dry ice) or at room temperature. However, when a dilute solution ($10^{-5}M$ in 0·1 *M Tris*, pH 7·5) of thymine or bromouracil was irradiated with 20kr there was about 50 per cent destruction of absorbancy at 260 mμ. This destruction caused a change in absorbancy of 0·246 optical density units, and probably resulted from the indirect effect of the X-rays on the water. In the concentrated solutions (optical density 33·936), a change of 0·246 optical density units would have been less than a 1 per cent change and may not have been detected. Using a much higher dose of X-rays (1200kr) Wacker and Lochmann[34] also found no dimerization of thymine-2-C-14 although other products were formed.

Irradiation of Dried Films of the Pyrimidines

Wang[37] found that dimers of uracil and thymine could be formed by the irradiation of dry films of the monomers as well as in frozen solution; however, the relative yields

TABLE 4. PER CENT PHOTOCHEMICAL CONVERSION OF PYRIMIDINES IN DRIED FILMS

U*	9%	(69)
T*	17	(85)
BU*	0	(0)
T*+U	17	(78)
U*+T	22	(68)
BU*+U	11	(64)
U*+BU	9	(44)
BU*+C	9	(32)
C*	0	(0)

Experimental conditions were as described in the section on Methods except that the solutions were first allowed to evaporate to dryness at room temperature before irradiation. Irradiations were carried on at room temperature where the intensity of the u.v. lamp is about five times greater than at —20°. Nevertheless, when a dry film of thymine was irradiated in the freezer (and thus at the dose given in Table 2) the photochemical conversion was only reduced by 3 per cent (to 14 per cent). Abbreviations and symbols are identified in the legend to Table 2. The values in parentheses are taken from Table 2 and are the per cent photochemical conversion in frozen solution.

under the two conditions were not given. We investigated the use of dry films to see if this might increase the yield of photoproducts or cause the formation of photoproducts not formed in frozen solution. The results are given in Table 4. For uracil only the material with an R_f of 0·12 was detected. Similarly for thymine only the material whose R_f was 0·24 was apparent. The other mixtures showed all of the photoproducts that were formed in frozen solution. It would appear that the only real difference in the results between the dried film and the frozen solution experiments is that the yield of photoproducts is greatly reduced with dried films.

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ADDENDUM

Having recently obtained a more sensitive radioactive strip scanner we decided to re-examine several of our early chromatograms that had shown no evidence of photoproduct formation. Cytosine now showed the formation of a photoproduct to the extent of 3–5 per cent at R_f 0·12 (uracil dimer appears at R_f 0·12) whether irradiated by itself or in the presence of bromouracil, thymine, or adenine. In the presence of uracil there was about twice as much conversion of the cytosine to this same photoproduct. This product would appear to be the dimer of uracil. When thymine-2-C-14 was irradiated in the presence of cytosine, a third photoproduct was apparent besides the two normally produced by thymine. The R_f of the new peak is 0·20 which is the R_f for the mixed dimer of uracil and thymine. Thymine irradiated in the presence of adenine also exhibits a new product at R_f 0·34. Bromouracil by itself or with thymine still showed no photoproducts; however, in the presence of adenine three photoproducts were evident, with about 10 per cent conversion of bromouracil.

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