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The Response of Uracil-2-^{14}C to X-Irradiation under Nitrogen and Oxygen and to Treatment with Ascorbic Acid[1]

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SMITH, KENDRIC C., AND HAYS, JAMES E. The Response of Uracil-2-^{14}C to X-Irradiation under Nitrogen and Oxygen and to Treatment with Ascorbic Acid. *Radiation Res.* **33,** 129–141 (1968).

The following compounds were produced when solutions of uracil-^{14}C were irradiated with X-rays: dihydrouracil, 6-hydroxy-5-hydrouracil (under nitrogen but not oxygen), isobarbituric acid, *cis*- and *trans*-glycols of uracil, alloxan, alloxantin, and dialuric acid. When uracil was treated with ascorbic acid–$FeSO_4$, the results were similar in many respects to those obtained after X-irradiation except that more dihydrouracil was produced relative to the more oxidized products (glycols, etc.), as compared to the results after X-irradiation. Auto-irradiation of an aged sample of uracil-^{3}H also mimicked in certain respects the effect of X-irradiation on uracil. The major products identified were the *cis*- and *trans*-glycols of uracil.

INTRODUCTION

It has been demonstrated that deoxyribonucleic acid is the target of primary radiobiological importance within a cell (*1*, *2*). If we are to understand the chemical basis by which ionizing radiation exerts its effects on cells, we must understand the mechanism of action of ionizing radiation on the nucleic acids. However, our understanding of the primary effects of ionizing radiation on the nucleic acids within cells is hampered by the paucity of knowledge concerning the chemical reactions that the purine and pyrimidine bases undergo when exposed to ionizing radiation *in vitro*. Since the pyrimidines are more radiation-sensitive than the purines (*3*), relatively more data are available on the pyrimidines. In the few papers that have been published on the radiation chemistry of the pyrimidines, however, there is

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frequently a lack of agreement in both the qualitative and the quantitative data from the different laboratories (*3–12*). The extreme dependence of G values (number of molecules altered per 100 eV absorbed) on pH, oxygen, and impurities is probably contributory. Also, much of the work published on the radiation chemistry of the nucleic acid constituents was performed before the ready availability of purines and pyrimidines labeled with radioactivity. Previous analyses were based on ultraviolet absorption and classical chemical procedures, and many products would have been undetected or lost by using only these techniques.

We have selected isotopically labeled uracil as a model compound for the study of the radiation chemistry of the pyrimidine bases. We have used paper chromatography to study the changes in uracil produced by X-irradiation under nitrogen and oxygen. We have also studied the fate of uracil when incubated in the presence of the hydroxylating agent ascorbic acid and the products formed by auto-irradiation, using an aged sample of tritium-labeled uracil.

MATERIALS

Uracil-2-^{14}C (22.7 mCi/mmole) was obtained from Calbiochem, and uracil-^{3}H (9.4 mCi/mg) from New England Nuclear. Uracil, alloxan, alloxantin, dialuric acid, and dihydrouracil were obtained from Calbiochem. Isobarbituric acid was obtained from Sigma Chemical Company.

METHODS

Chromatography

Descending chromatography was performed on 1½-inch-wide strips of Whatman No. 1 paper. Two solvents were used: (1) *n*-butanol–water (86:14) and (2) *n*-butanol–acetic acid–water (80:12:30) (*13*). The strips were chromatographed in solvent 1 for about 22 hours, air-dried, and then resubmitted for a second time in this solvent. This procedure gave a better separation of some of the adjacent materials. On these chromatograms, the position of a particular material was located relative to that for uracil taken as 1.0 and is referred to as R_U. The movements of various marker materials relative to the solvent front (R_f values) are also recorded for these solvents (Tables I and II). Ultraviolet-absorbing materials were located by photography by using ultraviolet light (*14*). Selected color-producing spray reagents were also used to locate and identify certain of the radiation products of uracil (see below). The distribution of the radioactivity on the strips was determined with a Vanguard strip scanner and automatic data system. The principle means of identifying the various radiation products of uracil relied on rechromatography of selected radioactive spots from the original chromatogram with approximately 100 μg of a known marker material. The center of the spot in question was cut out, overlayed with a known marker material, and sewn onto a second strip. The identical behavior of the two materials in several chromatographic solvents constituted identification.

Spray Reagents

Selected spray reagents and chemical treatments were used to locate and identify certain of the radiation products of uracil.

Detection of pyrimidines saturated at the 5–6 bond. The strips were sprayed with 0.5 *N* NaOH, dried at room temperature, and again sprayed with a solution containing 1 gm of *p*-dimethylaminobenzaldehyde (Eastman), 10 ml of concentrated HCl, and 100 ml of ethanol (*15*). A yellow color develops almost immediately for dihydrouracil but slowly for other compounds (isobarbituric acid, alloxan, dialuric acid, and alloxantin). Barbituric acid gives an orange color. Uracil and the *cis*- and *trans*-glycols of uracil give no color with this spray reagent.

Detection of the cis- and trans-glycols of uracil; alloxan, alloxantin, and dialuric acid. The strips were sprayed with 0.5 *N* NaOH and allowed to dry at room temperature for about 1 hour. A fresh solution of 0.5 *M* $AgNO_3$ in 2 *M* NH_4OH was then sprayed on the chromatogram (*16*). Brown to black spots develop rapidly for the glycols but much more slowly for the other compounds. The rate of development of the color also depends on the light intensity in the room and on the isomeric configuration of the glycols (see below).

Detection of hydration product of uracil and the cis- and trans-glycols of uracil. The strips were photographed with ultraviolet light (UV) and then hung in a cabinet saturated with hydrochloric acid fumes for 90 minutes. After air drying, the strips were again photographed with UV light. The hydration product of uracil is dehydrated by this process to yield uracil (*17*), and the glycols are dehydrated to yield isobarbituric acid, both of which are UV-absorbing. Confirmation of this identification was accomplished by rechromatographing these UV-absorbing materials (labeled with ^{14}C) along with unlabeled uracil or isobarbituric acid.

Irradiation

Samples (usually 10 ml) of uracil-2-^{14}C (10^{-4} *M*; 0.5 μCi/ml) were bubbled with Hi-Pure Nitrogen (99.996%) at a high rate for 30 minutes or with therapeutic oxygen (99.5%) for 15 minutes, and the vials were closed with a tight-fitting screw cap. Samples were irradiated with a Philips X-ray machine (no added filter, 15 mA, 250 kV, HVL 5.9 mm Al; exposure dose rate $\sim$1190 R/min). After irradiation, the samples were dried under vacuum in a rotary evaporator. The samples were then dissolved in 0.3 ml of water, spotted on 1½-inch strips of Whatman No. 1 paper, and chromatographed in solvent 1.

Treatment with Ascorbic Acid

Ten micromoles ascorbic acid, 5.0 μmoles of $FeSO_4$, 2.5 μmoles of ethylenediaminetetraacetic acid (EDTA), and 1.2 μmoles of uracil-2-^{14}C (1.8 μCi) in a total volume of 12.8 ml was adjusted to pH 5.4 with approximately 0.1 ml of *N* NaOH and then held at 33°C for 25.5 hours. These conditions are similar to the model hydroxylating system developed by Udenfriend *et al.* (*18*, *19*). The sample was

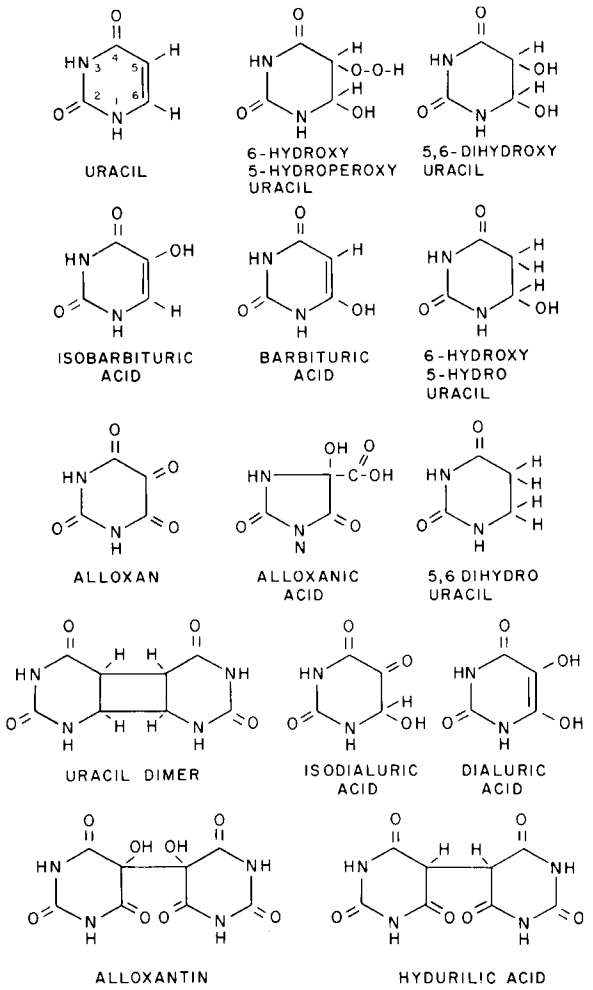

FIG. 1. The structure of various derivatives of uracil

dried in a rotary evaporator, dissolved up in a small volume of water, spotted on a 1½-inch strip of Whatman No. 1 paper, and chromatographed in solvent 1.

RESULTS

The structures of several of the known derivatives of uracil are given in Fig. 1. The 5–6 double bond of the pyrimidines is very sensitive to chemical attack, and the compounds shown involve principally the addition of various amounts of hydrogen, oxygen, or hydroxyl groups at this position. Since the main action of X-rays on water is to produce hydroxyl radicals, hydrogen radicals, and hydrated electrons,

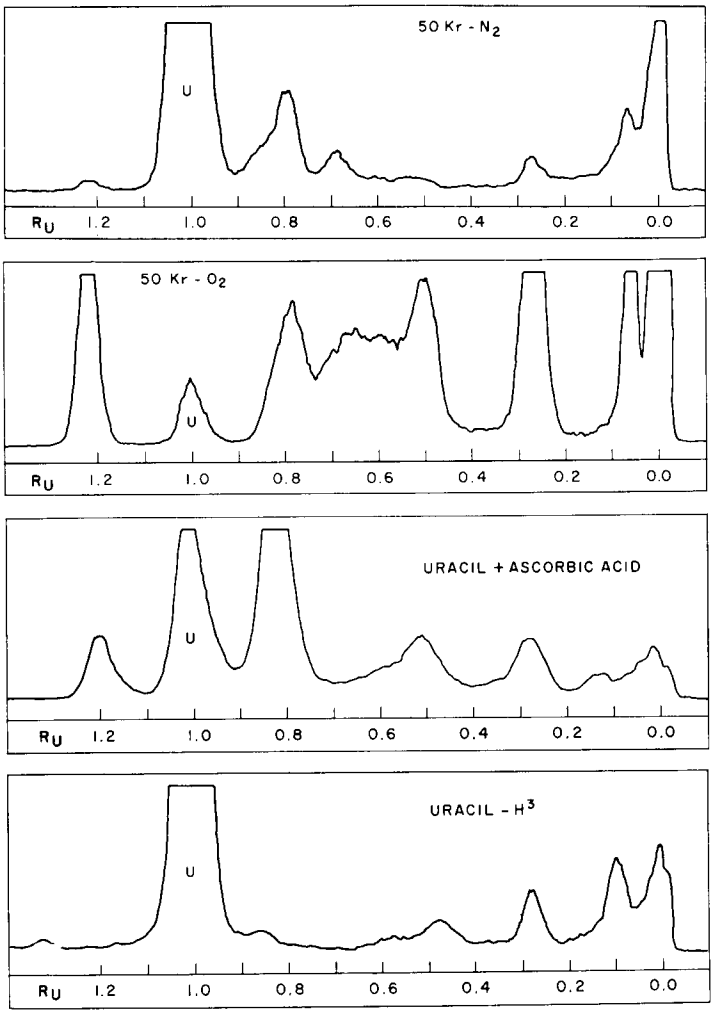

FIG. 2. Distribution of radioactivity on chromatograms of uracil-^{14}C (or ^{3}H) after various treatments. A plot of the distribution of radioactivity versus R_U (distance traveled relative to uracil, U, as 1.0) for uracil-^{14}C (or ^{3}H) treated under different conditions and chromatographed twice in *n*-butanol–water (86:14). 50 kR–N_2:50 kR of X-irradiation under nitrogen. 50 kR–O_2:50 kR of X-irradiation under oxygen. Uracil + ascorbic acid:uracil incubated in the presence of ascorbic acid. Uracil-^{3}H:aged sample (3.5 years) of tritiated uracil.

it is to be expected that many of the products illustrated will be produced by the X-irradiation of an aqueous solution of uracil.

Effect of X-Irradiation on Uracil

Figure 2 shows the distribution of radioactivity along chromatograms of uracil-

TABLE I

R_f VALUES FOR URACIL AND ITS DERIVATIVES

Compound	Solvent 1[a]		Solvent 2[b]
	R_f[c]	R_U[d]	R_f
Alloxan	0.40	1.17	0.46
Alloxantin	See Table II		See Table II
Dialuric acid	See Table II		See Table II
Dihydrouracil	0.28	0.82	0.47
6-Hydroxy-5-hydrouracil	0.23	0.68	0.37
Isobarbituric acid	0.25	0.74	0.36
Uracil	0.34	1.0	0.49
Uracil dimer	0.03	0.08	0.12
Urea	0.28	0.82	0.53

[a] Solvent 1: *n*-butanol–water (86:14).
[b] Solvent 2: *n*-butanol–acetic acid–water (80:12:30) (*13*).
[c] R_f = chromatographic mobility relative to solvent front as 1.0.
[d] R_U = chromatographic mobility relative to uracil as 1.0.

2-^{14}C that had been irradiated with an exposure dose of 50 kR in the presence of nitrogen or of oxygen. It is well known that cells X-irradiated in the presence of oxygen are more sensitive to killing than are cells irradiated under nitrogen. This oxygen effect is also demonstrated here for uracil irradiated *in vitro*. Not only was more of the uracil destroyed by 50 kR in the presence of oxygen than in the presence of nitrogen, but also the products produced differed both qualitatively and quantitatively. We have identified many of these products (see below), but many are still to be identified.

Dihydrouracil and Isobarbituric Acid

The material at R_U 0.79 from the nitrogen experiments has been identified as dihydrouracil. It has the same R_f as authentic dihydrouracil in the two solvent systems and gives a positive test with the *p*-dimethylaminobenzaldehyde reagent. The radioactive peak at this location on the oxygen strips contained dihydrouracil, but the major product was isobarbituric acid. These two compounds are well resolved in solvent 2 (Table I).

6-Hydroxy-5-hydrouracil.

The material at R_U 0.69 from the nitrogen strips has been identified as 6-hydroxy-5-hydrouracil (the water addition product of uracil). This compound was first produced when solutions of uracil and its derivatives were irradiated with ultraviolet light (*20*). The technique of identification relied on the known lability of this compound in acid and its consequent conversion back to uracil by a dehydration reaction (*17*). Thus, before treatment with acid it had no UV absorption and an R_f

TABLE II

R_f VALUES FOR DERIVATIVES OF ALLOXAN

Compound	Solvent 1[a]				Solvent 2[b]			
	PDABA[c]		$AgNO_3$[d]		PDABA		$AgNO_3$	
	R_f[e]	R_U[e]	R_f	R_U	R_f	R_U	R_f	R_U
Alloxan	0.17[f]	—	0.0–0.5[g]	0.0–1.4	—	—	0.04	0.07
	—	—	0.40	1.18	0.46	0.94	0.46	0.94
Alloxantin	0.01	0.03	0.0–0.5[g]	0–1.5[g]	0.07	0.14	0.04	0.08
	0.41	1.20	0.41	1.20	—	—	0.46	0.94
	—	—			0.56	1.14		
Dialuric acid	0.0	0.0	0.0–0.5[g]	0–1.4[g]	0.11	0.22	0.07	0.14
	—	—	0.22	0.65			0.45	0.92
	0.41	1.20	0.43	1.26	0.56	1.14	—	—
Alloxanic acid	—	—	—	—	—	—	0.0[h]	—

[a] Solvent 1: *n*-butanol–water (86:14). Uracil R_f = 0.34.
[b] Solvent 2: *n*-butanol–acetic acid–water (80:12:30). Uracil R_f = 0.49.
[c] *p*-Dimethylaminobenzaldehyde spray (see Methods).
[d] $AgNO_3$ spray (see Methods).
[e] For definition, see Table I.
[f] Very faint spot even after 24 hours.
[g] A streak of material between these R_f values.
[h] Data of Rupp and Prusoff (*29*).

different from uracil. After acid treatment is was UV-absorbing and co-chromatographed with uracil.

The yield of this compound was very dependent on the pH of the solution of uracil during irradiation. In a water solution of uracil (pH 6.6) only about 1% of the uracil was converted to this product. When the pH was adjusted to 4.8 with hydrochloric acid, about 4% of the uracil was converted to this product. This may reflect that the hydration product is more stable at about pH 5 (*21*) rather than that this pH favors the formation of the product. In other experiments at pH 5 as much as 17% of the uracil was converted to this product. We do not know the reason for this variation.

Although a considerable amount of radioactivity (but no distinct peak) was at this R_U on the oxygen strips, no 6-hydroxy-5-hydrouracil could be detected, regardless of the pH used.

5,6-Dihydroxyuracil.

The materials in the peaks at R_U 0.28 and 0.51 have been identified as the *cis*- and *trans*-glycols, respectively. These materials show no UV absorption originally but after acid treatment are converted to a UV-absorbing product that cochroma-

tographs with isobarbituric acid (see Methods). The assignment of the *cis*- and *trans*-isomers is based on the fact that the *cis*-isomer is expected to dehydrate more easily than the *trans*-isomer (22; but contrast with *8*) and the R_U 0.28 material is converted more rapidly to isobarbituric acid than is the material at R_U 0.51. At pH 9.5 and 61°C the R_U 0.28 material increased in optical density at 276 nm at the rate of 0.007 unit/min, while the R_U 0.51 material increased at 0.004 unit/min. Both compounds gave a positive test with ammoniacal silver nitrate (see Methods). The R_U 0.28 material yielded an intense red-black spot that developed very rapidly. The R_U 0.51 material yielded a red-brown spot that developed slowly. By comparison, *meso*-tartaric acid (a *cis*-glycol) yielded a dark brown spot that developed rapidly, while *d*-tartaric acid (*trans*-glycol) yielded a brown spot that developed slowly. Furthermore, treatment of uracil-2-^{14}C with less than stoichiometric amounts of $KMnO_4$ in the presence of a stoichiometric amount of acetic acid led to the formation of the R_U 0.28 material in good yield. $KMnO_4$ is known to direct the synthesis of *cis*-glycols (*23*). The major product formed was at R_U 0.06 and may be oxaluric acid (*24*), but we have not made a positive identification.

Typically the R_U 0.28 material was about 75% pure glycol, as judged by the extent of conversion to isobarbituric acid by exposure to HCl fumes. There were also several small peaks (each containing less than 4% of the total radioactivity) at R_f values (in solvent 2) other than that for isobarbituric acid. The material at R_U 0.51 ranged from 20 to 60% glycol, with two major peaks of radioactivity appearing after acid treatment at R_f 0.26 and R_f 0.43 (in solvent 2), and with minor amounts at other R_f values.

Material at R_U 0.0, 0.8, and 1.2

These materials will be considered together, since the results of cochromatography show that they all belong to a group of closely related compounds (alloxan, alloxantin, dialuric acid, and alloxanic acid; for structures see Fig. 1). An understanding of the chemical properties of these compounds is necessary before the results of the radiation experiments can be interpreted. Alloxantin dissociates in water to form alloxan and dialuric acid (*25*), and a mixture of alloxan and dialuric acid gives rise to alloxantin (*25*). Dialuric acid spontaneously oxidizes to alloxan (*26*), and this conversion is stimulated by the presence of iron (*27*). Alloxan in neutral or alkaline solution decomposes rapidly to form alloxanic acid (*28*). From these considerations it seems obvious that, if any one of these compounds were produced by the action of ionizing radiation, their ease of interconversion would yield multiple products when assayed by our chromatographic procedures, and the relative proportions of each product would depend on the pH and the presence of trace metals and of oxygen.

In Table II are presented the R_f values for these compounds run in two solvent systems. Different conclusions about the R_f of a given compound are reached in

some cases, depending on which spray reagent is used. Furthermore, it is difficult to make a precise assignment of R_f for certain of these compounds, since a common family of R_f values is usually obtained regardless of which compound one starts with (Table II).

Nevertheless, we have tentatively identified the radiation-produced material at R_U 1.2 as alloxan on the basis of its R_f. When the chromatograms were treated with HCl vapors and this material was submitted to chromatography in solvent 2, an R_f of 0.46 was obtained. In the absence of HCl treatment, the resubmitted R_f was 0.56. These latter R_f values were observed when alloxantin and dialuric acid were chromatographed in this solvent (Table II).

The materials at R_U 0.0 and 0.08 also have R_f values similar to certain members of the alloxan family of compounds, but because of the problems discussed above in conjunction with the results of Table II a more precise identification is not possible at this time. In view of the results with $KMnO_4$ (see section on 5,6-dihydroxyuracil), the peak at R_U 0.08 may also contain oxaluric acid.

Effect of Ascorbic Acid on Uracil

The chromatograms of uracil-^{14}C that had been treated with ascorbic acid mimicked, with significant quantitative differences, the results for the X-irradiation of uracil (Fig. 2).

The material near the origin separated into several peaks when rechromatographed in solvent 2. The major material present has been tentatively identified as alloxantin.

The chemical identity of the material at R_U 0.12 is unknown.

The material at R_U 0.27 is the *cis*-glycol of uracil and is converted to isobarbituric acid by acid treatment.

The material at R_U 0.51 contains the *trans*-glycol of uracil and four other products, as yet unidentified.

The material at R_U 0.82 is composed primarily of dihydrouracil but also contains isobarbituric acid and three unidentified products.

The material at R_U 1.2 appears to be alloxan, as judged by the same criteria used to identify the R_U 1.2 material produced by X-irradiation.

Auto-irradiation (Aged Sample of Uracil-^{3}H)

There is some similarity between the chromatograms of an aged sample of uracil-^{3}H and those for X-irradiated uracil-^{14}C (Fig. 2). The uracil-^{3}H had been stored in the freezer for nearly four years before it was used in these experiments. The glycols of uracil were present at R_U 0.28 and 0.50. These were identified by virtue of their conversion to isobarbituric acid on treatment with acid. The material at R_U 0.0 and 0.10 has not been identified.

DISCUSSION

When a solution of uracil was exposed to X-rays, the following compounds were produced: dihydrouracil, 6-hydroxy-5-hydrouracil, isobarbituric acid, *cis-* and *trans-*glycols of uracil, alloxan, alloxantin, and dialuric acid. We did not observe the formation of the hydroperoxide of uracil, but this can probably be explained by the extreme lability reported for this compound (*7*, *8;* compare *5*). A positive test for peroxide (*30*) was obtained immediately after irradiation but not after the solution was evaporated to dryness and redissolved. The breakdown product of the hydroperoxide is expected to be the glycol (*7*, *8*). The high yield of the two isomers of the glycol observed by us could be due both to the direct formation of the glycol by the sequential attack by two hydroxyl radicals and/or to the decomposition of the hydroperoxide.

The mechanisms involved in radiation chemistry are quite different from those observed in photochemistry, yet it is of interest that one product of uracil produced by ultraviolet irradiation was also produced here by X-irradiation in the presence of nitrogen but not in the presence of oxygen. 6-Hydroxy-5-hydrouracil results from the photochemical addition of a molecule of water across the 5–6 double bond of an excited uracil molecule. The radiation chemical formation of this compound would presumably be through the sequential attack of uracil by a hydroxyl and a hydrogen radical. An analogous derivative of dimethyluracil has been produced by X-irradiation under nitrogen but not in the presence of oxygen (*6*).

Ascorbic acid is known to be an active hydroxylating agent (*18*, *19*, *31*). One author has postulated that the mechanism of action of ascorbic acid on DNA is through hydroxyl radicals (*32*). This suggests that treatment of uracil with ascorbic acid should yield the same products as are produced by the hydroxyl radicals formed by ionizing radiation. Ascorbic acid, however, is also known to be an active reducing agent (*33*), a fact that is ignored in the papers dealing with the hydroxylating ability of ascorbic acid (*18*, *19*, *31*). Therefore, having both oxidizing and reducing properties, it should mimic the action of X-rays on uracil. This was indeed what was observed, but there were some quantitative differences. The most significant of these was the greater yield of dihydrouracil (R_U 0.8) relative to the yield of the *cis*-glycol (R_U 0.28) or of alloxan (R_U 1.2) as compared with the results after X-irradiation.

Alloxan can substitute (at reduced efficiency) for ascorbic acid in this hydroxylating system (*18*). Since alloxan is one of the products produced when uracil is X-irradiated, it is interesting to speculate on the possible effect that this radiation-produced alloxan might have on the further alteration of uracil both during and after irradiation. One might also speculate on the possible contribution of radiation-produced alloxan to "radiation sickness." It has been reported that X-irradiated animals (rats) were more susceptible to alloxan injections than were unirradated animals (*34*).

Auto-irradiation of an aged sample of uracil-^{3}H also mimics in some respects the

effect of X-irradiation on uracil. The *cis*- and *trans*-glycols of uracil have been identified. Tritium-labeled alloxan is absent, as would be expected, considering the location of the tritium on the uracil molecule (5 and/or 6 position). Two peaks of slightly greater magnitude than the *cis*-glycol peak were shown to contain several compounds when chromatographed in a second solvent, but these products have not been identified.

We have not attempted to establish G values for the reactions of uracil because of the extreme dependence of the radiation chemistry of the pyrimidines on pH, oxygen, and impurities. This dependency is apparent from the lack of agreement of the published G values for uracil destruction (*4*, *10*, *12*), and the discrepancy in the nature of the products formed (*4*, *5*, *7*, *8*, *10*, *11*). We have found marked quantitative differences whether the uracil solutions were irradiated before or after bubbling with compressed air, and marked qualitative differences in the types of products produced if phosphate buffer was present.

One goal of these studies was to isolate and identify the major products produced by X-irradiation, so that the same techniques could be used for the investigation of the products when the nucleic acids were irradiated *in vitro* or *in vivo*. The situation, however, would appear to be more complicated than anticipated. The multiplicity of the products produced by X-irradiation and the lack of stability of several of these to acid treatment will undoubtedly make it difficult to isolate them from DNA samples by methods analogous to those used to study the products produced in DNA by ultraviolet irradiation (*35*).

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