DIASTEREOISOMERS OF URACIL GLYCOSIDE PHOTOHYDRATES

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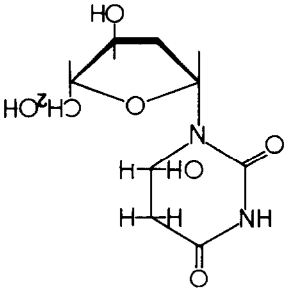
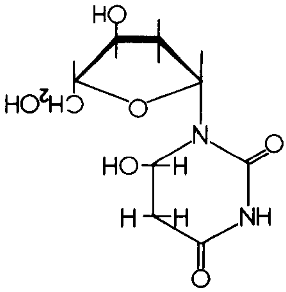
Summary: The two diastereoisomers of various uracil glycoside photohydrates have been separated by thin-layer chromatography, and one of the isomeric photohydrates of deoxyuridine isolated in crystalline form. The ratios of the two isomers formed are independent of the wavelength of irradiation. The dark dehydration of each of the isomers follows a different pathway, both in acid and alkaline media, the nature of which is described.

Ultraviolet irradiation of uridine (or other uracil glycosides) in aqueous medium leads largely to formation of the photohydrate, 5-dihydro-6-hydroxyhydrouridine (Shugar, 1960). The water adduct may subsequently be eliminated in the dark, in neutral or acid medium, by what is essentially an acid-base catalyzed reaction (Wierzchowski and Shugar, 1961) to regenerate the parent glycoside. In alkaline medium the dark elimination reaction is more complex and, in the presence of Na^+ or K^+ cations, is not quantitative, a fact made use of by Schuster (1964) to estimate the number of uracil hydrates formed in an irradiated polynucleotide. Catalysis by NH_4^+, however, leads to quantitative regeneration of the parent glycoside by two different pathways (Fikus and Shugar, 1966): (a) direct elimination of the water adduct, and (b) via an intermediate with an absorption band at 290 mμ, due to opening of the N_1-C_6 bond of the uracil ring, followed by dehydration and subsequent ring closure.

No consideration has been given, in the foregoing, to the possible formation of two diastereoisomers of the photohydrates, as follows:





The first evidence for the existence of such a mixture of stereoisomers was provided by Chambers (1968) for hydrated UMP on the basis of its NMR spectrum in D_2O. This was subsequently confirmed for hydrated uridine (U) by Wechter and Smith (1968). We have now found that the diastereoisomers of various uracil glycoside photohydrates may be separated by thin-layer chromatography, and have isolated one of these in crystalline form. The two isomers differ in stability under various conditions, and each undergoes alkali-catalyzed elimination of the water adduct by one of the two pathways referred to above.

A 10^{-3}M aqueous unbuffered solution of 2'-deoxyuridine (dU) was irradiated with a mercury resonance lamp (254 mu), from which radiation below 230 mu was eliminated by means of a sodium acetate filter (McLaren and Shugar, 1964), until 80-90% of the dU had been photochemically transformed. The irradiated solution was concentrated at room temperature and chromatographed on HF_{254} silica gel 3 times in the same direction with chloroform-methanol (85:15, v/v). Under these conditions, only one spot, corresponding to unchanged dU, was visible under a dark UV lamp, with an R_f of 0.47. Exposure of the plate to NH_4OH fumes for 20-30 minutes brought out two additional spots with R_f values of 0.22 and 0.67 which, on elution, proved to be dU. These two spots must, therefore, have been formed by the alkali-catalyzed elimination of H_2O from the two $dU \cdot H_2O$ isomers I (R_f=0.22) and II (R_f=0.67)[x].

Irradiation of dU in heavy water also gave the D_2O adducts, the two isomers being formed in the same ratio as in H_2O. Irradiation at longer wavelengths gave almost identical results, either in H_2O or D_2O.

By direct elution and spectral estimation, the ratio of the two isomers was found to be I:II = 70:30. However, due to the slight alkaline reaction of the silica gel, and the greater alkaline lability of isomer II (see below), about 60% of the latter reverts to dU during deposition of the mixture of isomers on the plate. The 70:30 ratio of the two isomeric photohydrates is to be compared with that inferred indirectly by Wechter and Smith (1968) from the NMR spectrum of a mixture of the isomers of uridine photohydrate, 60:40.

The more pronounced stability of isomer I pointed to the possibility of isolating it in purified form. About 40 mg. dU, at a concentration of 10^{-3}M in water, was irradiated in a reactor at 254 mμ until the absorption at 262 mμ had decreased almost to zero. The solution was then concentrated at room temperature under reduced pressure, and developed three times in the same direction on thick TLC HF_{254} plates with chloroform-methanol as above. The same solvent was used for elution of isomer I from the chromatograms. Following removal of solvent under reduced pressure, the residue was washed with anhydrous ethanol, dissolved in the minimal quantity of anhydrous ethanol, and left overnight in the cold room to give a deposit (20 mg.) of tiny platelets which were extremely hygroscopic. On a microscope hot stage the crystals did not exhibit a defined melting point, but, at 53° suddenly eliminated water to form droplets of a highly concentrated solution of dU.

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[x] Prolonged exposure of the chromatogram to the UV lamp brought out an additional spot, with an R_f of 0.10, corresponding to dU photodimer, which accounted for about 8% of the photochemically transformed dU, in agreement with previous observations on dimerization of uracil glycosides in aqueous medium at a concentration of 10^{-3}M (Fikus and Shugar, 1966).

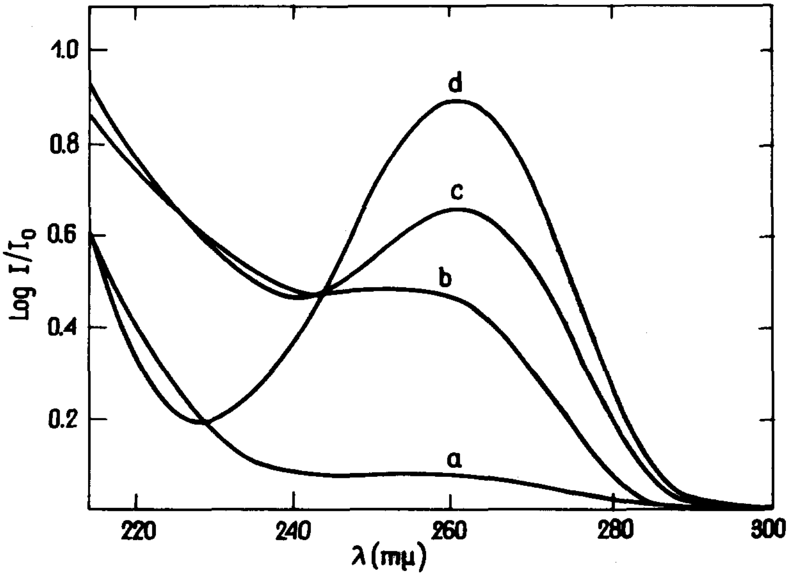

Fig. 1: Alkali-catalyzed dehydration of isomer II of dU•H_2O in 0.001N NaOH at 22°; (a) absorption spectrum of dU•H_2O at pH 7; (b) absorption spectrum of partially regenerated dU after 1 min. exposure to alkali; (c) absorption spectrum of fully regenerated dU after 2 mins. in alkali; (d) neutral absorption spectrum of regenerated dU.

The difference in stability, and in pathways for elimination of the water adduct, between the two isomers is strikingly illustrated by their behaviour in alkaline medium. At pH 12, at room temperature, isomer II reverts directly and rapidly (in less than one minute) to dU; this is illustrated in Fig. 1, where the pH is 11, in order to reduce the rate of dehydration, which is a first-order reaction. Elimination of the water adduct is quantitative in the presence of either Na^+ and K^+, or NH_4^+, cations; so that the ratio $\varepsilon_{max(pH\ 12)}/\varepsilon_{max(pH\ 6)}$ of the regenerated dU is 0.76-0.78 as compared to 0.76 for control dU.

Under the same conditions, the rate of dehydration of isomer I is not only much slower, but the reaction proceeds <u>via</u> an intermediate with an absorption maximum at 290 mμ which is, in turn, transformed to dU. This is shown in Fig. 2, at a pH of 11 to slow down the reaction sequence.

Furthermore, the alkali-catalyzed dehydration of isomer I is quantitative only in the presence of NH_4^+, but not Na^+ or K^+, ions (cf. Fikus and Shugar, 1966).

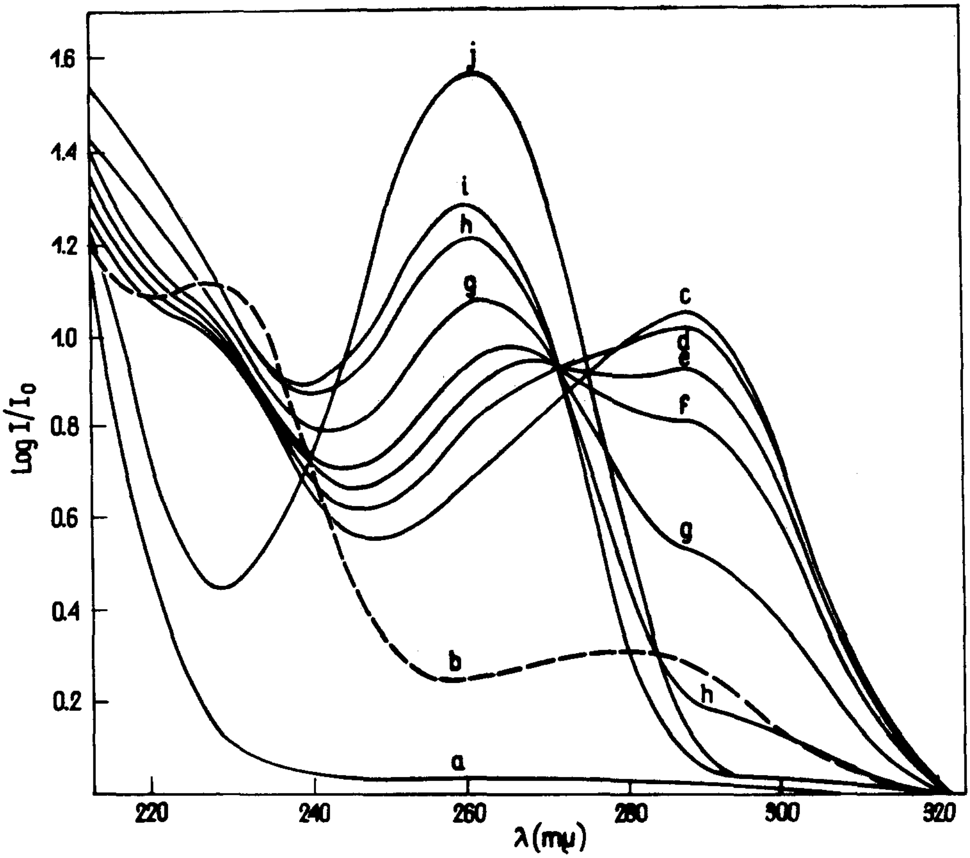

Fig. 2: Course of alkali-catalyzed dehydration of isomer I of dU•H_2O in 0.001N NaOH at 22°: (a) absorption spectrum of dU•H_2O at neutral pH; (b) to (i) are spectra after exposure to alkali for 0.5, 2, 3, 4, 5, 8, 15, 30 mins., respectively; curve (i) is the alkaline absorption spectrum of fully regenerated dU which, on neutralization, gives curve (j), the neutral absorption spectrum of dU.

In acid medium (1 N HCl) at room temperature (22°), isomer I appears to revert directly to dU with a first-order rate constant of $7{,}8\times10^{-4}$ sec^{-1} (Fig. 3). The reaction is readily followed by measuring the rate of appearance of the dU absorption maximum at 262 mμ. By contrast, isomer II exhibits a pronounced initial lag under the same conditions, the rate of dehydration gradually increasing until it approximates the value for isomer I (Fig. 3).

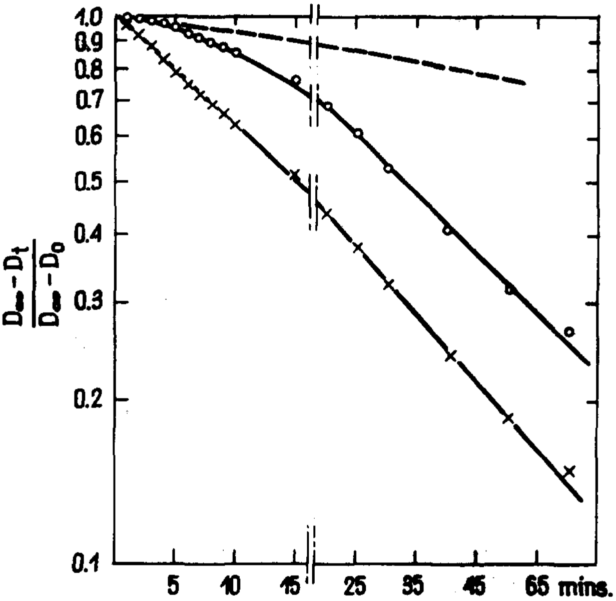

Fig. 3: Acid-catalyzed dehydration of $dU{\cdot}H_2O$ diastereoisomers I and II in 1N HCl at 22°. Course of reaction followed by rate of appearance of 262 mμ absorption maximum of regenerated dU.

A reasonable interpretation of the foregoing is that isomer II is unable to eliminate the water adduct directly, and that the lag period is due to its isomerization to I, which then dehydrates directly. The following experiment supports such an interpretation. An aqueous neutral solution of isomer II was acidified to pH 0 and kept at this pH for 10 mins. at room temp.; during this period only about 10-15% reverts to dU (see Fig. 3). The solution was then brought rapidly to pH 12, and the course of alkaline dehydration examined. This was now found to correspond to the behaviour of a mixture of the two isomers of $dU{\cdot}H_2O$: about 60% of the photohydrate reverted very rapidly to dU; the remaining 40% was initially transformed to the intermediate with an absorption maximum at 290 mμ, following which the latter was slowly converted to dU, i.e. 40% of what was initially isomer II now behaved in alkali like isomer I.

Furthermore, it has been shown that the formation of isomeric photohydrates is by no means confined to dU, but is the rule amongst uracil glycosides. This is illustrated in Table I. Note, in particular that, whereas there is little difference in the proportion of the two isomers between uridine and deoxyuridine, the ratio is appreciably modified on N-methylation of the aglycon or by a change in the anomeric configuration from β to α. The α-dU in Table I was not obtained by direct synthesis, but by photochemical de-ethylation (Pietrzykowska and Shugar, 1968) of α-5-ethyldeoxyuridine (Świerkowski and Shugar, 1969).

TABLE I

Percentages of diastereoisomeric photohydrates I and II of uracil glycosides formed by irradiation in aqueous neutral medium at 254 mμ, or at wavelengths to red of 265 mμ.

Isomer	Uridine	N-methyluridine	α-dU	β-dU
I	65	25	45	70
II	35	75	55	30

It is clear from the foregoing that photohydrate isomers will almost certainly be formed on irradiation of cytosine glycosides. But these will be considerably more difficult to identify, and even more so to isolate, because of their lower stability, i.e. their tendency to rapidly dehydrate. In this instance, however, resort may be had to alkylaminocytosine glycosides, the photohydrates of which are enormously more stable (Fikus et al., 1962).

It remains to establish which of the two isomeric dU photohydrates is I, and which is II. Attempts are therefore under way to isolate the latter on a preparative scale. On the basis of the observed behaviour of the isomeric dU photohydrates in alkaline medium, in particular as regards the absence of quantitative dehydration of the isomer we refer to as I in the presence of Na^+ or K^+ (cf. Fikus and Shugar, 1966), it may readily be inferred from the data of Schuster (1964) that this isomer is formed in ultraviolet irradiated nucleic acids. It will be of interest to see whether isomer II is also formed.

Acknowledgments

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