Gamma Irradiation of Cytosine in an Aerated Aqueous Solution, I

Identification of Radiolysis Products of Cytosine Resulting from the Deamination Pathway

M. Polverelli and R. Teoule

Département de Recherche Fondamentale, Laboratoire de Radiobiologie Centre d'Etudes Nucléaires,
Grenoble, France

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Radiolysis products resulting from the deamination pathway were isolated and identified after irradiation of cytosine by gamma rays in neutral aqueous solutions. They were identical to those identified in uracil radiolysis *i. e.* 5,6-dihydroxy-5,6-dihydro-uracil (*cis* and *trans* forms), *iso*-dialuric acid, alloxan, 5-hydroxy-hydantoin, parabanic acid and oxaluric acid.

The absence of 4,4′-di-*iso*-barbituric acid, alloxantin or *iso*-barbituric acid could be explained by the good stability of uracil glycols. The identification of buiret proof of potential degradation mechanisms different from these involving of the free radical attack on the 5,6 double bond.

In 1960, the degradation of cytosine after X-irradiation in an aerated aqueous solution was described by Ekert and Monier [1]. According to these authors, after the radical attack on the 5,6 double bond, there are two parallel pathways: The first one leads to 5-hydroxy-cytosine (no deamination occurred); the second one leads to deamination during the saturation of the 5,6 double bond, and ends in isobarbituric acid.

In a polarographic study of the effects of gamma irradiation on cytosine, Pleticha-Lansky and Weiss [2] have demonstrated the presence of the end product described by Ekert and Monier, *i. e.* isobarbituric acid.

Using radioactive tracers, chromatographic and spectroscopic techniques, such as nuclear magnetic resonance, UV and micro-infrared spectrometry, we have isolated and identified products belonging to the deamination pathway.

Results: Identification of Radiolytic Products

1. Six-membered heterocycles

Peroxides

After irradiation of cytosine in an aqueous aerated solution at neutral pH (6.50), peroxides were not detected.

When the solution was acidified with hydrochloric acid at pH 2 prior to irradiation, peroxides were observed on the chromatographic plate, but they were too unstable to be isolated and were obtained with non-reproducible yields.

Iso-barbituric acid and uracil

In our hands, only small amounts of these products were obtained. They were observed at 254 nm by fluorescence. A brown spot developed rapidly after spraying an ammoniacal solution of silver nitrate with isobarbituric acid.

5,6-Dihydroxy-5,6-dihydro-uracil (*cis* and *trans*)

These two isomeric forms of uracil "glycols" could be readily seen on the chromatographic plate as brown spots after spraying with a silver nitrate solution in ammonium hydroxide. The *trans*-form reacted more easily than the *cis*-form within 5 min.

The main bands in infrared spectra for the *cis*- and *trans*-forms, respectively were $\nu_{CO} = 1730 - 1770\ \text{cm}^{-1}$ and $\nu_{CO} = 1700 - 1730\ \text{cm}^{-1}$.

The NMR spectrum of the *cis*-form in dimethylsulfoxide with TMS as an internal reference, gave a doublet for H-5 $\delta = 4.20$ ppm, JH-5-H-6 = 4 cps, and H-6 a pseudotriplet $\delta = 4.66$ ppm which became a doublet after proton exchange with D_2O: JH-5-H-6 = 4 cps [3].

In high resolution mass spectrometry, the molecular ion was not obtained but characteristic peaks

Requests for reprints should be sent to M. Polverelli, Departement de Recherche Fondamentale, Laboratoire de Radiobiologie, Centre d'Etudes Nucléaires, BP 85, Centre de Tri 38041, *38-Grenoble,* France.

Table I. Chromatographie data. Silicagel MN-N-HR/UV (Macherey-Nagel Co, Duren R.F.A. (20 × 20 cm — 0.5 mm).

Products	Silice MN-N-HR		CelluloseF/Merck	
	A	B	C	D
5,6-dihydroxy-5,6-dihydro-uracil *cis*	0.27	0.35	0.24	0.78
5,6-dihydroxy-5,6-dihydro-uracil *trans*	0.32	0.46	0.24	0.72
iso-dialuric acid	0.40	0.45	0.34	0.85
alloxan	0.63	0.84	0.73	0.74
5-hydroxyhydantoin	0.51	0.67	0.49	0.77
parabanic acid	0.68	0.93	0.61	0.61
formylurea	0.68	0.71	0.62	0.69
urea	0.34	0.30	0.33	0.70
biuret	0.54	0.56	0.43	0.60
$C_4H_7N_3O_4$	0.41	0.56	0.39	0.60
cytosine	0.24	0.14	0.32	0.58

A = Chloroform-methanol-water (4:2:1) lower phase v/v/v with 5% methanol (R_F cited were obtained after two successive runs) [6]. B = Ethyl acetate-methanol-water (v/v/v,75:16:20) upper phase [6]. System A is used in the first dimension and the B system in the 2nd dimension. Cellulose F/Merck (precoated plates Darmstadt) (R.F.A.) (20 × 20 cm — 0.1 mm). C = 1st dimension propanol-water (10:3, v/v). D = 2nd dimension *iso*-propanol-ammonium sulphate saturated solution water (2:79:19, v/v/v). These chromatography media and systems of solvents were used and prepared according to Cadet and Teoule [6].

were identified as $m/e = 101$ ($M^{+\cdot} = 45$), 89 $(C_3H_5O_3)^+$, 46 $(CH_4NO)^+$.

Iso-dialuric acid and alloxan(traces)

The two substances were the final products of a higher degree of oxydation than that of glycols [3].

Alloxan gave a blue fluorescence at 254 nm and a brown spot after spraying of an ammoniacal solution of silver nitrate. The frequencies of the main bands, such as C=O were $\nu = 1720$ and 1760 cm^{-1} for alloxan, and 1715 and 1745 cm^{-1} for *iso*-dialuric acid.

In dimethylsulfoxide with TMS as an internal reference, proton H-6 of *iso*-dialuric acid gave a doublet ($\delta = 4.36$ ppm) JH-5-H-1 = 5 cps, proton H-1 doublet ($\delta = 8.03$ ppm, JH-1-H-6 = 5 cps), proton H-3 singulet ($\delta = 10$ ppm). The molecular ion $M^{+\cdot}$ was important at 144, but noticeable peaks were also measured at $m/e = 101$, 58, 44 and 43 [3].

2. Five-membered heterocycles

In this class of radiolysis compounds one could describe 5-hydroxy-hydantoin and parabanic acid. These compounds were obtained to a significant extent for high doses of gamma radiation above $5 \cdot 10^4$ rads.

While the ammoniacal solution of silver nitrate was being sprayed, 5-hydroxy-hydantoin appeared as a dark brown spot and then parabanic acid developed as a white one. Parabanic acid could be recognized thanks to its conjugated resonance system giving a blue fluorescence at 254 nm.

The frequencies of the carbonyl bond in infrared spectra were 1735 cm^{-1} for 5-hydroxy-hydantoin and 1750 cm^{-1} for parabanic acid.

3. Ureides and acids

N-glyoxyl-*N'*-formyl-urea*

This unstable ureide was obtained by the cleavage of the *cis*-5,6-dihydroxy-5,6-dihydro-uracil by sodium metaperiodate [3]. In an aqueous medium it gave spontaneously 5-hydroxy-hydantoin. Only a small amount of this product was obtained in neutral irradiated solutions of cytosine in contrast to irradiation in the acidic pH range.

Oxaluric acid, formylurea, urea

These ureides could be considered as end products of the radiolytic degradation of cytosine. They became major products only for very high doses of radiation. They rapidly developed as yellow spots after spraying of Fink's reagent. Microinfrared spectrometry gave carbonyl frequencies for oxaluric acid: $\nu_{CO} = 1715-1770$ cm^{-1}; and for formylurea: $\nu_{CO} = 1715-1770$ cm^{-1}.

Glyoxylic acid

This keto-acid was identified as a radiolysis product thanks to its isomeric forms of 2,4-dinitrophenylhydrazones (DNPH). The *cis* and *trans* isomeric forms were separated on silicagel Merck G-HR or Machereiy Nagel N-HR/UV with one bidimensional chromatography.

1st run: Petroleum ether-ethyl-formiate-propionic acid (140 : 70 : 15; v/v/v) [4]; R_F *cis*: 0.41; R_F *trans*: 0.35. 2nd run: Benzene-acetic acid (80 : 20; v/v); R_F *cis*: 0.56; R_F *trans*: 0.47. Spectral characteristics: *trans* λ_{max}: 451 nm, *cis* λ_{max}: 371 nm (in NaOH 0.5 M); *trans*: $\nu_{CO} = 1700$ cm^{-1}, *cis*: $\nu_{CO} = 1672$ cm^{-1}.

These values are in good agreement with those of Katsuki [5]. Unambigous identification of the 2,4-dinitrophenylhydrazone was given by the molecular peak $M^{+\cdot} = 254$ obtained by mass spectrometry.

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* N-glyoxyl-N'-formylurea exists probably in equilibrium with its cyclic form N-formyl-5-hydroxyhydantoin according to Ducolomb *et al.* [3].

Remarks: Among the products resulting from the non-deamination pathway, we identified mainly biuret as a new radiolysis product. This compound rapidly reacted with Fink's reagent giving a yellow spot on the chromatographic plate ($\nu_{CO} = 1710 - 1600\ cm^{-1}$) ($M^{+\cdot} = 103$).

Discussion

The poor yield of *iso*-barbituric acid can be explained by the good stability of uracil glycols under the present experimental conditions.

4,4′-Di-*iso*-barbituric acid, identified in the radiolysis of uracil and cytosine by Pleticha-Lansky and Weiss[2] and in the radiolysis of 5-bromouracil by Gilbert *et al.*[7], might be produced only when *iso*-barbituric is present in sufficient yield.

We did not find dialuric acid.

As a matter of fact, according to Elston *et al.*[8] and Dea[9] dialuric acid is thought to be transformed into alloxan with a concomitant production of hydrogen peroxide, if air is bubbled constantly through the aqueous medium.

Chatmara and Jones[11] had found biuret to be a permanganate oxidation product of cytosine. They admitted "cytosine glycols" as possible intermediates, but they also suggested degradation of cytosine by "alternative pathways".

The identification of biuret as a radiolysis product of cytosine gives proof of the stability of the amino group during the ring opening between carbon four and five.

We conclude that besides the classical explanation of radiolytic degradation of cytosine by ionizing radiations in neutral aerated aqueous solutions. *i. e.*, saturation of the 5,6 double bond and concomitant deamination, other mechanisms exist which keep the amino group associated with the molecule during ring cleavage between carbon four and five.

Experimental Methods

1. *Extraction of radiolysis products from the irradiated solution*

Solutions of cytosine (10^{-2} M to 10^{-3} M at pH 6.50) were subjected to gamma rays at a dose rate of 7500 rads/mn with permanent air bubbling.

The doses administered were measured according to Fricke[13].

After irradiation the solution was evaporated under vacuum at 30 °C. The residue was taken up with methanol-water (v/v).

The radiolysis products were separated on silicagel plates MN-N-HR (20 × 20 cm – thickness 0.5 mm) with the B system. After radiochromatography or chemical development, the silicagel was scraped off the plate. The investigated compounds were eluted three times from the silicagel with methanol (analytical grade). The solutions were filtered through Millipore GSWPO 1300 filter (0.22 μ pore size after elimination of the greatest part of the silicagel by centrifugation.

Methanol was evaporated under vacuum at room temperature. The radiolysis product was then submitted to different analytical methods (infrared, NMR, mass spectrometry, etc.).

2. *Detection of the products on the chromatographic plates*

All chromatographic plates contained a fluorescence indicator permitting vizualization of resonating-conjugated products at 254 nm. After spraying with Fink's reagent (paradimethyl-aminobenzaldehyde in alcoholic acid solution)[14], ureides rapidly developed as yellow spots. Silver nitrate in ammoniacal solution sprayed on the chromatographic plates was used for visualization of 5,6 saturated double bond products, such as glycols[15]. The products appeared as dark brown spots within fifteen minutes. Compounds with a free carbonyl function gave reddish yellow spots by spraying an acidic solution of dinitrophenylhydrazin. When present, peroxides gave black blue spots, due to the liberation of iodide which reacted with starch by spraying an alcoholic solution of potassium iodide. When radioactive cytosine was used, the positions of the labeled products were located with a Kodak emulsion – (Kodirex type).

3. *Analytical methods*

Radiolysis products were first submitted to micro infrared spectrophotometry in KBr pellets with a Perkin-Elmer-257 instrument. Electronic absorption spectra were registered on a differential Cary 15 spectrophotometer. NMR spectra were analysed on a Varian A 60 instrument (with TMS as an internal reference). Mass spectrometric fragmentation and identification of radiolysis products were performed on a AEI-MS9 high resolution spectrometer.

4. *Standard products*

The chromatographic standards were obtained as commercial products from Fluka or synthesized in the laboratory. *Cis* and *trans* glycols of uracil were prepared according to Baudisch and Davidson after the modifications of Ducolomb *et al.*[3].

Iso-dialuric acid was prepared by the method of Behrend and Roosen[17]. 5-Hydroxy-hydantoin was obtained by condensation of urea with glyoxylic acid[18].

Isomeric forms of 2,4 dinitrophenylhydrazones of glyoxylic acid were crystallized according to El Hawary and Thompson[19].

Glyoxylic acid was extracted from the irradiated solution as its DNPH derivatives in diethyl ether in an acidic medium. Radioactive cytosine (cytosine [2-^{14}C]), with a specific activity of 43 mCi/mmole), was purchased from CEA (Saclay-France).

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