Identification of Radiolysis Products of Cytosine Resulting from the Deamination Pathway

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Gamma Irradiation, Cytosine, Radioproducts, Identification

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 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. 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 iso-barbituric acid.

In a polarographic study of the effects of gamma irradiation on cytosine, Pleticha-Lansky and Weiss 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.

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Table I. Chromatographic data. Silicagel MN-N-HR/UV (Macherey-Nagel Co, Duren R.F.A. (20 x 20 cm—0.1 mm).

<table>
<thead>
<tr>
<th>Products</th>
<th>Silicagel MN-N-HR</th>
<th>Cellulose F/Merck</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>A</td>
<td>B</td>
</tr>
<tr>
<td>5,6-dihydroxy-5,6-</td>
<td>0.27</td>
<td>0.35</td>
</tr>
<tr>
<td>dihydro-uracil cis</td>
<td></td>
<td></td>
</tr>
<tr>
<td>5,6-dihydroxy-5,6-</td>
<td>0.32</td>
<td>0.46</td>
</tr>
<tr>
<td>dihydro-uracil trans</td>
<td></td>
<td></td>
</tr>
<tr>
<td>iso-dialuric acid</td>
<td>0.40</td>
<td>0.45</td>
</tr>
<tr>
<td>alloxan</td>
<td>0.63</td>
<td>0.84</td>
</tr>
<tr>
<td>5-hydroxyhydantoin</td>
<td>0.51</td>
<td>0.67</td>
</tr>
<tr>
<td>parabanic acid</td>
<td>0.68</td>
<td>0.93</td>
</tr>
<tr>
<td>formylurea</td>
<td>0.68</td>
<td>0.71</td>
</tr>
<tr>
<td>urea</td>
<td>0.34</td>
<td>0.30</td>
</tr>
<tr>
<td>biuret</td>
<td>0.54</td>
<td>0.56</td>
</tr>
<tr>
<td>C4H7N3O4</td>
<td>0.41</td>
<td>0.56</td>
</tr>
<tr>
<td>cytosine</td>
<td>0.24</td>
<td>0.14</td>
</tr>
</tbody>
</table>

A = Chloroform-methanol-water (4:2:1) lower phase v/v/v with 5% methanol (RF cited were obtained after two successive runs). B = Ethyl acetate-methanol-water (v/v/v, 75:16:20) upper phase. System A is used in the first dimension and the B system in the 2nd dimension. Cellulose F/Merk (precoated plates Darmstadt) (R.F.A.) (20 x 20 cm — 0.1 mm). C = 1st dimension propanol-water (10:3, v/v). D = 2nd dimension iso-propanol-ammonium sulphate saturated solution water (2:7:9:19, v/v/v). These chromatography media and systems of solvents were used and prepared according to Cadet and Teoule.

were identified as m/e = 101 (M+ = 45), 89 (C6H5O3)2+, 46 (CH4NO)+.

Iso-dialuric acid and alloxan (traces)

The two substances were the final products of a higher degree of oxidation than that of glycols.

Alloxan gave a blue fluorescence at 254 nm and a brown spot after spraying of Fink’s reagent. Microinfrared spectrometry gave carbonyl frequencies for oxaluric acid: νCO = 1715–1770 cm⁻¹; and for formyl-urea: νCO = 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 Macherey Nagel N-HR/UV with one bidimensional chromatography.

1st run: Petroleum ether-ethyl-formiate-propionic acid (140 : 70 : 15; v/v/v) ; RF cis: 0.41; RF trans: 0.35. 2nd run: Benzene-acetic acid (80 : 20; v/v); RF cis: 0.56; RF trans: 0.47. Spectral characteristics: trans λmax = 451 nm, cis λmax = 371 nm (in NaOH 0.5 M); cis: νCO = 1700 cm⁻¹, trans: νCO = 1672 cm⁻¹.

These values are in good agreement with those of Katsuki. Unambiguous identification of the 2,4-dinitrophenylhydrazone was given by the molecular peak M+ = 254 obtained by mass spectrometry.
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 ($\nu_{CO} = 1710 - 1600 \text{ cm}^{-1}$) ($M^+ = 103$).

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, and in the radiolysis of 5-bromouracil by Gilbert et al., 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. and Dea, 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 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 ($10^{-2} \text{ M}$ to $10^{-3} \text{ M}$ 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.

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 silica gel plates MN-N-HR ($20 \times 20 \text{ cm} - \text{ thickness } 0.5 \text{ mm}$) 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 visualization of resonating-conjugated products at 254 nm. After spraying with Fink’s reagent (paradimethyl-aminobenzaldehyde in alcoholic acid solution), 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. 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 dinitorphenylhydrazin. 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 an 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.
Iso-dialuric acid was prepared by the method of Behrend and Roosen\textsuperscript{17}. 5-Hydroxy-hydantoin was obtained by condensation of urea with glyoxylic acid\textsuperscript{18}.

Isomeric forms of 2,4 dinitrophenylhydrazones of glyoxylic acid were crystallized according to El Hawary and Thompson\textsuperscript{19}.

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

We wish to thank Mr. Cadet and Ulrich, Miss Ducolomb for helpful discussion and spectrometric analysis, Miss Pouchot, Mr. Mazza, Guy, and Gauci for their skilful technical assistance.