Reinvestigation of the Modification of Nucleic Acids with Malonaldehyde

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The reaction of yeast ribonucleic acid (RNA) with malonaldehyde, a product of lipid oxidation, at pH 4 and 37°C produced modified RNA with absorption at 325 nm and fluorescence. The fluorescence intensity was extremely low as compared with that of bovine serum albumin modified similarly. Torula yeast transfer RNA (tRNA), calf thymus deoxyribonucleic acid (DNA), polycytidylic acid (polyC) and polyadenylic acid (polyA) underwent similar modification. The modification took place at cytosine, adenine and probably guanine residues. The absorption maximum at 325 nm may be due to the modified cytosine and adenine residues, which form 1 : 3 adducts with a methylene cyclopropane ring and a six-membered ring (Nair et al., J. Am. Chem. Soc., 106, 3370 (1984)). These modifications did not produce any significant fluorescence. The present data are inconsistent with those of Reiss et al. (Biochem. Biophys. Res. Commun., 48, 921 (1972)), who reported that the modification of nucleic acids with malonaldehyde produced fluorescent cross-links due to conjugated Schiff bases.

Keywords — malonaldehyde; nucleic acid; RNA; DNA; cytidine; adenosine; fluorescence

Malonaldehyde is considered to be one of the secondary products formed during oxidation of polyunsaturated fatty acids. Its formation has been regarded as significant because the aldehyde is toxic, carcinogenic and mutagenic. Reactions of proteins with malonaldehyde have been investigated with respect to the formation of fluorescent components in lipofuscin. Tappel and his associates suggested the formation of fluorescent cross-links due to conjugated Schiff bases between the amino groups of proteins and malonaldehyde. Our recent studies demonstrated that the fluorescence was due to the formation of 4-methyl-1,4-dihydropyridine-3,5-dicarbaldehyde moieties and the cross-links to the formation of the less fluorescent conjugated Schiff bases.

Malonaldehyde can react with deoxyribonucleic acid (DNA) to cause various biological and physicochemical changes. While several reaction modes of nucleic acids have been postulated, they are not consistent. Formation of fluorescent cross-links due to conjugated Schiff bases between the amino groups of base moieties and malonaldehyde has been emphasized. Several papers demonstrated that the reactions of guanosine, cytidine and adenosine afforded 1 : 1, 1 : 2 and 1 : 3 adducts other than the conjugated Schiff bases. We reinvestigated the reaction of nucleic acids with malonaldehyde, in order to clarify the fluorescence characteristics and the reaction modes.

Materials and Methods

Adenosine, uridine-2′(3′)-monophosphate (2′,3′-UMP), adenosine-2′(3′)-monophosphate (2′,3′-AMP), guanosine-2′(3′)-monophosphate (2′,3′-GMP), polycytidylic acid sodium salt (polyC) and polyadenylic acid sodium salt (polyA) were obtained from Yamasa Shoyu Co., Ltd., Tokyo, Japan. Uridine, cytidine, arabinosylcytosine, guanosine, cytidine-2′(3′)-monophosphate (2′,3′-CMP) and ribonucleic acid (RNA) from yeast and transfer RNA (tRNA) from Torula yeast were obtained from Kohjin Company, Ltd., Tokyo, Japan. Calf thymus DNA and bovine serum albumin (BSA) were obtained from Sigma Chemical Company, St. Louis, Mo., U.S.A.

Malonaldehyde solution at 1M concentration was prepared by acid hydrolysis of malonaldehyde
bis(dimethylacetal) as described elsewhere.60 The solution was diluted into the appropriate buffer for use.
Phosphorus was determined according to the method of Gerlach and Deuticke.11 Paper chromatography was
performed on Toyo Roshi No. 51A paper with a solvent system of 1-butanol–water (84:16). Spots were detected
under ultraviolet (UV) light. Cellulose column chromatography was performed with Toyo Roshi cellulose powder
(above 100 mesh) for chromatography. UV absorption spectra were taken with a Shimadzu UV-200S double-beam
spectrophotometer. Nuclear magnetic resonance (NMR) spectra were taken in dimethylsulfoxide-d$_6$ with Me$_4$Si as an internal standard using a JEOL PS-100 machine. High performance liquid chromatography (HPLC) was performed by the use of a Shimadzu LC-2 liquid chromatograph equipped with a column of Zorbax ODS (4.6 mm i.d. × 25 cm), and the peaks were
detected at 260 nm with a Shimadzu SPD-1 spectrophotometric detector. The chromatograph was operated with a
solvent system of methyl alcohol–0.05 M ammonium phosphate buffer (pH 6.0) at a flow rate of 0.5 ml/min.

**Nucleic Acids of Polynucleotides Modified with Malonaldehyde**—A mixture of RNA (tRNA, DNA, polyC or polyA), and malonaldehyde or malonaldehyde/acetaldehyde in 0.1 M acetate buffer (pH 4.0) or 0.1 M phosphate buffer (pH 7.0) was incubated. Aliquots were periodically removed from the reaction mixture and dialyzed against 0.9% sodium chloride solution. The dialyzate was mixed with 2 volumes of ethyl alcohol, and the precipitate thus formed was collected by centrifugation at 3000 rpm for 10 min. The precipitate was redissolved in 1.0 ml of 0.9% sodium chloride solution and recovered by addition of ethyl alcohol. The precipitate was dried over calcium chloride. Under these conditions, no precipitate was obtained from the incubation mixture of malonaldehyde or malonaldehyde/acetaldehyde alone.

**Reaction Products of Nucleosides with Malonaldehyde**—A 100 ml reaction mixture of 40 mm cytidine and 100 mm malonaldehyde in 0.1 M acetate buffer (pH 4.0) was incubated at 37 °C for 6 d, then evaporated at below 40 °C. The residue was applied to a column (2 × 75 cm) of cellulose powder and eluted with 1-butanol–water (84:16). The fractions containing product CR’ were collected and evaporated. The residue was crystallized from ethyl alcohol–water to afford a pale yellow crystalline powder (41 mg). UV $\lambda_{\text{max}}$ (phosphate, pH 7.0): 238, 322 nm; $\lambda_{\text{max}}$ (HCl): 238, 322 nm. Fluorescence $\lambda_{\text{max}}$ (excitation): 380 nm; $\lambda_{\text{max}}$ (emission): 453 nm. Relative fluorescence intensity: $4 \times 10^{-3}$ with respect to the intensity of quinine sulfate in 0.1 N sulfuric acid. 1H-NMR (dimethylsulfoxide-d$_6$) ppm: 9.37 (1H, s, CHO), 9.20 (1H, s, CHO), 8.61 (1H, d, C$_6$H, $J=7$ Hz), 8.39 (1H, br. s), 7.71 (1H, s), 6.84 (1H, br s), 6.75 (1H, d, C$_6$H, $J=7$ Hz), 5.78 (1H, s, C$_6$H), 2.00 (1H, d, gem-H, $J=12$ Hz), 1.81 (1H, d, gem-H, $J=12$ Hz).

Product AC’ from arabinosylcytosine was isolated as a pale yellow crystalline powder (73 mg). UV $\lambda_{\text{max}}$ (phosphate, pH 7.0): 237, 324 nm; $\lambda_{\text{max}}$ (HCl): 237, 324 nm. 1H-NMR (dimethylsulfoxide-d$_6$) ppm: 9.39 (1H, s, CHO), 9.21 (1H, s, CHO), 8.39 (1H, d, C$_6$H, $J=7$ Hz), 7.72 (1H, s), 6.82 (1H, br s), 6.72 (1H, d, C$_6$H, $J=7$ Hz), 6.08 (1H, d, C$_6$H), 2.03 (1H, d, gem-H, $J=12$ Hz), 1.80 (1H, d, gem-H, $J=12$ Hz).

Product AR’ from adenosine was isolated as a pale yellow crystalline powder. UV $\lambda_{\text{max}}$ (phosphate, pH 7.0): 240, 270, 330 nm; $\lambda_{\text{max}}$ (HCl): 240, 270, 330 nm.

**Results and Discussion**

The extents of fluorescence formation in the reactions of RNA and BSA with malonaldehyde were compared (Fig. 1). When 0.04 or 0.1% BSA was reacted with 10 mm malonaldehyde at pH 7.0 and 37 °C, fluorescence with an excitation maximum at 404 nm and an emission maximum at 467 nm increased. The fluorescence can be attributed to the formation of 4-methyl-1,4-dihydropyridine-3,5-dicarbaldehyde moieties at the N and the ε-amino groups of BSA.60,61 In contrast, the reaction of 0.04 or 0.1% RNA with malonaldehyde at pH 7.0 or 4.0 at 37 °C produced much less fluorescence. The increase in fluorescence was only a little higher than that in the incubation mixture of malonaldehyde alone.

RNA, tRNA and DNA were reacted with malonaldehyde at 37 °C, and the modified nucleic acids were recovered. While the RNA modified at pH 7 did not show any change in the absorption spectrum, the RNA modified at pH 4 showed an absorption maximum at 325 nm. The extinction coefficients at 325 nm increased as the modification time was increased, and the ratio of $\varepsilon$ (p)$^{325 \text{ nm}}$/ε (p)$^{260 \text{ nm}}$ increased to 0.26 after 90 h of modification (Table I). The fluorescence spectrum of the modified RNA revealed an excitation maximum at 325 nm and an emission maximum at 500 nm. Relative molar fluorescence intensity with respect to quinine sulfate increased with the modification time, but the relative intensity reached only less than 0.1% after 90 h of modification (Table I). The modified tRNA and DNA showed lower ratios of the extinction coefficients, indicating that tRNA and DNA were resistant to the modification. When the nucleic acids were reacted at 70 °C, a marked increase in absorbance
TABLE I. Extinction Coefficients and Fluorescence Intensity of Nucleic Acids and Polynucleotides Modified with Malonaldehyde

<table>
<thead>
<tr>
<th>Nucleic acids and polynucleotides</th>
<th>Extinction coefficient</th>
<th>Relative molar fluorescence intensity</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>$e(260\text{nm})$</td>
<td>$e(325\text{nm})$</td>
</tr>
<tr>
<td>Unmodified RNA</td>
<td>9500</td>
<td>0</td>
</tr>
<tr>
<td>Malonaldehyde-modified RNA</td>
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<td></td>
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<tr>
<td>37°C, 24 h$^a$</td>
<td>9400</td>
<td>860</td>
</tr>
<tr>
<td>37°C, 48 h$^a$</td>
<td>9800</td>
<td>1400</td>
</tr>
<tr>
<td>37°C, 90 h$^a$</td>
<td>10100</td>
<td>2600</td>
</tr>
<tr>
<td>70°C, 5 h$^a$</td>
<td></td>
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</tr>
<tr>
<td>tRNA 37°C, 90 h$^a$</td>
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<td></td>
</tr>
<tr>
<td>70°C, 5 h$^a$</td>
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<td></td>
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<tr>
<td>DNA 37°C, 90 h$^a$</td>
<td></td>
<td></td>
</tr>
<tr>
<td>70°C, 5 h$^a$</td>
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<td></td>
</tr>
<tr>
<td>PolyC 70°C, 5 h$^a$</td>
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<td>PolyA 70°C, 5 h$^a$</td>
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<tr>
<td>Malonaldehyde/acetaldehyde-modified RNA</td>
<td>8100</td>
<td>0</td>
</tr>
<tr>
<td>37°C, 24 h$^a$</td>
<td>9100</td>
<td>820</td>
</tr>
<tr>
<td>37°C, 48 h$^a$</td>
<td>9800</td>
<td>1500</td>
</tr>
<tr>
<td>37°C, 90 h$^a$</td>
<td>10000</td>
<td>2400</td>
</tr>
<tr>
<td>Acetaldehyde-treated RNA 37°C, 90 h$^d$</td>
<td>8100</td>
<td>0</td>
</tr>
<tr>
<td>Quinine sulfate</td>
<td></td>
<td></td>
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</tbody>
</table>

A solution of a) 1% nucleic acid and 60 mm malonaldehyde, b) 0.25%, nucleic acid or polynucleotide and 250 mm malonaldehyde, c) 1% nucleic acid and 60 mm malonaldehyde/60 mm acetaldehyde, or d) 1% nucleic acid and 60 mm acetaldehyde, in 1m acetate (pH 4.0) was incubated, and the modified nucleic acid or polynucleotide was recovered. e) $e(325\text{nm})/e(260\text{nm} \text{ for } \text{polyC})$. f) The intensities determined at the excitation and emission maxima are expressed relative to that of quinine sulfate.

Fig. 1. Time Course of Fluorescence Formation in the Reaction Mixture of RNA and BSA with Malonaldehyde

A mixture of RNA or BSA, and 10 mm malonaldehyde in 0.1 m phosphate buffer (pH 7.0) (-----) or 0.1 m acetate buffer (pH 4.0) (-----) was incubated at 37°C. Relative fluorescence intensities of the reaction mixtures at the maximum wavelengths of excitation and emission were expressed with respect to 0.1 μm quinine sulfate in 0.1% sulfuric acid (excitation at 350 nm and emission at 450 nm). ○, 0.04% RNA or BSA; ○, 0.1% RNA or BSA.

Fig. 2. Paper Chromatography of the Reaction Mixtures of Nucleosides with Malonaldehyde

A solution of 40 mm nucleoside (10 mm in case of guanosine) and 100 mm malonaldehyde in 0.1 m acetate buffer (pH 4.0) was incubated at 37°C for 5 d. Chromatography was done with a solvent system of 1-butanol-water (84:16). Spots were visualized under UV light. CR, AC, AR, GR and UR indicate the reaction mixtures of cytidine, arabinosylcytosine, adenosine, guanosine and uridine, respectively.
at 325 nm was observed. PolyC and polyA produced modified polynucleotides with the same absorption maximum at 325 nm when treated at 70 °C. At elevated temperature, their secondary structures were disrupted and the molecules could be readily modified.

Malonaldehyde may coexist with other aliphatic aldehydes as secondary degradation products of polyunsaturated fatty acids, and the formation of fluorescent 4-methyl-1,4-dihydropyridine-3,5-dicarbaldehyde was enhanced by the presence of acetaldehyde in the reaction of amino acids and malonaldehyde.12) RNA was reacted with malonaldehyde/acetaldehyde at pH 4 and 37 °C, but the modified RNA showed the same absorption spectrum as malonaldehyde-modified RNA with similar ratios of the extinction coefficients (Table I). Fluorescence spectra and relative fluorescence intensity (Table I) were essentially similar to those of the malonaldehyde-modified RNA. Thus, the presence of acetaldehyde did not affect the formation of fluorescence.

The malonaldehyde-modified RNA was hydrolyzed with alkali and the 2',3'-nucleotides were fractionated by HPLC. While no modified nucleotides could be detected, probably owing to their instability to alkaline treatment, the contents of most of the nucleotides except for 2',3'-UMP were decreased, and the lost nucleotide had presumably been modified (Table II).

The reaction mixtures of cytidine, arabinosylcytosine and adenosine showed new spots corresponding to CR', AC' and AR', respectively (Fig. 2). The UV absorption spectra of the extracts of these spots had maxima at around 325 nm. All the spots corresponding to the parent nucleosides showed spectra similar to those of the parent nucleosides. Each reaction mixture was fluorescent, but the intensity was extremely low as compared to the standard quinine sulfate and did not exceed that of the control reaction mixture of malonaldehyde alone (Table III).

Product CR' which was isolated by the use of a cellulose column exhibited absorption maxima at 238 and 322 nm. It is fluorescent but its intensity is much less than that of quinine sulfate. The 1H-NMR spectrum of the product suggested that two signals at 9.37 and 9.20 ppm were assignable to two aldehyde protons, three signals at 8.39, 7.71 and 6.84 ppm to six-membered ring protons and two signals at 2.00 and 1.81 ppm to two geminal cyclopropane ring protons. This compound appears to be the hypermodified cytidine with a methylene cyclopropane-ring and a six-membered ring reported by Nair et al.10) Product AC' showed characteristics similar to those of CR'. Product AR' showed an absorption spectrum which coincided with that reported by Nair et al.10)
The present data demonstrated that malonaldehyde modifies nucleic acids without forming significant fluorescence at cytosine, adenine and probably guanine residues. Absorption maxima at around 325 nm of the modified nucleic acids may be due to the modified cytosine and adenine residues. The modified cytosine and adenine residues may be the 1:3 adducts of the bases and malonaldehyde with a methylene cyclopropane ring and a six-membered ring. Brooks and Klamerth suggested that guanine residues were more susceptible to the modification than the other bases. Seto et al. obtained the 1:1 adduct of guanosine with a cyclic structure showing an absorption maximum at 253 nm, and Marnett et al. obtained the 1:2 adduct with a bicyclic structure having an absorption maximum at 249 nm. While the former adduct has been reported to be fluorescent, its intensity was not elucidated. Since the content of guanine residues in the modified nucleic acids was decreased, the residues are presumably modified in either way. The reaction products of guanosine could not be detected on paper chromatography, and might not be separated under the conditions used.

Reiss et al. demonstrated that the reaction of nucleic acids with malonaldehyde produced significant fluorescence with maxima at 390 nm (excitation) and 460 nm (emission) and characteristic absorption with a maximum at 325 nm, and suggested that the alterations were due to the formation of the conjugated Schiff bases of the amino groups of the base moieties and malonaldehyde. However, they did not measure the fluorescence intensities of the modified nucleic acids. Nevertheless, the formation of fluorescent cross-links due to conjugated Schiff base has been regarded as a clue to the biological actions of malonaldehyde. Several attempts were made to detect the cross-links of DNA by fluorescence measurement or radiolabeling through borotritide reduction of the conjugated Schiff bases. On the other hand, no cross-linked base pairs due to conjugated Schiff bases could be detected under a variety of conditions, and it was suggested that cross-linking was not prerequisite for the biological activity. The present data are consistent with those of Fujimoto et al. who did not detect the formation of significant fluorescence in the reaction of DNA and malonaldehyde. It is suggested that fluorescent cross-links due to conjugated Schiff bases are hardly produced, but much less fluorescent and uncross-linked hypermodified bases are preferentially produced in the interaction of nucleic acids and malonaldehyde.

References


