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Chemical Reactivity of the Nucleic Acid Bases. I. Antioxidative Ability of the Nucleic Acids and Their Related Substances on the Oxidation of Unsaturated Fatty Acids

SETSURO MATSUSHITA, FUMIO IBUKI AND AKIJI AOKI[1]

From the Research Institute for Food Science, Kyoto University, Kyoto, Japan

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The nucleic acids and their related substances exhibit antioxidative effects on the oxidation of linoleic acid by air. Especially adenine, guanosine, xanthine, hypoxanthine, uric acid, uracil, orotic acid, and ribonucleic acid were as effective antioxidants as α-tocopherol, nordihydroguaiaretic acid, and butylhydroxyanisole. Nucleic acids and their related substances may be used as antioxidants for lipids.

INTRODUCTION

Unsaturated fatty acids are readily oxidized by atmospheric oxygen with the formation of the corresponding peroxides. There are naturally occurring substances which prevent this phenomenon. These substances are found in native tissues or in crude lipids; upon elimination of these protective substances, the fatty acids are then readily oxidized. Among the naturally occurring antioxidants are tocopherol, gallic acid, eugenol, sesamol, ascorbic acid, norconidendrin, and nordihydroguaiaretic acid. In addition, certain synthetic compounds such as butylhydroxyanisole, ethyl protocatecuate, propylgallate, trihydrobutylphenone, and dibutylhydroxytoluene have antioxidative ability.

The mechanism of action of these antioxidants is thought to be as follows (1):

$$ROO^{\cdot} + AH_2 \rightarrow ROOH + AH^{\cdot}$$
$$2AH^{\cdot} \rightarrow AH_2 + A$$
$$ROO^{\cdot} + AH^{\cdot} \rightarrow ROOH + A$$

AH_2 represents the antioxidant which is converted to A by oxidation. In the processes in which unsaturated fatty acids are oxidized and their peroxides are formed, free radicals are generated (2); the unsaturated fatty acids are rapidly oxidized in the chain reaction which these free radicals start. Therefore, the low concentration of free radicals formed at the early stage must be trapped by the antioxidant and the chain reaction of oxidation prevented in order to protect against the oxidation of unsaturated fatty acids.

Low concentrations of metal ions, particularly copper and iron, accelerate these oxidations. Hence, chelating agents such as polybasic organic acids, which can bind metal ions or phosphates which form insoluble salts, are generally used as auxiliary agents.

The observations that nucleic acids and nucleoproteins contain free radicals (3) and nucleic acids and their related substances show peroxidase activity (4), suggest that these substances have the tendency to release electrons and form free radicals; that is, they have the ability to trap free radicals. Furthermore, since purine and pyrimidine bases possess nitrogen and oxygen atoms in their own structures, they are thought to form chelate compounds with metals easily. If the nucleic acids and their related substances possess the two properties described above, they may be considered to be the ideal antioxidants.

Hence, we tested these substances as antioxidants for the oxidation of unsatu-

[1] Present address: Kokoku Rayon and Pulp Co., Ltd., Saeki, Japan.

rated fatty acids and have obtained satisfactory results.

MATERIALS AND METHODS

Nucleic acids and their related substances were purchased from Nutritional Biochemicals Corporation and Sigma Chemical Co. Nordihydroguaiaretic acid (NDGA), butylhydroxyanisole (BHA), α-tocopherol, linoleic acid, and thiobarbituric acid (TBA) were purchased from Tokyo Chemical Co. α,α′-Diphenyl-β-trinitrophenylhydrazyl (DPPH) was synthesized by the method of Goldschmidt (5).

α-Tocopherol, NDGA, and BHA were added to buffer solutions after they were dissolved in a small amount of ethanol. Nucleic acids and their related substances were dissolved in buffer solutions.

The modified TBA method (6) was used for the determination of peroxide. Linoleic acid, 200 mg., was dissolved in 0.7 ml. of 1 *N* sodium hydroxide solution and then diluted to 50 ml. with 0.2 *N* borate buffer, pH 9.0 or 0.2 *N* phosphate buffer, pH 7.0. Then 0.5 ml. of the solution of nucleic acids and their related substances and 0.5 ml. of the buffer solution or another solution were added to 1 ml. of the linoleic acid solution. The complete mixture was placed at 37° for the designated number of hours in air of high humidity; then 1 ml. of 35% trichloroacetic acid and 2 ml. of 0.75% TBA solution were added, and the tube was placed in a boiling water bath for 15 min. to develop colors. After this solution was cooled, 1 ml. of glacial acetic acid and 2 ml. of chloroform were added, stirred, and centrifuged (1000) × *g* for 5 min.). The optical density of the aqueous layer was measured at 535 mμ (TBA value).

The values in the tables except Table I are shown as the mean values of duplicate experiments. The values can be compared with those only in the same table, because the conditions of linoleic acid solutions used in every experiment were different.

EXPERIMENTAL AND RESULTS

1. The Effects of Addition of Nucleic Acids and Their Related Substances

Linoleic acid is oxidized spontaneously in air. Nucleic acids and their related substances were added to the solution of linoleic acid in an effort to determine which of these substances had antioxidative ability. For incubation periods up to 18 hr. at 37°, all substances showed antioxidative ability. Substances effective for 48 hours at 37° were: cytosine, adenine, adenylic acid, guanine, guanosine, xanthine, hypoxanthine, and uric acid at pH 7.0; uracil, dihydrouracil, adenine, guanosine, orotic acid, uric acid, hypoxanthine, xanthine, and ribonucleic acid at pH 9.0 (Table I). In these experiments, the linoleic acid remained in solution at pH 9.0, but was emulsified at pH 7.0.

TABLE I

Antioxidative Ability of the Nucleic Acid Related Substances

The values of peroxide are expressed as the percentage of that for no addition. The concentration of the added substances was 5×10^{-3} *M* except 4 mg./2 ml. ribonucleic acid. The mixtures were incubated at 37° for 48 hr.

Added substances	Peroxide value	
	pH 7.0	pH 9.0
Uracil	105	80
Uridine	107	98
3′-Uridylic acid	107	195
Dihydrouracil	—	61
Cytosine	72	173
Cytidine	89	164
3′-Cytidylic acid	98	176
Adenine	70	68
Adenosine	99	137
3′-Adenylic acid	73	182
Guanine	70	111
Guanosine	80	65
3′-Guanylic acid	153	127
Thymine	94	110
Orotic acid	93	62
Inosinic acid	87	101
Uric acid	59	52
Hypoxanthine	67	56
Xanthine	37	55
Ribonucleic acid	106	57
No addition	100	100
	(O.D., 0.281)	(O.D., 0.335)

From Table I, the substances which appeared to be most effective were chosen and their effects were reexamined. As shown in Table II, all showed obvious effects.

Those that appeared to be effective were then tested directly for possible interference in the color reaction itself. To the linoleic acid solution containing the peroxide which had been already formed during incubation at 50° overnight, the substances shown in Table II were added, and the colors were developed at once. A few kinds of substances increased the color yield, but most of the

substances did not cause interference. Only ribonucleic acid caused appreciable increase of color. However, when ribonucleic acid was added to a solution which contained no preformed peroxides, it showed antioxidative ability. Only when ribonucleic acid was added to the solution in which the peroxide had already been generated, was increase of color found.

2. The Effect of the Concentration of Added Substances

Varying levels of the substances which showed antioxidative ability in the experiment described above were tested to determine the effects of concentration of these substances. As shown in Table III, adenine, uracil, ribonucleic acid, and α-tocopherol were less effective below 10^{-3} *M*, but uric acid was more effective at the lower concentration, and less effective above 2.5×10^{-3} *M*. With α-tocopherol, at the lower concentration, the color became higher than that of the sample with no addition. It thus appeared to accelerate the rate of oxidation. This phenomenon of reversal of effects at different concentrations of the added substances may be due to the general properties of antioxidants. The mechanism of action of antioxidants is thought to be that they act as the acceptor of the free radicals produced at the initial stage and as the accelerators of oxidation by making peroxides produced in the course of the induction period. The antioxidative ability is revealed by the balance of these reactions. On the other hand, they can become accelerators of the oxidation over a certain range of their concentrations.

TABLE II

Antioxidative Ability of the Nucleic Acid Related Substances

The values are expressed as the TBA value. The concentration of the added substances was 5×10^{-3} *M* except 4 mg./2 ml. ribonucleic acid. The mixtures were incubated at 37° for 45 hr.

Added substances	pH 7.0	pH 9.0
Adenine	0.366	0.189
Guanosine	0.216	0.121
Xanthine	0.204	0.099
Hypoxanthine	0.240	0.108
Uracil	0.243	0.127
Orotic acid	0.350	0.242
Uric acid	0.200	0.104
Ribonucleic acid	0.278	0.085
α-Tocopherol	0.325	0.219
No addition	0.435	0.438

3. The Effects of the Nucleic Acid Related Substances in the Presence of Copper Salts

The oxidation of unsaturated fatty acids is accelerated by the addition of copper salts. Hence, the effects of the nucleic acid related substances were tested in the presence of copper salts. As shown in Table IV, the accelerating effect of copper ions was depressed by the added substances, and the autooxidation of unsaturated fatty acids in

TABLE III

The Effects of the Concentration of Added Substances

The values are expressed as the TBA value. The pH of the mixtures was 9.0, and they were incubated at 37° for 48 hr.

Added substances	Concentration (*M*)					
	5×10^{-4}	2.5×10^{-3}	10^{-3}	5×10^{-4}	2.5×10^{-3}	10^{-3}
Adenine	0.138	0.130	0.137	0.172	0.210	0.277
Uracil	0.226	0.148	0.167	0.273	0.370	0.367
Hypoxanthine	0.116	0.087	0.095	0.149	0.227	0.273
Uric acid	0.163	0.134	0.075	0.088	0.062	0.063
Ribonucleic acid	0.128[a]	0.105	0.105	0.144	0.166	0.208
α-Tocopherol	0.224	0.234	0.205	0.517	0.559	0.519
No addition	0.381					

[a] Ribonucleic acid, 4 mg./2 ml., in the case of 5×10^{-4} *M*.

TABLE IV

THE EFFECTS OF THE NUCLEIC ACID RELATED SUBSTANCES IN THE PRESENCE OF COPPER SALT

The values are expressed as the TBA value. The concentrations of added substances were: nucleic acid related substances, 5×10^{-3} *M*; ribonucleic acid, 4 mg./2 ml.; and copper sulfate, 5×10^{-6} *M*. The pH of the mixtures was 9.0, and they were incubated at 37° for 24 and 48 hr.

Added substances	24 hours		48 hours	
	No Cu	+ Cu	No Cu	+ Cu
Adenine	0.125	0.315	0.247	0.884
Uracil	0.104	0.300	0.290	0.683
Hypoxanthine	0.067	0.112	0.181	0.253
Uric acid	0.140	0.112	0.213	0.173
Ribonucleic acid	0.113	0.253	0.141	0.615
α-Tocopherol	0.070	0.561	0.300	1.000
No addition	0.267	0.568	0.684	0.990

the absence of copper ions was also depressed. This observation was thought to indicate that the nucleic acid related substances had the ability to chelate metal ions and trap free radicals at the same time. In contrast, α-tocopherol showed antioxidative ability in the absence of copper ions, but could not protect against the accelerating effect of added copper ions. In this experiment, uric acid proved to be the most effective antioxidant. The effect of addition of auxiliary agents was tested as shown in Table V. However, they did not show any additional effects in the presence of nucleic acid related substances.

4. THE EFFECT OF URIC ACID

Since uric acid was the most effective in overcoming the acceleration of oxidation of fatty acids due to copper ions, experiments were carried out to determine optimal concentration of uric acid under these conditions. As shown in Table VI, 10^{-4} *M* was the most effective in the presence of copper. Above 10^{-4} *M*, the data are rather confusing; that is, 10^{-3} *M* was less effective than 10^{-4} *M* or 5×10^{-3} *M*. However, this tendency was reproducible in several experiments. The same phenomenon is also found in Table VII. Table VII shows the comparison of the

TABLE V

THE EFFECT OF THE ADDITION OF AUXILIARY AGENTS

The values are expressed as the TBA value. The concentrations of added substances were: pyrophosphate and citrate, 10^{-3} *M*; uric acid, 10^{-4} *M*; xanthine, uracil, and adenine, 5×10^{-3} *M*; copper sulfate, 10^{-6} *M*. The pH of the mixtures was 9.0, and they were incubated at 37° for 65 hr.

Copper sulfate	1.231
Copper sulfate + uric acid	0.132
Copper sulfate + xanthine	0.200
Copper sulfate + uracil	0.530
Copper sulfate + adenine	0.444
Copper sulfate + pyrophosphate	0.804
Copper sulfate + pyrophosphate + uric acid	0.091
Copper sulfate + pyrophosphate + xanthine	0.210
Copper sulfate + pyrophosphate + uracil	0.205
Copper sulfate + pyrophosphate + adenine	0.563
Copper sulfate + citrate	1.107
Copper sulfate + citrate + uric acid	0.263
Copper sulfate + citrate + xanthine	0.244
Copper sulfate + citrate + uracil	0.766
Copper sulfate + citrate + adenine	0.750
No addition	0.988

TABLE VI

THE EFFECT OF URIC ACID

The values are expressed as the TBA value. The concentration of copper sulfate was 5×10^{-6} *M*. The pH of the mixtures was 9.0, and they were incubated at 37° for 48 hr.

Concentration of uric acid *M*	Expt. I		Expt. II	
	No Cu	+ Cu	No Cu	+ Cu
5×10^{-2}	0.225	0.182	0.244	0.203
2.5×10^{-3}			0.194	0.271
10^{-3}	0.119	0.510	0.084	0.468
5×10^{-4}			0.046	0.241
10^{-4}	0.090	0.173	0.037	0.082
5×10^{-5}			0.037	0.087
10^{-5}	0.198	0.845	0.075	0.383
10^{-6}			0.253	0.640
No addition	0.615	0.887	0.493	0.690

effects of uric acid and, the synthetic substances NDGA and BHA, which are commonly employed antioxidants. Uric acid was more effective than these substances.

Time course of the reaction is shown in Fig. 1, in which the effects of uric acid were compared with those of α-tocopherol in the presence or absence of copper.

TABLE VII

COMPARISON OF THE EFFECTS OF URIC ACID, NDGA AND BHA

The values are expressed as the TBA value. The concentration of copper sulfate was 5×10^{-6} *M*. The pH of the mixtures was 9.0, and they were incubated at 37° for 48 hr.

Added substances	Concentration of added substances (*M*)					
	10^{-3}		5×10^{-4}		10^{-4}	
	No Cu	+ Cu	No Cu	+ Cu	No Cu	+ Cu
Uric acid	0.103	0.595	0.065	0.508	0.066	0.163
NDGA	0.073	0.109	0.091	0.134	0.105	0.580
BHA	0.101	0.202	0.089	0.210	0.059	0.700
No addition	0.630					
Only Cu added		0.890				

5. THE REACTION BETWEEN NUCLEIC ACID RELATED SUBSTANCES AND α,α'-DIPHENYL-β-TRINITROPHENYLHYDRAZYL

DPPH is a dye which has a free radical (7) in itself and is stable between pH 5 and 6.5. This dye loses its purple color when it takes an electron from other compounds or reacts with other compounds which have free radicals. Nucleic acid related substances were added to the dye dissolved in a small amount of ethanol. Upon the addition of uric acid to the dye solution at 37°, the color faded at once. However, the other nucleic acid related substances did not exert a similar effect. If they were incubated overnight at 37° with hydrogen peroxide, solutions of uracil and cytosine also caused the dye to fade. These results may indicate that uric acid can react easily with free radicals in water and release an electron, whereas uracil and cytosine can react with free radicals only when treated with hydrogen peroxide; i.e., they can produce free radicals in the presence of hydrogen peroxide. This result

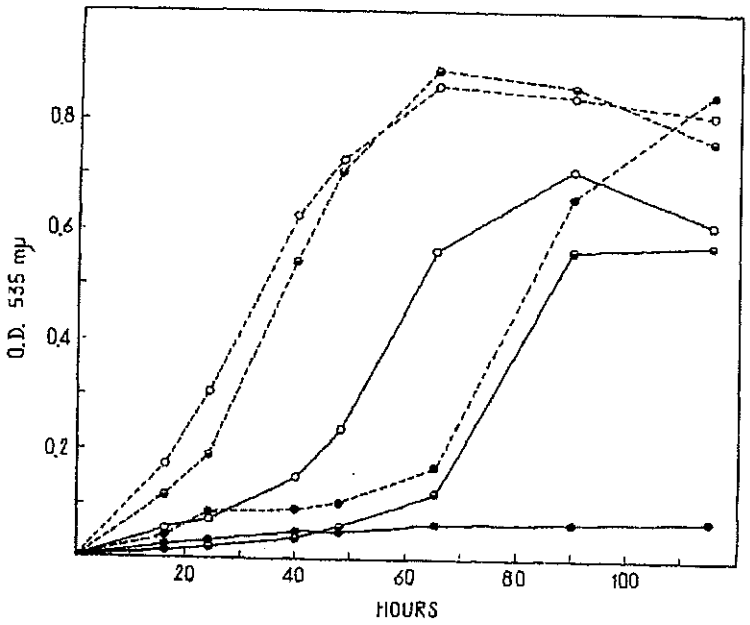

FIG. 1. Time course of the reaction. ○, linoleic acid only; ●, linoleic acid plus uric acid; ◒, linoleic acid plus α-tocopherol; solid line, no Cu; dotted line, Cu added. The concentrations of added substances were: uric acid, 10^{-4} *M*; α-tocopherol, 10^{-3} *M*; copper sulfate, 5×10^{-6} *M*. The pH of the mixtures was 9.0, and they were incubated at 37°.

may have some bearing on the observation that purine and pyrimidine compounds show peroxidase activity (4). The substances which could react with DPPH showed rather effective antioxidative ability among nucleic acid related substances.

DISCUSSION

The results presented here show that most of the nucleic acids and their related substances possess antioxidative ability toward linoleic acid oxidation. The effect may depend upon their ability to trap free radicals formed in the course of the oxidation of linoleic acid and to chelate certain metal ions which serve to accelerate the oxidation. The experiments described were performed only at pH 7 and 9, because of the insolubility of linoleic acid in acidic media. Certain of the nucleic acid related substances react at acidic pH with a dye which has a free radical in itself and, therefore, it is possible that the nucleic acid related substances possess antioxidative ability even at acidic pH.

In this paper, only linoleic acid was used as the unsaturated fatty acid, but the results should be applicable to other unsaturated fatty acids such as linolenic and arachidonic acids. Furthermore, one would expect that ribonucleic acid related substances might be used as antioxidants for the natural lipids. However, for this purpose, they should be used with an emulsifier because the nucleic acid related substances cannot be dissolved in lipids when they are to be used in foods. It, therefore, would be necessary to synthesize derivatives of the nucleic acid related substances which can be dissolved in lipids. These base analogs are considered dangerous because of the possibility that they may be incorporated in the body; therefore, they should have structures which can be readily degraded in the body to the native bases. On the other hand, the nucleic acid related substances which are added to foods could be easily digested, because they are natural substances. Purine can be excreted as uric acid (8), and pyrimidine can enter the TCA cycle (8) in the course of catabolism. Therefore, they should be harmless.

According to the reasons which are mentioned above, the nucleic acids and their related substances may be used as antioxidants for lipids.

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