Some Properties of Nitrosation-Accelerating Substance(s) in Sodium Chondroitin Sulfate Preparations

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Contaminant(s) in sodium chondroitin sulfate (SCS) preparations can strongly accelerate formation of nitrosamines from secondary amines and nitrite. About 70% of nitrosation-accelerating activity (NAA) was recovered in the filtrate through a DIAFLO YC 05 ultrafiltration membrane. The results of ultrafiltration and column chromatography using Sephadex G-10 identified that the molecular weight of the main component that showed NAA was several hundreds. It reacted with nitrite and gradually lost its NAA. It did not accelerate formation of nitrosomethylurea. It accelerated formation of nitrosodimethylamine additively with thiocyanate, a well known accelerator of nitrosamine formation.

Keywords—acceleration; chondroitin sulfate; contaminant; nitrite; nitrosamine; nitrosamine formation

Introduction

There are many substances in the environment and in foods that inhibit or accelerate carcinogenic nitrosamine formation from secondary amines and nitrite. As accelerators that might be ingested by humans, thiocyanate¹⁾ contained in saliva, catechins,²⁾ ingredients of Japanese tea,^{2a)} other flavonols such as kaempferol and quercetin,^{2c)} and iodide¹⁾ are known. As inhibitors, ascorbic acid³⁾ and some phenols,⁴⁾ components of foods, are well known.

During work on the effect of food additives on nitrosamine formation, we found that the dialyzable fraction of sodium chondroitin sulfate (SCS) preparations strongly accelerated nitrosamine formation.⁵⁾ The amount of nitrosodimethylamine (NDMA) formed from dimethylamine (DMA) and nitrite when the dialyzable fraction was present was about 18 times that formed in the presence of a similar weight of sodium thiocyanate or potassium iodide.⁵⁾ As thiocyanate and iodide are very potent

accelerators for nitrosamine formation, it seemed interesting to investigate the nature of the accelerator contained in the dialyzable fraction.

This report describes the further fractionation of the dialyzable fraction and also the nature of the substance(s) with nitrosation-accelerating activity (hereafter abbreviated as NAA).

Materials and Methods

SCS used in the experiments was purchased from Tokyo Chemical Industry Co., Ltd. It was dissolved in water and dialyzed overnight against water, and the dialyzable fraction was concentrated to dryness.⁵⁾ Other reagents used were of reagent grade.

Ultrafiltration was performed by using a 50 ml ultrafiltration cell and a DIAFLO YC 05 ultrafiltration membrane (Amicon Corp.). The dried dialyzable fraction obtained from 8 g of SCS preparation was dissolved in 50 ml water and filtered until the volume in the cell was reduced to approximately 5 ml. The retained solution was again diluted with water to 50 ml and filtered. This procedure was repeated three times.

The potency of acceleration of nitrosamine formation was measured by either of the following methods.

Method A—Ten milliliters of reaction mixture containing the test material, morpholine (MOR, 25 mM), and nitrite (2 mM) in 0.1 M sodium acetate-HCl buffer (pH 3.0) was incubated at 37°C for 30 min. After the decomposition of nitrite with 1 ml of 0.6 N sulfamic acid, the absorbance at 260 nm was measured and designated as A. The absorbance of the control solution without the test material was also measured and designated as B. The difference between A and B was considered to represent the increment of nitrosomorpholine (NMOR) formation by the test material. If the difference between A and B exceeded 2.0, the measurement was repeated using a reduced amount of the test material. The difference between A and B thus obtained was defined as the unit of the potency of acceleration of nitrosamine formation. This method cannot be applied when the reaction mixture has absorption at 260 nm in addition to that of NMOR. If the reaction mixture had such absorption, the following method was used.

Method B—The potency of acceleration was measured according to the previous paper.⁵⁾ The test material with DMA and nitrite was incubated at pH 3.5 and 37°C, and the amount of NDMA formed was determined by a gas chromatograph with a thermal energy analyzer after dichloromethane extraction. (In the case of Method B, the unit of potency of acceleration was not defined.)

The amount of ninhydrin-positive material was determined according to Yemm and Cocking,⁶⁾ using alanine as a standard. The amount of hexose was determined by the anthrone-sulfuric acid method using glucose as a standard. The amount of hexosamine was determined by means of the Elson-Morgan reaction using glucosamine as a standard.

Reaction of the ultrafiltration filtrate with nitrite was performed as follows. Reaction mixture containing 1 ml of 20 mM sodium nitrite, 2.5 ml of 0.4 M sodium acetate-HCl buffer (pH 3.0) and the filtrate was incubated at 37°C. After a definite period, 1 ml of 250 mM MOR was added to start the nitrosation reaction under the same conditions as used for measurement of the potency of acceleration of nitrosamine

formation, method A.

The effect of ascorbic acid on acceleration of nitrosamine formation by the filtrate was examined using 8 mM DMA, 2 mM sodium nitrite, 1.5 units of the filtrate and 0 to 4 mM ascorbic acid. The potency of acceleration was measured after a 30 min incubation, by method B. The effect of thiocyanate or iodide on acceleration by the filtrate was examined in a similar manner, where 50 mM sodium thiocyanate or 10 mM potassium iodide was used instead of ascorbic acid.

Column chromatography was carried out either on Sephadex G-10, SP-Sephadex C-25, DEAE-Sephadex A-25 (Pharmacia Fine Chemicals), Dowex 50 X-8, or Dowex 1 X-8 (Dow Chemicals). A Sephadex G-10 column (2.0 cm i.d. × 60 cm) was eluted with water and 1.8 ml portions were collected.

Conditions for liquid chromatography were as follows. Apparatus, LC-6 A (Shimadzu Seisakusho Co., Ltd.); column Shim-pack CLC-ODS column or two Shim-pack CLC-CN (6 mm i.d. × 15 cm each) columns (Shimadzu Seisakusho Co., Ltd.) connected in series; mobile phase, water containing 0.05% trifluoroacetic acid; flow rate, 0.4 ml/min; detection, ultraviolet absorption measurement at 210 nm.

Acceleration of nitrosomethylurea formation was examined according to Yamamoto *et al.*⁷⁾ Methylurea (20 mM), sodium nitrite (5 mM) and the filtrate were incubated at pH 2.5 and 37°C for 15 min and the absorbance at 392 nm was measured after decomposition of nitrite with sulfamic acid.

Results and Discussion

1. Ultrafiltration

As shown in Table I, about 70% of the NAA passed through the membrane. About 30% of NAA was still retained in the cell, and it would not pass through the membrane even when dilution and filtration were repeated. Therefore, there were at least two components that accelerate nitrosamine formation in the dialyzable fraction. The amounts of hexose and hexosamine were negligible in the ultrafiltration filtrate of the dialyzable fraction (simply called the filtrate). A small amount of ninhy-

TABLE I. Effect of Ultrafiltration on Nitrosation-Accelerating Substance

	Weight (mg)	Nitrosation- accelerating ability(unit) ^{a)}	Amino acid ^{b)} (mg)	Hexose ^{c)} (mg)	Hexosamine ^{d)} (mg)
Dialyzable fraction	236	749	9.60	10.00	3.20
Ultrafiltration					
Retained on membrane	119	275	4.08	8.24	3.84
Filtrate	90	501	6.12	<0.1	0.09

Dialyzable fraction obtained from 8 g of a sodium chondroitin sulfate preparation was filtered through an Amicon YC 05 ultrafiltration membrane.

a) Determined by measuring acceleration of nitrosomorpholine formation.

b) Determined by the ninhydrin method with alanine as a standard.

c) Determined by the anthrone-sulfuric acid method with glucose as a standard.

d) Determined by means of the Elson-Morgan reaction with glucosamine as a standard.

drin-positive material (6.12 mg) was contained, but it comprized only a small part (less than 7%) of the weight of the filtrate. It is possible that inorganic salts were contained in it.

By the use of ultrafiltration, many co-existing substances could be removed from the filtrate, and the specific activity (nitrosation-accelerating ability divided by the weight in Table I) of the filtrate became 1.75 times that of the dialyzable fraction. Therefore, the following experiments were carried out using the filtrate. The molecular weight cut-off of the membrane is nominally 500.

2. Reaction with Nitrite

As shown in Fig. 1, NAA of the filtrate was lost during incubation with nitrite at pH 3.0, and after 4 h of incubation, almost all

Fig. 1. Loss of Nitrosation-Accelerating Activity of the Ultrafiltration Filtrate by Reaction with Nitrite

NAA was lost. It is possible that nitrite was decomposed in the acidic medium during incubation and this caused the apparent loss of NAA of the filtrate. In a control experiment omitting the filtrate, nitrite loss during the 4-h incubation was negligible. It is also possible that NAA of the filtrate was decomposed in the presence of MOR or was heat-unstable. In the experiment omitting nitrite, however, NAA of the filtrate was stable for at least 4 h. Therefore, it is concluded that the substance which showed NAA was decomposed by nitrite.

The initial rate of nitrosamine formation from secondary amines and nitrite in the absence of an accelerator is proportional to the square of the nitrite concentration.⁸⁾ In our experiments, the amount of nitrosamine formed was measured instead of the initial rate of nitrosamine formation, and it was linear with time during our incubation period. The effect of varying the concentration of nitrite on nitrosamine formation was reported in the previous paper.⁵⁾ In a reaction mixture containing a secondary amine, nitrite and the filtrate, nitrosation occurred proportionally to about the 0.5 th power of nitrite concentration (the kinetics of nitrosamine formation in the presence of the filtrate is unknown) in one hand, and the filtrate reacted with nitrite and lost its activity as shown in Fig. 1 in the other hand. This

may explain the complex relationship between nitrite concentration and amount of nitrosamine formed that we reported in the previous paper. In the experiments shown in Fig. 1, MOR as a secondary amine and the filtrate were used, while DMA and an SCS preparation were used in the previous paper. These differences probably did not affect the characteristics of the relationship between nitrite concentration and amount of nitrosamine formed.

3. Effect of Ascorbic Acid on the Acceleration of NDMA Formation by the Filtrate

Ascorbic acid is known to inhibit nitrosamine formation⁹⁾ by reacting with nitrite.⁹⁾ The mechanism by which the substance in the filtrate exhibited NAA is not known yet, it is interesting to know the co-existing effect of the filtrate with ascorbic acid on it. As shown in Table II, ascorbic acid, equimolar with respect to nitrite, showed a strong inhibitory effect on NDMA formation, while a half that amount of ascorbic acid had little effect. The results were comparable to those in the absence of an accelerator, where a 2 : 1 molar ratio of ascorbic acid to nitrite resulted in almost complete inhibition of nitrosamine formation.^{3,10)}

4. Effect of Thiocyanate and Iodide on the Acceleration of NDMA Formation by the Filtrate

Thiocyanate and iodide are reported to

TABLE II. Effect of Ultrafiltration Filtrate and Ascorbic Acid on NDMA Formation

Ascorbic acid (mM)	Filtrate (unit)	NDMA formed (10^{-8}M)
0	0	15.1
0	1.5	256.8
0.5	1.5	251.1
1.0	1.5	249.3
2.0	1.5	9.7
4.0	1.5	<4.5

Reaction mixture (20 ml) containing dimethylamine (8 mM) and NaNO_2 (2 mM) was incubated at pH 3.5 and 37°C for 30 min with various concentrations of ascorbic acid.

TABLE III. Effect of Thiocyanate or Iodide on Nitrosation-Acceleration by the Filtrate

Added	NDMA formed (10^{-8}M)
Filtrate ^{a)}	290.7
Thiocyanate ^{b)}	484.5
Filtrate+thiocyanate	779.6
Iodide ^{c)}	285.7
Filtrate+iodide	333.0

a) The ultrafiltration filtrate (1.5 units of nitrosation-accelerating ability) was used.

b) Sodium thiocyanate (50 mM) was used.

c) Potassium iodide (10 mM) was used.

Dimethylamine (8 mM) and NaNO_2 (2 mM) were incubated with the filtrate and/or thiocyanate and/or iodide at pH 3.5 and 37°C for 30 min.

accelerate nitrosamine formation as described before.¹⁾ As shown in Table III, thiocyanate and the filtrate had an additive effect on NDMA formation while iodide and the filtrate did not. It is possible that iodide partially destroyed the substance (s) showing NAA in the filtrate or that iodide was partially destroyed by a component in the filtrate, and did not show the acceleration effect additively. When reacted with nitrite, both thiocyanate ion and other anions such as halide ion were reported to form nitrosyl thiocyanate and nitrosyl halide, and they are potent nitrosating agents.¹¹⁾ In this experiment, however, iodide added to the filtrate showed a different effect from that of thiocyanate on NDMA formation.

The filtrate accelerated the formation of nitrosamines from nitrite and secondary amines such as DMA, dibutylamine, pyrrolidine, piperidine and MOR.⁵⁾ An experiment to see whether the filtrate accelerated nitrosomethylurea formation from methylurea and nitrite was carried out. However, 1.5 or 3.2 unit of the filtrate did not show any accelerating effect on nitrosomethylurea formation. Thiocyanate accelerated formation of nitrosamines but did not accelerate formation of nitrosooureas,^{7,12)}

whereas organic acids accelerated formation of nitrosoureas but did not accelerate the formation of nitrosamines.^{7,12,13} The filtrate seemed to have a similar effect to thiocyanate on the acceleration of formation of nitroso compounds.

5. Column Chromatography with Sephadex G-10

The chromatogram of the filtrate on Sephadex G-10 is shown in Fig. 2. As the filtrate did not have any ultraviolet absorption maximum, absorption measurement at 230 nm was used for detection. The filtrate contains many ultraviolet-absorbing substances but almost all NAA measured by method A was eluted in only 3 tubes, tubes 47 to 49. The elution position of the substance(s) showing NAA was behind that of glycylglycylglycine (tube 45), being similar to those of glycylglycine, sucrose and sodium chloride (tube 49), and ahead of those of *N*-acetylglucosamine and glycine (tube 57).

Therefore, the molecular weight of the substance that showed NAA was at most 400.

Fig. 2. Column Chromatography of the Ultrafiltration Filtrate with Sephadex G-10

The filtrate obtained by ultrafiltration was chromatographed on a Sephadex G-10 column (2.0 cm i.d. $\times$ 60 cm). The eluent was water, and 1.8 ml fractions were collected.

●—●: absorbance at 230 nm, ○—○: absorbance (at 260 nm) of the reaction mixture consisting of a portion of the fraction, morpholine and nitrite incubated at pH 3 and 37 °C for 30 min.

Fig. 3. High-Performance Liquid Chromatography of the Ultrafiltration Filtrate

The ultrafiltration filtrate was chromatographed on Shimpack CLC CN columns (6 mm i.d. $\times$ 30 cm). The mobile phase was water containing 0.05% trifluoroacetic acid and detection was done by ultraviolet absorption measurement at 210 nm.

—: absorbance at 210 nm, $\square$: nitrosation-accelerating activity measured in terms of acceleration of nitrosomorpholine formation from morpholine and nitrite. The activity before application to the column was defined as 100%.

6. HPLC

When applied to Shim-pack CLC ODS column using water as a mobile phase, the substance(s) showing NAA was not retained on the column. With a Shimpack CLC CN column, it was retained a little. Two Shimpack CLC CN columns were connected to get better separation. A chromatogram obtained by injecting 2 μ l of the filtrate (about 40 μ g in dry weight) onto the columns is shown in Fig. 3. The eluates in each 2-min period were fractionated during the retention times of 14 to 34 min. The HPLC procedure described above was repeated 10 times and NAA in the collected fractions was measured with method A. A fraction eluted at the retention time of 18–20 min showed the highest NAA (about 30% of that of 20 μ l of the filtrate). The peak at a retention time of about 19 min seemed to be the main NAA, and NAA in the following fractions might represent tailing of the peak.

The substance(s) was not adsorbed on cation exchangers such as Dowex 50 X-8 or SP-Sephadex C-25. It was not eluted from anion exchangers such as Dowex 1 X-8 or DEAE-

Sephadex A-25 either with 0.1 N hydrochloric acid or with 0.1 N sodium hydroxide. It was stable for at least 4 h in these eluents. Thus, it may have a strongly acidic moiety.

The structure of the compound that showed NAA has not yet been elucidated. It was not a phenolic compound because the filtrate did not show any characteristic absorption in the ultraviolet region. Its molecular weight is less than 400 judging from the elution position from Sephadex G-10. Small compounds that may be present in the filtrate were checked for NAA. Some ninhydrin-positive compounds were contained in the filtrate, as shown in Table I. Therefore, amino acids such as glycine, alanine, and glutamic acid were examined. None of them showed NAA like that of the filtrate.

Decomposition products of chondroitin sulfate such as glucosamine, *N*-acetylglucosamine, glucosamine 2-sulfate and glucosamine 6-sulfate also did not show NAA.

Further investigations are required to elucidate the nature of the substance that showed NAA in the filtrate and to establish the significance of the ingestion of the substance from the point of view of human health, because, owing to the action of substances that show NAA, the amount of nitrosamine formed in the human body may be more than that predicted by calculation from the amounts of precursors.

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