in the intestine reflects to a certain extent that seen in the pancreas with significant elevation of trypsin and chymotrypsin and a reduction in amylase activities in the heat-treated groups (Table IV). The lower intestinal amylase activity is most likely due to the lower level in the pancreas available for secretion. The elevation in trypsin and chymotrypsin activity in the intestine could be due to increased secretin, decreased degradation as observed earlier (Percival and Schneeman, 1979b), or, most likely, a combination of these two factors.

Chichester and colleagues have argued that the poor nutritional quality of browned proteins is not simply due to the loss of amino acids but that other antinutritional effects appear to be involved in the physiological response to diets containing browned proteins (Kimiagar et al., 1980). The present study with heat-treated NFDM appears to support that argument. Enlargement of the kidney and of parts of the gastrointestinal tract represents not only a response to the presence of the poorly digested protein but also an increased demand for the protein needs of the animal. The changes in the pancreatic enzymes are similar to what one might expect if a high-protein diet were fed (Schneeman et al., 1977; Corring, 1977) yet in fact protein is less available in these groups and more of the dietary protein and perhaps the endogenously secreted protein is lost in the feces or by microbial degradation to unusable nitrogenous compounds (Pecival and Schneeman, 1979b; Varnish and Carpenter, 1975).

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Registry No. Trypsin, 9002-07-7; amylase, 9000-92-4; chymotrypsin, 9004-07-3; lipase, 9001-62-1.

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Acceleration of Nitrosamine Formation by Contaminant(s) in Sodium Chondroitin Sulfate Preparations

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Formation of nitrosamines from secondary amines and nitrite was markedly accelerated by sodium chondroitin sulfate (SCS) preparations. The nitrosation of dimethylamine in the presence of the SCS preparation was proportional neither to the nitrite concentration nor to the square of the nitrite concentration. Among dimethylamine, dibutylamine, pyrrolidine, piperidine, and morpholine, nitrosation of dimethylamine was most strongly accelerated. The nitrosation-accelerating activity was inhibited by cupric and silver ions. The nitrosation-accelerating substance in the SCS preparation was dialyzable and not chondroitin sulfate itself. The potency of the accelerating effect was about 18 times of that of a similar weight of sodium thiocyanate or potassium iodide. The importance of the substance in human exposure to carcinogens was suggested.

Secondary amines and other nitrosatable compounds easily react with nitrite in acidic medium and form nitroso compounds. The secondary amine that was most frequently found in foods was dimethylamine (DMA) (Neurath et al., 1977). Although the nitrosation rate of DMA by nitrite is rather slow (Mirvish, 1975), the rate is affected by many coexisting substances in the reaction mixture. Anions such as thiocyanate and iodide were reported to

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accelerate nitrosation of secondary amines by nitrite (Boyland et al., 1971; Fan and Tannenbaum, 1973) in acidic medium. In neutral and alkaline medium, formaldehyde catalyzed the conversion of various secondary amines to nitrosamines by nitrite (Keefer and Roller, 1973). Decyltrimethylammonium bromide and micelle-forming surfactants were reported to accelerate the nitrosation of dihexylamine by nitrite (Okun and Archer, 1977). In human gastric juice, an unknown factor that accelerated nitrosation reaction was reported (Tannenbaum et al., 1981). Among food constituents, chlorogenic acid in coffee (Challis and Bartlett, 1975) and catechins in green tea (Nakamura and Kawabata, 1981) were known to accelerate nitrosamine formation by nitrite.

In Japan, sodium chondroitin sulfate (SCS) is approved for use as a food additive (Ministry of Health and Welfare, Japan, 1978). The maximum approved levels were 20 g/kg for mayonnaise and dressings and 3 g/kg for fish sausage.

In this paper, a strong catalytic effect on nitrosamine formation from secondary amines and nitrite by a commercial SCS preparation is described.

EXPERIMENTAL SECTION

SCS usually used in the experiments was purchased from Tokyo Chemical Industry Co. Ltd.

Determination of Nitrosamines. Nitrosation reaction was carried out in 0.1 M acetate buffer (pH 3.5) at 37 °C for 15 min unless otherwise specified. The reaction conditions usually used were as follows. A mixture containing the buffer, DMA, and the SCS preparation was readjusted to pH 3.5 with hydrochloric acid and maintained at 37 °C. Sodium nitrite solution was added to start the reaction. The volume of the reaction mixture was 20 mL. After 15 or 120 min, the reaction was stopped by the addition of sulfamic acid. The reaction mixture was extracted with dichloromethane and determined by a gas chromatograph with a thermal energy analyzer. Each experiment was repeated at least twice and the figures in this report were the average.

To study the effect of pH, 0.1 M sodium acetate–hydrochloric acid buffers were used at pHs 2 and 3, and 0.1 M sodium acetate–acetic acid buffers were used for the experiments above pH 3.5.

To study the effect of silver ion, dibutylamine (DBA) was used as a secondary amine and acetic acid was used for the readjustment of pH.

Measurement of Denitrosation and Transnitrosation Ability of the SCS Preparation. Nitrosodimethylamine (NDMA) (5×10^{-5} mol·L^{-1}) with and without a 0.5% SCS preparation was incubated at pH 3.5, 37 °C, for 15 or 120 min. After the incubation, the amounts of NDMA were determined as described above. In other reaction mixtures, 2.0 mM morpholine was added besides NDMA and the SCS preparation to check the transnitrosation activity of the SCS preparation.

Fractionation by Dialysis. SCS (4 g) was dissolved in 200 mL of distilled water and dialyzed against 2 L of distilled water. The nondialyzable fraction was lyophilized and the dialyzable fraction was concentrated to dryness by a rotary evaporator. Then the nitrosation-accelerating ability of each fraction was examined. The reaction mixture consisted of 2.0 mM DMA, 2.0 mM nitrite, and (a) the undialyzed SCS preparation (100 mg), (b) the dialyzable fraction obtained from the 100-mg SCS preparation, (c) the nondialyzable fraction obtained from the 100-mg SCS preparation, (d) (b) plus (c), or (e) nothing.

RESULTS AND DISCUSSION

The reaction mixture fortified at 20 and 250 ppb of NDMA and 50 ppb of nitrosodibutylamine (NDBA) gave recoveries of 63, 71, and 94%, respectively. All data shown in this paper were not corrected by recoveries.

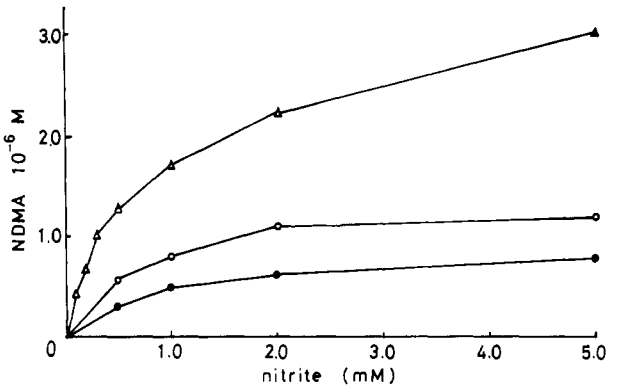

Figure 1. Effect of concentration of nitrite on NDMA formation. The reaction mixture (pH 3.5) contained 2.0 (Δ), 1.0 (○), or 0.5 mM dimethylamine (●), besides nitrite. It was incubated at 37 °C for 15 min in the presence of 0.5% SCS preparation.

Effect of Concentration of the SCS Preparation, DMA, and Nitrite. The reaction mixture containing 1.0, or 2.0 mM DMA, 2.0 mM nitrite, and varying concentrations of the SCS preparation was incubated. The amounts of NDMA formed were proportional to the concentration of the SCS preparation at least up to 0.5%. When the reaction mixture containing 2.0 mM nitrite, 0.5% SCS preparation, and DMA was incubated, the amounts of NDMA formed were proportional to the DMA concentration. Formation of NDMA under the conditions of 0.5, 1.0, or 2.0 mM DMA, 0.5% SCS preparation, and varying concentrations of nitrite was measured. As shown in Figure 1, the amounts of NDMA formed were proportional neither to the concentration of nitrite nor to the square of the nitrite concentration.

To confirm if the SCS preparation contained any nitrosatable compounds, 2.0 mM nitrite and the 0.5% SCS preparation were incubated for 120 min. About 2×10^{-7} mol·L^{-1} NDMA was found and no other volatile nitrosamine was detected. Though in the SCS preparation only a small amount of DMA or a precursor of NDMA was contained, the amounts of NDMA after incubation were so small that no correction was carried out for calculation of amounts of NDMA in the reaction mixture incubated with DMA. From the incubation mixture containing 2.0 mM DMA and 0.5% SCS preparation incubated for 120 min, no NDMA was detected. Thus, the SCS preparation did not contain any nitrosating agent.

Denitrosation or Transnitrosation Ability of the SCS Preparation. The amount of NDMA recovered from the incubation mixture containing NDMA and the SCS preparation was same as those recovered from the reaction mixture without the SCS preparation. No nitrosomorpholine was found in the reaction mixture containing NDMA, the SCS preparation, and morpholine. Thus the SCS preparation has neither denitrosation nor transnitrosation activity under the experimental conditions. We examined SCS preparations from a few companies and some of them had no nitrosation-accelerating ability and others showed different potency.

Optimal pH for NDMA Formation. Figure 2 shows the amounts of NDMA formed from 2.0 mM DMA, 2.0 mM nitrite, and 0.5% SCS preparation at various pH values. At pH 4, maximum amount of NDMA was formed. Mirvish (1970) reported that the optimum pH for the nitrosation of DMA was 3.4, and Lane and Bailey (1973) reported the optimum pH was in the range of pH 2.5 in human gastric juice. In the pH range of 2~5, the amount of NDMA formed was smallest at pH 2 in the presence of

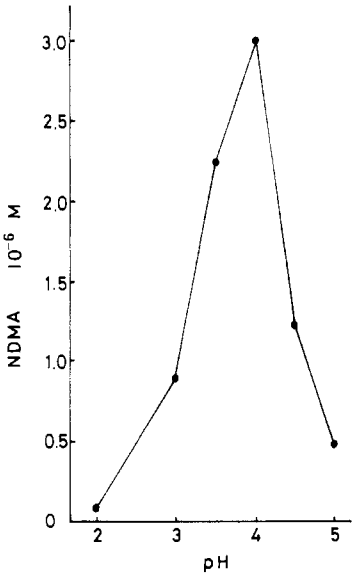

Figure 2. Effect of pH on NDMA formation. The reaction mixture contained 2.0 mM dimethylamine, 2.0 mM nitrite, and 0.5% SCS preparation and was reacted for 15 min at 37 °C.

Table I. Effect of the SCS Preparation on Nitrosamine Formation from Various Secondary Amines[a]

amine	nitrosamine formed, 10^{-8} mol·L^{-1}		
	−SCS		
	15 min	120 min	+SCS, 15 min
dimethylamine	ND[b]	27	220
dibutylamine	ND	10	28
pyrrolidine	ND	6	34
piperidine	ND	6	36
morpholine	49	–[c]	540

[a] SCS = sodium chondroitin sulfate. The concentration of amines was 2.0 mM, and the amines were incubated with 2.0 mM nitrite and the 0.5% SCS preparation for 15 or 120 min at 37 °C and pH 3.5. [b] ND means below the detection limit (5×10^{-8} mol·L^{-1}). [c] Not determined.

the SCS preparation, but it was still about 2.5-fold compared with one at pH 3.5 (near the optimum pH) in the absence of the SCS preparation.

Nitrosamine Formation from Various Secondary Amines. Nitrosamine formation from 2.0 mM DMA, dibutylamine, pyrrolidine, piperidine, or morpholine, and 2.0 mM nitrite was accelerated by the 0.5% SCS preparation as shown in Table I. The amounts of nitrosamine formed in 15 and 120 min in the absence of the SCS preparation were also shown. In this case, nitrosamines formed except nitrosomorpholine were below the detection limit (5×10^{-8} mol·L^{-1}) with a 15-min incubation. The amounts of nitrosamines formed from DMA and morpholine in the presence of the 0.5% SCS preparation were proportional to the incubation period at least up to 15 min.

Influence of Divalent Cations, Silver Ion, and EDTA. The incubation mixtures containing zinc sulfate, calcium chloride, barium chloride, or cupric sulfate besides 2.0 mM DMA, 2.0 mM nitrite, and the 0.5% SCS preparation were incubated. One millimolar Zn^{2+}, Ca^{2+}, Ba^{2+}, and 5 mM EDTA had little effect on NDMA formation in the absence (data are not shown) and presence of the SCS preparation. Cupric ion inhibited the acceleration by the SCS preparation (Table II). To elucidate further the effect of heavy metal ions, experiments using silver ion were also carried out. NDBA formation from 2.0 mM DBA, 2.0 mM nitrite, and the 0.5% SCS preparation was

Table II. Effect of Divalent Cations and EDTA on NDMA Formation[a]

substances	concentration, mM	NDMA formed,[b] 10^{-8} mol·L^{-1}
none	1.0	220 ± 16
Zn^{2+}	1.0	204 ± 15
Ca^{2+}	1.0	247 ± 18
Ba^{2+}	1.0	235 ± 6
Cu^{2+}	1.0	21 ± 3
Cu^{2+}	0.1	77 ± 5
EDTA	5.0	201 ± 11

[a] The reaction mixture (pH 3.5) contained 2.0 mM dimethylamine, 2.0 mM nitrite, and the 0.5% SCS preparation and was reacted for 15 min at 37 °C. [b] Average of five analyses and standard deviation.

Table III. Inhibition of the Acceleration Effect of the SCS Preparation on NDBA Formation by Silver Ion[a]

concentration of Ag^+, mM	NDBA formed, 10^{-8} mol·L^{-1}
1.0	15
0.5	14
0.1	14
0.05	13
0.01	99
0	110
0.1 (−SCS)	11
0 (−SCS)	11

[a] The reaction mixture (pH 3.5) contained 2.0 mM dibutylamine, 2.0 mM nitrite, and the 0.5% SCS preparation and was reacted for 2 h at 37 °C.

Table IV. Comparison of Accelerating Potency of the Dialyzable Fraction of the SCS Preparation with That of Thiocyanate or Iodide for NDMA Formation[a]

added	concentration		NDMA formed, 10^{-8} mol·L^{-1}
	mM	mg/tube	
none		0	3.4
dialyzable fraction		3.0	257
sodium thiocyanate	100	162	232
sodium thiocyanate	20	32	66
sodium thiocyanate	2	3.2	14
potassium iodide	20	66	147
potassium iodide	1	3.3	15

[a] The reaction mixture contained 2.0 mM dimethylamine, 2.0 mM nitrite, and the 0.5% SCS preparation and was reacted for 15 min at 37 °C.

strongly inhibited by silver nitrate, as shown in Table III. Silver ion, more than 0.05 mM, completely counteracted the acceleration effect of the SCS preparation and 0.01 mM Ag^+ inhibited it only 10%. In the 0.5% SCS preparation, X equivalent to 0.01–0.05 mM Ag^+ might be contained. In this experiment, DBA was used as a secondary amine instead of DMA, because DMA is usually supplied as hydrochloride and silver ion easily binds to chloride. Silver ion did not inhibit NDBA formation in the absence of the SCS preparation. Though the mechanism of inhibition of the accelerating effect is not known, it is possible that X was bound to the metal ions and the complex was not an accelerator any more.

Effect of Dialysis. After dialysis, dry weights of the dialyzable fractions were 34 ± 5 mg/g of SCS. Then, the fraction containing X was examined according to the method described in under Fractionation by Dialysis. In the reaction mixture containing (a), (b), (c), (d), or (e), 2.2, 2.6, 0.027, 2.1, or 0.027 μmol/L NDMA was formed, re-

spectively. It is clear that X was contained in the dialyzable fraction. Therefore X has a small molecular size and is not chondroitin sulfate itself.

Comparison of the Potency of Acceleration. Table IV shows the comparison of nitrosation-accelerating potency of X with that of thiocyanate or iodide. The reaction mixture containing the similar weight of sodium thiocyanate or potassium iodide formed only 5~6% of NDMA compared with the reaction mixture containing X. Phenols such as gallic acid sometimes showed an accelerating effect and sometimes showed an inhibitory effect depending on their concentrations or pHs of the media (Walker et al., 1975; Yamada et al., 1978). X, however, strongly accelerated the nitrosation reaction under all conditions in our experiments and was different in many ways from known nitrosation-accelerating substances. Nitrosamines and nitrosatable compounds are contained in some foods, and nitrosamines may also be formed in vivo. X or unknown substances having a similar function as X may play an important role in nitrosamine formation in vitro and in vivo.

The knowledge concerning the molecular structure of X is very limited so far, and separation and identification of the nitrosation-accelerating principle are in progress.

Registry No. Sodium chondroitin sulfate, 9082-07-9; dimethylamine, 124-40-3; dibutylamine, 111-92-2; pyrrolidine, 123-75-1; piperidine, 110-89-4; morpholine, 110-91-8; cupric ion, 15158-11-9; silver ion, 14701-21-4.

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Heat Inactivation of Ovoinhibitor in the Alkaline pH Region

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Heat inactivation of ovoinhibitor was studied at the pH region between 8.0 and 9.5. When ovoinhibitor was heated at higher pH and higher ionic strength, the protease inhibitory activity greatly decreased. To study the heat inactivation of the ovoinhibitor molecule, partially heat inactivated ovoinhibitor was applied to either an insolubilized-trypsin column or an insolubilized-chymotrypsin column and both the trypsin and chymotrypsin inhibitory activities were measured in the absorbed and unabsorbed fractions. Trypsin inhibitory activity of the insolubilized trypsin absorbed fraction or chymotrypsin inhibitory activity of the insolubilized chymotrypsin absorbed fraction was the same as that of native ovoinhibitor, and both trypsin and chymotrypsin inhibitory activities remained in the unabsorbed fraction. Circular dichroism and difference scanning calorimetry measurements suggest that heat treatment produces many kinds of denatured ovoinhibitor molecules of which domain structure is destroyed in a different degree.

Many kinds of protease inhibitors have been found in natural food materials. Egg white contains three kinds of protease inhibitors (Osuga and Feeney, 1977), namely, ovomucoid, ovoinhibitor, and cystatin [formerly called ficin–papain inhibitor (Barrett, 1981)]. It is interesting that these three protease inhibitors are relatively heat stable; ovomucoid is resistant to heating at 100 °C (Deutsch and Morton, 1961), cystatin survives even after exposure to 80–100 °C (Sen and Whitaker, 1973), and ovoinhibitor is stable under heating at 100 °C for 30 min when the solution pH is rather low (Matsushima, 1958). The heat stability of ovoinhibitor, however, is pH dependent and decreases rapidly at alkaline pH (Matsushima, 1958). Since the pH of native egg white is between 8.0 and 9.5, this ovoinhibitor property is very interesting. Furthermore, it has been shown that each molecule has independent binding sites for trypsin, chymotrypsin, and elastase (Davis et al., 1969). It is interesting to know whether the effect of heating in alkaline pH region is the same for both activities. In the present study, we extensively investigated the heat inactivation of ovoinhibitor in the pH region between 8.0 and 9.5.

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MATERIALS AND METHODS

Materials. Ovoinhibitor was prepared from fresh egg white by the method described by Davis et al. (1969) with a slight modification. Trypsin (Type XI, DPCC treated) was obtained from Sigma Chemical Corp. α-Chymotrypsin (bovine, 3 times crystallized) was obtained from ICN Pharmaceuticals Inc. (Cleveland, OH). Both insolubilized trypsin and insolubilized chymotrypsin were prepared by the method described by Porath et al. (1973).

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