Studies on *In Vivo* Formation of Nitroso Compounds (VI)

In Vitro and *In Vivo* Formation of Dimethylnitrosamine by Bacteria Isolated from Human Saliva

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In vitro and *in vivo* formation of dimethylnitrosamine (DMNA) by bacteria isolated from human saliva were studied.

Six in 7 strains reduced nitrate to nitrite. Significant amount of DMNA was detected after 24 hr incubation (37°C) of bacteria in the medium containing sodium nitrate, dimethylamine hydrochloride (DMA·HCl), glucose and brain heart infusion. The highest concentration of DMNA was observed in the culture solution of *Staphylococcus aureus*, isolate A-1 and *S. epidermidis*, isolate E-2, being 13.8 ppm and 13.7 ppm, respectively. In the case of short term incubation (37°C, 2 hr) using *S. epidermidis*, isolate E-2, pH lowered to 5.7 (initial pH was 6.5) and the concentration of nitrite increased to 310 ppm. When glucose was omitted from the incubation mixture, pH did not change, but the concentration of nitrite increased. The concentration of DMNA showed 16.7 ppm after 2 hr incubation with glucose and 0.3 ppm without glucose.

In the animal experiments, DMNA was detected at the level of 0.2~1.9 µg/animal in the stomach contents of 5 in 8 guinea-pigs when cell suspension of *S. epidermidis*, isolate E-2, sodium nitrate, DMA·HCl and glucose were orally administered.

1. Introduction

It is well known that the nitroso compounds have carcinogenicity towards specific target organs. Among them, dimethylnitrosamine (DMNA) has potent carcinogenicity, and has been shown to cause malignant tumors in liver, kidney and lung of the rat when given orally. Dimethylamine (DMA) which is one of the precursors of DMNA has been widely detected in seafoods, especially in sea fishes and fish roes. Kawamura *et al.*¹⁾ and Ito *et al.*²⁾ determined the amount of DMA in raw and roasted fishes, and many other kinds of foods. Nitrite, the other precursor, was widely distributed in natural products³⁾ such as vegetables and fruits, and also used as a color fixative in meat products or fish roes.

Moreover, the existence of nitrite in human saliva has been known. Tsujitsuka *et al.*,^{4), 5)} Okabe,⁶⁾ and Tannenbaum *et al.*^{7), 8)} reported that salivary nitrite might be produced from

nitrate by the action of microorganisms in oral cavity. These reports, however, did not clarify the relationship between the concentrations of nitrite and nitrate in saliva and those in the meal ingested. In the previous studies in this series, it was found that the concentrations of nitrate and nitrite in human saliva increased remarkably when sodium nitrate⁹⁾ or some vegetables containing high amount of nitrate^{9), 10)} were ingested, depending on the amount of nitrate ingested.

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Table 1. Components of Growth Media

	Medium I	Medium II
DMA·HCl (g)	0.5	0.5
NaNO ₃ (g)	1.0	1.0
Glucose (g)	—	2.0
BHI (g)	3.7	3.7
Water (ml)	100	100
		(pH 7.0)

Fig. 1. Growth of bacteria, accumulation of nitrite and change of pH in the culture solution of bacteria isolated from human saliva
 ●, Medium I; ○, Medium II; —, nitrite; ---, pH; —·—, growth of bacteria.

Thompson *et al.*,¹²⁾ Howksworth *et al.*,¹³⁾ Ayanaba *et al.*,¹⁴⁾ and Inoue¹⁵⁾ reported that nitrosamines were formed from nitrite or nitrate and secondary or tertiary amines by various microorganisms. The present authors also observed the formation of DMNA in the incubated solution containing sodium nitrate, DMA·HCl, glucose and brain heart infusion (BHI) when human saliva was added to the solution as the source of microorganisms.¹⁶⁾

In the present paper, 7 strains of bacteria were isolated from human saliva, and their abilities of nitrite and DMNA formation *in vitro* were compared. Furthermore, the formation of DMNA in the stomach of guinea-pig was tested by using *S. epidermidis*, one of the strains isolated.

2. Experimental Methods

2.1 Growth media

The components of growth media are shown in Table 1.

2.2 Test organisms

Staphylococcus aureus, isolate A-1, *S. aureus*, isolate A-2, *S. epidermidis*, isolate E-1 and *S. epidermidis*, isolate E-2 and unidentified nitrate-reducing bacteria (strain B-9, strain B-14 and strain C-13) were isolated from the saliva of one of the present authors (man, 31 years old). *S. aureus* IFO 12732 and *S. epidermidis* IFO 12993 were obtained from Institute for Fermentation, Osaka.

a) *Isolation of Staphylococcus* The sediment of human saliva was spread on mannitol salt agar (Eiken Chemical Co., Ltd.) plate, and incubated at 37°C. Each 2 yellow and 2 white colonies were again spread on the agar plates. The selective culture was repeated several times for the isolation. The main properties of the yellow colonies were gram-stain positive, cocci, coagulase positive, acid formation from mannitol positive, and O/F test F-type. These properties coincided with those of *S. aureus* IFO 12732 and the description of *S. aureus* by Cowan *et al.*¹⁷⁾ The main properties of the white colonies were gram-stain positive, cocci, coagulase negative, acid formation from mannitol negative, and O/F test F-type. These properties coincided with those of *S. epidermidis* IFO 12993 and the description of *S. epidermidis* by Cowan *et al.*¹⁷⁾

b) *Isolation of nitrate-reducing bacteria*

The sediment of human saliva was spread on BHI (Difco Lab.) plates, and few colonies were spread on new BHI plate. The selective culture was repeated several times for the isolation. Three nitrate-reducing strains were selected from the colonies isolated. They were not identified enough, but strain B-9 was gram-stain positive and rod-shaped; strain B-14 was gram-stain positive and rod-shaped; strain C-13 was gram-stain positive and spherical.

2.3 Formation of nitrite and DMNA *in vitro*

a) *Twenty-four hours incubation* The test organisms were cultured in the media shown in Table 1 at 37°C with shaking. Growth of bacteria, pH and nitrite concentration in the culture solution were measured at definite intervals. DMNA was measured at 24 hr.

b) *Two hours incubation* The mixture of 20 ml of cell suspension of *S. epidermidis*, isolate E-2 in 3.7% BHI solution ($1.8-2.8 \times 10^9$ cells/ml) and 2 ml of the reagent solution containing 0.5 g of sodium nitrate, 0.25 g of DMA·HCl and 0.5 g of glucose was incubated at 37°C. Nitrite concentration and pH were determined at definite intervals. DMNA was measured at 2 hr.

2.4 Formation of DMNA *in vivo*

The experiments were carried out according to the method of Inoue.¹⁵⁾

Ten ml of cell suspension of *S. epidermidis*, isolate E-2 in 3.7% BHI solution ($1.8-2.8 \times 10^9$ cells/ml) and 2 ml of the reagent solution described above were given simultaneously by a stomach tube to the guinea-pig (Hartley, female, body weight 600~800 g) which had been fasted from the day before. The stomach was exenterated after 2 hr, and the contents were collected. The stomach was washed with a small amount of water, and the washings were combined to the contents. Clear supernatant was obtained by centrifugation (3000 rpm, 10 min). The precipitate was washed with a small amount of water and centrifuged. Both supernatants were combined and used for the sample solution for the determination of DMNA.

2.5 Analytical procedures

Nitrite was determined by the method described previously.¹⁰⁾ Growth of bacteria was measured at the wave length of 540 nm by a

spectrophotometer. DMNA in the sample solution was extracted twice with three portions of dichloromethane. Dichloromethane layer was dried over night with anhydrous sodium sulfate, and concentrated to 0.1~1 ml. Detection and determination of DMNA were carried out by thin-layer chromatography and gas chromatography, respectively, under the conditions described previously.¹⁰⁾

3. Results and Discussion

3.1 Accumulation of nitrite by isolated bacteria

The accumulation curves of nitrite of 7 strains isolated from saliva and 2 strains obtained from IFO were shown in Fig. 1, together with the growth curves and the changes of pH.

The accumulating patterns of nitrite were considerably different in the individual strains, but they were mostly classified to the following three types. (1) The concentration of nitrite increased with growth of bacteria and the maximum value was maintained up to 24 hr. Such a type was observed in three unidentified nitrate-reducing bacteria, strain B-9, strain B-14 and strain C-13. (2) The accumulation curve of nitrite had a sharp peak at 8~12 hr and nitrite was almost completely lost at 24 hr. Two strains of *Staphylococcus* obtained from IFO belonged to this type. (3) The third was the intermediate type of the first two. *S. aureus*, isolate A-1 and A-2, and *S. epidermidis*, isolate E-2 corresponded to this type. In the case of *S. epidermidis*, isolate E-1, nitrite was not detected in both medium I and II, showing the lack of nitrate-reducing ability.

The changes of pH during the growth of bacteria were also different in the individual strains. When glucose was omitted from the medium, pH value first dropped and then gradually rose to about 8 at 24 hr, except three unidentified nitrate-reducing bacteria and *S. epidermidis* IFO 12993. However, when glucose was added, pH value lowered from the beginning to reach about 5 at 24 hr, except strain B-14 which showed no pH change.

3.2 Accumulation of DMNA by isolated bacteria

The amount of DMNA accumulated in the medium was determined after 24 hr incubation. As shown in Table 2, the highest concentration of DMNA was detected in medium II of

Table 2. Concentration of DMNA in the Culture Solution after Incubation for 24 hr

	Medium	DMNA (ppm)
<i>S. aureus</i> , IFO 12732	I	N.D.
	II	7.2
<i>S. aureus</i> , isolate A-1	I	N.D.
	II	13.8
<i>S. aureus</i> , isolate A-2	I	N.D.
	II	9.0
<i>S. epidermidis</i> , IFO 12993	I	N.D.
	II	11.8
<i>S. epidermidis</i> , isolate E-1	I	N.D.
	II	N.D.
<i>S. epidermidis</i> , isolate E-2	I	0.4
	II	13.7
Strain B-9	I	3.9
	II	7.0
Strain B-14	I	0.1
	II	0.1
Strain C-13	I	0.5
	II	6.7
—	I	N.D.
	II	N.D.

S. aureus, isolate A-1 and *S. epidermidis*, isolate E-2, being 13.8 and 13.7 ppm, respectively.

More acidification of the medium and more accumulation of DMNA were observed in medium II compared with medium I, except *S. epidermidis*, isolate E-1 and strain B-14. *S. epidermidis*, isolate E-1 might have no nitrate reductase, so DMNA was not formed in spite of acidification of the medium. As the change of pH was not observed in medium I and II of strain B-14, the amount of DMNA accumulated was the same in both cases. In the medium without bacteria, the change of pH and the accumulations of nitrite and DMNA were not observed. This result shows that glucose does not directly participate to the formation of DMNA, but that it may accelerate the formation of DMNA by acidifying the medium. It has been reported that the optimum pH of chemical formation of DMNA from nitrite and DMA is 3.3¹⁸⁾~3.4.¹⁰⁾

Tannenbaum *et al.*⁸⁾ reported that *S. aureus* and *S. epidermidis* isolated from human saliva have the ability of nitrite formation, and they discussed a possibility of formation of nitrosamines in mouth or stomach. However, the

present authors examined the formation of DMNA when the reagent solution containing sodium nitrate, DMA·HCl, glucose and BHI were kept in human mouth for 15 min, but DMNA was not detected in the spat out solution.¹⁶⁾ This fact seems to open another possibility of DMNA formation in the stomach.

Fig. 2. Formation of nitrite and change of pH in the incubation mixture of *S. epidermidis*, isolate E-2

●—●, A; △···△, B; ○—○, C; ×—×, D; ×···×, E; ○···○, F.

Nitrite was not detected in incubation mixture B, E, and F. The components of A~F were shown in Table 3.

3.3 Formation of DMNA by short term incubation

Before the experiment of DMNA formation in the guinea-pig stomach was started, *in vitro* formation of DMNA by *S. epidermidis*, isolate E-2 was carried out under the same condition as the *in vivo* experiment. Formation of nitrite and change of pH in the incubation mixtures shown in Table 3 were determined at definite intervals (Fig. 2). The amount of DMNA accumulated for 2 hr was shown in Table 3.

In the complete incubation mixture, pH was acidified linearly to reach 5.7 at 2 hr, and the concentration of nitrite increased linearly to 310 ppm (Fig. 2, curve A). The amount of DMNA was 16.7 ppm at 2 hr (Table 3). When glucose was omitted from the incubation mixture, no change of pH was observed regardless of the accumulation of 317 ppm of nitrite (Fig. 2, curve C), and the accumulation of DMNA was only 0.3 ppm (Table 3).

Inoue¹⁵⁾ reported that a trace amount of DMNA was detected in 1 hr incubation of the mixture of sodium nitrate, DMA·HCl and the cell suspension of *Escherichia coli* (final pH 6.6), but that 0.56 mg of DMNA was detected when glucose was added to the mixture (final pH 5.4~5.5). Our present result obtained by *S. epidermidis* was similar to the case of *E. coli*.

3.4 Formation of DMNA in the stomach of guinea-pig

DMNA in the stomach contents of guinea-pig was determined 2 hr after the oral administration of cell suspension of *S. epidermidis*,

Table 3. Components of the Incubation Mixture and Formation of DMNA by *S. epidermidis*, isolate E-2

Incubation mixture		A	B ^{b)}	C	D	E	F
Cell suspension ^{a)} (20 ml)		+	—	+	+	+	+
Reagent solution (2 ml)	NaNO ₃ (0.5 g)	+	+	+	+	—	—
	DMA·HCl (0.25 g)	+	+	+	—	+	—
	glucose (0.5 g)	+	+	—	+	+	—
Incubation at 37°C for 2 hr							
DMNA (ppm)		16.7	N.D.	0.3	N.D.	N.D.	N.D.

^{a)} 1.8–2.8×10⁸ cells/ml of 3.7% BHI solution

^{b)} Cell suspension was replaced with 3.7% BHI solution.

Table 4. Formation of DMNA in Guinea-pig Stomach

Group	No.	Contents in stomach (ml)	pH of the contents	DMNA (μ g)
A	1	24	6.0	0.3
	2	15	4.9	0.6
	3	18	4.0	0.2
	4	20	6.3	N.D.
	5	18	5.6	N.D.
	6	7	6.9	N.D.
	7	25	3.4	0.2
	8	28	4.9	1.9
B	1	3	3.0	N.D.
	2	8	1.7	N.D.
	3	1	2.1	N.D.
	4	9	1.7	N.D.
C	1	21	5.4	N.D.
	2	13	6.6	N.D.
	3	38	3.5	N.D.
D	1	33	4.2	0.6
	2	23	3.0	0.5
	3	11	6.9	N.D.
	4	20	3.1	0.3

Dose: Group A, cell suspension of *S. epidermidis*, isolate E-2 and reagent solution; Group B, reagent solution; Group C, cell suspension of *S. epidermidis*, isolate E-2; Group D, cell suspension of *S. epidermidis*, isolate E-2 and reagent solution after the pretreatment with cell suspension of *S. epidermidis*, isolate E-2 and 25% sodium nitrate solution on the day before

isolate E-2 and the reagent solution containing sodium nitrate, DMA·HCl and glucose. 0.2~1.9 μ g of DMNA was detected on the 5 in 8 guinea-pigs (Table 4, group A). In the group C administered distilled water instead of the reagent solution, no DMNA was detected. In the group B administered 3.7% BHI solution instead of the cell suspension, DMNA was not detected, the contents of stomach were only 1~9 ml and pH was 1.7~3.0, showing the normal function of stomach. On the other hand, in the case of the guinea-pig administered the cell suspension (group A, C and D), the contents of stomach were 7~33 ml and pH was 3.1~6.9, showing the abnormal function of stomach. In the group D which was administered 10 ml of the cell suspension and 2 ml of 25% sodium nitrate solution on the day before the experimental day, and which was further administered 10 ml of the cell suspension and the reagent solution on the experimental day, according to the method of Inoue,¹⁵⁾ 0.3~0.6 μ g of DMNA was detected.

Inoue reported¹⁵⁾ that DMNA was not detected in the stomach of guinea-pig 1 hr after the oral administration of sodium nitrate, glucose, DMA·HCl and cell suspension of *E. coli*, but that it was formed only when the animal had been administered with the cell suspension and sodium nitrate the day before of the experimental day in order to accumulate nitrite in the stomach. He also pointed out that such a pre-treatment caused the lowering of stomach function, accompanying the decrease of gastric juice and the stagnation of stomach contents.

However, in our present experiments, DMNA was significantly formed in the stomach of guinea-pig 2 hr after the oral administration of the reagent solution and cell suspension of *S. epidermidis*, isolate E-2. This discrepancy suggests that our organisms have a strong ability of nitrate reduction and also caused the stomach function to lower immediately resulting in the increases of stomach contents and pH value. Such a state of stomach may bring about the favorable condition for the

growth of bacteria and the formation of DMNA.

In the experiments of DMNA formation from sodium nitrite and DMA·HCl, Sakai *et al.*¹⁸⁾ demonstrated that the yield of DMNA was 10.75% in simulated gastric juice and only 0.09~1.10% in the stomach of rabbit. In the present experiments of DMNA formation from sodium nitrate and DMA·HCl by the action of bacteria, the amount of DMNA formed in the stomach of guinea-pig was also lower than that of *in vitro* experiment.

Staphylococcus is widely distributed in life environment, and isolated from skin and saliva of man or animals, air, sewerage, and almost every thing.²⁰⁾ In the case of stomach cancer or gastritis, various microorganisms including *Staphylococcus* were detected in the stomach because of high pH of gastric juice.^{21), 22)}

In human saliva, however, the population of *Staphylococcus* is not so much and there are many other microorganisms which possess nitrate-reducing activity. In the present study of DMNA formation in the stomach of guinea-pig, only *S. epidermidis*, isolate E-2 was used, but the same phenomena may be expected in other nitrate-reducing bacteria.

Conclusively, there is a possibility of DMNA formation in the stomach by the combined action of nitrate, DMA and oral nitrate-reducing bacteria, if the stomach function is lowered to provide some favorable condition for DMNA formation.

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