Occurrence of Psychrophilic and Halophilic Histamine-Forming Bacteria (N-Group Bacteria) on/in Red Meat Fish*¹

Masayo OKUZUMI*², Shozo OKUDA*², and Masaishi AWANO*²

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In a previous paper, the authors reported that psychrophilic and halophilic histamine-forming bacteria, which could produce histamine at 5°C, could be isolated from the common mackerel flesh homogenates stored at 5°C and 20°C, these organisms were named "N-group bacteria" for the time being.

The present study was undertaken to obtain further information as to the occurrence of N-group bacteria on/in red meat fish, common mackerel, saury pike, and sardines. These fish samples were allowed to stand at 2.5°C. N-group bacteria occurred on/in all samples; the number of them in the fresh ones were 1 to 10² per gram of the flesh (0.01 to 1% of total bacterial number), while those in the spoiled ones were 10⁶ to 10⁸ (1 to 10%). N-group bacteria seem to occur as a part of the normal surface microflora of marine fish.

Scombroid poisoning (histamine toxicosis) has occasionally broken out as a result of ingesting scombroid fish, such as saury, tuna, mackerel and bonito, which are characterized by having high levels of free histidine in their tissues. High levels of histamine in the incriminated fish are generally formed via microbial decarboxylation of histidine.¹⁻⁴⁾ A considerable number of food-borne microbial species have been reported to possess the enzyme, histidine decarboxylase. However, in the few instances of microbiological examinations of seafood actually incriminated in food poisoning, only *Proteus morgani*,^{3,5)} *Hafnia alvei*⁶⁾ and *Klebsiella pneumoniae*⁷⁾ have been implicated as causative organisms in the formation of toxicologically significant levels of histamine.

In the course of the studies on freshness of homogenates of red meat fish stored at 5°C and 20°C, the authors⁸⁾ found that large amounts of histamine were accumulated in these homogenates as reported in the previous paper, and further microbiological examinations of the homogenates resulted in the isolation of a new type of histamine-forming bacteria. These bacteria grew at 5°C but not at 35°C; they grew well in a medium with 1 to 3 percent NaCl; they were capable of producing large amounts of histamine at a temperature of 5°C. They could not be identified with any of the known genera, and so the authors⁸⁾ have named the unidentified organisms "N-group

bacteria". It may be possible to consider that this group bacteria inhabit in seawater. If they are distributed in seawater, marine fish may always be contaminated with them, and they may grow and produce histamine from histidine in the fish during chill storage after being caught. Accordingly, this bacteria group may be important for the problem of food hygiene.

This study was undertaken to obtain further information on occurrence of psychrophilic and halophilic histamine-forming bacteria (N-group bacteria) on/in some red meat fish.

Materials and Methods

Fresh and Spoiled Fish Samples

Red meat fish used for this experiment were common mackerel *Scomber japonicus*, saury pike *Cololabis saira*, and sardine *Sardinea melanostica*. Five bodies in each species were examined. They were purchased in excellent condition from a local retail stores in Tokyo, in October and November, 1977. The samples were packed in a polyethylene bag and were allowed to stand in a constant temperature box at 2.5°C. The examinations were carried out at the start of storage and at the early stage of spoilage of the samples. After 10 to 12 days, the fish were obviously spoiled.

*¹ A New Type of Histamine-Forming Bacteria—II.

*² Laboratory of Food Microbiology, Tokyo University of Fisheries, Konan-4, Minato, Tokyo 108
(奥積昌世・奥田昭三・栗野正石: 東京水産大学).

Preparation of Homogenates from Fresh and Spoiled Fish Samples

Ten grams of fish with 10 cm² (2 cm × 5 cm) of skin was homogenized in a blender with 45 ml of sterile distilled water and an equivalent amount of artificial seawater⁸⁾ for two minutes.

Preparations of Fish Infusion Broths with and without NaCl for Examinations of Bacterial Histamine Formation

Fish infusion broth with 2.5% NaCl was prepared as follows. Two hundred fifty grams of fresh common mackerel flesh was homogenized with 2 volumes of water (W/V), steamed at 100°C for 1 h and filtered. The filtrate was enriched with 1% D-glucose and 2.5% NaCl, adjusted to a pH value of 6.0, distributed into test tubes, and sterilized. Another infusion broth (without NaCl) was prepared in the same manner except for the addition of NaCl.

Measurement of Viable Counts of Bacteria

Enumeration of bacteria was conducted with a smear plate method using 50% seawater agar medium (50% SW agar) described in the previous paper.⁸⁾ Multiple decimal dilutions of the homogenate were carried out with a diluent of 50% sea water. Aliquots (0.1 ml) of the diluted suspensions were spread over replicate agar plates with Conradi rods. After incubation for 10 days at 20°C, colonies on the plates were counted.

Measurement of Number of Psychrophilic and Halophilic Histamine-Forming Bacteria

One ml aliquots of the diluted suspensions described above were inoculated into both infusion broths with and without NaCl, and incubated at 2.5°C for 12 days. After incubation, histamine was examined by a circular paper-chromatography as described in the previous paper.⁸⁾ It is usually assumed that the highest dilution that yielded histamine in subculture contained at least one histamine-forming bacterium per milliliter. If histamine formation was found in the infusion broth with NaCl but not in the broth without NaCl, this histamine former can be considered as a halophilic organism. The number of psychrophilic and halophilic histamine formers on/in the samples was therefore calculated from this dilution.

Isolation of Psychrophilic and Halophilic Histamine-Forming Bacteria

The infusion broth culture with 2.5% NaCl, in

which the highest dilution yield histamine, was inoculated by streaking onto a 50% SW agar plate. After incubation at 20°C for 48 h, 30 colonies per plate were picked up at random and subcultured to the same medium to assure purity. Nine hundred pure cultures were isolated from the 30 fish samples (15 fresh ones and 15 spoiled ones). In order to confirm whether these isolates are histamine-forming bacteria or not, they were inoculated into fish infusion broth with NaCl, and incubated at 2.5°C for 12 days. Histamine formed in the cultures was examined by the paper chromatography as mentioned above.

Identification of Isolates as N-Group Bacteria

In order to confirm whether or not the histamine-forming strains are identical to N-group bacteria, the morphological and biochemical examinations (shape, Gram stain, motility, flagellation, cytochrome oxidase activity, catalase activity, acid and gas formation from glucose, H₂S production, casein hydrolysis, pigmentation, luminescence, and sensitivity to 0/129 reagent and penicillin) were carried out as described in the previous paper.⁸⁾

In addition, 6 cultures (N27B, N47B, N74B, N78B and N82B) of N-group bacteria, which were isolated and named by OKUZUMI *et al.*⁸⁾ in the previous study, were used as control of the tests.

Effect of NaCl Concentration, Temperature and pH Value on Growth

Effect of NaCl concentration and temperature on growth of the histamine-forming strains were examined as reported in the previous paper.⁸⁾ Effect of pH value was studied by the use of nutrient broth (Polipeptone (Diago) 1%, Beef Extract (Kyokuto) 0.5%) containing 2% NaCl. The pH range studied was from 4.02 to 8.96. These broths were inoculated with 22 to 44 cultures, and the extent of visible growth was recorded after 1, 2, 3 and 6 days.

Quantitative Analysis of Histamine in Fish Samples

Thirty five ml of the homogenate prepared as above was taken for histamine analysis, and 10 ml of 20% trichloroacetic acid solution was added. The mixture was allowed to stand for 30 min after shaking well and then filtered through a filter paper. The residue was reextracted with a cold 5% TCA solution. Histamine in the filtrate was determined by the method of KAWABATA *et al.*⁹⁾

Results and Discussion

Numbers of Psychrophilic and Halophilic Histamine-Forming Bacteria on/in Fresh and Spoiled Fish Samples

The results obtained are shown in Table 1.

Psychrophilic and halophilic histamine formers were found in all of the fresh samples. Viable counts of the histamine formers ranged from 1 to 10^2 microorganisms per gram, that is, the ratios of those to the viable count of bacteria were 0.001 to 0.1%. The numbers of these formers increased during the storage of the samples, and they occurred to the extent of 0.1 to 10% of the viable count of bacteria in the majority of the spoiled samples. Accordingly, the percentage levels of the histamine formers of the spoiled samples were higher than those of the fresh ones.

As shown in Table 1, the spoiled fish containing more than 100 mg/100 g of histamine was sample No. 7 alone among all samples, while five samples No. 1, 2, 5, 8 and 9 had less than 10 mg/100 g. There seemed to be a causal relationship between content of histamine and number of histamine former but a few exceptional cases were also observed.

Although the number of histamine formers of sample No. 13 was 100 times that of sample No. 12, histamine content of the latter was about 3 times that of the former. The discrepancy between their concentrations and their numbers

may have resulted from one or more of the following reasons: (1) histamine-forming bacteria in sample No. 12 died; (2) histamine-decomposing bacteria in sample No. 13 reduced the histamine level.

Morphological, Biochemical and Physiological Characteristics of Psychrophilic and Halophilic Histamine-Forming Bacteria.

Nine hundred strains were isolated from 30 fish samples and examined on formation of histamine with fish infusion broth with NaCl at 2.5°C. In consequence, only 26 strains of 900 isolates produced histamine. In order to investigate whether or not these 26 strains are identical to N-group bacteria, the morphological, physiological and biochemical tests were conducted by using 6 strains of known N-group bacteria described above as control of these tests.

As shown in Table 2, these 26 strains were Gram-negative coccobacilli and had motility by one polar flagellum, and also they possessed the same biochemical characteristics except for two properties, cytochrome oxidase activity and luminescence productivity. Ten of the 26 strains were able to emit light, and 2 strains were cytochrome oxidase negative. Six N-group strains exhibited similar behaviors to the 26 strains in all biochemical reactions.

Effects of NaCl concentration, temperature, and pH value on growth are presented in Tables

Table 1. Numbers of psychrophilic and halophilic histamine-forming bacteria on/in the fresh and spoiled fish samples

Species	Fresh samples		Spoiled samples		
	Viable counts (/g)	Numbers of PHHB (/g)	Viable counts (/g)	Numbers of PHHB (/g)	Histamine (mg/100 g)
1 Common mackerel	5.7×10^3	1—10	3.8×10^8	10^5 — 10^8	4.6
2 "	9.4×10^3	1—10	9.2×10^8	10^5 — 10^8	4.2
3 "	1.1×10^5	10 — 10^2	5.5×10^8	10^7 — 10^8	87.8
4 "	2.4×10^4	1—10	1.5×10^8	10^7 — 10^8	66.4
5 "	2.1×10^5	1—10	3.1×10^8	10^6 — 10^7	2.0
6 Pacific saury	1.2×10^5	10^3 — 10^4	2.5×10^9	10^7 — 10^8	61.0
7 "	2.9×10^5	10 — 10^2	1.0×10^9	10^7 — 10^8	144.0
8 "	1.9×10^6	10 — 10^2	7.2×10^8	10^5 — 10^6	3.6
9 "	4.9×10^4	1—10	8.3×10^8	10^4 — 10^5	2.0
10 "	5.5×10^4	1—10	2.2×10^9	10^7 — 10^8	19.2
11 Sardine	1.2×10^4	1—10	5.8×10^8	10^7 — 10^8	41.7
12 "	3.0×10^4	10 — 10^2	4.1×10^8	10^6 — 10^7	67.4
13 "	1.9×10^4	10 — 10^2	5.7×10^8	10^8 — 10^9	26.7
14 "	1.1×10^4	10 — 10^2	1.2×10^8	10^6 — 10^7	25.7
15 "	2.4×10^4	1—10	1.7×10^9	10^7 — 10^8	34.2

PHHB: psychrophilic and halophilic histamine-forming bacteria.

Table 2. Morphological and biochemical characteristics of psychrophilic and halophilic histamine-forming bacteria

Strains	Shape	Gram stain	Motility	Flagellation	Cytochrome oxidase	Catalase	Acid from glucose		Gas from glucose	H ₂ S production	NO ₃ ⁻ reduction	Casein hydrolysis	Pigmentation	Luminescens	Sensitivity to	penicillin
							(Aerobically	(Anaerobically								
1	CR	-	+	PI	+	+	+	+	+	-	+	-	-	+	+	-
2	CR	-	+	PI	-	+	+	+	+	-	+	-	-	+	+	-
3	CR	-	+	PI	+	+	+	+	+	-	+	-	-	+	+	-
4	CR	-	+	PI	+	+	+	+	+	-	+	-	-	+	+	-
5	CR	-	+	PI	+	+	+	+	+	-	+	-	-	+	+	-
6	CR	-	+	PI	+	+	+	+	+	-	+	-	-	+	+	-
7	CR	-	+	PI	-	+	+	+	+	-	+	-	-	+	+	-
8	CR	-	+	PI	+	+	+	+	+	-	+	-	-	+	+	-
9	CR	-	+	PI	+	+	+	+	+	-	+	-	-	+	+	-
10	CR	-	+	PI	+	+	+	+	+	-	+	-	-	+	+	-
11	CR	-	+	PI	+	+	+	+	+	-	+	-	-	+	+	-
12	CR	-	+	PI	+	+	+	+	+	-	+	-	-	+	+	-
13	CR	-	+	PI	+	+	+	+	+	-	+	-	-	+	+	-
14	CR	-	+	PI	+	+	+	+	+	-	+	-	-	+	+	-
15	CR	-	+	PI	+	+	+	+	+	-	+	-	-	+	+	-
16	CR	-	+	PI	+	+	+	+	+	-	+	-	-	+	+	-
17	CR	-	+	PI	+	+	+	+	+	-	+	-	-	+	+	-
18	CR	-	+	PI	+	+	+	+	+	-	+	-	-	+	+	-
19	CR	-	+	PI	+	+	+	+	+	-	+	-	-	+	+	-
20	CR	-	+	PI	+	+	+	+	+	-	+	-	-	+	+	-
21	CR	-	+	PI	+	+	+	+	+	-	+	-	-	+	+	-
22	CR	-	+	PI	+	+	+	+	+	-	+	-	-	+	+	-
23	CR	-	+	PI	+	+	+	+	+	-	+	-	-	+	+	-
24	CR	-	+	PI	+	+	+	+	+	-	+	-	-	+	+	-
25	CR	-	+	PI	+	+	+	+	+	-	+	-	-	+	+	-
26	CR	-	+	PI	+	+	+	+	+	-	+	-	-	+	+	-
N27B	CR	-	+	PI	+	+	+	+	+	-	+	-	-	-	+	-
N47B	CR	-	+	PI	+	+	+	+	+	-	+	-	-	-	+	-
N64B	CR	-	+	PI	+	+	+	+	+	-	+	-	-	+	+	-
N74B	CR	-	+	PI	+	+	+	+	+	-	+	-	-	+	+	-
N78B	CR	-	+	PI	+	+	+	+	+	-	+	-	-	-	+	-
N82B	CR	-	+	PI	+	+	+	+	+	-	+	-	-	+	+	-

CR: coccoid rod.

PI: single, polar flagellum.

[3, 4 and 5, respectively.

All 26 strains grew well in the media containing 1 to 3% NaCl, and similar results were also obtained from the cultures of the N-group strains. The most abundant growth was observed in 2% NaCl, while no growth with 0 and 7% (Table 3).

All 26 strains grew at 5°C, but not 35°C. Their optimum temperatures were about 20°C. The

results were comparable to those obtained from the cultures of the N-group strains (Table 4).

All 26 strains and the N-group strains used as control grew well in a range of pH 4.51 to 8.96, but not at pH 4.02. Their optimum pH values were ranging from 6.00 to 7.98 (Table 5).

It was revealed that all 26 histamine formers were identified as N-group bacteria. Accordingly,

Table 3. Effect of NaCl concentration on growth of psychrophilic and halophilic histamine-forming bacteria

Strains	NaCl (%)						
	0	0.5	1	2	3	5	7
1	—	—	+	++	++	+	—
2	—	—	+	++	+	±	—
3	—	±	+	++	+	±	—
4	—	±	+	++	+	±	—
5	—	±	+	++	+	±	—
6	—	±	+	++	+	±	—
7	—	±	+	++	+	±	—
8	—	—	+	++	+	±	—
9	—	±	+	++	+	±	—
10	—	±	+	++	+	—	—
11	—	±	+	++	+	±	—
12	—	±	+	++	+	±	—
13	—	±	+	++	+	±	—
14	—	±	+	++	+	—	—
15	—	±	+	++	+	—	—
16	—	±	+	++	+	—	—
17	—	±	+	++	+	±	—
18	—	±	+	++	+	±	—
19	—	±	+	++	+	±	—
20	—	±	+	++	+	±	—
21	—	±	+	++	+	±	—
22	—	—	+	++	+	±	—
23	—	—	+	++	+	±	—
24	—	—	+	++	+	±	—
25	—	—	+	++	+	±	—
26	—	—	+	++	+	±	—
N27B	—	—	+	++	±	—	—
N47B	—	—	±	+	+	±	—
N64B	—	—	±	+	+	—	—
N74B	—	—	+	+	±	—	—
N78B	—	—	+	++	+	±	—
N82B	—	—	±	+	±	—	—

The extent of visible growth was recorded after 24 hours incubation.

++ very abundant growth, + abundant growth, ± slight growth, — no growth.

Table 4. Effect of temperature on growth of the psychrophilic and halophilic histamine-forming bacteria

Strains	Temperature (°C)						
	5	10	15	20	25	30	35
1	++	±	+	++	++	—	—
2	++	±	+	++	+	—	—
3	++	±	+	++	++	+	—
4	++	±	+	++	++	+	—
5	++	±	+	++	++	+	—
6	++	±	+	++	++	—	—
7	++	±	+	++	+	—	—
8	++	—	+	++	+	—	—
9	++	±	+	++	+	+	—
10	++	±	+	++	++	+	—
11	++	—	+	++	++	+	—
12	++	±	++	++	+	+	—
13	++	±	+	++	+	+	—
14	++	—	+	++	++	+	—
15	++	±	+	++	++	+	—
16	++	±	+	++	++	+	—
17	++	±	+	++	++	+	—
18	++	—	+	++	++	+	—
19	++	±	++	++	+	+	—
20	++	±	++	++	++	+	—
21	++	±	++	++	++	—	—
22	++	±	++	++	++	—	—
23	++	±	++	++	—	—	—
24	++	±	++	++	—	—	—
25	++	±	++	++	—	—	—
26	++	±	+	++	—	—	—
N27B	++	±	+	++	+	±	—
N47B	+	—	+	++	—	—	—
N64B	+	—	++	++	+	—	—
N74B	++	—	+	++	±	—	—
N78B	++	—	+	++	+	+	—
N82B	++	—	++	++	+	—	—

The extent of visible growth at 5°C and the other temperatures was recorded after 6 days and 19 hours, respectively.

++ very abundant growth, + abundant growth, ± slight growth, — no growth.

it is considered that N-group bacteria were present on/in both fresh and spoiled fish samples.

According to KAWABATA *et al.*¹⁰⁾ and FERENICK,⁶⁾ histamine levels in excess of 100 mg per 100 g of fish flesh have often been associated with clinical illness. In the present study, only sample No. 7 contained histamine above the clinical concentration for the poisoning. Consequently, it is suggested that this bacteria group may be important for the problem of food hygiene.

It seems to be very difficult to isolate a large number of N-group bacteria from fish, because the number of this group bacteria may be less than 1% of total bacterial number in fish and

moreover there is no selective medium for isolation of this group bacteria. In our preceding paper,¹¹⁾ it was reported that almost all of the bacterial strains isolated from fresh and spoiled fish could not grow at pH 4.9. In the present study, on the other hand, all of the N-group strains were able to grow at pH 4.51. It may be difficult to detect the responsible N-group bacteria because they generally comprise a minority of the bacterial flora on/in marine fish, but they can be selected by using a medium of pH about 4.5. To facilitate the detection of N-group bacteria, a selective enrichment medium with pH 4.5 should be devised for their isolation.

Table 5. Effect of pH values on growth of psychrophilic and halophilic histamine-forming bacteria

Strains	pH							
	4.02	4.51	5.02	6.00	7.01	7.98	8.47	8.96
1	—	++	+	++	++	++	+	+
2	—	++	+	++	++	++	+	+
3	—	++	+	++	++	++	++	++
4	—	++	++	++	++	++	++	++
5	—	++	++	++	++	++	++	++
6	—	++	++	++	++	++	++	+
7	—	++	++	++	++	++	++	++
8	—	+	+	++	++	++	++	+
9	—	++	+	+	++	++	++	+
10	—	++	+	+	++	++	++	++
11	—	++	++	++	++	++	++	++
12	—	++	++	++	++	++	++	++
13	—	++	++	++	++	++	++	++
14	—	++	+	++	++	++	++	++
15	—	++	+	++	++	++	++	++
16	—	++	+	++	++	++	++	++
17	—	++	+	++	++	++	++	++
18	—	++	+	++	++	++	++	++
19	—	++	++	++	++	++	++	++
20	—	++	++	++	++	++	++	++
21	—	++	+	++	++	++	++	+
22	—	++	+	++	++	++	++	++
23	—	++	+	++	++	++	++	+
24	—	++	+	++	++	++	++	++
25	—	++	+	++	++	++	++	+
26	—	++	+	++	++	++	++	++
N27B	—	++	+	++	++	++	++	++
N47B	—	±	±	++	++	++	+	+
N64B	—	±	+	++	++	++	++	±
N74B	—	++	+	++	++	++	++	+
N78B	—	++	+	++	++	++	++	++
N82B	—	++	+	++	++	++	++	++

The extent of visible growth at pH 4.02 and 4.51 was recorded after 4 days incubation and the others after 19 hours.

++ very abundant growth, + abundant growth, ± slight growth, — no growth.

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