STUDIES ON THE FORMATION OF AMMONIA AND TRIMETHYLAMINE IN SHARK

STUDIES ON THE FORMATION OF AMMONIA AND TRIMETHYLAMINE IN SHARK

By

Yasuhiko TSUCHIYA, Iwao TAKAHASHI and Seiki YOSHIDA

Institute of Fisheries, Faculty of Agriculture,
Tohoku University, Sendai, Japan

(*Received July 20, 1951*)

The annual Japanese production of shark was estimated for the representative postwar years to have been about 120 thousand tons (1). Shark is therefore an important food fish and its liver is also in great demand for vitamin oil. However, it has been generally thought to be inferior to the normal food fish in trade, because unfavorable metallic odour and flavour are given off from shark meat at a relatively early stage of spoilage.

In 1909, Suwa (2) has shown that trimethylamine develops from the reduction of trimethylamine oxide by the bacterial action in shark spoilage. In recent years, Wood (3) has shown that shark spoilage is due to the production of ammonia from the urea and other nitrogenous bodies present in the shark meat by bacterial action and autolysis. Namely, he has found the natural occurring urease in blood and meat and at the same time isolated from shark meat bacteria which can liberate ammonia from the urea agar, autoclaved shark meat, shark muscle juice, meat extract, peptone, and asparagine. Mori and Kobayashi (4) and Fujikawa (5) have also found the urease in shark meat, although in the case of the latter worker it was not ascertained. On the other hand, Simidu and Oisi (6) failed to find any appreciable amount of the urease in shark meat.

In the present investigation the authors studied the shark spoilage with special reference to the formation of ammonia and trimethylamine.

Here the authors express their gratitude to Mr. A. Takata, Laboratory of Food Technology, Nippon Reizo Kabushikikaisha, for his help in sampling the shark used in the experiments. Their hearty thanks are also due to Dr. C. H. Castell, Atlantic Fisheries Experimental Station in Canada, who cordially sent to us many useful papers concerning this experiment. Financial support, in part, was received from the Ministry of Education.

The occurrence of urease in the shark muscle

Primarily it is very important to decide whether ammonia is derived from the urea by the action of urease in shark meat. Therefore the following experiments have been made.

Preparation of urease solution.

To determine urease, the following procedure was adopted. Place 25 grams of the shark muscle into a mortar, add one teaspoon of quartzsand and wholly grind and then thoroughly mix with 75 ml. of water. After allowing to stand, with occasional stirring for about 3 hours at 10°C, clearly filter off the solution on pulp layer by means of suction. The filtrate was poured into 150 ml. of ethanol-ether mixtures (ethanol 4 : ether 1) with vigorous stirring. The precipitates filtered off were dissolved in a volumetric flask of 200 ml. with about 100 ml. of water, and measured up to the mark.

Determination of urease.

Pour 5 ml. of the urease solution thus obtained into a test tube and add 1 ml. of 1.5 per cent solution of urea and adjust to pH 6.8 with 5 ml. of phosphate buffer solution. The ammonia which developed therefrom was determined after incubating for half an hour at 30°C by the distillation method. The unit of urease activity was conveniently established as follows: 1 mg. of ammonia nitrogen liberated from the urea was substituted for 1 unit of urease in 100 grams of dry material.

The occurrence of urease.

The amounts of urease in the dog fish (*Squalus sucklii*), raja (*Raja kenojei*) and soy bean were determined according to the method described above, and

Table I.

The Occurrence of Urease in the Shark Muscle

No.	Sample	Days after catching	Total length (cm)	Body weight (g)	Sex	Part	Urease unit
1	Dog fish	3(iced)	37.5 35.0	247 220	male male	front dorsal back dorsal ventral	19.75 + −
2	〃 〃	7(frozen for 5 days, at −15°C)	84.0	1863	male	front dorsal back dorsal ventral	9.01 + −
3	〃 〃	9(frozen for 7 days, at −30°C)	85.0	2437	female	front dorsal back dorsal ventral	− − −
4	Raja	4(iced)	49.5 48.3	568 525		dorsal ventral	26.31 10.16
5	Soy bean					without oil extraction with oil extraction	652.4 1131.6

the results obtained are given in Table I.

Table I shows that minute amounts of urease were generally present in the dorsal muscle but not in the ventral, while in raja it was found in both. However, the rate of production of ammonia by this urease does not seem sufficient to explain the amount normally found in spoilage. The urease activity was variable in each sample of shark as shown in number 1, 2 and 3. It is considered to be due to the difference of physiological action in sex or age of the shark, and also to the degree of denaturation of urease during the storage or the defrosting of the frozen shark at room temperature. This point has not been investigated.

The biochemical change during shark spoilage

In the above experiment, it was found that the ammonia may partly split off from the urea by autolysis, though this is not the sole cause. Therefore the biochemical change during shark spoilage was studied with special reference to pH, ammonia, urea, trimethylamine and trimethylamine oxide. The latter ones, however, are supposed to be the constituent of unfavorable odours and its precursor, the trimethylamine oxide has been found to be large quantities ranging from 1000 to 1500 mg. per cent in the muscles of marine elasmobranchs (7).

Materials and methods.

In the experiment the whole gutted dog fish used was male, 88.4 cm. in total length, 2381 grams in body weight, and iced for 3 days after catching.

The spoilage temperature was 8 to 15°C.

Periodically small blocks of approximately 25 grams were cut off from the dorsal back and ventral part of the shark in the lot being examined. These were macerated together with a teaspoon of quartzsand in a mortar and mixed thoroughly with one and a half times their weight of acetate buffer solution of pH 5.3. Then about 100 ml. of hot water was added to the minced meat and warmed on a water-bath for 15 minutes. Thereafter, the filtrate in a volumetric flask of 250 ml. was filled up to the mark. This solution was used for the determination of total volatile nitrogen, urea, trimethylamine and trimethylamine oxide.

Urea was determined by the urease method of Takeuchi (8), and trimethylamine and trimethylamine oxide were determined by the method of Lintzel (9).

pH of muscle juice was colorimetrically measured.

Biochemical change in spoilage.

The results obtained are given in Table II and shown in Figure 1. They show that the amounts of the total volatile nitrogen, ammonia and trimethylamine increased, while the urea and trimethylamine oxide inversely decreased during the spoilage. It is also observed that the amounts of ammonia developed

Table II.
Biochemical Change during the Shark Spoilage

	Days in spoilage							
	Dorsal muscle				Ventral muscle			
	1	3	5	7	1	3	5	7
pH	6.2	6.3	7.0	8.0<	6.5	6.6	7.2	8.0<
Total volatile-N (mg. per 100g. of material)	22.72	81.36 (+58.64)	185.48 (+162.76)	298.60 (+275.88)	22.71	110.37 (+87.66)	222.51 (+199.80)	325.30 (+302.59)
Ammonia-N (mg. per 100g. of material)	22.50	62.65 (+40.15)	145.08 (+122.98)	230.29 (+207.79)	22.26	88.34 (+66.08)	165.10 (+142.84)	250.49 (+228.23)
Urea-N (mg. per 100g. of material)	553.28	529.97 (−23.31)	437.34 (−115.94)	363.84 (−189.44)	542.45	493.20 (−49.25)	390.21 (−152.24)	330.36 (−212.09)
Trimethylamine-N (mg. per 100g. of material)	0.22	18.71 (+18.49)	40.40 (+39.18)	68.31 (+68.09)	0.45	22.03 (+21.58)	57.41 (+56.96)	74.81 (+74.36)
Trimethylamineoxide-N (mg. per 100g. of material)	84.66	57.99 (−26.67)	42.73 (−41.93)	26.10 (−58.56)	80.16	50.27 (−29.89)	38.30 (−41.86)	32.55 (−47.61)
Sum of decreasing amounts of urea-N and trimethylamineoxide -N by		49.98 / 58.64	157.87 / 162.16	248.00 / 275.88		79.14 / 87.16	194.10 / 199.80	259.70 / 302.59
Sum of increasing amounts of ammonia-N and trimethylamine-N		(0.854)	(0.956)	(0.899)		(0.900)	(0.972)	(0.858)

from the dorsal muscle were less than that from the ventral. On the contrary, the urease content, as already found in the preceding experiment, was shown to be higher in the dorsal muscle than in the ventral.

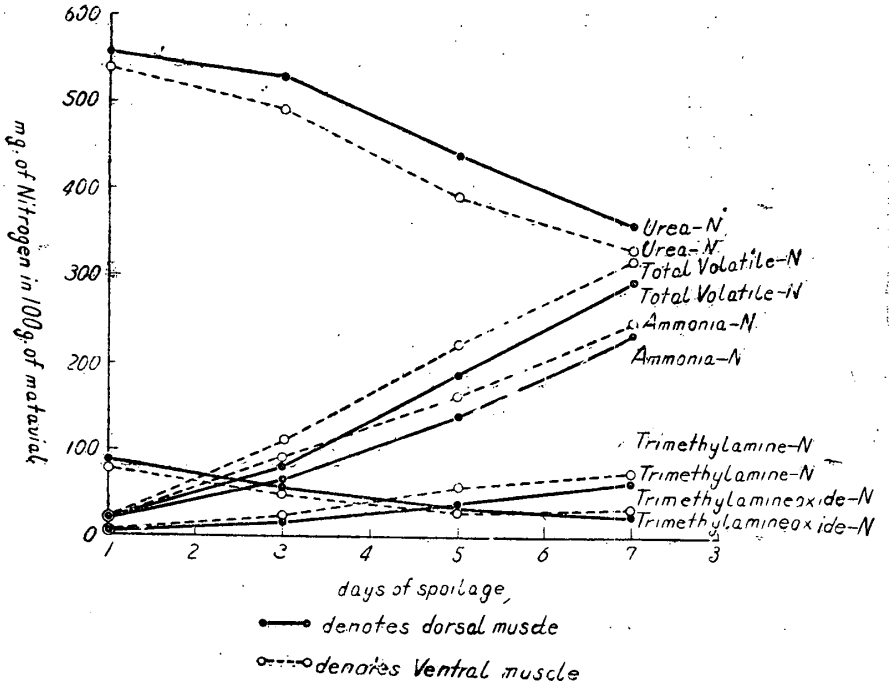

Fig. 1 Biochemical change during the shark spoilage.

The spoilage of shark is, therefore, evidently considered to be due to autolysis and bacterial action as Wood has already found, and from this experiment, it is ascertained that the microbial action was a main factor on the shark spoilage.

It is noteworthy that the sum of the amounts of increasing nitrogen in ammonia and trimethylamine were almost equivalent to the total amounts of dereasing nitrogen in the urea and trimethylamine oxide during the spoilage. This fact shows that the unfavorable odour and flavour in the early stage of shark spoilage are mainly due to ammonia and trimethylamine which are derived from urea and trimethylamine oxide.

Urease in bacteria isolated from shark muscle

As already pointed out, the autolysis plays a minor role in the formation of ammonia from the urea. Here the occurrence of urease in bacteria isolated from shark muscle was studied.

Cultures:

Sterilize the surface of fillets cut from iced shark (Prionace grauca) with a diluted formalin and take some small blocks from their inner part. Then mince

finely in a mortar and mix thoroughly with sterile water. The resulting suspension is then plated to nutrient agar. A total of 14 microorganisms were isolated from the dilution plates.

Qualitative test for urease in bacteria.

Pour 0.5 ml. of cell suspension of nutrient broth into a test tube containing 10 ml. of 1 per cent solution of urea and 2 drops of phenolphthalein as an indicator. After adding a few drops of toluol, close tightly with a rubber stopper. After incubating for 24 hours at 37°C, the appearance of pink color indicates the presence of urease.

Quantitative test for urease in bacteria.

Take bacterial colonies grown on nutrient agar in a test tube and mix thoroughly with sterile water.

1 ml. of the resulting suspension containing 8 to 10 mg. of dry weight of bacteria was poured into another test tube and 5 ml. of 1 per cent solution of urea and a few drops of toluol were added. After incubating 24 hours at 37°C, the ammonia developed from the urea is determined by the method of Conway (10).

Ammonia formation by isolated culture.

According to the method described above, the results obtained are given in Table III, which shows that 4 of the 14 organisms isolated from the shark muscle contained an active urease as indicated in culture number 5, 6, 7, and 8.

Table III.

Urease in Bacteria isolated from the Shark Muscle

Culture No.	Genus	Phenolphthalein test	γ of ammonia nitrogen in 1 ml. of culture solution
0	Uninoculated (control)	−	7.7
1	Micrococcus	−	11.3
2	〃 〃	−	7.7
3	Bacterium	−	19.2
4	Proteus	−	3.8
5	Corynebacterium	+	738.0
6	Proteus	+	34.8
7	Micrococcus	+	422.0
8	〃 〃	+	240.0
9	Corynebacterium	−	15.4
10	Achromobacter	−	21.2
11	〃	−	7.7
12	Corynebacterium	−	5.5
13	Flavobacterium	−	1.8
14	Corynebacterium	−	17.2

\+ denotes ammonia production
− denotes no ammonia production

Triamineoxidease in bacteria isolated from shark muscle

Suwa (2) was the first to show that the trimethylamine oxide is reduced to the corresponding amine in the course of bacterial action on the muscle. This discovery led to the observation that the specific bacterial species possessed enzyme triamineoxidease. (11), (12). Recent investigation (13) proves that the power to reduce trimethylamine oxide generally occurs in microorganisms, especially in the family Enterobacteriaceae. Therefore, the occurrence of triamineoxidease in bacteria isolated from shark muscle was studied.

Methods.

Microorganisms used in this experiment belonged to the same genera as isolated from the shark muscle in preceding experiment.

The anaerobic reduction test for the triamineoxidease in these bacteria was carried out in a Thunberg tube. Tarr's methods (12) were employed. The incubation time, however, was 20 hours at 25°C, and a sterile shark muscle extract was used in place of halibut as in his experiment.

Reduction by isolated cultures.

The results of this experiment are given in Table IV, from which, it is apparent that 3 of the 14 cultures studied, namely culture number 1, 4, and 6, formed significant amounts of trimethylamine from the shark muscle extract. On the other hand, in the culture solution of the mixture of trimethylamine oxide and glucose, a large proportion of the trimethylamine oxide was reduced by one organism, such as number 1 but not so large by the other two organisms as number 4 and 6.

Table IV.
Triamineoxidease in Bacteria isolated from the Shark Muscle

Culture No.	Genus	γ of trimethylamine nitrogen in 1 ml. of culture solution. (Bacterial cells +shark muscle extract)	γ of trimethylamine nitrogen in 1 ml. of culture solution (Bacterial cells +0.1 M of trimethylamineoxide +0.1 M Glucose)
0	Uninoculated (control)	13	1
1	Micrococcus	167	199
2	〃	12	6
3	Bacterium	13	4
4	Proteus	81	29
5	Corynebacterium	13	6
6	Proteus	56	14
7	Micoccoccus	15	4
8	〃	13	4
9	Corynebacterium	17	3
10	Achromobacter	13	6
11	〃	11	4
12	Corynebacterium	11	1
13	Flavobacterium	14	5
14	Corynebacterium	10	1

Summary

Under the conditions employed in these experiments the following results were established:

(1) Natural urease was found to occur in minute amounts in the dorsal muscle of the dog fish, but uncertainly in the ventral. Therefore, the ammonia may, in part, develop from urea by autolysis in the early stage of shark spoilage.

(2) During the shark spoilage, the amounts of total volatile nitrogen, ammonia and trimethylamine increased, while the urea and trimethylamine oxide decreased.

(3) In spite of a comparatively high content of urease in the dorsal muscle, the amounts of ammonia developed therefrom were smaller than from the ventral muscle. Therefore, it is ascertained that the autolysis plays a minor role in the formation of ammonia from urea and that the microbial action is the main factor.

(4) The unfavorable odour and flavour in the early stage of shark spoilage are mainly due to ammonia and trimethylamine which are derived from the urea and trimethylamine oxide.

(5) Of the 14 organisms isolated from the shark muscle, 4 powerfully developed ammonia from the urea.

(6) The bacteria isolated from the shark muscle have been tested for their ability to reduce trimethylamine oxide to trimethylamine. It was found that this property is present in 3 of the 14 cultures studied.

References

(1) Fisheries Agency of Japan: (1951) Fisheries Statistical Bulletin. Vol. 1, No. 1.

(2) Suwa. A.: (1909) Arch, Pflüger. Physiol., 129, 231.

(3) Wood, E. J. F.: (1950) Austl. J. Mar. Freshw. Res., 1. 129.

(4) Mori. T. &. T. Kobayashi: (1949 and 1950) Read at the annual meeting of Jap. Soc. Sci. Fish.

(5) Fujikawa. K.: (1935) Bull. Govern. Fish. Exp. St. Chosen., Vol. 6, p. 78.

(6) Simidu. W. &. K. Oisi: (1951) Bull. Jap. Soc. Sci. Fish., 16, 47.

(7) Reay. A. G. A. et al.: (1943) J. Soc. Chem. Ind., 62, 77.

(8) Takeuchi. : (1936) Nogei-Kagaku-Bunsekisho. Vol. 2, p. 196. Maruzen Book Company, Tokyo.

(9) Lintzel. W.: (1934) Bioch. Z. 273, 243.
Lintzel. W. et al.: (1939) ibid., 301, 29.

(10) Conway. E. J. &. A. Byrne: (1933) Bioch. J. 27, 419.

(11) Watson. D. W.: (1939) J. Fish. Res. Bd. Canada. 4, 252 & 267.

(12) Tarr. H. L. A.: (1939) ibid., 4, 367.

(13) Sigurdsson J. G. &. A. J. Wood: (1942) ibid., 6, 45.
Wood. A. J. &. E. J. Baird.: (1943) ibid., 6, 194.