Chemical Changes of Fish Muscle during Preservation with Ammonia

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In ammonia vapor (1 ml of liquid NH_3/100 g of fish) Mrigal fish can be stored without spoilage over 3 months at 4–6°, whereas the control and iced fish are found in edible condition for 6 and 13 days, respectively. Ammonia-sprayed (1 ml of liquid NH_3/100 g of fish) fish can be preserved without spoilage for a month at 28–30°, in comparison to the control which is markedly spoiled within 24 hr. The quantity of IMP as the quality index of fish flavor in the process of preservation with ammonia is decreased to half that of the control fish, but there is no increase of hypoxanthine and ammonia. The bacterial load is lower than that of the control fish due to ammonia action.

In the tropics, spoilage of fish starts within a few hours after the catch, and there is a considerable time lag before the fish reaches the consumer or is used for processing. Serious deteriorative changes can take place and the period of deterioration is limited. If these changes could be prevented and the fish material could be stored in bulk without spoilage, it could then be used for mechanical or even sun drying or processed into fish flour (fish protein concentrate) in a factory that could operate well beyond the fishing season.

The nucleotide of primary interest in the muscle of fish is inosine 5′-monophosphate (IMP). IMP can occur in fresh fish muscle and meat extracts at concentrations at which solutions are strongly meat flavored (Saito, 1960; Jones, 1960, 1961; Wood, 1961). The adenosine 5′-triphosphate (ATP) of fish muscle breaks down, either during the death struggle or subsequently. This breakdown results in liberation of IMP, the contributor of the pleasant flavor of fresh fish. The degradation of IMP to hypoxanthine is a factor in the progressive loss of desirable flavor and in the development of bitter off-flavor (Jones, 1961). Consequently, procedures for the course of reactions in the sequences of nucleotide degradation possess the basic prerequisites for valid and useful indices of quality. Most of the currently available chemical indices measure essentially bacterial spoilage, whereas the assay of hypoxanthine and ammonia also measures certain initial autolytic phases of deterioration (Kassemsarn *et al.*, 1963). Since the main value of this test lies in its monitoring of both autolytic and bacterial activities in a general sense, hypoxanthine measurement as a general index of quality appears to be more valuable than others (Jones *et al.*, 1964).

With the above objective, we have been exploring the possibility of preserving fish with some material that is safe and comparatively easy to obtain, which has a good preservative action, and which can, at the same time, be removed easily during drying or other processing. Among the preservatives, ammonia in the gaseous form has proved to be promising. It has already been reported by Subrahmanyan *et al.* (1963) that ammonia can be used successfully for the preservation of eviscerated oil sardines for more than 2 months without deterioration of their nutritive value. Also Mitchell and W. R. Grace, and Co. (1969) have a food patent covering the spraying of aqueous ammonia over fish for preservation up to 2 weeks without refrigeration. The changes of IMP, AMP, hypoxanthine, ammonia, and bacterial load as the quality index of freshness and flavors of fish are studied in the present paper.

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EXPERIMENTAL SECTION

Sample. Small fresh-water Mrigal fish (100 g each) were collected from local ponds of the Jadavpur area of West Bengal (India) and used throughout this study.

Methods of Treatment. To preserve the collected fish at different temperatures we have attempted different procedures, such as: (a) immersion of each 100-g fish in an equal volume of several concentrations (0.05, 0.1, 0.25, and 0.5 *N*) of ammonia for 30 min and then transfer to air-tight containers having different amounts (0.5, 1, 2.5, 5, 7.5, and 10 ml) of 2 *N* ammonia as a source of ammonia vapor; (b) immersion of each 100-g fish in an equal volume of several concentrations (0.05, 0.1, 0.25, and 0.5 *N*) of ammonia each along with varying small amounts of 8-hydroxyquinoline sulfate, the sodium salt of ethylenediaminetetraacetic acid (EDTA), and 1,10-phenanthroline separately; (c) spraying of each 100-g fish with different amounts (0.5, 1, and 2 ml) of 5 *N* ammonia in air-tight containers; (d) transfer of each 100-g fish after spraying with 1 ml of 0.5 *N* ammonia to air-tight containers having different amounts (0.5, 1, 2.5, 5, 7.5, and 10 ml) of 2 *N* ammonia as a source of ammonia vapor; (e) transfer of each 100-g fish to air-tight containers having different amounts (0.5, 1, 2.5, 7.5, and 10 ml) of 5 *N* ammonia as a source of ammonia vapor; (f) spraying well each 100-g fish with the mixture of 1 ml of 2 *N* ammonia separately with varying amounts of 8-hydroxyquinoline, the sodium salt of EDTA, and phenanthroline.

Among the methods tried, we have selected procedures c and e as the most promising and suitable for analyzing the quality index of fresh-water Mrigal fish. In method e, each 100-g Mrigal fish was placed on glass wool (treated with concentrated hydrochloric acid and washed with distilled water) about 5 cm above the bottom of an air-tight glass container having 1 ml of 5 *N* ammonia on the bottom for vapor and was kept in a cold room at 4–6°. Process c describes a method in which each 100-g fish, placed on the purified glass wool about 5 cm above the bottom, was sprayed with 1 ml of 5 *N* ammonia in an air-tight glass container and was kept at 28–30°.

Each fresh-water Mrigal fish (100-g each) was packed in an air-tight transparent polyethylene bag and iced in boxes kept in the cold room (4–6°). The samples also were kept in glass bottles as the control both at 4–6 and 28–30°.

Organoleptic Analysis. After repeated washing with water, the preserved fish were treated with 0.1 *N* HCl to improve texture and make them firmer (Subrahmanyan *et al.*, 1963). For each taste panel evaluation two pieces each of control and treated samples were cooked and served to a panel of five members. Coded samples were assessed for odor and flavor and the results were scored on a ten-point scale, using a score of 5 as the limit of acceptibility according to the method described by Bhadra *et al.* (1973).

Table I. Chemical Changes of Fish (Mrigal Weighing 100 g) without any Treatment during Storage at 4–6°

Storage, days	μmol/g of App. IMP	AMP	IMP	App. nucleosides + bases at 250 mμ	Hypoxanthine	Ammonia, mg/g	Bacterial load in terms of formazan, μg/g
0	1.47	0.046	1.42	1.89	0.470	0.099	84
3	1.05	0.042	1.01	2.06	0.470	0.099	84
6	0.51	0.034	0.48	2.69	0.500	0.099	138
7	0.45	0.028	0.42	2.76	0.816	0.134	420

Preparation of Tissue Extracts. Muscle from the anteriodorsal portion of the fish was extracted with chilled 3% perchloric acid (Spinelli and Kemp, 1966). The homogenate was filtered and 20 ml was immediately neutralized with 10% KOH to pH 6.5. The neutralized extract was stored approximately for 30 min at 0° to permit crystallization of potassium perchlorate.

Purification of Nucleotides. One procedure used by Spinelli and Kemp (1966) was followed by us. Dowex 1-X4 (Cl) was washed first with 10% NH_4OH and then with 4 *N* HCl. The neutralized extract (20 ml) was passed over a 1 × 2 cm resin bed to separate the nucleotides from nucleosides, purines, pyrimidines, and free sugar (Shewan and Jones, 1957). Distilled water (35 ml) was passed over the column until the effluent was free from ultraviolet-absorbing material. The column was then eluted with 25 ml of 1 *N* H_2SO_4 followed by 5 ml of 6 *N* H_2SO_4. This removed 99% of the ultraviolet-absorbing materials. All this work was done at 0–2°.

Measurement of Apparent IMP. An appropriate dilution was made of the second column eluate (usually 1:10) with distilled water so that the acid concentration of the final solution would be equivalent to 0.1 *N* sulfuric acid. The absorption of the solution was taken at 250 mμ, since at this wavelength and the acid concentration (0.1 *N*) the AMP and IMP have approximately the same molecular extinction coefficient (Pabst Laboratories, 1956). Apparent IMP was then calculated from a standardized curve prepared by dissolving IMP in 0.1 *N* H_2SO_4. All absorption measurements were made with a Ziess spectrophotometer.

Measurement of AMP and Actual IMP. The amount of adenosine nucleotides in the column eluate was accurately determined by the method described by Davis and Morris (1963). The amount of actual IMP was calculated by subtracting the number of moles of adenine found from the moles of apparent IMP.

Measurement of Total Nucleosides and Bases (Apparent). The first column effluent after the absorption of all the nucleotides was diluted (usually 1:10) with 1 *N* H_2SO_4. The absorption maximum was read at 250 mμ and the value of apparent nucleosides and bases was calculated in terms of equivalent adenine.

Estimation of Hypoxanthine and Ammonia. The extract that had not been neutralized was taken and hypoxanthine content was estimated by the silver salt method as described by Jones *et al.* (1964). Ammonia was estimated on aliquots of the acid extract, before neutralization, by the microdiffusion method of Conway (Conway, 1939; Bendell and Davey, 1957), followed by colorimetric determination of the color as developed with Nessler's reagent, using the blue filter in a Klett-Summersion photoelectric colorimeter.

Determination of Bacterial Load. The 2,3,5-triphenyltetrazolium salt has been used to detect spoilage in fish (Shewan and Liston, 1957; Rao *et al.*, 1956; Kamasastri, 1957). The method described by Moorjani and Iyengar (1957) and Moorjani *et al.* (1957) consists briefly of adding tissue suspension (5 g of abdominal tissue blended with 30 ml of distilled water) to a conical flask containing 0.5 ml of 0.5% 2,3,5-triphenyltetrazolium chloride (TPTZ) and 5 ml of phosphate buffer of neutral pH. After initial shaking of the contents of the flasks, the flasks were incubated at 38 ± 1° for 7 hr and the formazan formed was extracted by shaking with 30 ml of *n*-butyl alcohol; rapid separation of the *n*-butyl alcohol was effected by centrifugation. The color of butanol eluate (diluted if necessary) containing the red formazan was read in a Klett-Summersion photoelectric colorimeter using the green filter. The quantity of the dye reduced was read off from a calibration curve obtained by plotting the Klett reading against known concentrations of formazan prepared according to Fairbridge *et al.* (1951).

RESULTS AND DISCUSSION

Though emphasis has been placed in the present work on IMP, AMP, nucleosides and bases, ammonia, and bacterial load, organoleptic assessment, which still is the best quality evaluation method in spite of its limitations, has been used to determine the acceptable shelf-life of treated and untreated fish. The quality of the preserved fish was evaluated from the standpoint of the morphological appearance of the fish and by the organoleptic taste on a ten-point scale, using a score of 5 as the limit of acceptability. Fish thus preserved by procedures a, b, d, and f can be stored with a score of 5 for 10–15 days at room temperature and for about 1 month at 4–6°. In most of the cases of immersion, the head of the fish had been spoiled before the body started deterioration. Moreover, during immersion of the fish in ammonia solution the normal texture of the fish body was greatly affected due to excessive softening of the flesh.

In process c at 28–30° and e at 4–6°, the fish was scored on a ten-point scale from 8 to 5 up to 32 days and from 8 to 6 up to 83 days. The borderline of acceptability in the control at 4–6° and in the iced fish is reached in 7 and 13 days, respectively, while the control at 28–30° is far below the level within 24 hr. In the process at 4–6°, the well-acceptable condition of fish tested by the organoleptic taste up to 83 days would suggest that a much longer storage life may be expected.

The changes in the concentration of IMP, AMP, nucleosides and bases, hypoxanthine, and ammonia and in the micrograms of formazan formed on treatment with 2,3,5-triphenyltetrazolium salt as an index of the bacterial load during preservation of the fish (Mrigal) treated with ammonia at 4–6° (temperature of the cold room) and 28–30° (ambient temperature) and of the untreated fish (Mrigal) at 0 (iced), 4–6, and 28–30° are shown in Tables I–V.

Table I shows that the inosine 5-monophosphate of the control fish (4–6°) is reduced significantly on the sixth day of storage and remains more or less the same on the seventh day. Within 24 hr after the sixth day the freshness of fish is found to be destroyed with the increase of hypoxanthine, ammonia, and a heavy bacterial load. There is a continuous degradation of IMP and AMP as evident from the results shown in Table I and a continuous increase in concentration of apparent nucleosides and bases.

Table II. Chemical Changes of Iced Fish (Mrigal Weighing 100 g) in Boxes Kept in a Cold Room Maintained at 4–6°

Storage, days	μmol/g of App. IMP	AMP	IMP	App. nucleosides + bases at 250 mμ	Hypoxanthine	Ammonia, mg/g	Bacterial load in terms of formazan formed, μg/g
0	1.47	0.046	1.42	1.89	0.470	0.099	84
4	1.05	0.039	1.01	1.99	0.470	0.108	80
10	0.96	0.034	0.93	2.06	0.544	0.108	85
13	0.84	0.030	0.81	2.31	0.794	0.121	89
18	0.45	0.025	0.42	2.63	1.000	0.162	128

Table III. Chemical Changes Occurring during Preservation of Fish (Mrigal Weighing 100 g) with Ammonia as a Source of Vapor at 4–6°

Storage, days	μmol/g of App. IMP	AMP	IMP	App. nucleosides + bases at 250 mμ	Hypoxanthine	Ammonia, mg/g	Bacterial load in terms of formazan formed, μg/g
0	1.47	0.046	1.42	1.89	0.470	0.099	84
6	0.75	0.045	0.71	2.38	0.485	3.15	Nil
10	0.75	0.045	0.71	2.41	0.500	3.15	Nil
16	0.72	0.033	0.69	2.52	0.500	3.06	Nil
21	0.72	0.029	0.69	2.50	0.492	2.84	Nil
27	0.66	0.025	0.63	2.55	0.485	2.84	Nil
36	0.45	0.022	0.43	2.62	0.485	2.58	Nil
43	0.39	0.019	0.37	2.73	0.500	2.40	Nil
83	0.15	0.005	0.14	2.90	0.500	1.58	Nil

Table IV. Chemical Changes Occurring in Fish (Mrigal Weighing 100 g) during Preservation without any Treatment at 28–30°

Storage, days	μmol/g of App. IMP	AMP	IMP	App. nucleosides + bases at 250 mμ	Hypoxanthine	Ammonia, mg/g	Bacterial load in terms of formazan formed, μg/g
0	1.47	0.046	1.42	1.89	0.470	0.099	84
1	0.18	0.034	0.15	3.26	5.147	0.162	570

Table V. Chemical Changes Occurring in Fish (Mrigal Weighing 100 g) during Preservation with Ammonia at 28–30°

Storage, days	μmol/g of App. IMP	AMP	IMP	App. nucleosides + bases at 250 mμ	Hypoxanthine	Ammonia, mg/g	Bacterial load in terms of formazan formed, μg/g
0	1.47	0.046	1.42	1.89	0.470	0.099	84
4	0.57	0.039	0.53	2.62	0.470	5.28	Nil
7	0.57	0.039	0.53	2.62	0.485	5.52	Nil
15	0.42	0.037	0.38	2.80	0.500	5.52	Nil
22	0.33	0.037	0.29	2.92	0.515	5.60	Nil
28	0.24	0.034	0.21	3.11	0.868	5.52	Nil
32	0.21	0.021	0.19	3.11	0.956	5.52	Nil

Table II indicates that iced fish up to the tenth day are good from the quality index of hypoxanthine and bacterial load. After 10 days, enzymatic spoilage is found to begin with the increase of hypoxanthine, ammonia, and bacterial load. From the very beginning of the storage there is a continuous increase in content of apparent nucleosides and bases and a decrease in concentration of AMP and IMP.

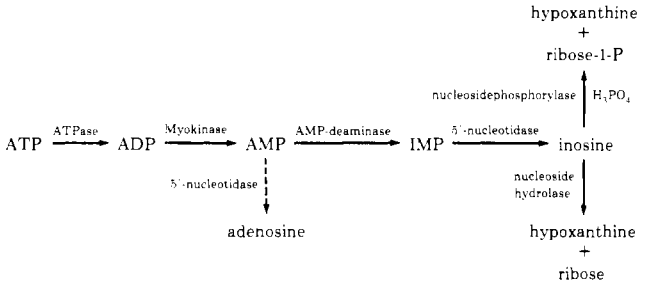
Table III shows that the IMP content of ammonia preserved fish at 4–6° is reduced to half that of the control fish after 6 days of treatment, remains constant for 27 days, and then gradually decreases at a slow rate while there is continuous degradation of AMP at a very slow rate from the beginning of the storage. There is no increase of hypoxanthine and ammonia even up to 83 days. It is interesting to note that the bacterial load is lower than that of the control fresh fish due to the preservative action of ammonia. At the beginning of the storage the ammonia content of fish muscle is suddenly increased to a high value due to the absorption of ammonia in muscle and is found to decrease gradually with the days of preservation passed. The increase in the concentration of appar-

ent nucleosides and bases appears to be closely related to the degradation of IMP and AMP as evidenced from the results of Table III.

From Tables IV and V it is found that treated fish at 28–30° can be kept in an edible condition up to 22 days, whereas the control fish at that temperature is greatly spoiled within 1 day to a nonedible condition with the bitter off-flavor and high bacterial load. In spraying ammonia, absorption is more than that of preservation in ammonia vapor at 4–6°. After 22 days its hypoxanthine content begins to increase, but there is no bacterial load up to 32 days of preservation as evidenced by the TPTZ-test value. The increase in the content of apparent nucleosides and bases is related to the decrease in concentration of IMP and AMP of fish muscle as evidenced from the results of Table V, which are similar to those of Table III. In process c at 28–30°, the preserved fish has pH 9.5–10.0 with an ammonia content of 5.52 mg/g of fish, whereas fish flour made from the preserved fish after washing according to the method described by Ismail *et al.* (1968) contains trace amount of ammonia with pH 7.0.

Analytical studies by different groups of workers (Jones and Murray, 1957, 1960, 1961a,b, 1962; Creelman and Temlinson, 1960; Kassemsarn *et al.*, 1963) on nucleotides and bases suggest a schematic course of the major pathways of ATP degradation in fish muscle as shown in Scheme I (where the broken line indicates a route of minor importance).

Scheme I. Pathways of Degradation of ATP in Fish Muscle



In our present paper it is noted that during preservation with ammonia, AMP and IMP gradually decrease and the absorption maximum at 250 mμ for nucleosides and bases increases with the days passed. It is also interesting to note that there is no increase of hypoxanthine and ammonia, and that bacterial load is lowered more than that of the control. From these data it may be assumed that ammonia inhibits the enzymatic systems involved in the degradation pathway for the conversion of inosine to hypoxanthine. From the evidence of the *in vitro* studies by Embden (1927) that ammonia arises from the deamination of AMP to IMP in fish muscle, we can also assume from our analytical data for ammonia that ammonia preservative also retards the deamination of AMP to IMP.

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LITERATURE CITED

Bendell, J. R., Davey, C. L., *Biochim. Biophys. Acta* **26,** 93 (1957).
Bhadra, R., Chaudhuri, D. R., Bose, A. N., "Radiation Preservation of Food," International Atomic Energy Agency, Vienna, 1973.
Conway, E. J., "Micro-diffusion Analysis & Volumetric Error," Crosby, Lockwood and Sons Ltd., London, 1939.
Creelman, V. M., Temlinson, N., *J. Fish. Res. Bd. Can.* **17,** 449 (1960).
Davis, J. R., Morris, R. N., *Anal. Biochem.* **5,** 64 (1963).
Embden, G., *Klin. Wochenschr.* **6,** 628 (1927).
Fairbridge, A., Willis, K. J., Booth, R. G., *Biochem. J.* **49,** 423 (1951).
Ismail, P. K., Madhavan, P., Pillai, V. K., *Fish Technol.* **5,** 53 (1968).
Jones, N. R., *New Sci.* **7,** 783 (1960).
Jones, N. R., "Proceedings of the Flavor Chemistry Symposium," Campbell Soup Co., Camden, N. J., 1961, pp 61–81.
Jones, N. R., Murray, J., *Biochem. J.* **66,** 5 (1957).
Jones, N. R., Murray, J., *Biochem. J.* **77,** 567 (1960).
Jones, N. R., Murray, J., *Z. Vergl. Physiol.* **44,** 174 (1961a).
Jones, N. R., Murray, J., *Biochem. J.* **80,** 26 (1961b).
Jones, N. R., Murray, J., *J. Sci. Food Agr.* **13,** 475 (1962).
Jones, N. R., Murray, J., Livingston, E. I., Murray, C. K., *J. Sci. Food Agr.* **15,** 763 (1964).
Kamasastri, P. V., *Curr. Sci.* **26,** 214 (1957).
Kassemsarn, B. O., Perez, B. S., Murray, J., Jones, N. R., *J. Food Sci.* **28,** 28 (1963).
Mitchell, F. W., Grace, W. R., and Co., U. S. Patent 3,442,661 (1969).
Moorjani, M. N., Iyengar, J. R., *Food Sci.* **6,** 203 (1957).
Moorjani, M. N., Iyengar, J. R., Bhatia, D. S., Subrahmanyan, V., *Food Sci.* **6,** 275 (1957).
Pabst Laboratories, Circular 02-10 (1956).
Rao, S. V. S., Nayer, M. R., Valsan, A. P., *Curr. Sci.* **25,** 396 (1956).
Saito, T., *Kagaku (Kyoto)* **15,** 101 (1960).
Shewan, J. M., Jones, N. R., *J. Sci. Food Agr.* **8,** 491 (1957).
Shewan, J. M., Liston, J., *J. Sci. Food Agr.* **8,** 222 (1957).
Spinelli, J., Kemp, B., *J. Agr. Food Chem.* **14,** 176 (1966).
Subrahmanyan, V., Lahiry, N. L., Moorjani, M. N., Nair, R. B., Krishnaswami, M. A., *Science* **142,** 233 (1963).
Wood, T., *J. Sci. Food Agr.* **12,** 61 (1961).

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