Biochemical Basis of Postmortem Nucleotide Catabolism in Cod (*Gadus morhua*) and Its Relationship to Spoilage

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The biochemical events associated with the postmortem breakdown of ATP-related compounds were investigated in Atlantic cod (*Gadus morhua*). The hydrolysis of inosine and formation of hypoxanthine were found to result from both autolytic and bacterial enzymes. Both *Pseudomonas spp.* and *Proteus spp.* were responsible for the production of intracellular inosine nucleosidase. Hypoxanthine production was more pronounced in fillets than in gutted whole fish. Hydrolysis of inosine in sterile fillets was also more rapid when compared to nonsterile intact fish. Nucleotide catabolite data suggested that any single compound could not be considered a reliable index of quality.

Fresh fish generally deteriorate by one of two mechanisms: bacterial spoilage and autolysis. Autolytic degradation occurs as the result of enzymatic changes within the muscle. Enzyme decompartmentalization or the loss of respiratory capability necessary for homeostasis are factors promoting autolysis. Nucleotide degradation in postmortem fish muscle has been examined by a number of groups and has been well reviewed by Hiltz et al. (1972), Watts and Watts (1974), and Martin et al. (1978). In a live fish, adenosine diphosphate (ADP) is produced in muscle contraction and can be rephosphorylated either by respiratory action or by phosphocreatine; therefore, the adenosine monophosphate (AMP) level remains low (Watts and Watts, 1974). After death, inosine monophosphate (IMP) accumulates via dephosphorylation and deamination of adenosine triphosphate (ATP) (Figure 1). This reaction usually goes to completion within 1 day and is believed to be totally autolytic (Jones, 1965; Hiltz et al., 1972). Jones and Murray (1964) suggested that the subsequent production of inosine (Ino) was rate-limiting in the ultimate formation of hypoxanthine (Hx), xanthine, and uric acid.

Several suggestions have been made for the utilization of nucleotide catabolites for the measurement of seafood quality. Although the extent of purine catabolism is obviously an indication of time after death, the compounds themselves may contribue to flavor (off-flavor) development. Inosine monophosphate (IMP) acts as a flavor enhancer while Hx is bitter (Kassemsarn et al., 1963). The use of a single catabolite as a freshness indicator has not been successful because of the many factors involved in purine decomposition and since the rates of breakdown of the various intermediates differ from one species to another (Dingle and Hines, 1971). Even though rapid methods for the determination of Hx have been developed in England (Burt et al., 1979) and the United States (Jahns et al., 1976), they have not received wide acceptance due to the considerable autolytic "lag" observed by various authors and the apparent disappearance of Hx as a result of bacterial action. For these reasons, the concept of "K" freshness factors was introduced by Saito et al. (1959) and later a Ki index by Karube et al. (1984), where

$$\mathrm{Ki} = \frac{\mathrm{Hx} + \mathrm{Ino}}{\mathrm{IMP} + \mathrm{Ino} + \mathrm{Hx}} \times 100$$

The objective of the present project was to study the enzymatic changes involved in the postmortem breakdown of nucleotides in cod and, in particular, the properties of nucleoside phosphorylase (NP) (EC 2.4.2.1) and inosine nucleosidase (IN) (EC 3.2.2.2) with regard to spoilage.

MATERIALS AND METHODS

In a previous study (Gill, 1983), four methods of chromatographic analysis of nucleotides in decomposing fish were compared. The method described here was selected as the best. Three types of fish (*Gadus morhua*) were obtained for the study: aquarium fish, which were immediately bled, gutted, placed on ice, and held at 2–5 °C; commercial headless, gutted fish obtained from a local fish market; commercial fillets obtained from a local supermarket.

Fish samples were prepared by blending a 50-g sample of skinless fillet in two volumes of 6% perchloric acid for two, 1-min intervals in a Waring Blendor at full speed. The extracts were filtered through Whatman No. 1 filter paper and stored in sealed plastic containers at −30 °C or analyzed immediately. Just before analysis, samples of the extracts were neutralized to pH 7.0 with a measured volume of 30% KOH to precipitate potassium perchlorate. After precipitation, a sample was filtered through a 0.22-μm Millipore filter.

Studies of sterile cod muscle were performed by aseptic removal of tissue from freshly killed fish and subsequent storage of samples in Petri dishes at 3 °C. Samples were taken as needed for analysis. Prior to acid extraction of samples, sterility was verified by stomaching tissue in a Stomacher Lab Blender 400 (A. J. Seward, Edmunds, U.K.) using 1% peptone water and plating on standard methods agar containing 0.5% NaCl. Plates were incubated at 21 °C for mesophilic counts and at 5 °C for psychrophiles. Samples showing any growth were discarded. The acid extractions were performed as above except that 10-g samples were used.

Chromatography was carried out on a Waters HPLC system equipped with a Model 720 controller, two Model 6000 A pumps, and a Model 450 variable-wavelength detector set at 254 nm. Determinations of AMP, IMP, Ino, and Hx were accomplished on a Waters μBondapak, C-18 radial compression column using pH 4.5, 0.01 M potassium phosphate buffer pumped at 2 mL/min. Run time between injections was 20 min. External calibration was used with standards obtained from Sigma.

The HPLC analysis of the sterile and nonsterile muscle tissue extracts was carried out as above except that a 0.05 M, pH 7.0 potassium phosphate buffer was used. These conditions resulted in better separations of the compounds involved.

Activity assays for nucleoside phosphorylase were performed as described by Boehringer Mannheim (1975). Activity assays for inosine nucleosidase were performed

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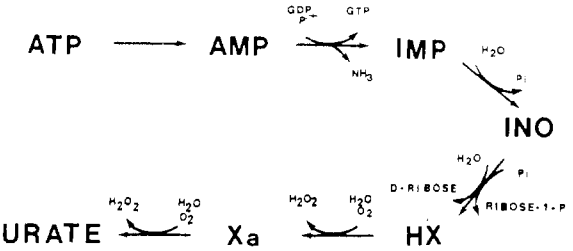

Figure 1. Sequence of postmortem nucleotide catabolism in fish muscle tissue: ATP = adenosine triphosphate; AMP = adenosine monophosphate; IMP = inosine monophosphate; INO = inosine; Hx = hypoxanthine; Xa = xanthine.

on bacterial cultures, cell-free extracts, and muscle tissue. The reaction mixtures contained 3.0 mL of 0.05 M veronal or TES buffer, 0.10 mL of 7.3 mM inosine solution, and 0.01 mL of a xanthine oxidase solution (Boehringer Mannheim) containing 20 units/mL. Reactions were initiated by the addition 0.10 mL of crude inosine nucleosidase. Activity was measured as the increase in absorbance at 293 nm and 22 °C. Protein concentrations of enzyme preparations were determined by the Lowry et al. (1951) method.

Preparation of crude inosine nucleosidase extracts from cod tissue using Tarr's (1955) method was carried out on fresh fish purchased at a local market or aquarium fish sacrificed immediately before enzyme extraction. This method involved blending 250 g of muscle tissue with 3 volumes of 0.6 M NaCl for 2 min. The solution was adjusted to pH 4.6 and spun in a bench top centrifuge at full speed for 1 min. The upper layer containing tissue was removed, followed by an additional 2 min of centrifugation. Following filtration through Whatman No. 1 paper, the supernate was adjusted to pH 7.0, and ammonium sulfate was added up to 40% saturation. This solution was stirred for 1 h and then centrifuged at 19000*g* for 10 min. The resulting supernate was removed and brought to 60% saturation with ammonium sulfate. This was followed by stirring and centrifugation as above. The pellet was resuspended in double-distilled water and concentrated by ultrafiltration (Amicon PM 10 filter). Furthermore, ammonium sulfate fractions of the filtered supernate were taken at 0–20, 20–30, 30–40, 40–50, and 50–60% saturation, and the IN activity was monitored at each step. Crude extracts were also prepared by blending cod tissue in three volumes of 0.05 M veronal buffer, pH 8.6. Extracts were centrifuged in the cold (5 °C) at 25000*g* for 45 min, and the supernate was subjected to ultrafiltration through a series of membrane filters (Amicon) with exclusion limits of 10 000, 50 000, and 100 000 Da.

Preparation of NP and IN was carried out from mixed and pure bacterial cultures obtained from spoiling fish. Mixed cultures were grown in either trypticase soy or brain heart infusion broth for 3 days. Cells were harvested by centrifugation. Cell-free extracts were prepared by ultrasonic disruption for seven 2-min intervals at the maximum setting (Biosonik III, Bronwill Scientific, Rochester, NY). Crude enzyme extracts were also prepared from individual colonies selected at random from trypticase soy agar plates and identified with the api 20E enterobacteriaceae identification kit (api Analytab Products, Plainview, NY). Broth cultures prepared from individual colonies isolated from spoiled cod were harvested and disrupted when at the peak of the exponential growth phase as measured by absorbance readings at 660 nm. Clear cell-free extracts were prepared by centrifugation of the disrupted cell suspensions at 10000*g* for 45 min. Extracts were stored at −80 °C until use.

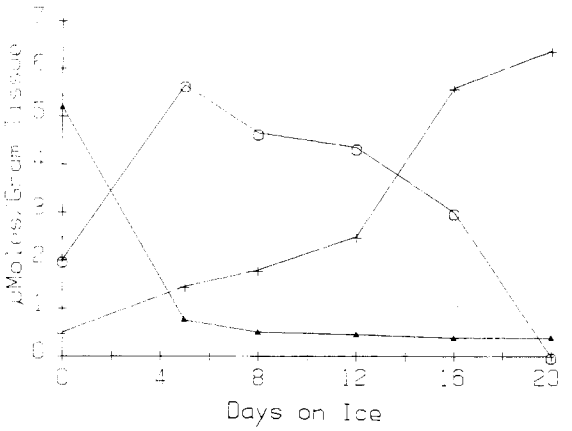

Figure 2. Changes in IMP (▲), inosine (○), and hypoxanthine (+) in commercial gutted whole code stored on ice.

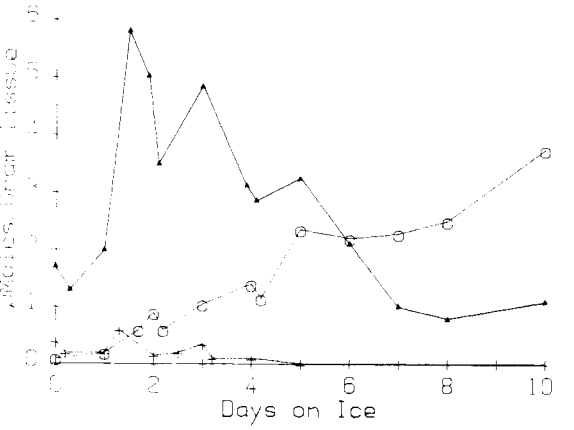

Figure 3. Change in IMP (▲), inosine (○), and hypoxanthine (+) in an aquarium cod fish that was gutted, bled, and stored on ice.

RESULTS AND DISCUSSION

Figure 2 illustrates the changes in I[illegible], and Hx in commercial fish stored on ice. Note th[illegible]vels of IMP and Ino are about equal at day 2. T[illegible]h were purchased as fresh, gutted whole cod on day 0. Figure 3 illustrates the changes in the same three compounds in fish procured from an aquarium. These fish were bled, gutted, and chilled immediately upon death (day 0), and the curves for IMP and Ino did not intersect until day 6. One explanation for this apparent discrepancy could be the effect of handling. Since both IMP and Ino levels evidently rise and fall during iced storage, it is not likely that one single compound could be used for the evaluation of freshness. Although hypoxanthine has been reported to be a good indicator of freshness (Jahns et al., 1976), the present cod data do not agree with this suggestion. Hypoxanthine would have perhaps been useful in predicting shelf life for fish in Figure 2 but of little use for the evaluation of the quality of the fish in Figure 3.

Efforts were made to isolate IN from cod tissue. Attempts to isolate IN by the method of Tarr (1955) were fraught with difficulty. Little or no IN or NP activity was measured in crude extracts of fresh cod nor in ammonium sulfate fractions ranging from 0 to 60% saturation. In fact, the levels of IN in the present study of crude extracts were much lower (1000× less) than the activity reported by Tarr for Pacific lingcod. A crude extract centrifuged at 25000*g* and subsequently passed through a series of membrane filters exhibited no significant purification.

Measurable levels of IN were detected in commercial cod fillets (1.5×10^{-3} μmol/min), suggesting that perhaps the production of Hx in stored cod may be due to bacterial IN.

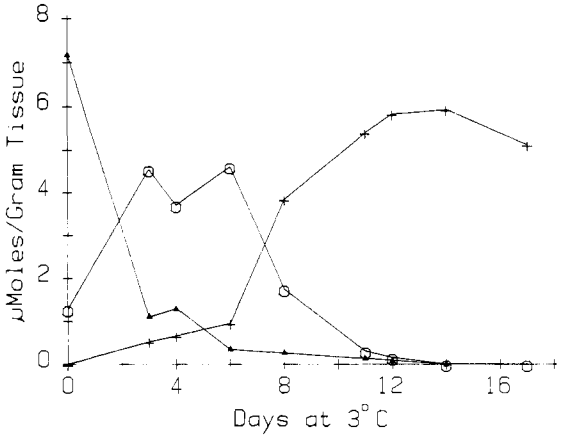

Figure 4. Changes in IMP (▲), inosine (○), and hypoxanthine (+) in sterile cod fillet stored at 3 °C.

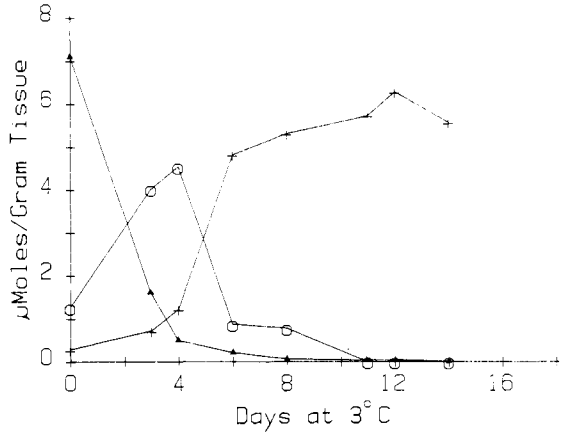

Figure 5. Changes in IMP (▲), inosine (○), and hypoxanthine (+) in nonsterile cod fillet stored at 3 °C.

Individual bacterial colonies isolated from spoiled tissue were cultured in trypticase soy broth, harvested at the peak of exponential growth, ultrasonically disrupted, centrifuged, and tested for activity. No IN activity was detected prior to cell disruption, suggesting that IN is produced primarily within the cells of spoilage bacteria. Seven cultures were screened for IN activity, with only *Proteus vulgaris* and *Pseudomonas fluorescens* containing any measurable activity (0.03 and 0.015 μmol/min per mg of protein, respectively).

In order to investigate the hypothesis that rates of nucleotide catabolism were partly attributable to bacterial enzyme action, paired samples of sterile and nonsterile tissue were removed from individual fish and incubated in Petri dishes at 3 °C. The paired samples were taken from the same fish in order to negate any differences in enzyme activity that may exist from one individual to another. Sterility was verified prior to acid extraction with standard plate counting procedures.

The changes in IMP, Ino, and Hx in the sterile and nonsterile samples are shown in Figures 4 and 5, respectively. The rate of IMP degradation was similar in both sterile and nonsterile samples taken from the same fish. One explanation may be that one would not expect high numbers of spoilage bacteria to be present prior to day 4 and therefore the biochemical changes encountered during the first few days postmortem are primarily due to autolysis. Beyond day 4, however, the disappearance of inosine coupled with the accumulation of Hx is accelerated in the nonsterile samples.

The data suggest that bacterial IN is responsible for the premature accumulation of Hx on day 4. The pattern of autolytic production of Hx in sterile tissue was similar to nonsterile tissue except that the process was delayed by approximately 2 days at 3 °C.

Notable were the differences in rates of nucleotide catabolism observed between intact aquarium fish that were gutted and stored whole and sterile fillet tissue prepared from asceptically removed fillets. One explanation for the accelerated rate of decomposition in the latter may be a result of mechanical damage to the tissue during the filleting operation. Enzyme decompartmentalization of both 5′-nucleotidase and IN could be a factor in the increased rate of spoilage observed in the commercial fish (Figure 2) over the aquarium fish (Figure 3).

This study presents results supporting the use of nucleotide catabolites as an index of quality in Atlantic cod. Evidence suggests that the role of nucleotide degradation is affected by both spoilage bacteria and mechanical handling of the fish. The inability to detect any significant IN or NP activity in extracts from fresh cod muscle tissue seemed to suggest that Hx accumulation may be due to bacterial enzymes rather than by autolytic mechanisms. However, in the absence of bacteria, nucleotide degradation leading to Hx accumulation was shown to proceed to completion in Atlantic cod fillets. Nevertheless, the rate of inosine degradation to Hx is increased in cod fillets in the presence of spoilage bacteria, indicating that bacterial enzymes do contribute to the degradative process.

Although it is difficult to account for the autolytic accumulation of Hx based on the low levels of activity observed for IN and NP in the extracts, as suggested by Dingle and Hines (1971), it is not always appropriate to draw conclusions about enzyme activities in tissue based on results obtained from extracts. The differences in the rates of IMP, inosine, and Hx accumulation and degradation observed in aquarium cod, commercial whole cod, and sterile fillets indicate that, in addition to bacterial load, the handling and subsequent mechanical damage to muscle tissue may accelerate both IMP and inosine degradation due to the enzyme decompartmentalization, thus rendering their substrates more accessible.

The results presented here clarify the respective contributions of autolytic and bacterial enzymes in nucleotide catabolism of postmortem cod muscle tissue. Also, the apparent relationship between rate of catabolite degradation and both bacterial spoilage and mechanical damage support the use of nucleotide catabolites in freshness evaluation of Atlantic cod. Future work will emphasize the development and testing of a diagnostic test kit for the measurement of IMP, Ino, and Hx using immobilized enzymes.

Registry No. ATP, 56-65-5; Ino, 58-63-9; Hx, 68-94-0; NP, 9030-21-1; IN, 9030-95-9.

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Demethylation and Conjugation of Formononetin and Daidzein in Sheep and Cow Liver Microsomes

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Cattle are considered to be less susceptible than sheep to plant estrogens. Therefore, this study was designed to evaluate possible differences in microsomal metabolism of formononetin and daidzein, two isoflavones responsible for estrogenic symptoms in ruminants. The demethylation of formononetin to daidzein was very low in both species. Conjugation, the major mechanism, showed only a slight difference in activity between cow and sheep. About 20% formononetin and 40% daidzein were removed by glucuronidation in cow liver compared to 30 and 50% in sheep liver. When the conjugation enzyme UDP-glucuronosyltransferase was activated, no significant differences occurred. However, the conjugation was augmented, and the ultimate activity in cow and sheep liver rose to the same final level. In addition, NADPH-cytochrome *c* reductase, 7-ethoxycoumarin deethylase, and α-naphthol UDP-glucuronosyltransferase were about 2–6 times higher in sheep liver compared to cow liver. With the two isoflavones, however, the differences in capacity of the liver to metabolize these substances are not sufficient to explain the observed differences in estrogen susceptibility between cow and sheep.

Plant estrogens are substances found in certain plants. Their effect is similar to that of estrogen hormones. The most common types are the isoflavones formononetin, daidzein, genistein, and biochanin A. Another group possessing estrogenic activity is the coumestans, the best known compound in this series being coumestrol.

High levels of the estrogenic isoflavones formononetin and biochanin A have been found in certain cultivators of subterranean (*Trifolium subterraneum*) and red clover (*Trifolium pratense*) (Shutt et al., 1967, 1968, 1970; Francis et al., 1967). Many reports, especially from Australia, have described reproductive abnormalities in ewes associated with the content of plant estrogens in clover pasture [see for example Bennets et al. (1946), Lightfoot (1974), and Adams and Nairn (1983)]. Infertility can occur in animals grazing estrogenic clover or given clover silage during the mating season (Morley et al., 1966; Thomson, 1975). A return to normal fertility usually occurs after the ewes revert to nonestrogenic pasturage (Morley et al., 1966). A longer pasture period can even result in permanent infertility (Turnbull et al., 1966). Formononetin is the major plant constituent responsible for reproductive dysfunction in sheep (Millington et al., 1964).

In contrast to the many reports of the deleterious effects of plant estrogens on sheep, their effect on cattle has not been extensively studied. It has been reported, however, that reproductive disturbances can occur in cattle grazing on estrogenic clover (Kallela et al., 1968, 1984; Adler and Trainin, 1960) and other herbage (Rankin, 1963; Thain, 1965). The influence of these plant estrogens has been shown to cause a high frequency of ovarian cysts (Kallela et al., 1984; Adler and Trainin, 1960; Thain, 1965) and increased uterine weight in ovariectomized heifers grazing on red clover (Kallela, 1968). Even abortions in late gestation have been reported and suggested to be associated with estrogenic pasturage (Rankin, 1963). However, it has been found that the effect of plant estrogens on cattle is generally weaker than on sheep (Lightfoot, 1974).

The metabolism of formononetin and biochanin A is qualitatively similar in sheep and cattle (Figure 1), but the circulating isoflavones and their metabolites seem to be more efficiently conjugated in cattle (Braden et al., 1971). It has been suggested that the differences in degradation and conjugation rate may explain the apparently lower susceptibility to plant estrogens of cattle vis-à-vis sheep (Braden et al., 1971).

Relatively high concentrations of isoflavones have been detected in Swedish pasturage (Pettersson and Kiessling, 1984). Silage of red clover has also been more frequently

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