MICROBIOLOGICAL DETERIORATION OF FISH POST MORTEM, ITS DETECTION AND CONTROL

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It was recognized over four decades ago by Bruns (1), and has since been reaffirmed repeatedly by other investigators, that the flesh and internal organs of healthy freshly caught fish are sterile bacteriologically. In contrast, the external slime and the digestive tracts of feeding fish support a qualitatively and quantitatively variable flora, while the digestive tracts of fasting fish are frequently free from viable bacteria (2, 3).

It is evident from certain reviews that in the past almost invariably there has been full agreement among different investigators regarding the bacterial genera which are usually associated with, and are responsible for, fish spoilage (4, 5, 6, 7, 7a). There would seem to be a general similarity between bacteria occurring in fish and those of their marine environment (8). The organisms which are encountered most frequently on external surfaces of fish are those commonly occurring in water, air and soil. Bacteria of the *Achromobacter*, *Pseudomonas*, *Flavobacterium* and *Micrococcus* genera usually predominate, while members of the *Sarcina*, *Proteus* and *Bacillus* genera occur less often. However, recent work has indicated that hitherto many marine bacteria which cause fish spoilage have been improperly classified. Thus many of the organisms classified as *Achromobacter* by investigators prior to 1948 would now be automatically recognized as species of *Pseudomonas* (9, 10). More important still is the recent finding, in three widely separated laboratories, that a large proportion of bacteria of marine origin which are associated with spoiling fish are members of the genera *Corynebacterium* and *Mycoplana* (R. A. MacLeod, private communication; 10a, 10b). In the digestive tracts of fish, beside the above genera, *Clostridium*, including *C. botulinum* (11, 12) *C. tetani*, *C. sporogenes*, *C. capitovalis* (13), and certain unidentified species occur (2, 14). Organisms of the colon-aerogenes group appear to occur only in fish taken from contaminated waters and are not usually associated with the normal intestinal flora (15, 16). Pigment producing bacteria have been isolated from marine sources quite frequently and are occasionally instrumental in occasioning objectionable discolorations of chilled fish (16a).

The generic distribution of the most commonly occurring fish spoilage bacteria found on freshly caught fish as reported by different investigators has been extremely variable (4, 5, 17). In spite of this the existence of a definite "generic succession" during bacterial spoilage has been proposed (3, 18): initially there appears to be a preponderance of micrococci and flavobacteria which, as spoilage progresses, are overgrown by the more strongly proteolytic and putrefactive *Pseudomonas* or *Achromobacter* species. However, a more recent finding has indicated that this generic succession may apply to teleost fishes but not to elasmobranchs, and the suggestion that metabiosis is involved has been made (10b). Whether the same generic succession occurs in fish stored under different temperature conditions has not been made clear.

BIOCHEMICAL ACTIVITIES OF MICROORGANISMS IN SPOILING FISH

The development of microorganisms during the storage of fresh fish is accompanied by decomposition of the muscle carbohydrates, proteins, and lipids; and because of variations in the composition of the muscle of different species and the complex nature of the bacterial populations involved, no consistent degradative pattern can be expected. There is, indeed, a paucity of information relative to the chemical changes which occur during bacterial spoilage of fish, and much of that which is available has accumulated as a result of investigations in which attempts have been made to evolve chemical tests for following its bacterial deterioration.

Fish muscle is composed largely of proteins of the globulin and albumin classes (actomyosin, myogen, myoalbumin and globulin X) which are excellent sources of essential and other amino acids (18a). The internal organs and the muscle to a lesser extent contain all of the known vita-

mins of the "B complex". Fish muscle tissue, exclusive of lipid material, contains about 80 per cent water and soluble nitrogenous constituents such as free amino acids, carnosine, anserine, creatine, trimethylamine oxide, amines and, in elasmobranch fishes, quite large amounts of urea (19). Fish tissues, therefore, provide an ideal nitrogenous substrate for the growth of the proteolytic and putrefactive organisms which are normally associated with them. It appears that, under normal conditions, autolysis occurs to a limited or negligible extent in sterile fish flesh or press juice prepared from it, bacterial action being responsible for all, or nearly all, of the breakdown of proteins, nitrogenous extractives or fats. Unfortunately, no information appears to be available concerning the actual course of bacterial degradation of fish muscle proteins. Apparently no really severe proteolysis occurs until after the flesh has become organoleptically stale. In the early stages of bacterial spoilage amino nitrogen gradually decreases and ammonia correspondingly increases, presumably through bacterial deamination of amino acids; in the later stages a rise occurs both in amino nitrogen and ammonia (20). Ammonia has been reported in spoiling fish muscle by a number of investigators (21, 22, 23).

Fish muscle contains variable amounts of glycogen, the content depending largely on the amount of struggling which has occurred during capture of the fish (24, 25, 26). Glycogen levels as high as 0.85 per cent have been recorded for fish muscle. Glycogenolysis occurs fairly rapidly *post mortem* at 0 C and in frozen fish is most marked at about −3 C (27). Though there may be some relation between the glycogen content of fish muscle and the lactic acid which is formed from it, it has been pointed out that frequently no such agreement is observed, and the possibility that the latter may arise through degradation of some other carbohydrate has been considered. Attempts to determine total reducing sugars in fish muscle by customary biochemical procedures have not met with much success because of the presence of interfering substances. Both reducing sugars and lactic acid are apparently broken down by bacteria during the early stages of fish spoilage (20). Evidence has been presented which indicates that the lactic acid is oxidized by bacteria in fish muscle with the simultaneous reduction of activated trimethylamine oxide; acetic acid, carbon dioxide and trimethylamine being formed (28). Naturally this exact course of events will not occur in fish which have little or no trimethylamine oxide in their muscle tissue (29, 30, 31); in such cases alternative oxidative routes must be followed. Acetic acid and carbon dioxide appear to be the only volatile acids produced in cod muscle press juice as a result of the action of the mixed bacterial flora on the lactic acid present, and are apparently also formed from some other uncharacterized precursor (32). Washed suspensions of cells of certain fish spoilage bacteria were found to produce lactic acid, acetic acid, formic acid, ethyl alcohol, acetylmethylcarbinol (acetoin) and, in certain instances, carbon dioxide from glucose (33).

It has been shown recently that free D-ribose commonly occurs in fish muscle *post mortem*, quantities in excess of 800 μg per gram being present in some instances. This pentose probably arises from enzymic degradation of the nucleic acid of fish muscle nucleotropomyosin by muscle enzymes (34). Free glucose is also usually present, though in very variable amounts (34a). The occurrence in fish muscle of certain of the known phosphorylated intermediates of carbohydrate metabolism has also been reported (35). No information appears to be available regarding the possible degradation of any of these compounds other than glucose by the natural spoilage organisms of fish flesh.

The lipid content of fish flesh varies from less than 1 to more than 20 per cent according to species, season, age, environment and degree of sexual maturity. Since these fats contain a very high proportion of unsaturated fatty acids, oxidation occurs readily, but in general this appears to be due rather to oxidation catalyzed by haem compounds of the flesh in conjunction with muscle enzymes rather than to bacterial action (103). It is probable that the fatty acid hydro peroxides formed exert a bacteriostatic action (37). Since fish spoilage organisms very often possess lipolytic enzymes, extensive hydrolysis usually occurs in advanced bacterial spoilage with formation of free fatty acids and, probably, other decomposition products. It is possible that fatty acids, such as butyric and isobutyric acids (38), arise through the action of bacteria on the fish fats rather than on muscle carbohydrates.

EFFECT OF PHYSICAL CONDITIONS ON GROWTH OF MICROORGANISMS IN FISH FLESH

Nearly all marine microorganisms and certainly those associated with fish are facultative psychophiles, having a very wide temperature range of growth which, with few exceptions, is from about −7.5 C to 30 C. It was found by one investigator that, of 71 organisms isolated from the waters of the North Pacific Ocean, 10 grew at −7.5 C, 22 at −5 C and 65 at 0 C (39). Experiments with *Pseudomonas fluorescens*, *Flavobacterium deciduosum* and *Bacillus vulgaris* showed that, though growth was most rapid at 20 C to 25 C, the maximum yield of bacterial cells occurred in cultures incubated at −5 C or −3 C (40). The temperature coefficient of growth (Q_{10}) of marine psychophilic bacteria has been shown to be much greater at −4 to 5 C than at higher temperatures (40, 41, 41a). The practical implication of this finding is extremely important, for it has been demonstrated that fish will spoil about twice as fast at 2.5 C as at −1 C (42). This observation was verified recently in studies with both whole dressed fish and fillets when it was found that spoilage proceeded about twice as rapidly at 2.8 C as at −0.3 C (43). One of the best, and probably most neglected, methods of retarding bacterial spoilage of fish is, therefore, to store it at a temperature as close as possible to the freezing point of the muscle (−1.1 C). This can be accomplished conveniently by storing the fish, or shellfish, in circulating sea water maintained at about this temperature by addition of ice (42). Undoubtedly, at least one of the reasons that fish usually spoils more rapidly than meat at normal cool storage temperatures is that the former is richly contaminated with psychophilic bacteria whereas the latter is more likely to be contaminated with a higher proportion of mesophilic bacteria (44).

All available evidence indicates that the muscle of living fish is approximately neutral in reaction and that, after capture, especially if there has been much struggling, the pH falls due to the formation of lactic acid (25, 45, 46). In general, the muscle pH values which have been reported for fish *post mortem* are distinctly higher than those recorded for well-fed cattle *post mortem*. For fish, the pH values most commonly encountered are between pH 6.0 and 6.6, though not infrequently exceptions occur. Thus in halibut pH values of 5.57 (46) and 5.9 (Tarr, unpublished data) have been recorded, and occasionally pH values of slightly over 6.6 are encountered. This initial *post rigor* pH undoubtedly depends largely on such factors as the glycogen content of the muscle of the living fish and the buffering power of the muscle (47). As bacterial spoilage proceeds, the pH rises because of formation of volatile bases such as ammonia and trimethylamine (*vide infra*). However, the rate of pH change differs markedly with different species of fish during storage. With species such as Atlantic cod and haddock the initial *post rigor* pH values are usually between 6.5 and 6.8, and these rise quite rapidly during storage in ice so that values of over 7.0 are usually encountered (48, 49). With many commercial species of Pacific coast fish the initial *post mortem* pH values are considerably lower than 7.0, and extensive bacterial spoilage may occur with only small rises in pH. Thus with species of salmon bacterial growth with attendant spoilage was found to occasion either only slight or no measurable increase in the initial values of about pH 6.2 (50). The usual slightly acid reaction of fish flesh may have an important retarding influence on the rate of bacterial spoilage and on the formation of trimethylamine, especially if it is maintained at about pH 6.0 or below (50, 51, 52). It is probable that the pH values of the flesh of fish have a considerable influence upon the types of organisms which develop and consequently upon the whole course of biochemical changes which occur.

Artificial adjustment of flesh pH with a view to delaying bacterial spoilage has been attempted by several investigators, but this method is usually impractical because with pH values much below 6.0 the muscle proteins are near their isoelectric zone and no longer retain water readily (36, 52). There is no doubt that, as has been pointed out above, a decrease in pH has a valuable deterrent effect on microbiological spoilage (51). This effect is definitely apparent when pH adjustment is employed in conjunction with the use of certain chemical bacteriostats which function best in mildly acidic environment (*vide infra*). Acid resistant organisms such as the micrococci, sarcina, and lactobacilli usually predominate in acidic fish products (53).

The effect of freezing bacteria in various environments on their viability has been investigated from time to time, and the whole subject

has been reviewed (54, 55). It appears to be generally agreed that freezing causes an initial decrease in numbers of viable bacteria, and that the rate of freezing has little influence on this. In stored frozen materials bacteria are killed more rapidly at the higher than at the lower storage temperatures. Some types of organisms appear to be more resistant to freezing than others. Thus in haddock (56) and in mackerel (57) *Achromobacter* species were less resistant than *Flavobacterium* or *Micrococcus*. Though the numbers of viable bacteria in fish flesh are somewhat reduced through freezing, apparently little change occurs in numbers during storage for a year at −18 C (58), and thawed fish spoil about as rapidly as similar fish which have not been frozen (59). From the bacteriological point of view it would probably be preferable to store frozen fish at higher temperatures (*e.g.*, −7 C) at which bacterial mortality is most pronounced. However, the slight beneficial effect which might result would be far outweighed by the marked acceleration of undesirable alterations such as myosin denaturation and oxidative rancidity at these higher temperatures.

The terms drying and dehydration are somewhat loosely employed to distinguish respectively the natural and artificial removal of water from food materials. Much of the value of common salt as a fish preservative (*vide infra*) is undoubtedly associated with its dehydrating effect. Experiments showed that with precooked dehydrated fish stored at 25 C visible mold developed only when the relative humidity was 80% or higher (16.8 to 18.3% water content), but that slight mold development, which was not visible, occurred at 75% relative humidity (13.2 to 13.6% water content). The corresponding critical point at which bacterial growth was liable to occur was about 85% relative humidity (19.0 to 19.7% water content). An increase in both total volatile bases and trimethylamine occurred in samples of dehydrated fish stored under humidity conditions which did not permit an increase in microorganisms. *Micrococcus* species predominated in properly dehydrated samples, while *Pseudomonas* and *Achromobacter* species appeared to be absent (60). In other independent experiments carried out at about the same time (61) it was found that dehydrated fish contained up to 10^5 organisms per gram, most of which were micrococci. A variable proportion of these cocci were considered potentially enterotoxigenic since they were capable of growing at 37 C and gave a positive coagulase test. In both the foregoing studies bacterial contamination was observed throughout the processing, the initial cooked fish being sterile or almost sterile bacteriologically. If the drying procedure was carried out at temperatures much below 50 C, active bacterial growth occurred.

The value of ultraviolet irradiation of fish flesh in reducing bacterial contamination has been studied. One group of investigators observed that a definite decrease in the viable bacterial content of fillets occurred invariably following exposure for two hours to ultraviolet light (62). At about the same time other workers found that a thirty minute treatment with ultraviolet light occasioned a decrease in the viable bacterial population of fillets and, in some instances, an organoleptic improvement following subsequent storage (63). None of the results indicated an outstanding improvement in keeping quality, and it was found by the latter investigators that, because of the poor penetrating qualities of ultraviolet light, a sharp increase in bacterial count occurred after seven hours in fillets which were continually exposed to the rays.

Recent investigations have shown that ionizing radiations, because of their marked penetrating power, have much greater possibilities than have ultraviolet rays for sterilization of fish. Experiments carried out several years ago showed that fish and shell fish may be completely sterilized bacteriologically by an extremely brief exposure to penetrating electrons generated by a specially designed capacitron (64). Other work showed that, after exposure to cathode rays, mackerel fillets remained sterile bacteriologically for 30 days at room temperature, but that oxidative rancidity and some autolysis with an increase in free amino groups did occur (65). The possibility of overcoming certain of these and other undesirable side reactions by irradiation at very low temperatures or *in vacuo* (66), or by the addition of harmless free radical acceptors such as ascorbic acid and closely related compounds (67) is being investigated.

EFFECT OF CHEMICAL AGENTS ON GROWTH OF MICROORGANISMS CAUSING FISH SPOILAGE

Sodium chloride is undoubtedly the most widely applied chemical preservative used on

fish. Its bacteriostatic or bactericidal action is probably due to a number of effects prominent among which are osmotic action and resulting dehydration, the toxic effect of chloride ions, the relative insolubility of oxygen in strong brines and depression of activity of proteolytic enzymes. When salt is used as a fish preservative, factors such as its concentration, rate of penetration, the degree of contamination with salt tolerant organisms and storage temperature have an important influence on its relative effectiveness (68, 69). When the effect of commercial salts on preservation of fish press juice was investigated in detail (70), it was learned that pure sodium chloride was the most effective preservative as judged by depression of bacterial growth and trimethylamine formation, and that the remaining salts studied were progressively less effective depending on their content of magnesium or calcium salts. The difference in the effectiveness of the salts was much more marked at 15.5 C than at 21 C or 25 C. Much of the spoilage of salt fish is occasioned by development of halophilic or halotolerant microorganisms. It is now well recognized that the red discoloration of salted fish is caused by development of salt tolerant members of the *Serratia* and *Sarcina* genera (6, 71, 72). The effect of both environment and of various preservative agents on development of these organisms on salt fish has been studied in detail (71, 73). It was found that growth did not occur below 10 C or 15 C, that the organisms were very resistant to ultraviolet light, and that many grew in a medium saturated with respect to sodium chloride (27%). Though all strains investigated could be adapted to grow in media containing 27% sodium chloride, none would grow when the concentration was less than 6%. It is of particular interest that sodium chloride appeared to be essential for growth of the true red halophilic organisms studied, and that the chlorides of potassium, lithium, magnesium, calcium or barium could not replace it in any of the concentrations studied. With one facultative halophilic organism growth occurred to a variable extent with all the cited chlorides except that of barium, and it was found that they could be arranged in a typical Hofmeister cation series in order of increasing toxicity as follows: Na^+, K^+, Li^+, Mg^{++}, and Ca^{++}.

Another objectionable condition of salted fish which appears to affect its appearance adversely but not its nutritive value is that described as "dun", for which halophilic brown molds of the genus *Sporendonema* are responsible (74, 75). Experiments showed that *Sporendonema epizoum* would not grow in a medium containing less that 5% NaCl, and that growth occurred in saturated NaCl solutions between 5 C and 30 C. and from pH 3.3 to 7.4. As with the halophilic bacteria these molds proved extremely resistant to ultraviolet light. Inhibition of growth in culture media was obtained with propionic and butyric acids in 0.2 or 0.3% concentration, and with n-caproic, n-caprylic, n-capric and lauric acids in 0.1% concentration.

From the practical standpoint, control of fish spoilage which is occasioned by halophilic microorganisms still presents a difficult problem. Sterilization of the salt which was first advocated years ago is said to be impractical (6), and in any event halophilic organisms occur in the fish slime (76). Disinfection of premises in which the fish are prepared with agents such as chlorine, formaldehyde, lye or sulfurous acid and use of salt which is fairly free from halophilic organisms are apparently helpful. Of various fatty acids tested propionic acid in about 0.1 or 0.2% final concentration in the fish flesh proved useful from several standpoints in protecting salted fish from development of red and "dun" discolorations. However, the most effective combination for preventing both these discolorations has been a curing salt containing 3% of sodium acid phosphate plus 0.25% sodium benzoate. Whether or not this rather acid salt mixture caused a more adverse affect than sodium chloride itself in denaturing muscle myosin and in altering palatability was not stated (70).

Several attempts have been made to reduce the rate of bacterial spoilage of fish by the use of substances which may be applied in the gaseous state. Of these substances carbon dioxide, which has proven useful in extending the storage life of chilled meats, has been studied most extensively (77, 78, 79). In all instances storage in high partial pressures of carbon dioxide extended the keeping quality markedly at 0 C or at higher temperatures. Optimum bacteriostatic effects were apparent in an atmosphere which contained about 50% of carbon dioxide. At concentrations below 40% the preservative effect was negligible, while concentrations above 60% were usually no more effective. Unfortunately, storage in carbon

dioxide does affect the external appearance of whole iced fish adversely, and autolysis proceeds and may even be accelerated. The possible effect of carbon dioxide on the myosin protein of fish muscle does not appear to have been investigated, though there has been a suggestion that its denaturation may be accelerated. The soluble coagulable nitrogen of fish muscle stored in 30% carbon dioxide showed about the same slow rate of decrease as was apparent in control samples (80). Storage under anaerobic conditions effected by use of nitrogen did not exert the pronounced improvement in keeping quality noted with fish stored in carbon dioxide (77). The mechanism by which carbon dioxide exerts its bacteriostatic action is not clear, though it is extremely improbable that the lowering of the muscle pH which occurs is entirely responsible. Carbon dioxide does not as yet seem to have been applied commercially to fish (6).

The bacteriostatic effect of several substances other than carbon dioxide when applied in the gaseous state to fish flesh has been investigated (81). It was found that methyl bromide either partially or entirely inhibited growth on agar medium of a number of bacteria or yeasts responsible for fish spoilage, but that exposure to rather high partial pressures of the gas for thirty minutes was required to do this. Similar exposure of fish muscle to the gas did not delay bacterial spoilage during subsequent storage, and it was suggested that this may have been due to absorption of the methyl bromide by the flesh lipids. Both ethylene oxide and propylene oxide retarded bacteriological spoilage of fish muscle markedly, the former being more effective than the latter when applied in similar concentration. Both compounds accelerated development of oxidative rancidity in treated fish and also tended to denature muscle proteins. Methyl ether, ethyl ether, and ethyl chloride were ineffective, and methyl formate, while effective, caused coagulation of the muscle proteins due to dissociation with formation of formic acid.

From time to time a very large and diverse assortment of chemical compounds has been tested on fish products to test their ability to delay bacterial deterioration. Most of these have proven ineffective, adversely affected the appearance or odor of the treated material, or have not complied with pure food regulations (81, 82, 83, 84). Benzoic acid has been used to a limited extent as a preservative for fresh or salted fish, but neither it nor its esters are appreciably effective when the muscle pH is nearly neutral. Its value probably lies more in its ability to retard trimethylamine formation than in its bacteriostatic action (83, 84). Sodium nitrite, first used experimentally as a fish preservative in 1939 (85), has probably been applied more widely than most other preservatives for fish. In initial work it was found that sodium nitrite was effective in retarding bacterial spoilage of several, but not all, samples of fish studied (84). Later this was explained by the fact that the *post rigor* pH of fish flesh varies considerably, and that incorporated sodium nitrite was very effective at pH 6.0, but at higher pH values it became progressively less effective until it possessed no bacteriostatic activity at pH 7.0 (52, 85). After finding that sodium nitrite was a cheap and effective bacteriostat for fish, it became widely employed as a mild preservative especially in the Canadian Maritime Provinces, and its use when added in concentrations not exceeding 200 μg per gram concentration in fresh fish flesh was recently legalized (86). Its rather wide spread use prompted further investigations concerning its bacteriostatic properties and its stability in fish flesh. With certain Atlantic fish, particularly cod and haddock, in which the flesh pH is initially rather high and rises rapidly during spoilage (*vide supra*), sodium nitrite exerted little or no bacteriostatic action but suppressed formation of trimethylamine strongly (87, 88). In certain Pacific coast fish it was found that neither added $NaNO_2$ nor $NaNO_3$ was appreciably reduced after storage of the samples for 15 days at 0 C. However, there was slight reduction of both after 3 days when a storage temperature of 10 C was employed (89). With Atlantic cod fillets stored at 4 or 5 C it was found that both added nitrate and nitrite were reduced steadily with a corresponding increase in pH which was initially about 6.5 (88). The discrepancies in these results are probably explained by differences in bacterial flora and effect of pH, for at pH 6.0 there is little reduction of $NaNO_3$ to $NaNO_2$ by fish spoilage organisms (89). Recent Norwegian work has indicated that sodium nitrite is an effective preservative for herring which are stored prior to being used in manufacture of fish meal (90).

The mechanism of the action of nitrites in

inhibiting growth of fish spoilage organisms has not as yet received a satisfactory explanation but is apparently not due to the inhibition of aerobic respiratory catalysts (52). Reference to literature concerning the mode of action of nitrites in inhibiting certain biological processes has been made in several publications concerned with the action of nitrites in retarding fish spoilage (52, 88, 91). It appears probable that in nitrite solutions of slightly acid pH the amino groups of certain enzymes, or of enzyme activators such as cocarboxylase, combine with the free nitrous acid present by means of a diazo reaction and are thereby inactivated.

It is known that those fish spoilage organisms of the genus *Achromobacter* which reduce trimethylamine oxide to trimethylamine invariably reduce nitrate to nitrite, but that certain species of the genus *Flavobacterium* reduce nitrates but not trimethylamine oxide (28, 92). The bacterial reduction of trimethylamine oxide to trimethylamine has been shown to be due to a specific enzyme (triamineoxide*ase*), which activates only trialkylamine oxides making them susceptible to reduction by various dehydrogenase systems of lower potential level (93, 94). Early attempts to prepare a cell-free triamineoxide*ase* preparation by autolysis, freezing and thawing, and grinding with powdered glass failed (94, Tarr and Ney, unpublished data), but such a preparation has been obtained recently (95) by exposing the bacterial cells to ultrasonic vibrations (550 kc at 0–10 C).

The mechanism by which nitrite inhibits reduction of trimethylamine oxide has not as yet been explained satisfactorily. It seems likely that direct inactivation of triamineoxide*ase* itself is involved, and this could probably be tested with cell-free enzyme preparations. Competition of the triamineoxide*ase* enzyme system with that which activates nitrite (nitritase?) for the various dehydrogenase systems of lower potential level is also possible, particularly since it has been shown that such a competition exists between the triamineoxide*ase* system and nitratase (92).

Hydroxylamine was observed to retard the bacterial spoilage of fish. It was active at pH values both above and below 7.0 and in certain instances was as effective as sodium nitrite when used in one-twentieth the concentration. The possible mechanisms by which it exerts its activity have been reviewed (96). Hydroxylamine appears to have a cumulative toxic effect not possessed by nitrites (97).

During the past few years some interest has been taken in the possibility of preserving foods with antibiotics. Preliminary attempts to preserve fish with antibiotics such as penicillic acid, penicillin and streptomycin were unsuccessful. Further trials were made with the following fifteen antibiotics: penicillin, streptomycin, neomycin, polymyxin B, gramicidin, metholyl gramicidin, subtilin, circulin, bacitracin, terramycin, aureomycin, chloromycetin, rimocidin, tyrothricin and one unnamed antibiotic. The concentrations used varied from 1 to 50 μg per gram (parts per million) of fish. The only antibiotics which exerted a significant bacteriostatic action were aureomycin, terramycin and, to a less marked extent, chloromycetin. Rimocidin inhibited the yeast growth which often occurred in samples containing aureomycin or terramycin in concentrations sufficient to strongly suppress bacterial multiplication. In some experiments marked preservative effects were secured with 0.5 or 1.0 μg per gram of aureomycin, and bacteriostatic effects were observed at 0 and 20 C (98). Experiments showed that aureomycin and terramycin are apparently not destroyed to any appreciable extent by muscle tissues but are, in fact, more stable in them than in aqueous solutions at similar pH values. Marked destruction of both antibiotics occurred at 100 C during heating of tissues containing them (99).

From time to time the possibility of retarding bacterial spoilage of fish by using ices containing incorporated germicidal substances has been considered (44, 83, 100). Few of the compounds used have frozen uniformly through the ice since not many have possessed convenient eutectic concentrations (100, 101), and hardly any have contributed a really significant improvement to the keeping quality of fish treated with such ices. Ices containing added antibiotics have been prepared and studied recently. Ices containing only 1 to 4 μg per gram of aureomycin (101a) and about 0.1% sodium nitrite (101) have proven definitely beneficial in delaying bacterial spoilage of fish. However, ices containing nitrite are not entirely satisfactory, for difficulty has been experienced in obtaining uniform distribution of this salt in ice without use of special manufacturing methods (100), and in obtaining uniform dis-

tribution in fish flesh because of uneven penetration (101, 102).

The smoking of fish serves a dual function, namely to aid in its preservation and to add desirable flavor. The various factors which contribute to the preservative effects of the smoking process have been reviewed (72). Probably the bacteriostatic effects of the salt added during the usual brining procedure and the dehydration which occurs during smoking are at least as important as the germicidal constituents of the smoke itself. Apparently the phenolic constituents of smoke are rather more effective bacteriostatically than is the formaldehyde which it contains. Though heavily smoked fish will keep much longer than fresh fish under similar conditions, the modern types of lightly smoked fish do not keep well (102a).

Certain compounds, particularly sodium nitrite and esters of gallic acid, exhibit both bacteriostatic and antioxidant activity in fish flesh stored at about 0 C (103).

TESTS FOR DETECTING BACTERIAL SPOILAGE OF FISH

Nearly all tests for quality of fish which have been proposed have taken into account only bacteriological deterioration. Important as this is the effect of other undesirable storage alterations such as myosin denaturation (69) and fat oxidation (103) must not be forgotten. Tests designed to determine the bacteriological quality of fish are of two kinds; namely, those which rely on a determination of the size of the bacterial population present, and those which measure products of the metabolism of the microorganisms. The entire subject of bacteriological spoilage of fish is complicated, for the predominating microflora is not always the same, the types which develop depending on the variety of fish, its chemical composition, the storage temperature, whether the fish are whole, eviscerated or cut as in filleting, and other factors. In addition the total bacterial population of different portions of the flesh of single spoiling fish may vary enormously (83, 104, 105).

The obvious, and one of the most frequently employed, experimental methods of ascertaining the degree of bacterial spoilage of fish is a determination of the number of bacteria present. This general method is naturally open to objections; thus, it has been stated categorically that, "Bacterial counts are valueless as a measure of the degree of spoilage of fresh fillets" (106). As a general statement this may hold true, but no more so than it does for chemical tests for products of metabolism of the organisms causing spoilage. It is extremely doubtful if any single test so far proposed for determining bacterial spoilage is absolutely reliable under all conditions even with one variety of fish. It is suggested that a given test will be reliable and give reproducible results only under certain defined conditions, and that any alteration of these conditions may render the results of such a test invalid. If this statement is accepted, it is obvious that most tests when applied to similar material held under identical conditions, as in experimentally treated and untreated samples, will usually give a satisfactory comparison of the relative rates of spoilage. On the other hand, attempts to compare, for example, organoleptic rates of spoilage of whole dressed fish with fillets cut from them by means of a single test such as a bacterial count will probably fail, for it is highly likely that the whole fish may have a very much lower count than the fillets at a stage when both are considered inedible.

A direct determination of the numbers of bacteria in fish flesh was found to be an extremely rapid test for spoilage and yielded results which compared well with those obtained by a viable count (107). This method was only useful with samples containing over 10^5 bacteria per gram, and this fact has been cited as a possible objection to use of a direct count procedure (6). However, this objection is by no means entirely valid, for, certainly with cut fish such as market fillets, counts of 10^5 or 10^6 per gram are more the rule than the exception. One valid objection to the use of a viable count in determining the quality of very fresh fillets is that there is, due to the initial stationary growth phase, no relation between the count obtained and the probable keeping quality of the fish. Thus, it was shown that in exceptional instances the ratios of the viable bacterial counts of different fish samples before storing them at 15 C for 24 hours to those obtained after such a storage period varied from 40 to 28,000 in extreme instances. This finding prompted the suggestion that the only really valid criterion of bacteriological quality of fresh fish might be an estimation of its potential keeping quality, such as might be ascertained by

storing a sample for one day at 15 C and making a direct bacterial count or other test (107). The finding that the rate of spoilage of cod fillets may depend more upon the degree of bacterial contamination during their preparation than on the *post mortem* age of the iced fish from which they are cut is also important (108). The suggestion was made that a bacterial count based on the number of organisms of the genus *Pseudomonas* and *Achromobacter* present might prove a more reliable indication of fish quality than a total count (3, 106). No method of making such a differential count appears to be available.

A number of tests for quality of fish based on chemical, or, less frequently, on physical alterations in the flesh have been described from time to time. Some of the compounds which serve as a basis for certain of these tests may play an important part in determining the four very general stages of organoleptic spoilage which have been suggested for fish (6). It would seem extremely doubtful whether all species of edible fish, fatty and otherwise, would undergo similar stages of organoleptic spoilage. One of the earliest proposed chemical tests for bacteriological spoilage of fish was a determination of the indole content of raw and of canned salmon (109). This test does not appear to have come into general use, but it is of interest that recent studies indicate that a test for this compound may prove of value in determining the quality of iced shrimp (110). A determination of the total volatile acids and of formic acid in canned salmon and tuna was suggested as a test for quality (38), and a test for total volatile acids was also found valuable in following spoilage of herring stored at 0 C (111). Several early investigators employed a determination of the hydrogen sulfide content of fish as an index of quality, but recent data published for stored herring (111) and some other fish (112) indicate that certain other tests are much more sensitive. A so-called "tyrosine" test was also studied to a limited extent, but this is not specific and the results were not always promising (49, 83, 111).

The volatile bases ammonia, dimethylamine and trimethylamine are normally produced in increasing concentrations during spoilage of sea fish, and their measurement, either collectively or singly, has been suggested by a number of investigators as a means of following its microbiological deterioration. Trimethylamine is formed by bacterial reduction of the trimethylamine oxide present in the muscle of sea fish (28, 92, 93, 113). A bacterial enzyme which activates only trialkylamine oxides, rendering them capable of reduction by various dehydrogenase enzyme systems of lower potential level, is responsible (93, 94). Some of the properties of this enzyme as it exists in intact bacterial cells have been described (93, 114). A determination of the amount of trimethylamine formed during storage of sea fish has been used by a number of investigators as an index of its quality (21, 23, 115, 116); this method was used to a limited extent in Canada to grade fish for export (117). Although the test appears to be applicable to certain fish stored under defined conditions, as, for example, dressed Atlantic cod stored in ice, it is practically valueless with some other species. It was suggested some years ago that a nonuniform distribution of trimethylamine reducing and nontrimethylamine reducing bacteria in fish muscle might cause irregular results in the application of the test (113), and it was shown that it was possible to obtain very stale fish flesh without the formation of appreciable amounts of trimethylamine (84). Very irregular distribution of trimethylamine in different portions of the muscle of whole spoiling fish was also observed (83). It has been shown repeatedly that the amounts of trimethylamine formed during spoilage of many varieties of fish taken from the North Pacific ocean (50, 112) are usually very much lower than those described for Atlantic cod (104, 108). There is probably no single satisfactory explanation of this fact, but there is little doubt that the difference in pH values of the spoiling muscle plays an important role in determining the amount of trimethylamine formed during spoilage (50, 88, 118). The fact that the content of trimethylamine oxide in the muscle tissues of different varieties of sea fish is quite variable (29, 30, 31) may also have an important bearing on the amount of trimethylamine formed during its bacterial deterioration. With swordfish it was found that the quantity of trimethylamine formed during spoilage depended to an important extent upon the storage temperature (119) and with dogfish that the high concentrations of ammonia formed from urea during the initial stages of spoilage inhibited trimethylamine formation because of the resultant alkaline reaction of the muscle (120).

The dimethylamine content of the flesh of iced haddock was found by Shewan to be a useful index of their bacteriological quality, especially during the early stages of spoilage (23). It appeared to arise as a result of bacterial action on some natural constituent of fish muscle, but its actual precursor was not established (121). Other investigators reported that the results with this test were too inconsistent as applied to cod and haddock to warrant its application (117). A recent report indicates that a determination of the dimethylamine content may prove a useful indication of the degree of spoilage of shrimp (110).

In general, a test for ammonia is not considered to be a very reliable indication of the state of bacteriological preservation of fish (6, 111). However, as with most spoilage tests there is some disagreement regarding this point, and recent Danish work [cited in (6)] indicates that a determination of ammonia correlates well with quality of certain fish.

A method for determining the total volatile substances which, when aerated from fish extracts, reduce alkaline permanganate solutions was proposed some years ago as a test for quality (122). This test was recently applied to several kinds of raw and canned Pacific fish with very favorable results (112). The method, which determines a number of chemical substances volatile at normal temperature, and at the natural pH values of the fish, appears to offer much greater possibilities than many other proposed tests which are based on the determination of only a single chemical compound.

Several physical methods for detecting fish spoilage have been described. Stansby and Lemon (123) developed a test based on changes in the buffering power of fish muscle during bacterial deterioration and applied it experimentally with some success (123). However, subsequent work indicated that it was not satisfactory (22, 124). A suitable biochemical explanation of the changes involved in causing alterations in the buffering capacity of flesh was given subsequently, and the claims of the original investigators refuted (47). Another physical test for freshness of fish which was proposed was a rapid determination of the surface pH values of fillets (49, 125). Though this test may be reliable under very limited conditions, more extensive (48) and recent (126) experiments have shown repeatedly that the test is usually completely unreliable. Geiger (127) considered the possibility of employing a bioassay method for determining the histamine content of fish flesh and relating this to the degree of spoilage.

The foregoing is by no means an exhaustive survey of all tests which have been proposed for determining the rate of bacterial spoilage of fish. It can be seen readily that it will prove difficult, and probably impossible, to evolve a single objective test which will prove a universal panacea in detecting such spoilage. A recent statement by one worker who has contributed much valuable and original work in this field is illuminating. He declared that, "In practice the application of any of the above objective methods for assessing quality is beset with many difficulties and none, so far as its known, has yet been tested sufficiently with regard to the effects of sampling error, species difference, method of catching, season and size and character of bacterial load, for it to be applied with confidence" (6).

In conclusion, it is important to note the paucity of information which exists with respect to the exact nutritive requirements of the organisms causing bacterial deterioration of fish. Some interesting general findings have been made. Thus it appears that those organisms which are normally associated with a marine environment will not grow readily in media made with ordinary fresh water, but require sea water and *vice versa* (128, 129, 130). No really adequate explanation has yet been offered of this effect. A more satisfactory knowledge regarding the exact nutritive requirements of organisms causing fish spoilage might well lead to some better means of inhibiting their growth, perhaps by application of the general principles of structure relationship of metabolites and antimetabolites (131). At present there is a totally inadequate amount of information on this subject, and very little is known regarding the amino acid, vitamin or mineral requirements of these organisms. It appears that, in some instances at least, their amino acids requirements are extremely simple, for, in one survey, all *Achromobacter* species isolated from prawns would grow with any one of 19 amino acids studied or with ammonium chloride as nitrogen sources (132). It is anticipated that much more information regarding the nutritional requirements of marine bacteria will be available within the next few years. In at

least one laboratory, research along these lines is proceeding actively, and already it has been found that the requirements are extremely diverse. Thus for those cultures which require sea water in their growth medium species have been found which will grow with the following nutrients: glucose, ammonium sulfate, phosphate and sea water; amino acids (mixture of eighteen); phosphate and sea water; amino acids plus a mixture of certain known vitamins of the "B complex"; and amino acids and glucose. For those capable of growing in media containing fresh water the following nutrients have been found suitable for different species: glucose and inorganic ions (whether or not nitrogen fixation occurs has as yet not been definitely established); glucose, ammonium sulfate and inorganic ions; amino acids (mixture of eighteen), ammonium sulfate and inorganic ions; amino acids, ammonium sulfate and biotin; and amino acids, biotin, thiamin and inorganic ions (R. A. MacLeod, private communication).

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