CHAPTER 2

Freshness Tests

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I. Introduction

One of the primary aims of investigators of food spoilage has been to find some method by which deteriorative changes could be accurately and quantitatively measured throughout their course. An independent, objective scale of deterioration would make available an impersonal reference standard that, when supplemented with sensory observations, would permit judgments of greater reliability than those based on subjective evaluations alone. A brief survey of various appraisal methods for objective testing of the quality of fish, raw unfrozen, frozen, or otherwise processed, as well as an evaluation of their particular merits, was made by Piskur (1956).

Several earlier reviews on the evaluation of freshness in seafoods as well as on the spoilage of fish are available, including those of Notevarp et al. (1942), Hjorth-Hansen (1943), Allison (1948), Reay and Shewan (1949), Partmann (1951), Tarr (1954, 1955a), Tomiyasu and Zenitani (1957), Wojciech and Varela (1958), and Montefredine and Testa (1960).

In the present review are discussed as many as practicable of the different types of methods that have been proposed and investigated.

II. Organoleptic or Sensory Tests

The oldest and still most widespread means of evaluating the acceptability and edibility of fish are the senses: smell and sight, supplemented by taste and touch. The reasons for the preferential use of sensory tests are obvious: no special laboratory equipment is needed; the fish can be examined wherever they happen to be; the tests can be carried out quickly; and many samples can be evaluated in a relatively short time. These obvious advantages, however, are to a great extent counterbalanced by a number of disadvantages inherent in the organoleptic method that significantly detract from its usefulness. The use of the senses is a subjective procedure and at best only very roughly quantitative. The impressions registered are the result of the interaction of a number of physiological, psychological, environmental, and even economic factors, including state of health, personal prejudices, preferences and interests, sensory acuity, freedom from disturbing and influencing conditions in the examining environment, and motives of possible profit or loss. The demand upon the senses becomes most critical and difficult when they are required to distinguish and assess the so-called borderline stage of freshness or the stage of incipient spoilage (that is, when a fish sample to be judged is in the last stages of freshness or in the first stages of
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spoilage). This task is well recognized as a difficult one, where even experienced judges often differ in their evaluations.

The criteria associated with freshness of fish have been known for many years. Among the early studies is that of Anderson (1908), who accurately and carefully described the various criteria. He recognized, among other signs, condition of the eyes, texture of the flesh, odor, and appearance of the abdominal walls. Stansby (1944) and the Ohio Department of Health (1953) have also discussed the problem of the organoleptic evaluation of fish from a number of points of view. Boury (1945) has discussed the sensory judgment of canned fish. The importance of sensory judgment as a primary standard with which to compare other tests has been pointed out by Allison (1948).

The uncertainty and difficulties of sensory judgment of fish freshness have been recognized by those in the field. Castell et al. (1956) have expressed similar ideas by stating that "in actual practice the grading of fresh fish is, and for some time will remain, an art and not a science." To overcome some of the disadvantages associated with sensory judgment, attempts have been made to minimize the personal uncertainties and to overcome the qualitative nature of the test by means of trained or experienced panels to judge the samples, and by the elaboration of numerical systems of scoring and recording the sensory judgments. Young (1938) has described the use of a taste panel for the evaluation of halibut samples. Griffiths and Stansby (1934), Castell et al. (1956, 1958), Hansen (1956), Shewan et al. (1953), and Shewan and Ehrenberg (1956) have described systems of grading and of numerical scoring of raw and cooked fish based on the evaluation of such factors as odor, general appearance, taste, and texture of raw and cooked fish. Score sheets and a point system for the numerical scoring of the samples have been elaborated as a basis for the sensory judgments. Soudan et al. (1957) have also described a special system for the organoleptic estimation of fish spoilage. The relation between sensory and chemical tests has been discussed by Jensen (1956); some general aspects of sensory testing procedures have been discussed by Harries (1953).

All of these systems are based on the summation and numerical representation of a number of sensory judgments of odor, texture, general appearance, etc., of raw fish, and of the taste of cooked fish. They have proven fairly serviceable, providing reasonably good sensory yardsticks of quality and freshness of fish samples within the limitations of such procedures.

A variation of the sensory test, in which the changes in the appearance of the lenses of fish eyes are used as the criterion of the condition
of the fish, has been described by Love (1954, 1956). This test has proven of some value under controlled conditions, but its general usefulness and applicability are influenced and limited by the treatment of the fish, such as freezing and other storage conditions.

Whereas sensory or organoleptic tests of the state of freshness of fish and fish products have proven of general value and are used widely throughout the world (particularly for samples of either unquestioned freshness or definite spoilage), their value is markedly diminished and they become less exact and less consistent when applied to fish at the stage between the end of freshness and the beginning of spoilage, the area of so-called incipient spoilage. It is precisely in this state of preservation that the sensory judgments of individuals become more variable, more dependent on subjective factors, and therefore less reliable. Even experienced persons have great difficulty in making an accurate and quantitative estimation of the condition of fish in this state. The line dividing fish that are still fresh from those with some early signs of spoilage is not very well defined and is most often subject to differences in personal opinions. As a result, investigators for about three-quarters of a century have attempted to devise tests that would be less dependent on personal factors and subjective opinion. This review is essentially an account of the attempts through the years to develop more objective criteria, based on physical or chemical tests, that would overcome those disadvantages inherent in the sensory, more subjective criteria.

III. Physical Methods

Several studies have been directed toward the development of practical deterioration tests based on changes in the physical properties of the fish flesh. In general these tests have not proven applicable, although a few, under certain limited and circumscribed conditions, have shown some correlation with the freshness of fish.

A. Texture Changes

Forbes (1926–1927) reported studies on the effect of various treatments on the tensile strength of fish muscle. He found a steady decrease in the breaking point of muscle strips after rigor, the rate of decrease varying with the temperature.

In 1931 Tauti et al. described a method for measuring the force needed to deform the surface of raw fish. The degree of deformation and the time needed for the return of the surface after removing the force were used as a measure of the freshness of the fish. Yamamura (1932, 1933a) reported further tests with this method and on the cor-
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relation of the loss of rigidity of the flesh with the increase in total volatile nitrogen content during spoilage. Charnley (1936) and Charnley and Bolton (1938) tested the firmness or softness of canned salmon as a measure of its condition. Takahashi et al. (1952) described experiments on the relationship between the depth of penetration of a plunger into fish jellies and the changes in total volatile nitrogen and pH during spoilage. No marked change in rigidity was found at the point where a marked ammoniacal odor was noted. Love (1960) described a new procedure for optically measuring the texture of fish muscle and its changes during storage. Buttkus (1963) recently described an apparatus for measuring texture changes based on the energy needed to cut muscle fibers.

B. Refractive Index

The possible use of the refractive index to follow the changes in fish during storage and spoilage has been studied, without much success, by Tillmans et al. (1927), Boury and Schvinte (1935), Riddell et al. (1937), and Sidaway (1941). A recent modification of this test has been reported by Proctor et al. (1959). These authors studied changes in the refractive index of the fluid of fish eyes during storage of whole haddock under different conditions. They reported a good correlation between the value of the refractive index and the organoleptic judgment. Further tests of this procedure will determine its practical use for raw whole or eviscerated fish.

C. Electrical Conductivity

The possible value of the determination of the electrical conductivity of fish flesh as a measure of its condition has been studied at various times. Tillmans et al. (1927), Riddell et al. (1937), and Labarre and Fougère (1942) reported that conductivity was of no practical value as an index of fish spoilage since it varied with storage time and temperature, passing through a minimum during the early stages and rising again as definite spoilage developed. Yamada and Kitano (1948), Ito et al. (1949), and Asakawa (1956) reported that the electrical resistance of carp muscle changed stepwise during storage as the fish passed through the stages of pre-rigor, rigor, and post-rigor to definite spoilage. Amano (1954) concluded, however, that the practical application of the test was unwarranted. The consensus of the value of this test is that it is not reliable for determination of the change from fresh to not-fresh.

D. Optical Tests

A variety of tests based on optical properties of fish flesh and its products have been studied over the years. In some instances favorable
results have been reported. The general conclusion, however, has been that these tests depend upon too many uncontrollable factors that reduce their value as reliable methods for measuring freshness. In 1933 Langstroth found no direct relation between the absorption of white light and the amount of bad-smelling products in fish. Boury and Schvinte (1932, 1935), Hinard (1932), van de Velde (1937), Leskov (1946), Adamova and Spector (1947), and Wittfogel (1952) studied the ultra-violet fluorescence and luminescence of dispersions of fresh and spoiled fish flesh. They reported that changes in the color of the fluorescence occurred during spoilage, but that such factors as the state of dispersion and the time of extraction affected the colors observed. Lünburg (1951) studied the possibilities of determining the green or blue fluorescence appearing in fresh-water fish and caused by bacterial activities. Quite fresh fish showed no fluorescence at all. No conclusive results were obtained. Tomiyama et al. (1955) reported a turbidimetric method for determination of the freshness of the cooked fish paste, "kamaboko." As the surface of the fish loaf underwent bacterial decomposition, it became more water-dispersible and hence increased the turbidity of the surface washings.

Recently Kurtzman and Snyder (1960) reported an optical test for estimating the freshness of crabmeat, based on the turbidity of alcohol extracts that developed upon the addition of saturated aqueous picric acid solution. This test is more suited for establishing the presence of spoilage rather than the degree of freshness.

E. SURFACE TENSION, VISCOSITY, AND INTERNAL FRICTION

A number of investigators have attempted to follow the changes occurring during deteriorative storage of fish by measuring several other physical properties. For example, Tillmans et al. (1927) showed that surface tension did not change markedly during deteriorative storage. The viscosity was of no value as an indicator of spoilage in fish flesh; it underwent a cyclic change, increasing somewhat at first, dropping to a minimum, and finally, with the onset of definite spoilage, mounting again (Labarre and Fougère, 1942). Hotani (1951) studied the internal friction of fish flesh by a damping oscillation method. He found that this quantity varied with the freshness of the samples, and that the test could be used for frozen fish without defrosting. No further reports have apparently been published on this method, and its significance for the detection of the early stages of spoilage remains undetermined. From its similarity to the physical properties just mentioned, particularly viscosity, it is, however, somewhat doubtful if this method could serve as a generally reliable index of incipient spoilage.
IV. Physicochemical Methods

Many reports have appeared during the past 35 years or so on the correlation between certain physicochemical measurements and the early stages of spoilage. The bulk of these has been concerned with pH changes, and several have dealt with buffering capacity and redox potential.

A. pH

There has been an extensive series of studies on the value of pH as a measure of fish spoilage. Although some of these reports have been contradictory or conflicting, the majority of them have been consistent in their conclusion that pH has little or no significance as a reliable index of the state of freshness of a sample (Yamamura, 1933b, and others).

Benson (1928), Poluektov (1933), Lücke and Geidel (1935), Okolov and Shavski (1936), Zwilling (1936), Riddell et al. (1937), Zakhar'evskii (1939b), Bradley and Bailey (1940), Hjorth-Hansen (1943), Elliott (1947), Sigurdsson (1947), Kondrup (1948), Rasmussen (1950), Cutting (1953), Higasa (1953), Uchiyama and Yokoyama (1953a), Yamamoto and Sonehara (1953), Luijpen (1954a,b), Simidu and Hibiki (1954b,c,d), Varela and Wojciech (1956), Wojciech and Varela (1958), and Zakhar'evskii (1939a,b) have reported that the pH varied initially and that it showed no real or significant correlation with the onset of spoilage in fresh fish (e.g., cod, haddock, perch, herring, halibut, and hake), salted fish (e.g., herring, carp, pike, and rock), fish loafs, or shellfish. On the other hand, van Deurs and Hoff-Jørgensen (1936) proposed an upper limit of pH 7.5 for acceptability of cod fillets. Strohecker et al. (1937) reported that the pH increased in fish parallel to the increase in the content of volatile oxidizable substances of steam distillates. Charnley and Goard (1942) suggested the use of pH as a measure of the freshness of fish muscle, such as salmon. Finally, Wood et al. (1942) and Dyer et al. (1944) maintained that the pH of the surface could be used as a rapid and simple test for the degree of freshness of white-meat fish, such as cod and haddock. In an extensive study of the fishes of the Adriatic Sea, Misericordia (1954) established the upper limit for pH in spoilage of each individual species and found that spoilage ran parallel to the mounting pH of the muscle tissue. Montefredine (1955) also concluded that pH was a reasonably reliable indicator of the degree of freshness.

The consensus from the above studies is that the pH determination cannot be used as a reliable index of the state of freshness or of the onset of spoilage, but that under certain restricted conditions pH limits for definite levels of spoilage may sometimes be set. The usefulness of pH determination is often greatly restricted or vitiated by its variability.
from sample to sample and by its cyclic fluctuations during the storage process.

B. BUFFERING CAPACITY

In 1933 Stansby and Lemon published a paper in which a differential titration to two levels of pH (A and B values) was suggested as a measure of the spoilage of haddock. They ascribed the changes in values to the breakdown of proteins and the formation of protein decomposition products, mainly of a basic nature. Griffiths and Stansby (1934) elaborated on this method and combined it into a single value. This procedure was tested by Riddell et al. (1937), Fitzgerald and Conway (1937), and Cutting (1938, 1953). These authors reported that the changes in titration values for the two pH levels were too irregular and variable to form the basis of a reliable spoilage test and that no correlation between organoleptic judgment or bacterial content and the buffering capacity was found.

A decreased buffering capacity was noticed during spoilage of cod muscle press juice; it was ascribed to a reduction of trimethylamine oxide (TMAO) to the free base (Collins et al., 1941).

Hacker (1950) has checked the Stansby and Lemon method of determining buffering capacity of haddock muscle on the muscle of cod as well as of three fresh-water fish (trout, roach, and sunfish). The B values were found to increase for all four fishes, confirming the earlier findings, whereas the A values, in contrast to the previous observations, declined in all cases. In separate tests it was found that lactic acid and TMAO had strong buffering capacity in the pH range 6.0–4.3, but that trimethylamine (TMA) had none.

The method was also tested on fresh-water fish by Bose and Dutt (1954). They found that the titration values A and B showed a rough correlation with the sensory grades but that they overlapped in other respects. The trend of the results for fish stored at higher temperatures was different from those found for fish stored in ice. As a result no definite limiting values could be established for fish judged to be spoiled. The premise and application of this method have also been discussed by Nickerson and Proctor (1935) and Beatty and Gibbons (1937).

The buffering capacity of fish muscle and muscle extractives was restudied by Suyama and Tokuhiro (1958) in order to test its value as a criterion of the degree of freshness of fish muscle. The method proved less reliable, although titration to the B-level seems to have possibilities. The muscles of elasmobranchs show a higher degree of buffering than those of the teleosts because of the abundance of TMAO.
C. Oxidation-Reduction Potential

The possible correlation between changes in the redox potential and the sensorily determined condition of fish has been studied a number of times (Tillmans et al., 1927; Riddell et al., 1937; Zakhar'evskii 1939a,b). It was established that the redox potential was of no practical use as a spoilage indicator for fish. Proctor et al. (1957) also found the redox potential too variable to be of use as a fish spoilage test.

D. Freezing Point Depression

Tillmans et al. (1927) studied the depression of the freezing point of fish flesh during spoilage. They found that the entire range of values was quite small and that the freezing point decreased to a minimum value at the beginning of spoilage and then increased on further spoilage, reaching the original value of the fresh samples. They concluded that its estimation was an unsuitable test of spoilage.

V. Chemical Methods

A. Volatile Basic Nitrogen Compounds

1. Total Amount

More data have probably been accumulated on this test than on any of the others suggested as spoilage indicators. Furthermore, there probably are more conflicting results for this test than for any others. For example, of 64 papers reviewed, 38 reported favorable results for the use of the total volatile bases as a spoilage indicator, 17 stated that it was of no use, and 9 presented data showing the variable nature of the test, which is useful for some species or products and useless for others.

The earliest report found on the use of ammonia or volatile bases as an index of spoilage was that of Eber (1891), who suggested that the fuming of a dilute HCl solution in ether-alcohol could be employed as a qualitative test for the spoilage of meat. Clark and Almy (1917a, b, 1920) studied a number of possible methods and reported that the total volatile basic nitrogen content increased during storage of shucked oysters and of white-meat fish. Weber and Wilson (1919, 1920) studied the volatile basic nitrogen compounds in canned sardines during decomposition of small sea herring. Hinard (1922) suggested the ratio of the content of total volatile basic nitrogen to that of total nitrogen as a useful index of fish spoilage. Tillmans and Otto (1924) found that the total volatile bases increased with the onset of spoilage of such fish as cod, haddock, eel, and sea pike; they suggested an upper limit of 30 mg. nitrogen per 100 g. for acceptability.
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Rochwarger (1929) also reported favorable results for the volatile basic nitrogen content as determined with Nessler's reagent and suggested 20 mg. N per 100 g. as the upper limit for good fish. Reed et al. (1929) and Gibbons and Reed (1930) compared the production of volatile basic nitrogen from haddock, clams, and lobsters during autolysis with that during bacterial decomposition. They found that only during the latter process were appreciable amounts of volatile nitrogen produced. Yamamura (1933b) and Tanikawa (1935) also found the content of volatile basic nitrogen useful as a measure of spoilage, and suggested 30 mg. N per 100 g. as the upper limit for acceptability. Favorable results for the increase of the total volatile basic nitrogen content with fish spoilage were also found by Boury and Schwinte (1932, 1935), Boury (1934, 1936, 1937), Dobrovskii and Novikova (1935), Salmon and LeCall (1936), Smorodintzev and Krulova (1936), Zwilling (1936), Firsov (1937), Labrie and Gibbons (1937), Crooks and Ritchie (1938), Shewan (1938, 1939a, 1942), Holmov (1939a), Stansby et al. (1944), Ota (1958a, b), Riemann (1952), Tomiyama et al. (1952), Pierangeli et al. (1954), Tanikawa et al. (1955).

Lücke and Geidel (1935) suggested an upper limit for the beginning of spoilage of 30 mg. volatile basic N per 100 g. Kimura and Kiamakura (1934) for salmon recommended volatile basic nitrogen levels per 100 g. of 10 mg. or less for fresh, 20–30 mg. for beginning of spoilage, and over 30 mg. for spoiled fish. Tanikawa and Akiba (1955) for crabmeat suggested 20 mg. volatile basic nitrogen per 100 g. as the upper limit for fresh meat.

The content of total volatile bases has in general been found a rather unsatisfactory indicator of spoilage. Variable results have been reported by several authors, including Bökman (1918), Tillmans et al. (1927), and Okolov (1932) for herring; Poluektov (1933) and Pershin (1935) for salted herring; Beatty and Gibbons (1937) for white-meat fish; Tanikawa (1938a, b) for carp, salmon, sardines, crabs, and oysters; Labarre and Fougère (1942) for salted and dried cod; Tarr (1942) for canned herring; Boury (1945) for canned fish; Rasmussen (1950) for minced fish; Tomiyama and Yone (1953) for “kamaboko” (fish loaf); Uchiyama and Yokoyama (1953) for fish cakes; Farber (1952) and Farber and Ferro (1956) for dark-fleshed, raw, and canned fish; Vaisey (1956) for cod; and Moorjani et al. (1958) for fresh-water fish.

Clark and Almy (1917a), although they observed some increase in the content of volatile bases during fish spoilage, nevertheless suggested that the determination of the content of other volatile substances associated with the development of off-odors offered a more promising means
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To assess spoilage. The conflicting and contradictory results that have been reported, partly with meat other than that of fish, are presumably explained by differences in composition, bacterial flora, handling methods, etc., between commodities (see further Volume I, Chapter 14). Waksman and Lomanitz (1925) found that if little or no carbohydrate were present, the content of ammonia or volatile bases increased appreciably during meat spoilage, but that if carbohydrates were present, protein decomposition with ammonia formation was depressed. A similar phenomenon was reported by S'midu and Hibiki (1957).

Kawabata (1953b) found that during the spoilage of such red-meat fish as albacore tuna and mackerel no marked increase in total volatile bases occurred, in contrast to the marked increase during spoilage of white-meat flatfish. (For the latter he suggested 30 mg. of volatile basic nitrogen per 100 g. as the upper limit for freshness.) Bose and Dutt (1954) reported that for spoilage of fresh-water fish at temperatures of 80-90°F the content of volatile bases showed a rough correlation with the freshness, but that at 30-40°F no correlation was found between the sensory judgment and the content of volatile bases. Farber and Cederquist (1953) found that the content of volatile basic nitrogen was of some value for white-meat fish spoilage but not for that of red-meat fish. Buffa and Ambanelli (1954) reported that total volatile basic nitrogen content could be used to judge the condition of raw unfrozen and frozen tuna and mackerel, but that it was not suitable as an indicator of the condition of the canned fish. This index of spoilage furnished a reasonably accurate and rapid method for appraising the keeping quality of cured fish products according to Indian studies (Pillai and Nayar, 1957), and it was found to be superior to the method based on determination of TMA by Valenkar (1952).

Wierzchowski (1956) found that the content of total volatile base was useful for estimating the freshness of lean fish, such as cod, and suggested 30-40 mg. N per 100 g. as the upper limit for fresh-water fish and 60 mg. N per 100 g. as the limit for marine fish. However, the total volatile base content was of no value for herring. Lythgoe (1913, 1938) and Fellers et al. (1957) recommended the ratio of volatile basic nitrogen to the total nitrogen as a useful index of the quality of fish.

Sato (1958, 1960) studied the volatile basic nitrogen and amino nitrogen in fresh market fish and the changes in spoilage. Employing Conway's microdiffusion methods, he found this method useful but noted a consistent difference between bottom fish and surface fish, the latter always having a lower value for volatile amino nitrogen in relation to volatile basic nitrogen.
2. Ammonia

In the paper by Lythgoe (1913) referred to earlier, the ratio between ammonia and total nitrogen was found to be an adequate test of decomposition in fish. Additional data supporting this conclusion were presented by Lythgoe in 1938. Since then very little further information has accumulated along this line.

Only in 1952 did Ota and Nakamura establish that the quantity of ammonia in raw and in precooked meat of various fishes heated under pressure was proportional to the freshness of the raw meats. They inferred that the grade of freshness of the original raw material could be estimated on the basis of the ammonia content found in canned fish products.

No real difference was established between thawed frozen and raw unfrozen fish muscle as to increase in ammonia, whereas histamine formation was largely suppressed in thawed muscle (Ota and Kaneko, 1958). Differences prevail as to species and freshness stages in several Italian fishes (Testa, 1955). No conclusive results were reached as to the usefulness of this method for determining degree of spoilage.

3. Trimethylamine (TMA)

The situation for determining spoilage by TMA is somewhat analogous to that of the other volatile basic nitrogen compounds. A number of reports have appeared recommending this method, others have claimed it is of little value, and some have reported equivocal results. As with the volatile basic nitrogen studies, the conflicting situation for trimethylamine is partly the result of the use of different species of fish with varying compositions and of the employment of different storage temperatures and conditions.

Poller and Linneweh (1926) reported that during fish spoilage trimethylamine oxide (TMAO) was reduced by bacteria to trimethylamine. Boury was one of the early investigators to report that trimethylamine could serve as an index of spoilage for raw fish (Boury and Schvinte, 1932, 1935; Boury, 1936). The initial impetus to the use of trimethylamine as a measure of spoilage, which has survived to the present, was provided, however, by the first report of Beatty and Gibbons (1937), who also presented the rather simple Conway microdiffusion technique for its determination. In later papers Beatty (1938, 1939) and Watson (1939) showed the probable origin of TMA in cod muscle press juice
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and worked out likely chemical reactions for its formation from the precursor, TMAO. Dyer (1943, 1945) described a colorimetric procedure for the determination of TMA; this has been modified by Hashimoto and Akaichi (1957). The increase in TMA during spoilage has also been studied by Holmov (1939a, b), Dyer and Mounsey (1945), Dyer et al. (1946), Proctor et al. (1950), Takahashi et al. (1952), Simidu and Hibiki (1954a, 1955), Castell and Triggs (1955), Bailey et al. (1956), Horie and Sekine (1956), and Shewan and Jones (1957). Hess (1941) suggested that the value of the TMA determination could be enhanced by determining its increase in fish fillets after an incubation period. A measure of the keeping quality during subsequent storage could also be obtained in this manner.

TMA is generally not found in fresh-water fish (see Volume I, Chapter 6); consequently this method cannot be employed with this category of fish.

A series of papers on the grading of whole and filleted fish by the content of TMA has been published by Castell et al. (1958), Hoogland (1958), and Castell and Greenough (1958). Furthermore, a number of reports have appeared in which it was found that the content of TMA was not a sensitive, reliable, or reproducible index of fish spoilage. Either the increase in TMA occurred during the latter stages of spoilage, or there was a variation in levels between species, or the ranges of values were so wide and overlapping as to preclude setting up definite standards, or no appreciable increase took place. Among the latter studies the following may be consulted: Tillmans and Otto (1924); Tillmans et al. (1927); Okolov (1932); Boury (1934, 1945); Riddell et al. (1937); Sigurdsson (1947); Anderson and Fellers (1949); Tarr and Ney (1949); Farber (1952); Velankar (1952); Kawabata (1953a); Simidu and Hibiki (1954b, d); Ehrenberg and Shewan (1955); Good and Stern (1955); Luijpen (1954a, b); Varela and Wojciech (1956); Farber and Ferro (1956); Shewan and Ehrenberg (1957); Luijpen (1958).

It has also been suggested that TMA is a product of the early stages of spoilage (Collins, 1938; Hess, 1941) and that it may be lost indiscriminately during storage. Dussault (1957) found that spoilage levels of TMA were not the same for rosefish as for cod fillets and that the TMA values were a good basis for grading rosefish fillets, using different spoilage levels from those that would be applicable for cod fillets. The relation of bacterial population to TMA content has been studied by Tarr (1938, 1939, 1940), Shewan (1939b), Wood and Baird (1943), Neilands (1945), Dyer and Wood (1947), and Robinson et al. (1952).
Ronold and Jakobsen (1947) found that TMA could also be produced in canned fish by a chemical reduction of the TMAO. Castell (1949) and Castell and Greenough (1957b) showed that nitrite suppressed TMA formation, whereas the tetracycline antibiotics did not.

4. Other Amines

Most of the determinations on volatile nitrogenous bases have either measured the total content of these substances or have involved the estimation of TMA with whatever amount of dimethylamine (DMA) may have been present. A few investigators have measured the amount of the individual amines formed during spoilage. Guareschi (1917) and Tillmans et al. (1927) found a positive test for carbamylamine in spoiled fish, which is an indication of the presence of primary amines. Reay (1938), Shewan (1938), Reay and Shewan (1949), and Shewan (1949) reported that during spoilage small amounts of mono- and dimethylamine were formed. Beatty and Collins (1940) found that in spoiling cod muscle press juice some dimethylamine was produced, and that this occurred before any increase in trimethylamine but to a smaller extent than that of trimethylamine. Varela and Wojciech (1956) and Wojciech and Varela (1958) reported that the content of dimethylamine ranged from 0.2 mg. N per 100 g. (in fresh hake) to 1.5 mg. N per 100 g. (in spoiled hake). The rather large differences often found between the content of total volatile base and that of TMA suggests that other intermediate amines could possibly be present in addition to the ammonia. The above data tend to substantiate this conclusion.

Steam heating for a short time produces insignificant quantities of formaldehyde from fish flesh, but heating under pressure produces measurable amounts of formaldehyde, together with dimethylamine, according to Japanese findings (Ota, 1958b). The amount of DMA was almost proportional to the TMAO content in the flesh, whereas that of formaldehyde was not. Thermal breakdown of TMAO was accelerated by the presence of fish extracts or certain kinds of amino acids. The degree of decomposition of TMAO in the presence of cysteine was proportional to the concentration of TMAO and cysteine. But the quantitative ratio between these two decomposition products was not the theoretical one. This might be attributed to some subordinate reaction of formaldehyde with certain kinds of amino acids (Ota, 1958b).

B. Volatile Acids

Next to the total volatile nitrogenous compounds, the content of total volatile or steam-volatile acids has probably received more attention as a possible index of spoilage than any other group of substances. In 1927
Tillmans et al. reported that the content of steam-volatile acids was not a reliable criterion of spoilage even though in certain cases it increased with spoilage. Boury (1934, 1935) reported similar results for fish spoilage. The most consistent proponents of steam-volatile acids as a spoilage index during the past 20 years have been Hillig and his co-workers. Starting with the first paper by Clark and Hillig (1938), a long series of results have been presented on the value of the content of steam-volatile acids as an index of spoilage of raw and canned tuna, mackerel, and sardines, of canned herring roe, and of raw cod: Hillig and Clark (1938); Hillig (1939a, b); Hillig and Knudsen (1942); Hillig (1944, 1945); Hillig et al. (1950a); Hillig (1954, 1956a, 1957); Hillig et al. (1958); Hillig (1958). Malin (1939) reported that the volatile acid number of ether-extracted fatty acids could serve as an indicator of fish spoilage. Collins (1941) found that the presence of acetic acid could be used as a guide to the state of preservation of cod, but that the determination was too time-consuming to be used as a routine procedure. Clague (1942) reported that for canned Maine sardines the content of volatile fatty acids was a useful indicator of spoilage, even though there was no correlation between the bacterial count and the content of volatile acids. Beacham (1946) studied the increase in total and individual volatile fatty acids in oysters and clams canned during spoilage. He found that formic and acetic acids increased during spoilage, and that butyric acid was not present in good shellfish but was formed during spoilage.

Sigurdsson (1947) concluded that above a storage temperature of 32°F. (0°C.) the content of total volatile acids was a satisfactory measure of the condition of raw and canned herring. Farber (1952) presented data showing that even though the content of steam-volatile acids increased somewhat during spoilage of raw tuna and mackerel, the increase was often small or variable, or else did not correlate well with sensory findings. In a series of canned tuna samples judged sensorily to be in various states of freshness or spoilage no correlation was observed between the content of steam-volatile acids and the organoleptic judgments. Higasa (1953) reported that the volatile acids increased to a greater extent during spoilage than did other criteria, such as pH or volatile bases. Suzuki (1953a) reported that volatile acids could be used to judge the freshness of fish. Uchiyama and Yokoyama (1953b) reported that for fish cakes the presence of propionic, butyric, and valeric acids could generally be used as an index of spoilage, but that sometimes these volatile fatty acids were found even in fish cakes with no sensorily detectable deterioration. Tomiyama and Yone (1953) found no consistent values for the content of volatile acids at the onset of spoilage of
“kamaboko” fish loaf. Luijpen (1954a, b) reported that for salted herring there was a relation between the content of volatile acids and the state of spoilage. However, the practical application of the volatile acids content as a spoilage indicator was doubtful since volatile acid (e.g., acetic), was added to many herring preparations. Furthermore, the type of spoilage was influenced by the salt content. Asakawa (1953, 1954, 1957), in a series of papers, described apparatus for the distillation of volatile acids; studied the volatile acid recovery in different amounts of distillate; studied the volatile acid production in different anatomical parts of fish and in different fish; proposed a numerical scale for the volatile acid content based on the ratio of the amount of acid in successive portions of distillate; and concluded that with advancing spoilage of fish flesh the higher volatile fatty acids became more prominent. Fieger and Friloux (1954) found no relation between the volatile acids content and the early stages of shrimp spoilage.

Procedures for the determination of the content of volatile fatty acids have generally been based on or are similar to those described by Clark and Hillig (1938) and by Friedemann (1938). Procedures for the chromatographic separation and identification of individual volatile fatty and organic acids have been described by Ramsey and Patterson (1945, 1948a, b), Ramsey and Hess (1950) and van Dame (1957). Vaisey (1956) found that in nitrite-treated cod fillets the volatile acids content was too scattered, in relation to the sensory spoilage assessment, to serve as a useful index of spoilage. Orlandi (1956) reported that volatile acids constituted an acceptable indicator of the degree of freshness in fish.

C. Succinic Acid

Hillig and his co-workers (Hillig, 1949, 1954, 1956a, 1959; Hillig et al., 1950b, 1958) studied the production of succinic acid during spoilage of tuna and cod. For tuna it was suggested that the presence of succinic acid could serve as an index of spoilage, whereas for cod the content of succinic acid did not correlate with the state of spoilage.

D. Indole and Skatole

Over the years various attempts have been made to use the content of indole and of its derivative methylindole or skatole as a measure of the state of freshness or spoilage in flesh foods. Technical procedures for its determination have been studied by, among others, Fellers and Clough (1925) and Chernoff (1940). The changes in the content of indole and skatole that occur in fish during spoilage have been reported by Ottolenghi (1913), Guareschi (1917), Clough (1922), Tillmans and Otto
2. FRESHNESS TESTS

(1924), Shostrom et al. (1924), Pershin (1935), Smorodintzev and Diskina (1936), Shtenberg et al. (1938), King et al. (1945), Beacham (1946), Duggan and Strassburger (1946), Duggan (1948), Amano and Tomiya (1950a), Campbell and Williams (1952), Farber (1952), Wierzychowski and Severin (1953), and Barry et al. (1956). The general conclusion that may be drawn from these data is that whenever indole and skatole were present, the fish was organoleptically judged to be definitely spoiled, but that during early stages of spoilage the absence of indole and skatole could not be used as a criterion of freshness. Foods that were judged sensorily to show some spoilage were not found to contain any or significant amounts of these substances.

E. HYDROGEN SULFIDE

The situation with respect to the determination of hydrogen sulfide as an index of spoilage is similar to that for indole and skatole. Various attempts have been made to use the content of hydrogen sulfide as a reliable measure of the condition of fish and the results have been variable. Among these studies are those of Eber (1897a, b), Fellers et al. (1924), Almy (1925, 1927), Budagyan (1932), Dobrovskii and Novikova (1935), Tilik (1935), Okolov and Shavskii (1936), Boury (1937), Boury and Schvinte (1932, 1935), Riddell et al. (1937), Tanikawa (1938a), Stansby and Lemon (1941), Sigurdsson (1947), and Farber (1952). Castell and Greenough (1957b) found that tetracycline antibiotics had no effect on the production of hydrogen sulfide by spoilage bacteria. Guareschi (1917) reported the presence of mercaptans in spoiled fish. The general conclusion from all the reported data is that wherever hydrogen sulfide was found, definite spoilage could be established sensorily, but that spoilage could occur with no hydrogen sulfide production.

F. CARBONYL COMPOUNDS

Among the possible products of bacterial action and of the chemical breakdown of unsaturated fats are the carbonyl-containing substances, including keto acids, ketones, and aldehydes. Diacetyl, for example, is a well-known product of lactobacilli and certain lactic streptococci. Mono- and dicarbonyl compounds have been used as indicators of fat rancidity. An attempt to use the content of carbonyl compounds reacting with bisulfite was made many years ago (Farber, 1952). It was found that the content of the bisulfite-binding substances increased with spoilage and varied directly with the sensory findings. In canned fish, however, the correlation was not as close as for raw fish. Proctor et al. (1957)
found carbonyl compounds in haddock flesh but reported no difference between fresh and spoiled fish. Since many of the carbonyl substances are odoriferous and could contribute to the sensory stimulation, further studies on their production might be of value. They probably contribute to and are included in the determination of total volatile substances discussed below.

G. Steam-Volatile Oxidizable Substances

As an approach to the chemical determination of odoriferous compounds formed during spoilage of foods, investigators have used the oxidizability of steam distillates as a measure of their condition. They have compared the values thus obtained with sensory judgments. Among the oxidizing agents used were acid potassium permanganate, acid potassium dichromate, and, less commonly, alkaline potassium permanganate. The relation of the oxidizability of steam distillates to the state of freshness has been investigated in various foods, but only a few studies refer to fish: Strohecker et al. (1937); Ergorova (1939); Tomiyasu et al. (1952); Hillig et al. (1958). Holaday (1939) and Hillig (1958) have used the same general procedure with specific refinements to estimate the content of ethanol as a measure of spoilage.

This procedure yielded some interesting correlations with the sensory judgments and showed definite utility as a means of measuring spoilage of various foodstuffs. But the method has certain disadvantages that limit its use: one, that a protein-free filtrate must be prepared for the distillation; another, that the steam distillation operation itself could conceivably remove many steam-volatile substances that are not essential to the odor. Heat-labile compounds may also be broken down with the formation of steam-volatile products. These would then be included in the evaluation of the total oxidizable substances. Finally, the physiological act of smelling and the steam distillation of a markedly altered extract of the original sample are not strictly comparable operations. Hence, the results of the two processes would not be expected to agree very closely. The rather divergent results reported for steam-volatile oxidizable substances may be due to these circumstances.

Tomiyama et al. (1960a) avoided foaming by removing the proteins through magnesium sulfate. The steam distillation was carried out in an alkaline reaction at pH 9.3. This method gave a more steeply mounting curve for the incipient spoilage of mackerel. The authors claimed that this modified form constituted the most sensitive way of measuring spoilage in mackerel flesh. A subsequent study confirmed these findings and it was maintained that this modified version of the steam-oxidizable
substances method gave a better coverage of available volatiles (Tomi­yama et al., 1960b).

H. Reducing Substances That Are Volatile at Room Temperature

A procedure that eliminated the objections enumerated above for the steam-volatile oxidizable substances was first reported by Lang et al. (1944, 1945). In it, the sample tested was a press juice squeezed from either raw, processed, or canned fish. Air at room temperature was passed first through the sample juice, where it picked up and entrained any substances that were volatile at ambient temperature, and then through a solution of potassium permanganate in normal sodium hydroxide. The amount of permanganate reduced was used as the measure of the content of volatile material in the air which had passed through the sample. This procedure was designed to duplicate chemically as closely as possible the physiological process of smelling or odor perception. In both processes air at ambient temperature is passed first over or through a sample; it then comes into contact with the detecting mechanism, either the olfactory nerve endings or the alkaline solution of potassium permanganate. In both processes the sample is not altered or changed, excepting that the soluble constituents are separated from the supporting structure for the chemical detection process. The reagent chosen was found to be the most sensitive oxidizing agent of a number tested, including acid permanganate, acid dichromate, and ceric sulfate. It reacted more rapidly at room temperature than any of the other oxidants tested, and with a wide variety of organic and inorganic compounds that could be expected to be present in fresh or spoiled fish. The main exception was ammonia, which did not reduce the reagent. The early exploratory studies indicated that the method would give useful results and its further development was promising. Subsequent reports by Farber (1949, 1952) and Farber and Cederquist (1953) showed the applicability of the volatile reducing substances (VRS) procedure to a wide variety of odoriferous foods and food products in addition to fish. Farber and Ferro (1956) described a modification of the method (the air purification train was eliminated and a recirculating pump inserted to make a self-contained unit), and showed the applicability of the modified method to a wide variety of canned fish. A review of this procedure as a useful index of spoilage of different kinds of raw and processed fish has been published by Farber and Lerke (1958). Some data for the mechanism of oxidation by alkaline permanganate solution have been reported by Karel et al. (1957). It may be of interest to point out that the VRS procedure for spoilage evaluation is essentially a relative one, the amount of reduction for any sample being compared to that for fresh samples of the same species determined under
the same conditions. The theoretically possible total reducing capacity and total recovery of all reducing oxidizable substances are therefore irrelevant concepts.

Reports of the usefulness of this method as a spoilage indicator for marine and fresh-water fish have been published by Moorjani et al. (1958), by Wittfogel (1956, 1958a, b), and by Wittfogel and Gebhardt (1957). Riemann (1952) reported on the content of total volatile nitrogen bases, trimethylamine, volatile reducing substances, and total bacterial count, as well as on the pH of cod fillets stored at 0–2°C. He also compared their values with the organoleptic judgments on a numerical scale of 1–5, from very fresh to putrid. He concluded that the determination of total volatile basic nitrogen gave better results than the other methods. A careful examination of the charts given, however, do not show the experimental basis for this conclusion. All the ranges for the various values at the corresponding sensory grades overlap and do not permit conclusions about the usefulness of the methods studied. Schmidt and Mayoh (1955) reported data for some salmon spoilage in ice and at 38°F in air. No marked odors developed in ice and the increase in VRS was small, whereas in air there was a more rapid spoilage with the emission of marked odors and a greater production of VRS.

Combining determinations VRS with that of TMA and of the percentage of pigmented bacteria before and after a 5-hour incubation period at 30°C gave a useful indication of the degree of freshness. This allows a reasonable prediction of the storage capability or keeping quality of a raw fish sample (Farber and Lerke, 1961).

A method for determining VRS by microdiffusion analysis was devised by Suzuki (1959b). When comparing the VRS values of canned mackerel (boiled) with those determined by sensory judgment, a good correspondence was observed. It was concluded that the freshness of the original raw fish can be satisfactorily established from the VRS values of canned fish, but not from the amount of volatile basic nitrogen (Suzuki, 1959b).

Inasmuch as the VRS determination gives a measure of the total odoriferous substances present in a sample, it could be expected that in general results from this method would correlate with the sensory evaluation, which is also to a large extent based on the presence of detectable odors. The studies carried out in the author's laboratory over the past ten years or more have led to the conclusion that determination of the content of volatile reducing substances is a useful and practical means to evaluate chemically the amount of spoilage in a wide variety of raw and canned fish and fish products.
2. FRESHNESS TESTS

I. HISTAMINE, HISTAMINE-LIKE SUBSTANCES, AND OTHERS

In recent years, interest in histamine as an index of spoilage of fish has been revived. A special chapter (Volume I, Chapter 10) is devoted to this intriguing subject with its many evasive aspects. Several causal relationships still remain to be elucidated. Until this has been done, the usefulness of histamine as a spoilage indicator is limited. A few studies pertaining to its applicability for this purpose are available (Williams, 1954−1959; Hillig, 1954, 1956a; Yamanishi et al., 1954; Torres-Acero Fernández, 1956). A chemical method for the determination of histamine has been described by Sager and Horowitz (1957). Earlier methods were of a biological nature, which reduced their dependability. Ota and Kaneko (1958) noted differences in histamine production between spoiling fresh and thawed muscles, even when differences in deterioration between the two products, measured in amount of ammonia and the mercuric chloride reaction, were insignificant.

Concerning the possibility of histamine as a poisoning agent, see Volume I, Chapter 10.

J. IODIMETRIC TITRATION AND IODINE UPTAKE

Truttwin (1953, 1954, 1955) presented an iodimetric titration procedure as a means to determine the extent of spoilage of various fish, including cod, hake, haddock, whiting, plaice, herring, and salmon. The originally recommended aqueous suspension was modified later to an aqueous acetone suspension of the fish. An iodine solution in potassium iodide was used as the titrant. Levels for various grades of fish were also given. Orlandi (1955) reported favorable results with this procedure in tests on seven major marine fishes. He recommended as a freshness limit an iodine uptake of 7 ml. of 0.01 N iodine solution per gram of fish. This method was also reported to have given good results by d'Orazio (1956). Varela and Wojciech (1956) found that there was an iodine uptake by hake during spoilage but that the amount depended on the physical state of the fish and the extent of its comminution. They concluded that this test was not a reliable spoilage indicator. Wojciech and Varela (1958) found that the iodine uptake varied with the species of fish or shellfish. (This principle of iodine uptake as a spoilage indicator had been tried on fish many years ago with little or no success. Among the previous reports on the subject are those by Poluektov, 1933; Okolov and Shavskii, 1936; and Shavskii and Vikoulov, 1936.)

The method apparently is based upon the idea that during spoilage substances that react with iodine solution are formed. This premise is
open to criticism, however, since an uptake of iodine is possible (e.g., by proteins and fats) without any relation to spoilage development.

K. PROTEIN HYDROLYTIC PRODUCTS

1. Amino, Carboxyl, and Sulfhydryl Group; Nonprotein Nitrogen, and Biuret Reaction

Over the years the contents of amino nitrogen and of nonprotein nitrogen have been studied as spoilage indicators in fish with variable results. Clark and Alby (1917a, b) studied the increase in amino nitrogen and found some increase with spoilage, but concluded that other more sensitive and reliable tests were needed. Bökman (1918), van Driest (1920), Riffart (1922), Aleev et al. (1936), and others have reported a favorable correlation between the sensory judgment of freshness and the content of amino nitrogen. Nevertheless, the usefulness of the content of amino nitrogen or that of nonprotein nitrogen as a measure of early fish spoilage was questioned by the majority of the reports, e.g., Tillmans and Otto (1924), Bouri (1934), Bouri and Schvinte (1932, 1935), Nickerson and Proctor (1935), Okolov and Shavskiï (1936), Smorodintzev and Kruilova (1936), Salmon and LeGall (1936), Riddell et al. (1937), Beatty and Collins (1939), van de Velde (1940), Labarre and Fougère (1942), Sigurdsson (1947), Partmann (1951), Tanikawa and Akiba (1955), Tanikawa et al. (1952a, b, 1953a, b, c), Alm (1956), Vaisey (1956), Amano and Bito (1951), and Suyama and Konosa (1957).

Among the methods that have been widely used to determine the content of amino groups are the formol titration of Sørensen, Van Slyke's gasometric method, and the copper precipitation procedures of Pope and Stevens (1939). The biuret reaction in relation to spoilage determination has been studied by Proctor et al. (1957), who concluded that it was of no significance for this purpose.

The reaction of ninhydrin with such compounds as amino acids and peptides, to give colored compounds, has been used for studying protein-breakdown products during fish spoilage. Free amino acids and other ninhydrin-reactive substances in cod muscle were determined by twodimensional paper chromatography to determine what changes in the ninhydrin-reactive substances occur as the muscle deteriorates during refrigerated storage (Miyauuchi and Malins, 1957). In ground samples stored for 6 days there occurred a decrease in the amounts of valine and of leucine. Except for these changes, no other ninhydrin-reactive compounds were found by this experimental procedure.

Employing chromatographic procedures, Ranke (1960) noted several changes in the relative amount of free amino acids during
spoilage, some of which possibly could be used for developing freshness tests (see further Volume I, Chapter 16).

The increase in sulfhydryl groups during spoilage has been studied by Okolov and Shavskii (1936) and by Mori and Hata (1949) with divergent conclusions. The latter proposed the determination of sulfhydryl groups by Anson and Mirsky's ferricyanide procedure as a method to estimate fish spoilage. The total sulfhydryl content of shrimp has been followed during spoilage by Kurtzman et al. (1960). They found some increase in the cystine content of spoiled shrimp as well as in the total sulfhydryl content. Neither of these determinations, however, could be considered a sensitive, reliable, and easily carried-out test for freshness or early spoilage.

Related to the possible increase in amino groups during protein breakdown by bacteria is the liberation of carboxyl groups. Tillmans and Otto (1924) followed the increase in carboxyl groups by the Willstätter and Waldschmidt-Leitz double aqueous and alcohol titration procedure in a number of fish species. They found some increase in each case, even though it was rather small.

2. Tyrosine Value

The liberation of substances reacting like tyrosine with the phenol reagents of Folin and Denis (1912a, b) and of Folin and Ciocalteu (1927) and with their modifications has been studied and proposed as a means to measure fish spoilage. Tarr and Bailey (1939) reported that the tyrosine values for halibut "showed such a variation that it would be difficult to establish a level above which fish could be considered unfit." Bradley and Bailey (1940) made a more extensive study of the method for carp, herring, and salmon spoilage and recommended it as a useful indicator of the condition of the fish. Since this work a number of other investigators have used the method with varying results. Wood et al. (1942), Sigurdsson (1947), Rasmussen (1950), Partmann (1951, 1954, 1957), Luijpen (1954a, b), Varela and Wojciech (1956), and Wojciech and Varela (1958) have reported data for the changes in tyrosine values during spoilage of cod, herring, hake, crab, and shrimp at various temperatures. The consensus from all these observations is that the tyrosine value generally increases with advanced spoilage but that it is not very sensitive to changes occurring during the early stages of spoilage. This observation has also been made by Soudan (1950) in a review on spoilage and preservation of fish. At low temperatures the tyrosine formation is very insignificant even if spoilage becomes evident (Bradley and Bailey, 1940; Luijpen, 1954a, 1958).
Vaisey (1956) reported that the increase in tyrosine value of cod fillets after a 3-hour incubation period at ambient temperature appeared to be a more satisfactory index of their condition than the original tyrosine value, which was quite variable. Basically this agrees with the findings of Ota and Ajisaka (1953), in several Japanese fishes, that the relative increase in amount of free tyrosine was, together with the changes in the ammonia values, most useful for the assessing of freshness of fish.

3. Mercuric Chloride precipitation

Amano and Uchiyama (1948, 1949), Amano et al. (1949), Amano and Tomiya (1950a), and Amano (1950) tried a freshness test described by Walkiewicz (1936) employing mercuric chloride and reported some favorable results. Amano (1954) stated that the test was used by local health inspectors as a measure of spoilage. Buffa and Ambanelli (1954) reported that for canned tuna and mackerel this test was useful for distinguishing slight from advanced spoilage by the degree of turbidity and precipitation. Tanikawa et al. (1952a, b) reported favorable results with mackerel but inconsistent results with fresh crab meat (Tanikawa et al., 1955). It was concluded to be more of a qualitative than a quantitative method in studies on hake, squid, and shrimp (Varela and Wojciech, 1956; Wojciech and Varela, 1958).

L. Nucleotides and Derivatives

The importance of adenosinetriphosphate (ATP) and related nucleotides and of sugar phosphates in tissue metabolism and muscle contraction has been known for a long time. In recent years interest has increased in the content of these compounds in fish muscle and in their fate during rigor mortis, low temperature storage, and spoilage. The relation of the nucleotides and their derivatives to the flavor of fish flesh and its changes during storage and to the development of discolorations during heat processing has also been pointed out in the last few years (see Jones, 1961). From the work reported to date on the nucleotides and their changes during storage (including that of Shewan and Jones, 1957; Saito et al., 1959; Jones and Murray, 1960, 1962; Burt, 1961; Bito and Amano, 1962; Tomlinson and Geiger, 1962; and Kassemern et al., 1963) it appears that ATP breaks down rapidly during the early stages of storage with the formation of such derivatives as adenylic acid, inosinic acid, inosine and hypoxanthine, and ribose. It has been suggested that the content of hypoxanthine may be a useful index of quality and freshness of fish muscle during the early stages of chill storage, before bacterial breakdown of the constituents becomes predominant and important.
Further work on this approach to the determination of freshness will reveal its general applicability and usefulness.

M. Fat Spoilage Methods

There appears to be a widespread acceptance that spoilage of fish is indicative of and predominantly the result of bacterial action on the protein constituents. Changes in the fatty constituents of seafood have therefore received much less attention than they deserve.

A number of reviews and general papers on the subject of fat spoilage in food and its mechanism have been published. This entire field was reviewed by Lea in 1938 and by Tarr in 1955b. Extensive bibliographies are appended to these studies. The changes in the fats and oils of fish are further discussed and evaluated in Volume I, Chapter 7, Section II. Khan (1952) studied the possible presence of the enzyme lipoxidase in the flesh of herring and its relation to rancidification. The presumptive role of bacteria in fat rancidification has been reviewed by Jensen and Grettie (1937).

1. Peroxide Tests

a. Iodimetric Titration

The iodimetric estimation of the content of fatty peroxides or hydroperoxides has been used by many as a measure of rancidity (for further references see Lea, 1938, 1952).

Innumerable studies, also of a comparative nature, refer to the methods employed for early detection of oxidative rancidity and further measurement of the progressive fat degradation. A variety of foods have been investigated in this respect. In a great many cases the findings apply to extracted oils or fats. Since this review is primarily concerned with ways of evaluating freshness of fish, only papers conveying relevant information and elucidating early changes in freshness as they can be traced in the fat constituents are considered here.

Regarding the history of particular methods with modifications and improvements, reference is made to a number of analytical surveys, such as Lea (1938), Ishikawa et al. (1957), and Ishikawa and Matsunaga (1958). For studies on fish see Fiedler (1941b), Banks (1937, 1944), and Hartman (1954). Since the existence of fat peroxides or hydroperoxides is of a transient nature and these substances represent but one stage in the chain reaction comprising unsaturated fatty acid and fat oxidation, the presence of peroxides in significant amounts is not always detected in fats that are sensorily judged to be rancid. Indeed, the fat peroxides may be regarded as among the early participants in the oxidative chain of events and often may be detected before any rancidity becomes
definitely evident. Furthermore, the correlation between the content of peroxides and sensorily detectable rancidity is often variable. Depending on conditions of storage and the type of fish undergoing change, the level of peroxides, determined iodimetrically or otherwise, at which rancidity becomes sensorily detectable may vary within rather wide limits. Notwithstanding the foregoing and other disadvantages, iodimetric estimation of the content of fat peroxides is probably the most widely used chemical test of rancidity.

b. Ferrimetric Method

Another procedure to determine the content of fat peroxides is based on the oxidation of ferrous ions to ferric ions by the peroxide present and the estimation of the amount of ferric ions formed by their reaction with thiocyanate to give rise to red ferric thiocyanate. Lea (1952) compared this procedure with iodimetric titration and concluded that the ferrimetric method was more sensitive and potentially more useful for studies on a micro scale or for the earliest stages of oxidation. Smith (1952) found this method most useful in studying changes in lean and fatty tissue of whales.

c. Leuco-dichlorophenol-indophenol Oxidation

This method is based on the reoxidation to a colored dye of reduced dichlorophenol-indophenol by the peroxides present and of its spectrophotometric determination. Hartman and White (1952) and Lea (1952) compared it with both the iodimetric and ferrimetric procedures and concluded that it was less useful than either since (1) high values were found in the presence of oxygen, (2) it was susceptible to interference by traces of copper, and (3) it was less reproducible.

2. Kreis Test

This method was described as early as 1902 but has since undergone many modifications (for further references, see Lea, 1938, p. 98). Since the original paper, many attempts have been made to improve the test so as to make it more quantitative, and to explain it. Jones (1924) suggested that it was based on the presence of epichlorhydrin in the rancid fat. Kerr (1918) concluded that the Kreis test was only roughly proportional to the degree of rancidity. Holm and Greenbank (1923) studied the quantitative aspects of the Kreis test; Lea (1931) investigated this test along with others; Walters et al. (1938) proposed a modification. Watts and Major (1946) reported a simplified quantitative Kreis test technique and compared the results with the peroxide values of oxidizing fats. Patton et al. (1951) studied the mechanism of the reaction. In
2. FRESHNESS TESTS

general, however, this test has not proved to have widespread applicability, since negative results have been found for sensorily rancid fats and vice versa.

3. Aldehyde Tests

a. Schiff’s Test

This is a test in which fuchsin, decolorized by sulfur dioxide, is re-colored by any aldehyde that may be present in a rancid fat. Von Fellenberg (1924) and Lea (1931) reported on the usefulness of this test. Fiedler (1941b) studied fish oils and compared the iodimetric peroxide determinations with a Schibsted (1932) modification of Schiff’s test.

b. 2-Thiobarbituric Acid Test

Patton and Kurtz (1951, 1955) suggested 2-thiobarbituric acid as a reagent to detect fat oxidation in milk. This was followed by a number of studies of the application of this color reaction as a measure of fat rancidity in other foods. Findings on fish have been reported by Yu and Sinnhuber (1957), Sinnhuber and Yu (1958), Sinnhuber et al. (1958), and Andresson and Danielson (1961). Sinnhuber et al. (1958) showed that malonaldehyde was the likely compound in fats which condensed with 2-thiobarbituric acid to form the red product. Only about 2% of the potential malonaldehyde, however, was free in the fat. The remainder was liberated from some precursor by a preliminary acid treatment. Ryan and Stansby (1959) and Andresson and Danielson (1961) reported favorable results for the correlation of this test with the sensory judgment of herring rancidity. Schwartz and Watts (1957) showed that the deterioration of refrigerated cooked oysters, but not that of raw oysters, could be followed satisfactorily by this method.

4. Acid Value and Free Fatty Acid Content

Not all fat changes are mainly oxidative in nature. Some involve the hydrolytic production or liberation of fatty acids as well as of other organic acids. Brocklesby (1932, 1933a, b) studied the hydrolysis of salmon oil. Ono (1935) showed that fatty acids increased in sardines and mackerel at low temperatures. Charnley and Davies (1944) suggested that the acid value of the oil could be used as an index of the condition of canned herring. Rockwood et al. (1947) studied the hydrolytic changes leading to free fatty acids.

Boury (1945) stated that the acid value of fish fat could serve as a supplementary but not primary measure of spoilage. Luijpen (1954a, b) reported that the acid value of salted herring fat showed no direct relation to the storage period or temperature. Dyer and Fraser (1959)
investigated the lipid hydrolysis and its relation to protein changes during low temperature storage of cod.

5. *Steam-Volatile Reducing Substances*

Mayrhofer (1898) reported on a method for the determination of rancid butter in which a steam distillate was treated with potassium permanganate in an alkaline solution. The amount of reduction found was a measure of the condition of the original fat sample. Issoglio (1916) used this procedure to obtain an "oxidation index" for fats, whereby the amount of permanganate reduced in acid solution per gram of fat was used as a measure of the condition of the fat. Strohecker *et al.* (1937) found that the content of oxidizable steam-volatile substances was a useful measure of the condition of fish fat.

6. *Carbonyl Compounds*

The presence of various carbonyl compounds inclusive of special ketones has been used in a variety of foods for the study of quality deterioration due to fat rancidity. Little has been done with fish in this respect. Ota (1958a, b) presented a simplified chromatographic method for measuring volatile carbonyl compounds (VC) in fish. In fresh fish, VC were very small in amount. Their contents increased with the length of storage time, and tended to mount in stages of advanced spoilage. The rate of VC formation varied according to the state of flesh stored and also with different portions of the fish. The gradual formation of acetaldehyde, butyraldehyde, and acetoin in fish flesh during storage was established.

Other changes related to fat deterioration, such as "rusting" or yellowing, and freezer or "belly burn," are discussed from the point of view of their etiology in Volume I, Chapter 7, p. 238. Special mention will be made here only of the studies on rusting by Tester (1941) on Canadian herring and by Ando (1956) on Japanese boiled and dried fishes.

N. *Miscellaneous Methods*

1. *Ammonium Vanadate Reaction*

Lassandro-Pepe and Maraglino (1954) described a procedure for the appraisal of the freshness of fish based on the color resulting from the interaction of a 1% sulfuric acid solution of ammonium vanadate with a fish extract. It was claimed that fresh flesh gave an emerald green color, which decreased to a light green at a stage of incipient spoilage and to white with the onset of definite spoilage. Proctor *et al.* (1957) found no direct correlation of the color with the stage of fish spoilage.
2. Lanthanum Blue Reaction

Demour's reaction of acetic acid with a lanthanum solution in which a blue color resulted was modified and extended by Krüger and Tschirch (1929, 1930a, b, c). Caselli and Ciaranfi (1942) reported that the formation of the lanthan blue color was inhibited by fish extracts, and that the inhibition increased as the fish decomposed. Suzuki (1953b) further tested this procedure and reported that the extent of inhibition of the lanthan blue color formation became greater as spoilage developed. He proposed that the amount of fish extract necessary to impede formation of the blue color could be used as a measure of the extent of spoilage. In a recent study Suzuki (1959a) could show that the time elapsed after capture could be satisfactorily determined for snapper and flatfishes.

3. Paraquinone Reaction

Obata and Zama (1950a, b) and Obata and Ichida (1950) reported the formation of piperidine and pyrrolidine during fish decomposition. They determined these substances by the reddish brown color of pyridyl quinone or pyrrolidyl quinone formed with paraquinone. They claimed this test was of value in the examination of canned fish and vitamin oils. Bortone and Testa (1956) also tested this procedure for judging the freshness of fish and found it satisfactory for teleosts, but not for mollusks and crustaceans. Proctor et al. (1957) stated that this test and others for piperidine did not correlate with the sensory judgment of spoilage.

4. Phthalein Value

Miyake and Hayashi (1955) reported a study of the relation between the post-mortem storage time and the change in the so-called phthalein value of aquatic animals. The dilutions of aqueous fish extracts found to recolor reduced alkaline Phenolphthalein solutions in the presence of acetic acid and hydrogen peroxide became less as spoilage developed. This procedure was first described by Hattori and Akiba (1952).

5. Lactic Acid

Macleod and Simpson (1927) studied the post-mortem production of lactic acid in fish muscle. For further reference see Volume I, Chapter 12, pp. 397-403.

Fiedler (1941a) reported on the formation of lactic acid as an index of freshness of frozen fish. The content of lactic acid, a product mainly of carbohydrate glycolysis, probably depends too much upon the state of nutrition and circumstances of capture of the fish or shellfish to offer a useful means of evaluating the early stages of spoilage.
6. Other Tests

Bukowska (1955) proposed using the change in color produced by the addition of bromthymol blue to fish extracts as a measure of the freshness of fish. Proctor et al. (1957) attempted to use the color changes of malachite green oxalate and bromphenol blue with a fish extract as a measure of piperidine formed during decomposition of haddock. These authors also attempted to use the color formed when sulfosalicylic acid was added to fish extracts and the determination of creatinine as measures of fish spoilage. None of these tests showed a correlation with the development of spoilage in haddock.

VI. Biological Methods

A. Nitrate Reduction

The reduction of nitrate has been used as a means of evaluating the extent of bacterial contamination and of bacterial growth during fish spoilage (Tillmans et al., 1921; Tillmans and Otto, 1924). No clear relationship between the amount of reduction and the state of spoilage was evident, and it was not found to be an accurate measure of bacterial growth.

B. Oxygen Consumption

The oxygen uptake by fish during spoilage has been suggested as a freshness test but was found unacceptable for this purpose (Tillmans and Mildner, 1916; Tillmans et al., 1921; Tillmans and Otto, 1924).

C. Dye Reduction Tests

Another means to obtain a measure of the extent of bacterial growth and of the bacterial content as a gauge of the degree of spoilage was the reduction of various dyes.

1. Methylene Blue

Tillmans et al. (1921), Tillmans and Otto (1924), Okolov (1936), van de Velde (1937), and Rasmussen (1950) all found some decrease in the reduction time with the onset of fish spoilage and the increase in bacterial numbers. Cavallone (1959) found this method unreliable as a freshness test of fish.

2. Resazurin

Resazurin, originally used to test the quality of raw milk, has been used a number of times as a measure of the bacterial population of perishable foods, including fish and shellfish. Among the reports of its use are those by Waldbauer (1931), Mundinger and Wolff (1933),
2. FRESHNESS TESTS

The color attained by resazurin in milk of low bacterial content depends upon the redox potential value of the equilibrium set up by the mixing of the reducing systems of the dye and milk and is not directly related to the number of bacteria in the milk (Thornton et al., 1941). Similar considerations could apply to other foods, including fish, and could help to explain the general observation that the reduction rate of the dye was of value as a measure of a high bacterial action but was of little significance for the early spoilage stages where the bacterial numbers and their products were only beginning to increase. Uno and Tokunaga (1954) studied the relationship between the reduction time of resazurin and the freshness of fish flesh. They found this test useful for herring, but not for mackerel. The pH had a definite effect on the reaction. Cavallone (1959) reports good results with Italian fresh-water fish.

3. Tetrazolium Derivatives

Colorless substituted tetrazolium (TZ) salts are reduced in neutral solutions by living cells which then become stained red by the formation of formazan. These salts have been used extensively for viability tests and in microbial and metabolic studies. Shewan and Liston (1957) reported on several years of investigation of the usefulness of this dye test for the purpose of assessing the quality of iced white fish. They found that a special new derivative, 2-p-iodophenyl-3-p-nitrophenyl-5-phenyl tetrazolium chloride, was more useful as a sensitive indicator compared with the traditional TZ salts. They described a technique of using filter paper impregnated with the dye and either judging the color visually or, after extraction, spectrophotometrically. The general value of this test for whole fish and perhaps for fillets will become clearer as more data become available for various species stored under different conditions.

Moorjani and Iyengar (1957) and Moorjani et al. (1957) measured the quality of Indian fresh-water fish and studied the effect of pH, incubation period, and fish species on the reduction time. It was claimed that this test was of less value in fishes with a high content of TMAO, since this substance apparently exerted a poising effect on the redox potential of the system and thus tended to delay the reaction.

4. Other Dyes

Okolov (1936) tested the reduction rate of indigo carmine as a measure of spoilage and concluded it was not as useful as methylene blue. In 1937, van de Velde used the reduction of Janus green to a red
color as a measure of spoilage, in addition to methylene blue and resazurin. He found that the reduction time for all the dyes increased with the progress of spoilage.

D. BACTERIAL COUNTS

1. Direct Count

Tarr (1941, 1945) published a technique designed to give a direct evaluation of the number of bacteria in a sample. This method was useful where the number of bacteria was above a certain low level, which represented the limit of sensitivity of the procedure. Tarr also recommended a short incubation period to increase the sensitivity. The direct count included any nonviable organisms that may have been present. Wittfogel (1953, 1955a, b) used the direct count to assess the condition of marine fish. Direct bacterial counts have been used by Tarr (1944a, 1945) to measure the condition of fish and were advocated by him in a recent paper (Tarr, 1958) as other methods of a chemical nature fail to offer guidance as to freshness.

2. Total Viable Count

The most commonly used test for bacterial contamination is the total viable count and its many variations and modifications. This has been used by many individuals to obtain a measure of the condition of a sample of fish. Total aerobic bacterial counts have been used by many investigators to follow the deterioration of fish flesh and shellfish flesh, among whom are Griffiths and Stansby (1934), Boury and Schvinte (1932, 1935), Aleev et al. (1936), Fitzgerald and Conway (1937), Salmon et al. (1937), van de Velde (1937) (who considered the count of little value), Proctor and Greenlie (1939), Castell et al. (1948), Rasmussen (1950), Luijpen (1954a), Partmann (1954), Tanikawa et al. (1955), Farber and Lerke (1956a, b, 1957, 1958), Novak et al. (1956), Fieger et al. (1958), and Moorjani et al. (1958).

Sekine and Nakakubo (1953) described a procedure for placing a sterilized parchment paper strip on the surface of the fish and then on the surface of a solid medium to obtain a measure of the bacterial contamination.

The type of bacterial flora and its relation to fish spoilage has been studied by Hunter (1920a, b, 1922a, b, c), Schönberg (1930), Schönberg and Debelic (1933), Tarr (1938), Shewan (1938, 1944, 1953), Snow and Beard (1939), Notevarp et al. (1942), Kiser (1944), Kiser and Beckwith (1944), Liston (1957), Nikkilä (1955), Lerke et al. (1963), and Adams et al. (1964).

Reviews covering the bacteriology of fish spoilage have been pub-
lished by Griffiths (1937), Shewan (1949), and Tarr (1954). Furthermore, special chapters in Volume I of this treatise deal with the bacteriological aspects of spoilage in sea-water fish by Shewan (Volume I, Chapter 14), in fresh-water fish by Bramstedt and Auerbach (Volume I, Chapter 16), and on shellfish by Fieger and Novak (Volume I, Chapter 15).

E. Enzyme Activity Tests

1. Catalase

Tillmans et al. (1927) tested catalase activity as a criterion of spoilage and found it too variable or irregular to be relied on. Poluektov (1933) also reported that the increase in catalase activity of fish was too variable to be used as a criterion of spoilage. Tomiyama et al. (1951) and Tomiyama and Yone (1953) used the catalase activity of gill tissue and of surface washings of "kamaboko" (see Volume II, Chapter 8) as a measure of bacterial activity and of the condition of the preparation. They reported that the catalase activity of the surface washings paralleled the growth of bacteria on the surface.

2. Peroxidase

Poluektov (1933) found the peroxidase activity of fish too variable to be of use as a spoilage indicator. Okolov (1936), and Okolov and Shavskiï (1963) reported that the peroxidase activity could be used as a criterion of the quality of salted fish and of the brines surrounding them.

3. Succinic Dehydrogenase

Fukuda, in a series of papers (1957, 1958), reported on the estimation of freshness of fish by the succinic dehydrogenase activity of the flesh and viscera. Even though the individual values fluctuated, there was in general a reduced activity with the decrease in freshness of the samples.

4. Other Enzymes

Tillmans et al. (1927) found no change in the activities of diastase or of proteolytic enzymes as spoilage progressed. Fiedler (1941a) reported that there was an indication of a relationship between the age of fish and their autolytic proteolytic enzyme activity; enzymes in older fish reacted more slowly than those in younger fish. He suggested that this may have a bearing on their subsequent keeping quality in cold storage.

VII. Concluding Remarks

From the extensive literature reviewed it is evident that the sensory judgment, particularly that based on the olfactory sense, has been and still is the most frequently and widely used means for the evaluation of
the state of freshness of fish, shellfish, and their products. It is also the reference criterion against which the usefulness of other methods is judged. Nevertheless, everyone concerned with freshness evaluation realized long ago the limitations inherent in the use of the senses as measures of quality, and the need for an objective and quantitative yardstick with which to compare the subjective results of the sensory tests was recognized. Methods to accomplish this purpose fall into two possible categories: (1) freshness tests to detect the early stages of the progressive deteriorative process up to the formation of the first perceptible odors not occurring in the original material, and (2) spoilage tests to detect and measure those changes in the transition stage from fresh to spoiled that are associated with the presence of undesirable and unpleasant odors, mainly resulting from bacterial actions which render the product unacceptable. At the present time not one of the reviewed methods is accepted as a true freshness test, that is, one which would enable the determination, while the sample was still judged usable, of the degree of change which had occurred in the muscle constituents since removal of the fish or shellfish from the original environment. The report by Farber and Lerke (1961) on the use of an incubation procedure along with the determination of substances volatile in air at ambient temperatures offered a means of assessing freshness not otherwise. Recent work on the changes in the nucleotides of fish muscle during the early stages of storage before any off-odors are detectable offers a more hopeful possibility for a true freshness test. Future studies on a large number of species stored under diverse conditions will show whether this approach will develop into a generally applicable procedure for estimating the freshness of a sample as compared with its condition on removal from its normal habitat.

The chemical methods proposed to date for quality evaluation depend mostly on the presence of one or more products of degradation of the muscle constituents, e.g., volatile nitrogenous bases, volatile acids, indole and skatole, hydrogen sulfide, and carbonyl compounds. Since the formation of these products, largely volatile and odoriferous, is the result of bacterial action, and since the bacterial flora of fish and shellfish may vary, chemical tests based on the presence of a single compound or type of compound have proven unreliable and less sensitive than the sensory judgment. The only chemical tests which can be expected to have a more general applicability and to give results correlating with the sensory evaluations are those based on the determination of the aggregate of the volatile constituents in a sample. The measurement of steam volatile substances by Mayrhofer (1898), Issoglio (1916), Strohecker et al. (1937), and Tomiyasu et al. (1951) was an approach toward this goal.
This procedure has the drawback that it determines not only the substances which are volatile in air (those mainly responsible for the odor of a sample) but also higher boiling compounds volatilized in steam as well as volatile products arising from the breakdown of nonvolatile precursors. The determination of substances volatile in air at ambient temperatures (e.g., see Farber, 1963) does not have this disadvantage, more closely approximates the olfactory test, and affords a measure of the odor intensity of a sample relative to that of a reference standard, such as very fresh material. This procedure has the further advantage that it can be used for all types of marine products, both raw and processed, including those preserved by salting, pickling, smoking, and canning. This test, together with the more restricted determination of trimethylamine in white-fleshed marine fish species and in shellfish, of all the available spoilage tests, gives the best correlation with sensory judgment and has the greatest range of application.

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2. FRESHNESS TESTS


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Hennings (1963, 1964) has recently described a new apparatus and procedure for the estimation of freshness of whole fish. This depends upon the measurement of the AC resistance or impedance at two frequencies of a fish between two electrodes connected to the measuring device. The impedance difference or "Q" value decreases with age and deterioration. However a number of investigators have reported variations in the values resulting from physical breaks of the skin and tissues, removal of scales, and other environmental factors. Further studies on this method will reveal its practical significance and place in the testing of freshness of whole fish.