trate in cotyledons from nonbitter peanuts on the supposition that these substances occur there normally, but at low levels. Substances that stain reddish purple with sulfuric acid plus heat and which chromatograph like the components of the bitter concentrate from hearts were found in the cotyledons. It took 20 times as much cotyledons as hearts to give roughly an equivalent amount of material as measured by glass paper chromatography. Small fragments of the peanut hearts which had adhered to the cotyledons could account for the substances observed. This possibility was ruled out by carving out and discarding the region of the cotyledon joining the heart and cotyledon together.

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TUNA MEAT PIGMENT STUDIES

Spectral Reflectance Studies of the Heme Pigments in Tuna Fish Flesh. Some Characteristics of the Pigments and Discoloration of Tuna Meat

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During investigations on the "greening" in certain yellowfin tuna on precooking, information regarding the characteristics and reactions of the heme pigments in tuna meat was revealed, largely through application of the technique of spectral reflectance. Spectral evidence indicated a predominance of hemoglobin rather than myoglobin in the flesh pigments, an increase in methemoglobin and denaturation on storage, and a greater solubility of methemoglobin in aqueous media in contrast to oxyhemoglobin. Greening is an actual color condition due to an anomalous heme protein oxidation. Related are high concentrations of methemoglobin, some denaturation, and a slightly high fat peroxide content in the raw meat. Oxygen starvation due to the exhaustion of the fish during landing does not appear to produce factors that lead to greening, but changes that occur even in the frozen state seem to be responsible.

THE DISCOLORATION of meatstuffs is a problem familiar to the packers and processors of such products. Present knowledge of the cause and probable mechanisms producing a variety of such conditions has been summarized by Watts (*24*). One similar off-color variation recognized in fish meat is the undesirable "green" color that develops in the flesh of certain tuna on precooking, prior to canning, and results in considerable economic loss to the fisherman and to the industry (*19*).

The problem has been the subject of intensive research by the Japanese (*16*). Matsuzaka and Takahashi (*15*) have reported a correlation between the greening of tuna and the concentration of a flesh pigment identified spectrophotometrically by them as myoglobin. More recently Brown, Tappel, and Olcott (*5*) have reported on work which indicates that the pigment responsible for greening is a hemichrome.

The advantages of spectral reflectance applied to biochemical problems have been pointed out (*4*, *18*). Application of this method to the present investigation was obvious, because it made possible the study of the normal or anomalous protein pigments in raw and cooked flesh, and of the changes induced by the variation of experimental conditions.

Materials and Methods

Samples used in this research were carefully trimmed sections of the loins of yellowfish tuna (70 to 200 pounds), *Neothunnus macropterus* (Temminck and Schlegel), caught on long line by research vessels of the Pacific Oceanic Fishery Investigations of the U. S. Fish and Wildlife Service in the central equatorial Pacific. The fish were frozen on board, as soon as possible after capture. Yellowfin up to 15 pounds were caught by trolling in local waters and sampled while fresh.

To obtain precooked samples for the evaluation of color, frozen fish were thawed in tanks at Hawaiian Tuna Packers, Ltd., and cut into loins. One loin from each fish was retained as a raw sample. The remaining loins were put through the regular commercial precooking process of that company. Color grade of the precooked samples was judged by the company's food technologist. Specific samples characteristic of their types (normal, green, pink, etc.) as subjectively judged, were used in much of the work.

Reflectance spectra in the visible range (400 to 700 mμ) were obtained with a Beckman DU spectrophotometer equipped with a standard reflectance attachment. All chemicals used were reagent grade.

The meat was forced through a 16-mesh stainless steel screen and packed it into a $1^1/_4 \times {}^1/_{16}$ inch aluminum planchet. Chemical treatment was accomplished by adding the reagents to the meat in the planchet. A glass plate was then pressed firmly upon the meat until a uniformly smooth surface was produced, to obtain a diffuse surface and eliminate gloss or directional color. Immediately after preparation the planchet was introduced into the reflectance attachment of the spectrophotometer, and relative reflectance was measured by comparison with a pure high-fired alumina disk, likewise covered with a glass plate. Apart from oxidative variations, well ground and thoroughly homogenized samples gave readings that were reproducible to ±0.5% reflectance unit. Replicate, nonhomogenized samples from the same fish loin gave an average precision of ±1.5% reflectance unit. Absorbance (optical density) was used in the curve plots, because Beer's law was obeyed in the systems studied.

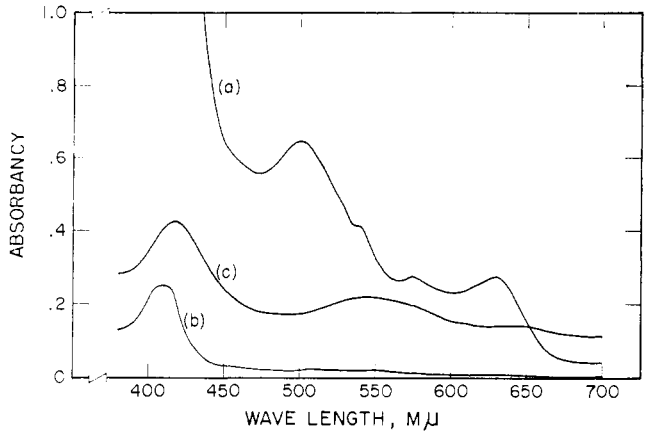

Figure 1. Separation of aqueous extract of raw tuna into methemoglobin and metmyoglobin by phosphate fractionation

a. Transmission curve for original extract
b. Transmission curve for residual solution after methemoglobin precipitation with phosphate
c. Reflectance curve of phosphate precipitated material

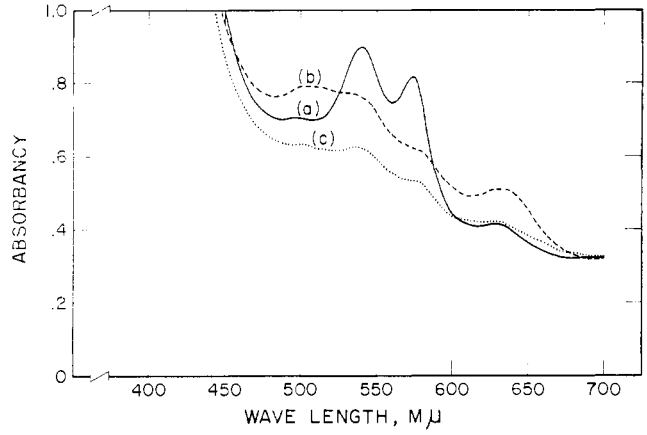

Figure 2. Reflectance curves showing pigment mixture found in raw tuna flesh

a. Largely oxyhemoglobin
b. Largely methemoglobin
c. Mixture of met- and oxyhemoglobin

SOME CHARACTERISTICS OF THE PIGMENTS

Nature of Pigments in Raw Tuna Meat

A natural assumption is that myoglobin, or muscle hemoglobin, is a major pigment imparting color to the tuna flesh. Certain experiments have cast doubt on the validity of this assumption. Bowen (*2*) and others have found that absorption peaks for mammalian myoglobin are displaced to longer wave lengths in the case of certain derivatives of the pigment. The peaks for the corresponding derivatives of tuna pigments were carefully measured and no such shift was observed. Instead, the curves obtained were identical with those characteristic of hemoglobin and its derivatives. To substantiate these results, the solubility of the extracted pigments in buffered phosphate was checked according to the procedure of Morgan (*17*) as modified by Ginger, Wilson, and Schweigert (*10*). The separation of the myoglobin from the hemoglobin by this method is based upon the solubility of the former and the insolubility of the latter compound in the phosphate buffer. The myoglobin was estimated as metmyoglobin through transmission measurements and the hemoglobin as methemoglobin by the examination of the residue through spectral reflectance.

Assuming that these procedures separate myoglobin from hemoglobin on the basis of their solubility in the differentiating medium, it was found that the pigments in tuna flesh were about 95% hemoglobin, with myoglobin comprising a minor fraction of the pigment system (Figure 1). Matsuura and Hashimoto (*14*) report all pigment in ordinary muscle of yellowfin tuna as hemoglobin (140 mg.%). The experiments described have not been sufficiently exhaustive to warrant a definite conclusion regarding this point. The main muscle pigment in tuna meat, if not hemoglobin, may be different from the corresponding pigment in mammalian flesh (*20*). Thus, the actual nature of the pigment in tuna meat is not yet definitely established. The distribution of the pigments, however, between myoglobin and hemoglobin is not pertinent to the color changes in tuna meat, because these involve the porphyrin moiety which is identical for both. The pigments in tuna flesh are referred to hereafter as hemoglobin or its derivatives.

Examination of the raw tuna meat by means of spectral reflectance seemed to indicate that oxy- and methemoglobin were the principal heme pigments present. Some typical curves characteristic of the oxy- and methemoglobin mixtures encountered are shown in Figure 2.

Pigment Changes in Raw Flesh

Conversion of Oxyhemoglobin. Fairly rapid oxidative deterioration was observed in tuna meat even when stored in a freezer. The concomitant conversion of oxyhemoglobin with time was observed by following the change in the reflectance spectra periodically (Figure 3). Complete conversion to methemoglobin was accomplished quickly by oxidation with potassium ferricyanide (Figure 3,*B*,*e*). That the changes correspond to a decrease in oxyhemoglobin can be verified by comparison of these data, presented as simplified curves, with similar changes in absorption obtained by Austin and Drabkin (*1*) for oxy- and methemoglobin mixtures of known composition (Figure 3,*A*).

Examination of these results indicated that the pigment systems in tuna meat were extremely susceptible to environmental changes. The major change that takes place at freezer temperatures is a conversion of oxyhemoglobin to methemoglobin which is not desirable, because it is accompanied by a certain degree of discoloration. This, in turn, might decrease the value of the meat from the commercial standpoint.

Deoxygenation of Oxyhemoglobin. An attempt at reducing oxyhemoglobin to hemoglobin by exposing the tuna meat to evacuation and a purified nitrogen purge, a procedure which normally removes the oxygen of the oxycompound, resulted in a rapid conversion of oxyhemoglobin to methemoglobin. Typical reflectance spectra showing this change are presented in Figure 4. The results may be an example of the phenomenon noted by Brooks (*3*), who found that the conversion of oxyhemoglobin to methemoglobin proceeded more

rapidly at low partial pressures of oxygen, with a maximum at 20 mm.

Pigment Denaturation. Examination of the spectral reflectance curves of raw tuna meat reveals that some degree of denaturation of the protein moiety of the heme pigment molecule takes place even at low temperatures. Such denaturation produces denatured globin hemichromes (*12*) more easily identified in the reduced condition as the denatured globin hemochromes, because their absorption curves exhibit pronounced peaks at 528 and 558 mμ. Figure 5 shows representative spectral curves obtained with tuna meat. Fresh tuna meat, chemically reduced with dithionite, gives the characteristic absorption peak of hemoglobin at 555 mμ (Figure 5, *a*). When subjected to mild denaturation by heating to 50° C. for 20 minutes the same reduced meat exhibits the distinct hemochrome peak at 528 mμ (Figure 5,*b*). Similar denaturation can be observed in curve *c* for a reduced raw sample which is rich in methemoglobin and in curve *d* for a second sample of the same type of meat commercially precooked and reduced. It is remarkable that precooking, as in the last instance, does not appear to have increased the denaturation of the pigment markedly as judged by the hemochrome peak at 528 mμ. There is some indication from a number of such raw, reduced samples that the degree of denaturation in tuna meat is directly related to the amount of methemoglobin originally present.

Solubility of Methemoglobin. The unexpectedly greater solubility of methemoglobin in aqueous media when compared to oxyhemoglobin in tuna meat is an example of unusual pigment changes and behavior encountered in this research. Aqueous extracts of tuna flesh gave absorption curves characteristic of methemoglobin. Examination of the reflection spectra of tuna meat obtained before and after subjecting samples to leaching action showed that methemoglobin is characterized by a rather great solubility in water, whereas oxyhemoglobin exhibits a high degree of insolubility. A typical case demonstrating this difference is shown in Figure 6. This suggests that pigment leaching during thawing and precooking might be a factor in producing a washed-out condition in tuna and in other meats with a high methemoglobin content. It might also be applied as a means of lightening meat color, and of removing flesh pigments that might be decolorized during precooking.

Pigments in Cooked Tuna Meat

Cooking of tuna meat results in the denaturation and coagulation of the proteins present with concomitant lightening of the meat color. Denaturation of the heme proteins leads to the formation of the hemochrome of the denatured globin. Characterizing absorption, aside from the Soret region, occurs at 545 and 575 mμ (Figure 7, curves *a* and *b*), in agreement with the values listed for the denatured hemochrome of hemoglobin (*12*). Because the absorption of this substance is weak and poorly defined, the more easily characterized curve of the reduced, or ferrous, denatured globin hemochrome previously mentioned was used for identification purposes. The contrast is evident in the examples given in Figure 7. The color of the reduced hemochrome is the normal pink which is considered commercially desirable.

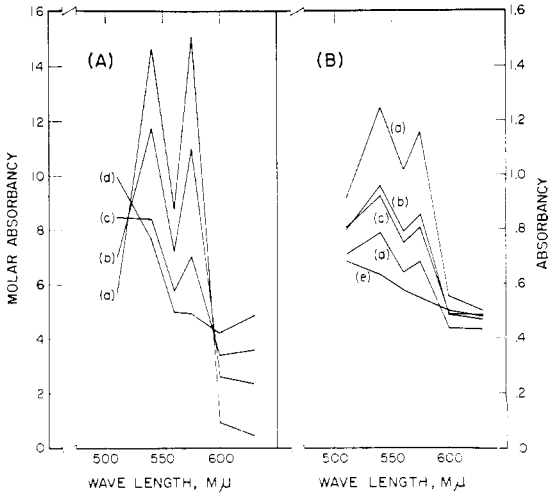

Figure 3. Absorption curves

A. Simplified curve shows decreasing concentrotion of oxyhemoglobin, in mixture with methemoglobin decreasing from *a* to *d* (*1*)
B. Similar simplified curve taken in reflection for tuna meat after various periods of freezer storage, showing decreasing oxyhemoglobin content

a. Fresh tuna meat
b. After 24 hours of freezer storage
c. After 36 hours of freezer storage
d. After 84 hours of freezer storage
e. After complete conversion to methemoglobin by oxidation with potassium ferricyanide

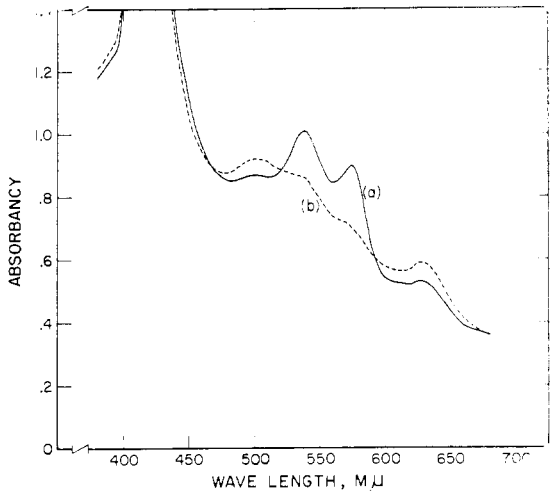

Figure 4. Oxidation of flesh pigment on treatment with nitrogen

a. Reflectance curve of fresh tuna meat
b. Reflectance curve after alternating evacuation and treatment with nitrogen for 3 hours

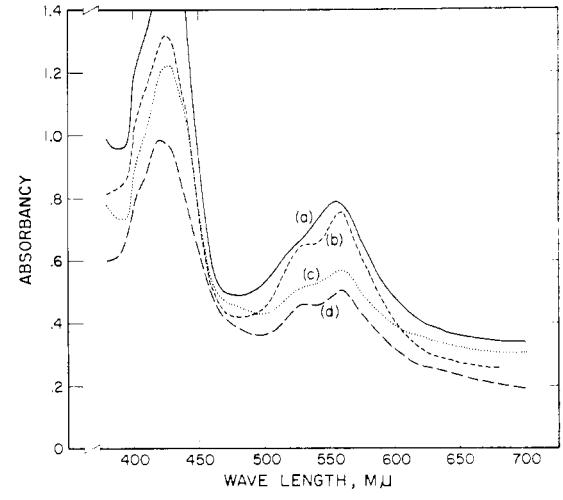

Figure 5. Reflectance curves showing denaturation by appearance of hemochrome band at 528 mμ after reduction with dithionite

a. Reduced raw fresh tuna hemoglobin
b. Tuna flesh of curve *a* after denaturation by heating 20 minutes at 50° C.
c. Reduced raw methemoglobin rich sample
d. Methemoglobin rich sample, commercially precooked, and reduced

DISCOLORATION OF TUNA MEAT

Color of Precooked Tuna Meat. Visual examination of different samples of cooked tuna meat reveals the complexity of the color system involved. Variations from highly bleached to abnormally red meat have been encountered which demonstrate the fluctuations of the absolute content of pigment in the flesh of different fish. It has not been determined whether these variations are physiological or post-mortem in nature. A brown condition is recognized

which appears to be a true post-mortem or postcooking phenomenon, due to oxidation of the natural hemochrome on exposure to atmospheric oxygen (*4*). The greening phenomenon, the main object of this research, and an orange coloration are also frequently observed. Both discolorations permeate the fish flesh rather than being associated with exposed surfaces as in the brown condition. The distribution of pigment within a single fish loin, seemingly uniform to the eye, was variable. This was confirmed by a superficial microscopic examination which showed the pigment to be present in bands within the metal. Spectral reflectance measurements gave variable estimates of the amount of pigment present. Uniformity could be assured only by grinding and thoroughly mixing all the meat required in a given sample sequence. This was not usually done because finely ground meat changed color even when held in a frozen condition.

A method used to evaluate greenness objectively was to compare absorption in the red and green parts of the spectrum (640 and 540 mμ, respectively) for cooked, green, and normal meat by reflectance. A green sample would be expected to show greater absorption of light in the red end of the spectrum than in the green, when compared with normal meat. The ratio of the absorbancies at the 540/640-mμ absorption peaks should be greatest for normal and least for green meat. Absorbancy ratios have been used by other investigators (*6*) in evaluating, objectively, the color of cured meats.

It became evident that pigment content was a factor affecting the degree or type of alteration that might occur. Upon the assumption that the height of the most characteristic absorption peak for heme pigments (the Soret peak, at about 415 mμ) can be used as an indication of the absolute pigment content of the cooked meat, several significant relationships were observed by plotting this peak against the above indicated greenness index (540/640 absorption peak ratio) (Figure 8). Normal samples, in general, occupy the portion of the figure indicative of higher 540/640 peak ratio as would be expected, and lower pigment content. It is noteworthy that both the albacore white color resulting from low pigment content and the fleshy pink color associated with a high 540/640 peak ratio are accepted as normal by observers, thus emphasizing the complexity of the systems encompassed by the term normal. The overlapping position of some of the points is due, at least in part, to the uncertainty and ambiguity inherent in the subjective evaluation of precooked flesh.

Green Pigmentation in Precooked Tuna Flesh. It was impractical to extract and identify the green component in precooked green flesh by the use of simple solvent systems. Attempts were made to cleave the pyrrole ring segment from the protein moiety of the pigment molecule by alcohol– or acetone–hydrochloric acid treatment, and to examine the extract by spectral transmission, and the residue by spectral reflection. Green pigmentation in meat has been attributed to an oxidative attack on the pyrrole ring of the heme pigments producing choleglobin or verdohemochrome (*24*). Removal of the oxidized or disrupted ring by the acid-alcohol or acetone treatment, and spectral examination should reveal the nature of the parent pigment. Verdohemochrome, and its postulated precursor choleglobin, for example, would produce biliverdin-like compounds (*12*).

The acid alcohol extracts gave an absorption curve in transmission which showed no characteristic absorption peaks and was very similar to that obtained from the aqueous residue left on cooking the fish (Figure 9, *b*). Extraction of this material with chloroform, however, gave in the extract an absorption curve with transmission peaks at 382, 510, 540, and 640 mμ (Figure 9, curves *a* and *c*). These absorption maxima are compared in Table I with the peaks reported for some of the products of heme protein disruption.

The coincidence of the absorption peaks with those reported by Lewis (*13*) for hemin chloride in acetone with excess hydrochloric acid added is striking. It indicates that this substance may be present in the extract. Microscopic examination of the evaporated residue of these extracts failed to reveal the characteristic hemin chloride platelets. No evidence of oxidative disruption of the pyrrole ring was obtained in the soluble fraction from acid-acetone or acid-alcohol treatment.

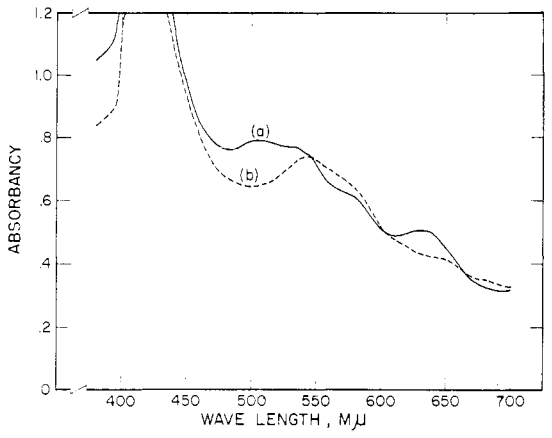

Figure 6. Reflectance curves showing solubility of methemoglobin and insolubility of oxyhemoglobin in raw tuna flesh

a. Raw tuna flesh
b. Residual flesh after aqueous extraction at pH 6.8. Note disappearance of 500 and 630 mμ methemoglobin absorption peaks

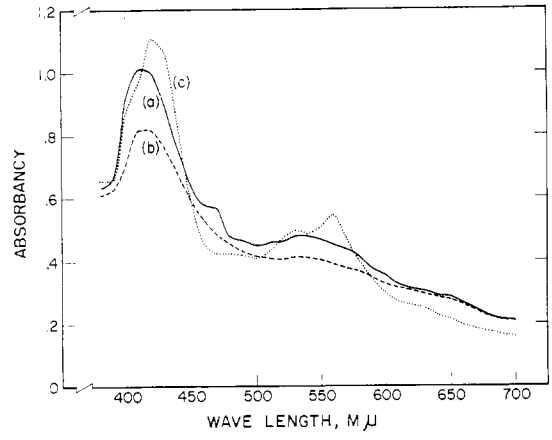

Figure 7. Reflectance curves showing pigments of cooked tuna flesh

a, b. Denatured globin hemochrome of cooked tuna flesh
c. Denatured globin hemochrome of reduced, cooked tuna flesh

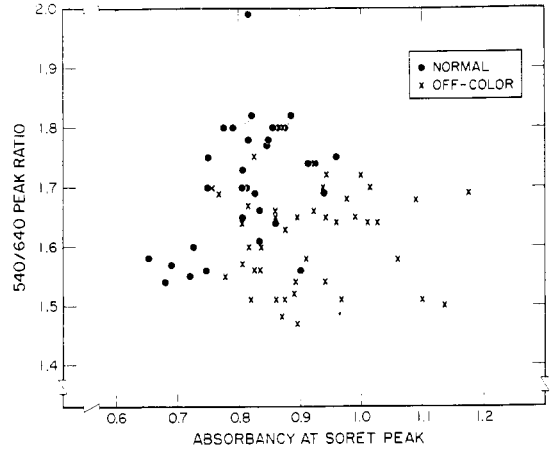

Figure 8. Variation of 540/640-mμ peak ratio with absorbancy at Soret peak (410–415 mμ) an index of pigment content

The residues left after treatment of the flesh with acid-acetone and acid-methanol were examined by spectral reflectance. The main characteristics of the curves obtained were very similar to those reported for acid hematin and acid-stable hemin (*13*).

Of interest was the slight, but definite absorption peak in the red end of the spectrum at 625 mμ for acid-acetone–treated meat (Figure 10, *a*, *b*, and *c*). The curves show a decrease in this peak height from very green meat (curve *a*) to less green (curve *b*) to normal (curve *c*), where the absorption peak has disappeared. This peak is a more direct point of evidence for the green pigment, the existence of which had previously been deduced more or less indirectly. A similar pigment, absorbing between 610 and 620 mμ, has been reported by Ginger, Lewis, and Schweigert (*9*) in both transmission measurements on green-colored extracts and spectral reflectance curves obtained from discolored beef that had been exposed to gamma radiation from a cobalt-60 source. The pigment noted here is present in a very much lower concentration and in an insoluble coagulated form, rendering it difficult to detect in the presence of other pigments, and impossible to extract into a solvent.

A pigment present in beef, which may be similar, has been identified as being largely sulfmyoglobin (*8*).

Relationship between Pigments of Raw and Precooked Tuna Flesh. The examination of many spectral reflectance curves for the flesh of raw tuna revealed the tendency for flesh with a high content of methemoglobin, characterized by a prominent 630-mμ absorption peak, to exhibit an off-color on precooking. The picture is complicated by the presence of oxyhemoglobin, which seems to modify the effect of methemoglobin and, when present in high concentration, produces pink meat that browns rapidly on exposure to air. Frequently a high concentration of both oxyhemoglobin and methemoglobin is found. The possible combinations result in a gamut of gray to brown to orange pigmentation on precooking, depending on the relative amounts of each parent pigment.

An evaluation of the relationship of both subjectively and objectively judged color to methemoglobin content is shown in Figures 11 and 12. The height of the absorption peak in the red region at 630 mμ, characteristic of methemoglobin, was used as an indication of the amount of this pigment in the raw meat and was measured by means of the ratio of 630/660-mμ absorption. Off-color of the same meat on precooking was judged subjectively by the food technologist of Hawaiian Tuna Packers on a scale which included: plus one (excellent), zero (normal), and minus value (for increasing degrees of greenness and off-color) (Figure 11). There seems to be a definite relationship between these factors.

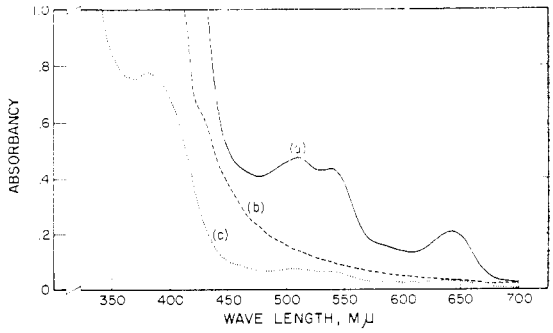

Figure 9. Spectral absorbance measured in transmission of extracts of precooked green tuna flesh

a. Chloroform extract of hydrochloric acid–acetone extract
b. Original hydrochloric acid–methanol extract
c. Chloroform extract of *b*

Table I. Characteristic Absorption Peaks of Heme Pigment Derivatives

Pigment	Absorption Maxima, Mμ I	II	III	IV
Muscle pigment in chloroform	640	540	510	382
Hemin chloride and hydrochloric acid (*13*)	640	540	512	382
Green skeletal pigment of skipjack in chloroform (*7*)	660	..	..	380
Methanolic ester of biliverdin in chloroform (*23*)	685	..	..	380
Biliverdin hydrochloride in hydrochloric acid–methanol (*12*)	680	..	..	377
Protoporphyrin in ether–acetic acid (*12*)	632.5	537	502	395
Coproporphyrin in chloroform (*11*)	622.5	533	499	405

Objective evaluation of greenness in precooked meat was made through the use of the absorption ratio at 540/640 mμ. The comparison of this color rating with the methemoglobin content is shown in Figure 12. An interrelationship is indicated, complicated by the orange color abnormality that is frequently encountered. It is surprising that this also seems to be related to a high methemoglobin content. The large number of coexisting flesh pigment forms and colors (pink, orange, green, and brown) gives a complicated system in which correlating factors are readily obscured. Nonetheless, the evidence seems to point rather definitely to methemoglobin as a dominating factor in the discoloration of tuna flesh.

Relationship of Fat Peroxides to Discoloration. The suspicion of oxidation, as one factor related to greening, has led to speculation on the role of fat peroxides in this phenomenon. The coupled oxidation of hemoglobin and unsaturated fats in mammalian meat tissue has been the subject of much research (*24*). The oxidation of unsaturated fats to peroxides is accelerated by heme compounds and is accompanied by the oxidation of the heme. The resulting fat peroxide may, in turn, play a role in furthering the deterioration of heme pigments. Hydrogen peroxide forms unstable addition compounds with hemoglobin, which subsequently decompose with destruction of the heme moiety of the molecule (*11*). A greening of tuna meat has been observed on treatment with dilute solutions of hydrogen peroxide.

To test the possibility of a correlation between peroxide formation and oxidation of the heme pigment, the fat content of tuna flesh has been determined by a modification of the cold extraction method of Sperry (*22*), carried out in deaerated solutions under a blanket of nitrogen. The peroxide content was determined by titration of iodine with standard thiosulfate solution (Table II). The iodine was released from an iodide solution by the peroxide present in the extracted fat. Appropriate blank determinations were made.

For the samples run, there is an indication of a high peroxide content in green and off-color flesh, which is in agreement with the postulated oxidation of fats along with the oxidation causing greening. The increased peroxide is most pronounced for abnormally pink or orange samples, and less so for green.

The investigation of the fat content and the peroxide values, related as they seem to be to the oxidation of heme compounds, is particularly pertinent, because it is known that fish derive their fat mainly, if not entirely, from dietary fat. The fat is deposited in the tissues more or less unchanged (*21*). The highly speculative conjecture could be made that the differences among fish of the same species, which result in greenness being exhibited by certain specimens, are related to differences in dietary fat intake. The presence of fats susceptible to oxidation—i.e., linoleic and linolenic acid fats—would in turn render the heme pigments present subject to easy oxidation.

Fish Exhaustion and Methemoglobin Content. As a result of the accelerated conversion of oxyhemoglobin to methemoglobin in the absence of oxygen (nitrogen and evacuation), anoxia, or lack of oxygen, in the muscle of the fish brought on by exhaustion might lead to a high methemoglobin content with consequent increased incidence of greening. Such a condition is probable for certain

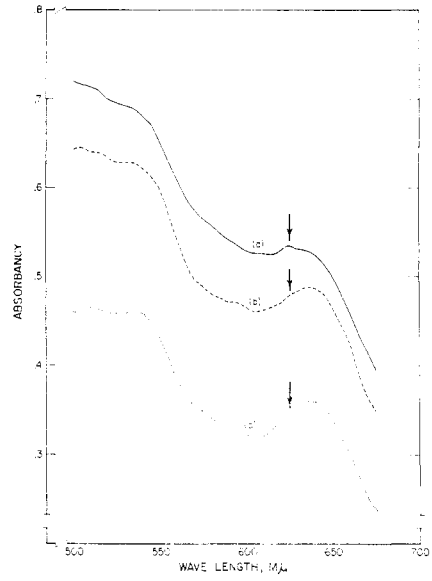

Figure 10. Reflectance curves of acid-acetone – extracted precooked tuna of decreasing greenness

a, b, c. Absorption decrease with decrease in greenness in the 610–620-mμ range

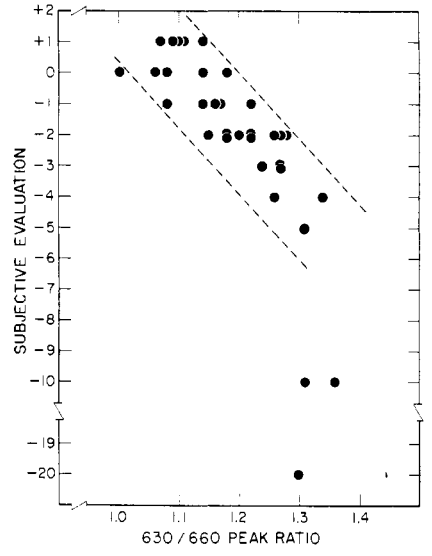

Figure 11. Relationship of methemoglobin content of raw tuna flesh to color rating as subjectively judged

fish caught by the long-line method of fishing.

The theory was tested through the facilities of the Coconut Island Marine Laboratory of the University of Hawaii. A fishing boat was available with a live-well in which it was possible to maintain small tuna alive. In addition, small yellowfin tuna abound in local waters in certain seasons. With the cooperation of the staff of the Hawaii Marine Laboratory, it was possible to catch tuna with lures and to kill certain of these within a few minutes of capture while maintaining others in a wounded and dying condition for many hours in the live well. The latter were finally killed and the flesh of both types was analyzed for methemoglobin by the reflectance technique. Two sets of such paired samples were obtained. Differences in methemoglobin content were very slight and seemed to be randomly distributed between the fish killed under the two conditions.

Table II. Fat and Peroxide Values of Raw Tuna Flesh

Samples	Description	Fat, %	Meq. Peroxide/1000 Grams Fat
Green			
1	Green	1.29	40
2	Pale green	0.47	14
3	Slightly green	0.63	24
4	Green	0.82	14
		0.79	12
Very pink			
1	Abnormally pink	0.55	27
2	Abnormally pink	0.92	20
		1.07	40
3	Orange	0.46	26
		0.44	22
Normal and Pale			
1	Normal	0.50	13
2	Washed out	1.57	9
3	Normal	0.59	9
4	Normal	0.58	4
5	Normal	0.54	10
		0.51	8

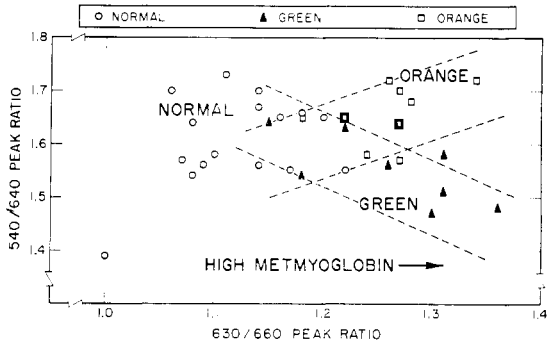

Figure 12. Relationship of methemoglobin content of raw fish flesh to color of meat after precooking as objectively judged (540/640 ratio)

Measurements made from absorption in spectral reflectance

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