Further Studies on Green or Offcolor Condition in Precooked Yellowfin Tuna

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FURTHER STUDIES ON GREEN OR OFFCOLOR CONDITION IN PRECOOKED YELLOWFIN TUNA

By

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ABSTRACT

Results are reported from a study of the "greening" condition that appears in certain samples of yellowfin tuna on precooking. Evidence is presented that this is an actual color condition similar to discoloring processes that occur in other meats, and is caused by an anomalous heme protein oxidation. Related to this tendency to turn green on precooking are the presence of high concentrations of metmyoglobin, some denaturation, and a slightly high fat peroxide content in the raw meat. In addition, green meat generally has a high flesh pigment content. Oxygen starvation due to the exhaustion of the fish that might occur in the process of catching does not seem to produce the factors that lead to greening, but rather a deterioration that goes on even in the frozen state seems to be responsible. Spectral reflectance was employed in much of the work and revealed important *in situ* changes or processes in flesh pigments that would have been impossible to note by other means.

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FURTHER STUDIES ON GREEN OR OFFCOLOR CONDITICN IN PRECOOKED YELLOWFIN TUNA

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An undesirable "green" color which develops in the flesh of certain tuna on precooking prior to canning, results in considerable economic loss to the fisherman and to the industry through rejection of such fish. The cause of this offcolor condition has been the subject of studies at the Chemistry Department, University of Hawaii, under contract No. 14-19-008-2475 with the Pacific Oceanic Fishery Investigations (POFI), U. S. Fish and Wildlife Service. The background and the results of preliminary studies are embodied in a previous report (Naughton, Frodyma, and Zeitlin, 1956).

The extension of the work herein described represents an attempt to establish more definitely the nature and genesis of the pigment or pigments responsible for the offcolor.

We are most grateful to Mr. Fred Jermann of Hawaiian Tuna Packers, Ltd., and to Dr. Albert L. Tester, Director, and other members of the staff of POFI for suggestions and help in the investigation.

SAMPLES

The fish used in this study were yellowfin tuna (Neothunnus macropterus) which, for the most part, were caught on longline by POFI research vessels in the central equatorial Pacific in 1955 and 1956. The fish were frozen on board ship as soon as possible after capture and were held in a solid frozen condition until they were needed. Sizes ranging from about 70 to 200 pounds were selected as it had been noted that the larger fish showed a greater tendency to turn green on precooking. In addition small yellowfin (up to 15 pounds) were caught by trolling in local waters by the staff of the Hawaii Marine Laboratory, University of Hawaii, and were sampled while fresh. Samples of fresh, prime fillets ("ahi" samples) of yellowfin caught in the local longline fishery were purchased from local markets for study.

In order 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. Judgment of color grade of the precooked samples was made by Mr. Fred Jermann, the company's food technologist. Specific samples noted to be characteristic of their types (normal, green, pink, etc.) as subjectively judged, were used in much of the work.

REFLECTANCE

Early in the research on the color of tuna flesh, an opaque material, the need was recognized for a device for measuring spectral reflectance, thus making possible an objective evaluation of the actual color of the samples. A reflectance attachment to the Beckman DU spectrophotometer was secured. With the development of techniques for reflectance measurement, it became apparent that the method had unique value as an analytical tool in addition to its recognized use in the specification of color. Curves exhibiting optical absorption peaks could be obtained by spectral reflection measurements. These were characteristic of the pigments present in tuna flesh and were identical with the absorption curves obtained from transmission measurements made on the same pigments in solution. Beer's law was obeyed, thus justifying the use of absorbancy or optical density in our curve plots. The obvious advantages of being able to make measurements in situ, and on insoluble systems, such as those encountered in this research, were points that were vital. A separate communication has been published (Naughton, Frodyma, and Zeitlin, 1957) which describes in greater detail the advantages of spectral reflectance as an analytical tool, especially in biological systems.

PRECISION OF MEASUREMENTS

The variability of the pigment content throughout a sample of meat (see below) introduces a difficulty when making reflectance and pigment-content measurements on a series of samples, even from the same fish. Ground and thoroughly mixed (homogenized) samples gave readings that were reproducible to $\pm$ 0.5 percent reflectance units or within the error of the measuring instrument. On standing, however, oxidative changes took place in such samples

with consequent changes in spectral absorption. Replicate nonhomogenized samples from a given fish loin gave an average precision of ± 1.5 percent reflectance units.

THE COLOR OF COOKED TUNA

A visual examination of different samples of cooked tuna flesh immediately reveals the complexity of the color system involved. First, the variability of the absolute content of pigment in the flesh of various fish should be recognized. We have encountered variations from highly bleached meat to a very high pigment concentration with resultant abnormal redness. Whether these variations are physiological or due to postmortem changes has not been determined. Secondly, the condition of "browning" can be recognized. As usually understood, this seems to be a postmortem and even a postcooking phenomenon which is due to oxidation on exposure to atmospheric oxygen. Thirdly, the greening phenomenon, which is the main subject of this research, is observed. This is a color phenomenon that permeates the flesh of the fish; its characteristics will be described later in this report. Also an orange coloration is frequently noted which seems to occur in much the same manner as the green color. Finally, it became very evident after a modicum of work on the pigment systems of the meat that the distribution of pigment within a single fish loin was quite variable, although it might seem uniform to the eye. This was confirmed by microscopic examination which showed the pigment to be present in bands within the meat. Spectral reflectance measurements gave widely variable estimates of the amount of pigment present. Uniformity could be assured only by grinding and thoroughly mixing all the meat needed in a given sample sequence. This was not usually done, however, since finely ground meat was found to change color even when held in a frozen condition.

At the beginning of this research the reality of the green of certain offcolor samples of tuna flesh was questioned by one of the investigators. The color is of such a subtle shade and exhibits so many variations that it was supposed that the so-called greenness might be a lack of pigment. In order to test the reality of the color, the ICI (International Commission of Illumination) system of color specification was plotted for typical cooked green and normal samples. The results are shown in figure 1. It will be noted that loci of the coordinates for both samples are in a region of lightness or brightness indicative of the general light color of cooked meat. The green flesh is situated nearer the

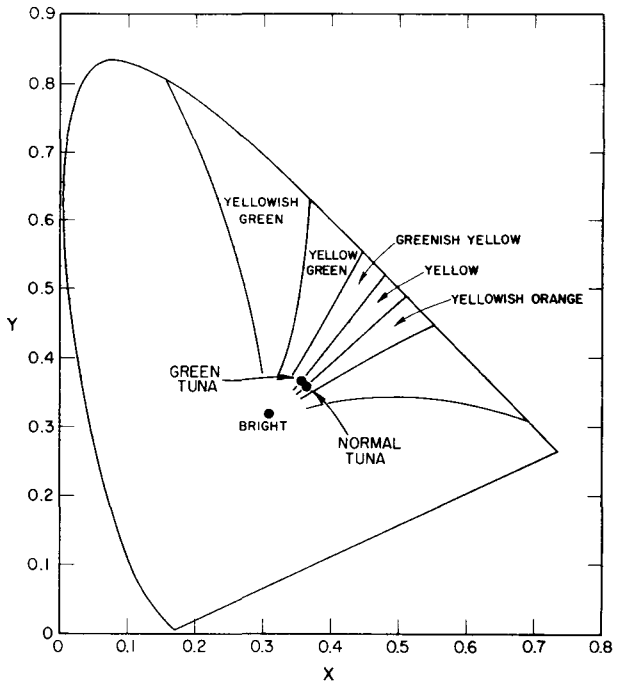

Figure 1.--Chromaticity coordinates for green and normal tuna meat. ICI system.

yellow-green color region. The normal, on the other hand, has more of the orange component present. The reality of the green cast of certain samples seems to be indicated.

Another method used in an attempt to evaluate greenness objectively was to compare absorption in the red and green parts of the spectrum (640 and 540 millimicrons, respectively) for cooked green and normal meat by reflectance measurements. A green sample would be expected to show greater absorption of light in the red end of the spectrum (640 millimicrons) when compared with normal meat. The ratio of the absorbancy at the 540/640 millimicron absorption peaks therefore should be greatest for normal and least for green meat if pigments that absorb in these regions are involved. In addition, we have been conscious throughout this work that pigment content must be a factor affecting the degree or type of alteration that might occur. If we assume that we can use the height of the most characteristic absorption peak for heme pigments (the Soret peak, at about 415 millimicrons) as an indication of the absolute pigment content of the cooked meat and plot this against the above indicated greenness index (540/640 absorption peak ratio) it is possible to observe several significant relationships. Such a plot has been made in figure 2, and it will be noted that normal samples, in general, occupy the portion of the

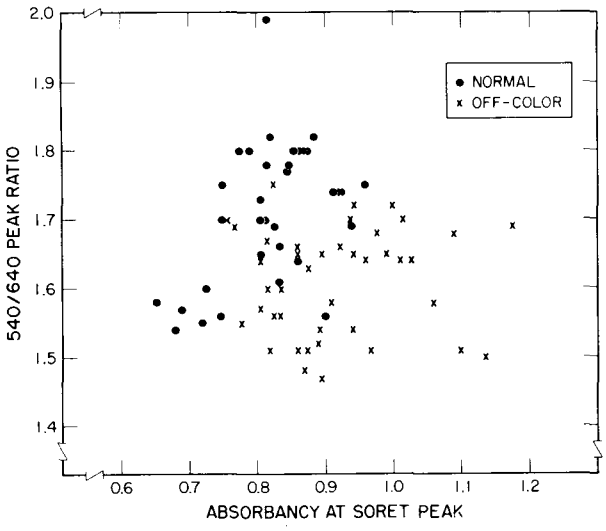

Figure 2.--Variation of the 540/640 millimicron peak ratio with absorbancy at Soret peak (410 - 415 millimicrons), an index of pigment content.

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." Another relationship which can be noted here, namely the association of offcolor with a high pigment content, is completely contrary to the supposition expressed above; that is, that the appearance of greenness is due to a lack of pigment. It should also be noted in passing that the overlapping position of some of the points is due to the uncertainty inherent in the subjective evaluation of precooked flesh.

NATURE OF THE PIGMENTS IN TUNA FLESH

It is obvious that in an investigation of pigment changes it is necessary first to understand the nature of the pigment. It is to be expected, in a muscle system such as tuna flesh, that muscle hemoglobin (myoglobin[1/]) would be the major pigment present and contribute the largest part of the flesh color. We had assumed this in the investigations herein described, but certain experiments have cast some doubt on the validity of the assumption. It was found by Bowen (1949) and others that the absorption peaks for mammalian myoglobin were displaced to longer wave lengths in the case of certain derivatives of the pigment. No such shift was observed for fish flesh.

The curves in either transmission or reflection for the systems studied were identical with those that we have measured for hemoglobin and its derivatives. For verification, the solubility of the extracted pigment in buffered phosphate solutions was checked according to the procedure of Morgan (1936), with the incorporation of the modifications of Ginger, Watson, and Schweigert (1954). The absorption curves for the separated pigments are given in figure 3. Assuming that these procedures would give separation of myoglobin from the hemoglobin of tuna flesh through differences in solubility, it was found by measurement of absorption in transmission and reflection that the tuna pigment was about 95 percent hemoglobin. Myoglobin seemed to comprise only a minor fraction of the pigment system. In figure 3 note the residual myoglobin (curve b) in relation to the total pigment content of the extract (curve a). It is felt that these experiments were not exhaustive enough to be conclusive. As far as color changes are concerned, the division of the pigment between myoglobin and hemoglobin is not too pertinent since these changes involve the porphyrin moiety of the pigment molecule which is identical for the two types. For convenience, we shall continue to ascribe the color to myoglobin

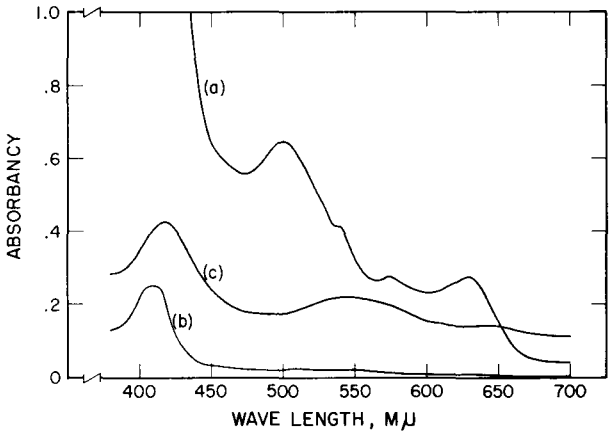

Figure 3.--Separation of aqueous extract of raw tuna (No. 6) into hemoglobin and myoglobin by phosphate fractionation. (a) Transmission curve for original extract. (b) Transmission curve for residual solution after hemoglobin precipitation with phosphate. (c) Reflectance curve of phosphate precipitated material.

[1/] Myoglobin is similar to hemoglobin except for its lower molecular weight.

until the point has been settled. In this connection, it might be noted that a recent report (Rossi-Fanelli and Antonini 1955) cites work in which myoglobin was separated from tuna flesh giving a compound differing from the corresponding compound found in mammalian flesh. No details of this difference were given.

PIGMENT DERIVATIVES PRESENT IN TUNA FLESH

As has been previously noted (Naughton, Frodyma, and Zeitlin, 1956), extraction of raw flesh gave a colored solution tentatively identified as being largely met- or ferric myoglobin. When reflectance measuring techniques were brought to bear on the problem, it became evident that the raw meat used in these studies contained a mixture of oxy- and metmyoglobin. Examples of the reflectance curves obtained can be seen in figure 4.

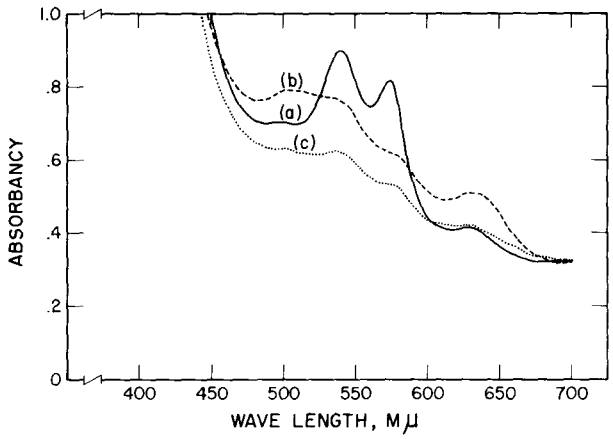

Figure 4.--Reflectance curves showing pigment mixtures found in raw tuna flesh. (a) Largely oxymyoglobin. (b) Largely metmyoglobin. (c) Mixture of met- and oxymyoglobin.

PIGMENT CHANGES IN RAW FLESH

Oxymyoglobin to Metmyoglobin

With the continuation of the measurements on raw meat, it became evident that we were dealing with a pigment system that was extremely changeable and evanescent. It was possible to recognize rather rapid oxidative deterioration in meat even when held under refrigeration. An example involving the change from oxymyoglobin to metmyoglobin can be seen in figure 5. That the change corresponds to an increase in metmyoglobin content can be verified by comparison with the "A" portion of figure 5, which shows

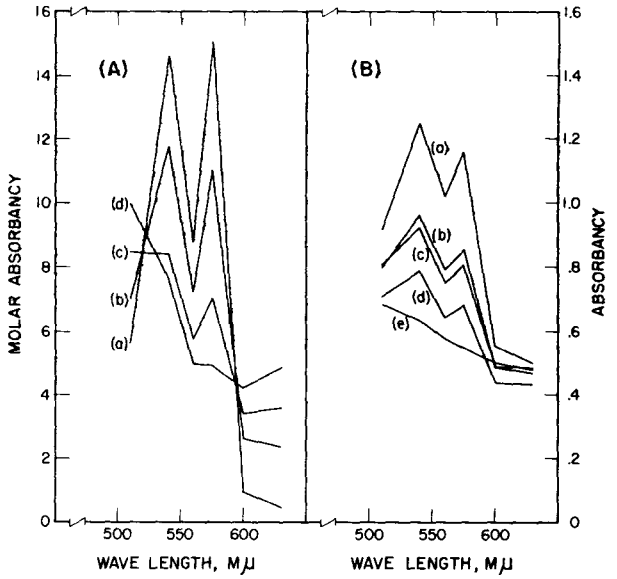

Figure 5.--A. Simplified absorption curve showing increased concentration of methemoglobin in mixtures with oxyhemoglobin increasing from curve (a) to (d). (From Austin and Drabkin 1935-36) B. Similar simplified absorption curves taken in reflection for tuna meat after various periods of freezer storage, showing increased metmyoglobin content. (a) Fresh tuna (sep. sample). (b) After 24 hours' freezer storage. (c) After 36 hours' freezer storage. (d) After 84 hours' freezer storage. (e) After complete conversion to metmyoglobin by chemical oxidation.

in a similar fashion the changes in absorption obtained by Austin and Drabkin (1935-36) for mixtures of oxyhemoglobin and methemoglobin of known content.

Deoxygenation of Oxymyoglobin

An attempt to bring about the reduction of oxymyoglobin to myoglobin by successive evacuation and purging with inert gas (nitrogen)--a technique that works well with oxyhemoglobin in blood--resulted in a more rapid conversion to metmyoglobin (figure 6). This rather surprising behavior may result from the very active form of oxygen released in situ in the flesh, which readily oxidizes the ferrous iron in the myoglobin to the ferric form in metmyoglobin. Lemberg and Legge (1949, p. 395) have discussed a similar reaction for hemoglobin in the presence of proton donors. The implications of this reaction in the greening of tuna flesh will be discussed subsequently.

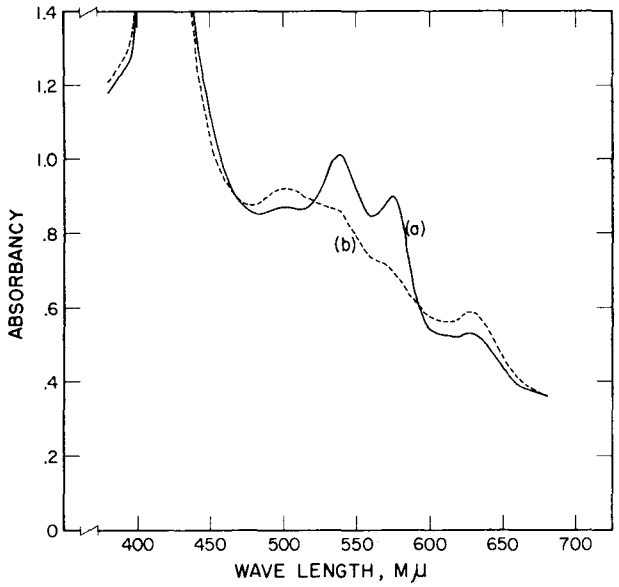

Figure 6.--Oxidation of flesh pigment on treatment with nitrogen. (a) Reflectance curve of fresh tuna flesh. (b) Reflectance curve after alternating evacuation and treatment with nitrogen for 3 hours showing increase in metmyoglobin content.

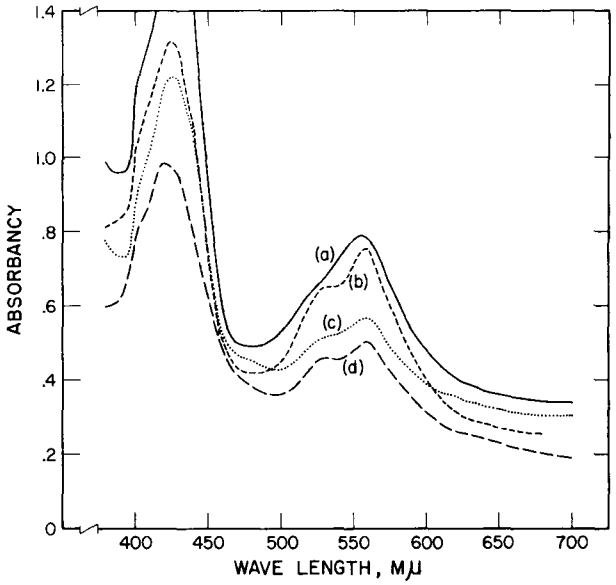

Figure 7.--Reflectance curves showing denaturation by appearance of hemochrome band at 528 mμ after reduction with dithionite. (a) Reduced raw fresh tuna--myoglobin; (b) Tuna flesh of curve (a) after denaturation by heating 20 minutes at 50°C.; (c) Raw green tuna, reduced; (d) Commercially precooked green tuna reduced.

Pigment Denaturation

Another change that takes place in raw flesh pigments is the denaturation of the protein moiety of the heme pigment molecule. Such denaturation produces so-called denatured globin hemichromes (Lemberg and Legge 1949, p. 228). These are more easily identified from their absorption curves in the reduced condition as the denatured globin hemochromes with pronounced peaks at 528 and 558 millimicrons. Figure 7 shows such spectral reflectance curves obtained with tuna flesh. Strictly fresh meat gave the characteristic single absorption peak of myoglobin in the green region at 555 millimicrons (curve a). Mild intentional denaturation of this meat by heating to 50°C. for short periods (20 minutes in the case illustrated) gave on reduction the appearance of the distinct hemochrome peak at 528 millimicrons (curve b). Similar denaturation can be noted in the absorption curve (curve c) for raw green tuna flesh, and, as would be expected, for cooked green tuna flesh (curve d). It is remarkable that intense commercial cooking in the last instance does not seem to markedly increase the denaturation of the pigment when so judged. There is some indication from a study of a number of such raw reduced samples that the degree of denaturation is related to the amount of metmyoglobin originally present in the samples and to the greening tendency.

Solubility of Metmyoglobin

Another example of unusual heme pigment changes and behavior noted in situ is the unexpectedly greater solubility of metmyoglobin relative to the oxymyoglobin present in tuna flesh. The phenomenon is illustrated in figure 8. It has been noted previously that aqueous extraction of tuna flesh gave absorption curves, measured in transmission, that were characteristic of metmyoglobin. It was always found that such extraction left a pigmented residue whose color showed but slight tendency to decrease with repetition of the extraction. This was initially assumed to be due to the inefficiency of the extraction procedure. Application of spectral reflectance measurements to the problem showed that the metmyoglobin in tuna flesh is characterized by a rather pronounced solubility, while the oxymyoglobin exhibited a high degree of insolubility (compare curves a and b, fig. 8). This suggests the possibility that pigments leaching during thawing and precooking might be a factor in producing a pale condition akin to greening in tuna with a high metmyoglobin

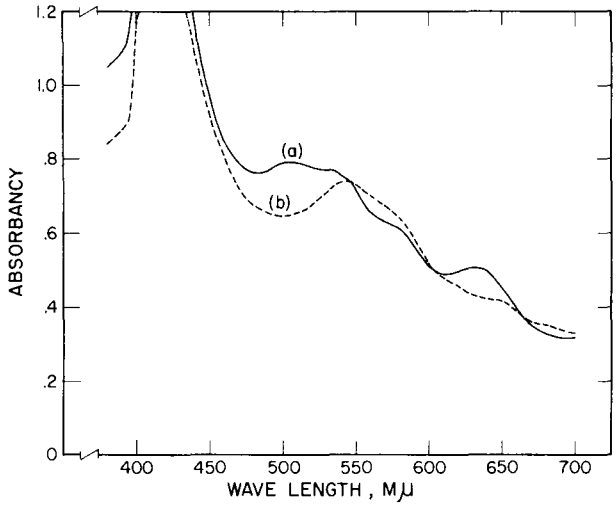

Figure 8. -- Reflectance curves showing solubility of metmyoglobin and insolubility of oxymyoglobin in raw tuna flesh. (a) Raw tuna flesh. (b) Residual flesh after aqueous extraction at pH 6.8. Note disappearance of 500 mμ and 630 mμ metmyoglobin absorption peaks.

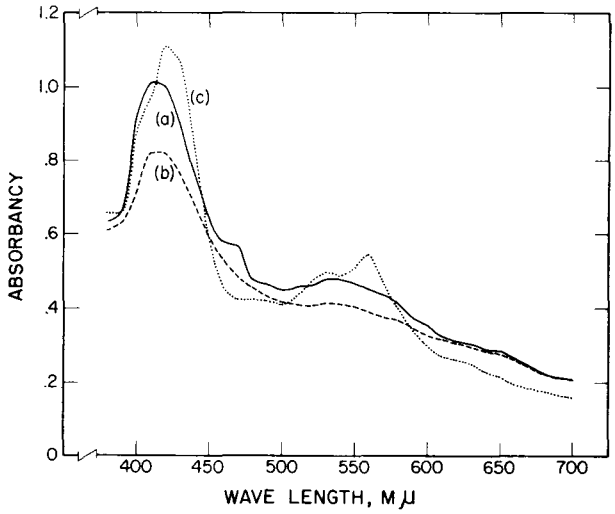

Figure 9. --Reflectance curves showing pigments of cooked tuna flesh. Curves (a) and (b) denatured globin hemichrome of cooked tuna flesh. Curve (c) denatured globin hemochrome of reduced cooked tuna flesh.

content. This effect may be one of the complex of factors related to greening, but it is probably more closely related to a "washed out" condition occasionally observed in tuna flesh.

PIGMENTS IN COOKED TUNA FLESH

The cooking of tuna meat results in the denaturation and coagulation of the proteins present, with consequent lightening of the meat color. Denaturation of the heme protein pigments leads to the formation of the hemochrome (ferrous form), and under oxidative conditions the hemichrome (ferric form) of the denatured globin. Characterizing absorption, aside from the Soret region, occurs at 545 and 575 millimicrons (fig. 8, curves a and b) in agreement with the values listed for the denatured globin hemichrome of hemoglobin (Lemberg and Legge 1949, p. 228). It will be noted by examination of figure 9 that absorption by this substance is rather weak and the peaks are ill defined. A more unique and more easily characterized curve is obtained for the reduced, or ferrous, denatured globin hemochrome, as has been discussed previously. The contrast is evident in figure 9. The color of the reduced hemochrome is pink and more desirable from the consumer's viewpoint. Brown and Tappel (1957) recently have identified this pink compound as being a mixed, denatured, globin nicotinamide hemochrome.

The difference between the spectral reflectance curves of green and normal fish flesh seems to lie in the greater absorption by the former in the red region (620 - 660 millimicrons) for a given absorption in the yellow-green region (520 - 580 millimicrons). Thus, in figure 9, curve (b) would be characteristic of flesh with a greener hue than the flesh represented by curve (a).

GREEN PIGMENT IN PRECOOKED FLESH

It proved to be impractical to extract and identify the green component in precooked green flesh, as has been pointed out previously. Another approach would be to cleave the pyrrole ring segment from the protein moiety of the pigment molecule by alcohol--or acetone--hydrochloric acid treatment, and then 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, with the resultant production of choleglobin or verdohemochrome (Watts 1954). Removal of the oxidized or disrupted ring by the acid-alcohol or acetone treatment, and spectral examination, would be expected to reveal the nature of the parent pigment. Verdohemochrome, and its postulated precursor choleglobin, would produce biliverdin-like compounds (Lemberg and Legge 1949, p. 458).

The acid-methanol or acetone extracts gave an absorption curve in transmission that showed no characteristic absorption peaks and was very similar to that obtained from the leachate of cooked fish (fig. 10, curve b). Solvent extraction

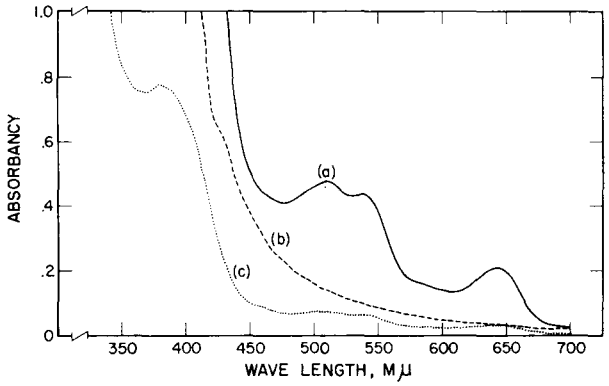

Figure 10.--Spectral absorbance measured in transmission of 1:9, 12 N HCl-methanol extract of precooked green tuna flesh (No. 7). (a) Chloroform extract of HCl-acetone extract. (b) Original HCl-methanol extract. (c) Chloroform extract of (b).

of this material, however, gave an absorption curve in transmission with peaks at 382, 510, 540, and 640 millimicrons (fig. 10, curves a and c). These absorption maxima are compared in table 1 with the peaks reported for some of the products of heme protein disruption that might be identical with the substance found in precooked tuna flesh.

The coincidence of the absorption peaks with those reported by Lewis (1954) for hemin chloride in acetone with excess hydrochloric acid added is particularly striking, and we may conclude that this substance is present in the extract. We should note, however, that we have failed to find the characteristic hemin chloride platelets on microscopic examination of these extracts.

DIRECT EVIDENCE FOR GREEN PIGMENT

The residues left after treatment of the flesh with acid-acetone and acid-methanol were examined by the spectral reflectance technique. The curves obtained were very similar to those reported for acid hematin (Lemberg and Legge 1949, p. 173) and "acid stable hemin" (Lewis 1954). The comparative values are listed in table 2.

Of far greater interest was the slight but definite absorption peak that appeared in the red end of the spectrum. This will be noted in figure 11 at 625 millimicrons for acid-acetone treated meat (curves a, b, and c), and at 610 millimicrons after acid-methanol treatment (curves d and e). The curves show a decrease in this peak height from very green meat (curve a) to less green (curve b) to normal (curve c),

Table 1.--Characteristic absorption peaks of heme pigment derivatives

	Pigment	Absorption maxima, millimicrons			
		I	II	III	IV
1	Muscle pigment in chloroform	640	540	510	382
2	Green skeletal pigment of skipjack in chloroform (Fox and Millott 1954)	660	-	-	380
3	Methanolic ester of biliverdin in chloroform (Tixier 1945)	665	-	-	384
4	Biliverdin hydrochloride in HCl-methanol (Lemberg and Legge 1949)	680	-	-	377
5	Protoporphyrin in ether-acetic acid (Lemberg and Legge 1949)	632.5	537	502	395
6	Coproporphyrin in chloroform (Lemberg and Legge 1949)	622.5	533	499	405
7	Hemin chloride and HCl (Lewis 1954)	640	540	512	382

Table 2.--Acid-hematin absorption peaks

Pigment	Absorption maxima, millimicrons			
	I	III	IV	Soret
1 Reflectance of residue in precooked tuna meat after HCl-methanol or HCl-acetone extraction	630	540	500	400
2 Acid hematin (Lemberg and Legge 1949, p. 173) glacial acetic acid	630-635	540	410	400
3 Acid stable hemin (Lewis 1954)	630	-	500	-

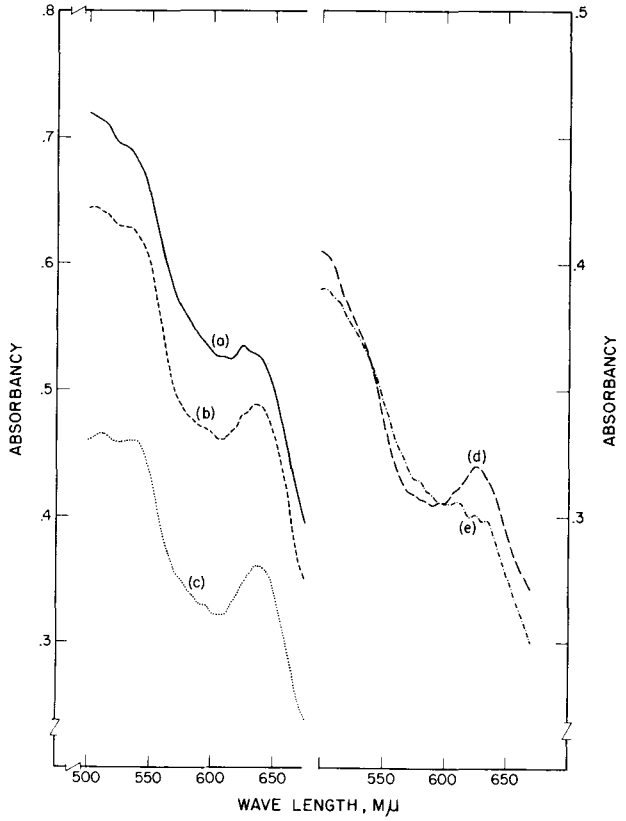

Figure 11.--Reflectance curves of acid-acetone (a, b, c) and acid-methanol (d, e) extracted precooked tuna of decreasing greenness (a, b to c; and e to d). The decrease in absorption with the decrease in greenness in the 610-620 millimicron range is evident.

where the absorption peak has disappeared. A similar effect is noted in the acid-methanol treatment (curve d, normal; curve 3, green meat). It is felt that this peak is direct 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 millimicrons, has been reported 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 (Ginger, Lewis, and Schweigert, 1955). The pigment noted here may be similar, although present in lower concentration and in an insoluble coagulated form. The exact nature of the pigment is still under investigation.

RELATION BETWEEN PIGMENTS OF RAW AND PRECOOKED TUNA FLESH

The examination of many spectral reflectance curves for the flesh of raw tuna reveals the tendency for flesh with a high content of metmyoglobin, characterized by a prominent 630-millimicron absorption peak, to give an offcolor on precooking. Thus, referring to figure 4, one would usually find that raw meat which gave a reflectance curve such as (b) would turn green or gray-green on precooking. The picture is complicated by the presence of oxymyoglobin, which seems to modify the effect of metmyologbin and, when present in high concentration, produces pink meat that browns rapidly on exposure to air. Frequently a high concentration of both oxymyoglobin and metmyoglobin is found. The oxymyoglobin predominates in effect, and the possible combinations seem to result in a gamut of gray to brown to orange pigmentation depending on the relative amounts of each parent pigment.

An evaluation of the relation of both subjectively and objectively judged color to metmyoglobin content is shown in figures 12 and 13. The height of the absorption peak in the red region at 630 millimicrons, which is characteristic of

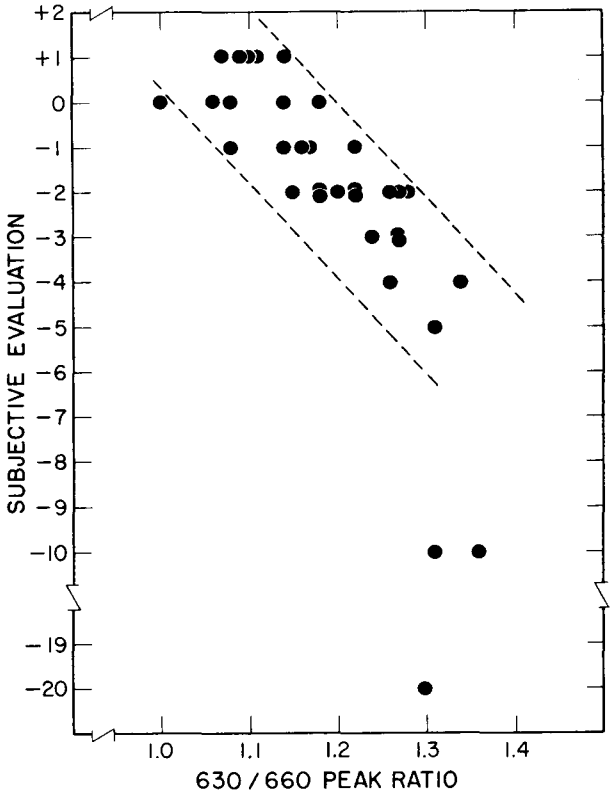

Figure 12.--Relation of metmyoglobin content of raw tuna flesh (as judged by magnitude of spectral reflectance at the 630/660 peak ratio) to the color rating of the same meat on precooking as subjectively judged.

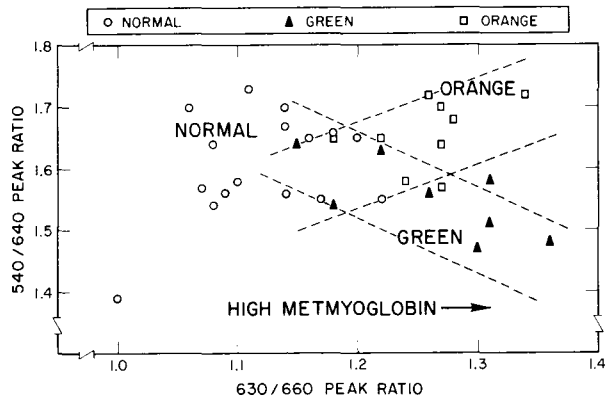

Figure 13.--Relation of metmyoglobin content of raw fish flesh to color as objectively judged (540/640 ratio). Measurements made from absorption in spectral reflectance.

metmyoglobin, 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 millimicron absorption. Offcolor of the same meat on precooking was judged subjectively by Mr. Jermann of Hawaiian Tuna Packers on a plus one (excellent); zero (normal); minus value (for increasing degrees of greenness and offcolor) scale. The results are shown in figure 12. There seems to be a definite relation between these factors when judged in this manner.

Objective evaluation of greenness in precooked meat was made through the use of the absorption ratio at 540/640 millimicrons described earlier in this report. The comparison of this color rating with the metmyoglobin content is shown in figure 13. Again we note the indication of an interrelation, complicated by the orange color abnormality that is sometimes noted. It is rather surprising that this also seems to be related to a high metmyoglobin 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 metmyoglobin as a factor in the discoloration of tuna flesh.

PEROXIDES IN FISH FATS

The suspicion of oxidation as one factor related to greening leads one to speculate 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, summarized by Watts (1954). 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. It is known that hydrogen peroxide forms unstable addition compounds with hemoglobin, which subsequently decomposed with destruction of the heme moiety of the molecule (Keilin and Hartree 1950). We have observed a greening of tuna meat on treatment with dilute solutions of hydrogen peroxide.

To test the possibility of a correlation between peroxide formation and oxidation of the heme pigment, we have determined the fat content flesh by a modification of the cold extraction method of Sperry (1955), carried out in deaerated solutions under a blanket of nitrogen. The peroxide content was determined by titration of the iodine released from an iodide solution by the peroxide present in the extracted fat with a standard thiosulfate solution. Appropriate blank determinations were made. The results are given in table 3.

For the samples run, there is an indication of a high peroxide content in green and

Table 3.--Fat and peroxide values of raw tuna flesh

Samples	Description	Percent fat	Milliequivs. per 1000 gms. of fat
Green			
No. 1	Green	1.29	40
No. 2	Pale green	0.47	14
No. 3	Slightly green	0.63	24
No. 4	Green	0.82	14
		0.79	12
Very pink			
No. 1	Abnormally pink	0.55	27
No. 2	do.	0.92	20
		1.07	40
No. 3	Orange	0.46	26
		0.44	22
Normal and pale			
No. 1	Normal	0.50	13
No. 2	Washed out	1.57	9
No. 3	Normal	0.59	9
No. 4	do.	0.58	4
No. 5	do.	0.54	10
		0.51	8

offcolor 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 since 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 (Shorland 1956). Therefore it is conceivable that the differences among fish, which result in greenness being exhibited by certain specimens, are related to differences in dietary fat intake. The presence of fats that are susceptible to oxidation (i.e., linoleic and linolenic acid fats) would render heme pigments subject to easy oxidation.

FURTHER INVESTIGATIONS ON LEACHING

It has been noted in previous reports that on precooking, a solution was drained from the fish flesh which was found to exhibit a degree of pigmentation. We have referred to this process as "leaching" and to the product as the "leachate."

Solutions of all leachate pigments gave noncharacterizing absorption curves when measured in transmission that are identical with curve (b), figure 10. In addition, the residues left on boiling aqueous extracts of raw flesh have the same color and give the same type of absorption curve. As has been indicated previously, extracts of the hemin of precooked meat with acetone or methanol-1N hydrochloric acid (Brückmann and Zondek 1940), gave the same types of absorption curves on measurement in transmission as those resulting from similar treatment of raw meat. In view of this, one would expect to find that heme was the pigmented substance cleaved from the myoglobin in the tuna flesh and leached out during precooking. Repeated efforts, however, to produce the rhomb-shaped crystals of "alpha-hemin" (chlorohemin, hemin), which are so characteristic of this compound when derived from mammalian blood, were not successful (Teichmann test). The identification of this substance is particularly pertinent because of the possible relationship between the phenomenon of leaching and the pronounced solubility of metmyoglobin reported above.

FISH EXHAUSTION AND METMYOGLOBIN CONTENT

As a result of the accelerated conversion of oxymyoglobin to metmyoglobin noted in the absence of oxygen (nitrogen and evacuation), it was hypothesized that a condition of anoxia, or

lack of oxygen, in the muscle of the fish brought on by exhaustion might lead to a high metmyoglobin content with consequent increased incidence of greening. Such a condition is probable for certain fish caught by the longline method of fishing.

An opportunity to test the theory was afforded through the utilization of 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 metmyoglobin by the reflectance technique. Two sets of such paired samples were obtained. Differences in metmyoglobin content were very slight and seemed to be randomly distributed between the fish killed under the two conditions.

DISCUSSION

Consideration in toto of the experimental work performed in the period covered by this report reveals that it was guided by the assumption, based on evidence gathered in earlier experiments, that there is an actual green pigment that produces the undesirable color in certain samples of precooked tuna. As a working hypothesis, it has been assumed that the green color is due to the pigments that result from the opening of the porphyrin ring of certain of the heme proteins of tuna flesh. This process, which takes place as a result of oxidation, is familiar to food scientsts in the meat industry.

A hypothetical picture of the processes occurring in the greening of precooked meat follows. Certain samples of raw tuna flesh contain a high concentration of metmyoglobin in proportion to the oxymyoglobin content. There is evidence that such tuna are prone to greenness on precooking. The high concentration of metmyoglobin is a result of the decomposition of oxymyoglobin which releases oxygen in an active form capable of oxidizing the ferrous iron of myoglobin, and also capable of producing a denaturation of the protein moiety of the heme protein molecule. This oxidation takes place largely during storage after the death of the fish, and may be catalyzed by the presence of certain fats ingested as a result of peculiarities of the fish diet. The denaturation of the pigment renders the heme moiety more susceptible to further oxidation, which takes place on precooking, and results in an opening of the porphyrin ring. This last process may result in the production of pigments similar to verdohemochromes, which impart a green color to the meat. Concomitant browning occurs due to the formation of denatured globin hemichromes.

Although this picture is hypothetical, there is some evidence for the steps indicated, and it is offered as a basis for further work.

SUMMARY

Continued work on the green or offcolor condition that appears in the flesh of certain specimens of tuna on precooking has indicated the following:

1. The greenness is an actual color condition, rather than a lack of pigment or offcolor.
   a. ICI (International Commission on Illumination) color evaluation of the light reflected from green meat shows it to be in the yellow-green region, with somewhat more green color than is present in normal meat.
   b. Study of spectral reflection shows generally a greater relative absorption of light in the red region of the spectrum for green meat--hence the green color--and indicates a higher concentration of a compound or mixture of compounds absorbing in this region (the green pigments).
   c. Evidence is offered for a green pigment in precooked meat that has been exposed to hemin cleavage by chemical means.
2. Spectral reflectance studies reveal oxymyoglobin and metmyoglobin as the chief pigments of raw tuna flesh. Mixtures are usually present with a high concentration of oxymyoglobin in the freshest meat, and increasing concentrations of metmyoglobin in older stored meat. Metmyoglobin content increases even on freezer storage.
3. The reactions of the pigments in situ in the raw tuna flesh were studied by reflectance methods and revealed the following:
   a. Interconversion from oxy- to met- to myoglobin, and reverse were readily achieved by chemical means.
   b. Metmyoglobin was found to be very soluble in aqueous media while oxymyoglobin was

not easily extracted into the same media.

c. Oxymyoglobin was converted to metmyoglobin on de-aeration.

d. Stored meat showed evidence of progressive denaturation of the pigment protein.

4. The characteristics of the pigments of cooked tuna flesh were investigated. One of the pigments, denatured globin hemichrome, was readily reduced to the hemochrome by chemical means.

5. Evidence is presented showing a relation between high metmyoglobin in the raw flesh and a tendency towards discoloration on precooking.

6. Data are cited indicating a low fat peroxide content in normal meat relative to meat inclined to become offcolor on precooking. It is felt that fat oxidation and heme pigment oxidation are interrelated.

The conclusion reached is that greening in tuna flesh is similar to the greening process that occurs in other meats, and is due to an anomalous heme protein oxidation. A hypothetical pathway for the production of the green condition in tuna flesh is outlined.

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