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BIOCHEMISTRY OF MYOGLOBIN

Production and Identification of a Green Pigment Formed during Irradiation of Meat Extracts

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Studies of the conditions under which the maximal discoloration of heme pigments occurs, by ionizing radiations, show a maximal relative production of green pigment with respect to red at pH 5.3. This production could be greatly enhanced by the addition of cysteine or other sulfhydryl reagents and inhibited by the addition of aldehydes. This evidence, plus the reactions it undergoes with dithionite and carbon monoxide identifies the green heme pigment as sulfmyoglobin. Evidence is presented that the sulfmyoglobin complex breaks down under either mild oxidizing or reducing conditions to form either metmyoglobin or myoglobin. The protein moiety of the green pigment is partially denatured, and the production of the green pigment is postulated to be one of the steps in the irradiation destruction of muscle color.

THE PRINCIPAL FORM of the heme pigments, myoglobin and hemoglobin, after irradiation is a bright red compound, which is spectrally similar to the oxygenated form of the pigments (*3*, *15*). This pigment is relatively stable and imparts a very satisfactory color to the irradiated meat in which it predominates. However, under certain conditions other pigments occur, principally the met or ferric form of the heme compounds. In this oxidation state, the heme pigments are brown, with absorption bands at 507 and 635 for myoglobin and 500 and 630 for hemoglobin. This condition imparts a brownish or sometimes greenish cast to the meat. The met form is an important component of the heme pigments in irradiated tuna and pork (*15*) and was observed by Hannan (*5*) and Ginger, Lewis, and Schweigert (*3*) in irradiated meats in which the oxygenated form predominated before irradiation.

While these two pigments are the major contributors to the color of irradiated meat, there is yet another detectable pigment produced, characterized by an absorption band in the 615- to 620-mμ region. This green pigment, under some conditions, contributes to the color of the irradiated product, particularly in comminuted meats (*3*). Although the greatest quantities are produced when meats or extracts thereof are irradiated in the presence of air, close examination of the spectra of meat samples irradiated in the absence of air show a slight absorption in the 615-mμ region. The identity of this green pigment has not been established, and it appeared possible that the isolation or identification of the compound could give some insight into the reactions which take place during irradiation, with particular reference to the changes which the heme pigments undergo.

Two chromatographically distinct compounds have been identified previously (*4*) from the acid-acetone cleavage of irradiated meat extract pigments. One had an R_f of about 0.75 and appeared to be protohematin, while the other compound had a somewhat lower R_f and fluoresced in the red portion of the spectrum. The fluorescence marks the pigment as an iron-free porphyrin, but whether it was produced as a result of gamma irradiation or was produced from hematin by the isolation procedure was not established.

The irradiation of beef muscle extracts resulted in color changes similar to

those observed in ground meat (*3*). The use of such extracts eliminates the necessity of making extracts from meat after irradiation at a time when time, temperature, and exposure to air are critical factors in the destruction of the pigments. The crude extracts all contained hemoglobin in addition to myoglobin and other tissue constituents, although the predominating pigment is the muscle heme pigment. Myoglobin was the only heme pigment present in any appreciable quantity in the 50% saturated ammonium sulfate solutions which were used for the bulk of the work reported in this paper. However, as some of the solutions did contain the blood pigment and as the term myoglobin will be used to designate a specific oxidation state, the term "heme pigments" shall be used hereinafter to refer to any or all of the soluble globin-heme complexes normally found in meat.

Experimental

Beef muscle extracts were prepared by extracting comminuted beef skeletal muscle with an equal weight of cold distilled water. After expressing the liquid from the tissue, the solutions were either centrifuged to clarify them and used thus, or, as was done in most cases, brought to 50% ammonium sulfate concentration and centrifuged. The formula as given by Taylor (*16*) was used to calculate the degree of saturation of ammonium sulfate. For irradiation, the resulting supernatants were then placed in appropriate glass containers, and stoppered tightly. Although no attempt was made to exclude air before irradiation, no further exchange with the atmosphere occurred, as attested to by the slight positive pressure which developed in the tubes from gases produced during the irradiation process.

Two different sources of ionizing radiations were used: the spent fuel element source at Argonne National Laboratories, Lemont, Ill., and the cobalt-60 source at Argonne Cancer Research Hospital, University of Chicago. The former source is operated at ambient temperatures around 20° C., while the latter is kept at 4° C. Spectra were taken both with a Beckman Model DU and a Beckman Model DK-2 (recording) spectrophotometer, the former instrument being used when the greatest accuracy was desired. Although both instruments were kept in a constant temperature room at 18° C., the instability of the irradiated proteins at elevated temperatures made it advantageous to cool the light source in the Model DU, to keep the temperatures of the solutions as low as possible while taking the spectra.

Sedimentation data were obtained using a Spinco Model E ultracentrifuge.

Spectra. The concentration of the heme pigments was determined from the spectra, using the extinction at 525 mμ to determine the concentration of the extracts before irradiation. This wave length proved to be approximately the isobestic point for oxymyoglobin and metmyoglobin as the former was converted to the latter upon standing. The absorption maxima of oxymyoglobin have been found to be at 544 and 582 mμ, while those of the met form occur at 507 and 635 mμ. The green irradiated pigment had a major peak at 616 mμ, as had sulfmyoglobin, which was prepared according to the method of Hoppe-Seyler (*8*). Cholemyoglobin, which was prepared according to Hart and Anderson (*6*), using ascorbate as the reductant, had an absorption maximum at 628 mμ.

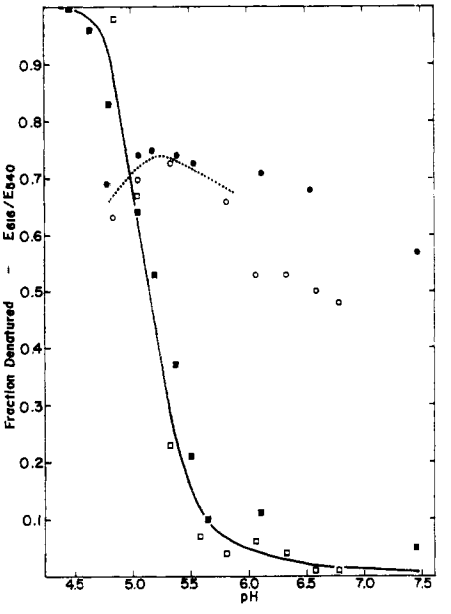

Figure 1. Fractions of pigment destroyed by irradiation and E_{616} to E_{540} ratios at varying pH. Ratio measures relative amount of green-to-red pigment

□, ■, —— Pigment destruction
○, ●, - - - E_{616} to E_{540}
Solid circles and squares for one experiment, open figures for another

Physical State of the Globin. It has been difficult or impossible to take accurate spectra of the irradiated solutions because of the development of turbidities when the solutions were brought to room temperature. The labilization of proteins by ionizing irradiations has been observed (*2*). These turbidities are apparently ascribable to the post-irradiation denaturation of protein, and involved the denaturation of the globin as evidenced by the observation that the total heme extinction was less after the precipitates had been removed from the solutions. This effect was particularly noticeable in the solutions irradiated in the spent fuel element source at 20° C., losses ranging from 40 to 100%, depending upon the irradiation dosage and the length of time the samples were in the source. Conversely, if the samples were irradiated in the cobalt-60 source at 4° C., pigment losses for corresponding dosages were from 5 to 50%, although the samples were in the source five to ten times as long. That the pigments remaining in solution were still heme-protein complexes could be demonstrated by heating the solutions to 100° C. for 5 minutes, thereby removing all pigmentation from the solutions.

Sedimenting the irradiated solutions to separate the two pigments proved inconclusive. Whereas, it was possible to distinguish the red pigment quite readily, it was not possible to identify any schlieren peak as being that of the green compound. However, when the green compound was produced in large quantities, centrifugation of the solutions in which it predominated showed that there was a definite difference between the globin of the green compound on the one hand, and the red irradiated pigment, or the native pigment, on the other. The green pigment was not only more polydisperse, but had a sedimentation value of 8.54×10^{-13} second corrected to 20° C. in water as compared with a value of 2.04×10^{-13} second for native myoglobin and the red irradiated heme pigment.

The bulk of the postirradiation pigment instability was at the expense of the green pigment. Irradiated solutions allowed to stand for 3 or 4 days in the cold developed further turbidities. Upon centrifugation and spectral examination, the 615 mμ absorption was found to be almost completely eliminated, with little or no diminution of the red pigment absorption. Ginger, Lewis, and Schweigert (*3*) found that the amount of green pigment that could be observed post-irradiation could be enhanced by the addition of ammonium sulfate to 50% saturation, but it was not determined whether or not this was a specific effect of the added salt during irradiation. The effect is due primarily to the "salting-in" of the unstable globin of the green pigment, as indicated by the observation that potassium chloride in concentrations of 0.1*M* or higher, when added after irradiation, stabilized the color effectively as did ammonium sulfate added before irradiation.

Effect of Varying the pH. Two important results of the effect of varying the pH of the solutions to be irradiated are shown in Figure 1 in which the results of two separate experiments are plotted. The solutions to be irradiated were buffered with McIlvaine's buffer and irradiated in the cobalt-60 source for total dosages of 5,300,000 rep. The first effect noted is the sharp increase in pigment destruction which occurs at pH 5.0 to 5.5. The inflection in this curve is at pH 5.2. The dotted curve represents the E_{616} to E_{540} ratio, which is a measure

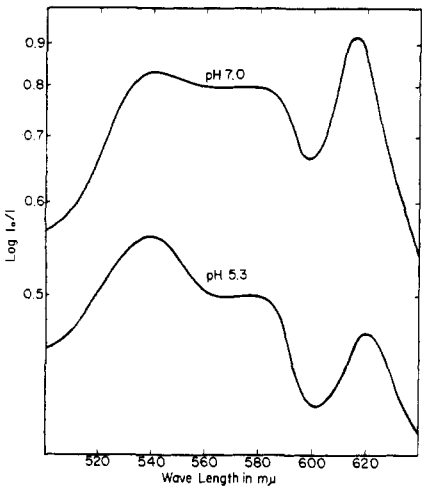

Figure 2. Spectra of heme pigments after irradiation in the presence of 1.0% cysteine. Muscle extracts 50% saturated with respect to ammonium sulfate

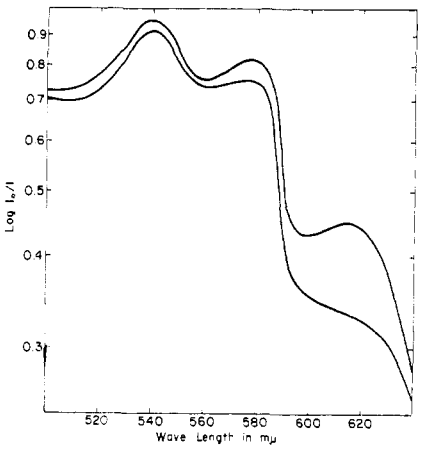

Figure 3. Effect of adding glyoxal to heme pigment extracts prior to irradiation

Upper curve. No glyoxal
Lower curve. Spectrum of heme pigments with 1% glyoxal

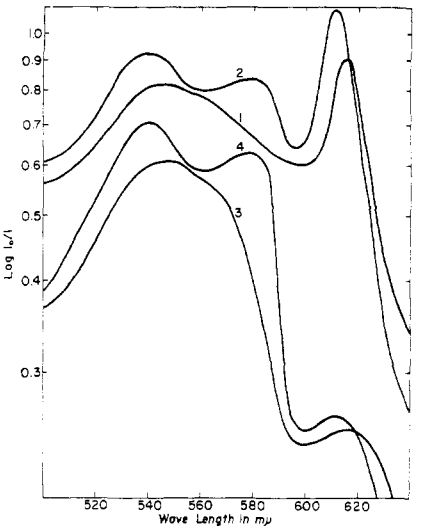

Figure 4. Effect of the addition of dithionite and carbon monoxide to irradiated muscle extracts

1. Spectrum of heme pigments with dithionite added to solution irradiated with cysteine
2. Solution of curve 1 gassed with carbon monoxide
3. Dithionite added to solution irradiated without cysteine
4. Solution of curve 3 gassed with carbon monoxide

of the relative amount of green pigment produced. Although the amount of green pigment produced at higher pH's varied somewhat from experiment to experiment as shown in the figure, the maximum ratios obtained for five different experiments were between 0.72 to 0.75 at pH 5.2 to 5.3. The inflection at pH 5.2 to 5.3 was always observed, but as the green pigment was somewhat more unstable at these hydrogen ion concentrations than was the red, the inflection is probably a reflection of increased destruction rather than decreased production. The increase in production of the green pigment is due to the variation with pH in the rates of reaction of hydrosulfide ion, oxidant, and myoglobin.

Effect of Added Reagents. If the saturated fractions were dialyzed before they were irradiated, the destruction of the heme pigments was almost complete. Resaturation of the dialyzed solutions to 50% before irradiation had no effect in protecting these pigments against irradiation destruction, and the effect of dialysis appeared to be the removal of protective agents in the crude muscle extracts and not merely to the removal of salt. However, attempts to remove and isolate the protective agents from crude muscle extracts by evaporating down dialyzates of the extracts were unsuccessful. The greatest pigment alteration and destruction occur on the surface of meats in the presence of oxygen (*3*), and the protective reagents appear to be readily oxidizable compounds. The following experiments have been carried out to test this hypothesis.

Several reagents were added to semipurified myoglobin extracts in order to determine which type of compounds exert the greatest protective effect. The myoglobin solutions used for this particular study were not crystalline preparations, but were free of impurities except for approximately 5% of extraneous protein. These solutions behaved like crystalline preparations toward gamma irradiation—i.e., the pigment was completely destroyed with irradiation doses of 5,000,000 rep. The following compounds were added to 0.2% metmyoglobin solutions, the final concentration of each being as indicated: ascorbic acid, 0.5%; potassium cyanide, sufficient to convert all the metmyoglobin to cyanmetmyoglobin; bovine plasma albumin, 2%; cysteine, 1.0%; and cysteine and bovine plasma albumin together. The pH of these solutions was adjusted to 5.3 and the solutions were irradiated in the cobalt-60 source overnight. Ascorbate had no protective effect as the pigment was completely destroyed. Ascorbate has a somewhat variable effect in comminuted meats (*3*), resulting occasionally in increased discoloration of the pigments. The production of choleglobin, a green pigment in which the porphyrin ring has been cleaved, is a result of oxidation in the presence of reducing agents, ascorbate commonly being used as the reductant.

The addition of cyanide to form cyanmetmyoglobin was suggested by the observation (*7*) that this latter compound inhibits the production of choleglobin. Protein loss was about 20%, but the procedure was successful in that the spectra of the irradiated solutions were essentially that of cyanmetmyoglobin with trace amounts of metmyoglobin.

The irradiation of metmyoglobin in the presence of cysteine was carried out at pH 5.3 and 7.0. These hydrogen ion concentrations were obtained by adjusting a rapidly stirred solution of the heme pigment with dilute acid or alkali. The spectra of the solutions after irradiation are shown in Figure 2. From the spectra, the green compound appears to be a sulfur derivative of myoglobin, probably sulfmyoglobin, as this compound has an absorption maximum at 616 mμ. There were, however, several considerations which made necessary more positive proof of the identity of the green heme pigment.

First, it must be considered that the physical state of the globin may determine the fine structure of the absorption spectra, for example, denatured globin hemochrome has absorption maxima at 558 and 528 mμ as compared with a single absorption maximum at 555 mμ for what may be considered the native parent compound, hemoglobin. Insofar as previous experience has shown the globin of the red irradiated compound to be in a native state, or very nearly so, the possibility of a spectral shift in this compound is rather remote. However, the globin of the green irradiated compound is in a state of partial denaturation and it was possible that the 616 mμ absorption maximum represented a peak lying farther toward the red, which had been shifted to shorter wave lengths by the state of the globin. That such a spectral shift might have occurred was further supported by the finding that the oxidation of myoglobin with hydrogen peroxide in the presence of hydrosulfide or cysteine resulted in the production of

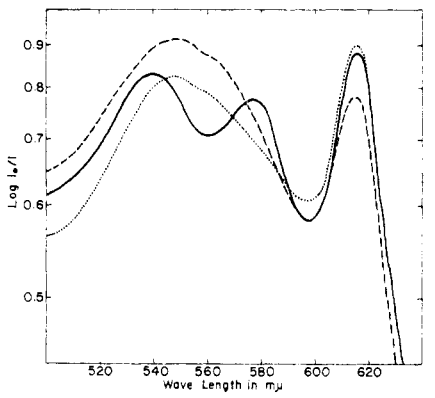

Figure 5. Effect of excess dithionite on the green heme compound

—— Spectrum of irradiated solution with added cysteine
.... Dithionite added
– – – 15 minutes later

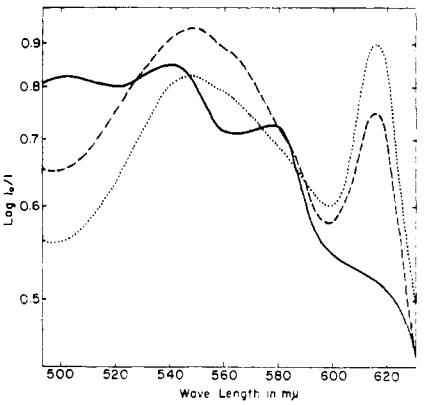

Figure 6. Effect of mild oxidative conditions on the green heme compound

——Spectrum of irradiated solution after dialysis (or electrophoresis)
.... Dithionite added to irradiated solution before dialysis
– – – Dithionite added to irradiated solution after dialysis

cholemyoglobin, a heme-protein complex in which the heme ring has undergone scission and which has an absorption maximum at 628 mμ. Nijveld (*14*) has shown earlier that a similar reaction takes place between hemoglobin, hydrogen sulfide, and hydrogen peroxide. As the oxidative driving force during irradiation is either hydrogen peroxide or the hydrogen peroxide radical, the possibility existed that the irradiated green pigment was a cholemyoglobin-like compound with the globin altered sufficiently to cause a shift of the peak to shorter wave lengths. Finally, the role of reducing agents in the irradiated solutions has not been fully defined and there is no *a priori* reason to assume that sulfhydryl reagents are not acting solely as reductants in these systems.

Effect of pH on the Reaction between Myoglobin and Hydrosulfide Ion or Cysteine. The reaction which takes place between myoglobin, cysteine, and oxygen follows two different courses depending upon the pH. At pH 5.3 the initial reaction is the reduction of metmyoglobin by cysteine and subsequent oxygenation of the reduced pigment. This reaction is quite rapid and, under the conditions used in this study, goes to completion in 10 to 20 minutes. If the solution is allowed to stand 24 to 48 hours a second slower reaction among these three compounds takes place to produce sulfmyoglobin. The sulfmyoglobin is in a more stable state than oxymyoglobin, under these conditions, and the reaction proceeds nearly to completion. Subsequent oxidation of the pigment and denaturation of the globin occur, removing the sulfmyoglobin from the solution. Earlier work with sulfhemoglobin has shown this pigment to be unstable (*9*); the observed instability of the myoglobin derivative at pH 5.3 may be due to the same causes, the process of denaturation being hastened for sulfmyoglobin by the instability of the globin at lower hydrogen ion concentrations, as reported by Lewis and Schweigert (*10*). The only apparent reactions at pH 7.0 among myoglobin, cysteine, and oxygen are the reduction and oxygenation of myoglobin. Solutions have been kept for a month or more and, although they fade slowly, the visible spectrum is that of oxymyoglobin, no evidence of any green compound appears. The fading of the solution implies oxidation of the heme ring, probably through the formation of quantities of cholemyoglobin too small to be detectable spectrophotometrically.

The reaction between hydrosulfide ion and myoglobin in the presence of oxygen is pH dependent. At pH 5.3, the reaction produces an absorption maximum at 616 mμ in only a few minutes; whereas, at pH 7.0 a period of several hours is required before an absorption maximum of the same intensity is obtained.

Irradiation of Myoglobin Solutions. Littman and coworkers (*12*) have shown that the addition of aldehydic reagents to sulfhydryl containing compounds protected the sulfhydryl group against irradiation destruction. The addition of several aldehydes to crude muscle extracts results in almost complete suppression of the 616 mμ absorption maximum, indicating that the formation of the green pigment is dependent upon the breakdown of sulfhydryl groups—liberation of hydrogen sulfide. Although myoglobin is denatured by the shorter chain aldehydes, glyoxal at the 1% level or disaccharides at the 5 to 10% level are effective in inhibiting the formation of sulfmyoglobin in crude extracts as shown in Figure 3.

The chemical reactivity of the pigment after irradiation has been studied, with the evidence again identifying the green irradiation-produced pigment as sulfmyoglobin. The reaction of dithionite and carbon monoxide with sulfmyoglobin results in characteristic spectral changes which may be used to identify this compound in mixtures of heme pigments (*9*). Figure 4 shows the effect of adding dithionite and gassing with carbon monoxide on myoglobin extracts irradiated with and without added cysteine. As the results of the dithionite-carbon monoxide test were the same for both the irradiated green pigment and sulfmyoglobin, only the results for the irradiated compounds are shown. As shown in the figure, the peak was increased by the addition of dithionite and shifted toward shorter wave lengths by the gassing with carbon monoxide. Because of the relatively small quantities of sulfmyoglobin in irradiated muscle extracts without cysteine, the observed changes are not as pronounced, but calculations of the extinctions of the major components present definitely show a spectral shift with the addition of carbon monoxide. Similar tests on cholemyoglobin showed neither the increase with the addition of dithionite nor the shift with the addition of carbon monoxide.

Oxidation and Reduction of Irradiated Pigments. Although the positive identification of the structure of sulfmyoglobin awaits the isolation of a homogeneous compound, certain deductions as to the chemical and physical state of the heme-globin complex may be made by studying derivatives of the compound. Thus, although claims for the conversion of sulfhemoglobin to hemoglobin have been discounted (*14*), the conversion of sulfhemoglobin to protohemochromes by alkali or pyridine denaturation seems certain, implying that the heme ring has not been oxidized or broken. Similar observations made in this study tend to corroborate the view that the heme ring in sulfmyoglobin has not undergone scission.

When a 100-fold excess of dithionite was added to a solution of sulfmyoglobin, made either by hydrogen sulfide and oxidation or by cysteine and irradiation, the initial effect was the increase in the 616-mμ extinction. If, however, the solution had been allowed to stand for 15 to 20 minutes, as shown in Figure 5, there was a decrease in the 616-mμ extinction and a concomitant increase in the 540- to 560-mμ region. The reaction had not gone to completion in this period, as evidenced by the remaining 616-mμ extinction, but it was impossible to follow the reaction further owing to the formation of turbidity. If the proportionate increase in the 540- to 560-mμ region is calculated, the greatest in-

crease is at 555 mμ—the wave length of maximum absorption of myoglobin.

Conversely, if the solution is subjected to mild oxidative conditions, the observed spectral changes indicate a conversion of the green pigment to metmyoglobin. The irradiation of myoglobin extracts to produce the green pigment was carried out in the presence of cysteine, and the pigments after irradiation were in a reducing atmosphere. If the reductants were removed from the solutions containing the green pigment, as by dialysis or electrophoresis, the spectrum of the mixture of heme pigments changed, with an increase at 500 mμ—the absorption maximum of metmyoglobin. When dithionite was added to either of these solutions, the 616-mμ absorption peak reappeared, not as intense as before the dialysis or electrophoresis, and the extinction in the 550-mμ region was proportionately increased. These changes are illustrated in Figure 6. The conclusion reached is that part of the sulfmyoglobin was converted under these conditions to metmyoglobin, which upon reduction was then converted to myoglobin. The intermediate fate of the remainder of the sulfmyoglobin cannot be determined from the spectra alone, but as neither procedure was able to eliminate completely the reappearance of reduced sulfmyoglobin, the sulfur appears to be firmly bound in a complex structure that is but slowly decomposed under these conditions.

Cleavage Products. Protoporphyrin IX can be derived from sulfmyoglobin (*6*), demonstrating that the porphyrin ring has not undergone scission. Similar results have been obtained in this study by the acid-acetone (1.0 ml. of concentrated hydrochloric acid to 20 ml. of acetone) cleavage of the green pigment produced by irradiation of myoglobin and cysteine. The brown acetone soluble pigment had the spectrum of acid hematin, and chromatography in a 2,6-lutidine–water system showed only one component. Acid-acetone cleavage of myoglobin extracts irradiated in the absence of sulfhydryl reagents yielded acid hematin primarily, with trace amounts of a red-fluorescent material, which moved in the lutidine-water system with the same R_f as free porphyrin. The source of these free porphyrins is in doubt although they are probably freed from heme pigments. In addition, small amounts of yellow chloroform soluble pigments are obtained from muscle extracts irradiated at 5,000,000 rep. These pigments separate in the 2,6-lutidine–water system; some of the material remains at the origin and some smears out at and behind the solvent front. These pigments are found in increasing quantities as the irradiation dosage is increased. No specific compounds have been identified but small peaks or shoulders in the absorption curves at 420 to 450 mμ may be indicative of bile pigments.

Discussion

The evidence obtained in this study identifies the major green pigment produced during the gamma irradiation of meat or meat extracts, as sulfmyoglobin. The reaction which takes place during irradiation to produce this green compound apparently takes place in two steps, of which one or both are dependent upon pH. The first of these two steps is the production of hydrosulfide from either glutathione and other thiol-containing compounds already present in meat or extracts thereof (*7*), or from added sulfhydryl reagents. As has been shown by Littman and Brady (*11*), this reaction is essentially independent of pH in the range studied here. The amount of hydrosulfide produced at sterilizing doses of gamma radiation was found by Marbach and Doty (*13*) to be approximately 0.2 μmoles per gram of muscle tissue. As myoglobin is present in muscle tissue in about the same concentration, this amount of hydrosulfide is adequate to produce the observed amounts of green pigment. The second reaction is that of hydrosulfide ion, oxidant, and myoglobin to produce sulfmyoglobin. The rate of this reaction is greater at pH 5.3 than at higher pH's, which apparently accounts for the greater relative amounts of green pigment produced at the lower pH during irradiation. However, the destruction of total pigments by irradiation in this region increases sharply with decreasing pH and the net amount of green pigment is somewhat less than at higher pH's. Experiments using hydrosulfide, metmyoglobin, and peroxide as oxidant have yielded only cholemyoglobin as the green pigment. Hence, either hydrogen peroxide is not the active oxidant during irradiation, or the hydrogen peroxide reaction during irradiation is modified by some other agent or condition present.

Lewis and Schweigert (*10*) found that beef myoglobin is cleaved between pH 4.0 and 4.5 and the native pigment is both soluble and stable in solution above pH 4.5 to 5.0. The observed denaturation of pigment is therefore due to the gamma irradiation, but the data do not determine whether the pH increases the radiation sensitivity of the heme compounds or vice versa. The denaturation curve has an inflection at pH 5.2 to 5.3, which is the pK value associated with the ionization of the imine nitrogen of the histidine molecule coordinated with the heme iron. Although the oxygenation of myoglobin is insensitive to the ionization of this group, the data suggest that putting a proton on the nitrogen affects the stability of the complex. As sulfmyoglobin is more unstable than are the native heme pigments from which it is prepared (*9*), the observed instability of the irradiation-produced sulfmyoglobin over that of the red pigment or native metmyoglobin may not necessarily be directly attributed to the process of irradiation. However, the instability of the green pigment suggests that it is in one of the pathways of breakdown of the heme pigments during irradiation.

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