Effects of Heat, Nitrite Level, Iron Salts, and Reducing Agents on Formation of Denatured Nitrosomyoglobin

A. J. SIEDLER and B. S. SCHWEIGERT

Division of Biochemistry and Nutrition, American Meat Institute Foundation, and Department of Biochemistry, University of Chicago, Chicago, Ill.

These studies were undertaken to clarify the sequence of reactions that occur during curing of meat products. Ascorbate is the most efficient reductant for desired pigment formation, followed by cysteine and hydrosulfite. Ascorbate and cysteine degrade the heme in the absence of nitrite, although ascorbate or iron salts allow lower nitrite levels to be used. Denaturation of pigment must precede or occur in conjunction with reducing activity and the lowest temperature allowing a complete denaturation of pigment is necessary for optimal yields of the desired pigment. The cured pigment is stabilized by heat. Very low nitrite levels may cause discolorations of cured meats.

THE CHEMISTRY OF THE MEAT CURING PROCESS has been reviewed by Urbain (*17*) and more recently by Watts (*19*), but the specific sequence of reactions which form the cured meat pigments is not well understood. To observe these reactions under controlled conditions, a model system using purified myoglobin was designed to approximate conditions found in meat. The influence of nitrite, reductants, and temperature on the pigment formation was studied in this model system. The results were compared with those of other investigators under actual curing conditions.

Procedures

Model System. Highly purified beef metmyoglobin preparations obtained by ammonium sulfate fractionation procedures (*14*) were used in all the studies reported. The metmyoglobin solutions contained 3% of ammonium sulfate (equivalent to 1.8% of sodium chloride on an ionic basis), and were buffered with acetate, at pH 5.5 to give a buffer concentration of 0.057*M* in the final reaction mixture. The concentration of metmyoglobin used in each system ranged between 0.19 and 0.23 μmole (3.2 to 3.8 mg.) in a total volume of 3.5 ml.

Reducing substances tested in this system included: sodium hydrosulfite (dithionite, $S_2O_4^{--}$) at a 30-μmole level, ascorbate at a 20-μmole level, and cysteine hydrochloride at a 40-μmole level. These levels were chosen assuming a one-electron oxidation equivalent for cysteine and a two-electron oxidation equivalent for ascorbate. The level of hydrosulfite was arbitrarily chosen to lie between ascorbate and cysteine, because the electron oxidation equivalent for hydrosulfite, in this system, is not known. The ascorbate and cysteine solutions were prepared prior to use and adjusted to pH 5.5 with sodium hydroxide. Hydrosulfite solutions were freshly made up, using water previously flushed with nitrogen.

The reactions were run in loosely capped 15-mm. polyethylene conical centrifuge tubes and temperatures were maintained by immersing the tubes in a relay-controlled water bath (±0.25° C.). All reactants were heated for 1-hour periods at the specific temperature studied. The test systems were studied in duplicate, and the tabulated results are the average of the duplicate results.

Table I shows the conditions, in the model system, compared to the approximate conditions encountered during the curing process in meat. The model system is aerobic, and therefore is more similar to curing conditions in comminuted meats than to curing conditions in intact meat.

Analytical Procedures. After heating, the reactants were cooled, and the precipitate was removed by centrifugation. Excess nitrite and hydrosulfite crystals were added to the supernatant solution, which converted all of the remaining soluble pigment to the nitrosomyoglobin derivative. The resulting nitrosomyoglobin spectrum is an estimation of the maximum amount of water soluble pigment with the heme intact.

The primary cured meat pigment, denatured nitrosomyoglobin, was determined in the precipitated residue by extracting the heat-denatured protein with 80% acetone in water as described by Hornsey (*10*). The amount of pigment present in the extract was determined spectrophotometrically immediately after the extraction.

The heme remaining in the precipitate, after extraction of the denatured nitrosomyoglobin, was determined as acid hematin using acid-acetone cleavage (*10, 14*). The acid hematin content of the residue is a measure of the amount of pigment, other than denatured nitrosomyoglobin, which was heat-precipitated with the heme intact. Control experiments using purified metmyoglobin which was heat denatured at 100° C. showed 90 to 100% recovery of the metmyoglobin as acid hematin.

The acid hematin, the reactive heme of the supernatant solution (as nitrosomyoglobin), and the denatured nitrosomyoglobin were converted to metmyo-

Table I. Comparison of Model System *vs.* Approximate Conditions Found in Meat during Curing

Constituent	Model System/3.5 Ml.		Approximation in 3.5 G. of Meat plus Curing Additions		
	Mg.	μmole	Mg.	μmole	%
Metmyoglobin, MetMb	3.2–3.8	0.19–0.23	3.5 pork 9 beef (*7*)		
Nitrite (NO_2^-)		1, 10		8.7	0.02
Salt					
Ammonium sulfate					
Sodium chloride		3%			2–5
Cysteine		40		±300 (*8*)	
Ascorbate		20		0.1–10[a] (*15*)	
Hydrosulfite ($S_2O_4^{--}$)		30			
O_2		+	+ (comminuted meats)		
pH	5.5		5.3–6.3		

[a] Ascorbate used as curing adduct in certain processes.

globin values by using the extinction coefficients shown in Table II. The difference between the total metmyoglobin recovered and the metmyoglobin content of the original preparation was assumed to be degraded or unreactive heme.

All spectrophotometric data were obtained using a Beckman DK-2 recording spectrophotometer. Complete spectral curves from 450 to 700 mμ were obtained, in most analyses, to facilitate interpretation of the spectra of solutions containing more than one heme pigment.

Table II. Extinction Coefficients of Heme Pigments

Compound	Solvent	Wave Length, Mμ	E mM^{-1} $Cm.^{-1}$	Reference
Acid hematin	Acid–acetone	512	9.52	Hornsey (*10*)
Nitrosomyoglobin, MbNO	Water	543	12.2	Walsh and Rose (*18*)
	Water	573	10.4	Walsh and Rose (*18*)
80% acetone-water extract of denatured MbNO	80% acetone-water	540	11.3	Hornsey (*10*)
MetMb	Water	505	9.1	Fox (*3*)
	Water	635	3.77	Fox (*3*)
Myoglobin, Mb	Water	555	12.9	Bowen (*1*)

Results

Heat Denaturation of Metmyoglobin. The percentage of metmyoglobin which was heat-precipitated at the temperatures used in these experiments is shown in Figure 1. Insolubility and/or heme destruction were used as the criteria of denaturation of the pigment. The results showed that maintaining a temperature of 70° C. (158° F.) for 1 hour will heat-denature the major portion of metmyoglobin at pH 5.5. Recovery of metmyoglobin, as acid hematin and soluble metmyoglobin, accounted for 90 to 105% of the initial metmyoglobin, showing that heme destruction was negligible.

Influence of Reductants and Nitrite on Denaturation of Pigment. The percentages of heme destroyed and of pigment rendered insoluble using the various experimental treatments are presented in Table III. As shown in Figure 1, the metmyoglobin remained soluble after heating for 1 hour at 60° C., while almost complete precipitation occurred on heating at 70° C. The presence of either 1 or 10 μmoles of nitrite did not influence the results at either temperature. The addition of reducing agents had a profound effect on the stability of metmyoglobin.

The addition of hydrosulfite caused immediate and complete reduction of metmyoglobin to myoglobin; in the presence of nitrite, it caused immediate formation of nitrosomyoglobin. Hydrosulfite also increased the amount of metmyoglobin which was precipitated at 60° C. A second preparation of purified metmyoglobin (preparation 2) was somewhat more stable toward excess hydrosulfite at 60° C., but at 65° C. the results obtained using preparation 2 were comparable to those obtained with preparation 1 at 60° C. Little or no loss of heme occurred in the presence of hydrosulfite at either 60° or 65° C.

The presence of ascorbate caused a significant loss of metmyoglobin heme at 60° and at 70° C. Ascorbate caused the characteristic green discolorations owing to choleglobin and/or verdoglobin formation, as reported by a number of investigators (*2, 13, 21*). After metmyoglobin had been heated in the presence of ascorbate, a green component with a spectrum similar to choleglobin (*13*) was extracted with acid-acetone, from the precipitated residue. The presence of this compound in the acid-acetone extract may have resulted in slightly higher acid hematin values owing to a slight absorption at 512 mμ. Nitrite was found to protect the heme from degradation in the presence of ascorbate. The protective effect of nitrite, when meat pigments are maintained in the presence of excess ascorbate, has been reported by Watts and Lehmann (*22*).

Table III. Denaturation of Metmyoglobin and Amount of Heme Destruction Occurring in Test Systems, pH 5.5

Reactants: MetMb	Added NO_2^- μmoles	Denatured MetMb[a], % 60° C. (MetMb Preparation No. 1)	Denatured MetMb[a], % 60° C. (MetMb Preparation No. 2)	Heme Destroyed,[a] % 60° C. (MetMb Preparation No. 1)	Heme Destroyed,[a] % 60° C. (MetMb Preparation No. 2)	Denatured MetMb[a], % 70° C. (MetMb Preparation No. 1)	Denatured MetMb[a], % 70° C. (MetMb Preparation No. 2)	Heme Destroyed,[a] % 70° C. (MetMb Preparation No. 1)	Heme Destroyed,[a] % 70° C. (MetMb Preparation No. 2)
Control	0	7	10(19)[b]	0	0(5)	95	72	0	0
	1	3	...	0	...	95	...	0	...
	10	17	14	0	7	97	70	17	5
+ $S_2O_4^{--}$ 30 μmoles	0	67	16(56)	0	0(11)	98	84	8	10
	1	88	29(66)	0	0(0)	95	94	8	0
	10	78	33(57)	15	0(18)	96	90	34	37
+ ascorbate 20 μmoles	0	50	33	37	23	95	73	30	39
	1	18	18	17	12	96	84	11	6
	10	5	22	5	15	97	78	8	0
+ cysteine 40 μmoles	0	55	40	48	35	99	77	28	37
	1	29	34	20	30	94	80	13	7
	10	5	20	5	15	95	82	4	3

[a] Percentage original metmyoglobin, denaturation includes acid hematin of precipitated protein and unrecovered heme.

[b] Parenthetical figures are results of heating the more stable metmyoglobin of preparation 2 at 65° C. for 1 hour.

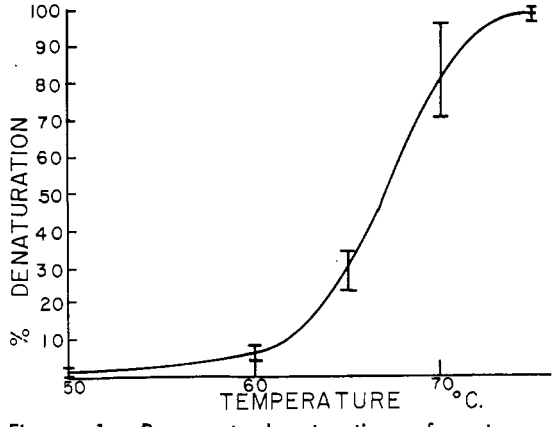

Figure 1. Per cent denaturation of metmyoglobin at pH 5.5, 1-hour heating

The addition of cysteine caused an increase in heme destruction at 60° and 70° C. After heating metmyoglobin with cysteine at 60° C. for 1 hour, a distinct absorption peak appeared in the region 615 to 620 mμ. The enhancement of this peak under reducing conditions was indicative of the presence of sulfmyoglobin (*4, 13*). The degradation of metmyoglobin heme in the presence of cysteine, however, did not form a green acid-acetone soluble component. Nitrite protected the heme in the presence of cysteine, but it was less effective in the presence of cysteine. The data

Table IV. Formation of Denatured Nitrosomyoglobin in Model Systems

Reactants: MetMb	Added NO_2^- μmoles	Denatured MbNO[a], % 60 C. MetMb Preparation No. 1	60 C. MetMb Preparation No. 2	70° C. MetMb Preparation No. 1	70° C. MetMb Preparation No. 2
Control	0	0	0	0	0
$+S_2O_4^{--}$ 30 μmoles	1	42	10	31	60
	10	27	7	12	26
+ ascorbate, 20 μmoles	1	0[b]	0[b]	60	66
	10	0[b]	0[b]	65	65
+ cysteine, 40 μmoles	1	0	0	52	51
	10	0	0	62	69

[a] Percentage original metmyoglobin
[b] See text concerning red soluble pigment formation.

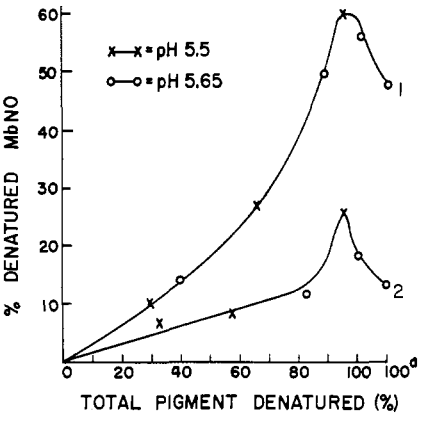

Figure 2. Relationship of pigment denaturation to yield of denatured nitrosomyoglobin in hydrosulfite-nitrite system

All pigment prior to heating was soluble nitrosomyoglobin.
1. Plus 1 μmole nitrite
2. Plus 10 μmoles nitrite.
100[a] heated at temperature 10° C. higher than that required to cause 100% denaturation

of Watts *et al.* have shown a stabilizing effect of nitrite, when cured meat is maintained in the presence of sulfhydryl groups (*20*). These results indicated that the degradation of metmyoglobin heme by cysteine results in different degradation products than those obtained in the presence of ascorbate. Oxygen was present in the model system and the degradation of metmyoglobin heme by ascorbate or cysteine is presumably dependent on the presence of oxygen (*13*).

Effect of Conditions on Formation of Pigment. Reductants and Nitrite Level. The yields of the denatured nitrosomyoglobin derivative obtained with the various systems are presented in Table IV. Only the systems containing hydrosulfite and nitrite were capable of forming denatured nitrosomyoglobin at 60° C. The systems containing hydrosulfite also caused appreciable denaturation of metmyoglobin with the heme intact at 60° C. The soluble nitrosomyoglobin derivative initially present in the hydrosulfite-nitrite systems was rapidly lost on heating and the soluble pigment was oxidized to metmyoglobin. The high level of nitrite was detrimental to the formation of denatured nitrosomyoglobin in all the systems containing hydrosulfite.

In contrast to hydrosulfite, neither cysteine nor ascorbate was able to cause immediate formation of the soluble nitrosomyoglobin derivative. The reductive activity of cysteine was exhausted after 1-hour heating at 60° C., the soluble pigment consisting of metmyoglobin. The systems containing ascorbate, however, had appreciable amounts of soluble, reduced metmyoglobin derivatives present after 1 hour at 60° C. The soluble pigments using ascorbate plus 1 μmole of nitrite consisted of a mixture of approximately 20 to 40% metmyoglobin (calculated by absorbance at 635 mμ) and a mixture of oxymyoglobin and nitrosomyoglobin. The soluble pigments in the systems containing ascorbate and the high nitrite level (10 μmoles) consisted of a mixture of approximately 50% metmyoglobin, and 50% soluble nitrosomyoglobin. Denatured nitrosomyoglobin was not formed using either ascorbate or cysteine with nitrite at 60° C.

All the systems containing nitrite and reductant were capable of forming denatured nitrosomyoglobin at 70° C. Hydrosulfite was the least efficient reductant tested for formation of the denatured nitrosomyoglobin derivative. A consistently low yield of denatured nitrosomyoglobin was obtained in the presence of the high level of nitrite, and a significant amount of heme was degraded under these conditions (see Table III).

In the presence of either cysteine or ascorbate a high percentage of denatured nitrosomyoglobin was formed at 70° C. Ascorbate gave consistently high yields of denatured nitrosomyoglobin (60 to 73%), independent of the level of nitrite present. Souci (*16*) has reported that lower levels of nitrite are required when ascorbate is used in the curing of meat. In the presence of cysteine, the yield of denatured nitrosomyoglobin was, however, dependent on the level of nitrite used. The presence of a high concentration of nitrite was the most efficient condition for denatured nitrosomyoglobin formation, using cysteine as a reductant (62 to 72% yield). A low level of nitrite decreased the yield of denatured nitrosomyoglobin to 50%.

Denaturation. Previous data by Winkler and Hopkins (*24*) on the formation of desirable color in bacon using different temperatures and heating periods indicated that denaturation temperatures are required for optimum color formation. Kampschmidt (*11*) has also reported that denatured nitrosomyoglobin is more stable to light at elevated temperatures than the soluble nitrosomyoglobin derivative. Because in the presence of hydrosulfite all of the pigment prior to heat treatment is the soluble nitrosomyoglobin derivative, a second series of experiments were performed at pH 5.65 using hydrosulfite as the reductant. The results of these experiments and the results obtained with hydrosulfite at pH 5.5 are shown in Figure 2. Optimal denatured nitrosomyoglobin formation occurred when 90 to 100% of the total pigment was denatured. Increasing the temperature beyond that required to cause 100% denaturation of the pigment decreased the yield of denatured nitrosomyoglobin. The small difference in pH (5.5 *vs.* 5.65) and the corresponding difference in the temperature required to denature the pigment did not cause a significant variation in the amount of denatured nitrosomyoglobin formed at any specific level of denaturation. These results indicated that the maximum yields of denatured nitrosomyoglobin are obtained at temperatures which totally denature the protein.

The ability of denatured metmyoglobin to form the nitrosomyoglobin derivative was also tested. Metmyoglobin was heat-precipitated at 70° C. at a pH of 5.5. The precipitated metmyoglobin was washed and excess nitrite and hydrosulfite added. The suspension was mixed vigorously, centrifuged and washed, and the denatured nitrosomyoglobin in the precipitate was determined. The previously denatured metmyoglobin was found to be capable of 50% conversion to denatured nitrosomyoglobin under these conditions. Allowing the reaction to proceed for 18 hours at 5° C. in stoppered, foil wrapped tubes yielded 30 to 40% denatured nitrosomyoglobin generation. These results are in accord with the observation by Kelly and Watts (*12*) on the ability of faded cured meat to regenerate cured meat color in the dark after addition of nitrite and/or reductant.

Iron Salts. An observation by Gibson that the reduction of methemoglobin was catalyzed by the presence of metal ions (*6*) led Weiss *et al.* to test this effect in the formation of nitrosohemoglobin (*23*). They found iron, copper, and

Table V. Effects of Iron Salts on Yield of Denatured Nitrosomyoglobin Obtained at 70° C.

Reactants			
MetMb	Added NO_2^- μmoles	Added Fe^{+2} μmoles	Denatured $MbNO^a$, %
+ cysteine, 40 μmoles	1		51, 46
	1	1	67, 67
	10	..	71, 71
	10	1	73, 78
+ ascorbate, 20 μmoles	1	..	60, 62
	1	1	60, 66
	10	..	65, 68
	10	1	76, 68

[a] Original metmyoglobin.

zinc ions to be effective in catalyzing nitrosohemoglobin formation, using a system consisting of methemoglobin, nitrite, and ascorbate. A patent, in which ferrous ions are reported to improve the color of meat, has been recently granted (*5*). Because ferrous salt solutions are known to be excellent trapping agents for nitric oxide gas (*9*), the activity of iron salts in this model system was tested (Table V). No differences were observed when ascorbate was used as the reductant or when cysteine was used with the high level of nitrite. The lower yields of denatured nitrosomyoglobin obtained with 1 μmole of nitrite and cysteine (50%) were, however, increased to maximum yields (60 to 70%) by the presence of 1 μmole of iron salts. This increase was obtained with either ferrous or ferric salts, probably because of the reducing activity of the system converting the ferric ion to the ferrous ion. The presence of equimolar amounts of citrate did not influence the results. These data show an apparent nitrite sparing effect by ferrous ion.

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FOOD COLOR CHANGES

Role of the Sugars in the Browning Reaction in Potato Chips

R. S. SHALLENBERGER[1] and ORA SMITH

Department of Vegetable Crops, Cornell University, Ithaca, N. Y.

and

R. H. TREADWAY

Eastern Utilization Research Branch, U. S. Department of Agriculture, Philadelphia, Pa.

This study was undertaken to elucidate the role of the sugars in potato chip color development, as a result of nonenzymatic browning. Both reducing sugars and sucrose react with amino acids to produce brown colors, at temperatures used to fry potato chips. Multiple correlation between potato chip color and the reducing sugar and the sucrose contents of the raw potatoes, yielded a correlation coefficient of 0.983. Both reducing sugars and sucrose must be considered in determining the suitability of a potato for chipping. The multiple regression equation may accurately predict chip color.

POTATOES HIGH IN SUGAR content are particularly susceptible to excessive browning, when processed as potato chips. Sweetman (*13*) found that potatoes stored at temperatures favoring sugar accumulation resulted in dark colored potato chips. Denny and Thornton (*3, 4*) found that the color produced during the frying of chips correlated best with the reducing sugar content of the raw stock, less well with the total sugar content, and not at all with the nonreducing sugar values. These authors substantiated their findings by demonstrating that filter paper disks impregnated with glucose and fried would brown, while filter paper disks impregnated with sucrose and fried would not brown. However, selecting and handling potato tubers to control chip color, based upon the premise that the color is a function of the reducing sugar content, have led to inconsistent results. Tubers low in reducing sugars may yield burnt or dark chips.

Patton and Pyke (*9*) observed that potato slices which had been leached until no color developed upon frying had to be impregnated with both reducing sugars and amino acids to develop color. More recently, Habib and Brown (*5*) found that multiple correlation among

[1] Present address, Department of Food Science and Technology, New York State Agricultural Experiment Station, Cornell University, Geneva, N. Y.