so that the same conditions as the previous operation were obtained, except that the solution contained D isomer in excess. As D isomer crystallized by the inoculation of D isomer, the composition changed from S to T. After the D crystals were filtered off, solid racemic modification was added to the filtrate until the gross composition reached point P. The entire cycle was repeated indefinitely and both L and D isomers were obtained reciprocally.

The results of successive resolution which was controlled with the fixed crystallization time are shown in Table I. The isomers obtained by this procedure had an optical purity of about 98% on the average. If the optical purity is not satisfactory and further purification is required, the crude products can be purified easily by recrystallization. The purification can be made without loss of the optically active isomer, according to the principle shown in Figure 3, indicating that the optically active isomer no longer dissolves in the saturated solution of the racemic modification. Namely, this purification can be performed by dissolving the mixture in a minimum amount of water required to dissolve the racemic modification in the crude crystals and allowing the pure crystals to crystallize out. However, the operation is not easy because the amount of water required to dissolve the racemic impurity is very small. Therefore, it was convenient to carry out the above operation by adding an appropriate amount of the solution saturated with the racemic modification. L-Lys-*p*-ABS thus obtained was decomposed with hydrochloric acid and converted into L-lysine hydrochloride. Insoluble *p*-aminobenzenesulfonic acid could be easily recovered quantitatively by filtration and used for resolution. On the other hand, D-Lys-*p*-ABS was completely racemized into DL modification by heating in an autoclave and used again for the resolution.

The yield of L-Lys-*p*-ABS from DL-Lys-*p*-ABS through the procedure combined resolution and racemization of Lys-*p*-ABS was 90%. The overall yield of L-lysine hydrochloride from DL-lysine base was 82% of theory. All processes and yields are summarized in Scheme I.

CONCLUSION

In the optical resolution presented here, the yield at each step is very high and the operation is simple. Also, all processes are expected to be operated automatically by sequence control system. Therefore, application of the present method for the industrial production of L-lysine is considered to be very promising if combined with a proper synthetic method for DL-lysine. In the present work we can not establish a theory to predict what kind of racemic modification forms a racemic mixture suitable for the resolution by preferential crystallization. However, it is possible to determine whether a given racemic modification is a racemic mixture or a racemic compound and to predict whether or not resolution of a given racemic modification is possible. In addition it suggests that the present simple method using aromatic sulfonates may be applied more generally for resolution of synthetic amino acids because aromatic sulfonic acids have a variety of properties and easily form salts with any kind of amino acids, so that it is very likely that some of their salts will form racemic mixtures suitable for preferential crystallization procedure. Its further application to other amino acids is under investigation.

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Possible Substitutes for Nitrite for Pigment Formation in Cured Meat Products

Arlene Howard, Patricia Duffy, Kathy Else, and W. Duane Brown*

Various concentrations of 24 nitrogenous ligands were examined for their ability to form ferrohemochromes with bovine myoglobin at pH 5.0 or 6.0. Methyl and hexyl nicotinate and *N,N*-diethylnicotinamide were particularly effective. Methyl and hexyl nicotinate and *N,N*-diethylnicotinamide produced stable pink pigments in cooked ground meat mixtures. These compounds were also effective, as was trigonelline, in combination with 10 or 20 ppm of nitrite in forming a stable and long-lasting pink cured meat color in such systems.

The curing of meat products generally involves the use of a mixture of sodium chloride, sugar, nitrate and/or nitrite, and often a reductant such as sodium ascorbate or sodium erythorbate. In this conventional method of curing, the nitrate and nitrite undergo reduction to nitric oxide. Nitric oxide then binds with the heme pigments myoglobin and hemoglobin to form the unstable bright red pigments nitric oxide myoglobin and nitric oxide hemoglobin. Upon heating, a relatively stable pigment, denatured globin nitric oxide ferrohemochrome, is formed.

The chemistry of the pigment formed during curing has been studied extensively and reviews are available (Fox,

Institute of Marine Resources, Department of Food Science and Technology, University of California, Davis, California 95616.

1966; Hornsey, 1959; Price and Schweigert, 1971; Watts, 1957).

Recently the use of nitrite as an additive has been questioned (Lijinsky and Epstein, 1970). Reduction of allowable sodium nitrite levels in finished products from 200 to 10 or 20 ppm has been urged (Food Chemical News, 1971; Intergovernmental Relations Subcommittee of the Committee on Government Operations, 1971). Should the allowable levels of nitrite or nitrate be substantially lowered there would be need for new color stabilizers because of the importance the consumer places upon the stable pink color that nitrite gives to cured meats, sausages, and other products such as pet foods.

The use of nicotinic acid has been patented as a preservative of the red color of meats (Coleman *et al.*, 1949). Other patents disclose the use of other pyridine derivatives (Dekker, 1958; Hopkins and Sato, 1971) and tetrazole (Duiven and DeVries, 1971). Another patent describes stabilizing the color of meat with the following heterocyclic compounds: purines, pyrimidines, imidazoles, pyrazines, triazines, and similar ring systems, as well as derivatives of these ring systems (Tarladgis, 1967). These patents generally apply to fresh meats, but some include cured products as well (*e.g.*, Duiven and DeVries, 1971; Tarladgis, 1967).

Unfortunately the pigments formed by the various nitrogenous ligands included in the patents just mentioned are substantially less stable than that formed from nitrite. Consequently it was the purpose of this study to examine a variety of other nitrogenous ligands for their ability to form stable pink pigments in models and cured meat systems.

EXPERIMENTAL PROCEDURE

Our earlier work on the chemistry of ferrohemochrome formation (Akoyunoglou *et al.*, 1963) provided the basis for the initial selection of compounds tested and for the design of models. Part of the work reported herein is included in a thesis (Howard, 1971).

Materials. Chemicals and other materials were obtained from the following sources. From Allied Chemical: sodium nitrite; from Baker Chemical: citric acid; from Bio-Rad: DEAE cellulose; from Calbiochem: *N,N*-bis-(2-hydroxyethyl)glycine (bicine), glucosamine; from C and H Sugar: sucrose; from Eastman: EDTA (ethylenediaminetetraacetic acid, sodium salt), isonicotinamide, isonicotinic acid, isonicotinic acid hydrazide, ethyl nicotinate, hexyl nicotinate and methyl isonicotinate; from Gallard-Schlesinger: 2-methylimidazole, 2,4-dimethylimidazole; from K & K Laboratories: *N*-ethyl nicotinamide; from Leslie Salt Co.: sodium chloride; from Mallinckrodt: sodium citrate; from Mann: trigonelline and L-leucine methyl ester; from Matheson Coleman and Bell: sodium dithionite; from Nutritional Biochemicals: nicotinic acid; from Sigma Chemical Co.: nicotinamide, duponol (sodium lauryl sulfate), methyl nicotinate, nicotinuric acid, L-leucineamide·HCl, L-histidine·HCl, L-histidine methyl ester, DL-phenylalanine, thiamine·HCl, *N,N*-diethylnicotinamide, sodium nicotinate, ascorbic acid, nicotinic acid hydroxamate, and D-gluconic acid lactone. All were used without further purification.

Myoglobin was isolated from water extracts of bovine top round muscle by repeated ammonium sulfate fractionation. It was purified on a DEAE-cellulose column (30 × 6 cm), using 0.1 *M* Tris buffer, pH 8.6 (Brown, 1961). The ratio of the absorbancy at the Soret (heme) peak to that at 280 nm (protein) was used as a guide to purity; bovine myoglobin had a Soret (409):280 nm ratio of about 5.5. The myoglobin concentration was calculated from recordings made on a Cary 15 spectrophotometer using an extinction coefficient of $\epsilon_{409}^{1\ mg/ml} = 9.3$.

Model System. The model system used for testing the potential of the various nitrogenous ligands for forming ferrohemochromes contained final concentrations of 0.22 mg/ml of metmyoglobin, 0.1% duponol, 0.4 *M* sodium citrate buffer of pH indicated in the tables and figures, and nitrogenous ligand at concentrations indicated in the tables and figures. After mixing the various constituents in a small beaker, about 2 mg of sodium dithionite was added, the contents were poured into a cuvette, and the spectrum from 700 to 480 nm was recorded in a Cary model 15 spectrophotometer. Preliminary experiments indicated that the absolute absorbance was not significantly affected regardless of whether 2–4 mg of dithionite was carefully preweighed or added by visual estimate. Reference cuvettes contained distilled water. Recording of the spectra was started within 10–15 sec after all of the reagents were mixed. Ferrohemochrome formation was ascertained by the presence of sharp peaks in the 555–560 and 525–530 nm regions. Whether or not a ferrohemochrome had formed could also be determined by visual inspection, *i.e.*, the presence of a pink color. With some ligands there were changes in spectral properties with time other than those due to oxidation. Such cases are noted in the Results Section.

At least duplicate analyses were done in every case with virtually identical results.

Oxidation Studies. In these studies samples were rescanned a number of times over a period of time, at hourly or longer intervals (1 hr to 1 month). The amount of oxidation was measured by the decrease in the absorbance of the peak in the 555-nm region with time. Rate constants were calculated and the graphs were plotted using a least-squares subroutine (program no. 09100-70820) designed for the Hewlett-Packard desk calculator (model 9100B) and line plotter (Model 9125A).

Sausage Model. The formulation used and the method for preparing, stuffing, and cooking the sausages was based on a procedure described by Fox *et al.* (1967). Percentages of the mixture used follow: equal parts of ground beef and ground pork, 76; sucrose, 1.5; sodium chloride, 2; ice water, 20. In addition, ascorbic acid was ordinarily employed at levels of 0.05% and in some trials glucono-δ-lactone was used, also at 0.05%. The ligands were tested at various levels as shown in the Results Section.

The sodium chloride, sucrose, and nitrogenous ligand were added to about one-half of the water and this solution was added to the ground beef and pork. These ingredients were mixed with either a meat grinder attachment of a Hamilton Beach mixer or an Oster meat grinder. The mixture was then reground with the reductant which had been added to the remaining water. For each condition tested, duplicate aliquots of approximately 30 g of the ground mixture were stuffed into labeled 35-ml disposable plastic syringes which had the needle tip end cut off. A rubber stopper was used to cover the cut end. Following mixing and stuffing, the sausages were stored at +5° or cooked immediately in the following ways: in a 70° water bath until reaching an internal temperature of 69° (45–51 min); in a 70° water bath for 2 hr (internal temperature 69°); in a 94° water bath until reaching an internal temperature of 70° (6 min); or in a 94° water bath for 2 hr (internal temperature 93 ± 1°). The samples were immediately cooled with ice and stored in the syringes in the refrigerator for several hours or else immediately removed from the syringes while still warm, placed on a tray lined with absorbant paper, covered loosely with Saran Wrap, and chilled for several hours prior to color evaluation. Analyses of residual levels of nitrite in a few of the samples were made by the method described in Pearson (1962).

Table I. Concentrations of Ligand Required for Ferrohemochrome Formation in the Model System and Spectral Data of the Resulting Ferrohemochrome

Ligand	Minimum concn,[a] mM		Maximum concn,[a] mM		Absorbance maxima,[b] α and $\beta\lambda$, nm
	pH 5	pH 6	pH 5	pH 6	
Sodium nitrite	30		40		567, 534
Sodium nicotinate	20	30	100	160	555–556, 525–526
Methyl nicotinate	0.05	0.07	2	10	555, 525 → 563, 531
Ethyl nicotinate	0.05	0.02	0.2	0.2	554–555, 523–524 → 562, 532
Hexyl nicotinate	0.01	0.01	0.1	0.1	555, 525
Nicotinic acid–hydroxamate	8	3	30	30	557, 529 → 561–562, 529–530
Nicotinuric acid	40	30	80	80	555, 525 → 563, 531
Nicotinamide	1.6	0.8	12	6	555, 524 at pH 5 559–560, 529–531 at pH 6
N-Ethyl nicotinamide	2	0.8	3	2[c]	555, 525
N,N-Diethylnicotinamide	0.4	0.2	3	2	555, 525
Isonicotinic acid	1	1	10	5	555, 525–526
Isonicotinic acid–hydrazide	0.6	0.3	80	40	555, 525–526
Isonicotinamide	2	1	30	20	554–555, 524–525

[a] Minimum concentration is that at which was first noted the development of a definite peak in the region of 550–560 nm (see, for example, Figure 2); maximum concentration was that beyond which the peak absorbance either falls or remains relatively constant. [b] Arrows indicate that the initial peaks shift to higher wavelengths. This generally happened within 2–3 min after dithionite was mixed into the sample. Associated with the shift is a change in color from pink to orange. [c] Approximate max; however, the OD_{555} tended to increase slightly up to 40 mM.

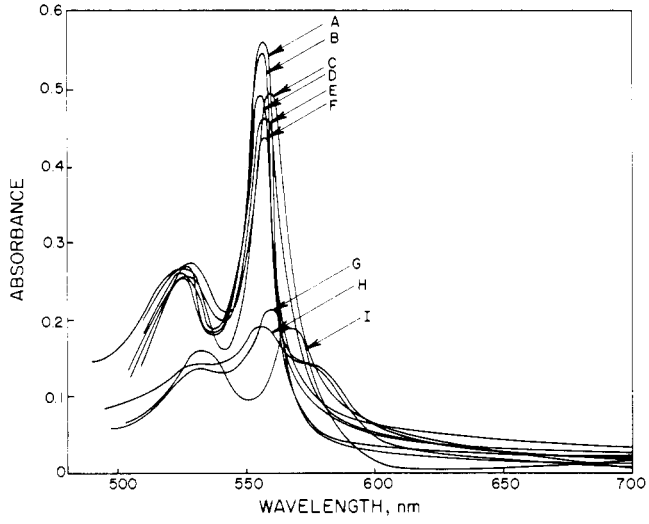

Figure 1. Absorption spectra of ferrohemochromes formed with various ligands and bovine myoglobin, pH 5. General conditions as described in Experimental Section of text. Ligands present: A, sodium nicotinate, 80 m*M*; B, methyl nicotinate, 40 m*M*; C, ethyl nicotinate, 40 m*M*; D, nicotinamide, 40 m*M*; E, nicotinic acid hydroxamate, 40 m*M*; F, *N,N*-diethylnicotinamide, 40 m*M*; G, isonicotinic acid, 40 m*M*; H, nicotinuric acid, 40 m*M*; I, sodium nitrite, 80 m*M*. Curves obtained prior to determination of maximum effective concentration of each ligand (see Table I for latter information).

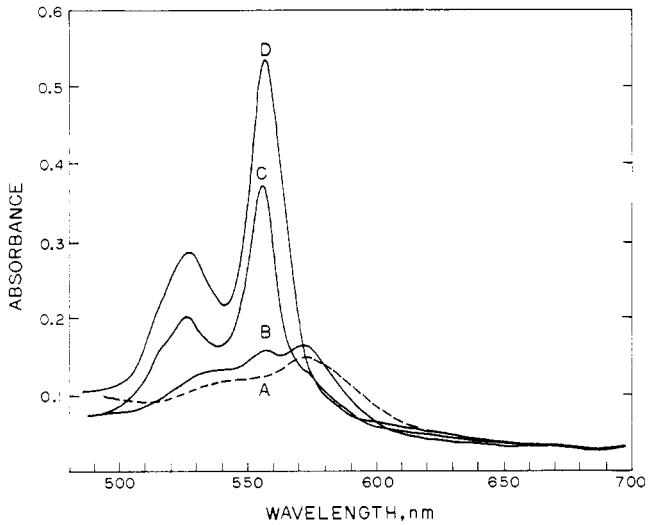

Figure 2. Effect of increasing concentration of methyl nicotinate on the absorption spectra of bovine myoglobin, pH 5. General conditions as described in Experimental Section of text. A, no ligand; B, 0.05 m*M*; C, 0.2 m*M*; D, 2 m*M*.

It should be emphasized that the sausages, by virtue of being cased in the syringes, were likely relatively anaerobic compared to sausages in frankfurter casings. Thus the rate of pigment oxidation may differ.

RESULTS AND DISCUSSION

Model Systems. A variety of nitrogenous compounds, including derivatives of pyridine, amino acids, and amino acid esters, was examined for their ability to form ferrohemochromes in model systems with bovine myoglobin at pH 5 and 6. Of the ligands examined, the following compounds were found to form ferrohemochromes: methyl, ethyl, and hexyl nicotinate; *N*-ethyl and *N,N*-diethylnicotinamide; nicotinamide, sodium nicotinate, nicotinuric acid, nicotinic acid hydroxamate, isonicotinic acid, isonicotinic acid hydrazide, and isonicotinamide. All of these ligands are derivatives of pyridine. All ferrohemochromes formed were pink in color. Their visible spectra showed peaks in the 555–560 (α peak) and 525–530 nm (β peak) regions. The exact wavelengths of these peaks for individual ligands are given in Table I. Figure 1 shows the absorption spectra of several of the ferrohemochromes formed with bovine myoglobin at pH 5 and 40 or 80 m*M* ligand concentration. The spectrum of the nitrite derivative is included for comparison. At the same ligand concentration, spectra appear similar for derivatives at pH 6.

The minimum concentration of ligand required for formation of a ferrohemochrome provides a good indication of relative reactivity. Such concentrations are given in Table I. It is evident that esters of nicotinic acid are quite reactive, as is *N,N*-diethylnicotinamide.

Gradually increasing the concentration of a ligand resulted in a pronounced change in the absorption spectra. This is illustrated in Figure 2. The amount of ligand necessary to effect a change in the shape of the absorption

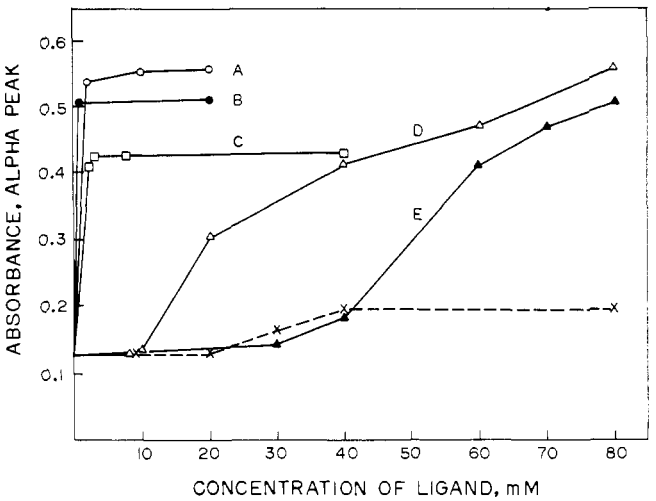

Figure 3. Association curves for ferrohemochromes formed with various ligands at increasing concentrations of the latter. pH was 5; other conditions as described in Experimental Section of text. Solid lines: A, methyl nicotinate; B, hexyl nicotinate; C, *N,N*-diethylnicotinamide; D, sodium nicotinate; E, nicotinuric acid. Dashed line, sodium nitrite.

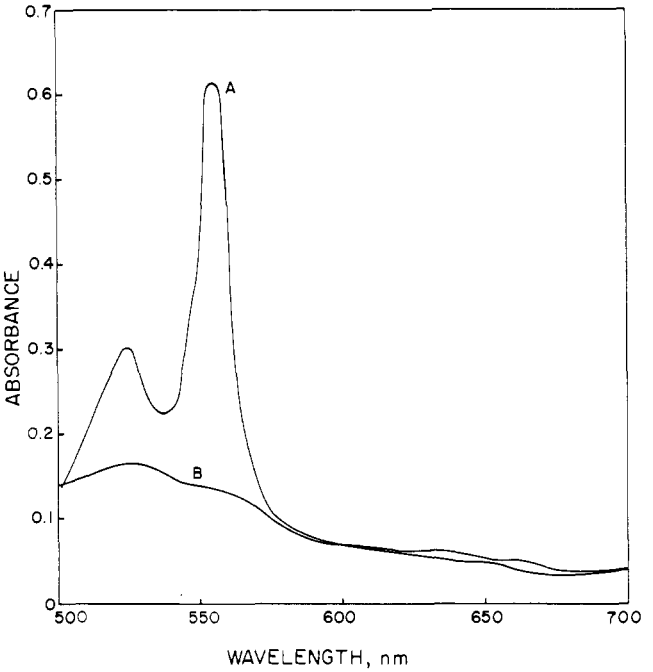

Figure 4. Effect of oxidation on spectrum of ferrohemochrome formed by bovine myoglobin and methyl nicotinate (40 m*M*) at pH 5. A, reduced sample, ferrohemochrome; B, oxidized sample, ferrihemochrome.

spectrum is dependent on the type of ligand and pH. At some point, further increase in concentration of ligand results in little or no change in the absorption spectrum; we have designated this the maximum concentration. Such values are included in Table I. With some ligands there was a decided shift in absorption peaks with time, as noted in Table I. The derivative formed with methyl nicotinate exhibited this behavior, resulting in a change from a red to orange color. Figure 2 was drawn from curves obtained with this ligand prior to the shift. Plotting absorbance of the α peak against ligand concentration shows an association curve; several of such are shown in Figure 3.

Ligands analyzed which did not form ferrohemochromes at pH 5 or 6 at 80 m*M* ligand concentration were glucosamine, 2-methylimidazole, 2,4-dimethylimidazole, L-histidine·HCl, phenylalanine, *N,N*-bisglycine, L-leucine methyl ester, L-leucineamide·HCl, L-histidine methyl ester, thiamine·HCl, methyl isonicotinate, and trigonelline. The spectra of systems containing 2,4-dimethylimidazole, glucosamine, L-histidine·HCl, phenylalanine, or *N,N*-bisglycine were nondescript and had small peaks at about 565–570 nm and flat shoulders at 535–550 nm. The ligands L-leucine methyl ester, L-leucineamide·HCl, L-histidine methyl ester, and thiamine·HCl caused immediate formation of cloudy brown precipitates; their visible spectra, therefore, could not be recorded.

Stability to Oxidation. In general, oxidation of ferrohemochromes to ferrihemochromes results in a color change from pink or red to brown and therefore is undesirable in cured meat and similar products (spectral change recorded in Figure 4). Hence, it seemed important to evaluate the stability to oxidation of the ferrohemochromes formed in the model systems. Rates of oxidation of several of these pigments are shown in Table II. It can be noted that rates of oxidation generally are lower at higher pH, although there are exceptions. It is clear that the presence of a nitrogen atmosphere is inhibitory.

Effectiveness of Ligands in Sausages. Based on findings in the model systems (and the oxidation studies), the most promising nitrogenous ligands were selected for testing as possible substitutes for nitrite in a frankfurter type preparation. The formation of a suitable ferrohemochrome in the final product was judged subjectively and was based on the formation of a pink or tannish-pink color within the range of colors currently found in commercial frankfurters. This evaluation was made by the authors of this paper, plus four additional laboratory personnel. Consideration was given to formalizing a panel for this purpose; however, the range of colors found in commercial frankfurters is very wide. It was quite easy for an observer of the sausages prepared in this study to note whether or not the color was within such a range.

The first compounds tested that satisfied the criteria just described were *N,N*-diethylnicotinamide and methyl nicotinate. Both compounds will form stable pink pigments in frankfurters heated to an internal temperature of

Table II. Factors Affecting the Oxidation Rate of Ferrohemochromes

Ligand				Oxidation rate[a]
Type	Concn, m*M*	pH	Oxidation atmosphere	(× 1000) hr^{-1}
Methyl nicotinate	40	5	Air	83
Methyl nicotinate	4	6	Air	43
Methyl nicotinate	4	6	N_2	8
Methyl nicotinate	40	6	Air	38
Methyl nicotinate	40	6	N_2	13
Hexyl nicotinate[b]	40	5	Air	2
Hexyl nicotinate[b]	20	5	Air	3
Hexyl nicotinate[b]	20	6	Air	<1
Ethyl nicotinate[c]	40	5	Air	7
Ethyl nicotinate[c]	40	6	Air	4
N,N-Diethylnicotinamide	80	5	N_2	7
N,N-Diethylnicotinamide	80	6	N_2	1
N,N-Diethylnicotinamide	40	5	Air	24
N,N-Diethylnicotinamide	40	6	Air	31
Nicotinamide	40	5	Air	33
Nicotinamide	40	6	Air	23
Sodium nitrite	80	5	Air	12

[a] Decreased absorbance of α peak with time. [b] 0.7% duponol was necessary to keep 40 m*M* hexyl ester in solution; 0.4% duponol required for 20 m*M*. [c] First peak positions about 524, 555. Shifts within 3–5 min to 532, 562. Oxidation measured only on 562 peak.

Table III. Effect of Type and Concentration of Ligand, and Time, Temperature, and Method of Cooking on the Formation of the Ferrohemochrome Pigment in Sausage Model

Ligand		Glucono-δ-lactone (GDL) and/or ascorbic acid		Ferrohemochromes[a]			
Type	%	Type	%	A[b]	B[c]	C[d]	D[e]
Methyl nicotinate	0.2	GDL	0.05	+	+	+	+
Methyl nicotinate	0.1	Ascorbic acid	0.05	NT	NT	NT	+
Methyl nicotinate	0.3	Ascorbic acid	0.05	+	+	+	+
N,N-Diethylnicotinamide	0.2	GDL	0.05	+	+	+	+
N,N-Diethylnicotinamide	0.1	Ascorbic acid	0.05	NT	NT	NT	+
N,N-Diethylnicotinamide	0.3	Ascorbic acid	0.05	+	+	+	+
N,N-Diethylnicotinamide	0.3	Ascorbic acid + GDL	0.05[f]	+	+	+	+
Hexyl nicotinate	0.05	Ascorbic acid	0.05	NT	NT	NT	+
Sodium nitrite	0.1	Ascorbic acid	0.05	+	+	+	+
Sodium nitrite	0.05	Ascorbic acid	0.05	+	+	+	+

[a] Ferrohemochromes formed: + = yes; NT = not tested. [b] Samples A were cooked in a 70° water bath until reaching an internal temperature of 69° (31 min). [c] Samples B were cooked in a 94° water bath until reaching an internal temperature of 69° (6 min). [d] Samples C were cooked in a 94° water bath for 2 hr (internal temperature of 92°). [e] Samples D were cooked in a 70° water bath for 2 hr (internal temperature of 69°). [f] Total 0.05%, half ascorbic acid and half GDL.

about 70° (Table III). Under the conditions usually used, a 45-min time period was needed to reach this temperature. A variety of heating times and of storage times of the frankfurter mixture prior to heating was employed. In general, heating for a longer period, *i.e.*, up to 2 hr, improved the final color. However, prolonged heating was not necessary to obtain a satisfactory color.

The various ligands were tested with and without ascorbic acid and/or glucono-δ-lactone. The latter constituents improved the color as well as the stability of the pigment formed. Increasing the ascorbic acid concentration from 0.05 to 0.5% was without benefit.

Subsequent similar studies revealed that hexyl nicotinate would also form a satisfactory pigment. This compound in model systems is effective at extremely low concentrations compared to the other ligands. It has limited water solubility but disperses well in water; quantitative addition was not a major problem. Stable pigments were not formed with the following compounds (all used at levels up to 0.3%): nicotinamide, nicotinic acid hydroxamate, and 2-methylimidazole. Trials were included with trigonelline in spite of the fact that it was ineffective in the model system because it is an excretory product of nicotinic acid, is excreted unchanged after ingestion, and is thus potentially an ideal food additive. It was not effective in the sausage model, but apparently worked synergistically with low levels of nitrite (see section following).

Effectiveness of Ligands in Combination with Nitrite. A number of ligands were tested in combination with low levels of nitrite (*i.e.*, 5-, 10-, and 20-ppm initial levels). In general, the results with 5 ppm of nitrite were unsatisfactory. At 10 and 20 ppm, however, the initial color of the frankfurters was quite satisfactory with nitrite alone or with nitrite in combination with *N,N*-diethylnicotinamide, methyl nicotinate, or trigonelline. After storage (5°) for periods of time up to 10 weeks, the combination of any of these ligands (initial concentration 0.1%) enhanced the color stability. That is, after storage, samples with only nitrite present faded on exposure to air, while those with combinations of nitrite and the ligands just mentioned (particularly *N,N*-diethylnicotinamide and trigonelline) showed more acceptable and stable pink colors.

Analyses of a few samples of frankfurters at 12 weeks following preparation with 10 or 20 ppm of nitrite showed residual levels of no more than 2 ppm of nitrite.

It should be noted that some derivatives of nicotinic acid and nicotinamide are known to have vasodilatory properties. We find no mention of pharmacological effects of nicotinic acid esters. *N,N*-Diethylnicotinamide has been used as a mild vasodilator but with suggested doses of several grams per day given intravenously (Woolf, 1970).

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