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The Determination of Methemoglobin in Beef Muscle Extracts

I. A Study of the Spectrophotometric Method
II. Factors Affecting Methemoglobin Formation in Frozen Beef

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INTRODUCTION

A major problem in food technology today is the development of objective, reproducible tests which will indicate changes in foods before any deterioration is recognizable by organoleptic means. Such tests are valuable not only for determining quality of food but for measuring the effectiveness of various methods of preservation.

In meats, particularly in beef, changes in palatability are accompanied by darkening of cut surfaces. The major cause of this darkening is considered to be the conversion of the bright red pigment, oxyhemoglobin, which forms on exposure of the meat surface to air, through reduced hemoglobin, into a brownish red pigment, methemoglobin. For this reason, some method of measuring the methemoglobin present in beef might be used as a measure of deterioration.

The hemoglobins, like many colored compounds, are most conveniently identified by their light-absorption characteristics. Fortunately, a given hemoglobin compound shows little species difference in this respect.

The problem is somewhat complicated by the fact that, even with modern methods of slaughter, meat pigments may be mixtures of blood hemoglobins designated by convention, hemoglobins, and muscle hemoglobins also called myoglobins, probably chiefly the latter. These two series of compounds differ slightly in their light absorption characteristics, due chiefly to a difference in the globin fraction of the molecule. The occurrence of hemoglobin in an associated form consisting of four iron-porphyrin-protein units does not seem to influence light absorption when one mole is defined as equivalent to one mole of porphyrin, as suggested by Austin and Drabkin. The standard absorption curves for the various blood hemoglobin compounds are quite well established. Those for the myoglobins are still being developed. For this reason, hemoglobin absorption constants have been used for work with meat pigments.

The method for estimation of the proportion of methemoglobin in a mixture assumed to contain only the two pigments, oxyhemoglobin and methemoglobin, is based on the fact that taken individually, at a standard concentration and pH, each of these two pigments will give a characteristic, reproducible, absorption curve.

At any given wave length the difference between these two curves may be measured. This difference is considered to be the total change which would occur in a reading at this wave length if the oxyhemoglobin were to be completely converted to methemoglobin. By converting all the pigments in the mixture to be tested to cyanmethemoglobin, a stable common derivative which has well established absorption constants, the spectrophotometric readings on the original mixture may be calculated to a standard concentration. The difference between the reading for oxyhemoglobin and that for the unknown mixture at the chosen wave length is considered to represent the partial change the pigment has undergone. The ratio of partial change to total change is used as an estimate of the proportion of methemoglobin formed.

This method was elaborated upon by Austin and Drabkin (1935) to produce their method of summation of partial change. In this method, several carefully chosen wave lengths were used and the ratio of the sum of the partial change to the sum of total change was used as the estimate of methemoglobin present. The wave lengths chosen for their study were 540, 560, 575 and 630 millimicrons. These represent maximum and minimum points on the oxyhemoglobin curve, in the visible range, and hence on the curves representing the mixture. Since the curve is horizontal at these points, errors in spectrophotometric measurements are much smaller than when the readings are taken on the slope of a curve.

The absorption curve for oxymyoglobin, while very similar to that of oxyhemoglobin, is displaced from two to four millimicrons to the right in the visible range. This means that the use of hemoglobin wave lengths for myoglobin studies results in measurements in areas where absorption curves begin to slope, and error in measurement is consequently greater. However, since myoglobin constants are not entirely agreed upon and since they are available for 540 but not for 560, 575 or 630 millimicrons, Austin and Drabkin's method of summation of partial change at the wave lengths mentioned, employing hemoglobin constants, should still be useful in evaluating relative changes in meat pigments.

While this method of estimation of methemoglobin in meats appears to have become an accepted procedure, very few results have been published. Ramsbottom and Koons (1941) have reported greater conversion of oxyhemoglobin to methemoglobin in beef steak stored at 10° F. than at -30° F. for one year.

The present series of experiments, undertaken in connection with a study of factors influencing changes in quality of frozen foods, was designed to determine spectrophotometrically, the extent of pigment change in frozen ground beef under different storage conditions and to relate this change to other evidence of deterioration such as the development of off flavors and odors. Certain peculiarities in the spectrophotometric readings have led to a re-examination of the method with somewhat unexpected results. Since the findings of this series of experiments are to be used in a relative sense only and since the study of the method has not invalidated them for this purpose, they will be reported as originally calculated.

Ground beef was the product chosen to work with for several reasons. Since grinding exposes a maximum surface to the air, a more nearly complete conversion of reduced hemoglobin to oxyhemoglobin may be assumed in ground than in unground beef. Moreover, ground meats deteriorate more rapidly than piece meats and thus present a special storage problem. Since oxyhemoglobin is converted most rapidly to methemoglobin at low oxygen tensions, the greatest change occurs not at the surface, but at about one to two millimeters beneath it. Thus, the matter of sampling presents a real problem in determination of pigment change in extracts from unground meats. Sampling procedure is much simplified by grinding and mixing in large lots.

EXPERIMENTAL

The ground beef used in the first year's experiments was part of a lot reserved for Food Preservation Laboratory use from animals of known history which had been graded as good quality. Rounds and trimmings of this beef were power-ground twice. Portions weighing 450 grams each were put into cellophane bags which were then heat-sealed, boxed and frozen at -24° C. Two series of these packages were put in the storage rooms held at -18° C. and -12° C. A third series was placed on the coils in the colder room, a fourth in a storage room allowed to fluctuate from -18° C. to -12° C., and a fifth series was held at -18° C. until taken to the home-type freezer for the thawing experiments. These last samples were allowed to thaw and refreeze from one to five times before being returned to the -18° C. room. Thermo-couples were placed in one package from each series so that temperature records of both meat and rooms might be kept.

One series of samples was treated with ascorbic acid solution as a reducing agent and wrapped and boxed as above. Three series of samples were packed in 400-gram portions in mason jars. These were equipped with specially designed stoppers so that they could be evacuated and flooded with the gases to be studied. Nitrogen, oxygen and carbon dioxide atmospheres were used with air as a control.

Since it was necessary to store the ground beef described above overnight at 0° C. before packaging and freezing, it was decided to do an additional study of the effect on the pigment of holding at this temperature. Beef for this purpose was purchased from a local packing house and ground as before. Packages were sealed immediately and 1, 2, 4, 6, 9, 12, 24, 30 and 72 hours after grinding. These packages were held at 0° C. until sealed and frozen at -24° C.

During the second year, experiments were undertaken to determine the effect of amount of fat on pigment change in ground beef. Good quality beef was purchased from a local packing house, together with additional suet. The beef was freed from most visible fat and ground once through a power grinder. Portions were then reground with no added fat, and 5, 10, 15, 20 and 25 per cent added ground suet. The ground meats were then divided into one-pound portions, packaged as before, frozen, and stored at -18° C. Packages from each series were analyzed for actual fat content.

One-pound pieces of the same beef were frozen unground for comparison with the ground to which no fat was added. For a study of variation in pigment change in market samples, nine samples of ground beef, one to two pounds each, were purchased at local markets. These were frozen and stored as described previously.

In adapting the method of Austin and Drabkin for use with tissue extracts, it was recognized that results of any study of the change of meat pigments would be relative, depending as they do on the particular meat used and upon the conditions under which the tests are conducted. In the absence of standardized test procedures, preliminary experiments were carried out to determine the effect of test conditions upon results. Since time and temperature seemed to be the most important variables in test procedure, these two factors were controlled as carefully as possible in the schedule which was established. Unfrozen samples showed much more pigment change when tested than frozen samples from the same lot, indicating the necessity for freezing the control samples before testing. The amount of light present during preparation of the extract had no apparent effect on the results. While the spectrophotometric examination of extracts may be made with a wide range of pigment concentration, a procedure which gave a final concentration of from 0.035-0.040 millimoles of hemoglobin per liter (as defined by Austin and Drabkin, where one millimole is equivalent to one millimole porphyrin) was found to be most convenient to use, due to the relative stability of the solution and to the ease in reading extinction coefficients in this range. While Austin and Drabkin worked with more concentrated solutions of the hemoglobins, Bowen working with myoglobins used from 0.03-0.07 millimoles.

The procedure established as the result of preliminary experiments was as follows. The lower one-third of the frozen meat from each rectangular package to be used in a day's work was cut from the package, wrapped in cellophane and put into the freezing compartment of the laboratory refrigerator. These one-third-pound portions were removed as needed. Enough one-fourth-inch slices were removed from one side to give 50 grams. These slices were put with 100 milliliters of boiled, cooled distilled water into a Waring blendor and mixed for three minutes. The resulting slurry was centrifuged for six minutes. The fatty portion was lifted off the top and the fairly clear red liquid was filtered through filter paper in the refrigerator. The resulting filtrate, except in a very few cases in which the meat was in very poor condition, was a clear sparkling liquid. Ten milliliters of this solution were diluted to 25 milliliters with boiled cooled distilled water for both treated and untreated solutions. The treated solutions contained potassium cyanide and potassium ferricyanide added, according to the method of Austin and Drabkin, to convert the pigments to cyanmethemoglobin. The treated extracts were read at 540, 545, and 551 millimicrons to determine concentration, and the untreated solutions were read at wave lengths of 540, 560, 575 and 630 millimicrons, in a Beckman model DU spectrophotometer. Cloudiness of the treated solutions occurred occasionally and since this interfered with determination of concentration, read-

ings from cloudy solutions have not been reported. The total period elapsing, from the time the samples were removed from the freezer until the first reading, was two hours. During the first year three-hour readings were also taken in order to note the rate of change of the pigments.

Near the end of the second year a Cary recording spectrophotometer was available. It was used to obtain curves for six presumably identical samples (from the 15 per cent fat series) for the wave length range from 500 to 700 millimicrons. In order to study the method, three sets of curves were obtained for each sample using the (A) extracts containing the mixture of pigments, (B) extracts in which the pigment mixture had been converted to metmyoglobin and (C) extracts in which the pigment had been converted to cyanmetmyoglobin. The readings were taken as soon as possible with the new equipment and again at two hours after removing from the freezer. All spectrophotometric results were calculated to the millimole-per-liter basis as described by Austin and Drabkin.

In addition to the spectrophotometric study, color estimates were made for most meat samples from the Dictionary of Color. These proved useful chiefly as a matter of record. The pH of both meats and extracts was determined, using the Beckman pH meter with glass electrode. The electrode was inserted directly into the cup of ground beef with no water added for the former determination. The pH of the extract was determined both after filtering and after dilution to volume. All determinations were made on samples at room temperature. Peroxide and iodine determinations were made on fat from samples studied the first year but these tests were discontinued when they were found to yield little information. Samples from the major series were analyzed for fat, moisture, nitrogen and ash. Samples in the series to which fat was added, were analyzed for fat and moisture.

A panel of four experienced judges rated the palatability of cooked samples of the meats. Upon removal of the one-third-pound portion from each package for spectrophotometric analysis, the remainder was rewrapped and kept frozen, for not more than three days, until the morning of judging. It was then thawed, sliced and broiled. The cooked slices were scored on appearance, odor, flavor, tenderness, juiciness and general condition. Each attribute was rated: 5, very desirable; 4, desirable; 3, acceptable; 2, slightly undesirable; 1, undesirable; or 0, inedible. No attempt was made to weight or combine scores of the individual attributes. The category "general condition" was the judge's estimate rather than an average.

RESULTS AND DISCUSSION

1. Study of the Spectrophotometric Method

Theoretically, the spectrophotometric reading at each wave length may serve as a separate estimate of the change in the pigment from the oxy- to the met- form. If the method is measuring only the proportion of one compound in a mixture of two, the values indicated at the different wave lengths should vary in a random manner. It was noted even in the preliminary experiments, however, that estimates of methemoglobin at 560

millimicrons were consistently lower than at any other wave length. In fact, a few readings at this wave length were higher than would be expected if the pigment were entirely in the oxy- form.

A systematic study of this problem was begun, using a series of eight samples from the experiment to determine the effect on the pigment of holding before freezing. Methemoglobin estimates were calculated from readings at each separate wave length and for each combination of two and three as well as four wave lengths. The values of methemoglobin indicated at the separate wave lengths were ranked, with the highest value given a rank of one. (Table 1.) The reading at 560 millimicrons ranked fourth in most cases. Moreover, the methemoglobin estimated at this wave length and at 630 millimicrons was quite variable for a series which had exhibited considerable uniformity in methemoglobin estimated at four wave lengths. The estimates from readings at 540 and 575 millimicrons were quite comparable to each other and to the four-wave-length estimates. It was concluded that readings at 540 or 575 millimicrons or both would serve nearly as well as the four-wave-length readings for determining relative methemoglobin formation in a series of samples.

TABLE 1.—Comparison of Methemoglobin Estimates at Individual Wave Lengths With Estimates by Austin and Drabkin's Method of Summation Using Four Wave Lengths[1]

Sample[2] number	Wave lengths $m\mu$								
	Summation of 4 wave lengths methemoglobin pct.	540 methemoglobin pct.	rank	560 methemoglobin pct.	rank	575 methemoglobin pct.	rank	630 methemoglobin pct.	rank
H50	31	35	2	13	4	37	1	24	3
H52	27	32	2	7	4	34	1	24	3
H55	30	37	1	15	3	36	2	13	4
H56	26	29	3	4	4	33	1	29	2
H57	26	30	2	5	4	32	1	26	3
H58	33	40	2	19	3	40	1	14	4
H59	27	31	3	5	4	34	1	31	2
H60	27	32	2	7	4	34	1	22	3

[1]This method gives a weighted average.
[2]From the holding time series

In order to minimize the possibility that the systematic variation in methemoglobin estimates indicated could be due to the sample of beef used or one particular set of test conditions, all determinations from experimental series were calculated and ranked as described above. Analyses of the results by the method of ranks were made for the eight samples studied first, for each of the two year's determinations, and finally for the total of 113 samples studied up to that time. (Table 2.) In each case, a systematic rather than a chance variation between estimates at the different wave lengths was indicated. A similar analysis, ranking only estimtes made at 540, 575 and 630 millimicrons showed a significant difference between estimates made at these wave lengths, indicating that elimination of

TABLE 2.—Analysis of Methemoglobin Estimates at Individual Wave Lengths by the Method of Ranks

Group analyzed	Number of samples	χ^2_r	Significance at 0.001 level (with 3 degrees freedom)
Holding time series	8	18.5	+
First-year samples	48	97.3	+ + +
Second-year samples	61	114	+ + +
All experimental samples	113	169	+ + +

readings at 560 millimicrons would not eliminate the systematic variation.

In trying to discover the cause for variation in estimates described above, two possibilities were considered. A third form of the pigment might be present in the extracts in varying amounts. An examination of the absorption spectra of the various forms of hemoglobin led to the conclusion that reduced hemoglobin which shows a maximum at 560 millimicrons could be responsible for the observed results. It had been assumed that, under the conditions of these experiments, this pigment would be present in negligible amounts.

The ratios of the extinction coefficients at two wave lengths have frequently been used to determine purity of pigment solutions as well as to estimate methemoglobin formation. Presumably, the ratio for a mixture would lie between that for the two pigment forms involved. Calculations of ratios for extracts from the holding time series showed this to be true for the 575/560 but not for the 540/560 ratio. (Table 3.)

Further investigation of the presence of a third form of pigment in the extracts is now being made.

A second possibility is that either the extinction coefficients chosen as constants for the pure pigments or the wave lengths used for the determinations were not the best ones for this particular type of experiment.

Examination of the mixed-pigment curves obtained with the recording spectrophotometer showed that the position of maxima and minima indicated the pigments to be chiefly myoglobin in nature rather than a variable mixture of muscle hemoglobins with blood hemoglobins as had been previously assumed. Since the oxymyoglobin absorption spectrum, while quite similar to that of oxyhemoglobin, shows maxima and minima to the right of the latter, a change in both wave lengths at which determinations were

TABLE 3.—Comparison of Extinction Coefficient Ratios of Pure and Mixed Pigment Extracts

Pigment form	Approximate ratios-mμ		Source of data
	575/560	540/560	
Oxyhemoglobin	1.73	1.67	Austin and Drabkin
Methemoglobin	0.96	1.67	Austin and Drabkin
Mixed pigment extracts	1.33	1.42	Holding time series

made and substitution of myoglobin for hemoglobin extinction coefficients seemed indicated.

Since the beginning of this experiment, information on myoglobin absorption spectra and extinction coefficients has been published both by the Medical Nobel Institute in Stockholm (Dr. Hugo Theorell et al) and by Dr. William J. Bowen of the Laboratory of Physical Biology, National Institutes of Health, Bethesda, Maryland. From the published data of, and by personal communication with the latter author, a series of myoglobin extinction coefficients, as well as a new series of wave lengths representing myoglobin maxima and minima, have been selected for use in calculation.

For the six determinations for which complete curves were available, it was possible to estimate methemoglobin using Bowen's extinction coefficients for myoglobin at the optimum wave lengths of 544, 564 and 582 millimicrons (Table 4), as well as Austin and Drakbin's coefficients for blood hemoglobin at 540, 560 and 575 millimicrons, and thus make a more direct study of the effect of choice of wave lengths and extinction coefficients on results of experiments with tissue extracts.

Methemoglobin estimates at all the individual wave lengths studied did not vary greatly between samples (with the exception of sample three, the curve for which was slightly displaced to the left). Methemoglobin esti-

TABLE 4.—The Effect of Choice of Extinction Coefficients and Wave Lengths on Methemoglobin Estimation in Tissue Extracts

Wave length mμ	Sample (from complete curves)						
	1	2	3*	4	5	6	Average
	Methemoglobin per cent						
Calculated from Austin and Drabkin's hemoglobin constants							
540	34	33	23	31	32	37	32
560	11	13	8	17	15	16	13
575	46	44	36	42	43	45	43
630	42	37	26	39	35	17	33
Summation 4	36	34	26	34	34	34	33
Calculated from Bowen's myoglobin constants							
544	29	26	19	27	26	30	26
564	18	18	10	20	18	24	18
582	29	27	21	27	27	26	26
Summation 3	27	25	18	26	25	27	25
Comparison of estimates							
Summation 4 (A.D.)	36	34	26	34	34	34	33
Summation 3 (B)	27	25	18	26	25	27	25
Difference	9	9	8	8	9	7	8

*This curve was displaced slightly to the left of the others in this group.

mates calculated using a summation of change at three or four wave lengths appeared to give somewhat less variable results. No obvious difference in this variability was noted between estimates using wave lengths and constants suggested by Austin and Drabkin and those suggested by Bowen. The methemoglobin estimates by the method of the former were approximately eight units or 30 per cent higher than by the method of the latter.

The systematic variation between estimates of methemoglobin made at individual wave lengths, which had been noted earlier, was still present though its effect was much less pronounced when myoglobin constants and wave lengths representing maxima and minima of the mixed pigments were used in the calculations. It seems possible that the use of beef myoglobin constants, which are not now available, might result in further improvement of estimates.

A comparison of the extinction coefficient ratios from values obtained in this series with those from the pure pigment solutions (Table 5) does not preclude the possibility of the presence of a third form of the pigment, although, as in the study of the extinction coefficients themselves, there is less indication of its presence when optimum wave lengths and constants for myoglobin are used. It will be noted that only the ratios 540/560 or 544/564 are useful for detecting this suggested form of pigment, since the other observed ratios are possible with a mixture of only the oxy- and met- form of pigments present.

An examination of the curves from which the above calculations were made shows the typical shape, variability and stability of the pigments under the conditions of this experiment.

Figure 1 shows the average curves for (A) the mixed pigments, (B) the pigments converted to methemoglobin and (C) the pigments converted to cyanmethemoglobin, on the millimolar basis, for the first reading of

TABLE 5.—Comparison of Extinction Coefficient Ratios for Mixed Pigment with Those for Pure Pigment Extracts at Critical Wave Lengths

Pigment	Approximate ratios mμ					
	575/560	540/560	575/540	582/564	544/564	582/544
Hemoglobin ratios (Austin and Drabkin)						
Oxyhemoglobin	1.73	1.67	1.03			
Methemoglobin	0.96	1.67	0.57			
Myoglobin Ratios (Bowen)						
Oxymyoglobin				1.66	1.64	1.01
Metmyoglobin				0.88	1.62	0.55
Mixed-pigment ratios						
Average curve (1 hour)	1.25	1.48	0.85	1.56	1.60	0.97
Average curve (2 hours)	1.22	1.45	0.84	1.50	1.56	0.96
Holding time data (2 hours)	1.33	1.42				

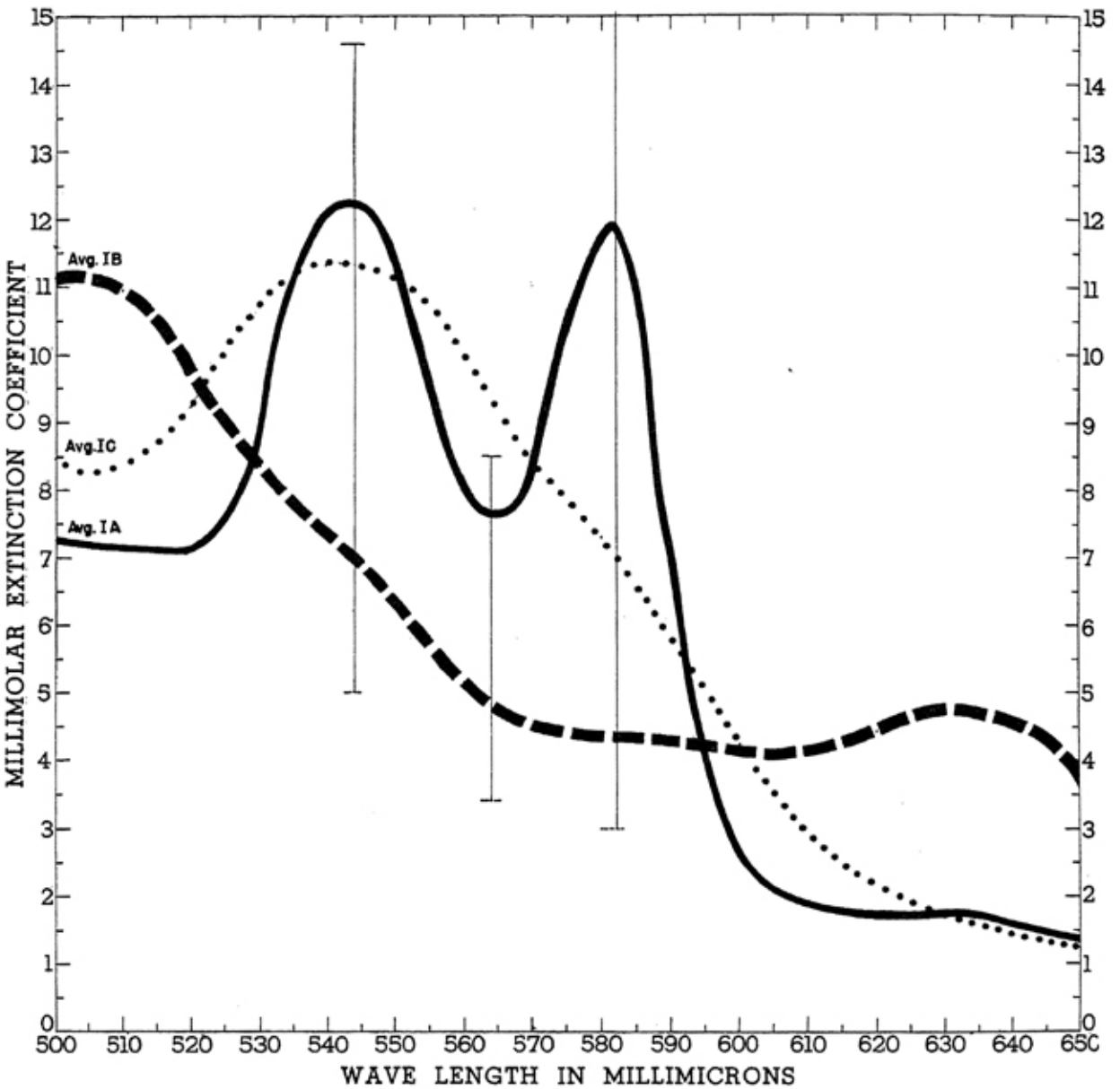

Figure 1.—Typical absorption spectra for (A) mixed pigment, (B) methemoglobin and (C) cyanmethemoglobin extracts. (Average of first readings for six determinations.) Bowen's extinction coefficients for oxymyoglobin are shown as solid, and for metmyoglobin as dotted horizontal lines at the critical wave lengths, 544, 564, and 582 millimicrons.

the six extracts. Bowen's extinction coefficients for oxy- and metmyoglobin are shown as horizontal lines at wave lengths of 544, 564, and 582 millmicrons. It will be noted that his coefficients for metmyoglobin are lower than those obtained in this series.

The small variation among curves from the six extracts is shown in Figure 2. Whether the slight shift of curve 3 to the left is due to an actual difference in the pigments in this extract or to the technique of the method is not known.

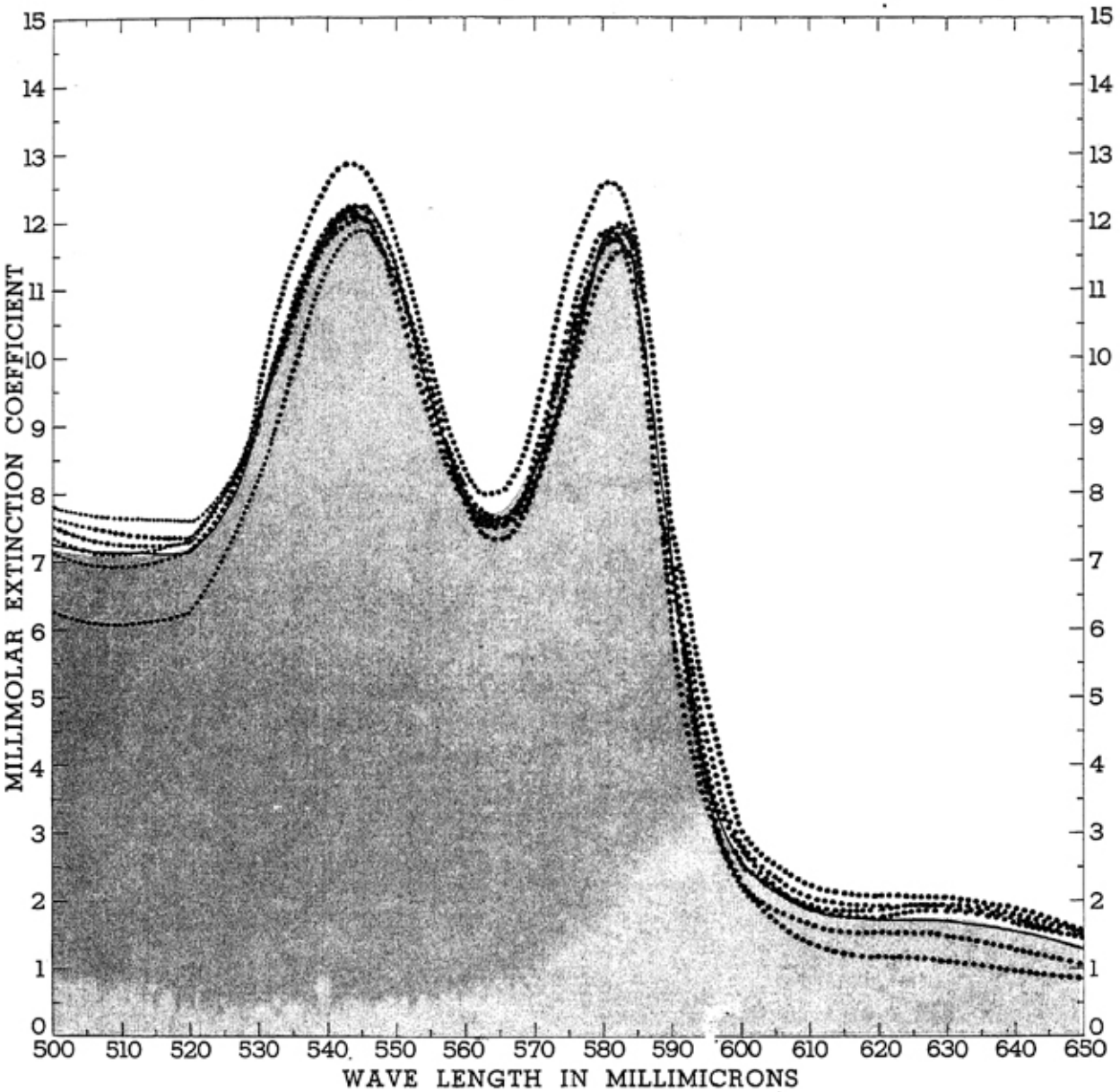

Figure 2.—Variation in absorption spectra of six mixed pigment extracts from similar samples. (First readings.) The area below the average curve is shaded.

The change in the three types of curves shown in Figure 3 from the readings made approximately one hour and ten minutes to those made two hours after removal of the sample from the freezer, indicate a gradual alteration in composition of the mixed pigment extract on standing but a much more rapid change of the met- form of the pigment which should be relatively stable at this pH. This instability may account in part for the failure in this experiment to obtain extinction coefficients comparable to those published by Bowen for metmyoglobin. The cyanmethemoglobin curve shows satisfactory stability at 540-545 millimicrons, the region from which concentration is determined.

Due to the choice of wave lengths at which extracts from the 113 de-

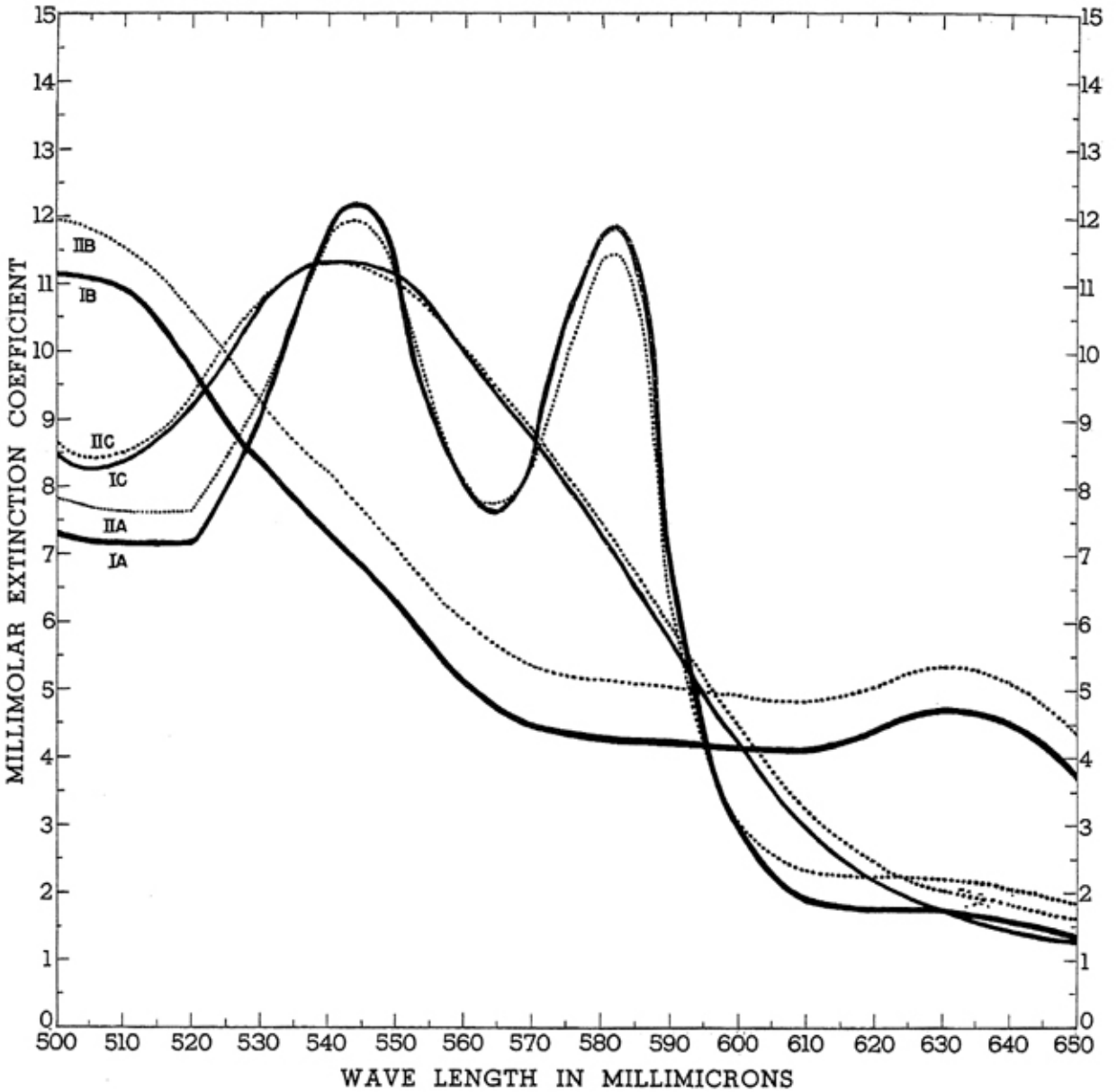

Figure 3.—Effect of time on the absorption spectra of (A) mixed pigment, (B) methemoglobin and (C) cyanmethemoglobin extracts. First readings (approximately one hour and ten minutes after removal of the sample from the freezer) are shown as solid lines. Second readings (two hour) are shown as dotted lines.

terminations made during the two-year study had been read, it was possible to check the effect of choice of constants on this series at only one wave length. Estimates of methemoglobin using Austin and Drabkin's extinction coefficients at four wave lengths were about 20 per cent higher than estimates of metmyoglobin using Bowen's extinction coefficients at 540 millimicrons. This difference was quite consistent and did not change the results in a relative sense nor influence the variability of results appreciably.

II. Factors Affecting Methemoglobin Formation in Frozen Beef.

The results of this part of the study are reported on the basis of readings made at wave lengths of 540, 560, 575 and 630 millimicrons, calculated from Austin and Drabkin's extinction coefficients for oxy- and methemoglobin at these wave lengths. It is estimated from the study on choice of wave lengths and constants that these results are approximately 30 per cent higher than if they had been determined from readings at 544, 564 and 582 millimicrons using Bowen's extinction coefficients for myoglobin.

Although preliminary work had established the ability of the experimenters to reproduce results quite closely with different portions of the same extracts and with different extracts from the same or similar samples, and although the established schedule was adhered to strictly, surprisingly large variations occurred among readings from supposedly comparable samples. These variations did not appear to be related to the surrounding temperature the first year, but summer methemoglobin estimates on the just frozen samples for the second-year experiments were higher than later estimates.

In the methemoglobin estimates for the storage times and temperatures for the first year (Table 6), the only trend noted was that the pigment change was consistently lower at the highest storage temperature, -12° C. An analysis of variance (Table 7) of the portion of the table for which complete data were available, the three steady storage temperatures

TABLE 6.—Effect of Time and Temperature of Storage on Pigment

Storage treatment	Methemoglobin (pct.)				
	Just frozen	Stored 8 wks.	Stored 16 wks.	Stored 24 wks.	Stored 32 wks.
None	42 44				
-12° C. (+10° F.)		35	37	34	41 42
-18° C. (0° F.)		48	43	39	—
-24° C. (-10° F.) Coils		44	43	40	—
-12° -18° C. (FLUCTUATING)					
(fluctuated 2 months)				38	53
(fluctuated 4 months)				34	49

TABLE 7.—Analysis of Variance of Blocked Portion of Data from Table 6 (Effect of Time and Temperature of Storage on Pigment)

Source of variation	Degrees of freedom	Sum of squares	Mean square	F	Significance
Between storage periods	2	35	17.5	3.68	at 0.20 level
Between storage temperatures	2	114	57.0	12.00	at 0.05 level
Residual	4	19	4.75		
Total	8	168			

and three storage times, indicated no significant difference between storage periods, but a difference, significant at the five per cent level, between storage temperatures. Since, however, this difference was not in the direction expected, some influence other than storage temperature might be considered to be responsible. Fluctuation between -12° C. and -18° C. for two months appeared to give methemoglobin estimates comparable to those from samples stored at steady temperatures after 24-week storage. While samples fluctuated between -12° C. and -18° C. for two or four months gave higher methemoglobin estimates after 32-week storage than samples held at -12° C., cloudiness of extracts from samples held at -18° C. and -24° C. resulted in an insufficient number of estimates for comparison of storage temperatures.

Palatability tests on these samples indicated that by the end of 32-week storage, samples stored at -24° C. were more desirable in appearance, odor and flavor than those stored at higher or fluctuating temperatures. The samples stored at -12° C. were considered undesirable in flavor and odor though not in appearance, while those fluctuated for four months were considered undesirable in all three respects by the end of 32 weeks. This does not confirm the theory that change in color may be used as an indication of change in palatability.

TABLE 8.—Effect on the Pigment of Thawing and Refreezing

Number of times thawed	0	1	2	3	4	5
Methemoglobin pct.	46 39 34 40 38 34	39	55	43	40	45

It was expected that the results of the radical-fluctuation experiment (Table 8), in which samples were allowed to thaw and refreeze from one to five times, would show a marked and regular increase in methemoglobin formation when compared with controls. This, however, was not the case. The samples thawed three and four times showed the lowest per cent of methemoglobin formation, while those thawed two and five times showed the highest. All samples were within the range shown by the controls except that thawed twice. It was only slightly higher. The change in all cases might have been more marked if some storage time had been allowed between the 35-hour thawing cycles. All samples thawed more than one time showed surface darkening and moisture accumulation. All cooked samples, however, were rated good to excellent in appearance although those allowed to thaw more than once were rated down in flavor.

Extracts from ascorbic acid treated samples were consistently cloudy and could not be used for spectrophotometric determinations even when the pH was adjusted and the extracts buffered. Moreover, the color of the cooked samples exhibited a greenish hue and the samples were considered unpalatable by the judges.

TABLE 9.—The Effect on the Pigment of Storage Under Different Gases

Gas used	Methemoglobin pct.	
	Stored 12 weeks	Stored 24 weeks
Air	43	61
Carbon dioxide	44	58
Nitrogen	44	54
Oxygen	39	52

TABLE 10.—Analysis of Variance of Data from Table 9 (Effect on the Pigment of Storage Under Different Gases)

Source of variation	Degrees of freedom	Sum of squares	Mean square	F	Significance
Between storage times	1	378.2	378.2	69.61	at 0.01 level
Between gases	3	49.4	16.47	3.03	at 0.20 level
Residual	3	16.3	5.43		
Total	7	443.9			

In the part of the experiment designed to determine the effect of various atmospheres on methemoglobin formation, a darkening of samples treated with oxygen was noted immediately. These samples showed lower methemoglobin formation on storage (Table 9) but an analysis of the data (Table 10) indicated no significant difference in influence among the gases used. A highly significant storage effect was noted under the conditions of this experiment. The exceptionally high methemoglobin formation after 24 weeks is difficult to explain. It might possibly be due to a difference in tightness of pack in the jars as compared to that of the cellophane packages. Cut surfaces of samples stored under all gases showed considerable spottiness and variation in color. No palatability tests were made on these samples since it was necessary to break the glass jars for testing.

When samples were held at 0° C. before freezing, no tendency toward increased methemoglobin formation was noted with up to 24-hour holding time, although after 30- and 72-hour periods methemoglobin estimates were slightly higher. The meat for this series had been purchased separately and was darker-cutting and was of lower fat content than the meat used for all other experiments during the first year (Table 11). All samples brightened quickly after cutting and were of similar color. The methemoglobin content was consistently lower than that found in the major series. All cooked samples were judged good in appearance but two judges found the flavor of those held more than 24 hours objectionable.

TABLE 11.—Effect on the Pigment of Holding Before Freezing

Hours held at 0° C. before freezing	0	1	2	4	6	9	12	24	30	72
Methemoglobin pct.	31	28	30	27	26	34	28	27	38	37

TABLE 12.—Composition of Samples from Series Tested the First Year

Series	Fat pct.	Moisture pct	Protein pct.	Ash pct.
Major	23.1	58.2	17.6	0.9
Holding time	10.1	68.2	20.9	1.0

Since the samples used in the preliminary experiments and in the major and holding time series, studied during the first year, were so different in fat content (Table 12), it was decided to investigate the possible effect of amount of fat on methemoglobin formation. The method as described was modified to give similar concentrations in all extracts. An analysis of variance (Table 14), using the method of unweighted means, of the data (Table 13), showed row and column effects significant at the 0.1 per cent level but no significant interaction. The row effect might be interpreted as increased methemoglobin formation in the presence of fat. This effect is noticeable at the 15 per cent level and above, and is in accord with a comparison of findings from the two series of different composition studied previously. Since there is no evidence of interaction between fat content and storage time, this effect of fat may be operative during the test procedure. While the determinations for a given storage period took

TABLE 13.—Effect of Fat and Moisture Content on Change of Pigment During Storage

Fat pct.		Moisture pct.	Methemoglobin pct.			
Calculated (added as suet)	Analyzed pct.		Just frozen	Stored 3 months	Stored 6 months	Stored 9 months
None	2.7	73	36 33	16 15 28	29 22 28	22 21
5	5.1	74	39 35	17 19	30 39	25 25
10	12.4	67	34 31	29 23	22 21	27 25
15	14.7	66	50 38	26 23	31 26	33 26
20	21.5	60	45 35	30 26	31 34	38 34
25	23.0	60	40 50	25 28	33 30	33 33

TABLE 14.—Analysis of Variance by the Method of Unweighted Means of Data from Table 13 (Effect of Fat on Change of Pigment During Storage)

Source of variation	Degrees of freedom	Sum of squares	Mean square	F	Significance
Between storage periods	3	718.2826	239.4275	28.0311	at 0.001 level
Between levels of fat	5	280.0938	56.0188	6.5584	at 0.001 level
Interaction	15	186.4577	12.4305	1.4553	not at 0.20 level
Residual	26	222.9122	8.5415		
Total	49	1406.9122			

four testing days, samples were selected for testing in such a way as to minimize the chance of day to day variations in results influencing the relation between fat content and methemoglobin estimation. There was, however, no apparent influence of the testing day on methemoglobin formation. Seemingly irregular results were obtained throughout the testing periods. The column effect cannot be interpreted as the influence of storage on methemoglobin formation since the highest methemoglobin estimates were obtained from the just-frozen samples, although the lowest were generally obtained after only three-month storage. The possible causes of such results are being investigated further.

Palatability tests on this series of samples indicated that while the sample with no added fat was rated as only acceptable in appearance and flavor when just frozen and the other samples were rated good, all samples were rated as acceptable at the end of nine-month storage except those containing 20 per cent fat. These were rated as slightly undesirable and were the samples which showed the highest methemoglobin formation at this time.

Extracts from unground samples had shown lower methemoglobin formation than those from ground samples in the first-year experiments. This effect is not observable in series studied the second year. (Table 15.)

TABLE 15.—Effect of Grinding on Change of Pigment During Storage

Treatment	Methemoglobin pct.			
	Just frozen	Stored 3 months	Stored 6 months	Stored 9 months
Unground	23 34	34 20	28	27 22
Ground (no added suet)	36 33	16 15 28	29 22 28	22 21

Nine market samples examined during the second year showed considerable variation in composition, although all would be classed as high in fat. (Table 16.) Methemoglobin estimates were quite variable also and showed no relation to fat content within this range. Samples five and six are included in the table to show the effect of unusually high pH. As was found in the preliminary experiments, it is possible to obtain only a milky pink emulsion from samples which have been allowed to stand too long either at room or refrigerator temperature. An elevation of pH accompanies this deterioration. This cloudiness is quite different from that occurring occasionally in treated solutions by flocculation of protein from a clear extract at lower pH.

A summary of the pH values of the different series (Table 17) shows the least variation during the first year. The two series studied that year are comparable to each other. The second-year series shows somewhat greater variation, due perhaps to differences in fat content. The variation as well as the mean pH of the market samples is influenced considerably by the two high values mentioned in the preceding paragraph.

TABLE 16.—Composition and Variation in Methemoglobin Formation of Market Samples

Sample number	Fat pct.	Moisture pct.	Methemoglobin pct.	pH
MS1	20.1	59.3	36	5.76
MS2	25.4	55.2	15 19 20	5.95
MS3	20.4	61.0	45 43 51	5.86
MS4	31.7	47.7	44 36	5.89
MS5	30.4	50.7	43* 46*	6.65
MS6	29.1	54.1	38* 42* 42*	6.57
MS7	32.8	46.1	40 43 53	5.90
MS8	26.9	52.9	25 26	5.73
MS9	21.7	58.8	37 38 37	5.94

*Cloudy extracts

TABLE 17.—Variation in pH of Samples Studied

Series	No. of determinations	pH								
		Tissue			Extract			Diluted extract		
		Mean	Range	S.D.	Mean	Range	S.D.	Mean	Range	S.D.
First year	54	5.75	5.44-5.95	0.06	5.78	5.49-5.95	0.06	5.81	5.61-5.97	0.08
Second year	64	5.82	5.64-6.70	0.11	5.86	5.64-7.00	0.16	5.89	5.68-6.65	0.16
Market samples	24	6.02	5.74-6.65	0.32	6.06	5.74-6.62	0.30	6.06	5.75-6.62	0.26

SUMMARY AND CONCLUSIONS

The findings of this series of experiments are reported under two headings.

I. A Study of the Spectrophotometric Method.

Study of methemoglobin formation from beef muscle extracts, using the four wave lengths and the extinction coefficients suggested by Austin and Drabkin for blood extracts, showed a significant variation between methemoglobin estimates made at individual wave lengths. Those made at 560 millimicrons were consistently low and were more variable than those made at 540 and 575 millimicrons.

It was considered that this effect might be due to the presence in the extract of a third form of the pigment. The presence of appreciable amounts of reduced hemoglobin along with the oxy- and met- forms of hemoglobin would give the observed effect both on the extinction coefficients and their ratios at critical wave lengths. This problem is being investigated further.

Another possible explanation of this systematic variation in methemoglobin estimates is that either the extinction coefficients or wave lengths selected or both are not good ones to use with muscle extracts. When continuous curves from 500 to 700 millimicrons were made for six extracts from presumably identical samples, they showed maxima and minima shifted to the right of those for oxyhemoglobin indicating them to be chiefly muscle hemoglobin or myoglobin. Calculations of methemoglobin formation estimated at 540, 560, 575 and 630 millimicrons with Austin and Drabkin's extinction coefficients for blood hemoglobin were compared with those for 544, 564 and 582 millimicrons using Bowen's extinction coefficients for myoglobin. The former gave methemoglobin estimates approximately 30 per cent higher than the latter. However, the variability in estimates was about the same for the two calculations and relative differences between samples were not influenced by choice of constants and wave lengths. The methemoglobin estimates at the minimum at 564 millimicrons were still consistently lower than those at the maxima 544 and 582, but the effect was much less pronounced.

II. Factors Affecting Methemoglobin Formation in Frozen Beef.

Estimates of methemoglobin formation in 113 samples have been reported as calculated from spectrophotometric readings at 540, 560, 575 and 630 millimicrons using the extinction coefficients of Austin and Drabkin for blood hemoglobin. These estimates should be comparable to those in the literature for tissue extracts, although, as indicated by the study of the method, they are consistently higher than if muscle hemoglobin constants and the wave lengths 544, 564 and 582 millimicrons had been used for calculating.

Experiments during the first year indicated that methemoglobin did not increase with storage time up to 32 weeks. Methemoglobin formation was slower when samples were stored at the highest temperature, -12° C., than when stored at -18° C., -24° C., or than when the temperature of the storage room was allowed to fluctuate from -12° C. to -18° C. The samples stored at -12° C. were considered the least palatable, however. When samples were allowed to thaw from one to five times the methemoglobin formation did not increase with repeated thawing although samples allowed to thaw more than once were considered undesirable in flavor and odor. Methemoglobin formation tended to be slower when the tissue was stored under oxygen than under nitrogen, carbon dioxide or air, although the difference was not significant. Holding at 0° C. up to 24 hours before freezing did not increase methemoglobin formation. Samples held 30 and 72 hours showed only slightly higher methemoglobin formation but were considered unpalatable. Apparently methemoglobin estimates do not reflect flavor changes consistently.

During the second year the influence of grinding and of the amount of fat on methemoglobin formation was studied. Samples containing more than 15 per cent fat showed higher methemoglobin content than those containing less than 15 per cent fat, but this tendency was not related to

storage time. No differences in methemoglobin formation between ground and unground samples could be observed in experiments the second year, although an increase in that of ground samples had been noted earlier. Nine market samples examined during this period showed considerable variation in composition and methemoglobin content. The pH values for experimental samples were lower than for market samples.

Investigation of the rather large, unexplained variations in results which occur at times even with trained workers and strict adherence to schedules, the possibility of the presence of a third form of pigment in the extracts, and absorption curves of tissues themselves in comparison to those of extracts, are now being studied.

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