developed a "beef" aroma when heated. Freeze-drying of the unheated extract yielded a water-soluble, friable powder. This dried powder, when heated, developed an odor reminiscent of roast beef; a water solution of the powder, upon heating, evolved a boiled beef aroma. As the extraction and lyophilization procedure concentrated the flavor precursors and simultaneously simplified the system under study by the removal of fat, water, and water-insoluble matter, this concentrate has been the starting point for this work, with the realization, however, that under usual cooking conditions where fat and moisture are present, the isolated products may differ in some respects from those obtained in the present study.

Acknowledgment

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Literature Cited

(1) Bouthilet, R. J., *Food Research* **15,** 322 (1950).
(2) *Ibid.*, **16,** 201 (1951).
(3) Crocker, E. C., *Ibid.*, **13,** 179 (1948).
(4) Davies, D. F., Wolfe, K. H., Perry, H. M., *J. Lab. Clin. Med.* **41,** 802 (1953).
(5) Gaddis, A. M., Ellis, Rex, *Anal. Chem.* **31,** 870 (1959).
(6) Gaddis, A. M., Ellis, Rex, *Food Research* **24,** 392 (1959).
(7) Kramlich, W. E., Pearson, A. M., *Ibid.*, **23,** 567 (1954).
(8) Marbach, E. P., Doty, D. M., J. AGR. FOOD CHEM. **4,** 881 (1956).
(9) Moulton, C. R., "Allen's Commercial Organic Analysis. Meat and Meat Products," Vol. **IX,** Blakiston, Philadelphia, 1932.
(10) Pippen, E. L., Eyring, E. J., *Food Technol.* **11,** 53 (1957).
(11) Pippen, E. L., Nonaka, M., Jones, F. T., Stitt, F., *Food Research* **23,** 103 (1958).
(12) Sastry, L. V. L., *J. Sci. Ind. Research (India)* **16B,** 508 (1957).

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MEAT AGING AND FREEZING

Post-Mortem Changes in the Water-Soluble Proteins of Bovine Skeletal Muscle during Aging and Freezing

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Alteration of the water soluble proteins of bovine skeletal muscle is of particular interest, because these proteins have enzymatic character and are involved in biochemical processes occurring in meat. The effect of aging meat for 7 days and of freezing at −20° C. for 5 weeks was studied by electrophoresis and ultracentrifugation, as well as by several chemical methods. Systematic differences in protein content and in the content of the enzyme aldolase were noted from muscle to muscle. Both aging and freezing of meat were shown to result in a decreased extractability of water soluble proteins, as well as in a loss of specific electrophoretic and ultracentrifugal components.

POST-MORTEM CHANGES occurring in meat are known to be rather profound. Without doubt, a number of biochemical changes identified with alterations in tissue properties are associated with the salt extractable, structural proteins such as actin and myosin. On the other hand, many processes are controlled by enzymes found in the water-soluble myogen fraction—e.g., lactic acid production. While such protein enzymes may comprise only a small weight percentage of meat, the significance of the reactions that they catalyze may render their alteration during aging or freezing of tissue of the greatest importance.

The effect of aging and freezing on the extractability of the enzyme aldolase and the other water-soluble proteins has been studied. These data, as well as more specific information as to the alteration of the water soluble proteins by ultracentrifugal and electrophoretic analysis of muscle extracts and subfractions derived from these extracts by fractional salt precipitation, are presented.

Experimental

Materials and Methods. Details of analytical and preparative methods have been described previously (*8*).

Four animals were used in this study. Animal 6 was a 5-year-old cow, while animals 7, 8, and 9 were 18-month-old steers of choice grade. Muscle dissected from the left side of the animal approximately 20 minutes post mortem—fresh muscle—was compared with muscle taken from the right side of the same animal held at 3° C. for 7 days. For the sake of brevity the latter muscle has been referred to as aged. Samples of the fresh muscle, which had been chilled in ice for transportation to the laboratory, were frozen in a deep freeze unit about 3 hours post mortem. Frozen muscle was stored at −20° C. for 5 weeks after which it was thawed for about 24 hours at about 5° C. prior to extraction. Aqueous extracts were prepared in the manner described previously (*8*).

In the case of animal 6, an average muscle sample was taken over much of its length. In the case of animals 7, 8, and 9, sections were made according to published diagrams (*4*) and individual muscles were then removed from the proper section (Table I). Shown in the remaining columns of Table I are the locations at which sections were made, the illustration plate number of the reference cited, and the commercial cut of meat corresponding to this section.

Moving Boundary Electrophoresis and Ultracentrifuge Measurements. The methods and equipment employed have been described previously (*8*). Both types of measurements were carried out on protein solutions in pH 8.17, 0.05 ionic strength tris(*N*,*N*,*N*-hydroxymethyl) methylamine (Tris) buffer. Prior to measurement, solutions were centrifuged in the Spinco Model L at 60,000 × G in order to remove trace amounts of insoluble protein.

Because it was virtually impossible to carry out electrophoresis or ultracentrifuge measurements at identical concentrations, it was necessary to normalize the curves for purposes of calculation. The areas of projected traced

Table I. Individual Muscles

Muscle No.	Muscle Name	Location of Section	Commercial Cut Corresponding to Section	Illustration Plate No. (4)
1	*Longissimus dorsi*	8th, 9th, 10th rib	Rib steak	XXIII–XXIV
2	*Psoas major*	4th, 5th lumbar vertebrae	Porterhouse or T bone steak	XXVII
3	*Semimembranosis*	Proximal extremity of femur	Round steak	IX
4	*Serratus ventralis*	8th, 9th, 10th rib	Rib steak	XXIII–XXIV
5	*Rectus abdominus*		Flank steak	XXI
6	*Semitendinosis*	Proximal extremity of femur	Round steak	IX
7	*Latissimus dorsi*	8th, 9th, 10th rib	Rib steak	XXIII–XXIV
8	*Trapezius*	8th, 9th, 10th rib	Rib steak	XXIII–XXIV

Table II. Absolute Aldolase and Soluble Protein Content of Fresh, Aged, and Frozen Bovine Muscle

Animal	Aldolase Units/100 G. of Wet Tissue × 10^{-6}			Grams of Protein/100 G. of Wet Tissue		
No.	Fresh[a]	Aged	Frozen	Fresh	Aged	Frozen
6	2.1 ± 0.6	...	2.5 ± 1.1	3.55 ± 0.45	...	2.82 ± 0.28
7	2.9 ± 1.0	2.8 ± 1.0	1.6 ± 0.4	2.60 ± 0.51	2.29 ± 0.47	2.81 ± 0.28
8	2.8 ± 0.9	3.3 ± 0.9	2.8 ± 0.8	3.65 ± 0.27	2.76 ± 0.32	2.62 ± 0.22
9	2.8 ± 0.6	2.9 ± 0.8	3.9 ± 0.6	3.19 ± 0.42	2.47 ± 0.29	2.90 ± 0.26
Av.	2.6	3.0	2.7	3.25 ± 0.27	2.51 ± 0.17	2.79 ± 0.08

[a] (*8*).

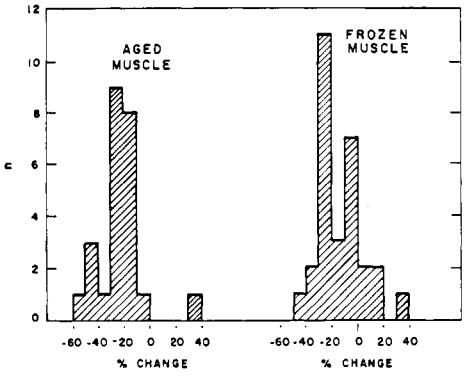

Figure 1. Per cent change in water-extractable protein for aged and frozen muscle

The change is relative to muscle taken 20 minutes post mortem. The number of samples having a given percentage change is given by *n*

patterns were determined with the planimeter. In the case of ultracentrifuge patterns, suitable solvent base line corrections were applied. All curves shown in this paper were constructed from the tracings using a pair of proportional dividers to reduce the patterns to the same area. In each case the standard area was that of the fresh muscle pattern. For calculation of per cent change in ultracentrifugal components, use was made of a normalized peak height—i.e., the height of the peak, h, divided by the area of that pattern, A. The per cent change in this ratio, h/A, was taken as the per cent change in the amount of that component.

Results and Discussion

Protein and Aldolase Content. Table II shows average values of the protein content for eight muscles with the exception of muscle 8, animal 6, and muscles 4 and 8, animal 9, which were not studied. Figure 1 illustrates the magnitude and variation in the change in protein contents as compared to fresh muscle. As Table II and Figure 1 indicate, aging beef muscle for 7 days renders the previously soluble protein less liable to extraction from the tissue in agreement with the results of Wierbicki *et al.* (*14*). Using solubility as a criterion of denaturation, one would conclude that from 10 to 30% of the soluble protein may be denatured during the 7 days of aging.

The fact that the aged values shown in Figure 1 are somewhat larger than those reported by Wierbicki *et al.* (*13, 14*) for 13 days of aging is probably due to differences in analytical methods. The biuret procedure employed in this study will distinguish highly degraded protein from that in the native state in contrast with the analysis for total nitrogen used by Wierbicki *et al.* (*13, 14*). Furthermore, the basis for their changes was a 3-day old sample in contrast with one of 3-hours employed in this study. The extent of protein alteration during this initial 3-day period may be sufficient to account for the observed differences.

As Table II and Figure 1 indicate, 5 weeks of freezing of the fresh muscle lead likewise to a decrease in the amount of extractable protein. These results are in marked contrast with those obtained by Artyukh (*2*), and Sadikov and Khaletzkaya (*11*) who reported that freezing had no effect on the extractability of the water-soluble proteins. Loss of protein solubility due to denaturation would appear to be a more reasonable explanation in the case of freezing than degradation of protein by proteolysis, because the latter process would probably be rather slow at −20° C.

The effect of aging or freezing of tissue on extractability of aldolase is not at all clear-cut. Examination of Table II might lead one to believe that, with the exception of animal 7—frozen tissue—these processes have no effect, or if anything, lead to an increase in extractability. This is, however, only a consequence of data averaging. Shown in Figure 2 are plots of the per cent change in aldolase content *vs.* the number of samples, n, exhibiting this change. The extreme variation observed for these changes cannot be due to assaying errors and must be regarded as being due to differences in extractability of different muscle samples. No systematic variation from muscle to muscle or animal to animal was observed with the exception of animal 7—frozen tissue—which showed a consistent decrease.

This apparent wide variation in extractability of aldolase from beef skeletal muscle may be related to the question of the extractability of aldolase from rat liver. Roodyn (*10*) found that after complete destruction of liver nuclei by ultrasonic vibrations, aldolase was strongly bound to cellular debris. It may be, therefore, that the extractability of muscle aldolase will depend upon the degree of disruption of the cellular structure during the aging or freezing process and that this disruption is a function of parameters which are not apparent at the present time.

These results, together with those of Roodyn, throw some doubt on the conclusion drawn by Andrews and others (*1*) that aldolase is not a rate-limiting glycolytic enzyme. The question that remains unanswered at present is whether their observed loss of activity is due to inactivation of the enzyme or its adsorption on the homogenized tissue.

Examination of the aldolase and protein content for a given animal under a fixed set of conditions indicates a greater variation of values from muscle to muscle than one would expect from experimental error. The significance of this variation can be more readily demonstrated by comparing relative quantities—e.g., relative protein content is defined as:

$$\frac{\text{(Grams of protein per 100 grams of tissue) animal } a\text{, muscle in question}}{\text{(grams of protein in 100 grams of tissue) animal } a \text{ av.}}$$

Table III. Relative Protein and Aldolase Contents of Individual Muscles

Muscle	Relative Protein Content			Relative Aldolase Content			Specific Activity = Relative Aldolase Content / Relative Protein Content		
	Fresh[a]	Aged	Frozen	Fresh[a]	Aged	Frozen	Fresh[a]	Aged	Frozen
1	1.2 ± 0.08	1.1 ± 0.06	1.2 ± 0.15	1.4 ± 0.05	1.1 ± 0.05	1.4 ± 0.12	1.2	1.0	1.2
2	1.0 ± 0.10	0.82 ± 0.13	1.0 ± 0.07	0.72 ± 0.09	0.92 ± 0.08	1.1 ± 0.05	0.72	1.1	1.1
3	1.2 ± 0.10	1.3 ± 0.07	1.1 ± 0.08	1.3 ± 0.10	1.5 ± 0.04	1.2 ± 0.13	1.1	1.3	1.1
4	0.86 ± 0.04	0.68 ± 0.07	0.96 ± 0.14	0.65 ± 0.28	0.38 ± 0.18	0.57 ± 0.41	0.76	0.56	0.59
5	0.86 ± 0.10	1.1 ± 0.07	0.90 ± 0.08	0.71 ± 0.07	0.92 ± 0.03	0.69 ± 0.30	0.83	0.84	0.77
6	1.1 ± 0.03	1.0 ± 0.04	0.94 ± 0.11	1.4 ± 0.15	1.4 ± 0.08	1.3 ± 0.15	1.3	1.4	1.4
7	0.96 ± 0.06	1.1 ± 0.08	0.96 ± 0.04	1.1 ± 0.16	1.0 ± 0.08	0.99 ± 0.17	1.1	0.91	1.0
8	0.87 ± 0.07	0.92 ± 0.05	0.93 ± 0.02	0.75 ± 0.13	0.64 ± 0.17	0.65 ± 0.10	0.86	0.70	0.70

[a] (*8*).

Use of this type of parameter tends to eliminate variations characteristic of a given animal. Shown in Table III are values of relative aldolase and protein contents and specific activities. Shown also are the values for fresh tissue taken from a previous paper (*8*).

The fresh muscles 4, 5, and 8 differ from the others on the basis of protein and aldolase contents (*8*). In spite of the wide variation of the magnitude of the change in protein and aldolase contents on aging and freezing, these data substantiate this previous observation. The specific activities for fresh, aged, and frozen tissue all indicate that for muscles 4, 5, and 8 aldolase makes up a smaller percentage of the total water soluble protein fraction. The differences in soluble protein content are not merely a consequence of differences in aldolase contents. Assumption of a specific activity of 5.4 units per γ (*8*) for pure aldolase, permits calculation of the aldolase protein content for each sample. When such a calculation is carried out and the aldolase content is subtracted from the total soluble protein content, the differentiation of muscles 4, 5, and 8 is not only maintained, but is made even more extreme.

pH Changes. The pH that a muscle attains post mortem is a very important factor in determining its characteristics as meat. Grau and coworkers (*6*) have shown that the water-holding capacity of meat can be modified by changing its pH. More recently, Swift and Berman (*12*) demonstrated for the same eight muscles studied that the higher the ultimate pH attained by the muscle, the higher the water-holding capacity.

Shown in Table IV are relative pH values at present obtained for the extracts of aged and frozen tissue. As for protein and aldolase content, the pH values serve to differentiate muscles 4, 5, and 8 from the others—i.e., these muscles have higher pH values than the others. Similar differences in pH have also been observed by Swift and Berman (*12*) for the same group of eight muscles. They also found that the total protein nitrogen contents of muscles 4, 5, and 8 were lower than those of the other muscles.

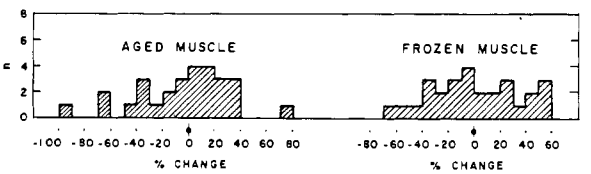

Figure 2. Per cent change in the amount of aldolase for aged and frozen muscle

The change is relative to muscle taken 20 minutes post mortem. The number of samples having a given percentage change is given by *n*

The post-mortem pH of a muscle will be determined chiefly by the rate of lactic acid production, the buffering capacity of the muscle, and the rate of inactivation of the significant glycolytic enzymes. The latter factor may be taken to be the same for all muscles. Thus, the fact that muscles such as 4, 5, and 8 yield relatively high pH values means either that the rate of glycolysis—lactic acid production—is slower in such muscles or that their total buffering capacity is higher. While there is no information as to the total buffering capacity, the protein buffering capacities of muscles 4, 5, and 8 are lower than the others (*12*). Furthermore, if one takes the total phosphorus content of such muscles as a measure of phosphate content, muscles 4, 5, and 8 have phosphate buffering capacities lower than or comparable to the other muscles (*12*).

While it must be conceded that the relatively higher pH values of muscles 4, 5, and 8 could be a consequence of higher anserine and carnosine contents (*3*), they may, on the contrary, be due to lower rates of lactic acid production. The fact that these three muscles have lower amounts of water-extractable protein—the enzyme rich protein—suggests that the cellular concentration of rate-limiting glycolytic enzymes is lower in these muscles. Aldolase, one of the important enzymes of glycolysis, makes up a smaller percentage of the soluble proteins in these three muscles than in the others. Until the question of the rate-limiting character of aldolase or other glycolytic enzymes is answered, it cannot be certain that the final pH value a muscle can attain will be a function of its soluble protein content.

Table IV. Relative pH, Average

Muscle No.	Aged Muscle[a]	Frozen Muscle[b]
1	0.996 ± 0.014	0.981 ± 0.021
2	0.989 ± 0.010	0.995 ± 0.006
3	0.976 ± 0.008	0.988 ± 0.012
4	1.056 ± 0.023	1.031 ± 0.026
5	1.008 ± 0.003	1.019 ± 0.005
6	0.981 ± 0.003	0.992 ± 0.008
7	0.987 ± 0.005	0.993 ± 0.005
8	1.007 ± 0.003	1.012 ± 0.016
Av. Absolute	5.51 ± 0.10	5.44 ± 0.09

[a] Av. for animals 7, 8, and 9.
[b] Av. for animals 6, 7, 8, and 9 with the exception of muscle 8, animal 6, and muscles 4 and 8, animal 9 which were not studied.

Furthermore, gross variations in glycogen levels could also be responsible for differences in rates of lactic acid production. Small differences in glycogen concentration apparently do not have much effect on ultimate pH of beef muscle (*7*). Until more detailed analytical data become available on the composition of these eight muscles, none of the factors considered can really be excluded.

Fractionation of Muscle Extracts. Solubility has commonly been taken as a criterion of protein denaturation. In order to determine what effect the aging and freezing of muscle has on the water-extractable proteins, such extracts were fractionated with ammonium sulfate in the manner described previously (*8*). Shown in Table V are the protein distributions obtained for fresh, aged, and frozen *longissimus dorsi*—rib eye.

As was true for the fresh muscle, a relatively large percentage of the pro-

Table V. Protein Distribution in *Longissimus Dorsi*, Rib Eye

Fraction No.	$(NH_4)_2SO_4$, Molarity	Total Soluble Protein, %											
		Animal 7			Animal 8			Animal 9			Average		
		Fresh	Aged	Frozen	Fresh	Aged	Frozen	Fresh	Aged	Frozen	Fresh	Aged	Frozen
FI	0–1.75	5.4	5.0	7.2	5.5	3.5	8.2	4.8	3.5	10.6	5.2 ± 0.3	4.0 ± 0.7	8.7 ± 1.3
FII	1.75–2.40	8.5	8.7	12.4	3.5	8.9	9.5	2.2	8.9	17.2	4.7 ± 1.8	8.8 ± 0.1	13.0 ± 2.8
FIII	2.40–2.65	10.1	12.4	8.2	4.9	10.4	14.9	8.9	5.2	17.9	8.0 ± 2.0	9.3 ± 2.8	13.7 ± 3.6
FIV	2.65–3.35	25.7	22.7	17.2	30.0	34.0	18.0	29.7	34.0	11.2	28.5 ± 1.8	30.2 ± 5.0	15.5 ± 2.8
SIV	>3.35	17.9	16.5	20.5	11.8	8.0	16.7	4.1	8.0	17.5	11.3 ± 4.8	10.8 ± 3.8	18.2 ± 1.5
	Unrecovered protein	32.4	35.0	34.4	44.3	33.0	32.6	50.3	40.6	36.1	42.3 ± 6.7	36.2 ± 2.9	34.3 ± 1.2

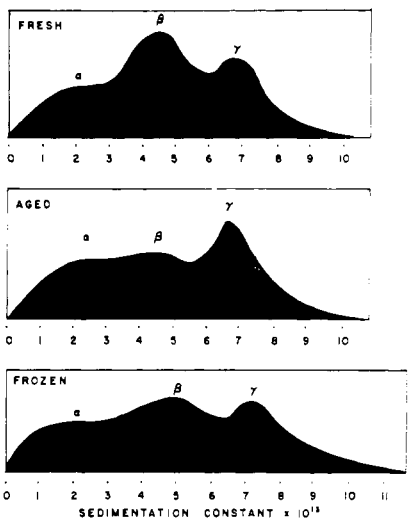

Figure 3. Normalized sedimentation patterns for extracts of fresh, aged, and frozen *longissimus dorsi*, animal No. 9

Speed. 59,780 r.p.m.
Temperature. 10° C.
Time. 80 minutes
Bar angle. 50°
Solvent. pH 8.17 Tris buffer, $\gamma/2 = 0.05$
Protein concentration. 1.3 grams/100 ml. for fresh; 1.2 grams/100 ml. for aged; 1.1 grams/100 ml. for frozen

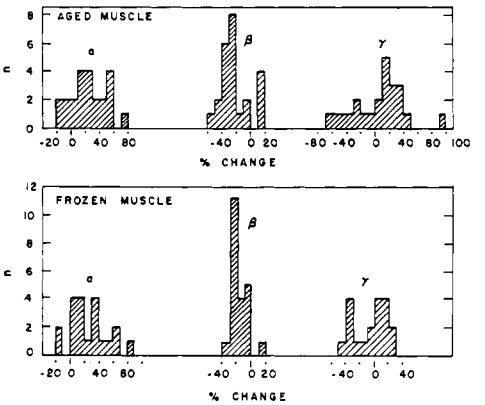

Figure 4. Per cent change in ultracentrifugal components, α, β, and γ aged and frozen tissue

The change is relative to muscle taken 20 minutes post mortem. The number of samples having a given percentage change is given by *n*

tein is not recovered in the fractions and presumably corresponds to the insoluble protein removed during the course of the fractionation. Whether this unrecovered protein was denatured before addition of ammonium sulfate or became so during the course of the fractionation cannot be answered with certainty.

The total amount of protein precipitating in fractions FI, FII, and FIII from frozen tissue is significantly greater than that derived from fresh tissue (Table V). Presumably, this increased amount of protein precipitating at lower ammonium sulfate concentrations is protein which has been altered during the storage at −20° C. so as to decrease its solubility. This increase in the first three fractions was obtained at the expense of a decrease in the amount of fraction FIV derived from frozen tissue.

As Table V illustrates, aging of tissue had little effect on the solubility of the myogen proteins in ammonium sulfate solutions. Nonetheless, as will be shown in a subsequent section, a rather drastic change in the myogen protein fraction has occurred.

Sedimentation and Electrophoretic Analysis. In order to obtain information as to the extent and freezing of muscle tissue, electrophoretic and sedimentation analysis was carried out on extracts and ammonium sulfate fractions. Both electrophoresis and sedimentation patterns can serve as a kind of fingerprint for protein components in complex mixtures, if the pure components have been previously studied. This approach has been considered in a previous paper (*8*).

Figure 3 shows three representative ultracentrifuge patterns obtained for extracts of muscle 1, *longissimus dorsi*. These patterns have been normalized in the manner described in the experimental section. The sedimentation patterns of extracts of aged and frozen muscle are qualitatively similar to those of fresh tissue in that they exhibit the characteristic α, β, and γ groups having average sedimentation constants of 2.3*S*, 4.7*S*, and 6.8*S*, respectively. Comparison of the patterns in Figure 2 indicates a change in the sedimentation pattern which means that the relative proportions of proteins having different molecular weights has changed.

Summarized in Figure 4 are data derived from ultracentrifuge measurements on extracts of all eight muscles from animals 7, 8, and 9. The muscles noted in the footnote to Table IV were not studied. The percentage change for a given component was calculated by comparison of the normalized peak heights in the manner described under Experimental. Considerable variation in the actual numerical value of the percentage change was observed from sample to sample without any apparent regularity from muscle to muscle as was the case for other properties considered previously. Nonetheless, the direction of change for both component α and β was quite evident.

Component α showed a significant increase on aging and freezing. Component β decreased in both cases, but probably to a greater extent in the case of the aged muscle. The experimental data for component γ are inconclusive, yielding increases apparently as often as decreases.

The average molecular weight of proteins in the α group will almost certainly be less than those of proteins in the β group. It therefore seems reasonable to associate an increase in component α with degradation of larger protein molecules—e.g., proteins of group β.

Examination of ultracentrifuge and electrophoresis patterns obtained from ammonium sulfate fraction FI indicated even more striking changes in protein composition. Sedimentation patterns (Figure 5) obtained for this fraction derived from muscle 1, animal 9 are quite representative of patterns obtained for all three animals. Superficially, the patterns for the fresh muscle fraction FI (Figure 5) and the fresh muscle extract (Figure 3) are comparable. More careful examination of these patterns, however, indicates an enrichment of components having sedimentation constants greater than 7.5*S*. The patterns of Figure 5 show that the bulk of protein having sedimentation

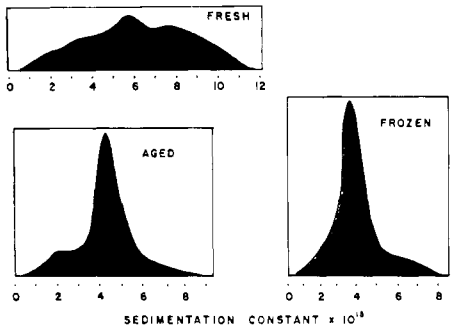

Figure 5. Normalized sedimentation patterns for fraction FI derived from extracts of fresh, aged, and frozen *longissimus dorsi*, animal No. 9

Speed. 59,780 r.p.m.
Temperature. 10° C.
Time. 80 minutes
Bar angle. 40°
Solvent. pH 8.17 Tris buffer $\gamma/2 = 0.05$
Protein concentration. 1.5 grams/100 ml. for fresh; 1.0 gram/100 ml. for aged; 1.2 grams/100 ml. for frozen

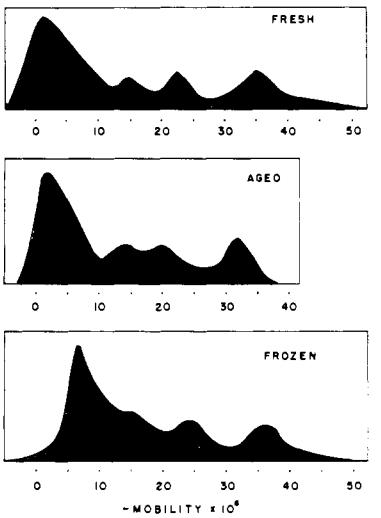

Figure 6. Normalized electrophoretic patterns for extracts of fresh, aged, and frozen *longissimus dorsi*, animal No. 9

Field strength. 14.3 volts cm.$^{-1}$
Time. 6000 seconds
Solvent. pH 8.17 $\gamma/2 = 0.05$ Tris buffer
Protein concentration. 1.3 grams/100 ml. for fresh; 1.2 grams/100 ml. for aged; 1.1 grams/100 ml. for frozen

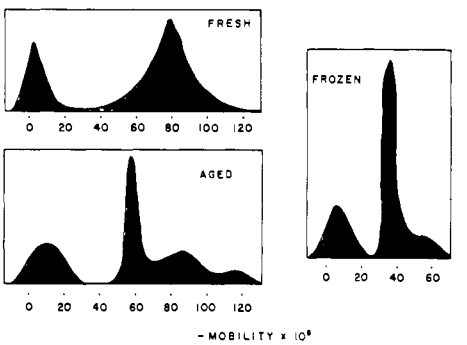

Figure 7. Electrophoretic patterns for fraction FI derived from extracts of fresh, aged, and frozen *longissimus dorsi*, animal No. 9

Field strength. 14.3 volts cm.$^{-1}$
Time. 4300 seconds
Solvent. pH 8.17 $\gamma/2 = 0.05$ Tris buffer
Protein concentration. 1.5 grams/100 ml. for fresh; 1.0 gram/100 ml. for aged; 1.2 grams/100 ml. for frozen

constants greater than 7.5*S* has been lost upon aging or freezing.

Changes in electrophoretic patterns usually occurred on aging or freezing, but were less clear-cut than those occurring in sedimentation patterns. Shown in Figure 6 are representative electrophoresis patterns of extracts of muscle 1, animal 9. While changes in the relative amounts of components were noted, these changes were not at all consistent. Similar nonregular changes were observed in electrophoresis patterns of ammonium sulfate fractions FII, FIII, and FIV. The reason for this lack of reproducibility is not clear.

Changes in patterns obtained for fraction FI, however, were quite clear-cut. Shown in Figure 7 are representative curves obtained from muscle 1, animal 9. Proteins having mobilities in excess of 50×10^{-6} are found exclusively in FI (*8*). Comparison of Figures 7, *b* and *c*, with 7, *a*, demonstrates that aging or freezing results in a loss of protein components having mobilities in excess of -65×10^{-6}. This effect is particularly pronounced in the case of the frozen tissue where components having mobilities in excess of -70×10^{-6} are completely absent. These losses are accompanied by an increase in the amount of material of lower mobility.

Identification of protein components which change on aging or freezing tissue is handicapped by the fact that many of the pure components have never been isolated and characterized and are only known to be present on the basis of their enzymatic activity. The situation is further complicated by the fact that the bulk of the work done has been with rabbit muscle proteins. The identification of some of these components of fresh muscle was considered in a previous communication (*8*). The tentative conclusions made are of some value in identifying components which change during the aging or freezing process.

The sedimentation constants of lactic dehydrogenase and phosphorylase *b* are 7.0*S* and 8.2*S*, respectively, and their solubilities are such that one would expect to find them in fraction FI (*8*). Thus, the material lost from FI may be partially composed of these two enzymes. The mobilities of these two enzymes place them in the main peak of Figure 4, *a*. Because this main peak is displaced on aging or freezing (Figure 5, *b* and *c*) there is support for the hypothesis that lactic dehydrogenase and phosphorylase *b* are two of the components lost during aging and freezing. The decrease in the β component in whole extracts (Figures 3 and 4) may be due to a loss of ATP–creatine transphosphorylase, which has a sedimentation constant of 5.0*S* and is probably present in relatively large amounts in beef muscle [10 to 38% in rabbit muscle (*8*)].

The changes in the soluble protein fraction of beef muscle are obviously less profound than those observed by Zender *et al.* (*15*) during the aseptic storage of lamb muscle at 25° and 38° C. In their experiments, 42 days of storage yielded significant amounts of low molecular weight peptides and gross alterations of electrophoretic patterns of extracts. In the experiments reported here, a maximum of 30% of the soluble protein may have been converted to amino acids and low molecular weight peptides on freezing and aging. This amount represents unextractable protein and as has been pointed out may consist of degraded protein undetectable by the biuret procedure.

It seems likely that degradative changes are associated in part with hydrolysis by the naturally occurring proteolytic enzymes of muscle (*15*). It should be emphasized that much more subtle changes in protein structure occur which can so alter a protein-enzyme as to completely halt any process in which it is involved. The splitting of pepsin (*5*) and phosphorylase *a* (*9*) by very gentle chemical treatment are good examples of this type of process. In the former case this is accomplished by increasing the pH beyond 7, while in the latter it occurs on blocking certain sulfhydryl groups. These considerations, as well as the experimental evidence presented, show that extractability or solubility of proteins does not provide a sufficiently sensitive criterion for protein alteration. Measurements of specific properties such as enzymatic activity, acid and base-binding ability, ultracentrifugal and electrophoretic behavior would appear to be more promising methods for elucidating post-mortem changes in the proteins of meat.

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Literature Cited

(1) Andrews, M. M., Guthneck, B. T., McBride, B. H., Schweigert, B. S., *J. Biol. Chem.* **194,** 715 (1952).
(2) Artyukh, I., *Myasnaya Ind. S.S.S.R.* No. 1, 25 (1940).
(3) Bate-Smith, E. C. B., *J. Physiol.* **92,** 336 (1938).
(4) Bratzler, L. J., ed., "A Cross Sectional Muscle Nomenclature of the Beef Carcass," Mich. State College Press, East Lansing, Mich., 1952.
(5) Edelhoch, H., *J. Am. Chem. Soc.* **79,** 6100 (1957).
(6) Grau, R., Hamm, R., Bauman, A., *Biochem. Z.* **325,** 1 (1953).
(7) Howard, A., Lawrie, R. A., Technical Paper No. 4, Division of Food Preservation and Transport, C.S.I.R.O. Melbourne, Australia, 1957.
(8) Kronman, M. J., Weinberger, L. E., Winterbottom, R. J., *Arch. Biochem. Biophys.*, in press.
(9) Madsen, N. B., Cori, C. F., *J. Biol. Chem.* **223,** 1055 (1956).
(10) Roodyn, D. B., *Biochim. et Biophys. Acta* **25,** 129 (1957).
(11) Sadikov, V., Khaletzkaya, E., *Proc. Sci. Inst. Vitamins Research (U.S.S.R.) (Trudy, Noi, i-ta Vitaminar)* **1,** 123 (1936).
(12) Swift, C. E., Berman, M. D., *Food Technol.* **13,** 365 (1959).
(13) Wierbicki, E., Kunkle, L. E., Cahill, V. R., Deatherage, F. E., *Ibid.*, **8,** 506 (1954).
(14) *Ibid.*, **10,** 80 (1956).
(15) Zender, R., Lataste-Dorolle, C., Collet, R. A., Rowinski, P., Mouton, R. F., *Food Research* **23,** 305 (1958).

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FOOD DISCOLORATION

Reddening of White Onion Tissue

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Factors affecting the formation of the water-soluble, red nitrogenous pigment in acidified white onion purée were investigated. The presence and absence in onions of several compounds are reported. Added acetic acid was not necessary for pigment formation, and its great enhancing effect was traced to the 1 to 3 p.p.m. of formaldehyde which occurs in reagent glacial acetic acid as an impurity. Other similar compounds such as diacetyl, acetoin, and glycolonitrile can replace formaldehyde in the reddening reaction, and studies were made in an effort to identify the naturally occurring carbonyl compound. A type-reaction mechanism for the pigment formation is postulated and discussed.

A WATER-SOLUBLE RED pigment is formed in macerates of white onion bulb tissue when the purée is allowed to stand after acidification with acetic acid. In an earlier paper (*5*), it was shown that the pigment formation in purées was affected by variety, storage conditions, and heat and acid treatments.

New evidence affecting pigmentation has been found, and it has been possible to develop the problem considerably. The acetic acid specificity had been fully explained and, along with this, a general type sequence of steps is postulated. These are presented in this paper.

Experimental

Southport White Globe onions obtained from the same sources as previously reported (*5*) were used, as well as some onions from the same lots used in past work. In addition, one lot grown in Washington State was obtained. No significant difference was found between those from Washington and those from the Stockton region of California.

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Methods of preparing the samples, adding reagents, extracting, and measuring the pigment formed were identical with those previously reported.

Properties of the Pigment

The mpure pigment, prepared in the usual manner, could be purified further by adding sodium hydroxide until the aqueous-alcoholic solution reached pH 9.5. The pigment remained in solution, but a colorless, amorphous nitrogen-free precipitate settled out. After filtering rapidly, the clear solution was reacidified to avoid excessive destruction of pigment at the high pH. Acetone was added and the precipitated salt filtered off. Then the pigment was precipitated by ether addition. The solid was redissolved in absolute ethyl alcohol, filtered, and reprecipitated with ether to remove the remaining traces of inorganic salts. The solid preparation was then lyophilized for 24 hours and used immediately.

The infrared absorption spectrum of the pigment, prepared as above, was determined by using the potassium bromide pellet technique. The data obtained are plotted in Figure 1.

The same pigment preparation was used for organic elementary analysis, with the following results:

Element	Per Cent
C	27.96
H	6.70
N	7.88
O	44.06
Ash	13.4

A comparison of the infrared data and the elementary analysis with the previously published data (*5*) shows the relative purity of the two preparations.

The pigment coupled readily with diazotized sulfanilic acid, forming a compound which was orange in acid and red in base. The diazonium salt was prepared in the classical manner by dissolving 10.5 grams of sulfanilic acid in 100 ml. of 2.5% sodium carbonate by boiling. After cooling the solution, 3.7 grams of sodium nitrite were added, stirred until dissolved, and poured into a beaker containing 50 grams of ice and 10 ml. of concentrated hydrochloric acid. For coupling, sodium hydroxide was added to the aqueous solution of the onion pigment, then the diazotized sulfanilic acid suspension was added, stirred, and allowed to stand. The red solid was filtered off, redissolved in 95% ethyl alcohol, giving an orange solution, and precipitated as a yellow oil by ether addition. After redissolving in absolute methanol, the material was precipitated as an orange solid by addition of ether.