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PROTEIN INVESTIGATIONS AT THE USDA  
BELTSVILLE MEAT LABORATORY

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INTRODUCTION

There has been a long standing interest in meat proteins in the Department of Agriculture. I need not remind this audience of the work of Hoagland, McBride, and Powick in the old Biochemic Division on the autolysis of sterile tissue held in cold storage, or of the later work at Beltsville of Paul Howe and of Hoagland on nutritive value. Recently, when we came to expand our studies on basic meat composition it was only natural that we should want to improve our knowledge of the muscle proteins. The objective of our present work is to provide a functional knowledge of muscle proteins which will establish a basis for improving the processing, preservation, and quality of meats and meat products.

Most of the previous research effort has been made to isolate and characterize proteins and to determine their role in the functions of living muscle. Much of our present knowledge is based on studies of small laboratory animals, such as the rabbit; and the current state of our knowledge is such that only a few concepts have been produced which are not subject to continual modification.

Classical studies of muscle contraction, such as those contributed by Szent-György, Mommaerts, Dubuisson, and others, have had a special place in elucidating muscle composition and muscle functions. Their work assumes special importance in meat research, since it involves work on expiring tissue, or tissue which is in the process of becoming meat as we use and know it. Work to date indicates that some of the isolated muscle proteins do not exist as such in living or strictly fresh animal tissue. Thus, information on some of the proteins of muscle actually pertains to precursors of the proteins in post rigor.

Our program started from a realization that the proteins of muscles of meat animals need not necessarily correspond exactly, or closely, with those of small laboratory animals; that variability of the proteins, an important consideration in meat research, has not been systematically studied; nor are the changes in the proteins after slaughter and rigor understood. Prior work has provided some concepts probably pertinent and certainly useful in muscle protein research. Other investigators have developed methods for use on tissues of small laboratory animals, but there was a dearth of information on large, meat-type animals. In the early phases of our work, emphasis has been given to isolating, purifying, and characterizing the proteins of freshly slaughtered meat animals, with primary attention given to the principal, or structural, proteins. Another important phase of the work involves investigation of the properties of the proteins.

Aspects of our protein studies which I will review here are those concerning:

1. Preparation and properties of bovine myosin.
2. Isolation and analysis of bovine L- and H-meromyosins, which are products of the fission of myosin and trypsin.
3. Characteristics of bovine tropomyosin.
4. Factors relating muscle protein components to the water-holding capacity of beef.

#### PROTEIN FRACTIONATION AND CHARACTERIZATION

There have been several mentions in the literature of studies on impure bovine muscle protein extracts. However, prior to our investigations, there was no information available on the actual isolation and purification of the proteins whose properties will be discussed in this paper. Therefore, one of our first tasks was to develop methods for isolating these muscle proteins before their properties could be determined.

Preliminary to an isolation of bovine myosin, we studied the characteristics of water-insoluble, salt-soluble extracts obtained from eight different beef muscles. Figure 1 shows the ultracentrifuge patterns of extracts from four of these muscles. The presence of two main peaks shows that the extracted proteins belonged to two main sedimentation classes. The variation in the area

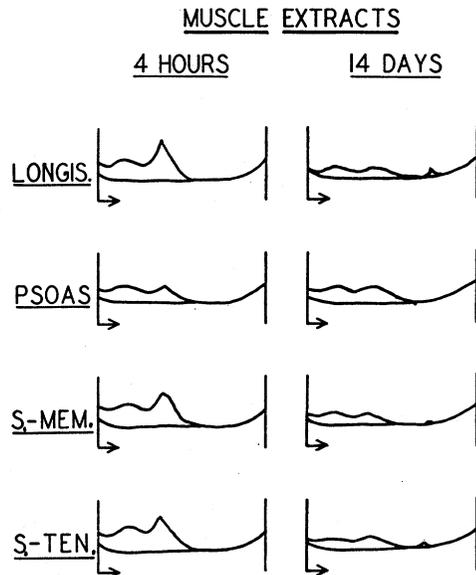


Figure 1. Ultracentrifuge patterns of extracts of four muscles.

under the peaks, from muscle to muscle, shows that the composition and the amount of protein dissolved varied with the different muscles. We have also found that, after rigor sets in, the amount of extractable material decreased sharply, especially in the fast peak which includes myosin. As we know, rigor occurs at different times with different muscles. The time when extractable protein diminished was different from one muscle to another and corresponded in general with the time of onset of rigor in the different muscles.

The onset of rigor also parallels the loss of extractability of myosin. The importance of myosin in the living muscle lies in the fact that it is the most abundant protein and has certain unique properties. First of all, myosin is a contractile protein and is primarily responsible for maintaining the structure of the muscle myofibrils and performing the contractions and relaxations by which muscles do work. Also, myosin contains an important ATPase (adenosine-triphosphatase) enzyme activity, which helps control the transformation of chemical energy to physical energy or work.

Myosin, either *in vitro* or *in vivo*, can combine with actin, to form the protein complex actomyosin. In this complex, ATPase activity is still present. After slaughter, myosin can be isolated separately from actin by rapid extraction with salt. However, after rigor sets in, the myosin-actin complex can no longer be split into its two protein components by ordinary extractions. The ATPase activity remains, although some chemical changes occur in the union of the two proteins.

Thus, to study myosin separately from actin, it is necessary to isolate the protein from meat in the pre-rigor state. To prepare bovine myosin, we therefore obtain samples from the hot carcass immediately after skinning and begin extraction within one to two hours.

The classical procedure for the isolation of rabbit myosin is that of Kessler and Spicer (Fig. 2). When it was applied to a one-kilogram sample of ground

## ISOLATION OF BOVINE MYOSIN

(FROM 1 KG. GROUND BEEF MUSCLES)

<u>MODIFIED K-S METHOD</u>	<u>NEW (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub> METHOD</u>
1. EXTRACT MUSCLE: <u>8 LITERS.</u>	1. EXTRACT MUSCLE: <u>6 LITERS.</u>
2. PRECIPITATE BY DILUTION WITH WATER: <u>40 LITERS.</u>	2. PRECIPITATE WITH SOLID (NH <sub>4</sub> ) <sub>2</sub> SO <sub>4</sub> : <u>6.5 LITERS.</u>
3. DISSOLVE; REMOVE ACTO-MYOSIN WITH ATP.	3. DISSOLVE; PRECIPITATE BY DIALYSIS vs. WATER.
4. PRECIPITATE BY DILUTION WITH WATER: <u>10 LITERS.</u>	4. DISSOLVE; PURIFY BY PRECIPITATION.
5. DISSOLVE; PURIFY BY PRECIPITATION.	
<u>YIELD: 4 GRAMS</u>	<u>YIELD: 8 GRAMS</u>

Figure 2. Isolation of bovine myosin.

beef muscle, about 8 liters of extract were obtained; the volume of the extract had to be increased to about 40 liters before any substantial amount of bovine myosin would precipitate. After high speed centrifugation and subsequent purification, the final yield of myosin was about 4 g./1 kg. meat.

Speed was essential in the purification of the protein because myosin gradually ages and denatures, even on storage in the cold. Unfortunately, with such large volumes of liquid to centrifuge and with time such an important factor, the amount of myosin we could prepare in each batch was quite limited.

We have now developed a rapid procedure for the isolation and purification of bovine myosin. We have also been able to apply this method to the preparation of rabbit myosin. With ammonium sulfate used during extraction and precipitation, the liquid volume is cut down at least sixfold and the ultimate yield is at least doubled. The final product has the same properties as bovine myosin which we obtained by other means, and we are now able to take better advantage of the large amount of tissue available from a carcass.

For the measurement of ATPase activity in myosin, we have modified Mommaerts' method. A Radiometer pH-stat is used to maintain constant pH in a solution containing myosin and ATP. As the ATP is split by myosin, yielding inorganic phosphate, the instrument adds increments of alkali to maintain the pH of the solution. The results are automatically recorded. This new procedure will permit the investigation of ATPase activity under conditions impossible by other procedures.

Our bovine myosin preparations have the usual ATPase activities of myosins from other animal sources. In the presence of calcium ions, the activity at 25° C. is maximal at about pH 9.0 (Fig. 3). At a higher pH, myosin rapidly denatures and is inactivated.

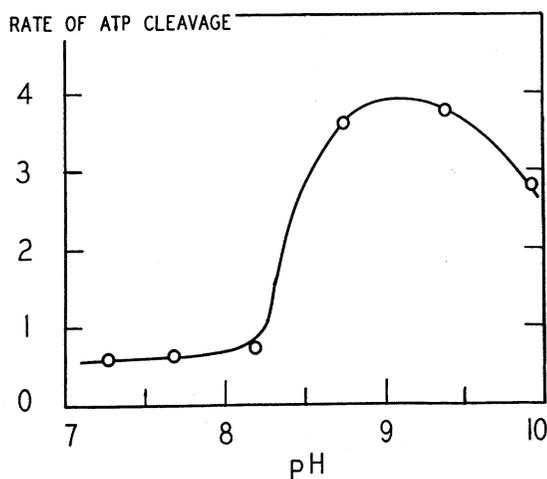


Figure 3. Adenosinetriphosphatase activity of bovine myosin.

Ultracentrifugal data show that bovine myosin, prepared in our new manner, is homogeneous by this criterion. Upon extrapolation (Fig. 4), the intrinsic sedimentation constant at 20° C. was 5.78 Svedberg units. Up to at least 0.4% bovine myosin concentration, the sedimentation constant was the usual linear function of concentration as follows:

$$S_{20} = S_{20,w} - 3.3 \times 10^{-13}$$

Assuming a diffusion coefficient of  $1.0 \times 10^{-7}$ , the sedimentation-diffusion molecular weight was 560,000.

Myosin from various sources has been described as a very long, thin protein molecule, 800 to 1000 Å. long and 16 Å. wide. The helical chains are arranged alternately, first two side-by-side and then four side-by-side. Our measurements on the change in optical rotation with wavelength (optical rotatory dispersion) showed that bovine myosin likewise has a very strong  $\alpha$ -hel-

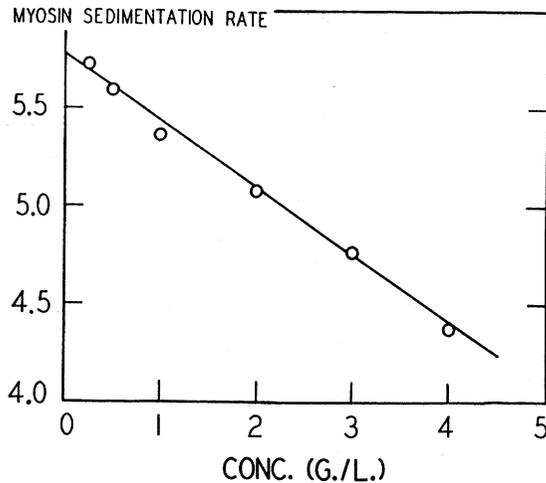


Figure 4. Myosin sedimentation rate.

ical character. The specific rotation at the sodium D line was  $-19.8^\circ$ , which was an unusually low value for a protein, and further indicated its helical nature.

From the ultraviolet absorption spectrum, we have determined that the tyrosine content of bovine myosin is 18 moles/100,000 grams and that there is but little tryptophane. On the addition of alkali to pH 13, the spectrum underwent a shift characteristic of the phenolate ion. However, there was then a rapid, time-dependent increase in absorbance, indicating denaturation of the myosin molecule. The pK of the phenol group in the tyrosine residues was about 10.8 at  $25^\circ\text{C}$ , which is a somewhat high value for this group in a protein. At  $280\text{ m}\mu$ ,  $E_{1\%}^{1\text{cm.}} = 5.73$ .

Myosin is an unusual protein in several additional respects. Although its amino acid composition is similar to that of most other proteins, it is insoluble in dilute salt solutions (below 0.1 ionic strength) and soluble to a concentration of about 3% in strong salt solutions. On storage in the cold, it gradually loses its solubility and its ATPase activity. At the same time, it forms aggregates, and solutions gradually become turbid.

On treatment with trypsin or other proteolytic enzymes, myosin very quickly splits into protein-sized fragments, which are called meromyosins (Fig. 5); the light one: L-meromyosin, and the heavy one: H-meromyosin. It is only after prolonged treatment that small peptide fragments are obtained. Each meromyosin fraction inherits some of the properties of the parent myosin. We have found that the bovine meromyosins have similar properties to those described for meromyosins from other sources. L-meromyosin has similar solubility and viscosity; H-meromyosin retains the ATPase activity and the instability of myosin, although it is soluble in water and low-salt solution. By careful fractionation, we have determined that, contrary to reports of earlier workers, the rabbit H-meromyosin fraction is not homogeneous. Bovine H-meromyosin is likewise heterogeneous. This finding must necessarily lead to a revision in the picture of the way in which the meromyosins combine to form the original structure of myosin.

We are currently studying certain of the properties of the bovine meromyosins to establish how these are derived from and contribute to the properties of myosin. We feel that this information is important in understanding the structure of meat, as well as the modification which occurs when meat

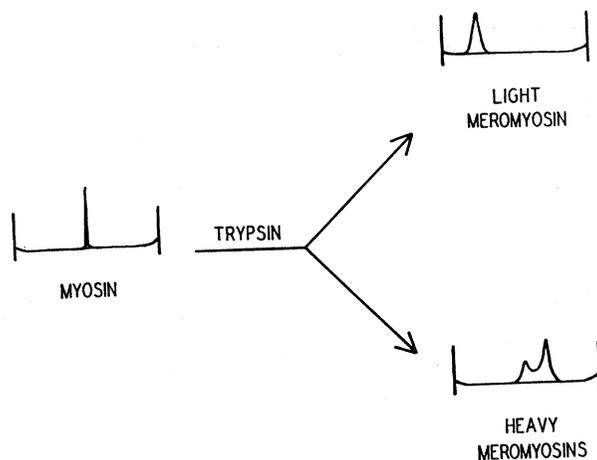


Figure 5. Separation of myosin, showing ultracentrifuge patterns of original protein and products.

undergoes rigor.

Another important structural protein is tropomyosin. This protein is obtained from meat only after the removal of the water-soluble and salt-soluble proteins, and after the dehydration of the meat fiber with acetone and ether. Tropomyosin is then isolated by extraction with molar KCl; it is then water-soluble. For bovine tropomyosin, the isoelectric point, as derived from electrophoretic measurements, is pH 4.95 (Fig. 6). The sedimentation constant is 2.9 Svedberg units.

Near the isoelectric point, its viscosity in water is very large. A 1% solution of bovine tropomyosin has somewhat the consistency of a thin mucilage. When the pH is raised to about pH 7, or when salts are added, the solution thins rapidly as the viscosity drops. As with myosin, optical rotatory dispersion measurements indicate a tight helical configuration. The specific rotation at the sodium D line is  $-21.0^\circ$  in acid and  $-70^\circ$  at pH 12. From spectroscopic studies, we have determined that the tyrosine content is 21 moles/100,000 grams. The pK of the tyrosine phenolate group is 10.6 at  $25^\circ\text{C}$ ., and, unlike myosin, the alkaline spectrum does not change rapidly with time. At  $280\text{ m}\mu$ ,  $E_{1\%}^{1\text{cm}} = 3.26$ .

#### STUDIES ON WATER-HOLDING

A major line of research in our Laboratory has been concerned with the water-holding capacity of meat. This has obvious implications concerning not only the properties of fresh meat, but also the structural nature of the binding forces in cured meats, especially in comminuted products such as emulsion sausages and similar products.

We have investigated the effects on the meat residues of the extraction of various protein components from the meat. Two hours after slaughter, a sample of ground beef muscle was treated with several volumes of liquid containing salt and acid or base, as needed, to adjust the total ionic strength and the pH of the meat-liquid mixture. After storage overnight in the cold, the percentage change in weight of the meat residue was measured and information was then gathered about the composition of the excess liquid which could

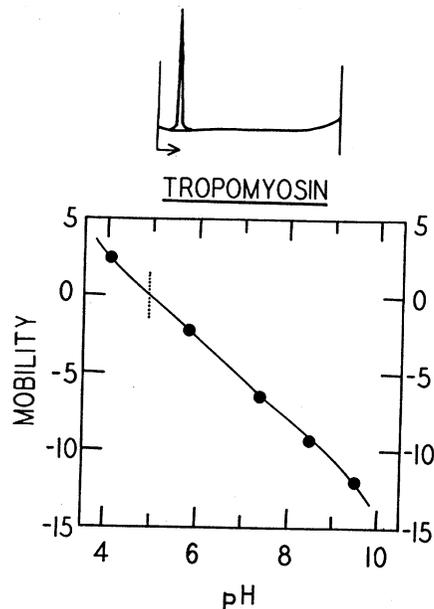


Figure 6. Tropomyosin, ultracentrifuge pattern and isoelectric point from electrophoretic mobility.

be separated from the mixture after centrifugation (15,000 r.p.m. for 15 minutes).

In the absence of added salt (Fig. 7), the weight of meat residue was at a minimum at about pH 5, but increased markedly as the pH was changed toward either pH 2.5 or 10.5. On the addition of salt, a minimum was found in about the same region (pH 4.5). In acid solution, the meat did not imbibe much water in the presence of salt. On the other hand, there was a very rapid increase in the water uptake in the region from pH 6 to 8.

In the region from pH 4 to 9, there was no significant variation in the amount of the non-protein nitrogen as a result of variation in salt content, even though there was some effect of pH on the extractability of this material (Fig. 8).

The pigment content of the extracts was also measured, to give an indication of the extractability of the water-soluble proteins. The salt level had very little effect on the quantity of pigment released. Also, pH was not a factor, except at the extremes of pH where pigment extraction decreased rapidly.

The total of the TCA-insoluble, extractable nitrogen compounds was also studied. In this case (Fig. 9), there were marked changes in the quantity of material extracted (which can be considered equivalent to the total extractable protein). The effects of both pH and salt content followed the general pattern of the water retention curves. What would appear to be most remarkable was the observation that the more protein was removed from the meat, the more water the residue would hold. One explanation of this is that the same conditions are optimal for maximum extraction and imbibition. Another possible conclusion is that certain proteins, by their presence in the meat, prevented other proteins from holding as much water as they were capable of retaining.

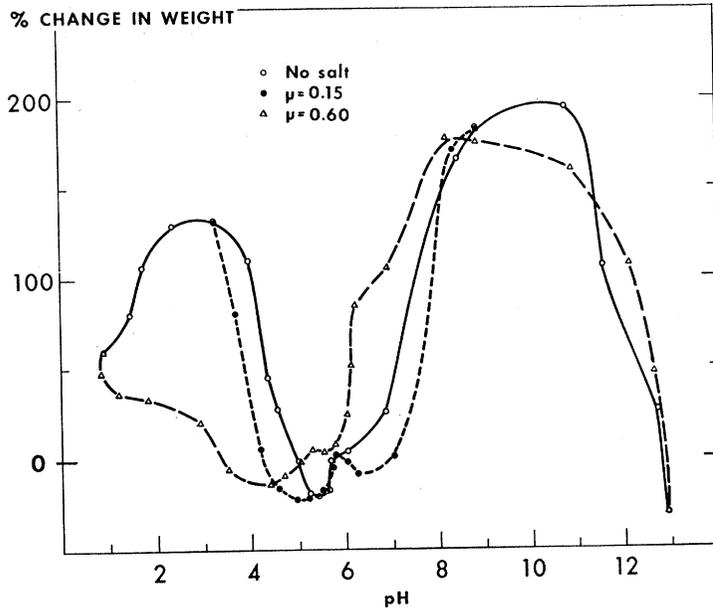


Figure 7. Effect of salts and pH on water holding

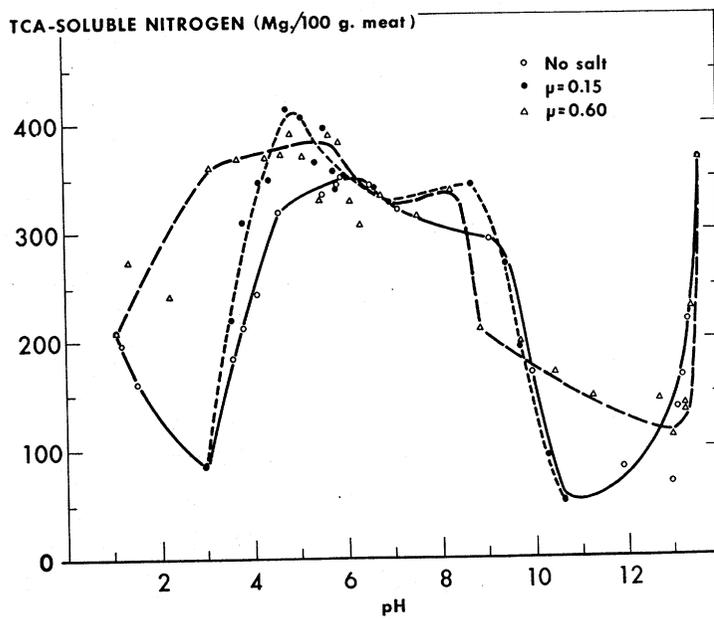


Figure 8. Relationship of pH to non-protein nitrogen extractability.

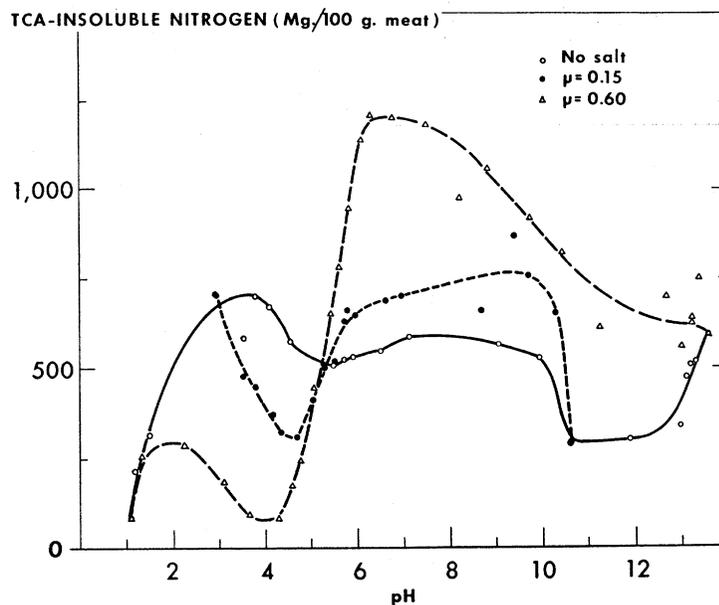


Figure 9. Relationship of pH to extractable protein.

When these proteins were removed, the water retention would then be able to rise, as was observed. Certain critical experiments are needed to elucidate this phenomenon.

The water in the structure of the meat residue cannot be simply water of hydration. In 100 g. of lean meat, there are no more than 21 g. of protein and over 70 g. of water. In alkaline solution (pH 10-11), the total weight of meat residue increased by 150-200%, while as much as one-third of the protein was extracted. This leaves no more than 15 g. of meat proteins firmly linked with 235-285 g. of water. As Edsall indicates, the upper limit for water of hydration is of the order of 0.6-1.1 g. water/g. protein. In the present case, then, water of hydration can only account for about 15 g.

The general pattern of the water-holding curves follows that found for the swelling of fibrous proteins such as collagen, gelatin, and wool fibers. The effects of pH and salt content are generally predictable in accord with the Donnan membrane theory. Since meat swelling can mostly be treated as a particular case of fiber swelling, a potential basis therefore exists for predicting the effects on the properties of comminuted products on treatment under various conditions.

An exception to the general pattern of fiber-swelling is found in the effects of large amounts of salt in the pH region from pH 6 to 8. Here, contrary to expectations, water-holding increases very rapidly with pH. To determine the reason for this, we have investigated the extracts in some detail.

When myosin is extracted from meat, the enzyme deaminase accompanies it. As a measure of salt-soluble components, the deaminase activity of the meat extracts was measured. It was found that there was only a small amount of activity in water-soluble extracts. However, in salt-soluble extracts, the activity was very marked in the pH region from 6 to 8. This is the same region in which the residues, after salt extraction, showed a marked increase in their tendency to retain water. Because of the inactivation of deami-

nase above pH 7.5, it was not possible to obtain information of a reliable nature above that pH.

Since deaminase accompanies myosin in a meat extract, the sudden increase in water retention which occurred between pH 6 and 8 may be related to the extraction of the myosin components from the meat. The structure of the myofibrils depends on myosin and actin. These proteins might be expected to resist the tendency of the fibers to swell by cross-linkage. After myosin has been extracted, not only would its restraining influence on the fiber structure be eliminated, but it is possible that additional binding sites for water molecules would also be made available. Future work will be designed to test this hypothesis and to investigate possible practical applications.

In this talk, I have attempted to review some of the protein work in which my colleagues are engaged. It is our conviction that progress along these lines of inquiry must precede intelligent improvements in meat processing and will provide a sound basis for an expanded meat technology.

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