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CHEMICAL CHANGES IN POSTMORTEM MUSCLE

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Meat may be defined as muscle that has passed through rigor mortis, if you believe in the resolution of rigor, or muscle that is in rigor mortis, if you believe that there is no resolution of rigor. Whichever your view, this definition states that differences between living muscle and meat are due to chemical changes that occur during rigor mortis. Since numerous studies have shown that tenderness, water-holding capacity, and emulsifying properties of muscle all undergo large changes during rigor mortis, chemical changes in postmortem muscle have been a subject of intense interest to the meat scientist. Most of the early studies on chemical changes in postmortem muscle emanated from the Low Temperature Research Station at Cambridge. In a series of studies that are now classical, E. C. Bate-Smith, J. R. Bendall, and coworkers (Bate-Smith, 1939; 1948; Bate-Smith and Bendall, 1947; 1949; 1956; Bendall, 1951) showed that the reactions of anaerobic glycolysis were among the important chemical changes that occur in muscle soon after death. In order to respond immediately to demands for contractile activity, living muscle cells normally store variable amounts of glucose as glycogen, and oxygen as oxymyoglobin; the oxygen is used to convert glycogen to carbon dioxide and water and thereby liberate energy that can be used by the contractile proteins. During and after muscular activity, oxymyoglobin is replenished by oxygen from the blood stream. Death interrupts the blood supply and hence also the oxygen supply to muscle. After oxygen in the stored oxymyoglobin has been exhausted, reactions of the citric acid cycle cease, and any glycogen remaining is then converted to lactic acid in the muscle cell's last desperate attempts to keep its life processes going. Bate-Smith and Bendall (1947; 1949) showed that this lactic acid production was responsible for the pH decline normally observed in postmortem muscle. When the muscle's

glycogen supply is exhausted, anaerobic production of ATP also stops, and creatine phosphate, the last reservoir of high energy phosphate, begins to disappear. When creatine phosphate is depleted, ATP concentration drops precipitously to less than 1 mM and the muscle is said to be in rigor mortis. Erdos (1943), in Albert Szent-Gyorgyi's laboratory in Hungary, suggested in 1943 that loss of ATP was responsible for the onset of rigor mortis, and this idea has persisted to the present time. Recent studies show that one of ATP's functions in living muscle is to keep the thick and thin filaments separated and therefore free to slide past one another. This molecular sliding is manifested macroscopically as extensibility of muscle strips. Therefore, postmortem loss of ATP in muscle leads directly to a loss of extensibility. The term "rigor mortis", however, means stiffness or rigidity, and rigidity is produced by attempted shortening of two muscles on opposing sides of the same bone. Consequently, it is clear that loss of extensibility is not itself the sole cause of the physical changes associated with the onset of rigor mortis, although loss of extensibility frequently occurs very soon after the onset of rigor mortis, and it is sometimes difficult to distinguish the time-course of the two events. The physical changes in postmortem muscle will be considered in more detail in the following paper, but this brief discussion may show how chemical and physical changes in postmortem muscle are related, often in subtle ways. The relationships normally observed among postmortem changes in muscle pH, creatine phosphate and ATP concentration, and extensibility are summarized in the first slide.

Slide 1 - Some Postmortem Chemical Changes in Muscle.

It should be obvious from the preceding discussion that depletion of a muscle's glycogen supply before death will diminish the extent of the postmortem pH decline, and that ATP and creatine phosphate concentrations and extensibility in these muscles will decrease when muscle pH is still near 7.0. Moreover, continued studies by Bendall, Lawrie, Marsh, and associates in England (Bendall, 1966; Marsh, 1953; 1954; Lawrie, 1953) together with recent studies by Briskey, Cassens, and their coworkers at Wisconsin (see Briskey, 1964, for a summary) and by Newbold and Scopes in Australia (Newbold and Scopes, 1971a; 1971b) have shown that rate

of postmortem glycolysis can vary widely among different animals, and that very rapid postmortem glycolysis frequently has severe adverse effects on meat quality. The exact reasons for these variations in rate of postmortem glycolysis are still unclear, but it is beginning to appear that rate of postmortem glycolysis depends critically upon the type of muscle cells present and the way metabolism in these different cells is regulated in the living animal. Since a logical discussion of these recent subtleties would require more time than that allotted for this presentation, I propose simply to mention the existence of these complexities and then to procede to some recent studies of chemical changes in postmortem muscle that have been of particular interest to us at Iowa.

Our interest at Iowa has centered on the nature of the postmortem changes in myofibrillar proteins and on the agents responsible for these changes. We have approached this problem by attempting to separate the changes that occur during the onset of rigor mortis from those that occur during the resolution of rigor; consequently, it is important before discussing our results to state clearly our definitions of the terms "onset" and "resolution" of rigor mortis. We routinely use isometric tension measurements to monitor the time course of rigor mortis in muscle. The unique advantages of isometric tension measurements for quantitatively monitoring the time-course of rigor mortis have already been discussed (Busch et al., 1972; Goll, 1968; Goll et al., 1970), and these arguments will not be repeated here. A typical example of the results of this kind of measurement is shown in the next slide.

Slide 2 - Postmortem Isometric Tension Development in Bovine Semitendinosus Muscle at 2 and 37°.

We define the period of increasing isometric tension as corresponding to the onset of rigor mortis; the time of maximum isometric tension development corresponds to maximum rigor or the height of rigor, and the period during which isometric tension declines is defined as the resolution of rigor (Busch et al., 1972). We have found that this pattern of increasing isometric tension followed by a decline in isometric tension occurs in muscle from all three species

(rabbit, porcine, bovine) and at all four temperatures (2°, 16°, 25°, and 37° C) that we have investigated (Busch et al., 1972).

These definitions for the onset and resolution of rigor mortis enable us to categorize the study of postmortem chemical changes in myofibrillar proteins into the study of two distinct problems: 1) what changes cause the onset of rigor mortis (i.e., initiation of isometric tension), and 2) what changes cause the resolution of rigor mortis (i.e., the decline of isometric tension)? Having posed these two questions, it may be easiest to simply give you our answers to these questions and then to discuss the evidence that led us to these answers.

Slide 3 - What Causes the Onset of Rigor Mortis?

Answer: A) Loss of Ca^{2+}-accumulating ability of the sarcoplasmic reticular membranes resulting in an increase in free intracellular Ca^{2+} concentration. Higher intracellular free Ca^{2+} concentration causes shortening or isometric tension development in either living or postmortem muscle. Possible underlying causes for loss of Ca^{2+}-accumulating ability:

1) Postmortem loss of ATP
2) Postmortem pH decline
3) Uncoupling of the Ca^{2+}-pump by proteolysis

Slide 4 - What Causes the Resolution of Rigor Mortis?

Answer: A) Modification of the actin-myosin interaction which results in a) changes in the nucleosidetriphosphatase activities of actomyosin; b) changes in in vitro contractile properties of actomyosin; c) lengthening of rigor shortened sarcomeres; and d) changes in dissociability of the actin-myosin complex. Possible underlying causes for modification of the actin-myosin interaction:

1) Limited proteolysis
2) Postmortem sulfhydryl alteration

3) Postmortem pH decline

B) Loss of Z-disk integrity which results in a) fragmentation of myofibrils, and b) loss of tensile strength of fibers. Possible underlying causes for loss of Z-disk integrity:

1) Proteolysis by a Ca^{2+}-activated enzyme

Lack of time will prevent us from discussing all the points in the preceding two slides in detail, and we will examine only four of those areas that are presently interesting to us. Before presenting our evidence for the answers given in the previous two slides, it may be useful to very briefly review a few salient features of the contractile system in muscle. The next slide shows an electron micrograph of vertebrate muscle together with a schematic illustration of the thick and thin filament array that is responsible for the unique striated features of skeletal muscle. Notice particularly the fibrillar and zig-zag-like structure of the Z-disk.

Slide 5 - Electron Micrograph plus Schematic of Skeletal Muscle
(from H. E. Huxley)

The next slide shows the arrangement of sarcoplasmic reticular membranes in the muscle cell. Numerous studies have shown that sarcoplasmic reticular membranes contain an ATPase that will use the energy of ATP to enable these membranes to accumulate Ca^{2+} against a concentration gradient. These membranes can be studied biochemically by mincing the muscle cell and breaking the sarcoplasmic reticular membranes into fragments; these fragments can then be isolated by differential centrifugation and purified by density gradient centrifugation.

Slide 6 - Electron Micrograph of Vertebrate Muscle Showing the Sarcoplasmic Reticulum.

We can now examine the evidence that supports our answer to the question, "what causes the onset of rigor mortis?" The first pieces of this evidence were obtained by Marion Greaser and his colleagues at the University of Wisconsin who showed that sarcoplasmic reticular membranes rapidly lose their ability to accumulate Ca^{2+} during postmortem storage. Subsequently,

Bruce Eason in our laboratory at Iowa showed that loss of Ca^{2+}-accumulating ability in sarcoplasmic reticular membranes occurs just before the onset of isometric tension development. Dr. Eason's findings are summarized in the next slide where it is shown that Ca^{2+}-accumulating ability in rabbit psoas strips after 2.4 hours at 37° (when these strips had just started to develop isometric tension) was less than 1/3 that of the same muscle immediately after death.

Slide 7 - Postmortem Changes in Ca^{2+}-Accumulating Ability and ATPase Activity of Fragmented Sarcoplasmic Reticulum

Since ATP is a required energy source for sarcoplasmic reticular membranes to accumulate Ca^{2+} against a concentration gradient, it seems likely that the postmortem decline in ATP concentration is responsible in many instances for the postmortem loss in ability of the sarcoplasmic reticular membranes to accumulate Ca^{2+}. Other investigators, however, have shown that either pH values below 6.5 or very brief tryptic treatment will also decrease the ability of sarcoplasmic reticular membranes to accumulate Ca^{2+}; therefore, it is possible that postmortem pH decline or very limited proteolysis by muscle cathepsins may also be involved in the postmortem loss of Ca^{2+}-accumulating ability by the sarcoplasmic reticulum, particularly in those muscles that undergo a very rapid pH decline postmortem.

What is the evidence supporting our answer to the question, "what causes the resolution of rigor mortis?" Let's begin with the first answer, i.e., modification of the actin-myosin interaction. Rich Robson in our laboratory showed that Mg^{2+}-modified ATPase activity of myofibrils isolated from postmortem muscle is 50-100% higher than the same activity of myofibrils prepared from at-death muscle. Similarly, Ca^{2+}-modified ATPase activity of myofibrils made from postmortem muscle is greater than the same activity of myofibrils prepared from at-death muscle. These results have subsequently been confirmed by numerous investigators and are summarized in the next slide.

Slide 8 - Effect of Postmortem Storage at 2° on the ATPase Activity of Myofibrils From Bovine Semitendinosus Muscle

Activator	Time of postmortem storage, hr		
	0	24	312
5 mM Mg^{2+}	0.051[a]	0.081	0.047
5 mM Ca^{2+}	0.106	0.133	0.187

[a]Assays were done in 120 mM KCl, 24 mM Tris, pH 7.6, 5 mM ATP at 25°. Figures are μmoles inorganic phosphate released per milligram myofibrillar protein per minute.

The agent causing ATPase activity to increase during postmortem storage is unknown, but we have recently shown that very limited tryptic digestion will increase the ATPase activity of myofibrils in a way that is qualitatively and quantitatively similar to that caused by postmortem storage.

Postmortem modification of the actin-myosin interaction is also indicated by our finding that natural actomyosin prepared from postmortem muscle undergoes an in vitro contractile response (as measured by the turbidity assay; see Goll et al., 1970) much more quickly than natural actomyosin prepared from at-death muscle. Again, the agent causing this

Slide 9 - Effect of Postmortem Storage on the Superprecipitation of Natural Actomyosin

increase in rate of in vitro contraction of natural actomyosin is unknown, but we have found that very brief tryptic treatment of actomyosin will qualitatively mimic this effect of postmortem storage.

We propose to spend the remaining time discussing our evidence that loss of Z-line integrity is at least partly responsible for resolution of rigor mortis, and that postmortem Z-disk degradation is caused by a Ca^{2+}-activated enzyme that we have recently succeeded in isolating from muscle. Our electron microscopic studies of muscle after varying times and temperatures of postmortem storage clearly show that postmortem storage causes progressive loss of Z-disk structure (Henderson et al., 1970). In general, postmortem Z-disk degradation

occurs more rapidly at higher (37°) storage temperatures than it does at lower (2°) storage temperatures, and is more rapid in rabbit or porcine muscle than in bovine muscle.

Slide 10 - Electron Micrograph of At-Death Porcine Muscle.

Slide 11 - Electron Micrograph of Porcine Muscle After 120 hr at 2°. The Z-disk is beginning to fragment after this time and temperature of postmortem storage, and has lost its fibrillar appearance.

Slide 12 - Electron Micrograph of Porcine Muscle After 24 hr at 25°. The Z-disk is very faint and amorphous, and appears to possess a trough of decreased density along its center.

Wayne Busch in our laboratory discovered that postmortem Z-disk degradation was related to intracellular Ca^{2+} concentration while he was doing some electron microscope studies on muscle strips that had been incubated in a Ca^{2+}-containing solution. Suspension of rabbit psoas strips for 9 hours at 37° in a saline solution containing 1 mM $CaCl_2$ causes about 25% of the Z-disks to become very diffuse and weak, causes another 25% to split along their center, and the remaining 50% of the Z-disks are totally removed. Substitution of 1 mM EGTA (a Ca^{2+} chelator) for Ca^{2+} in these saline incubating solutions results in complete preservation of the Z-disk structure after 9 hours at 37°; therefore, Z-disk disintegration in these strips is due specifically to the presence of Ca^{2+}. Dr. Busch's results are summarized in the following slides.

Slide 13 - Electron Micrograph of At-Death Rabbit Psoas Before Incubation. Z-disks are distinct and fibrillar.

Slide 14 - Electron Micrograph of Rabbit Psoas Muscle After Incubation in a 1 mM Ca^{2+}-Containing Solution for 9 Hours at 37°. Z-disks are very faint and diffuse, and lack their normal fibrillar appearance.

Slide 15 - Electron Micrograph of Rabbit Psoas Muscle After Incubation in a 1 mM Ca^{2+} - Containing Solution for 9 Hours at 37°. Z-disks are split along their center and Z-disk material can be observed adhering to the ends of thin filaments from adjacent sarcomeres.

Slide 16 - Electron Micrograph Showing a Higher Magnification View of the Split Z-Disks Seen in the Previous Slide.

Slide 17 - Electron Micrograph of Rabbit Psoas Muscle After Incubation in a 1 mM Ca^{2+}-Containing Solution for 9 Hours at 37°.

Z-disks have been totally removed in this sample, but M-lines remain intact.

Slide 18 - Electron Micrograph Showing a Higher Magnification View of the Sample Seen in the Previous Slide.

Thin filaments extend to the Z-disk region and end there.

Slide 19 - Electron Micrograph of Rabbit Psoas Muscle After Incubation in a 1 mM EGTA-Containing Solution for 9 Hours at 37°.

Z-disks are intact and fibrillar, and appear identical to Z-disks in at-death psoas muscle.

We have indicated that loss of Z-disk integrity is one cause of the resolution of rigor mortis. Since we define resolution of rigor as a decline in isometric tension development of postmortem muscle, it follows, therefore, that addition of Ca^{2+} should markedly increase the rate of postmortem isometric tension decline of muscle strips, whereas addition of EGTA should delay isometric tension decline under the same conditions. As the data in the next slide demonstrate, this is indeed the case. Incubation of muscle strips in a saline solution containing 1 mM Ca^{2+} at 37° results in complete loss of isometric tension within 7 hours postmortem. Postmortem isometric tension, however, does not decline perceptibly after 24 hours of incubation of muscle strips in the same saline solution but containing 1 mM EGTA substituted for 1 mM Ca^{2+}.

Slide 20 - Effect of Ca^{2+} and EGTA on Postmortem Isometric Tension of Rabbit Psoas Strips at 37°.

Our early experiments showed that Ca^{2+} had no effect on Z-disk structure when it was added to purified myofibrils after removal of sarcoplasmic proteins. This finding suggested that the sarcoplasm contains a Ca^{2+}-activated factor capable of degrading Z-disks, and Dr. Atsushi Suzuki in our laboratory has recently succeeded in isolating this factor. We have now purified this factor to the stage that it gives only 3-4 bands on polyacrylamide gel electrophoresis; we estimate that our preparations are 50-60% pure. The factor is a protein, and it possesses proteolytic activity as measured by its ability to hydrolyze either casein or hemoglobin. It requires at least 0.1 mM Ca^{2+} for activity and is maximally active in the presence of 1 to 10 mM Ca^{2+}, and in the pH range of 6.0-7.0. Very small amounts of our partly purified preparations will cause specific degradation of the Z-disk; none of the other structural features of the myofibril are affected by this enzyme. In this respect, the enzyme's action resembles the effects caused by incubation of muscle strips in Ca^{2+}-containing solutions. Consequently, we are convinced that this enzyme is the factor that is activated by incubation of muscle strips in Ca^{2+}-containing solutions, and that it is also the agent responsible for postmortem degradation of Z-disks in mammalian muscle. The effects of partly purified preparations of this enzyme on purified myofibrils are shown in the last three slides.

Slide 21 - Phase Micrographs Showing the Effects of the Ca^{2+}-Activated Enzyme on Purified Myofibrils in the Presence and Absence of Ca^{2+}.

Slide 22 - Electron Micrographs Showing the Effects of the Ca^{2+}-Activated Enzyme on Purified Myofibrils in the Presence and Absence of Ca^{2+}.

Slide 23 - Electron Micrograph Showing Z-Disk Removal by the Ca^{2+}-Activated Enzyme.

Note that the M-line is not affected by the enzyme.

To our knowledge, the Ca^{2+}-activated enzyme is the first protein that has been isolated from muscle cells and that has been demonstrated to have the ability to catalyze degradation of the myofibril. Since it seems highly probable (but not yet proven) that Z-disk removal, leaving sarcomeres 0.0025 mm in length, would cause a tremendous increase in meat tenderness, it is likely that the Ca^{2+}-activated enzyme is the agent that causes tenderization during "aging" or "hanging" of carcasses. It will be very interesting to determine whether the Ca^{2+}-activated enzyme has any effects on myosin or actin, or whether Z-disk removal by the Ca^{2+}-activated enzyme leaves the remainder of the myofibril susceptible to previously ineffective proteases. These studies are presently underway in our laboratory, and we can now begin to hope that the long debated role of proteolysis in postmortem muscle (Goll et al., 1971) will finally be understood.

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LITERATURE CITED

Bate-Smith, E. C. 1939. Changes in elasticity of mammalian muscle undergoing rigor mortis. J. Physiol. 96, 176.

Bate-Smith, E. C. 1948. The physiology and chemistry of rigor mortis with special reference to the aging of beef. Advan. Food Res. 1, 1.

Bate-Smith, E. C., and J. R. Bendall. 1947. Rigor mortis and adenosinetriphosphate. J. Physiol. 106, 177.

Bate-Smith, E. C., and J. R. Bendall. 1949. Factors determining the time course of rigor mortis. J. Physiol. 110, 47.

Bate-Smith, E. C., and J. R. Bendall. 1956. Changes in muscle after death. Brit. Med. Bull. 12, 230.

Bendall, J. R. 1951. The shortening of rabbit muscles during rigor mortis: its relation to the breakdown of adenosine triphosphate and creatine phosphate and to muscular contraction. J. Physiol. 114, 71.

Bendall, J. R. 1966. The effect of pre-treatment of pigs with curare on the post-mortem rate of pH fall and onset of rigor mortis in the musculature. J. Sci. Food Agr. 17, 333.

Briskey, E. J. 1964. Etiological status and associated studies of pale, soft, exudative porcine musculature. Advan. Food Res. 13, 89.

Busch, W. A., D. E. Goll, and F. C. Parrish. 1972. Molecular properties of postmortem muscle. Isometric tension development and decline in bovine, porcine, and rabbit muscle. J. Food Sci. 37, 289.

Erdos, T. 1943. Rigor, contracture, and ATP. Stud. Inst. Med. Chem. Univ. Szeged 3, 51.

Goll, Darrel E. 1968. The resolution of rigor mortis. Proc. Twenty-First Reciprocal Meats Conference, National Livestock and Meat Board, Chicago. p. 16.

Goll, D. E., N. Arakawa, M. H. Stromer, W. A. Busch, and R. M. Robson. 1970. Chemistry of muscle proteins as a food. In "The Physiology and Biochemistry of Muscle as a Food, 2" (E. J. Briskey, R. G. Cassens, and B. B. Marsh, ed.). The University of Wisconsin Press, Madison. p. 755.

Goll, Darrel E., M. H. Stromer, R. M. Robson, Joanne Temple, B. A. Eason, and W. A. Busch. 1971. Tryptic digestion of muscle components simulates many of the changes caused by postmortem storage. J. Animal Sci. 33, 963.

Henderson, D. W., D. E. Goll, and M. H. Stromer. 1970. A comparison of shortening and Z-line degradation in postmortem bovine, porcine, and rabbit muscle. Am. J. Anat. 128, 117.

Lawrie, R. A. 1953. The onset of rigor mortis in various muscles ofthe draught horse. J. Physiol. 121, 275.

Marsh, B. B. 1953. Shortening and extensibility in rigor mortis. Biochim. Biophys. Acta 12, 478.

Marsh, B. B. 1954. Rigor mortis in beef. J. Sci. Food Agr. 5, 70.

Newbold, R. P., and R. K. Scopes. 1971b. Post-mortem glycolysis in ox skeletal muscle: effect of adding nicotinamide-adenine dinucleotide to diluted mince preparations. J. Food Sci. 36, 215.

Newbold, R. P., and R. K. Scopes. 1971a. Post-mortem glycolysis in ox skeletal muscle: effects of mincing and of dilution with or without addition of orthophosphate. J. Food Sci. 36, 209.