The Influence of pH on the Protein Net Charge in the Myofibrillar System

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Introduction

During the last years before my retirement (1985), our group in Kulmbach was working on post-mortem biochemistry of bovine and porcine muscle and on some phenomena related to the subcellular distribution of certain muscle enzymes. This presentation, however, refers to results, which we obtained more than 30 years ago when we studied the effect of different treatments on protein charges in myofibrils and tissue. These studies had not yet been completely published, neither in English nor in German, and the method used might be almost forgotten. Nevertheless, the consequences derived from this investigation seem to be interesting enough to be critically discussed and perhaps to be proven.

Water-Holding Capacity (WHC) of Meat and the Electrostatic Theory of Swelling

The following considerations concern only WHC phenomena which are mainly related to the myofibrillar system. Remarkable changes or differences in drip loss or cooking loss can occur which are primarily due to a shift of tissue water between intracellular and extracellular spaces (Hamm, 1985, 1986) or to shrinkage of connective tissue (Offer and Knight, 1988) but not to alterations in the myofibrillar system.

The water-holding capacity (WHC) is one of the characteristics of meat quality. It is well known that the power with which tissue water and added water are bound by the protein systems of muscle is of great practical importance for the quality of meat and meat products. Water is held in the muscle, not because it is enclosed in the cells; it is held by the internal structure of the cell (Hamm, 1960, 1985; Offer and Knight, 1988).

Since myofibrils occupy about 70% of the volume of the lean muscle (more than 80% of the fibers), most of the tissue water must be located in the myofibrils. Therefore changes in the WHC of meat will be, in great part, due to variations in the immobilization of bulk phase water in the interfilamental spaces and the filaments themselves. Offer and Knight (1988) emphasize the hypothesis that water retention following uptake by the meat is due to myofibrillar expansion. I believe that the water-imbibing power of myosin in the thick filaments plays also an important role in WHC of meat, particularly after comminution; it is well known that myosin, which makes up about half of the myofibrillar protein, has an enormous capacity for imbibing water (Hamm, 1985, 1986).

Although even today we do not yet know exactly which forces restrict the mobility of water in the tissue, we know much more about the factors that influence the immobilization of water as it was already shown by my work carried out in the 50's and 60's (Hamm, 1960, 1972). I developed an electrostatic theory of swelling of the myofibrillar system which was supposed to be more appropriate for an understanding of some WHC phenomena than Donnan osmotic pressure effects, although those effects cannot be excluded.

The immobilization of water in the tissue is apparently determined by the spatial molecular or filamental arrangement within the myofibrils, whereby myosin might be of predominant importance. If the attraction between adjacent molecules or filaments decreases as it is caused by increasing electrostatic repulsion between similarly charged protein groups or by weakening of hydrogen bonds or hydrophobic bonds, the protein network is enlarged, the swelling increases and more water can be immobilized within the larger meshes; that is, there is an increase in WHC (e.g. in terms of expressible water or cooking loss). Of course, swelling of myofibrils is restricted by the restraining forces exerted by transversal structural elements (Z- and M-lines, rigor-bridges between myosin and actin filaments, etc.). If, however, the attraction between adjacent molecules or filaments is increased (as happens if the electrostatic attraction between oppositely charged protein groups is increased or interlinking hydrogen bonds or other bonds are formed), less space is available for the retention of immobillized water in the tighter protein network. Thus, during tightening of the network, shrinkage occurs and a part of the originally immobilized water becomes free to move and flows out by low pressure.

Influence of pH on WHC and Swelling of Meat

A good example for the importance of protein-protein interaction for the WHC and swelling of meat according to the electrostatic theory of swelling is the effect of pH (Figure 1). A loosening of the microstructure and, consequently, an increase of immobilized water is caused by rising the protein net charge by the addition of acid or base (Figure 2), which results in an increase of interfilamental spacing (April et al., 1972; Matsuda and Podolsky, 1986). The pH at which the WHC (or swelling) is at a minimum - at about 5.0 - corresponds to the isoelectric point (IP) of myosin as well as of the whole protein system of the myofibril (Offer and Knight, 1988). At the IP, the net charge of a protein is at a minimum; at this pH, we should expect a maximum of intermolecular (or interfilamental) "salt bridges" between positively and negatively charged groups (Figure 2);

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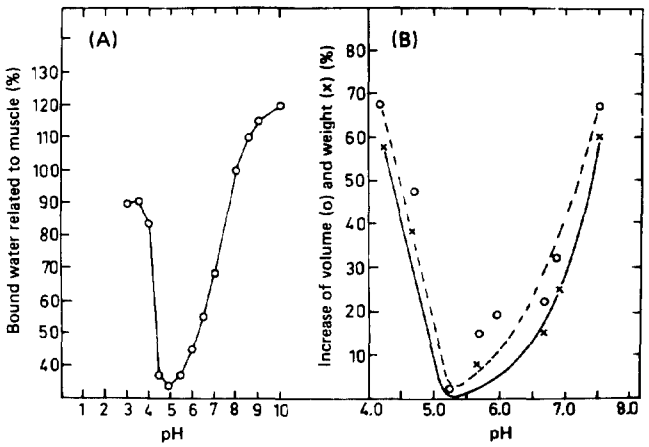
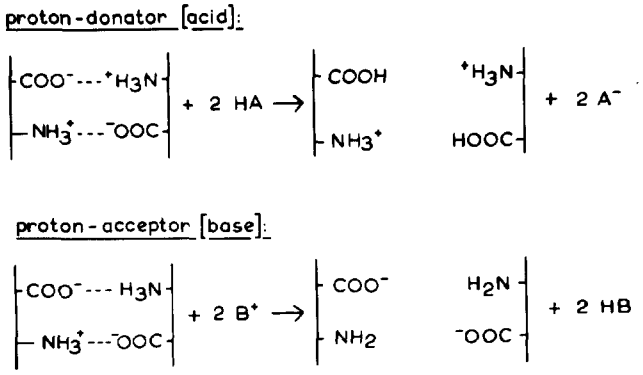
Figure 1



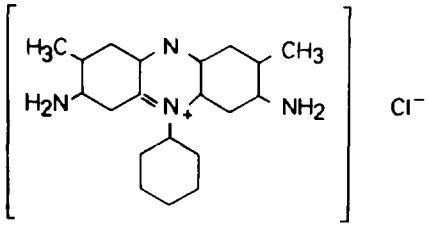
Effect of pH on water-holding capacity of muscle homogenates (filter-paper press method) (A) and on swelling of muscle cubes (3 mm) (B). Bovine *longissimus* muscle post-rigor (Hamm, 1986).

Figure 2

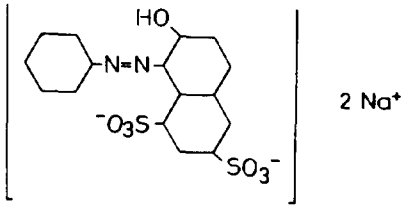


Influence of acid (HA) and base (B^+) on the interaction between protein charges. Left: isoelectric protein.

Figure 3



SAFRANINE



ORANGE G

Dyes used for the determination of charged protein groups.

this may explain the minimum of WHC and swelling at the IP (Figure 1).

The explanation of the WHC-hydration curve of muscle in terms of the electrostatic theory of swelling seems to be logical. But not everything which is logical is true. Therefore, we tried to prove the theory by studying the pH-dependence of charged protein groups in myofibrils. For this purpose, we used dye-binding methods which allow the measurement of basic and acidic groups more directly and in a better interpretable way than *the acid/base titration of the protein.*

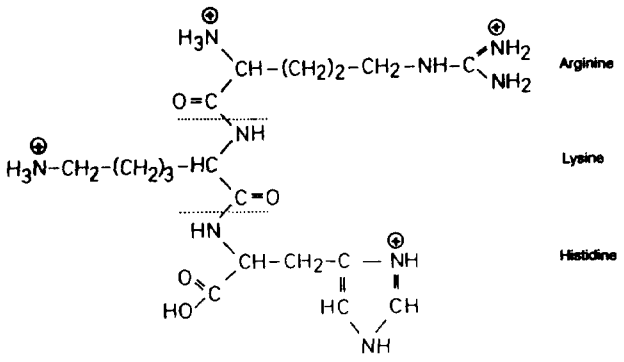
Total Amount of Acidic and Basic Groups in Myofibrils as Determined by Dye-Binding

Fraenkel-Conrat and Cooper (1944) developed a microanalytical method for the determination of acidic and basic groups in proteins by the formation of insoluble protein-dye complexes. The acid dye Orange G (Figure 3) binds stoichiometrically with basic groups in a buffer of pH 2.2. The basic dye Safranine (Figure 3) reacts with acidic groups at pH 11.5. As the authors showed, the number of protein groups binding these dyes corresponded well to the total number of basic (guanidyl, imidazol, amino) groups (Figure 4) and acidic (carboxyl, phenol, sulfhydryl) groups (Figure 5) of purified proteins.

Whereas Connel (1957) used the procedure of Fraenkel-Conrat and Cooper in experiments with fish muscle, I applied this method for the first time to mammalian muscle (Hamm and Deatherage, 1960) when I studied the effect of freezing, freeze-dehydration and heating on protein charges of beef in the laboratory of Dr. F. E. Deatherage at the Ohio State University in Columbus. The sample (25 mg) was shaken at +2°C for 18 hours with 2 ml buffer solution and 8 ml dye solution (0.1% Orange G and 0.2% Safranine respectively). After centrifugation, in the diluted supernatant the concentration of the non-bound dye was spectrophotometrically determined. Because of the insolubility of the sample under the conditions used, the procedure is comparable to an equilibrium dialysis.

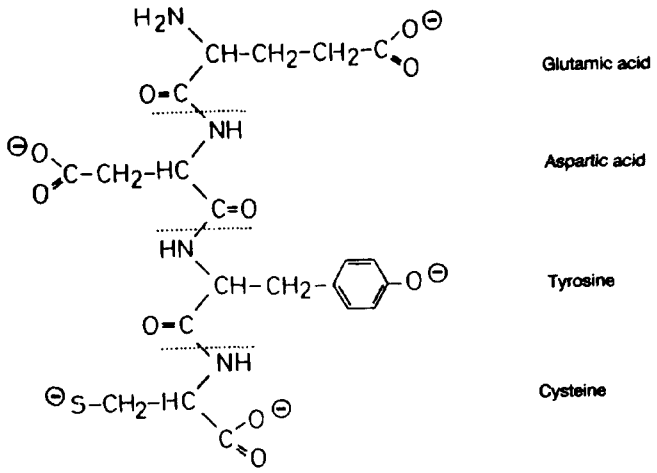
Later we studied the dye-binding of tissue, myofibrils and actomyosin more in detail (Hofmann, 1964). We found that the repeatibility of the method was very good, that the degree of the mechanical disintegration of the myofibrillar material has no detectable effect on dye-binding, that increase in the concentration of dye did not result in more bound dye, and that a reaction time of 12 hours for Safranine and of 4 hours for Orange G was sufficient to reach a maximum of dye bound by myofibrils. It could also be demonstrated that the uptake of both dyes follows the adsorption isotherme of Langmuir and is, therefore, a real adsorption and not simply a distribution of dye between protein and solution.

Figure 4



Basic groups in proteins, binding Orange G at pH 2.2.

Figure 5



Acidic groups in proteins, binding Safranine at pH 11.5.

Myofibrils (20 samples from different animals) contained in average 160 g equivalents acidic groups and 130 g equivalents basic groups per 10^5g protein. These values correspond roughly to figures calculated from the amino acid analysis of myosin and actomyosin (Kominz et al., 1954) or derived from titration curves with myofibrils (Bendall and Wismer-Pedersen, 1962; Connell and Howgate, 1964). Nevertheless, there was a remarkable variation from one sample to the other; this is not surprising because myofibrils represent a complicated protein complex and not a purified protein. The myofibrils contained significantly more acidic than basic groups. This is to be expected because the IP of most of the myofibrillar proteins is around 5 (see above).

It should be mentioned that several authors suggested using the binding of acidic dyes at low pH for a rapid determination of protein in meat and meat products. The dyes proposed are Orange G (Bunyan, 1959; Torten and Whitaker, 1964; Moss and Kielsmeier, 1967; Shiga et al., 1989), Acid Orange 12 (Ashworth, 1971; Statter, 1976; Heller and Sherbon, 1976; Seperich and Price, 1979), Amido Black 10B (Moss and Kielsmeier, 1967; Shiga et al., 1989), or Cochenille Red A (Meester and Krol, 1964; Moerman, 1966; Olsman, 1967).

In this context, it might be of interest that the affinity of muscle proteins for dye ions can be affected by contraction, heat denaturation (Kruchinina and Troshina, 1980; Troshina and Kruchinina, 1983) or by aging (Fujimaki and Arakawa, 1958; El-Badawi et al., 1971).

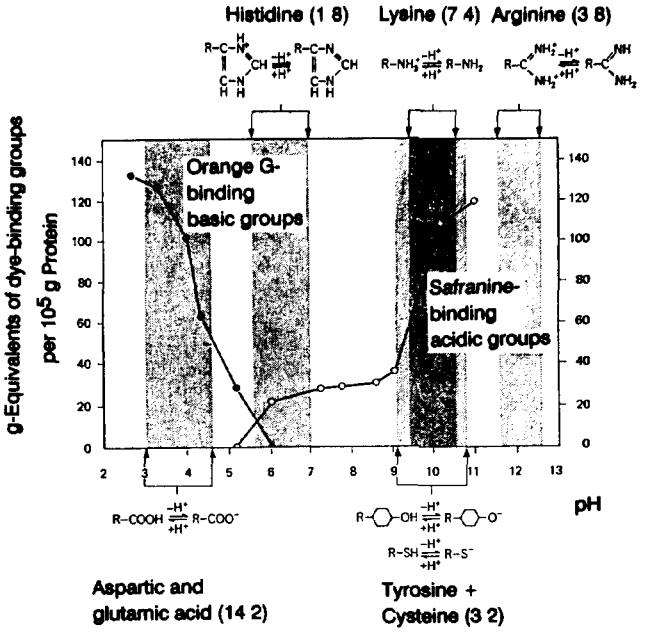
Effect of pH on Dye-Binding by Myofibrils

Of particular interest with regard to the electrostatic theory of swelling was the measurement of dye-binding by myofibrils at different pH values. Fraenkel-Conrat and Cooper (1944), who determined the dye-binding by proteins only at the extreme pH values of 2.2 and 11.5, mentioned in their paper: "Studies are in progress aiming at differential determination of the more strongly basic and acid groups by equilibration proteins with the dyes in buffers less acid or basic than 2.2 or 11.5. A method for determination of an approximate isoelectric point of insoluble proteins through measurements of dye bound at various pH levels will be described elsewhere." However, in the extensive research work of Fraenkel-Conrat, I could not find any publications of this type. Thus, I was probably the first who studied, 20 years later, the binding of Orange G and Safranine by a protein system at various pH levels.

The measurement of dye-binding by myofibrils (from bovine *longissimus* muscle) was carried out principally in the same manner as described with the exception that equilibration with the dye occured in buffers with different pH between 2.2 and 11.

Figure 6 shows the pH-dependence of dye-binding by myofibrils, including some information necessary for an interpretation of the curves (Hamm, 1972). The number of the Or-

Figure 6



Effect of pH on the number of dye-binding groups in myofibrils. The dissociation equilibria with the corresponding ranges of pK values are indicated for basic groups (top) and acidic groups (bottom). The shaded areas indicate the pH ranges in which the dissociation of protein groups is changing. In parentheses g-equivalents of amino acids per 10^5g myosin (Hamm, 1972).

ange G binding positively-charged protein groups decreases between pH 2.2 and 5.5 with rising pH, although the dissociation of these groups does not change in this range of pH because the pH values of the imidazolium group of histidine, of the -amino group of lysine and of the guanidinium group of arginine are lying above pH 5; all these groups remain dissociated between pH 2.2 and 5. At pH 6, the myofibrils do not bind Orange G at all. The strongest decrease in dye-binding takes place between pH 3 and 4.5, in which range the carboxylic groups of glutamic and aspartic acid are transformed from the non-dissociated into the dissociated state because the pK values of these groups fall in this range of pH. This means that with an increasing number of negatively charged groups, the binding of Orange G by the positively-charged groups is reduced.

The pH dependence of the Safranine binding follows a similar pattern. The dye-binding decreases by lowering the pH from 11 to 5. Only the negative tyrosyl and sulfhydryl groups are discharged (pK between 9 and 11), whereas the bulk of the negative charges remains dissociated because the pK values of the carboxylic groups of glutamic and aspartic acid are lying below pH 4.5.

Nevertheless, at pH 5 no Safranine is bound. The first strong decrease in Safranine binding coincides with the pH range in which the -amino groups of lysine are dissociating (pK between 10.5 and 9.5). In this pH range, also the negative charges of phenolic and sulfhydryl groups disappear, as already mentioned. Between pH 9 and 7, the change of dye-binding is only small; in this pH range, no alteration in the dissociation of protein groups is to be expected. However, between pH 7 and 5, a further fall of the number of Safranine binding groups can be observed which corresponds approximately to the increase of positive charges by dissociation of the imidazolium group of histidine (pK between pH 7 and 5.6). Thus the binding of Safranine by the negatively-charged groups in myofibrils decreases by lowering the pH from 11 to 5 essentially to the extent in which positive charges arise.

Although in the isoelectric range (around pH 5), a maximum of positively and negatively charged protein groups exists, a minimum of dye anions and cations is bound. At this pH, at which the number of positive and negative charges is equal, the interaction between oppositely charged groups is apparently so strong (formation of "salt bridges", see Figure 2) that they are not able to bind dye ions. Actually, the binding of dye ions depends on the protein net charge. At the IP, where the net charge is zero, no dye is bound. I think this is not self-evident. It would be conceivable that oppositely charged groups are located in the protein separated from each other in a way that they cannot interact. In this case, they should be able to bind oppositely charged dye ions also at the IP. This, however, is not the case.

These results support the electrostatic theory of swelling insofar as at the IP maximum interaction between oppositely charged protein groups exists and as this interaction decreases by lowering or rising the pH i.e. by increasing elimination of one type of charges (Figure 2).

The pH range between 5 and 6.5 is of particular importance with regard to quality of meat and meat products; pH changes in this interval affect strongly WHC and swelling of meat (Figure 1). From the results presented in Figure 6, it can be concluded that in this pH range the dissociation of the imidazolium groups of the histidine residue is decisive. Although myosin or actomyosin contain only 18 to 20 g equivalents histidine per 10^5 g protein, discharging of the imidazolium group by raising the pH apparently results in a remarkable increase in WHC and swelling of the myofibrillar system (compare Figure 1 with Figure 6!). In other words: The improvement of the binding by raising the pH seems primarily due to a release of protons from the histidine residue in the myofibrillar protein (Hamm, 1972). This hypothesis of the importance of histidine for meat quality still needs critical examination. It would be desirable if somebody could check this hypothesis by modern experimental methods.

Some Comments Concerning the pH-Dependence of Dye-Binding by Myofibrils

It should be mentioned that the dyes used are fully dissociated and do not change the color in the pH range studied (Seals and Rippie, 1956). Increasing dye concentration and prolonged reaction time did not increase the dye-binding in the isoelectric range of the myofibrillar protein. The type of the buffer used (acetate, glycin, phosphate, Michaelis) was of little influence on the results; an increase in ionic strength of the buffer from 0.1 to 0.4 had some effect on the dye-binding but did not result in a principal alteration of the results presented in Figure 6. It is not probable that, in the system investigated, a non-electrostatic interaction between dye and nonpolar groups of the proteins (Levin, 1969) plays a role because in this case some affinity of the dye to the protein also at the IP should be expected.

The question of how the spatial arrangement of the charged protein side chains, particularly in the rod of the LMM moiety of myosin, can allow such a strong interaction at the IP that no dye ions can be bound, is certainly of importance. But I cannot answer this question. Another question is whether a complete compensation of oppositely charged groups in the protein needs a spatial neighborhood or not. After the tremendous progress during the last years which brought about the knowledge of the complete three-dimensional structure of the subfragments of myosin (Rayment et al., 1993a), of the actin-myosin complex (Rayment et al., 1993b) and of actin (see Mannherz, 1992) and also of the charge distribution on the protein molecules as it is revealed by Fourier analysis, etc. (see e.g. Morris and Lu, 1987; Atkinson and Stewart, 1992), it seems to be conceivable that experts are able to answer these questions. But I doubt if such sophisticated investigations could allow better information on the actual protein net charge and the isoelectric range of the myofibril than was obtained by the simple dye-binding studies. I used the pH-dependence of dye-binding also as a tool for studying the effect of heating on the myofibrillar protein system (Hamm, 1966).

References

April, E.W.; Brandt, P.W.; Elliott, G.F. 1972. The myofilament lattice: Studies on isolated fibers. II. The effects of osmotic strength, ionic concentration, and pH upon the unit-cell volume. J. Cell. Biol. 53:53-65.

Ashworth, U.S. 1971. Proteins in meat and egg products determined by dye-binding. J. Food Sci. 36:509-510.

Atkinson, S.J.; Stewart, M. 1992. Molecular interactions essembly. Role of the 28 residue charge repeat in the myosin rod. J. Mol. Biol. 226:7-13.

Bendall, J.R.; Wismer-Pedersen, J. 1962. Some properties of the fibrillar proteins of normal and watery pork muscle. J. Food Sci. 27:144-159.

Bunyan, J. 1959. Orange-G binding as a measure of protein content. J. Sci. Food Agric. 10:425-430.

Connell, J.J. 1957. Some aspects of the texture of dehydrated fish. J. Sci. Food Agric. 8:526-537.

Connell, J.J.; Howgate, P.F. 1964. The hydrogen ion titration curves of native, heat-coagulated and frozen-stored myofibrils of cod and beef. J. Food Sci. 29:717-722.

El-Badawi, A.A.; Anglemeir, A.F.; Cain, R.F. 1971. Hydrative changes in two muscles during post-mortem aging. Alex. J. Agr. Res. 19:89-95.

Fraenkel-Conrat, H.; Cooper, M. 1944. The use of dyes for the determination of acid and basic groups in proteins. J. Biol. Chem. 154:239-246.

Fujimaki, M.; Arakawa, N. 1958. Chemical studies on the autolysis of meats. Part IX. On combination of muscular proteins with anionic and cationic dyes during aging of meats. J. Agr. Chem. Soc. Japan 32:858-862.

Hamm, R. 1960. Biochemistry of meat hydration. Advanc. Food Research 10:355-463.

Hamm, R. 1966. Heating of muscle systems. In "The Physiology and Biochemistry of Muscle as a Food" (Briskey, E.J.; Cassens, R.G.; Trautmann, eds.). Wisconsin Press. Madison, Milwaukee, London, pp. 363-385.

Hamm, R. 1972. "Kolloidchemie des Fleisches". 274 p. Paul Parey. Berlin, Hamburg.

Hamm, R. 1985. The effect of water on the quality of meat and meat products: Problems and research needs. In "Properties of Water in Foods" (Simatos, D; Multon I.L., eds.). NATO ASI Series E: Appl. Sci. No. 90. Martinus Nijhoff Publ. Dordrecht, Niederl. pp. 591-602.

Hamm, R. 1986. Functional properties of the myofibrillar system and their measurements. In "Muscle as Food" (Bechtel, P., ed.). Academic Press, Inc. pp. 135-199.

Hamm, R.; Deatherage, F.E. 1960. Changes in hydration, solubility and charges of muscle proteins during heating of meat. Food Research 25:587-610.

Heller, St.N.; Sherbon, J.W. 1976. Interlaboratory study of Acid Orange 12 dye binding for measuring protein in meat. J. Assoc. Off. Anal. Chem. 59:62-66.

Hofmann, K. 1964. Untersuchungen des Einflusses der thermischen Behandlung von Fleisch auf die funktionellen Gruppen der strukturellen Muskelproteine. Dissertation Universität Gießen (Germany), 136 p.

Kominz, D.R.; Hough, A.; Symonds, P.; Laki, K. 1954. Amino acid composition of actin, myosin, tropomyosin and the meromyosins. Arch. Biochem. Biophys. 50:148-159.

Kruchinina, N.G.; Troshina, V.P. 1980. Changes of sulfophtalein dye sorption by actomyosin filaments during contraction and heating. Tsitologiya 22:722-726.

Levin, J.V. 1969. Conformational changes of proteins studied by their interaction with organic dyes. Sb. Rab. Inst. Tsitol. Akad. Nauk. SSSR 1969 (No. 13):41.

Mannherz, H.-G. 1992. Die Molekülstruktur des Actins: Implikationen für Motilität und Kontraktilität. Nachr. Chem. Techn. Lab. 40:9-15.

Matsuda, R.; Podolsky, R.J. 1986. Ordering of the myofibrillar lattice in muscle fibers. J. Mol. Biol. 189:361-365.

Meester, J.; Krol, B. 1964. Schnellmethoden zur Bestimmung des Wasser-, Fett- und Eiweißgehaltes in Fleisch und Fleischwaren. III. Bestimmung des Eiweißgehaltes. Fleischwirtschaft 44:446-450.

Moerman, P.C. 1966. Schnell-Methoden zur Bestimmung des Wasser- Fett- und Eiweißgehaltes in Fleisch und Fleischwaren. Fleischwirtschaft 46:1130-1137.

Morris, E.P.; Lu, R.Ch. 1987. The distribution of the charged residues in myosin hinge region and its relationship to the distribution of charged residues in the rest of myosin rod. J. Muscle Res. Cell Motil. 8:297-302.

Moss, V.G.; Kielsmeier, E.W. 1967. Rapid determination of protein in meat by dye-binding. Food Technol. 21 (No. 3A):33A-36A.

Offer, G.; Knight, P. 1988. The structural basis of water-holding in meat. Part 1: General principles and water uptake in meat processing. In "Developments in Meat Science", Vol. 4 (R. Lawrie, ed.). Elsevier Applied Science. London, New York, pp. 64-190.

Olsman, W. 1967. Schnell-Methoden zur Bestimmung yon Wasser-, Fett- und Eiweißgehalt in Fleisch und Fleischwaren. V. Eine Modifikation der schnellen Eiweißbestimmung nach der Farbstoffbindungsmethode bei Fleisch und Fleischwaren. Fleischwirtschaft 47:137-141.

Rayment, I.; Rypniewski, W.R.; Schmidt-Bäse, K.; Smith, R.; Tomchick, D.R.; Benning, M.M.; Winkelmann, D.A.; Wesenberg, G.; Holden, H.M. 1993a. Three-dimensional structure of myosin subfragment 1: A molecular motor. Science 261:50-58.

Rayment, I.; Holden, H.M.; Whittaker, M.; Yohn, Ch.B.; Lorenz, M.; Holmes, K.C.; Milligan, R.A. 1993b. Structure of the actin-myosin complex and its implication for muscle contraction. Science 61:38-65.

Seals, R.; Rippie, E. 1966. Effect of pH on protein binding of Orange G and Amido Black dyes. J. Dairy Sci. 49:617-620.

Seperich, G.J.; Price, J.F. 1979. Dye binding procedure for the estimation of protein content of meat components and sausage emulsions. J. Food Sci. 44:643-645.

Shiga, K.; Kami, R.; Tamata, R. 1989. Dye-binding method for estimation of protein in meat. Rakuno Kagaku, Shokuhin no Kenkyu 38:A175-A178.

Statter, S. 1976. Protein analysis. US Patent 3936271.

Torten, J.; Whitaker, J.R. 1964. Evaluation of the biuret and dye-binding methods for protein determination in meats. J. Food Sci. 29:168-174.

Troshina, V.P.; Kruchinina, N.G. 1983. Changes in the parameters of equilibrium sorption of acidic dyes by contractile proteins during their functional activity and heat denaturation. Tsitologiya 25:1393-1397.