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Some aspects of protein changes in frozen foods

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With 4 figures

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Introduction

For some years the discussion has been going on in Germany about the alterations in proteins of foods which are caused by freezing and frozen storage. Before a well-founded judgement can be made on several categories of quality changes of such frozen products, particularly from the standpoint of nutrition physiology, we should know about what actually happens with the proteins. In this paper therefore the attempt shall be made to gain, from results hitherto obtained by many researchers, some insight into the causes underlying these protein changes, and into the nature of these phenomena, generally referred to as denaturation caused by freezing.

Definition and measurement of protein changes

The present conception of protein denaturation is substantially based on the definitions by *Neurath* and *Kauzmann* (1). According to a recent and more comprehensive definition, denaturation is any process that alters or destroys secondary or tertiary structures in a biopolymer without breaking covalent linkages to carbon atoms (23). Intermolecular aggregations are not necessarily involved; they may, however, occur at the same time or subsequently.

Freezing is one of the many influences under which proteins may be changed. Several methods are available to measure the alterations that proteins undergo in frozen foods; they all comprise the following criteria:

1. Loss of specific biological activities; e.g. changes of enzyme activity and loss of contractibility of muscle fibres in several kinds of meat.
2. Alterations of physical-chemical properties such as changes in viscosity, texture, ultrastructure, waterbinding capacity, solubility, isoelectric point, absorption in the UV range, optical rotation dispersion, sedimentation coefficient in the ultracentrifuge, and electrophoretic properties.

Apart from the fact that the physical, chemical and sensorial data, scatter over wide ranges due to inevitable biological variations, it is generally impossible to decide by the results of individual determinations whether the measured changes are indeed intramolecular in the sense of true denaturation, or whether they are merely caused by aggregation.

Consequences of freezing of water

The disappearance of liquid water was found to be one of the main reasons for the alteration of proteins during freezing. Particularly the breakdown or change of the structure of the so-called "ordered water" in the vicinity of hydrophobic groups of protein molecules is supposed to lead to a change of the native protein structure (2, 3, 4). Such conformational changes may be supported by the concomitant influence of various factors such as electrolyte concentrations, semipolaric compounds like phenylalanine and phenylpyruvate (5), and pH changes (6). In extreme cases a denaturated protein may exist as a random coil with all of its functional groups exposed. In this situation it is very reactive and can take part in the formation of new intra- and intermolecular crosslinks. Certain SH-groups seem to be of special importance for the intramolecular changes but also for intermolecular aggregations. According to *Connell*, aggregations of this kind can occur without evidence of structural changes (48). The formation of intramolecular SS-bonds by freezing for steric reasons seems to be possible only when the molecules have reached a certain size (7).

Small protein molecules generally found among the albumins are supposed to also have a small number of hydrophobic groups. Hence it is understandable that many of the proteins resistant to freezing belong to the albumins (7). Many globulins, on the other hand, are highly susceptible to freezing or at least to aggregation, as is the case with myosin in solution (8, 9, 10).

Numerous studies on isolated and purified proteins in solution, e.g. myosin (8, 9, 10, 11) and actomyosin (12), show that dissolved proteins are more apt to alterations by freezing than in situ, where apparently other compounds present in the tissue have a protecting effect.

Changes of enzyme activity

Among the proteins, considerable interest was devoted to enzymes in frozen foods. Whereas some enzymes retain their full activity, some show a decrease and others a striking increase (13).

As an example for such an "activating effect" of freezing the enzymatic breakdown of the acid-labile energy-rich phosphate (mainly ATP) in thin muscle strips of carp,, withdrawn immediately after death shall be presented in dependence upon temperature in the range from + 20 °C to – 25 °C. The phosphates were split more quickly at – 2 °C than in unfrozen muscle at + 10 °C (Fig. 1). It is assumed that such increases of activity as found for other systems, too (14, 15, 16), are generally due to removal of the natural barrier between enzyme, substrate or activator. This means that the separating membranes are affected by freezing. In the molecular domain these changes take place mainly in the lipoproteins of the membraneous system.

The difficulties arising in the freezing of fresh milk and eggs are also attributable mainly to the lability of lipoproteins. In fish meat and meat from warmblooded animals the membraneous systems are changed by

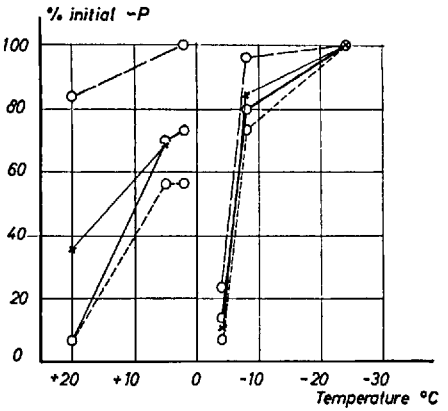

Fig. 1. Contents in acid-labile energy-rich phosphate (~P) of two carps (× and O) at various times post mortem (— — — 18 hours, ———— 24 hours, and - - - - - - 48 hours) in dependence on temperature.

freezing; their damage is of less *immediate* importance, however, for the sensorial quality of the frozen meat.

The so-called "denaturation" of fish muscle proteins

General viewpoints:

A great many scientific studies on protein changes of frozen foods were concerned with fish. The mere adequate freezing of fish muscle seems to be of negligible importance for the solubility (20) and water sorption ability (21) of fish muscle proteins. When the limits to storage times

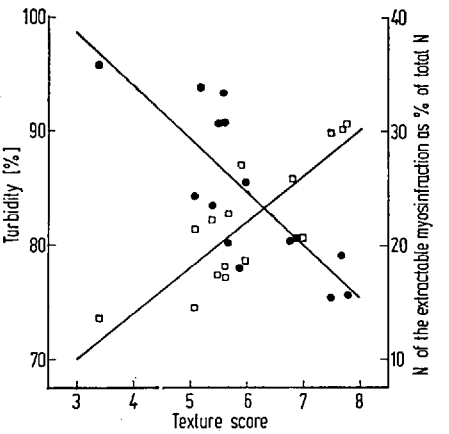

Fig. 2. Relation between consistency and extractability of the myosin fraction □, and between consistency and contractibility of the muscle fibres • obtained from slices of coalfish (Gadus virens L.) stored for different times at – 8 °C, – 18 °C and – 30 °C.

determined in time-temperature-tolerance studies have been exceeded, very undesirable textural changes appear in many fish species which may lead to complete toughening. At least in the temperature range above – 29 °C a fairly reliable correlation between protein extractability and toughness of the meat seems to exist (17, 18, 19) (Fig. 2).

During frozen storage the alterations of proteins generally increase with higher temperatures and extended storage times. For many proteins the temperature range between – 2 °C and – 10 °C is particularly critical. Several maximum reaction rates for the formation of undesirable compounds related to protein changes, like formaldehyde (Alaska pollack around – 10 °) fall in this range (22).

Research over the past thirty years has been able to show that the decrease of protein extractability is due to alterations of the myofibrillar proteins. Until now the role of the individual proteins has not been completely elucidated. Cross-links between elements of the sarcoplasmic reticulum and fibrillar proteins may also occur. It is generally held that the sarcoplasmic proteins do not undergo significant alterations which might be responsible for textural changes.

It is obvious that in fish meat, like in other tissues, the freezing of water and the correlated concentration of polar and semi-polar compounds may change the conformation in certain areas of fibrillar protein molecules, whereby particular aggregation reactions may be initiated (23). This alone does not explain yet the different rates of protein changes in frozen fish of related fish species with similar gross chemical composition (24).

Free fatty acids:

Dyer and his groups concluded from their first studies on frozen fish that fish species with lower fat contents had a higher reaction rate of protein changes than species with higher fat content (25). Further experiments gave rise to the assumption that free fatty acids support protein denaturation, whereas non-hydrolyzed fats or lipoprotein complexes have a stabilizing effect on proteins (26).

Olley et al. (27, 28) found that the main source of free fatty acids in many fish species were phospholipids which, so the authors assumed, particularly originated from the membraneous system of the muscle fibre. The results of this group and of other researchers (18, 29, 30, 31, 32, 33) suggest that mainly very specific free fatty acids and above all the oxidation products of unsaturated fatty acids react with the fibrillar muscle proteins rather than the total of all free fatty acids.

Recently *Sikorski* et al. (24) emphasized that free fatty acids may bind polypeptide side chains under formation of intermolecular hydrophobic-hydrophilic or hydrophobic-ionic linkages. This process is favoured by appropriate concentrations of inorganic ions. That hydrophobic adherences are mainly involved in the aggregation of flish proteins during frozen storage follows from the observation that solubility in sodium dodecyl sulphate (SDS) not breaking covalent cross-links decreases only slightly with the time of storage (34).

Oxidation products of lipids:

The products of lipid oxidation have been well known to produce aggregation of proteins, hence causing a decrease of solubility. According to recent ultrastructural and chemical studies, linoleic acid hydroperoxides were about 10 times more effective in decreasing the protein solubility in KCl solutions of incubated cod myofibrils than linoleic acid (33). This explains why fibrillar proteins in the surface layers of frozen blocks of cod fillets deteriorated much more rapidly during frozen storage of four years than those in deeper layers. This became manifest not only in the loss of extractability, but also in the decrease of contractibility of the muscle fibres (55).

Formaldehyde:

Among the substances contributing to protein changes of fish meat during frozen storage, certain aldehydes like malonaldehyde, which are formed during the autoxidation of fats, are of importance (35, 36). For some years formaldehyde originating, besides dimethylamine, from the enzymatic breakdown of trimethylamine oxide in certain fish species, has been the subject of intensive studies (54). *Castell* et al. found that the enzyme capable of formaldehyde formation and largely concentrated in the red lateral body muscle and blood (56) was highly active in gadoid species, but not in non-gadoid ones. In such species like halibut, wolffish and ocean perch the solubility changes of the proteins during frozen storage were considerably slower, and the values of the extractable proteins became not as low as in the gadoid fish (37).

Model experiments showed that among the proteins of cod muscle homogenates heavy meromyosin and tropomyosin were completely inextractable in buffered 5% NaCl solution after reaction with formaldehyde of 0.04% concentration. Actin, on the other hand, was least reactive (38). It is not surprising that formaldehyde concentrations of this order of magnitude lead to a time-dependent inhibition of the mainly

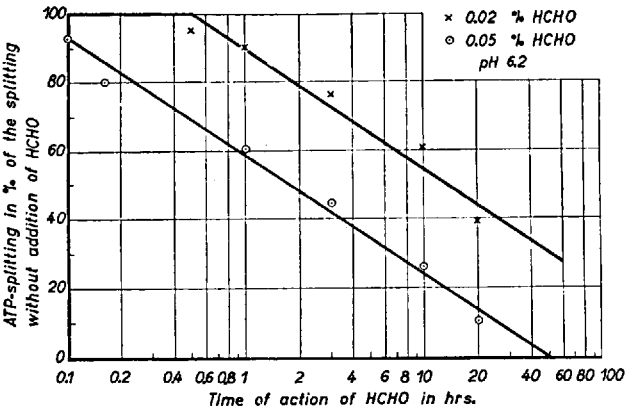

Fig. 3. ATP-splitting in ground muscle tissue of carp in dependence on reaction time with formaldehyde.

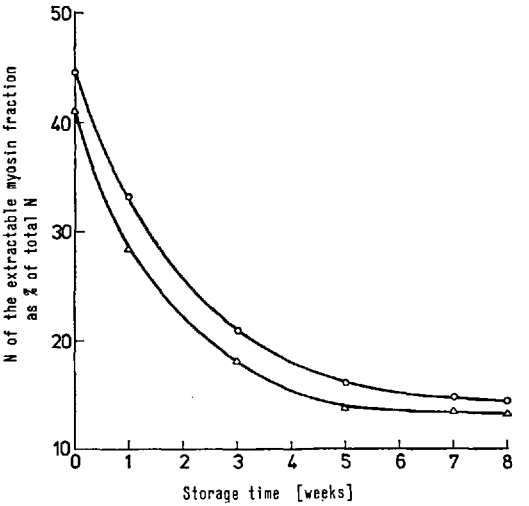

Fig. 4. Changes in the extractable myosin fraction in comparable parts of 5 cods (Gadus morrhua L.) stored in evacuated bags at – 8 °C; △ minced slices; O intact slices.

myosin-bound ATPase system as observed in own experiments with ground muscle tissue of carp (Fig. 3) (39). During frozen storage of comminuted fish meat formaldehyde and DMA are produced faster than in intact muscle tissue, leading to a higher rate of protein changes, as observed by several authors (40, 41, 42). The higher losses in extractability of fibrillar muscle proteins in minced fillets of cod than in corresponding intact fillets during frozen storage at – 8 °C may serve as an example (Fig. 4). In coalfish the differences in solubility are even more pronounced; the same applies to the contractability of muscle fibres (43). Recently, the causes of these findings and their own convincing results were reason enough for Canadian scientists to advise the fishing industry not to mix minced fish meat of the Gadidae family with that of other scarcely formaldehyde producing fish species like flatfish (56).

This conception of formaldehyde playing an essential role in protein alterations of certain fish species during frozen storage was doubted by *Connell* (34). His argumentation is based on the assumption that the polymerizing activity of formaldehyde leading to toughness is mainly caused by the formation of covalent methylene cross-links between protein molecules. In this case the formed aggregates should be insoluble in hydrogen-bond breaking solvents like sodium dodecyl sulphate. The author found, however, that myofibrillar proteins of cod stored for 29 weeks at – 14 °C, in contrast to fresh cod treated with formaldehyde, can be dissolved in 1 % SDS. *Connell* therefore regards the role of formaldehyde as a methylene cross-linking agent acting during frozen storage of Gadus species as questionable. He admits, however, that these experiments do not exclude the possibility that there may still be other ways of

irreversible reactions between formaldehyde and proteins during frozen storage, not necessarily producing covalent cross-links.

The changes leading to inextractability of fibrillar proteins of fish obviously are not initiated by only one of the factors mentioned. The effects of water freezing may support the reactions caused by certain free fatty acids and their oxidation products. In species of the family Gadidae the formation of formaldehyde may be the predominant factor for protein deterioration.

The molecular range:

There seems to be general agreement in the pertinent literature that the insolubilization of fibrillar proteins correlated with the toughening of the muscle results from aggregation reaction. *Connell* (44) and *Anderson* and *Ravesi* (45, 46) believe that most of the cross-links formed between the proteins during frozen storage result from formation of intermolecular hydrogen or hydrophobic bonds. *Sikorski, Olley* and *Kostuch* are of the opinion that lipid autoxidation products and formaldehyde lead at least to a limited number of covalent cross-links, although no direct experimental evidence has been furnished (24).

Connell found no significant changes for the highly reactive or total SH groups in frozen fish or frozen protein solutions (8), and no reduction of the number of acidic and basic groups in myofibrils of cod stored at – 14 °C (46). He therefore concluded that disulfides do not contribute to the aggregation of myosin (44), and that, in fact, the bulk of the protein in cod – until it is extremely tough – is not covalently cross-linked to a large extent (47, 48).

Buttkus, however, proposed a mechanism for the aggregation of myosin which involves disulfide-sulfhydryl exchange reactions between activated myosin molecules without a net change of existing SH groups (10). Neither can it be completely excluded that only a few scarcely detectable cross-links of this kind contribute considerably to toughening.

There has apparently been no answer to the question yet whether the single molecules of the fibrillar proteins which aggregate during frozen storage are still native or denaturated. *Connell* showed that, when the actomyosin fraction in the cod muscle became completely insoluble at a storage temperature of – 14 °C, there was only a very small change in the properties of the actin (49). Genuine myosin capable of combining with actin to an ATP-sensitive complex was extractable from cod muscle stored at – 14 °C for up to 52 weeks (50). Non-extractability hence cannot be regarded as a reliable indicator for denaturation in the classic sense of the aggregated fibrillar proteins of frozen fish muscle. All possibilities conceivable seem to exist: the molecules, or part of them, may still be native or be more or less changed in their conformation before or during aggregation.

Conclusive remarks

In other meats, e.g. poultry (51, 52) and probably in many other foods similar alterations of proteins during freezing and frozen storage may occur, frequently without aggregation. The single molecules may retain

their native state or undergo conformational changes to different extent. This is in agreement with the well-founded view of *Lewin* on protein denaturation in general (23). The large variety of possible alterations, so I believe, does not allow a general conclusion regarding the properties of the proteins concerned of frozen foods from the viewpoint of nutrition physiology, unless substantial evidence is furnished by experiments, for instance, by feeding tests in human volunteers. *Colvin* has already underlined that the different treatments of proteins leading to "denaturation" do not produce identical changes of the molecules and hence no identical alterations of their properties (53).

Summary

In the past decades many results on changes of food proteins caused by freezing and their relation to quality changes were found. Recently especially the nutrition physiological viewpoint of these alterations has aroused considerable interest. In the present paper the attempt is made to outline some aspects of these changes which are usually referred to as "denaturation" caused by freezing. After a description of the phenomena observed and their measurement, a survey of the main causes presumed for the "denaturation" of fish muscle proteins by freezing is given. With special consideration of the molecular range it can be said that generally aggregation phenomena prevail and that the participating molecules, or part of them, may still be native or more or less changed in their conformation before or during aggregation. Definite judgement of the nutritional properties of the altered proteins in frozen foods is possible only when convincing results of feeding experiments will be available.

Zusammenfassung

In den vergangenen Jahrzehnten wurden viele Ergebnisse über Veränderungen von Proteinen in Lebensmitteln durch Gefrieren und ihre Beziehungen zu Qualitätsveränderungen erhalten. Neuerdings hat speziell der ernährungsphysiologische Gesichtspunkt dieser Veränderungen besonderes Interesse gefunden. Im vorliegenden Bericht wird der Versuch gemacht, einige Aspekte dieser Veränderungen zu umreißen, die gewöhnlich als „Gefrierdenaturierung" bezeichnet werden.

Nach einer Beschreibung der beobachteten Kriterien und ihrer Messung wird auf die Ursachen eingegangen, die vermutlich für die „Denaturierung" der Fischmuskelproteine durch Gefrieren verantwortlich sind.

Unter besonderer Berücksichtigung des molekularen Bereichs kann gesagt werden, daß im allgemeinen Aggregationsphänomene vorherrschen und daß die beteiligten Moleküle oder ein Teil von ihnen noch nativ sein können oder vor oder während der Aggregation in ihrer Konformation mehr oder weniger verändert sind. Eine verläßliche Beurteilung der ernährungsphysiologischen Eigenschaften der Proteine in gefrorenen Lebensmitteln wird erst möglich sein, wenn überzeugende Ergebnisse von Fütterungsversuchen vorliegen.

References

1. *Kauzmann, W.*, J. Cell. Comp. Physiol. **47**, Suppl. 1, 113 (1956). – 2. *Baust, J. G.*, Cryobiology **10**, 197 (1973). – 3. *Mazur, P.*, Science N.Y. **168**, 939 (1970). – 4. *Meryman, H. T.*, Cryobiology **8**, 489, (1971). – 5. *Heber, U., L. Tyankova, K. A. Santarius*, Biochim. biophys. Acta **291**, 23 (1973). – 6. *Van den Berg, L.*, Cryobiology **3**, 236 (1966). – 7. *Levitt, J.*, Interactions. Cryobiology **3**, 243(1966). – 8. *Connell, J. J.*, Biochem. J. **75**, 530 (1960). – 9. *Connell, J. J.*, Nature **183**, 664 (1959). – 10. *Buttkus,*

H., J. Fd. Sci. **35**, 558 (1970). – 11. *Hanafusa, N.*, Contrib. Inst. Low Temp. Sci. J. Ser. B 1–20 (1972). – 12. *Matsumoto, J. J., S. Noguchi*, Proc. XIII Intern. Congr. Refrig. **3**, 237 (1973). – 13. *Partmann, W.*, The effects of freezing and thawing on food quality. In: Water relations of foods. Edit. *R. B. Duckworth*, p. 505–537 (London 1975). – 14. *Hamm, R., L. Körmendy*, Fd. Sci. **34**, 452 (1969). – 15. *Hamm, R., A. El-Badawi*, Z. Lebensm.-Unters. u. Forschg. **150**, 12 (1972). – 16. *Gould, E.*, J. Assoc. Offic. Analyt. Chem. **56**, 541 (1973). – 17. *Dyer, W. J.*, Fd. Res. **16**, 522 (1951). – 18. *Connell, J. J.*, Fish muscle proteins and some effects on them of processing. In: Proteins and their reactions. Edit. *H. W. Schultz*, and *A. F. Anglemier*, p. 255–293, AVI Westport-Conn. (1964). – 19. *Partmann, W.*, Quality changes of coalfish (Gadus Virens, L.) during frozen storage. Proc. XIVth Intern. Congr. Refrig. in press. – 20. *Love, R. M., J. I. M. Ironside*, J. Sci. Fd. Agric. **9**, 604 (1958). – 21. *Mao, W. W., C. Sterling*, J. Texture Stud. **1**, 338 (1970). – 22. *Tokunaga, T.*, Bull. Jap. Soc. Sci. Fish. **40**, 167 (1940). – 23. *Lewin, S.*, Displacement of water and its control of biochemical reactions, 367 S. (London and New York 1974). – 24. *Sikorski, S., J. Olley, S. Kostuch*, Critical Rev. Fd. Sci. and Nutrition **7**, 97 (1976). – 25. *Dyer, W. J., M. L. Morton*, J. Fish. Res. Bd. Can. **13**, 129 (1956). – 26. *Dyer, W. J., J. R. Dingle*, Fish proteins with special reference to Freezing. In: Fish as food. Edit. *G. Borgström*. Vol. **1** p. 275 (1961). – 27. *Olley, J., J. A. Lovern*, J. Sci. Fd. Agric. **11**, 644 (1960). – 28. *Olley, J., R. Pirie, H. Watson*, J. Sci. Fd. Agric. **13**, 501 (1962). – 29. *King, F. J., M. L. Anderson, M. A. Steinberg*, J. Fd. Sci. **27**, 363 (1962). – 30. *Olley, J., W. R. H Duncan*, J. Sci. Fd. Agric. **16**, 99 (1965). – 31. *Buttkus, H.*, J. Fd. Sci. **32**, 432 (1967). – 32. *Childs, E. A.*, J. Fish. Res. Bd. Can. **31**, 111 (1974). – 33. *Jarenbäck, L., A. Liljemark*, J. Fd. Technol. **10**, 437 (1975). – 34. *Connell, J. J.*, J. Sci. Fd. Agric. **26**, 1925 (1975). – 35. *Kuusi, T., O. E. Nikkilä, K. Savolainen*, Z. Lebensm. Unters.-Forschg. **159**, 285 (1975). – 36. *Babbitt, J. K., D. K. Law, D. L. Crawford*, J. Fd. Sci. **41**, 35 (1976). – 37. *Castell, C. H., B. Smith, W. J. Dyer*, J. Fish. Res. Bd. Can. **30**, 1205–1213 (1973). – 38. *Childs, E. A.*, J. Fd. Sci **38**, 1009 (1973). – 39. *Partmann, W.*, Fd. Research **22**, 51 (1957). – 40. *Babbitt, J. K., D. L. Crawford, D. K. Law*, J. Agric. Fd. Chem. **20**, 1052 (1972). – 41. *Sørenson, T.*, Separated cod mince. Deterioration of functional properties during frozen storage. Orsberetning 1975 Fiskeriministeriets Forsøgslaboratorium Lyngby, Denmark, p. 28–38. – 42. *Hiltz, D. F., B. Smith Lall, D. W. Lemon, W. J. Dyer*, J. Fish. Res. Bd. Can. **33**, 2560 (1976). – 43. *Partmann, W.*, Lebensm.Wiss. u. -Technol. **7**, 186 (1974). – 44. *Connell, J. J.*, J. Sci. Fd. Agric. **16**, 769 (1965). – 45. *Anderson, M. L., E. M. Ravesi*, J. Fd. Sci. **35**, 139 (1970). – 46. *Anderson, M. L., E. M. Ravesi*, J. Fd. Sci. **35**, 551 (1970). – 47. *Connell, J. J., P. F. Howgate*, J. Fd. Sci. **29**, 717 (1964). – 48. *Connell, J. J.*, The effect of freezing and frozen storage on the proteins of fish muscle. In: Low temperature biology of foodstuffs. Edit. *J. Hawthorn*, and *E. J. Rolfe*, p. 333 (Oxford 1968). – 49. *Connell, J. J.*, J. Sci. Fd. Agric. **11**, 515 (1960). – 50. *Connell, J. J.*, J. Sc. Fd. Agric. **13**, 607 (1962). – 51. *Kahn, A. W., L. van den Berg, C. P. Lentz*, J. Fd. Sci **28**, 425 (1963). – 52. *Kahn, A. W.*, Cryobiology **3**, 224 (1966). – 53. *Colvin, J. R.*, Denaturation: A requiem. In: Symposium on foods: Proteins and their reactions. Edit. *H. W. Schultz* and *A. F. Anglemier*, p. 69–83, AVI Westport. Conn. (1964). – 54. *Amano, K., K. Yamada*, The biological formation of formaldehyde in cod flesh. In: The technology of fish utilization. Edit. *R. Kreuzer*, p. 73–78 (London 1965). – 55. *Partmann, W., J. Gutschmidt*, Kältetechnik **24**, 327, 328, 331, 332, 343, 344, (1972). – 56. *Dingle, J. R., B. Lall, R. A. Keith*, Environment Canada, Fish and Marine Service, Halifax Laboratory New circular No 59 (1976).

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