*Food Chemistry* **34** (1989) 1–14

# The Effect of Heat-treatment and Frozen Storage on Histological and Cytological Changes in Smooth Muscles of Pig Stomachs*

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(Received 8 August 1988; revised version received and accepted 14 November 1988)

*ABSTRACT*

*Studies were carried out on stomach pylorus muscle layers: (a) fresh-chilled (control), (b) frozen-stored at −18°C for 6 months and (c) fresh-chilled, cooked at 90°C and sterilized at 121°C. The muscle and connective tissues were analysed using light microscopy as well as scanning and transmission electron microscopy. Frozen storage did not cause any significant changes in the tissue structure of the examined material. After heat-treatment, the muscles contained condensed and amorphous collagen, apart from native collagen. Ultrastructural changes in myocytes due to heat-processing were slight. Sterilization resulted in more significant structural changes.*

## INTRODUCTION

Rational utilization of less valuable meat raw material, constituting additional sources of cheap protein in the diet, has been a major problem investigated by many authors (Tyszkiewicz & Danielewicz, 1975; Young & Lawrie, 1975; Tederko, 1979; Lawrie, 1981; Kosiba, 1983).

A wide variety of assortments of less valuable muscle tissues of slaughter animals and versatile utilization of offals in processing are rather the effects of practical experience then anatomohistological, physico-chemical or technological studies (Pezacki, 1984). Although, pig stomach meat tissue is a

* The work included in CPBP 0509 programme.



*Food Chemistry* 0308-8146/89/$03·50 

quite common component of several, mainly comminuted, processed meat products, its ultrastructure has not been satisfactorily evaluated and therefore further detailed studies are required.

Modern processing technologies need more detailed data of these unconventional raw materials.

Rapid progress in the studies of structure of smooth muscles in the last decade has contributed greatly to the knowledge of this tissue. Usually, new experimental data are restricted to only a few model tissues, e.g. smooth muscles of intestines, deferent ducts and uterus. Very few reports have been published on chicken gizzard (Gabella, 1981).

Similarly, structural evaluation of cooked smooth muscles is rather obscure (Otwell & Hamann, 1979; Chen & Stinson, 1983).

Recent incidental publications contain some interesting data on the proteins present in smooth muscles (Kamm & Stull, 1986; Small *et al.*, 1986). Polymorphic properties of the skeletal muscle protein, collagen, have been well established (Aberle & Mills, 1983; Bailey, 1984; Light *et al.*, 1984; Burson & Hunt, 1986). The extent of rupture of collagenous fibrous structures upon heat-treatment is attributed to the quality of this protein, cooking temperature and time (Jones *et al.*, 1977; Aberle & Mills, 1983) as well as to the thickness of collagen fibres (Light *et al.*, 1984). At the same time, investigations point to a close relationship between quality of this protein and meat tenderness (Light *et al.*, 1984).

The quality of collagen present in smooth muscles has not been analyzed in detail. Data in the literature describe some traits of this protein, in reference to smooth muscle of blood vessels and poultry gizzards (Mayne *et al.*, 1978; Gabella, 1981; Rauntrakool & Chen, 1986).

Our studies were aimed at determination of structural and ultrastructural characteristics of pig stomach meat tissue and at assessment of the effects of frozen storage and heat-treatment on stomach muscle tissue and its collagenous fibres.

## MATERIALS AND METHODS

The experiment was carried out on pig stomach meat tissues of Large White Polish breed. Preslaughter weight of the animals ranged from 110 to 120 kg. The fattening system used in rearing was the same for all pigs.

The raw material was taken for studies immediately after slaughter. Next, after the mucous membrane and adhering fat had been removed, the stomachs were chilled at +4°C for 24 h.

The muscle layers of pylorus were examined in four replications using ten pig stomachs for each trial. The effect of freezing was assessed after 6 months

of frozen storage of the raw material at −18°C and after thawing at 4°C for 24 h.

The fresh-chilled muscles were cooked at 90°C for 90 min and sterilized at 121°C for 30 min.

The samples for a light microscopy analysis were fixed in buffered formalin at pH 7·0 (according to Lillie (1953)) and in the Carnoy liquid and then submerged in paraffin. The paraffin sections were stained by the azan Heidenhain reagent and resorcin-fuchsin (Burck, 1975). Histometric measurements of 100 samples of collagen fibres (selected at random) were made using an eyepiece micrometer.

The experimental material for the ultrastructural studies carried out using a transmission electron microscope (TEM) was fixed in glutaraldehyde, stained in 1% osmium tetroxide solution and dehydrated in ethanol (Burck, 1975). Ultrathin sections were stained according to Reynolds (1963) and examined in TEM ('Tesla' BS613). The tissues for ultrastructural studies carried out using a scanning electron microscope (SEM) were previously fixed in a 2·5% solution of glutaraldehyde and 1% osmium tetroxide solution (Kimoto, 1972). The material was dehydrated, dried and coated with silver and next examined in SEM ('Tesla' BS-300).

## RESULTS

### Fresh-chilled muscles

The meat tissue of pylorus of pig stomachs is built up of a thick layer of cellular bundles (myocytes) exhibiting multidirectional orientation. Myocytes can be stained orange by the azan Heidenhain method. The muscle layer of the examined part of pig stomach is rich in connective tissue. The connective tissue forms sheaths (perimysium) which unite the entire meat tissue. The large bundles of a muscle cell are surrounded by thick sheaths of the connective tissue (perimysium). Thinner sheaths, being a continuation of thick perimysium, penetrate into myocyte bundles, dividing them into smaller subunits which eventually form endomysium. Collagen fibres present in thicker perimysia form bundles whose thickness varies from 3·0 to 7·5 $\mu$m but the most common thickness is about 4·7 $\mu$m. These structures run in different planes, especially at the point where a few perimysia are joined together. In thinner sheaths the thickness of collagen fibrous bundles is more uniform. The thinnest bundles are found in endomysium. Collagen fibres can be stained blue according to the azan Heidenhain method (Fig. 1). Transmission electron microscopy (TEM) revealed that sarcoplasm of myocytes contains numerous myofilaments

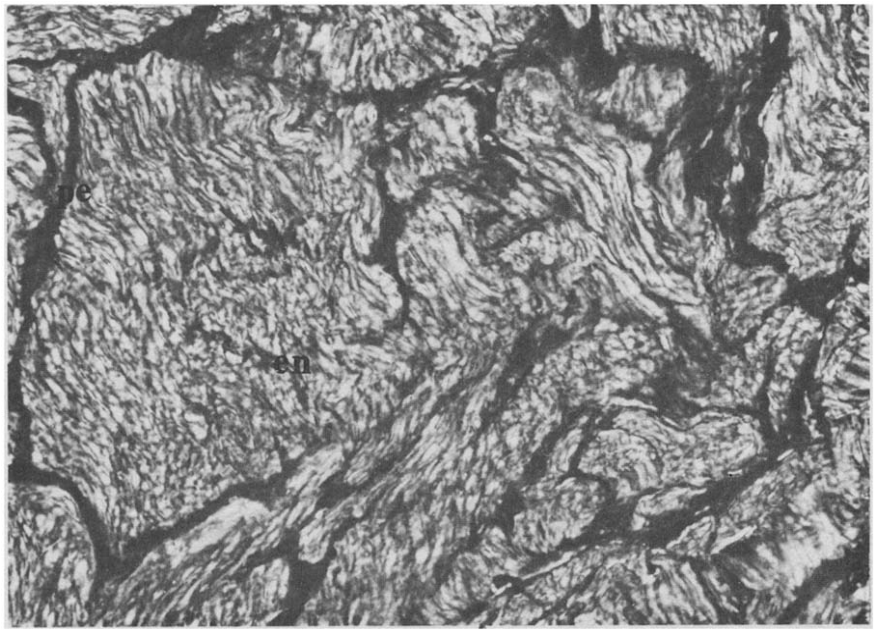


**Fig. 1.** Fresh-chilled muscles. Myocytes bundles of multidirectional orientation. Perimysium (pe), endomysium (en). Azan Heidenhain.

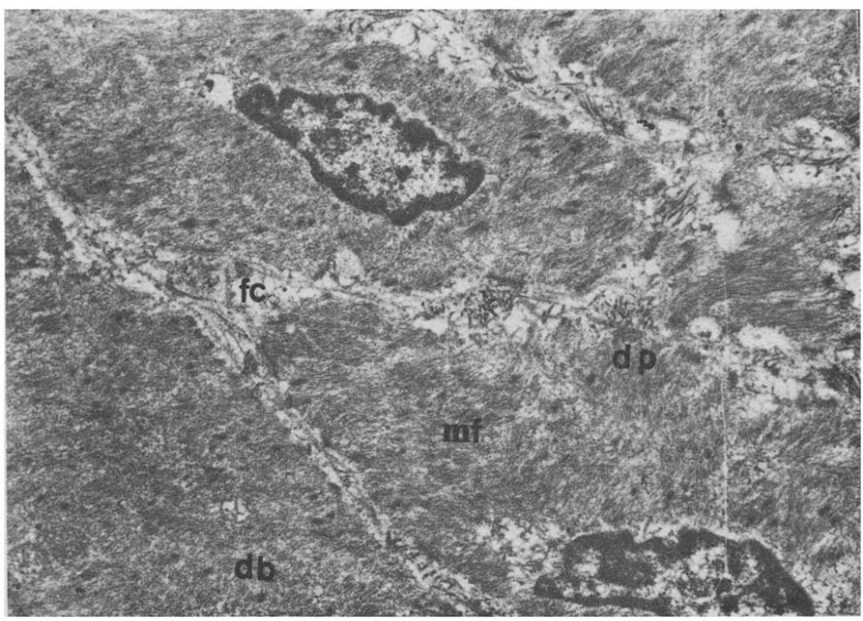


**Fig. 2.** Fresh-chilled muscles. Myofilaments (mf), dense bodies (db) and dense bands (dp) in myocytes. The bundles of collagen fibres (fc) between myocytes. TEM, × 7000.

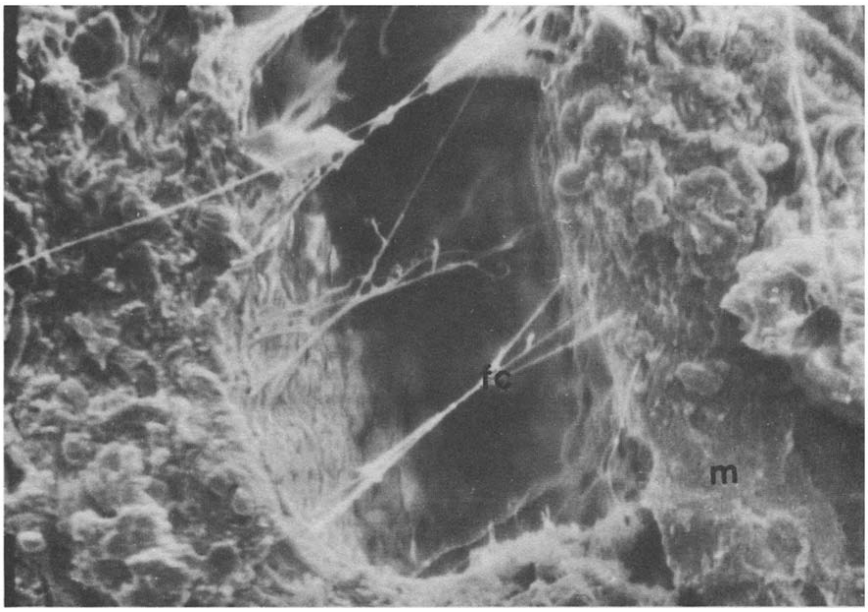


**Fig. 3.** Chilled muscles. Myocytes (m) linked by collagenous fibres (fc). SEM, × 220.

which run along the long cell axis. Moreover, myocytes contain structures typical of smooth muscle cells, i.e. dense bodies and dense bands. The bundles of collagen fibres are present in endomysium, between the myocytes (Fig. 2).

Scanning electron microscopy (SEM) confirmed the compact structure of the muscle tissue in which myocyte bundles are surrounded by collagen fibres (Fig. 3).

## Stomachs frozen stored at −18°C for 6 months

*Light microscopy*

The material frozen stored for 6 months does not show any significant changes in comparison with fresh-chilled muscles. The only difference is the occurrence of fissures between perimysium and myocyte bundles. It is very likely that such a picture is a consequence of physical changes affected by freezing (Fig. 4).

The muscle cells stain orange according to the azan Heidenhain method. Collagen fibres do not exhibit any morphological changes and do not change the affinity to aniline blue.

Furthermore, it was found that 6 months frozen storage did not change ultrastructure of the examined tissue. The muscle cells analyzed by TEM

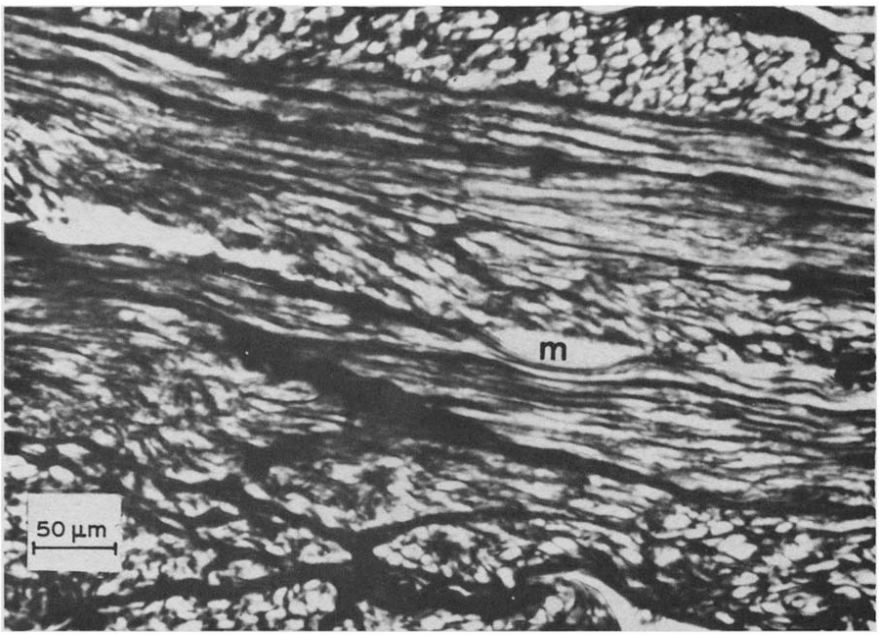


**Fig. 4.** Frozen muscles. Fissures between myocytes bundles (m) and perimysium. Azan Heidenhain.

contained myofilaments, dense bodies and dense bands. Endomysium contained collagenous fibrous bundles (Fig. 5).

The data of SEM analysis confirm the results of TEM analysis proving that neither freezing nor frozen storage can detrimentally affect the pig stomach tissue and its connective tissue (Fig. 6).

**Fresh-chilled stomach muscles cooked at 90°C and sterilized at 121°C**

Fresh-chilled muscles were cooked at 90°C and the cells were analyzed using light microscopy. No changes in the appearance of the muscle cells were observed, but their affinity to dyes in the azan method was changed from orange to aniline blue. Collagen fibres undergo substantial changes at high temperatures. Partial condensation of collagen is observed in perimysium in thick sheaths and at the perimysial junction, where collagen blocks are formed. In thinner sheaths fibrous collagen predominates. Fibrous collagen bundles, however, lose sharpness of their contours. Both fibrous and compact collagen stain aniline blue. Apart from compact and fibrous collagen forms, there are large areas filled with amorphous substance, being a gelatinized collagen. This substance stains blue (Fig. 7). At high temperatures, this protein exhibits unspecific staining effect with resorcin-

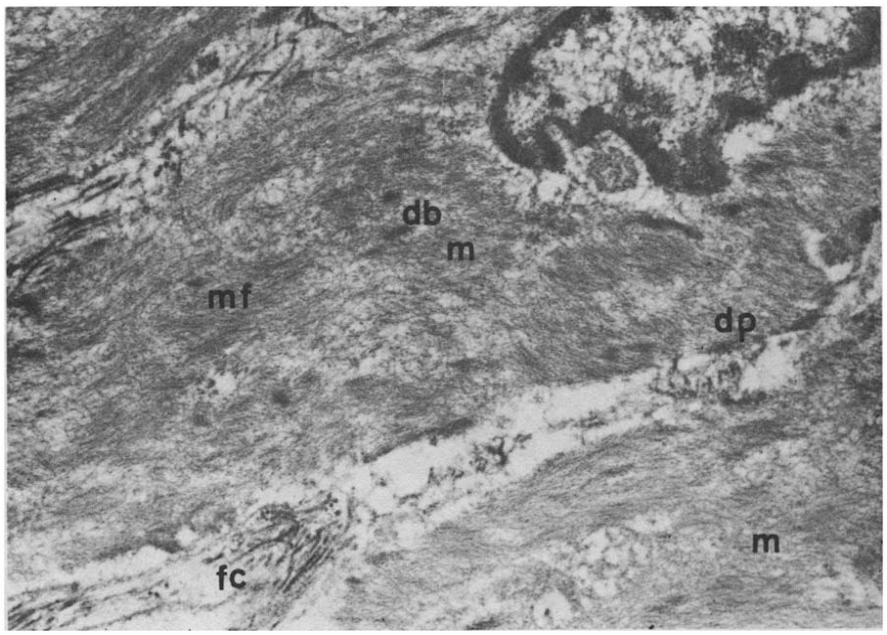


**Fig. 5.** Frozen muscles. Myocytes (m) contain myofilaments (mf), dense bodies (db) and dense bands (dp). Bundles of collagen fibres (fc) between myocytes. TEM, × 5000.

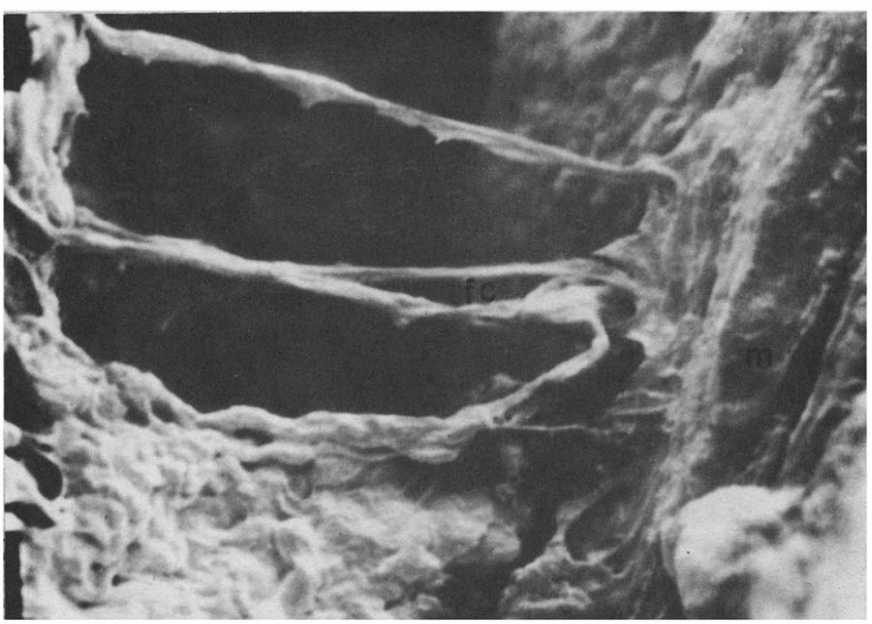


**Fig. 6.** Frozen muscles. Bundles of collagen fibres (fc) between myocytes (m). SEM, × 1250.

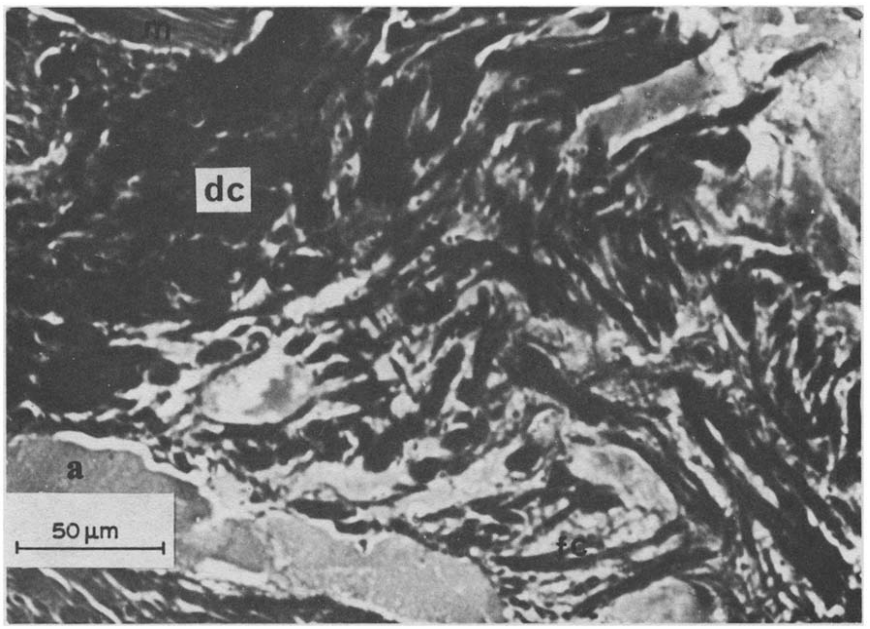


**Fig. 7.** Chilled muscles cooked at 90°C. Myocytes (m) stained unspecifically with aniline blue. Fibrous (fc) and condensed collagen (dc), Areas filled with amorphous substances (a). Azan Heidenhain.

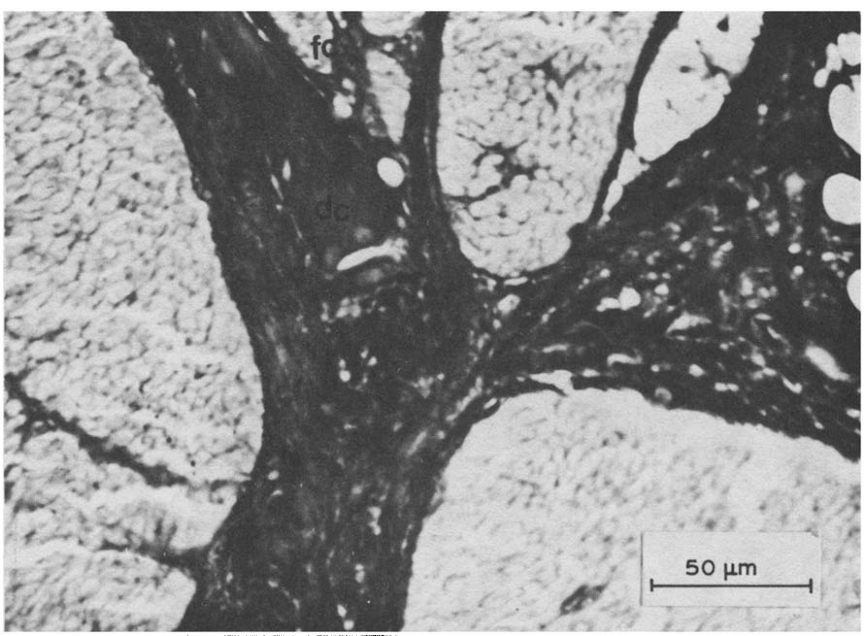


**Fig. 8.** Chilled muscles cooked at 90°C. Fibrous (fc) and condensed (cd) collagen unspecifically stained with resorcin-fuchsin.

fuchsin. Fibrous, condensed and amorphous collagen forms stain brown (Fig. 8).

The muscles sterilized at 121°C, analyzed under a light microscope, exhibit a higher degree of collagen gelatinization than those cooked at 90°C.

The picture of fresh-chilled muscles cooked at 90°C observed in the TEM shows that collagen is granular in form and fibrous collagen bundles occur sporadically, in thin sheaths (perimysium) and endomysium. This granular substance is a gelatinized collagen. Gelatinized collagen fibres are surrounded by a granular structure (Fig. 9); hence the contours of fibrous collagen bundles, observed in the picture of light microscopy, fade away. Myofilaments are present in the myocytes. The dense bodies and bands are less clear.

Structural changes observed in the muscles sterilized at 121°C, analyzed by TEM are characterized by a higher degree of collagen gelatinization than those observed in the muscles cooked at 90°C. The number of collagen fibres decreases, especially in thinner sheaths. Myocytes contain myofilaments. Dense bodies and dense bands are just visible (Fig. 10).

The pictures of scanning electron microscopy (SEM) demonstrating the muscles cooked at 90°C, reveal the presence of well preserved myocytes and transformation of collagen to gelatine (Fig. 11).

SEM of the muscles sterilized at 121°C exhibits a loose structure of the


**Fig. 9.** Chilled muscles cooked at 90°C. Gelatinization of collagen fibres (g). Collagen fibres (fc) and granular gelatine structure (g). TEM, × 7000.

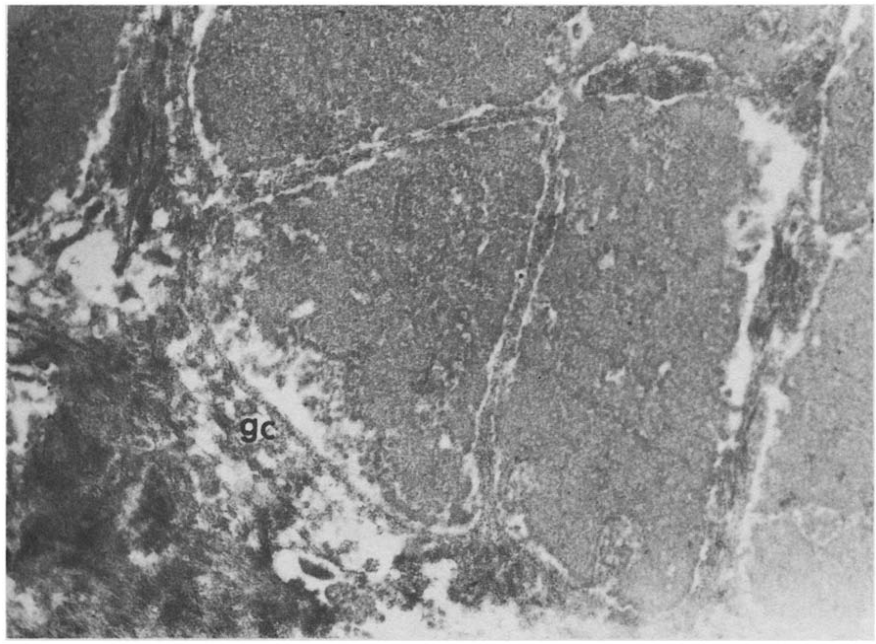


**Fig. 10.** Chilled muscles sterilized at 121°C. Gelatinization of collagen fibres (gc) in endomysium. TEM, ×9000.

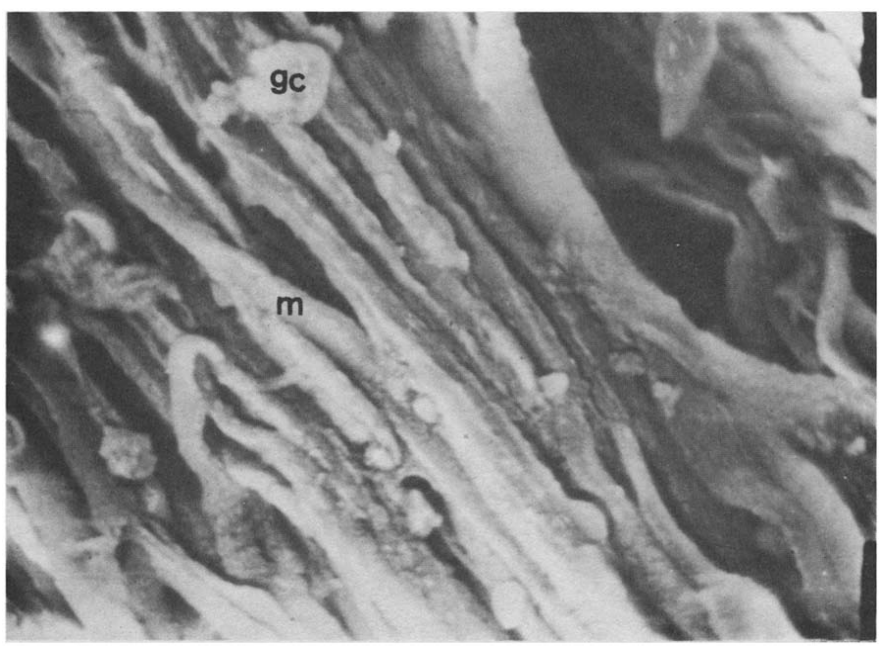


**Fig. 11.** Chilled muscles cooked at 90°C. Myocytes (m) coated with gelatinized collagen (gc). SEM, ×1250.

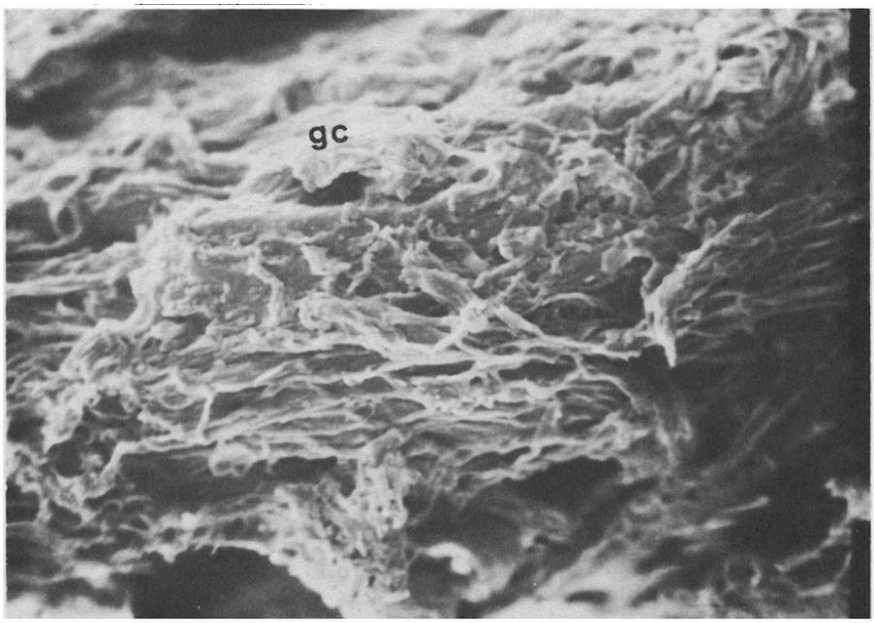


**Fig. 12.** Chilled muscles sterilized at 121°C. Gelatinization of collagen (gc). SEM, ×230.

muscle tissue, presumably resulting from gelatinization of collagen in endomysium (Fig. 12).

## DISCUSSION

The porcine stomach muscles do not exhibit any significant morphological changes after 6 months' frozen storage as compared to fresh-chilled muscles viewed with the light microscope, TEM and SEM.

The cells of smooth muscles contain three types of filament: thin-actin, thick-myosin and intermediate (Gabella, 1981).

The ratio of actin to myosin myofilaments in this tissue is 12:1 (Nonomura, 1976).

The myofilaments present in sarcoplasm of myocytes, should be referred to as actin myofilaments as they predominate over myosin filaments (Gabella, 1981). The presence of actin myofilaments in the muscles cooked at 90°C and sterilized at 121°C accounts for their high thermostability. The myocytes of smooth muscles observed in TEM show the presence of dense bodies and dense bands. These structures are equivalent to the Z-line of striated muscle fibres in which α-actinin has been detected (Gabella, 1981).

The dense bodies and dense bands are well preserved in fresh-chilled and also in frozen-stored material. However, after sterilization at 121°C, dense bodies and bands fade away.

Cooking at 90°C and sterilization at 121°C result in partial condensation of collagen, but a well-retained part of the native form is observed.

Our results are in agreement with those obtained by Forrest *et al.* (1975) who observed shrinkage of collagen upon heat-treatment at 61–64°C. The shrinkage of collagen is associated with its increased solubility (Tarrant, 1981). The condensation of collagen and the immense fields filled with amorphous substance observed in the smooth muscles (both cooked and sterilized) seem to confirm the relationship between collagen condensation and its solubility.

According to Forrest *et al.* (1975) temperatures higher than 64°C result in collagen transformation to gelatine. The resultant gelatine is an aggregate of monomers, dimers and trimers of unfolded collagen chain (Jones, 1984). Undoubtedly, gelatine is the amorphous substance detected in the stomach muscle tissue, cooked at high temperatures (90°C and 121°C) and examined by light microscopy. The lack of amorphous substance in other samples of fresh-chilled and frozen-stored muscles confirms the previous statement. Collagen fibres of the muscles cooked at 90°C and sterilized at 121°C exhibit unspecific staining with resorcin-fuchsin. This effect is in agreement with the results obtained by Puchtler & Sweat (1964). High temperature changes collagen behaviour (Burton *et al.*, 1955); hydrogen bonds collapse and are replaced by new resorcin-fuchsin linkages (Partidge, 1958). Unspecific staining with resorcin-fuchsin observed in the cooked and sterilized stomach meat tissue is exhibited by fibrous and condensed collagen as well as amorphous substance. The pictures reveal substantial physico-chemical changes in this protein due to high temperatures. In skeletal muscles about 10% of collagen fibres remain in the native form at 90°C (Cheng & Parrish, 1976). Stomach muscles cooked at the same temperature are higher in collagen fibres. It is very likely that collagen in the stomach muscle tissue is more resistant to high temperatures, since at 121°C a portion of this protein remains in fibrous form.

Particularly resistant to high temperatures are thick bundles of fibrous collagen, present in perimysium. These observations concur with the results obtained by Aberle & Mills (1983) who studied skeletal muscles.

Skeletal muscles contain collagen which is 'labile' and 'resistant' to high temperatures. The resistance of collagen to high temperatures is due to transverse covalent linkages between amino acids in adhering collagen molecules (Tarrant, 1982). The stomach muscles, cooked and sterilized (and examined in TEM) show that gelatinization in endomysium, and in perimysium, is alike. Varying susceptibility of collagen fibres to gelatinization can be attributed to 'labile' and 'resistant' collagen fractions. Different response of the collagen fibres of skeletal muscles to the temperatures from 70°C to 100°C is attributed to hydroxyproline content and the carbohy-

drates bound to collagen molecules and also to different spaces between the fibres (Otwell & Hamann, 1979).

Thermal denaturation of collagen is considered to be a structural change occurring irrespective of the changes in amino acid sequence. A triple-helical structure of collagen unfolds during denaturation (Jones, 1984). If we assume that condensed collagen in the pig stomach muscle tissue is a denaturated protein, then structurally different collagen maintains affinity to aniline blue.

The ultrastructural pictures of porcine stomach show that frozen storage does not have a significant influence on the structure of their tissues, while high temperatures have a profound influence on the structure of collagen fibres.

The cognitive data obtained in this study can be the basis for further evaluation of technological usability of porcine stomachs.

## ACKNOWLEDGEMENTS

The author thanks Dr Leokadii Kiełb for her contribution to our experiment and Professor Zbigniew Duda for this suggestions and valuable remarks which have helped us much in writing this paper. The author is also grateful to Mrs Jadwiga Bolechowska for linguistic help.

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