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Contributions and editorial correspondence should be sent to Dr E. C. Bate-Smith, A.R.C. Institute of Animal Physiology, Babraham, Cambs.

General correspondence (including items for the News Letter) should be sent to Mr S. H. Cakebread, Florasynth Ltd, 327 Oldfield Lane, Greenford, Middlesex.

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Meat science and technology—an industrial view of the present position

G. B. GALLIVER

I am glad to write an introductory paper to this number of the *Journal of Food Technology*, because it gives me an opportunity of looking at the present state of meat science and technology from an essentially industrial viewpoint. The industrial view of meat science and technology is naturally concerned with the benefits it can confer on the raw material producer, the processor, the seller and the consumer. It is perhaps because of the fact of having this broad span of outlook and concern that the industrial meat research man has a somewhat comprehensive view of the contemporary scene.

The main contributions to the progress of meat science and technology in this country may be expected to be made by university departments, government institutes, research associations and industrial laboratories. In general, one would expect the University contribution to be of a more basic or background nature and comparatively modest in volume. The main forward thrust in providing new information and knowledge should come from government institutions and research associations, and we would expect the major provider of funds for new meat science and technology to be the government. However, in this country government contribution has been relatively small (Cutting, 1967) and consequently the flow of information available to the meat trade has been less than that needed for rapid technological progress. It is pleasing to record the establishment of the Meat Research Institute at Bristol, where the funds are being provided in part by the Treasury and in part by Industry. The primary role of industrial laboratories is to exploit new knowledge in the development of new and improved products, and processes for the particular business in question. However, some companies feel it profitable and beneficial to their business to establish a balanced research and development programme, in which a proportion of background research is included. Much of this background research is published, thus contributing to the common pool, from which we all draw.

We in industry are looking at meat research for a number of advances or benefits such as a better return to the producer; a higher quality product; a more suitable and economic raw material for the processor; and an increased range of products. The pressure for new products is generated by the consumer's insistence on products which are more convenient, of uniform and dependable quality, and which offer variety and new gastronomical experience.

Author's address: Unilever Research Laboratory, Colworth House, Sharnbrook, Bedford.

What has been lacking in meat science and technology to date? As I have already said the volume of work has been insufficient, and I regret to say that until recently much of the work has been of an inferior standard. Of recent years, however, the biological sciences have attracted much attention, and meat research has benefited from this increase in standards. Perhaps the most serious deficiency of all was the fact that research on the animal 'stopped at the farm gate' and had no real bond with meat technology which took over at the factory gate. The major rewards today are for those people who understand that animal breed, management, nutrition, pre-slaughter handling, slaughtering conditions and post-slaughter treatments are a closely knit sequence of events which all have a significant effect on the final product, and the manufacturing processes used to produce that product.

In this country it is heartening to know that the Meat Research Institute programme takes this sequence into account. In our laboratory also we have provided ourselves with the facilities to study not just from 'the womb to the tomb', but from conception to product in the home. The opportunity then is for a real harmonizing and blending of the different disciplines contributing to the subject; a proper integration of projects from the pre-slaughter period to the product; and a better liaison between the scientists and technologists in research institutions and those in industry so as to obtain a speedy utilization of new technologies.

One can conveniently group meat research into a number of 'packages' and the following are the main areas for the future as I see them.

Animal production

Traditionally meat animal breeding has been concerned with producing strains with the best conversion of food into flesh. More recently the selection criteria have also included quality in the most meaningful objective terms possible and also the suitability of the animal for processing. Now attempts are being made to use such indicators as blood types and serum protein polymorphs to classify existing phenotypes or to identify new phenotypes. One can then look for correlations between the genotype so identified and economic factors such as the number of animals per litter, mortality rate of newly born animals, live weight gain, carcase and meat quality, etc. This search for correlations is only possible by using a computer because one could not sift the vast amount of data any other way, and because the speed of computation enables one to see correlations which would otherwise be well-nigh impossible. Thus the problem is essentially one of adapting modern biochemical techniques to handle large numbers of samples and feeding the data from these samples into suitable data handling systems developed by the statistician and the computer expert.

In addition to breeding new strains it is important to consider the effect of management and nutrition on the meat. Here I am thinking especially about the important period shortly before slaughter where the treatment and handling which an animal

receives can have a dramatic effect on meat quality. Similarly the final stages of the nutritional regime can have a big influence on quality.

Body and carcase composition

The value of a slaughtered animal to both the farmer and the processor depends on the obvious factor of weight, but in addition its value will depend on the relative properties of the various parts of the body and of the dressed carcase, as well as on the quality of the different cuts. Studies of body and carcase composition are tedious, time-consuming and expensive, but absolutely essential if an objective measurement of carcase value and quality is to be obtained. The difficulties of evaluation make it desirable to make the most of the animals concerned by using them for as many other purposes as possible, such as biochemical investigation and organoleptic assessment.

Muscle and meat structure and biochemistry

I think that the progress made in the biochemistry of muscle and meat, and in the structure of muscle and meat, is perhaps the single most significant development in the field of meat science and technology. If one is justified in thinking of a single symposium as being a milestone in the field, then the International Symposium on *The Physiology and Biochemistry of Muscle as a Food* held at the University of Wisconsin, 12–14 July 1965 is certainly such a milestone (Briskey, Cassens & Trautman, 1966).

There is a complete spectrum of meat quality, so that in pigs for example it ranges from the top-grade on the one hand to pale exudative muscle on the other, and the differences between these are of degree rather than of kind. Pale exudative muscle may well prove to be caused by an enzyme system 'going wild' and once this is shown we will have a method of screening our breeding stock so as to reduce the liability of pig herds to the condition. Perhaps more important is the hope that an unravelling of the biochemistry of muscle quality will lead to our ability to improve and control quality at the top end of the spectrum.

The molecular architecture of muscle is more than a preliminary plan on the laboratory drawing board, but quite a lot of work is required before the 'molecular architect's' dream is fulfilled. The technology in current use for using muscle as meat in highly processed products is largely empirical. I do not think that it will be too long before our technology will be based on the basic science which is now being forged.

Meat microbiology

Microbiology is another key discipline in meat research—its contribution can be 'negative' in the sense that preventing the incidence of pathogenic bacteria does not add anything to the product's discernible attributes, or it can be 'positive' in the sense

of giving longer shelf life, improved methods of preservation, or making new products available.

The role of the bacteriologist in ensuring freedom from pathogens is especially important in these days of rapid technological change when the comparatively simple change from an aerobic pack to a vacuum pack can completely alter the bacterial flora which survives and thrives. The bacteriologist must be vigilant in monitoring operational changes in the product, process or pack, because a food poisoning outbreak caused by a branded foodstuff will cause a far greater financial catastrophe than a similar outbreak by an unbranded product. Not only will the sales of the particular product involved drop, but also other products bearing the same brand-name or sold by the company involved. The methods of isolation and identification of pathogenic organisms in food are often too slow, laborious and expensive to serve as effective quality control procedures. The methods for *Salmonella* and staphylococcus isolation and identification have been much improved and speeded up, but similar improvements need to be made for other organisms. There is also much scope for automating bacterial analysis.

The role of microbiology in new product development has barely begun. One can envisage the commonsense application of existing knowledge conferring additional shelf-life to packaged semi-preserved meat products sold either at ambient temperatures or from the chill cabinet. However, to discover and develop new methods of preservation new basic information will be required. As the bacterial spore is the organism which any preservation method has to deal with in the ultimate it seems sensible to think that a basic study of the germination process of bacterial spores could lead to dramatic new preservation procedures.

Objective assessment methods

Whenever we apply the results of our research we need to find out how successful, or otherwise, we have been and this inevitably involves taste panelling and consumer testing. Anyone who has been involved in taste panelling knows how difficult it is to assemble groups of suitable, willing and enthusiastic participants, and having done so it is still a time-consuming operation. In many cases taste panel facilities not only dictate the speed of research, but also limit the total amount of research that can be carried out. Thus there is a great need for objective methods for the assessment of colour, texture, taste and aroma. These methods will not replace the human palate, but will act as a faster and easier screening procedure so that the human subject can be reserved for the critical experiments.

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A study of the histological changes in the growing muscles of beef animals

J. R. BENDALL AND C. A. VOYLE

Summary. 1. Histological studies have been made of the growth of the longissimus dorsi (LD) and semitendinosus (ST) muscles of eight Hereford and eight Friesian steers between the ages of 11 days and 24 months.

2. It was found that the average sarcomere lengths of the muscle fibres varied considerably from animal to animal and muscle to muscle, due to postural shortening during rigor. The lengths of the constituent filaments of actin and myosin were, however, constant within a narrow range (1·30 and 1·50 μ, respectively). Hence it is possible to define a standard sarcomere length for reference, as the length when the actin filaments from each half of the sarcomere just touch in the middle. This length is 2·60 μ, after correction for fixation. Cross-sectional areas and diameters of fibres were then corrected accordingly.

3. After correction in this way, it was found that the internal diameters of the muscle fibres increased from about 15 μ at 1 month to about 45 μ at 24 months. For the first 12 months of growth the cross-sectional areas of the fibres kept in step with the cross-sectional area of the muscle, but after that time the former parameter increased more rapidly than the latter, so that fibres appeared to be lost from the muscle as it aged further. In the ST muscles, this loss amounted to about 40% at 24 months.

4. The collagen content of the muscles was higher at the calf stage than later. From 6 months onwards however this content remained nearly constant (0·43 and 0·77% of the wet weight, for LD and ST muscles, respectively). These changes were easily observable in the histological sections.

5. The elastin content, both by semiquantitative histological estimation and by an exact chemical method, was found to be very low in the LD muscles (about 3% of the total connective tissue content), but much higher in the ST muscles (about 37%). In other muscles of prime cuts, such as biceps femoris, the elastin content is, however, of the same order as in the LD, so that its contribution to the toughness of most cuts of meat is probably insignificant.

6. More detailed conclusions of the results are given at the end of the paper.

Authors' address: A.R.C. Meat Research Institute, Low Temperature Research Station, Downing Street, Cambridge.

Introduction

Studies of the growth and differentiation of muscle fibres and connective tissue in the muscles of the larger meat animals, such as beef, seem to be extremely sparse, mainly because of the difficulty of obtaining sufficiently consistent material. We have, therefore, taken advantage of a large scale growth experiment, conducted by the Agricultural Research Council and the Royal Smithfield Club on pure-bred Herefords and Friesians, and the crosses between them, to obtain material from some twenty individual muscles at ages from 1 month to 2 years. In the event, we found that we had to make a drastic selection of the breeds and the muscles, because of the laborious procedures entailed in the measurement of fibre area and sarcomere length. We therefore chose for initial study the lumbar region of the longissimus dorsi (LD) and the 'leg' region of the semitendinosus (ST) muscles from pure-bred Herefords and Friesians. This selection seems to be justified by the finding that the histological changes during the growth of these two very different types of muscle were almost identical, thus making a more exhaustive investigation unnecessary, at least as a first approach to the problems of growth.

Unfortunately, owing to the changing regimen somewhat arbitrarily imposed upon the animals during the course of the experiment, it has not been possible to draw definite conclusions about the increase in a number of important parameters with age. Nevertheless we can salvage, from the confusion produced by these changes, a number of useful relations between the parameters, as the animals age. For instance, our results show that the fibres of the two muscles we have studied grow from the calf stage onwards by simple lengthening and enlargement, which is also the case with lambs, as Joubert has shown (1956); they fail to differentiate into distinct classes of large and small phase fibres, characteristic of the adult mouse biceps brachii, for example (Goldspink, 1962). They also differ from the mouse muscle fibres in that there is a complete absence of mitotic figures even at the 1-week stage, and hence it seems certain that no new fibres are laid down after the birth of the animal, whereas this is a noticeable feature of growth in the mouse, where the young are born very immature.

Another feature of our results which may be of general significance is the apparent loss of fibres from the muscles as they age. This loss can be very considerable, particularly in the longissimus dorsi (LD), where it amounts to almost 50% at 2 years of age. The problem would seem, therefore, to be worth re-investigating in other animals. To this end, we have begun a study of fibre growth in a pure strain of rat, where it is possible to count all the fibres in the muscle of choice.

An aspect of muscle growth of particular importance to meat quality is the development of the connective-tissue which, being the main load-bearing material, must necessarily keep in step with the growth of the contractile, work-producing elements of the muscle fibres. This aspect has been studied here both histologically and by chemical analysis. One interesting finding is that the percentage collagen content of both the

LD and ST muscles falls from the 1st to the 6th month after birth and then remains almost constant at the lower value during the next 18 months. From this it would appear that any toughening of the muscle which may occur with age can certainly not be accounted for by collagen content alone.

Materials and methods

I. Histological technique

Portions from the centres of the longissimus dorsi (LD) muscles in the lumbar region, and of the semitendinosus (ST) muscles in the round, were obtained from pairs of pure bred Hereford and Friesian steers, slaughtered at the ages of 1, 6, 12, 18 and 24 months (eighteen animals in all). For details of rearing see Smithfield Club (1966). The members of the pairs had been selected as the lightest and the heaviest in their particular age groups. The samples were fixed in 10% neutral formalin and subsequently embedded in paraffin wax. Transverse and longitudinal sections were cut at a thickness of 5 μ, and stained by Verhoff's method for elastin fibres, followed by Van Gieson's picro–acid–fuchsin. Elastin fibres appear black, muscle fibres yellow and collagen red by this technique.

The samples for electron microscopy, which were kindly prepared and photographed by Dr Sally Page of University College London, had been previously fixed in buffered glutaraldehyde (pH 7·0). They were taken from a pair of 24-month-old Herefords from another growth experiment.

II. Measurement of fibre area

Negative prints of selected fields in the stained sections were prepared by projecting the microscope image (magnification × 200), onto bromide paper which had already been exposed, by contact, to a negative image of millimetre squares as described by Voyle (1966). On development, a positive image of the squares was superimposed on the negative image of the muscle fibres, making the task of counting squares relatively simple (see Plate 1a). To obtain the fibre area distribution, the number of mm squares within the light area of each of 100 fibres was counted. We shall call this the *internal fibre area*, a_i. The corresponding *mean internal diameter* (d), assuming the fibres were originally circular, in cross-section, is given by:

$$d = \sqrt{(4a_i/\pi)}. \qquad (1)$$

It is these values of d which, after correction for sarcomere length, are used for the frequency distribution histograms of Fig. 3.

The internal fibre areas, measured by the above method, are minimal areas and do not take account of possible shrinkage of the constituent myofibrils and sarcoplasm during processing. As seen from Plate 1 such shrinkage must have been considerable, first because the endomysium (dark areas in Plate 1a) is far thicker than is normally seen in preparations from fresh post-mortem material, and secondly because individual fibres have become separated from one another (in Plate 1 b–c.) For this reason we have

also measured what we shall call the *mean fibre packing area* (a_p) of the 100 or so fibres which were selected for the measurement of a_1. This was done by measuring the total area of a counted number of fibres by planimetry and dividing that area by the number of fibres it contained. The square root of a_p is the *mean packing diameter* of a fibre, D.

III. Measurement of sarcomere length

The sarcomere lengths in the fibres of the various muscles were estimated in each case by counting the number of sarcomeres in a 20-μ stretch in each of four fibres in the microscope field, and repeating this on four more fields. The mean sarcomere length is then obtained by simple division, the number of sarcomeres contributing to each mean being about 200. The average standard error is about 1·5% of the mean value (20 degrees of freedom).

IV. Weight and length of the muscles

The lumbar portions of the LD, from the last rib to the sacrum, and the whole of the ST muscles were weighed in each case. No length measurements are available for the muscles themselves, but the loin lengths (L_2) from the last rib to the last lumbar vertebra, and the leg lengths (L_3) from the ankle to the symphysis pubis, were measured and give the nearest approximation to the resting lengths of the LD and ST muscles, respectively. These lengths are used here to compute the mean cross sectional areas of the two muscles (A_{LD} and A_{ST}), assuming a specific gravity of 1·0. Photographs were also taken of the cross-sections of most of the LD muscles at the last rib and from these the areas were measured by planimetry. Since the correlation coefficient between the measured and calculated areas was 0·99 for $n = 15$, it was considered that A_{LD} could be used as a good representative measure of the area of this muscle. As calculated, A_{LD} is 1·4 times the measured area at the last rib.

V. Carcase weight

Carcase weights were measured in each case. The animals could be divided, regardless of breed, into fairly narrow carcase weight categories, as shown in Fig. 1, and it is

PLATE 1

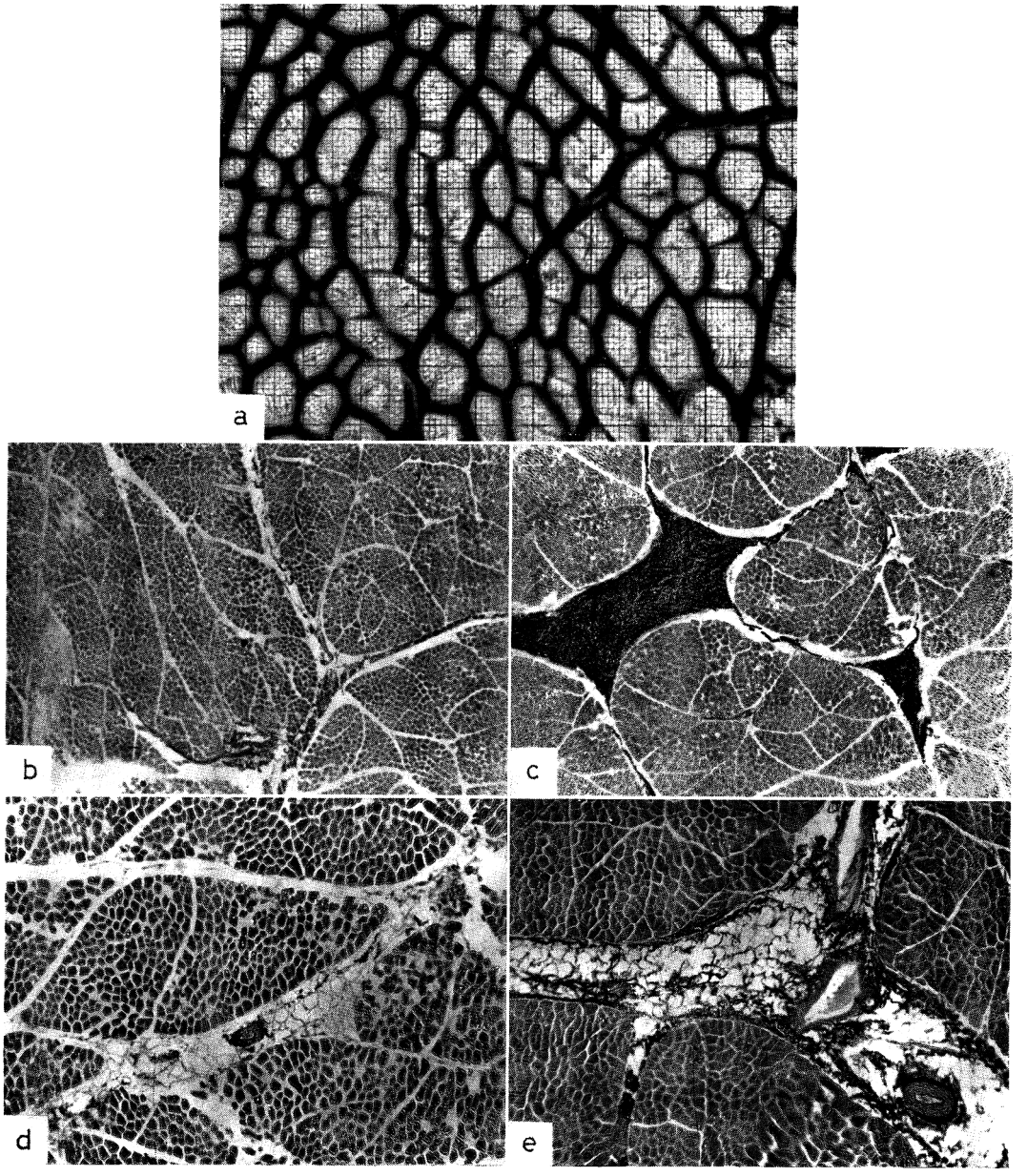
(a) 'Negative' print of transverse section of muscle fibres with grid of millimetre squares superimposed. ×200.

(b) Transverse section of longissimus dorsi (LD) muscle in Hereford (6 months) showing a small amount of connective tissue in the perimysium between the fibre bundles. ×41.

(c) Transverse section of semitendinosus (ST) muscle in Hereford (6 months), showing a much greater amount of connective tissue and a large proportion of dark-staining elastic fibres. ×41.

(d) Transverse section of LD muscle in Hereford (18 months) with deposition of adipose tissue in the perimysium between the fibre-bundles. ×41.

(e) Transverse section of ST muscle in Hereford (24 months) showing more marked deposition of adipose tissue. ×41.



(*Facing p.* 262)

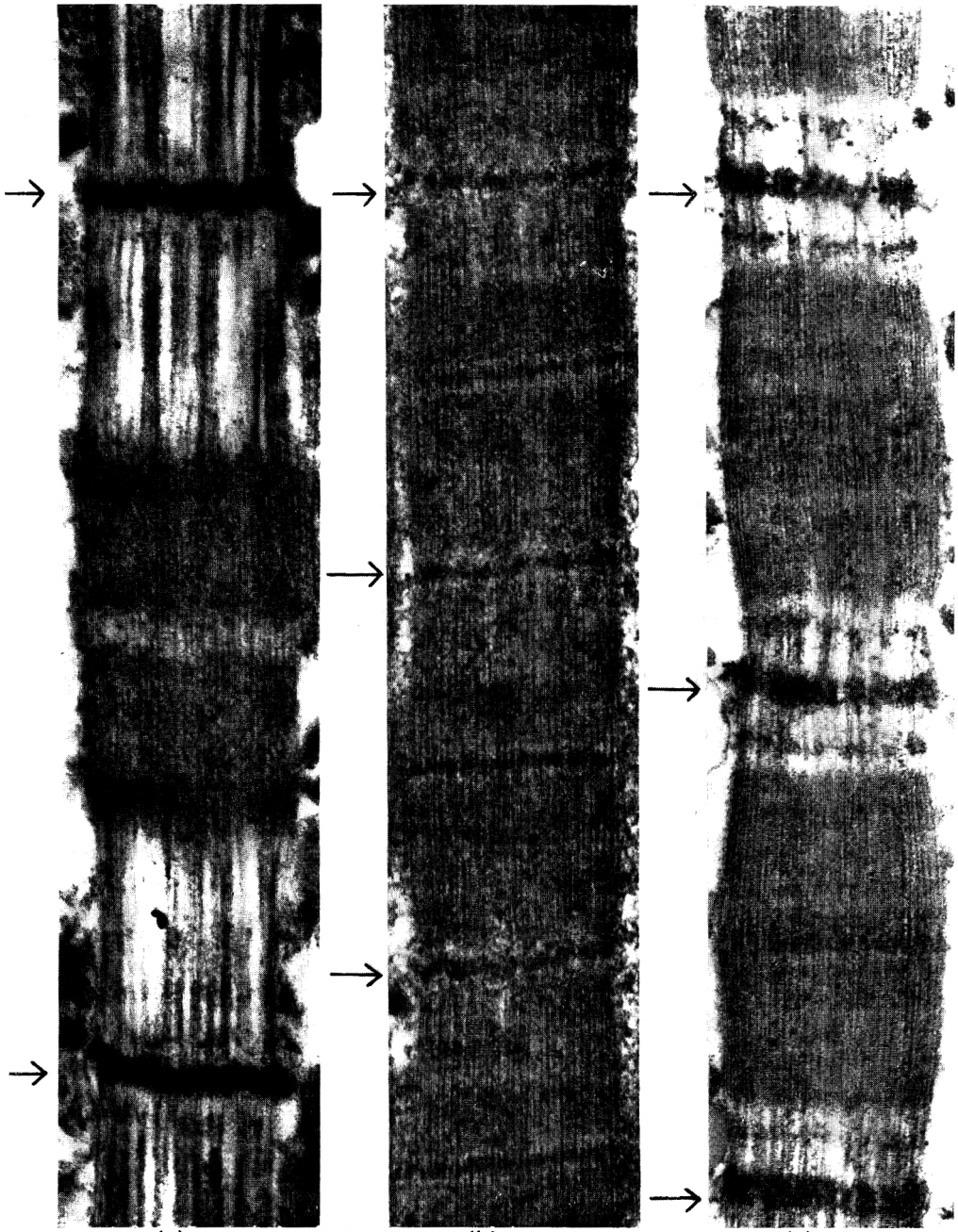


PLATE 2. Electron micrographs of longitudinal sections of: (a) psoas, (b) LD, and (c) ST muscles, from the same animal to illustrate the variability in sarcomere length, but constancy of actin and myosin filament lengths. Arrows indicate position of *Z*-lines. See Fig. 2 for diagrammatic representation of the plate.

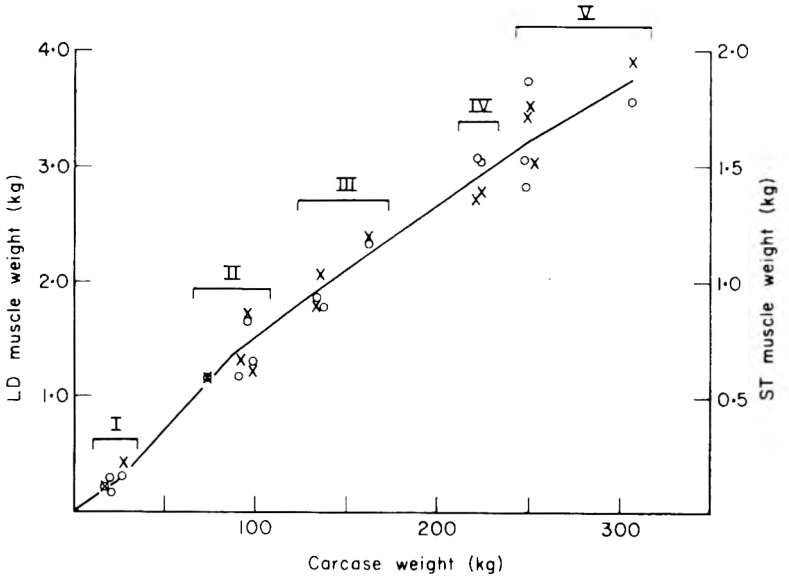


FIG. 1. Plot of the weights of the longissimus dorsi (LD) and semitendinosus (ST) muscles against carcase weights to illustrate the carcase weight categories used in Fig. 3. Ages from 11 days to 24 months. O, LD muscles; ×, ST muscles.

these categories which are used as the basis for the combined frequency distribution histograms of internal fibre diameter (*d*) in Fig. 3.

Values for the various carcase parameters are given in Table 1.

Results

I. General histological features and collagen content

At all ages there was noticeably less connective tissue present in LD than in ST muscles, as seen from Plate 1 (b–e). In the LD muscles most of the connective tissue consisted of collagen, with elastin, the darkest stained material, almost entirely confined to the blood vessels (Plate 1b). In ST muscles, however, a considerable proportion of the connective tissue, especially in the perimysium, was elastin (Plate 1c). Many of the elastin fibres were as thick as 5 μ in cross-section, whereas in longitudinal sections thinner branching fibres could also be discerned.

No adipose tissue was found in sections from the 1-month or 6-month-old muscles (Plate 1a and b), but at 12 months, areas of adipose tissue were present, especially in those parts of the section where connective tissue was fairly abundant. These areas of intramuscular fatty tissue became more widespread with increasing age, and were more numerous and extensive in ST muscles than LD (Plate 1d and e).

Chemical estimation of the apparent collagen content by the hydroxyproline

TABLE 1. Carcase dimensions of the animals used in the study

		Age (months)	Carcase weight (kg)	LD (lumbar) weight (g)	Loin length (L_2) (cm)	ST weight weight (g)	Leg length (L_3) (cm)
Herefords	I	0·25	22·7	304	15·4	169	34·6
	I	1·5	19·3	210	17·0	128	35·3
		6*	35·2*	384*	20·2*	203*	41·4*
	II	6	91·5	1200	26·2	660	50·7
	II	12	99·0	1310	29·5	610	54·3
	III	12	133·5	1860	30·5	890	56·5
	IV	18	224·0	3030	33·7	1380	65·0
	IV	18	222·0	3030	33·3	1360	56·1
	V	24	254·0	3730	37·0	1760	66·3
Friesians	I	1	22·1	174	18·9	125	40·5
	I	1	28·2	300	17·7	207	43·4
	II	6	96·0	1660	27·1	850	55·7
	II	6	74·0	1160	25·5	600	52·7
	III	12	138·0	1790	32·5	1030	61·9
	III	12	162·0	2330	34·0	1200	66·3
	V	18	249·0	2800	37·8	1710	73·3
	V	18	252·0	3060	35·3	1510	65·6
	V	24	307·0	3530	40·4	1950	75·1

*Animal held back by bloat — not included in calculations.

method* confirms that the content is higher in ST muscles than LD. For the twelve animals between 6 months and 2 years, the collagen content of the LD was 0·43 ± 0·02% of the wet weight, and that of the ST 0·77 ± 0·03%; the values were randomly scattered and there was no evidence in either case of any correlation with age within this range. All the values were higher for the calves under 1½ months old, available from this and another similar experiment: LD (seven animals) 1·30 ± 0·23%, and ST (six animals) 1·20 ± 0·08%. It is noticeable that the scatter in the LD is here higher than in any other case. These high values in calf muscles were also observed in the biceps femoris, psoas major and pectoralis major, all of which showed about double their later steady value, when they were at the calf stage.

Analysis of the elastin content, by an elaborate technique designed to remove the last traces of collagen (Gotte *et al.*, 1963), has shown that the elastin/collagen ratio of all the muscles mentioned above, except the ST, is very low and lies in the range 0·03–0·06 (including also semimembranosus), whereas the ratio for the ST muscles is

* Collagen calculated by assuming a content of 13·4% hydroxyproline. Note that this is true for the connective tissue actually present in muscle, according to our measurements.

more than ten times higher, often exceeding 0·60. This accords well with the histological findings, and also serves to eliminate elastin as a substantial contributor to toughness in most cuts of meat. This is also true, even when the elastin content is high, as shown by the results from a small consumer panel, which reported samples of ST muscle as being firm but fairly tender, in spite of their relatively high elastin content (0·50% on the wet weight basis).

II. Overall growth of the muscles

The weights of the muscles are plotted against carcase weights in Fig. 1. This seems to be a more realistic way of plotting muscle growth than that based on real age, in the sense that the two 12-month-old muscles included in Group II in the figure, for instance, are closer to the other four 6-month-old muscles in the group, in weight, cross-sectional area and fibre diameter, than they are to the muscles in Group III, which are all 12 months old. A particularly clear-cut example of this occurred by hazard in the present series, when one of the animals developed 'bloat', so that it was held back by its infirmity, when slaughtered at 6 months, to a developmental stage corresponding to a 1-month-old normal animal, in respect of all the parameters mentioned above, and, most interesting of all, collagen content as well (see Table 1).

Another feature of Fig. 1 is that the weight of the ST muscles increases almost exactly in step with the weight of the LD muscles, although there is a significant difference between the breeds in this respect, as shown by the following linear regression equations, for Herefords and Friesians, respectively:

$$W_{LD} = 2{\cdot}22W_{ST} - 0{\cdot}10 \text{ kg} \quad r = 0{\cdot}998 \text{ for } \mathcal{N} = 8; \tag{2a}$$

$$W_{LD} = 1{\cdot}87W_{ST} - 0{\cdot}001 \text{ kg} \quad r = 0{\cdot}989 \text{ for } \mathcal{N} = 9. \tag{2b}$$

Similarly, the loin length (L_2) keeps in step with the leg length (L_3), except in the early stages of growth before the animal is 1 month old, when L_3 is increasing faster than L_2 (see Fig. 8b). From these two relations, it follows that the mean cross-sectional areas of the two muscles, calculated as described on p. 261, should also keep in step as the animal grows. That this is indeed the case is shown from the following linear regression equations, for Herefords and Friesians respectively:

$$A_{LD} = 3{\cdot}93\ (A_{ST} - 0{\cdot}29)\ \text{cm}^2 \quad r = 0{\cdot}995 \text{ for } \mathcal{N} = 8; \tag{3a}$$

$$A_{LD} = 3{\cdot}404\ (A_{ST} + 0{\cdot}7)\ \text{cm}^2 \quad r = 0{\cdot}981 \text{ or } \mathcal{N} = 9. \tag{3b}$$

Thus, the overall growth of the two muscles we have chosen for study is very similar, and this is reflected at the histological level, as we shall see later.

III. Growth of the muscle fibres

The two parameters which predominantly determine the growth of the muscles themselves are, first, the increase in the diameter of their fibres, due to the increase in number and diameter of the constituent fibrils (Goldspink, 1965); second, the increase in the length of the fibres, due either to the laying down of new sarcomeres or to the lengthening of already existing ones. Of these parameters, the fibre area and the

sarcomere length are dependent on the relative length which the particular muscle under consideration assumes during the rigor process, and it is therefore necessary to be able to define a standard muscle length to which, in each case, these fortuitous changes can be referred back.

Variations in sarcomere length after the completion of rigor can be considerable, not only between the muscles on a carcase, but also between carcases, as the recent results of Herring, Cassens & Briskey (1965a, b) have demonstrated. This is particularly true of carcases allowed to go into rigor in the conventional hanging posture, as were those studied here. This posture has the effect of unnaturally stretching some muscles, e.g. the psoas major, and allowing others to become unnaturally slack, e.g. the semitendinosus and the longissimus dorsi, particularly when the back becomes markedly arched. Then, as rigor occurs, the sarcomeres of the psoas become fixed in the stretched state, whereas those of the slacker muscles are able to shorten and become fixed in rigor at this shorter length. Thus a simple count of the number of sarcomeres in a given length of muscle can lead to completely erroneous conclusions. The effect of sarcomere length on fibre area is equally important, because the volume of a muscle, whether stretched, at rest length, or contracted, remains virtually constant, so that the cross-sectional area of the muscle and of its constituent fibres and fibrils will vary inversely as its relative length.

The establishment of a suitable reference length, because of what we have said about normal slaughter, can unfortunately be achieved only by the very roundabout means described in the next two sections.

(*a*) *Apparent changes in sarcomere length and numbers during growth.* The sarcomere lengths of the muscles, measured as described on p. 262, are given in Table 2. Also shown are the relative increases in leg length (L_3) and loin length (L_2) for comparison with the sarcomere lengths of the ST and LD muscles, respectively.

Two points emerge from Table 2: first, that there is a great deal of variability in the sarcomere lengths of both muscles, which appear to be distributed at random, regardless of age or carcase dimensions; second, that the sarcomeres of the LD are considerably shorter than those of the ST muscles.

Bearing in mind that neither muscle can be considered to have been at an easily definable standard length when it passed into rigor, and taking into account the random variability of sarcomere lengths resulting from this, the simplest deduction from the results is that both these muscles in beef animals grow longer by the addition of *new sarcomeres*, and not by the addition of material lengthwise to already existing ones.

The precise meaning of the differences in average sarcomere length between the LD and ST muscles, at all stages of growth, can only be understood from the discussion of filament lengths to be given in the next section.

(*b*) *Lengths of the constituent actin and myosin filaments of the sarcomeres.* The sarcomeres of all striated muscles are made up of interdigitating filaments of actin and myosin, as Hanson & Huxley (1955) were the first to show. The arrangement of these filaments

TABLE 2. Sarcomere lengths (l_s) of fibres from LD and ST muscles at various ages

Type of animal	Age (months)	LD muscles		ST muscles	
		Mean l_s (μ)	Relative increase in loin length	Mean l_s (μ)	Relative increase in leg length
Hereford	1	1·64	1·00	2·35	1·00
	1	—	—	1·73	1·02
	6	1·61	1·31	—	—
	6	1·49	1·63	1·84	1·47
	12	1·28	1·91	1·91	1·57
	12	1·21	1·98	1·74	1·63
	18	1·80	2·19	2·15	1·88
	18	1·87	2·16	1·97	1·62
	24	1·74	2·40	1·90	1·92
Mean		1·58		1·95	
Friesian	1	1·58	1·00	1·81	1·00
	1	1·48	1·07	1·79	1·07
	6	1·62	1·44	2·09	1·38
	6	1·60	1·53	2·35	1·30
	12	1·49	1·84	2·13	1·53
	12	1·58	1·92	2·17	1·64
	18	1·55	2·00	2·12	1·81
	18	1·73	2·14	2·41	1·62
	24	1·54	2·28	1·72	1·86
Mean		1·57		2·07	

is shown diagrammatically in Fig. 2. An overall electron microscope view of them is given in Plate 2, but not showing as much detail as the diagram would suggest (Page & Bendall, unpublished observations). The length of the two sorts of filament in a particular muscle is remarkably consistent from animal to animal of a given species (Page & Huxley, 1963), so that once these have been established it is possible to define a standard length of sarcomere, independent of the actual length of the muscle.

In the case of the three 2-year-old beef muscles, illustrated in Fig. 2 and Plate 2, correction for magnification and shrinkage during fixation gives values of 1·3 μ for the length of the thin actin filaments in each half sarcomere, and 1·5 μ for that of the thicker myosin filaments in the sarcomeres of fresh muscle. These values are reduced by about 10% after fixation in glutaraldehyde, and it is these shorter values of about 1·17 and 1·35 μ, respectively, which probably best represent the lengths of the filaments in the sarcomeres in Table 2, which were measured on fixed samples by light microscopy. Although these results were obtained from only two mature Hereford animals, it is not

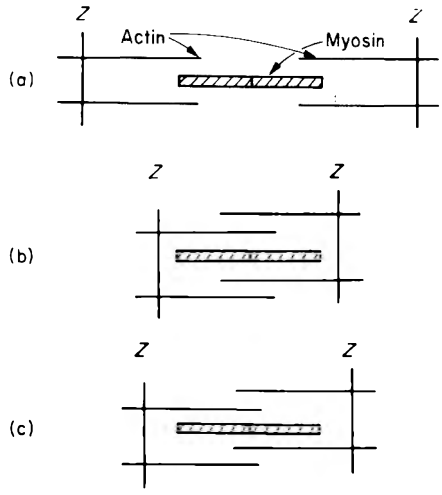


FIG. 2. Diagrammatic representation of Plate 2 to show how the actin and myosin filaments are disposed in relation to one another and to the Z-line, in: (a) psoas; (b) LD; and (c) ST muscles from the same animal.

likely that they will be found to vary significantly between other mature animals of the same breed, since as we have said, filament length seems to be genetically determined fairly exactly.

Plate 2 and Fig. 2 also illustrate what we have already noted about the variability of sarcomere length from muscle to muscle in a beef animal, e.g. the very long sarcomeres of the psoas and the shortened sarcomeres of the LD and ST muscles; we see, however, that this occurs without any alteration in the length of the constituent actin and myosin filaments themselves (cf. Fig. 2). This is because of the interdigitating mechanism whereby a muscle can be pulled out passively (during relaxation or in the pre-rigor state), and can actively shorten (during stimulation or the onset of rigor), merely by the two sorts of filament sliding over one another, passively in one case, actively in the other.

Knowing the filament lengths, it is now possible to define a standard sarcomere length as the point at which the actin filaments just touch in the middle of the A-band (point of no overlap). For the psoas major, LD and ST muscles in Fig. 2, and also in samples of sternomandibularis muscle from the same animal, this standard sarcomere length (l_{00}) is 2·6 μ for fresh muscle, or about 2·34 μ for muscle after fixing for histological sectioning. For the three muscles in the plate, therefore, the relative sarcomere lengths (l_s) are 1·40 l_{00} for psoas, 0·64 l_{00} for LD and 0·81 l_{00} for ST, whereas the average values in Table 2, after allowing for 10% shrinkage, are 0·67 l_{00} for LD and 0·86 l_{00} for ST muscles.

Using this standard sarcomere length, and making the simplifying assumption that the filament lengths of the muscles in Table 2 are identical and do not vary with age,

the observed area or diameter of the fibres in any muscle can be corrected back to standard conditions (a_{00} or D_{00}) as follows:

$$a_{00} = a_p l_s / 2{\cdot}35$$
$$D_{00} = D \sqrt{(l_s/2{\cdot}35)} \qquad (4)$$

where a_p is the observed packing area of the fibres (μ^2),

D is the observed packing diameter of the fibres (μ),

l_s is the observed sarcomere length (μ).

It is these corrected areas and diameters which are used in the following sections.

(*c*) *Changes in fibre diameter and area with growth.* The changes in the frequency distribution of fibre diameters with growth can best be illustrated by the frequency histograms of internal fibre diameters (*d*), shown in Fig. 3 for the five carcase weight categories defined in Fig. 1. To obtain these histograms, the frequency distribution of the fibre diameters, at 10-μ intervals, was summed for all the muscles in a category and then averaged. These mean values were subsequently corrected for differing sarcomere lengths, as above, which resulted in altering the width of the intervals, on average, to 8·2 μ in the case of the LD muscles, and to 9·3 μ in the case of the ST muscles (hence the difference in scales in Fig. 3). The numbers of muscles in each category are given in parenthesis.

It is seen that both the average fibre diameter and the spread of the distribution increase as the animal grows, as would be expected. Even in the heaviest group of animals, however, the distribution is of nearly normal form, and there is no evidence of splitting into two peaks, as happens in the case of the mouse biceps brachii (Goldspink, 1962, 1964).

The usefulness of the internal fibre diameter as a guide to growth is more or less exhausted by the histograms of Fig. 3, because of the variability of the shrinkage factor during fixation, which will affect these internal diameters more than it does the packing diameters. In the following, therefore, we shall mostly make use of the minimal fibre packing area, a_{00}, and the packing diameter, D_{00}, corrected for sarcomere length as described above. By the present methods of fixation D is a linear function of d and $= 1{\cdot}085\ d\ (\pm\ 0{\cdot}095$ for $N = 34)$.

A common way of illustrating the growth of muscle fibres is to plot mean fibre area against muscle weight (e.g. Joubert, 1956), although these parameters cannot in fact be linearly related, as Joubert evidently supposed they were, because no account has thereby been taken of the changing length of the muscle. It seems more logical, particularly in the case of a muscle such as the ST, where the fibres run almost parallel to the long axis, to plot either the fibre area (a_{00}) against the mean muscle cross-sectional area (A_{ST}) or the fibre packing diameter (D_{00}) against the square root of the muscle cross-sectional area. Here we have preferred the former, because it is a more sensitive measure and can reveal differences between breeds which are obscured by the other plot. The results are shown in Fig. 4, for the LD and ST muscles. It is apparent from the plot for the LD muscles that the relation between muscle cross-sectional area and

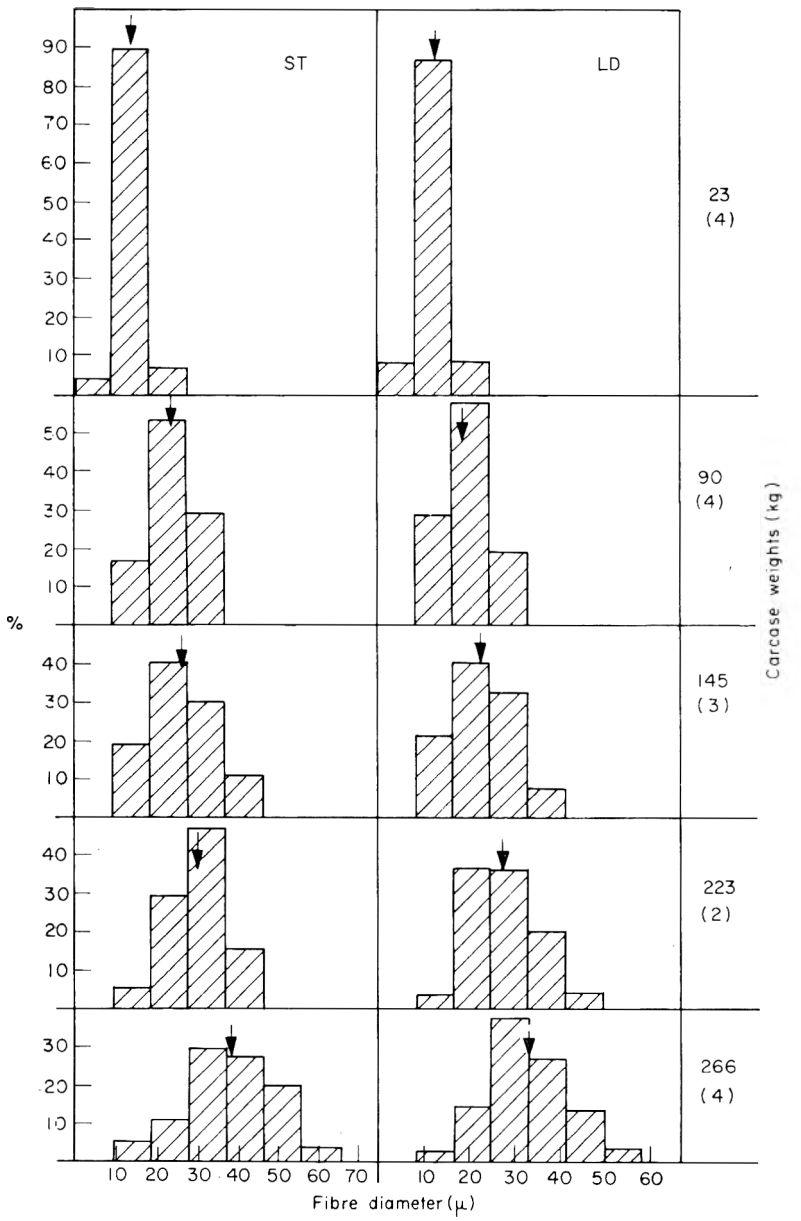


FIG. 3. Frequency distribution histograms of internal fibre diameter (d) of LD and ST muscle in the carcase weight categories, defined in Fig. 1. Vertical arrows indicate the mean diameter in each category.

corrected mean fibre area (a_{00}) has no real linear phase at any stage, the muscle area increasing faster than the fibre area in the earlier stages, until at a fibre area of about

1000 μ^2 a plateau begins to form, where the reverse obtains, that is, fibre area begins to increase out of proportion to muscle area. In the first phase the implication is that new fibres (but, see next section for statistical significance of this) are still being laid down in the LD after the birth of the animal, whereas in the later plateau stage one would

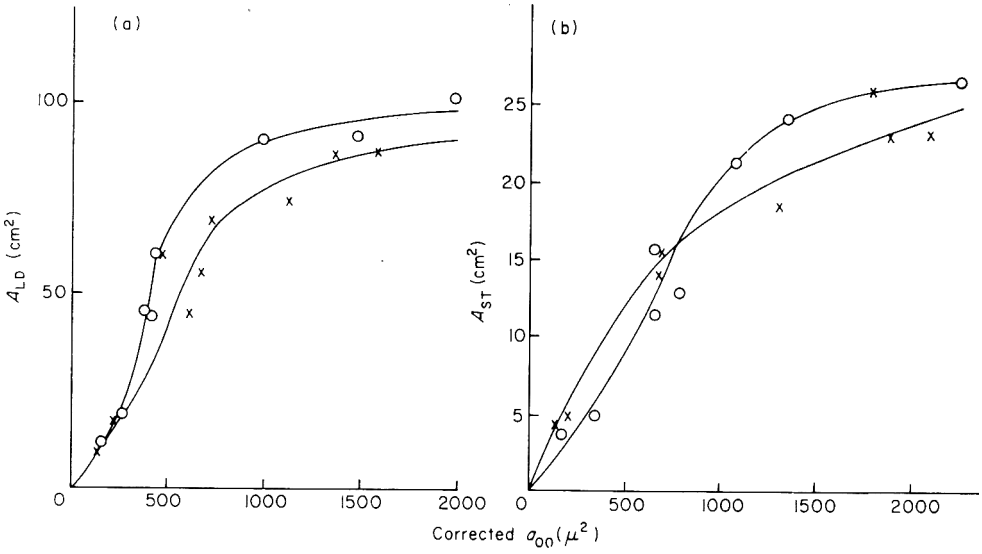


FIG. 4. Mean muscle cross-sectional area (A_{LD} or A_{ST}) plotted against corrected mean fibre cross-sectional area (a_{00}) for: (a) LD and (b) ST muscles. O, Herefords; ×, Friesians.

suppose that fibres are being lost, as the muscles age further. It will also be noted that there is a marked difference between the breeds, the Friesians having the larger fibres at any given muscle cross-sectional area.

The plot for the ST muscles, in Fig. 4(b), shows similar characteristics to that for the LD muscles, although in the early stages here there seems to be a linear relation between the two parameters. Nevertheless a plateau again begins to form after the fibres have attained an area of 1000 μ^2, and once again there is a difference between the breeds, the Friesian fibres being larger, at any given muscle area, than the Herefords, particularly in the later stages of growth.

The implied loss of fibres after the plateau stage is reached is further confirmed in the case of the LD muscles by two relations which follow from their peculiar geometry. In the LD muscles, unlike the ST, the fibres do not run parallel to the long axis, but forward and downward from spinous processes to ribs or transverse processes at an angle of about 45° or less to the long axis (cf. Eisenhutt *et al.*, 1965). Thus, here the long dimension of the muscle (L_2) should be closely related to the fibre diameter, and so should the breadth of the muscle, measured along the transverse process (= X in Fig. 5). These two relations are illustrated in Fig. 5, where again there is an early

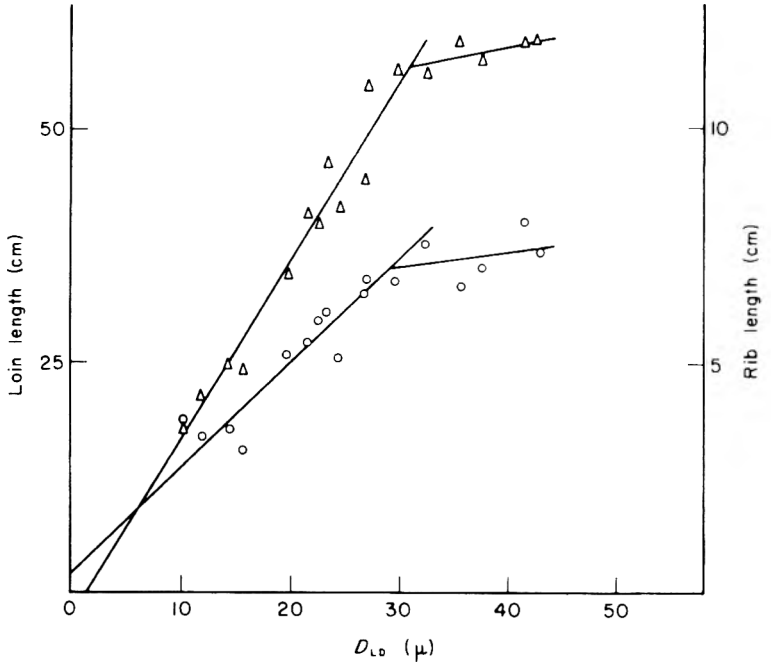


FIG. 5. The packing diameter (D_{00}) of the fibres of the LD muscles plotted against the loin length (L_2) and the breadth at the last rib (X), for Herefords and Friesians combined. O, Loin length (L_2); △, dimension (X).

linear portion passing almost through the origin, which is succeeded by a plateau during which D increases faster than either of the other two parameters. Note that here, due to taking square roots, it is not possible to distinguish the breeds. The relation during the early linear phase can therefore be fairly accurately represented by the following overall regression equations:

$$L_2 = 1{\cdot}18 \times 10^4 D + 1{\cdot}45 \qquad r = 0{\cdot}935 \text{ for } \mathcal{N} = 13; \tag{5a}$$

$$X = 3{\cdot}88 \times 10^3 D - 0{\cdot}45 \qquad r = 0{\cdot}972 \text{ for } \mathcal{N} = 13; \tag{5b}$$

where L_2, X and D are given in centimetres.

Another feature of the results which is worth noting is that the mean fibre diameters of the LD and ST muscles keep almost in step at all stages of growth, the average value for D_{LD}/D_{ST} being 0·89 (S.E. = ± 0·03 for $\mathcal{N} = 17$) after correction for sarcomere length, and 1·00 before correction.

We revert to the question of the apparent loss of fibres in the next section.

(*d*) *Changes in the numbers of fibres with growth.* To calculate the number of fibres in the mean muscle cross-sectional areas of the LD and ST, we have used the corrected fibre packing diameters (D_{00}). The numbers in the cross-section are then given by:

$$\mathcal{N} = A/D_{00}^2, \tag{6a}$$

where A is the mean muscle cross-sectional area.

The numbers in the loin length (L_2) are similarly given by:

$$N = L_2/D_{00}. \tag{6b}$$

We have calculated these values for all the muscles available, and then expressed them as percentage of the mean maximum value for the particular muscle, taking breed into account where possible. The summarized mean results in these relative terms are plotted against age in Fig. 6. It is now seen that the numbers in the cross-section of the

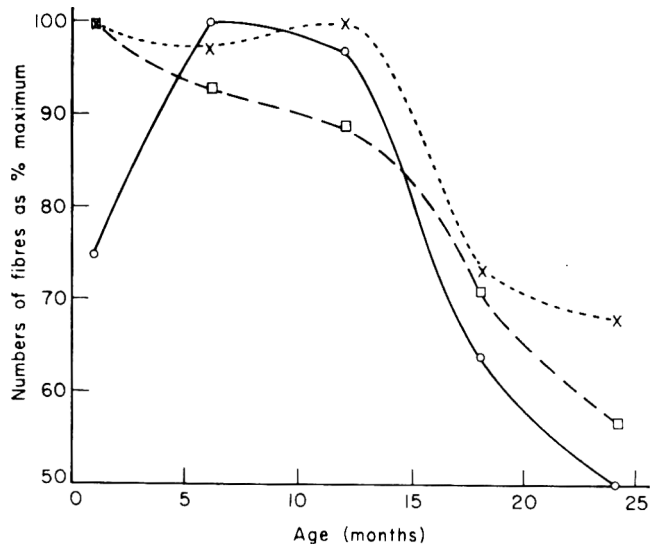


FIG. 6. Relative changes in numbers of fibres with age, in the cross-section of the LD and ST muscles, and in the loin length (L_2). Mean maximum values: LD cross-section, $11{\cdot}0 \times 10^6$ fibres; ST cross-section, $2{\cdot}1 \times 10^6$ fibres; Loin length, $1{\cdot}4 \times 10^4$ fibres (total in loin region of LD = $\sim 1{\cdot}5 \times 10^{11}$ fibres). ×, Loin length, □, ST area; ○, LD area.

LD tend to increase from the 1-month stage to the 6-month stage, then to remain constant until 12 months and afterwards to decline somewhat precipitously. In the ST on the other hand there seems to be a more or less steady decline from the 1-month stage, which becomes considerably sharper after 12 months. In the loin length, however, the numbers remain steady and maximal for 12 months, before they begin once again to decline precipitously.

The statistical significance of the decline in numbers after 12 months can probably best be assessed by applying the *t*-test to the difference between the combined mean numbers at 6 months and 12 months (seven values in each case) and those at 18 and 24 months (six values in each case). We then find, for the cross-section of the LD, that the combined means decline from $10{\cdot}8 \times 10^6$ to $6{\cdot}4 \times 10^6$, and the difference between them is significant at the 0·5% level of probability. For the ST, the combined means decline from $1{\cdot}93 \times 10^6$ to $1{\cdot}42 \times 10^6$, and the difference is significant at the 3% level

of P. Finally, the numbers in the loin length (L_2) decline from $1{\cdot}31 \times 10^4$ to $0{\cdot}98 \times 10^4$, and this difference is significant at the 0·1% level of P.

It remains to assess the significance of the sole case of an apparent increase in numbers of fibres, that in the LD section from 1 to 6 months. For this purpose we have compared the mean numbers at 1 month (four values) with the combined means for 6 and 12 months (seven values). The numbers appear to increase from $8{\cdot}3 \times 10^6$ to $10{\cdot}8 \times 10^6$, but this difference is only just significant at the 10% level of P. In the absence of any confirmatory data, and more particularly in view of the complete absence of mitotic figures in any of the samples or of a similar effect on the numbers of fibres in the loin length, we prefer to regard this single exception as due to some unexplained systematic error.

IV. Comparative changes of the growth parameters with age

It is of some interest to follow the changes of the various parameters with age, particularly the mean muscle and fibre cross-sectional areas and the lengths and weights of the muscles.

The fibre areas are plotted in Fig. 7(a) and (b), for LD and ST muscles, respectively.

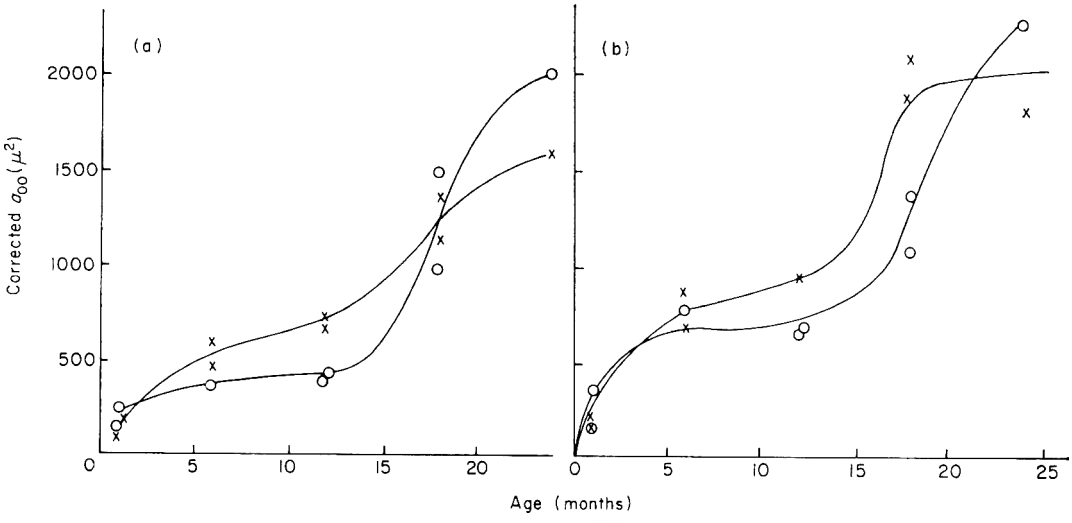


FIG. 7. Corrected mean fibre cross-sectional area (a_{00}) plotted against age, for LD and ST muscles. (a) LD; (b) ST. O, Herefords; ×, Friesians.

This figure shows up the differences between Friesians and Herefords quite clearly, and also those between LD and ST muscles. It also reveals that the growth of the fibres follows a very marked S-shaped curve, with a sharp increase from 1 to 6 months, a plateau from 6 to 12 months, and a further and even sharper increase from 12 to 18 months, followed by a decline in rate of increase between 18 and 24 months. It is the

sharp increase of fibre area between 12 and 18 months which accounts for the apparent decrease in fibre numbers at this time, because the mean muscle cross-sectional area (plotted in relative terms in Fig. 8a) although also following an S-shaped time-course, shows by no means such an abrupt rate of increase during this period. We also note from Fig. 8(b) that the length increases (L_2 and L_3) follow smooth curves of almost exponential shape, and show none of the abrupt changes of slope to be observed in the area/time plots. The effect of this on muscle weight ($= L \times A$) is to preserve the S-shape of the area plots, but to a much less marked degree.

The only other parameter, so far observed, which shows as marked discontinuities in growth as the fibre area is the fat content. Luckily, values are available for the muscles used in this study and they have been plotted in Fig. 8(a) as the percentage of the

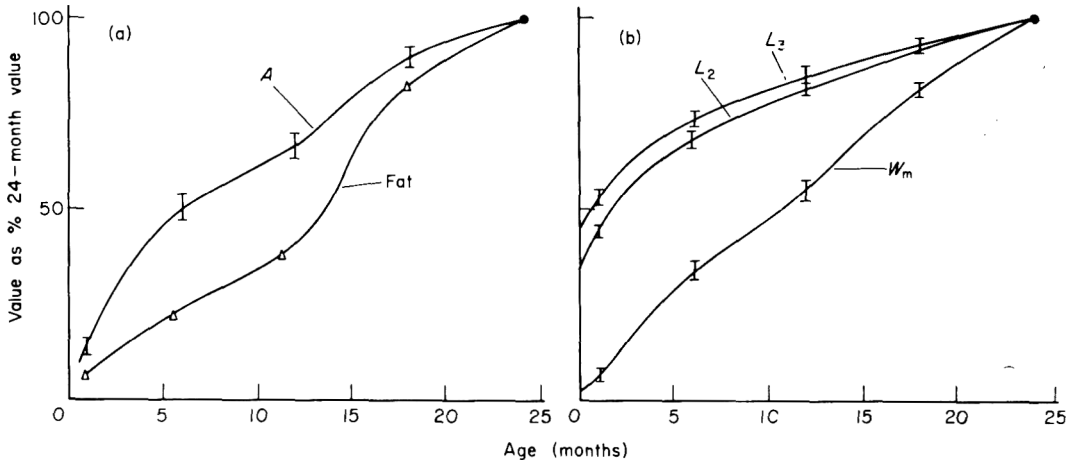


FIG. 8.(a) Plot of mean cross-sectional area of the muscles (A) and of the fat content, as the percentage of the 24 month values, against age. LD and ST muscles combined to give a total of eight muscles at each age. Vertical bars = overall SEs of the means.
(b) Plot of the LD and ST muscle weights (W_m), and the loin lengths (L_2) and leg lengths (L_3), all as the percentage of 24-month values, against age. Vertical bars = S.E.s of means. Eight values at each age for the weights and four for the lengths.

values at 24 months (as are all the other parameters in this figure and its companion). On this relative basis, as with muscle areas and weights, it is impossible to distinguish either the breeds or the LD and ST muscles from one another. The values in the figure are therefore shown with their overall standard errors. It is noticed that the fat content follows almost exactly the same pattern of increase as that of the fibre areas in Fig. 7.

We must, of course, be very cautious in drawing far reaching conclusions from the growth discontinuities we have noted above, because the regimen of the animals was changed back and forth during the course of the experiment from stall feeding on a diet of concentrates and silage to free-range feeding on grass. It is interesting, however,

to see that the phases of rapid growth (1–6 and 12–18 months) correspond to the periods the animals spent in the yards on concentrated feed, whereas the plateau phases (6–12 and 18–24 months) correspond to the periods they spent on grass.

Discussion

The investigation of the growth of muscle fibres reported here not only has interesting fundamental implications, but also an important bearing on the quality of meat as a foodstuff. It is appropriate to discuss this aspect first.

The elements which contribute most to the toughness of meat are the connective tissue, principally in the form of collagen or a collagenous-like protein, but also containing some elastin, and the contents of the muscle fibres themselves, that is the fibrils and the actin and myosin filaments which they contain. As the present study has shown, the relative proportions of these elements alter as the muscle grows, the collagen and elastin content being high for the first few weeks after birth and then declining at about 6 months of age to a steady value which remains virtually constant up to 2 years, and probably later (cf. Hill, 1966; Wilson, Bray & Phillips, 1954). This no doubt accounts for the fact that underdone veal is tougher than underdone meat from older animals, because then the collagen will not have been thoroughly denatured to gelatin, so that it remains in its highly inextensible native form as the principal contributor to toughness. We must constantly bear in mind that this applies to any underdone meat, such as 'rare' steaks, where the extreme toughness of the undenatured collagen overrides any of the other factors we shall discuss later. On the other hand, when the meat has been thoroughly cooked, the difference in collagen content between calves and older animals becomes a less important factor, because young collagen is solubilized to gelatin by cooking far more easily than older collagen (Sharp, 1964; Goll, Bray & Hoekstra, 1964; Hill, 1966). This has been demonstrated very elegantly by Hill who has shown that the solubility declines precipitously and linearly from 1 month to 2 years of age, and then tends to level out at later ages. The decline is clearly a reflection of the increasing cross-bonding of the collagen molecules as the animal ages.

Unlike collagen, elastin is not denatured by cooking, but remains in its native state. However, its content is so low in most of the important muscles of the hind limb and the loin that it would, in any case, be unlikely to contribute significantly to toughness. Moreover, the one muscle in the thigh which has an exceptionally high elastin content, the semitendinosus, is no tougher than the biceps femoris, which has almost no elastin, but a nearly identical collagen (cf. Herring *et al.*, 1965 a). Hence we can more or less eliminate elastin from further consideration.

The contractile filaments of the muscle fibres themselves, because they make up more than 50% of the total protein, must also contribute greatly to eating quality, particularly as they are not solubilized during cooking in the same way as collagen, but denature to form insoluble, longitudinally arranged aggregates. These aggregates will

be more complex at the points in the sarcomeres where myosin and actin filaments overlap, and it is tempting to suppose that this is the explanation of the marked increase in toughness which occurs in muscles such as the extensors of the hip joint, when they shorten during normal rigor (Herring *et al.*, 1965a, b). Locker & Haggyard in 1963 were the first to draw attention to this effect of shortening on toughness, which they were able to take to extreme limits by making use of the so-called 'cold-shock' phenomenon. 'Cold-shock' can be brought about, particularly in beef and lamb, by cooling the muscles to about 1°C while they are still in the pre-rigor state; this has the effect of stimulating a slow but active shortening process which derives its energy from accelerated splitting of adenosine triphosphate (ATP), and which in extreme cases can reduce the muscle to less than 50% of its slack length. Thus by loading muscles appropriately, any desired degree of shortening can be obtained, the length being subsequently fixed by allowing rigor to occur under the cold conditions.

Marsh & Leet (1966) have taken the original observations of Locker & Haggyard considerably further, and have shown that toughening first becomes obvious at about 85% of the slack length, and exponentially reaches a maximum at about 60% of this length. Further shortening below this point, however, suddenly produces a decrease in toughness, which these authors attribute to rupture of cell membranes. Although this latter observation is clearly of fundamental importance in interpreting the shortening effect, it plays little or no role in normal meat, where the fibres can rarely if ever shorten below 65% of their slack length.

Herring *et al.* (1965a, b) as we noted above, have measured sarcomere lengths in several different muscles of normal carcases, and have been able to show the same general trend as Marsh & Leet, that is, the shorter the muscle the tougher it becomes. However, their results clearly demonstrate that Marsh & Leet's generalization applies accurately only to the shortening of a particular muscle, and that the so-called background toughness defined by these authors, and also the absolute shear-values, vary considerably from muscle to muscle. The cause of the variation is evidently the collagen content: thus, the biceps femoris and semitendinosus muscles have about double the collagen content of the psoas major and longissimus dorsi, according to our own extensive investigations; they also have about double the shear value of the latter pair of muscles, i.e. they are twice as tough at all the sarcomere lengths measured by Herring *et al.* Similarly the muscle used exclusively by Marsh & Leet, the sterno-mandibularis, is extremely rich in collagen, the content being about four times that of the LD, and its toughness also appears to be proportionately greater at all the muscle lengths studied. For this reason, and also for the even more pertinent one that all the authors mentioned above have based their arguments on the shear values of the *cooked* meat plotted against the sarcomere lengths of the *fresh* material, it seems somewhat premature to jump to the conclusion that the effect of shortening on toughness is entirely due to the increasing overlap of the actin and myosin filaments in the sarcomeres.

The growth of the muscle fibres themselves presents some interesting fundamental problems. First, we find that there is no certain evidence of any change in the total numbers of fibres in either muscle, between the 4th week of post-natal life and the 12th month. In the case of the ST, and to some extent the LD, the mean cross-sectional area of both the fibres and the muscle keep reasonably well in step during this period (cf. Fig. 4a and b), and in the case of the LD, the diameter of the fibres keeps in step with both the length and breadth of the muscle (Fig. 5 and equations 5a and 5b). After the 12th month, however, there is evidence in both cases that the muscle grows to maturity by a further rapid increase in fibre area (see Fig. 7,) out of proportion to the increase in muscle area, which necessarily implies the disappearance of a considerable number of the smaller fibres, amounting to a loss of up to 50% by the age of 2 years. Loss of fibres in this way can also be deduced from the growth curves published by Joubert (1956) for the lamb, and by Goldspink for the mouse (Goldspink, 1962, 1964). In the former case, there is some evidence that the plateau of growth, where fibre area is increasing faster than muscle area, and where fibres must therefore be lost, is succeeded by a phase in which fibre area and muscle area once more increase, hand in hand. In the mouse biceps brachii, however, there is a contradiction between the fibre numbers estimated indirectly in this way and the actual counts in the cross-sections themselves. By the latter method, the total numbers in the centre of the mature muscle are 1600–1700. However, the counts do not reveal any decrease during the later stages, but rather a slight increase during the plateau stage from about 1620 to 1700 fibres, whereas on the basis of muscle area and fibre area the numbers should decrease from 1650 to 1200. This contradiction could perhaps be explained by the rather complex geometry of the muscle, leading to the ends of those fibres which run from tendon to fascial sheath becoming increasingly included in the later counts, as the author himself suggests. However, investigation of other muscles from the mouse, where the geometry is simpler, has shown that the fibre numbers by direct count, do in fact remain constant in the mature muscles within the errors of measurement (Goldspink, personal communication), so that the anomaly in the mouse biceps and beef LD and ST remains to be satisfactorily explained.

We should mention here that we have found support for the indirect observation that fibre numbers decrease in the beef muscles, in the appearance of distinctly stained macrophages around the fibres of muscles from the 18- to 24-month-old animals. Such cells seem to be completely absent from younger muscles. The suggestion therefore is that, as the fibres grow larger and larger, there comes a stage at which they become partially starved of nutrients and oxygen and begin to degenerate. As soon as this happens, macrophages appear to mop them up. It remains to be seen how far this observation can be applied in general to growing muscle.

The other aspect of growth where differences exist between beef and mouse muscle is the mode by which the muscles lengthen. In the mouse biceps brachii which was held extended by pinning down the forearm, and allowed to go into rigor at this standard

length, the average sarcomere length increases from 2·0 to 2·5 μ while the muscle is lengthening from about 3·5 mm to its mature length of about 7·0 mm. From this it follows that the number of sarcomeres in the muscle length increases from about 1800 to about 2800, during the same phase of growth as we are considering in the beef muscles. Hence, although most of the length change is here due to increase in sarcomere numbers, a significant proportion can be attributed to increase in length of the individual sarcomeres. In the beef muscles, on the other hand, there is no evidence that the sarcomere length itself increases from the 4-week stage onwards, but rather that the whole of the increase in muscle length is due to the laying down of new sarcomere. We must, however, be cautious in generalizing from the beef results, because as we have already pointed out, neither the LD nor ST muscles can be considered to have been at a standard length as they passed into rigor, so that the sarcomere lengths from muscle to muscle are subject to very considerable random variations (see Table 2), due entirely to variability in relative muscle length. At present there is no way of overcoming this difficulty apart from measuring the actual lengths of the actin and myosin filaments in individual muscles, by electron microscopy, and thus being able to define the limits within which a sarcomere can be stretched or contracted at each stage of growth. In fact, the latter is ultimately the only realistic way of describing length changes within the sarcomeres with growth because even at the best, definition of a standard muscle length is difficult, and at its worst can lead to apparent differences in sarcomere lengths between muscles, which are solely dependant on the posture chosen and bear absolutely no relation to the lengths of the constituent filaments (cf Herring *et al.*, 1965b). In this connection, we believe the actin and myosin filament lengths recorded in this study (Page & Bendall, unpublished observations) to be the first to have been measured in mature beef muscle. As we have pointed out, these lengths in fresh muscle are 1·3 μ for the actin filaments in each half sarcomere (i.e. 2·6 μ from tip to tip through the Z-line), and 1·5 μ for the myosin filaments. The former values are nearly the same as those in human muscle, but considerably longer than those in the rabbit, which are about 1·1 μ (Page, unpublished observations).

Although the histological data show us the mode by which the fibres lengthen and thicken, they do not reveal either the relation between the two processes or the detailed changes in protein composition as the muscles grow. The first of these relations has been illustrated in Figs. 7 and 8, where it is seen that both the fibre area and muscle cross-sectional area and weight show growth discontinuities which are most noticeable in the first case. Thus we find a distinct plateau of growth of the fibres between 6 months and 12 months of age, and then a sudden dramatic increase in fibre area from 12 to 18 months. The only other parameter we have found to vary in quite this manner is the fat content of the muscles, which also shows a fairly well-marked plateau between 6 and 12 months and a rapid increase from 12 to 18 months (Fig. 8). Reference to the length/time curves in Fig. 8(b) shows that the latter rapid phase coincides with a period when the length changes in the leg and loin have almost ceased.

As we have already noted, the discontinuities in the 'growth' pattern of fibre and muscle areas and of the intramuscular fat correspond to the changes of regimen imposed on the animals, the rapid phases of growth corresponding to the periods when the animals were being fed on concentrates and the slower plateau phases to the periods when they were on grass. It is interesting that these changes of regimen, which have such a dramatic effect on the girth of the muscles, scarcely perturb the smooth lengthening of the loin and the leg; in other words, bone growth can pursue its smooth exponential time course, unaffected by large changes in nutritional level.

We have so far discussed only the changes in the overall dimensions of the muscle fibres with growth, but it is also of importance to consider the density of packing of the fibrils within the fibres at the different stages, particularly because Goldspink (1965), in studies of the effect of low planes of nutrition on the mouse, has suggested not only that there are less fibrils within the smaller fibres which result from this treatment, but also that they are far less densely packed. Thus on going from a fibre of 49 μ diameter to one of 20 μ, his figures suggest the fibril packing density decreases by 200%, that is, the fibrillar protein content per gram falls by 200%. This raises the question whether such a low protein content is also to be found in the small fibres which are characteristic of young animals, and we have therefore studied the fibrillar protein fraction in a number of calves for comparison with older animals (LD and psoas muscles). We find, after correction for the collagen entrained in this fraction, that the mean value for calves is 12·5% fibrillar protein within the fibres at 3 days old (two muscles) and 12·9% at 3 weeks old (six muscles). This value rises to 14·1% for animals of 6 months or over (twenty-four muscles). All values are on the wet weight basis, after allowing that the collagen present outside the fibres entrains the same amount of water per gram as the fibrils within. Remembering that the fibres in the young calf have an average diameter of about 13 μ, whereas at 6 months the diameter has risen to about 23 μ and at 24 months to about 43 μ, our values for the changes in fibrillar protein content with age are therefore far less than those to be expected from Goldspink's results. In fact there can only be a maximum increase of about 13% in the fibril packing density from the calf to the adult. This observation also incidentally disposes of the idea that the percentage of extracellular space changes drastically from the new born to the adult animal, unless we make the unwarranted assumption that the water-relations within the fibre also change equally dramatically without any observable corresponding change either in protein, ash or non-protein nitrogen content.

A further interesting aspect of Goldspink's observations on the fibril packing density in small and large fibres was that the small fibres, produced by reducing the food intake of the adult mice, were capable of developing only about a third of the tension shown by the larger fibres of mice on normal rations. This he suggested was entirely due to the difference in packing density of the contractile fibrillar material in the two cases. It is clear that if this observation were of general validity we should expect the small fibres of young calves, for instance, to behave in the same way. It is therefore of interest to

make a rough calculation of the load per square centimetre a muscle, such as the ST in the hind limb, must bear as the animal grows. Taking this as being proportional to the ratio of carcase weight to mean muscle cross-sectional area, we find the load per square centimetre increases from an arbitrary value of 1 unit at 1 month to 1·25 units at 6 months, 1·40 at 12 months, 1·78 at 18 months and 1·82 at 24 months, for the seventeen animals included in the present study. The only stage at which there is any corresponding increase in the amount of fibrillar material in unit volume of muscle is between 1 month and 6 months, when it increases to about 1·13 times the calf value, but later remains constant at this higher value. We should therefore conclude, in this case, that calves and 6-month-old animals are capable of faster movement than older animals, which is more in accord with the facts than would be the case if the fibrillar material per unit volume did not lag behind, but kept in step with the increasing load per square centimetre as the animal grew, when we should expect older animals to be able to frolic as gaily as calves.

Conclusions

1. Histological studies have been made of the longissimus dorsi (LD) and semitendinosus (ST) muscles of eight Hereford and eight Friesian steers between the ages of 11 days and 24 months.

2. Cross-sections of the muscles show that the proportion of connective tissue is higher in calves than in older animals, and that this connective tissue becomes increasingly invaded with fat cells as the animals age. Most of this connective tissue in the LD is in the form of collagen or a collagen-like protein, with elastin confined to the blood-vessels, whereas in the ST there are large amounts of elastin, in the form of fine corrugated fibres, in the perimysium at all ages.

3. The histological observations on the connective tissue are confirmed by chemical analysis, which shows that the mean collagen content for calves is 1·3% of the wet weight in LD muscles, and 1·20% in ST muscles, falling to 0·43 and 0·77%, respectively, at 6 months, and thereafter remaining constant. The elastin/collagen ratio in the LD is very low (0·03), whereas in the ST it averages 0·60, and can reach values as high as 0·70. In this respect, the ST is exceptional, the other large muscles in the hindquarter all resembling the LD in their low elastin content.

4. The mean internal diameters of the muscle fibres increase from about 15 μ at 1 month to about 45 μ at 24 months. Their frequency distribution histograms are of normal form and show no evidence of splitting into two peaks, as the fibres enlarge. At first the histograms are very sharp, but later spread out to embrace a wider range of fibre diameters, e.g. at 24 months, from 24 to 50 μ. After correction for sarcomere length, the mean diameter of LD fibres is 89% of that of ST fibres at all ages.

5. The sarcomere lengths of the fibres of the two muscles are highly variable, but randomly distributed with age. On average, those of the LD are 1·57 μ long, and those

of the ST 2·02 μ, whereas the psoas, a muscle highly stretched on the hanging carcase, has sarcomeres of 3·3 μ, or more, in length (after fixation). The evidence suggests that beef muscles grow longer after the calf stage solely by the addition of new sarcomeres, and not by the lengthening of old ones.

6. It is shown that the differences between the sarcomere lengths of the LD, ST and psoas arise solely because the muscles go into rigor on the carcase in the unnatural hanging position, whereas the lengths of the fundamental filaments of actin and myosin, and thus the possible range of sarcomere lengths, are the same for all three muscles. As observed, these lengths are 1·17 μ for the actin filaments in each half sarcomere, and 1·35 μ for the myosin filaments (1·30 and 1·50 respectively, after correction for shrinkage). The 'standard' sarcomere length is here defined as 2·60 μ (= 2·34 μ after fixation), which is the length at which the actin filaments would just touch in the middle of the sarcomere. The observed fibre diameters and areas have been corrected accordingly.

7. Using corrected packing areas (a_{00}) as the criterion, it is found that the area of the ST fibres is linearly related to the mean muscle cross-sectional area, from 1 month up to 12 months, therefore indicating that the total numbers of fibres in the muscles do not change during this period. After 12 months, fibre diameter begins to increase more rapidly than the square root of the muscle area, therefore indicating that fibres are actually destroyed as the muscle ages further. This loss apparently amounts to about 40% at 24 months (significant at $P = 3\%$).

8. In the LD muscles (lumbar region), where the fibres run at 45° to the spine, fibre diameter is linearly related to muscle width and to the loin length, from 1 month to 12 months, but thereafter increases more rapidly than either of the other parameters, indicating that a total of about 50% of the fibres have been destroyed between 12 and 24 months (significant at $P = 0{\cdot}1\%$).

9. When the changes in the parameters with time are considered, a distinct plateau in the growth curve between 6 months and 12 months is found in the plots of mean muscle cross-sectional area, muscle weight and, most noticeably of all, mean fibre cross-sectional area. The latter runs an almost parallel course to the increase in fat content of the muscles, which also shows this plateau, followed by an abrupt rise up to 18 months of age, and then another decline in rate. These growth discontinuities can be explained by the changing regimen to which the animals were subjected.

10. Analysis for fibrillar protein content shows that the fibres contain within them about 12·9% fibrillar protein, in the calf stage, and that this increases to 14·1% at 6 months of age and thereafter remains constant (on the wet weight basis). Thus, neither the packing of the fibrils within the fibres, nor the percentage of extracellular space, change drastically as the animal grows from the young calf stage.

11. The contribution of the various structural proteins to toughness is considered, and it is shown that elastin can virtually be ruled out as a serious contributor in most cuts of meat. On the other hand, both the length of the sarcomeres and the collagen

content play a major role, the former being inversely and exponentially related to toughness, and the latter directly and probably linearly. When the age of the collagen is also taken into account, it is concluded that the toughness of any particular muscle can be entirely accounted for in terms of these three parameters only.

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Quality characteristics of pork with special reference to Pietrain, Pietrain × Landrace and Landrace pigs at different weights

D. B. MacDOUGALL* AND J. G. DISNEY†

Summary. The meat quality of Pietrain, Pietrain × Landrace and Landrace hogs and gilts at four finishing weights was studied with respect to post-mortem glycolytic rates, colour, wateriness and toughness.

The rate of post-mortem glycolysis was found to be indicative of the subsequent meat quality in the three breeds.

The longissimus dorsi of the Pietrain carcasses exhibited those characteristics associated with paleness, wetness and toughness. The Pietrain × Landrace were intermediate between the parent breeds, but more closely resembled the Landrace.

Muscle brightness was significantly related to the pH of the muscle measured at 45 min post mortem (pH_1) and also pigment concentration.

Significant correlations were found between pH_1, and protein solubility, compressibility and tenderness values; and also between compressibility ratios and tenderness values.

The light coloured part of the semitendinosus was significantly paler in the Pietrain than in the Landrace and Pietrain × Landrace, while the dark part became darker with increasing weight.

With increasing weight, the inner layer of subcutaneous fat became less pink and increased in brightness for gilts.

Introduction

Pietrain pigs have aroused considerable interest in Europe in recent years. In trials in the United Kingdom reported by Duckworth *et al.* (1966), the advantages of the breed with regard to leanness, large eye muscles and higher kill-out percentage have been shown. Various European workers, however, have given unfavourable reports on the quality of the meat from Pietrain pigs. For example, Kroes (1960), Kirsch *et al.* (1963), Clausen (1961) and Blair (1964) have all reported a pronounced tendency for Pietrain meat to be pale and wet.

* Agricultural Research Council, Meat Research Institute, Food Quality Department, Great Westminster House, Horseferry Road, London, S.W.1.

† J. Sainsbury Ltd, Stamford House, Stamford Street, London, S.E.1. Present address: Tropical Products Institute, 56–62 Gray's Inn Road, London, W.C.1.

The characteristics and causes of the 'watery pork' syndrome have been studied extensively in various breeds of pig and this subject has recently been reviewed by Briskey (1964), Bendall & Lawrie (1964) and Bray (1966). The development of the pale and watery condition has been related both to the ultimate pH (measured at 24 hr post mortem), and the rate of glycolysis which may be indicated by either the pH_1 (read at 45 min post mortem) or the pH_2 (measured at 2 hr post mortem).

The present work, which was started during the Spring of 1966, forms part of a breed assessment programme instituted by the Pig Industry Development Authority (PIDA). The quality of the meat from Pietrain pigs was assessed in terms of colour variation, wateriness and tenderness, and these aspects were compared with the meat from pure Landrace controls and with that from Pietrain × Landrace. The results obtained were examined in relation to the breed and age of the animals, and to the rate of glycolysis as indicated by the pH_1 of the longissimus dorsi muscle.

Materials

The animals were reared at Wye College, Kent. Litters of the Pietrain, Pietrain × Landrace and Landrace breeds were divided according to sex, and each individual pig was then allocated to one of four predetermined slaughter weight groups; namely 140, 170, 200 and 230 lb (live weight). The experiment involved a total of ninety-six pigs which were divided into four replicates of each sex for each breed and each weight group.

The animals were fed *ad libitum* and on reaching the required weight, they were transported to the abattoir. The journey from farm to abattoir by road was approximately 100 miles and after arrival the pigs were rested in covered lairage with access to water for at least 3 hr prior to slaughter. The animals received no special treatment and were electrically stunned and bled under the commercial conditions practised in this particular abattoir.

Methods

pH and temperature

Temperature was measured by insertion of a Thermophil probe and the pH of the intact muscle was measured with probe electrodes (Radiometer pH meter, model 24). The pH of a water extract was measured using an E.I.L. meter 23A. pH_1 and temperature were measured at 45 min post mortem in the longissimus dorsi muscle in the region of the last rib. At the same time it was observed whether the foreleg of the carcass had developed the stiffness which is symptomatic of the onset of rigor.

The carcasses were then passed into a cold store at 36–38°F and further pH readings were taken at 2 hr post mortem (pH_2) and 24 hr post mortem (pH_{ult}). The following morning the carcasses were transported 70 miles in an insulated vehicle to a meat manufacturing plant, where they were dissected and the required samples were excised. The latter included the entire semitendinosus muscle of the left side, and the

left longissimus dorsi muscle extending from the last rib to the sacral region. A sample of inner subcutaneous fat was also removed for colour measurement.

The sample of longissimus dorsi was divided, the anterior portion being used for colour and pigment analyses, the posterior portion for estimations of wetness, tenderness and the analysis of soluble protein.

The pH of a distilled water extract (pH_{ext}), prepared by homogenizing 1 g of muscle with 5 ml of water, was determined for the longissimus dorsi and for the pale and dark portions of the semitendinosus muscles.

Muscle preparation for colour and pigment analyses

(1) *Longissimus dorsi.* At the time of analysis, a 6-mm slice was removed to expose fresh tissue. A second 6-mm slice was then removed and homogenized in an M.S.E. Vortex Mixer for pigment analysis. The next 6-mm slice was placed in a partly covered Petri dish and held at 2°C for 1 hr. Evaporation losses were thus minimized while free access to air permitted oxygenation to proceed at the surfaces. When the slice had acquired a 'bloom' by this standardized procedure, a 1-in. diameter sample was cut from the central region and mounted in an aluminium holder for colour measurement.

(2) *Semitendinosus.* The muscle was cut transversely at a point half-way along its length. A slice, 6 mm thick, was removed and oxygenated as described for the longissimus dorsi sample. It was then divided subjectively into pale and dark portions which were weighed, in order to assess the percentage of pale muscle in the slice. Samples were then mounted in the aluminium holder for colour measurement, and other portions of pale and dark muscle were homogenized for pigment analysis.

(3) *Muscle colour.* Reflectance measurements of the muscle samples were taken with an automatic recording Optica CF4DR double beam spectrophotometer fitted with an integration sphere and light trap to eliminate the specular component. The region 380–710 nm was scanned at the rate of 12 Å/sec in comparison with a magnesium oxide standard. The C.I.E. tristimulus values, X, Y and Z were calculated from the reflectance spectra by the 10-selected-ordinate method as illustrated by Mackinney & Little (1962) for illuminant C. The chromaticity co-ordinates, x and y, were then calculated. Y measures brightness, and in conjunction with x and y locates the position of the colour in the colour space.

(4) *Metmyoglobin.* The proportion of pigment present as metmyoglobin was calculated by the method of Stewart, Zipser & Watts (1965) based on the ratio of K/S at 572 nm to K/S at 525 nm.

(5) *Fat colour.* The colour of the fat sample was measured using a Gardner Automatic Colour-Difference meter, Model AC-2a, scaled in L (visual lightness), a_L (positive red, negative green) and b_L (positive yellow, negative blue). a_L/b_L expresses 'hue', i.e. the redness or yellowness. The ratio a_L/b_L follows a tangential function and it is

more appropriate to use the actual angle (Francis, 1963). In this case cotangent $\theta = a_L/b_L$, i.e. the angle θ increases as the hue changes from red to yellow.

The meter was standardized using a white tile with the values $L = 93{\cdot}2$, $a_L = -0{\cdot}7$ and $b_L = +2{\cdot}9$.

Determination of total haematin pigment

The method used was a modification of the method of Hornsey (1956). Ten grams of the mixed, minced sample of meat was macerated with 2 ml of water plus 40 ml of acetone. One millilitre of concentrated hydrochloric acid was added and the solution filtered after 1 hr. The absorbance of the clear filtrate was read at 512 nm against a blank of 80% acetone.

Visual assessment of colour and wetness

After cutting a fresh surface, the sample was exposed to the atmosphere for a minimum of 30 min before assessment of colour and wetness. Each of these parameters was assessed separately by a panel of three people, who scored according to two 7-point systems (1 = pale, or wet; 7 = deep colour, or dry).

Soluble protein

Duplicate 1-g samples of the minced meat were homogenized in 10 ml of ice-cold 0·04 M potassium phosphate buffer (pH 7·0) according to the method of McLoughlin (1963). Forty millilitres of buffer were added to the homogenate, which was then allowed to stand for 15 min with intermittent shaking before decanting. The residue was re-extracted in a further 50 ml of buffer.

The combined extracts were centrifuged for 20 min at 25,000 *g* and the supernatant was retained for the determination of soluble sarcoplasmic protein. The residue, containing the myofibrillar fraction of protein, was extracted twice with 50-ml portions of 0·6 M potassium iodide buffered to pH 7·0. Fifteen minutes with occasional shaking was allowed for each extraction.

Soluble protein in the clear extracts obtained above was determined in duplicate by the colorimetric method of Snow (1949), reference being made to a calibration curve constructed for purified egg albumen.

Water holding capacity (W.H.C.)

The method used was essentially the filter paper press technique of Grau & Hamm (1953). Expressed water, or 'loose water', was calculated from the equation:

$$\text{Loose water (mg)} = \frac{\text{Wetted area (cm}^2) - 1{\cdot}0}{0{\cdot}09}.$$

The 'bound water', i.e. the water remaining in the sample after pressing, was determined by oven drying (12 hr at 101°C). To facilitate removal of the pressed

meat sample for this purpose, a thin layer of glass wool was introduced between the filter paper and the meat sample before pressing.

The total water content of the sample was obtained as the sum of loose and bound water (mg/g dry weight of meat). The expression:

$$\frac{\text{Bound water} \times 100}{\text{Total water}},$$

is used as the index of W.H.C. of the meat. For each sample, the value of W.H.C. is given as the mean of three determinations.

The areas on the filter paper obtained in the W.H.C. determination were used to calculate a compressibility ratio similar to that used by Grau & Fritz (1964). This ratio is the area of the meat sample after pressing divided by the weight of the sample taken.

The pressed meat areas are indicative of the compressibility or 'softness' of the meat and therefore of some aspects of the tenderness of the meat.

Tenderness values

Tenderness values were obtained with a Volodkevitch instrument, using a die and circular plunger (diameter 10 mm). The tenderness value is defined in this study as the slope of the stress–strain curve recorded by the instrument. Higher values are indicative of tougher meat. The value for each sample of meat is given as the average of six readings taken on a slice cut perpendicular to the axis of the muscle and with a standard thickness of 1·5 cm.

Results and discussion

Since one animal (Pietrain gilt, 140 lb) did not survive and one (Landrace gilt, 170 lb) was condemned at the time of slaughter, allowance has been made for these missing plots in the statistical treatment of the results. The means for each parameter for each of the three factors, breed, weight and sex, are presented in Tables 1–4. The degree of significance of the differences between comparable means is indicated and the occurrence of significant interactions noted. The levels of significance will be referred to as very highly significant ($P < 0{\cdot}001$), highly significant ($P < 0{\cdot}01$) and significant ($P < 0{\cdot}05$).

Prior to slaughter, it was observed that the Pietrain animals tended to be more excitable and nervous than the Landrace, with the Pietrain × Landrace intermediate.

pH and colour of the longissimus dorsi

pH_1 and pH_2 measurements in the longissimus dorsi indicate a very highly significant difference in glycolytic rates between the breeds (Table 1). Little difference was found, however, in the ultimate pH or the pH of the water extract. The glycolysis

TABLE 1. Means and significance of differences for appearance characteristics for the longissimus dorsi

		pH_1	pH_2	pH_{ult}	pH_{ext}	Y	x	y	$\%R_{525nm}$	Haematin (ppm)	Metmyoglobin (%)
Landrace	(L)	6·33	5·98	5·71	5·45	28·16	0·3601	0·3464	23·36	32·42	38·77
Pietrain ⁄ Landrace	(C)	6·16	5·77	5·65	5·42	30·06	0·3652	0·3475	25·07	34·98	41·60
Pietrain	(P)	5·70	5·74	5·73	5·47	33·63	0·3627	0·3488	28·52	37·12	42·94
D.S.A.											
$P<0·05$				*	P>C		N.S.	N.S.		P>L	N.S.
$P<0·01$		A.S. L>(C, P)			N.S.	P>(C, L)				N.S.	
$P<0·001$		(L, C)>P	L>P			P>L			P>(C, L)		
140 lb	(1)	5·94	5·73	5·66	5·45	32·84	0·3609	0·3454	26·86	31·70	43·14
170 lb	(2)	6·07	5·82	5·73	5·44	30·37	0·3628	0·3458	25·26	34·92	42·99
200 lb	(3)	6·06	5·87	5·68	5·46	29·99	0·3628	0·3490	25·57	36·03	39·68
230 lb	(4)	6·17	5·90	5·72	5·42	29·25	0·3641	0·3500	24·88	36·70	38·60
D.S.A.											
$P<0·05$		4>1	N.S.	*	N.S.	1>(3,4)	N.S.	N.S.	N.S.	(4,3)>1	N.S.
$P<0·01$		N.S.				N.S.				N.S.	
$P<0·001$											
Hogs	(H)	6·06	5·84	5·69	5·46	30·65	0·3607	0·3474	25·78	35·24	40·37
Gilts	(G)	6·06	5·82	5·71	5·43	30·58	0·3646	0·3477	25·51	34·44	41·83
D.S.A.											
$P<0·05$		N.S.	N.S.	*	N.S.	N.S.	N.S.	N.S.	N.S.	N.S.	N.S.

D.S.A., Differences significant at; A.S., all significantly different; N.S., no significant differences.

* Significant breed × weight × sex interaction. Hogs, 140 lb P>C; cross hogs (3, 4)>1 ($P<0·05$).

was much faster in the Pietrain pigs than in the Landrace, the Crosses being intermediate in this respect. Of the Pietrain carcasses, 77% showed stiffness in the forelegs at 45 min post mortem; comparable figures for the Pietrain × Landrace and Landrace being 22 and 13%, respectively. Temperatures recorded in the longissimus dorsi at 45 min post mortem were 1–3°F higher in the Pietrain than in the Landrace carcasses. The Pietrain carcasses were characterized by comparatively low pH_1 values, higher temperatures and rapid development of rigor. These features are known symptoms of watery pork.

The heaviest group of pigs (230 lb) averaged over all breeds showed a significantly higher pH_1 than the lightest group (140 lb).

The colour characteristics of the longissimus dorsi are also given in Table 1. The colour of the muscle is indicated in terms of the C.I.E. specification (Y, x and y) and also by the per cent reflectance (% R) at 525 nm. Both Y and % R showed very highly significant differences of brightness between breeds, the Pietrain muscle being paler than that of Landrace and Crosses. In brightness, the Pietrain × Landrace are more easily distinguished from the Pietrain than from the Landrace. By definition x and y are the ratios of the tristimulus values X and Y, respectively, to the sum $X + Y + Z$ of the three tristimulus values. x and y did not show significant differences attributable to breed, weight or sex; nor did the per cent metmyoglobin content of the muscle.

The means of the visual assessments did not show any significant differences, but samples scoring 3 or less for paleness and/or wetness (in a 7-point system) amounted to 55% for Pietrain and 32% for Landrace, indicating poorer overall quality in the Pietrain. These values may be compared with the results of Clausen & Thomsen (1960) and Ludvigsen (1960) who characterized the muscle as pale and watery in 89% of Pietrain hams, compared with 62% of Landrace hams.

Increase in pigment concentration would be expected to cause a loss of brightness, i.e. the meat would become less pale. This expectation is supported by the results in Table 1 which show that as the animal increases in weight, the concentration of pigment increases and Y correspondingly decreases. However, in the Pietrain muscle, where a significantly greater amount of pigment occurred than in the Landrace, the relative paleness of the Pietrain cannot be attributed to lack of pigment. At least two separate factors, independently of each other, are responsible for the variation in brightness, i.e. Y is influenced by: (1) those changes occurring in the muscle structure as indicated by pH_1, and (2) pigment concentration. A multiple correlation, with Y as the dependent variable and pH_1 and haematin as the independent variables was estimated using the data obtained for the longissimus dorsi. The regression coefficients for both pH_1 and haematin were found to be very highly significant and the straight line of best fit to these data was:

$$Y = 95{\cdot}9 - 9{\cdot}02\ (\pm\ 1{\cdot}02) \times pH_1 - 0{\cdot}305\ (\pm\ 0{\cdot}055) \times \text{ppm haematin.}$$

The standard errors of the regression coefficients are given in parentheses.

In this study, brightness was affected more by variation in pH_1 than by variation in pigment concentration. Thus, as is shown in Table 1, the Pietrain have the highest mean pigment concentration, nevertheless they are the palest owing to their low mean pH_1.

Pfau, Lohse & Schröder (1966) have shown that single wavelength reflectance readings are correlated with pH_1, and consequently % R at 525 nm may be used as a simpler alternative to Y. % R at 525 nm and Y were in fact found in this study to be very highly significantly correlated ($r = 0{\cdot}96$).

Protein solubility

The results of determinations of protein solubility are given in Table 2. Denaturation of sarcoplasmic and myofibrillar proteins (as measured by loss of solubility) were considerably greater in the Pietrain than in the Pietrain × Landrace and Landrace. These differences were very highly significant. A highly significant level of correlation also exists between the rate of glycolysis (as measured by pH_1) and the denaturation of sarcoplasmic ($r = 0{\cdot}37$), and myofibrillar ($r = 0{\cdot}61$) protein. These results are in accord with the work of Sayre & Briskey (1963) and Bendall & Wismer-Pedersen (1962). Briskey (1964) also found the development of pale and watery meat to be associated with rapid anaerobic glycolysis, low pH_1 and higher muscle temperature in the early post mortem period. Such conditions cause denaturation of sarcoplasmic

and myofibrillar proteins, with consequent changes in gross morphology of the muscle, and loss of colour and juice retention. The highly significant difference in brightness between the muscles of the Pietrain breed and those of Landrace or Pietrain × Landrace provide further evidence in support of this hypothesis.

The extent of protein denaturation observed in the Pietrain × Landrace was similar to that in the Landrace, as would be expected from the similarity of their respective rates of post mortem glycolysis. Thus the Pietrain × Landrace resembles Landrace rather than Pietrain in respect to those changes which are characterized by pH_1 and which affect protein denaturation.

The solubility of sarcoplasmic and myofibrillar protein was unaffected by the sex of the pig. The results show some tendency for myofibrillar protein denaturation to increase in the higher weight groups but this difference was not statistically significant.

W.H.C. and compressibility ratio

The mean W.H.C. values shown in Table 2 did not differ significantly between breeds. This is contrary to our earlier experience (unpublished) with Pietrain pigs

TABLE 2. Means and significance of differences for wetness and texture characteristics for the longissimus dorsi

		pH_1	Soluble sarcoplasmic protein (mg/g)	Soluble myofibrillar protein (mg/g)	Water holding capacity (% bound water)	Compressibility ratio (area/weight)	Tenderness value
Landrace	(L)	6·33	69·3	87·4	33·2	0·833	0·675
Pietrain × Landrace	(C)	6·16	69·6	80·8	33·7	0·820	0·773
Pietrain	(P)	5·70	60·4	56·6	33·1	0·717	1·187
D.S.A. $P<0·05$					N.S.		
D.S.A. $P<0·01$		A.S.					
D.S.A. $P<0·001$		(L,C) > P	(C,L) > P	(L,C) > P		(L,C) > P	P > (C,L)
140 lb	(1)	5·94	66·4	80·7	32·2	0·762	0·857
170 lb	(2)	6·07	63·5	76·8	33·0	0·783	0·946
200 lb	(3)	6·06	69·3	72·8	34·0	0·793	0·845
230 lb	(4)	6·17	66·5	69·4	34·2	0·822	0·865
D.S.A. $P<0·05$		4 > 1	N.S.	N.S.	(4,3) > 1;4 > 2	N.S.	N.S.
D.S.A. $P<0·01$		N.S.			(4,3) > 1		
D.S.A. $P>0·001$					N.S.		
Hogs	(H)	6·06	66·0	74·9	33·6	0·797	0·878
Gilts	(G)	6·06	66·9	75·0	33·1	0·783	0·878
D.S.A. $P<0·05$		N.S.	N.S.	N.S.	N.S.	N.S.	N.S.

D.S.A., Differences significant at; A.S., all significantly different; N.S., no significant difference.

where the Pietrain samples were found to have a significantly lower level of bound water than Landrace or Pietrain × Landrace.

W.H.C. appeared to be related to weight. In the two highest weight groups, W.H.C. was greater than in the lowest weight group and this difference was highly significant. It appears that W.H.C. tends to increase with increasing weight of the animal.

A highly significant correlation between pH_1 and W.H.C. was found in the Landrace ($r = 0{\cdot}47$) and Pietrain ($r = 0{\cdot}31$) samples, but not among the Pietrain × Landrace.

The W.H.C. results were also used to calculate rigidity or compressibility ratios and these are also shown in Table 2. Similar values have been determined by Hamm & Deatherage (1960) who estimated the rigidity of meat tissues from the results of a W.H.C. determination.

The mean compressibility ratio was smaller, indicating a greater resistance to compression, in the Pietrain samples than in either of the other groups ($P < 0{\cdot}001$). Compressibility was shown, by Grau & Fritz (1964), to be related to tenderness in beef, as measured by shear values. In the present work, compressibility ratios showed a very highly significant negative correlation ($r = -0{\cdot}63$) with tenderness values.

The means of the compressibility ratios showed no correlation with weight, and there was no significant difference between hogs and gilts. There was, however, a very highly significant correlation with pH_1 ($r = 0{\cdot}62$), indicating that compressibility is affected by the post-mortem glycolytic rate, possibly owing to changes associated with rigor mortis.

Tenderness values

The results shown in Table 2 indicate a substantial difference in the mean values for the three breeds. Pietrain meat showed a higher mean value (greater toughness), which was very highly significantly different from that of Landrace and Pietrain × Landrace. There was no relation between increasing weight and tenderness values of the raw meat.

The higher tenderness values of the Pietrain samples is probably related to the rapid post-mortem glycolysis which characterized the Pietrain muscles. The negative correlation between pH_1 and tenderness values was very highly significant ($r = -0{\cdot}76$). The relationship between tenderness and the rate of glycolysis, which determines the pattern of rigor mortis and loss of muscle extensibility, has been reported by Bendall (1963), Cover, Hostetler & Ritchey (1962) and de Fremery & Pool (1963). In Pietrain carcasses it would appear that these complex changes in the muscle occur at a faster rate than in the Landrace and Pietrain × Landrace carcasses. This results in the Pietrain meat tending to be tougher, as well as paler and wetter, than meat from the other two breeds.

Appearance of the semitendinosus

The semitendinosus muscle is two-toned, consisting of a light coloured distal portion

and a dark coloured proximal portion relative to the femur. Marked two-toning is considered an important defect in hams.

The semitendinosus was chosen in this experiment as being indicative of the extent of possible two-toning in the ham.

The appearance characteristics are shown in Table 3. Y was measured on both the light (l) and dark (d) portions. The light portion was found to be very highly

TABLE 3. Means and significance of differences for appearance characteristics for the semitendinosus

	Y_l	Y_d	$Y_l - Y_d$	$\frac{Y_l - Y_d}{Y_l}$	Haematin$_l$ (ppm)	Haematin$_d$ (ppm)	$pH_{ext.l}$	$pH_{ext.d}$	Light muscle (%)
Landrace (L)	23·48	11·57	11·92	0·500	40·51	106·00	5·90	6·23	57·5
Pietrain × Landrace (C)	23·73	12·59	11·14	0·455	49·31	119·25	5·83	6·08	58·8
Pietrain (P)	29·61	11·81	17·80	0·586	45·21	116·35	5·82	6·09	56·5
D.S.A.									
$P < 0·05$		N.S.				(C,P) > L	N.S.	L > (P,C)	N.S.
$P < 0·01$				P > (L,C)	C > L	C > L		N.S.	
$P < 0·001$	P > (C,L)		P > (L,C)	P > C	N.S.	N.S.			
140 lb (1)	27·35	14·29	13·06	0·461	38·84	96·92	5·75	6·03	59·2
170 lb (2)	26·67	13·09	13·59	0·492	44·38	114·52	5·84	6·12	56·8
200 lb (3)	24·30	10·47	13·84	0·543	47·84	115·93	5·92	6·19	57·5
230 lb (4)	24·11	10·12	13·99	0·599	48·98	128·10	5·90	6·20	56·8
D.S.A.									
$P < 0·05$	N.S.		N.S.	(4,3) > 1;4 > 2		4 > (3,2) > 1	N.S.	N.S.	N.S.
$P < 0·01$				(4,3) > 1	(4,3) > 1	4 > 2 > 1; 3 > 1			
$P < 0·001$		(1,2) > (3,4)		N.S.	N.S.	(4,3,2) > 1			
Hogs (H)	24·88	12·12	12·76	0·494	44·30	113·73	5·88	6·17	57·7
Gilts (G)	26·34	11·86	14·49	0·534	45·72	114·00	5·83	6·11	57·4
D.S.A.									
$P < 0·05$	N.S.	N.S.	N.S.	N.S.	N.S.	N.S.	N.S.	N.S.	N.S.

l, Light portion; d, dark portion; D.S.A., differences significant at; N.S., no significant difference.

significantly different between breeds, the Pietrain being much paler than the Landrace or Pietrain × Landrace. No significant effect of weight was found.

In the dark portion of the semitendinosus, no difference in Y was detected between breeds, but a very highly significant effect of weight was seen, those portions from the heavier animals being darker.

The results for concentration of pigment and brightness, show the expected relationship between high concentration and darkness, low concentration and lightness. This is in agreement with the observations of Beecher *et al.* (1965) who investigated the red and white fibre content of the semitendinosus, and the associated post-mortem properties of the muscle.

The two-toning of the muscles of the ham would appear to be dependent on two separate variables, i.e. those muscles modified by breed characteristics and those modified by increasing weight.

The contrast between the light and dark areas may be of more practical importance than the absolute measure of either. Brown & Mueller (1965), in their discussion on 'brightness contrast', show that contrast can be given a quantitative specification by using the ratio of the difference between the values to either the darker or the lighter or some function involving both. The ratio $(Y_l - Y_d)/Y_l$ has been used in this experiment since contrast defined in this way varies between zero and 1·0.

The advantage of studying contrast can be seen from the results in Table 3, where the difference, i.e. $(Y_l - Y_d)$ only illustrates the difference in breed, while contrast as expressed by the ratio shows the effects of both breed and weight. The results indicate the possibility that the Pietrain × Landrace is an improvement on the Pietrain, the Pietrain showing the greatest contrast ($P < 0{\cdot}001$). Increased weight also increases the level of contrast ($P < 0{\cdot}001$).

The pH_{ext} for both the light and the dark portions did not show any significant differences between breed or weight, except that the pH_{ext} of the Landrace was significantly higher in the dark portion. There was however a difference of about 0·2 pH units between the light and dark portions. This difference as well as the differences between the portions in respect to Y and pigment concentration were all very highly significant. This confirms the observations of Beecher *et al.* (1965) and also Topel *et al.* (1966), who studied the variations in the physical and biochemical properties of different ham muscles. The results of the subjective judgement of relative abundance of light muscle in the sample do not show any significant differences. This may well have been due to the difficulty experienced in judging where the demarcation between light and dark portions occurred. In some cases the border between the halves was distinct, while in others there was no definite boundary.

As an adjunct to this trial, the colour of the subcutaneous fatty tissue immediately above the longissimus dorsi was measured. The results are given in Table 4. The Gardner colour difference meter term for brightness (L) showed no difference between breeds, but a very highly significant effect of weight for gilts only. This difference, being apparent in gilts only, may be due to different fattening rates between the sexes. In the case of the younger animals with little fat, the increased proportion of connective tissue could lower the brightness value. There was no significant hue difference between breeds, but θ was significantly correlated to L ($r = 0{\cdot}24$). In the heavier animals, the appearance of the fatty tissue was lighter and less pink.

In conclusion it may be stated that the main Pietrain characteristic was the extremely rapid rate of post-mortem glycolysis. This resulted in the quality defects, paleness, wetness and toughness being more noticeable than in the Landrace. In addition, two-toning as measured by the contrast in the semitendinosus was accentuated in the Pietrain.

It has been substantiated that the rate of glycolysis, as deduced from the pH at 45 min post mortem, is indicative of the subsequent quality of the meat. This is evident from the relationship between pH_1 and protein solubility, compressibility, shear values,

TABLE 4. Means and significance of differences for colour of inner subcutaneous fat

		Brightness (L)	Hue angle (θ°)
Landrace	(L)	72·89	63·9
Pietrain × Landrace	(C)	72·61	63·9
Pietrain	(P)	72·69	61·7
D.S.A. $P<0·05$		*	N.S.
D.S.A. $P<0·01$			
D.S.A. $P<0·001$			
140 lb	(1)	71·63	57·3
170 lb	(2)	72·69	62·4
200 lb	(3)	72·87	67·1
230 lb	(4)	73·73	65·7
D.S.A. $P<0·05$		*	(3,4,2) > 1; 3 > 2
D.S.A. $P<0·01$			
D.S.A. $P<0·001$			(3,4) > 1
Hogs	(H)	72·94	62·9
Gilts	(G)	72·51	63·4
D.S.A. $P<0·05$		*	N.S.

D.S.A., Differences significant at; N.S., no significant difference.

* Significant weight × sex interaction. Gilts (4, 3) > (1, 2) ($P<0·01$); 4 > (1, 2) ($P<0·001$).

water holding capacity and paleness. Paleness was also shown to be more dependent upon the rate of glycolysis than upon the pigment concentration.

In the majority of the parameters studied, the Pietrain × Landrace gave values intermediate between those of the two parent breeds, but tended to be closer to those of the Landrace than in the Pietrain.

Presumably, if the rate of glycolysis in Pietrain carcasses could be reduced by some means, the quality would improve. One of the factors which may be important in this respect is the observed excitability of the Pietrain under stress. Improved pre-slaughter management planned to avoid stress ought to reduce some of the quality defects.

Against any commercial advantages the Pietrain carcass may have in conformation must be set the probability that there will be defects in the quality of the meat.

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Bovine collagen

I. Changes in collagen solubility with animal age

D. J. CARMICHAEL* AND R. A. LAWRIE

Summary. The changes with animal age in the concentrations of five forms of collagen of differing solubility characteristics were followed in bovine l. dorsi, skin and tendon. Those forms which were extractable in neutral salt and dilute acid were found to increase swiftly during the gestation period and fall to low levels between birth and 1–2 years of age in the three tissues. After birth there was a rapid rise in the concentrations of the more insoluble forms of collagen and these greatly predominated by 1–2 years of age in each tissue. The relative time relations of the changes were compatible with a transformation of the easily extractable forms into the more insoluble ones with increasing age.

In muscular tissue only, after about 2 years of age, there was some diminution in total collagen and also in the proportion of the latter which resisted extraction. Possible reasons were discussed.

Introduction

Recent research work at the Meat Industry Research Institute of New Zealand has implicated the myofibrillar proteins in accounting for the toughness of meat. Within certain limits, the degree of interdigitation of myosin and actin filaments during the onset of *rigor mortis* has been shown to be directly related to toughness (Locker, 1960; Locker & Hagyard, 1963; Marsh & Leet, 1966). Muscles cooked pre-rigor, however, are tender despite severe contraction (Marsh, 1964), possibly because post mortem glycolysis is arrested whilst the pH is high (R. A. Lawrie, unpublished observations). Nevertheless, it is important to appreciate that these considerations apply to a given muscle from animals of a given age and species. When the degree of post mortem contraction is not the major variable, muscles differ in toughness within a given animal according to their location; and a given muscle differs in toughness according to the age of the animal from which it is derived. Such differences might seem explicable on the basis of the relative connective tissue content; and, in particular, by the concentration of its main constituent, collagen. Nevertheless, analysis has shown that

Authors' address: Food Science Laboratories, University of Nottingham.

* Present address: Northwestern University, Chicago, Illinois, U.S.A.

the collagen content of the musculature of mature bovine animals is 50% less than that of veal calves, despite their much greater toughness (Orekhovitch, 1952; Wilson, Bray & Phillips, 1954; Lawrie, 1961). An indication that the nature of the collagen molecule might change with increasing animal age was reinforced by recent evidence showing that the connective tissue of the musculature of older bovines was less susceptible to breakdown by heat (Sharp, 1963–64) or by collagenase (Goll, Hoekstra & Bray, 1964). Moreover, Verzar (1963–64) observed that there was a steady increase in the tensile strength of the collagen from rat tail tendon with increasing age.

It is presently accepted that the collagen molecule consists basically of three polypeptide, left-hand helices, which are intertwined to form a right-hand super helix (Ramachandran & Kartha, 1954; Rich & Crick, 1961). This structure, with an approximate molecular weight of 300,000, is referred to as tropocollagen. Early chromatographic analyses of denatured soluble collagens indicated that the three polypeptide strands could be distinguished from one another (Piez, 1965), being identified as α_1, α_2 and α_3; and it was shown that α_2 was markedly different in amino acid composition from the α_1 and α_3 components which were closely similar. The latter two components could only be separated with difficulty. These results were supported by a number of other workers (Hollmen & Kulonen, 1964; Pikkarainen & Kulonen, 1964; Veis & Anesey, 1965; Heidrich & Wynston, 1965). Recent work by Piez and his colleagues, however, has suggested that α_1 and α_3 are in fact a single molecular species, although a minor variant of unknown origin may be detectable (Butler & Piez, 1967).

Ultracentrifugal studies of collagen preparations which have been denatured by heating to *ca.* 40°C indicate that the component chains may be cross-linked with one another to varying degrees. Such cross-linking may be either intramolecular or intermolecular in nature (Veis & Anesey, 1965). If two α chains (molecular weight *ca.* 100,000) are so linked a dimer of molecular weight *ca.* 200,000 is obtained: it is referred to as the β component. When three α chains are cross-linked a trimer of molecular weight *ca.* 300,000 is obtained (γ component), (Nishihara & Doty, 1958; Piez, Weiss & Lewis, 1960; Lewis & Piez, 1964).

Jackson & Bentley (1960) suggested that, in developing connective tissue, there is a continuous spectrum of collagen aggregates of varying degree of cross-linking, the exact nature of which is determined by the age of the animal. Subsequently, Veis & Anesey (1965) postulated that the cross-links are predominantly intermolecular in mature collagen. The insolubility of mature collagen has been explained by the covalent nature of such bonds (Reed, Stainsby & Ward, 1963); as well as by the highly cross-linked nature of this form of collagen. A review of this complex subject has recently been published (Harding, 1965).

The solubility of collagen reflects these various aspects of inter- and intramolecular cross-linking between the α chains of the tropocollagen molecules; and thus the changes occurring as maturation proceeds. A proportion of collagen is extractable by neutral

salt solutions (NSS collagen; Gross, Highberger & Schmitt, 1955): another portion requires dilute acid to make it soluble (AS collagen: Orekhovitch *et al.*, 1948). 'Mature' collagen which resists such solvents can be solubilized by enzymic, physical or more severe chemical means. Some destruction of intermolecular bonds must occur in these circumstances (Ward, 1959).

Against this background it was felt desirable to carry out a detailed study of NSS, AS and mature collagen in the bovine—a species in which the degree of tenderness is a significant aspect of eating quality—in relation to animal age; and to compare a tissue containing collagen more or less exclusively (tendon) with those of intermediate or low collagen content (skin and muscle respectively).

As an essential aspect of such an investigation it was clearly necessary to establish the relative changes in concentration of NSS, AS and mature collagens extractable from the three tissues and by which the collagen fraction of each of these tissues could be characterized.

Materials

The samples of bovine tissue studied were derived from l. dorsi (lumbar region), skin (mild ventral regions) and shin tendons. The age of the animals ranged from pre-natal to 8 years post-natal.

Methods

(i) *Determination of hydroxyproline*

Because collagen is the only animal protein known to contain appreciable amounts of hydroxyproline, the concentration of this amino acid may be used as a measure of the collagen content of a tissue. The hydroxyproline content of samples was taken to be proportional to their collagen content. Samples were hydrolysed for 3 hr at 130°C in 6 N HCl (sealed tubes). Losses were of the order of 2%. Hydroxyproline was determined in the hydrolysates by the method of Woessner (1961).

(ii) *Determination of tyrosine*

Samples were checked for contamination by non-collagenous proteins by determining the tyrosine content using the method of Ceriotti & Spandrio (1956). The acceptable upper limit was regarded as 1% tyrosine, since tyrosine has been reported present in collagen only up to 0·5%.

(iii) *Determination of nitrogen*

Nitrogen fractions were determined by the micro-Kjeldahl technique.

(iv) *Preliminary extraction of non-collageneous proteins*

A preliminary extraction of each tissue was carried out to remove non-collagenous protein. This procedure was particularly important with muscular tissue where collagenous protein constituted only a small proportion of total protein of the tissue.

In the procedure adopted the tissue was frozen in liquid nitrogen, comminuted by a pestle and mortar and extracted with excess 7 M urea at 0–4°C for 12 hr. The collagenous residue was removed by centrifuging at 1500 ***g*** for 30 min and washed four times with cold distilled water. The various collagen fractions could then be isolated by extraction of the residue using the appropriate medium.

In a separate series of experiments (Carmichael & Lawrie, 1967) the collagen fractions prepared here were further characterized using electrophoretic techniques. For this reason it was important that these collagen preparations were not contaminated by non-collagenous proteins. Consequently, although the method used here for the preliminary removal of non-collagenous proteins involved a small loss of NSS collagen, this was felt to be justified by the avoidance of contamination with sarcoplasmic and myofibrillar proteins. Subsequent purification procedures were then less complex.

(v) *Neutral salt soluble collagen*

It was found that 1 M NaCl in 0·01 M sodium phosphate buffer, pH 7·0, was the most satisfactory standard extractant for NSS collagen. As the literature indicated that there was considerable variability in the time of extraction regarded as effective, this was established in the present work for the three tissues studied. Although Gross *et al.* (1955) found that minute quantities of NSS collagen could still be removed from calf skin even after eighteen extractions, the present data (Table 1) suggested that it

TABLE 1. The extractability of neutral salt soluble collagen from bovine tissue as a function of time (extractant: M sodium chloride/0·01 M sodium phosphate buffer pH 7·0)

Extraction No.	Time (hr)	Muscle* (μg/g)	Skin† (μg/g)	Tendon (μg/g)
(a) Foetal (5 months gestation)				
1	24	73	189	175
2	72	77	182	308
3	72	18	57	154
4	24	10	29	46
Total		178	457	683
(b) Adult (4-year-old steer)				
1	24	29	63	42
2	72	13	24	16
3	72	10	16	5
4	24	3	1	0
Total		55	104	63

* The muscle used was l. dorsi.

† Figures refer to the hydroxyproline content of the extract expressed in μg/g of tissue on fresh weight basis.

would be realistic to limit the number to four (of duration 24, 72, 72 and 24 hr). This procedure was applied to the residues obtained after removal of non-collagenous protein as in (iv).

(vi) *Acid soluble collagen*

Although the ionic strength and pH of citrate buffer employed to extract AS collagen have varied widely in the literature, it was decided to use 0·15 M citrate buffer, pH 3·7, following Higgs & Reed (1963). Experiments to determine the optimum number of extractions required were carried out. While the results showed that AS collagen was less easily removed from the skin of adult bovines than from muscle or tendon, it appeared that four extractions (of duration 24, 72, 72 and 48 hr) would remove most of this fraction (Table 2). The routine procedure for the extraction of AS collagen was as follows:

TABLE 2. The extractability of acid soluble collagen from bovine tissue as a function of time (extractant: 0·15 M citrate buffer, pH 3·7)

Extraction No.	Time (hr)	Muscle* (μg/g)†	Skin (μg/g)	Tendon (μg/g)
(a) Foetal (5 months gestation)				
1	24	20	22	96
2	72	16	28	23
3	72	8	9	13
Total		44	59	132
(b) Adult (4-year-old steer)				
1	24	5	84	26
2	72	5	81	25
3	72	0	53	4
4	48	0	24	0
Total		10	242	55

* Muscle used was l. dorsi.

† Hydroxyproline content of tissue extract expressed as μg hydroxyproline per g of tissue.

(a) The tissue residue from the extraction of NSS collagen was washed four times with cold distilled water.

(b) The washed residue was extracted in 0·15 M citrate buffer, pH 3·7, for 24 hr, re-extracted for two further periods each of 72 hr, and finally for a fourth period of 48 hr.

(vii) *Mature collagen*

Mature collagen is the fraction remaining after extraction of NSS and AS collagens (Veis & Anesey, 1965). To permit a detailed study of mature collagen it was necessary to employ a method which would selectively destroy the bonds which cause its insolubility in salt and dilute acid. Pre-treatment with alkali appears to be milder than the use of strong acid (Veis, Anesey & Cohen, 1957). The product then extracted by citrate buffer is termed eucollagen (Ward, 1959).

The method used was essentially that of Higgs & Reed (1963):

(a) The tissue residue remaining after extraction of AS collagen was washed four times with cold distilled water.

(b) It was then soaked overnight at 20°C in saturated sodium sulphate, followed by 5 days at 20°C in saturated sodium sulphate containing 5% NaOH.

(c) The tissue was next soaked three times, each for 1 hr, in saturated NaCl, and, finally, with saturated NaCl containing acetic acid to bring the pH to 6.

(d) The tissue residue was freed from NaCl by soaking in distilled water for two 12-hr periods at 0–4°C.

(e) Finally, the eucollagen was extracted by soaking for five periods each of 48 hr in 0·15 M citrate buffer, pH 3·7.

Higgs & Reed (1963) suggested that (following the salt and alkali pre-treatment) the initial extracts by citrate buffer contained little eucollagen. The results obtained in the present work indicated, however, that the first extraction generally contained a significant amount of eucollagen.

Although the major portion of the 'mature' collagen of foetal tissue was solubilized by the procedure described, a substantial amount of the mature collagen from tissues of the adult bovine remained insoluble. It was found, however, that dissolution of a large portion of this residue could be achieved by an additional extraction at higher temperature. The residue from the fifth low temperature extraction of eucollagen by citrate buffer was heated to 45°C for 60 min in citrate buffer which effectively gelatinized a large proportion of the residue. A distinction has been made, therefore, between low temperature and high temperature extracted eucollagen. As used here, the term eucollagen denotes, therefore, low temperature extracted eucollagen (LTE) plus high temperature eucollagen (HTE) unless otherwise specified.

With tissue from adult bovines a small proportion of the mature collagen remained insoluble even after such high temperature treatment. It could only be dissolved by autoclaving and is here referred to as the insoluble fraction of mature collagen.

Results

In all cases concentrations of hydroxyproline have been used as equivalent to the concentrations of collagen. From Fig. 1 it may be seen that the concentration of NSS collagen increases swiftly during gestation in the three tissues studied and, just before birth, reaches a maximum which is greatest in tendon ($\sim$700 μg/g), inter-

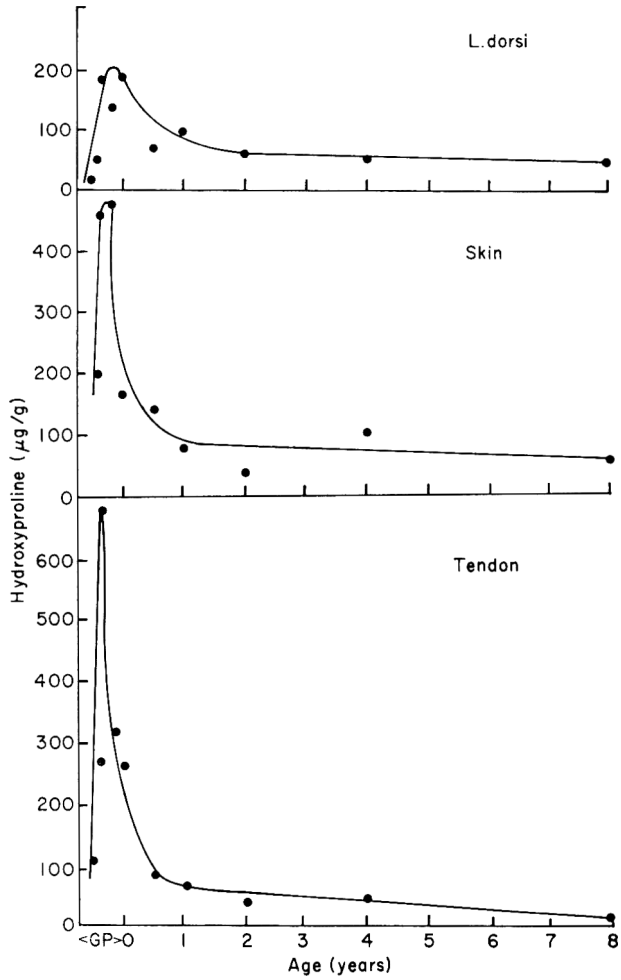

FIG. 1. The content of neutral salt soluble collagen (expressed as hydroxyproline) in tissues from bovines of different ages.

mediate in skin (~ 550 μg/g) and least in l. dorsi (~ 200 μg/g). After birth there is a rapid fall in NSS collagen to a low level which is fairly constant with increasing animal age above 1 year. This fall is especially rapid in tendon and in this tissue NSS collagen is virtually absent in the older bovine.

In the case of AS collagen there is again a swift increase in concentration during the gestation period in all three tissues studied (Fig. 2); but the maxima achieved (~ 120, 350 and 150 μg/g in l. dorsi, skin and tendon, respectively) are markedly less than the corresponding levels of NSS collagen. Moreover, while the level of AS collagen has fallen to relatively low value by birth in l. dorsi, the decreases in tendon

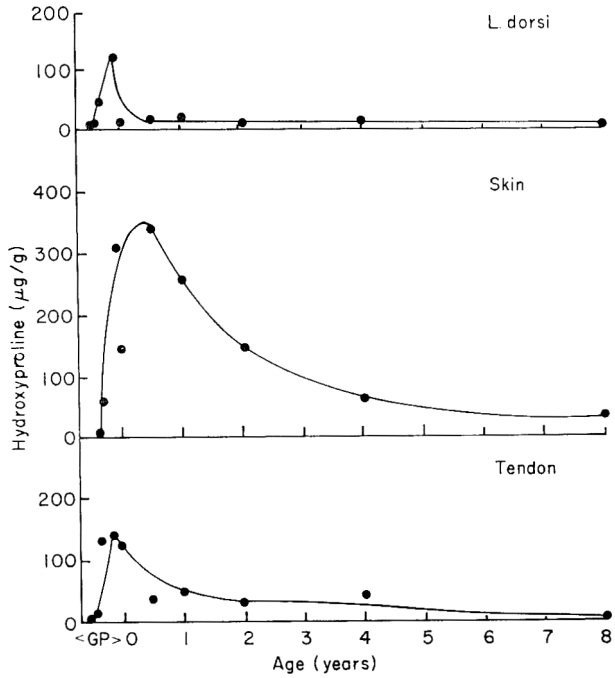

FIG. 2. The content of acid soluble collagen (expressed as hydroxyproline) in tissues from bovines of different ages.

and, especially, skin are much slower. There are appreciable quantities of AS collagen in tendon up to an age of 2 years and in skin to 4 years.

The data on eucollagen in Table 3 show, as general features, that the proportion of mature collagen solubilized as eucollagen (i.e. the sum of that extracted – after the alkali treatment specified above – at ambient temperature and at 45°C) is much less in l. dorsi than in skin or tendon; and that the level in tendon is greater than that in skin. Another general feature of the data recorded in Table 3 is the trend in extraction as eucollagen with age. These suggest that the amount of mature collagen extractable as eucollagen increases from the foetal stage to about 2 years of age: thereafter, there is some suggestion of a decrease – especially in the tissues of the 8-year-old bovine. The data in Table 3 show that, while most of the eucollagen extractable from mature collagen is obtained in the low temperature form in the three foetal tissues, the proportion extractable at 45°C increases as the animal grows older. This effect is especially marked in skin and tendon. There is some evidence that, in the 8-year-old bovine, the quantity of the more intractable form of mature collagen diminishes relatively to the more easily soluble variety.

As further evidence that the mature collagen in l. dorsi is more readily soluble than that in skin and tendon, the data in Table 3 show that, at most ages, the majority

TABLE 3. The eucollagen content (expressed as hydroxyproline) of the tissues from bovines of different ages

Age	Tissue	Eucollagen extracted at low temperature extract No. (μg/g)						Eucollagen extracted at high temperature (45°C) (μg/g)	Total eucollagen (μg/g)
		1	2	3	4	5	Total		
Foetal	L. dorsi	177	43	10	4	1	235	6	241
4–5 months	Skin	241	148	67	104	68	628	54	682
gestation	Tendon	609	840	410	270	92	2221	127	2348
6 months	L. dorsi	103	82	62	26	42	315	497	812
	Skin	760	358	636	401	210	2365	15900	18265
	Tendon	680	622	332	174	256	2044	16050	18094
1 year	L. dorsi	51	40	16	13	14	134	462	596
	Skin	445	261	165	194	138	1203	17400	18603
	Tendon	155	423	162	151	223	1114	20200	21314
2 years	L. dorsi	31	14	5	4	3	57	2312	2369
	Skin	537	410	274	197	40	1458	10200	11658
	Tendon	275	254	143	90	55	817	14700	15517
4 years	L. dorsi	19	14	9	5	4	51	192	243
	Skin	350	278	272	133	99	1132	11000	12132
	Tendon	562	273	348	195	202	1580	10600	12180
8 years	L. dorsi	5	193	1	1	2	202	177	379
	Skin	574	271	51	47	44	987	4670	5657
	Tendon	51	83	91	31	29	285	8325	8610

of the eucollagen which is soluble in citrate buffer without heat treatment appears in the first extract; whereas three or four extractions are required in skin and, especially, tendon to remove this component.

In Fig. 3 the percentage of the total mature collagen which requires treatment at 45°C before extraction as eucollagen has been plotted against animal age. In the three tissues studied there is a rapid decrease in solubility, as determined by this criterion, from gestation, when almost all the mature collagen can be extracted without prior treatment at 45°C, to 6 months after birth in skin and tendon and to about 2 years in muscle, when a high level of insolubility is found. Whereas in the former two tissues the insoluble nature of the mature collagen remains at a fairly constant high level, the more soluble form extractable as low temperature eucollagen appears to increase again in the l. dorsi of 4–8-year-old bovines.

The results recorded in Table 4 show the change with age, in the three tissues, of the total collagen soluble without heat treatment (i.e. NSS, AS and LTE collagens), the total of all forms of collagen in the tissues and the percentage of the latter which is insoluble. It may be seen that the level of total collagen is much greater in skin and

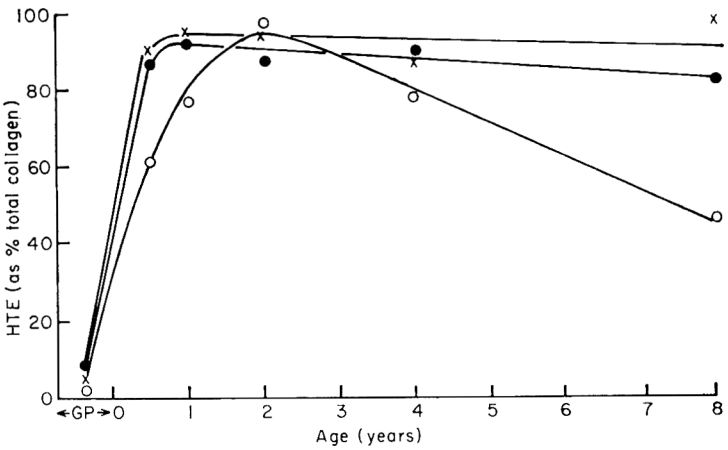

FIG. 3. Effect of bovine age on percentage of eucollagen requiring pre-treatment at 45°C before extraction. ○ = L. dorsi; ● = skin; × = tendon.

TABLE 4. Soluble* and total collagens (as hydroxyproline) in the tissues from bovines of different ages

Age	Tissue	Collagen soluble* (μg/g)	Total collagen (μg/g)	Total collagen insoluble* (%)
Foetal	L. dorsi	456	462	1
4–5 months	Skin	1142	1196	4
gestation	Tendon	3036	3163	4
6 months	L. dorsi	421	898	53
	Skin	2855	18755	75
	Tendon	2188	18238	88
1 year	L. dorsi	253	715	64
	Skin	1526	18926	92
	Tendon	1233	21433	94
2 years	L. dorsi	123	2435	95
	Skin	1640	11840	88
	Tendon	1188	15588	92
4 years	L. dorsi	123	426	71
	Skin	1300	19647	93
	Tendon	1630	19470	92

* Sum of NSS, AS and low temperature extracted eucollagen.

tendon than in l. dorsi; and that this difference becomes appreciably greater after birth. Moreover, the percentage of insoluble collagen swiftly increases to a plateau in all three tissues with increasing animal age—especially in skin and tendon. Nevertheless, it is noteworthy that the total collagen in the l. dorsi of the 4-year-old bovine is markedly lower than that in animals aged 6 months to 2 years.

Discussion

These data have confirmed that the total collagen content of muscle diminishes after the animal has attained about 2 years of age (cf. Wilson *et al.*, 1954; Lawrie, 1961) but that total collagen remains at a high level in skin and tendon (Smits, 1957). The results have also clearly indicated that there are marked differences in the solubility of bovine collagen with increasing animal age. Moreover, such differences are not merely reflected by an increased difficulty of extraction of a given molecular species. That there is a spectrum consisting of at least five independent entities follows from the changes observed in the concentrations of collagens which are characterized by their solubility in neutral salt, in acid, or in acid after alkali treatment both at ambient temperature and at 45°C; or by their insolubility in such extractants. Even in foetal tissue, despite the great predominance of readily soluble forms, the intractable species are represented.

The behaviour of these forms with time differs. On the one hand, in muscle, skin and tendon, both NSS and AS collagens are only present in appreciable quantities during the period of gestation, when they rise swiftly, falling to low levels between birth and 1–2 years of age. On the other hand, mature collagen, especially that form requiring prior exposure to 45°C for extraction as eucollagen and residual insoluble mature collagen, predominate in all three tissues in the animal by 1–2 years of age. The insolubility of mature collagen is said to reflect not only *intra*molecular cross bonds (Verzar, 1963, 1964), but also an increasing development of *inter*molecular bonding (Veis & Anesey, 1965).

The changes with animal age in the concentrations of collagen fractions in Figs. 1 and 2, and Tables 3 and 4 are compatible with the interpretation that both NSS and AS collagens are precursors of the various forms of mature collagen; and isotopic incorporation experiments have led to a similar conclusion (Harkness *et al.*, 1954; Jackson, 1957; Jackson & Bentley, 1960). Nevertheless, several studies have indicated that the turnover rate of AS collagen is too slow to serve as an intermediate between NSS and mature collagens (Nimni, de Guia & Bavetta, 1965); and Veis & Anesey (1965) suggest that the presence of *intra*molecular bonds in AS collagen would prevent its incorporation into mature collagen. Alternatively, AS collagen may represent a breakdown product, being a degraded form of mature collagen (Lapiere, 1966).

It is possible that the solubility characteristics of NSS, AS, LTE and HTE, and insoluble collagens represent a series of progressively greater *intra-* and *inter*molecular cross-bonding with increasing animal age—conceivably to accommodate the greater mechanical stresses of increasing bulk. The significance of the decrease in the concentration of total collagen, and of the percentage of mature collagen requiring prior exposure to 45°C for extraction as eucollagen in muscular tissue after about 2 years of age, is uncertain. In bovine musculature, the swift increase in the concentrations of sarcoplasmic and myofibrillar proteins, which constitute the major components of

the muscle cell, has ceased by about 12–18 months of age (Lawrie, 1961). It might thus be reasonable to explain the concomitant increase in total and insoluble collagens by the circumstances governing the general maturation of the tissue; and it might be supposed that the subsequent fall in the concentration of total collagen could be explained on the basis of dilution by myofibrillar and sarcoplasmic elements. There is no evidence, however, for an increased concentration of these elements in bovine musculature after maturity, when determined on a whole tissue basis. On the other hand the percentage of intramuscular fat *does* increase in this period (Lawrie, 1961); and the observed fall in the concentration of total collagen can be rationalized, at least partly, on the basis of dilution by fat. Moreover, it is conceivable that the deposition of fat in the connective tissue may well dilute the collagenous elements and this leads to a lower apparent concentration of collagen in this tissue. This may explain the observed fall in the proportion of mature collagen requiring pre-treatment at 45°C for extraction as eucollagen in the muscles of older animals. It is of interest that there should be a gradual increase in the myoglobin concentration of bovine muscle after maturity (Lawrie, 1961). This suggests that there is increasing difficulty of oxygen supply in the older animal. The deposition of fat, adduced to explain changes in mature collagen, may also interfere with blood distribution.

The results reported here provide suggestive evidence of an increase with chronological age in the number or strength of the cross-links of bovine collagen. It is for this reason that a consideration of the total amount of intramuscular collagen is inadequate to explain the toughness of meat with increasing chronological age. The degree of solubility of the collagen as well as the total amount should be considered when biochemical explanations of toughness in meat are sought.

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Bovine collagen

II. Electrophoresis of collagen fractions

D. J. CARMICHAEL* AND R. A. LAWRIE

Summary. Vertical starch gel electrophoresis was used to separate components of neutral salt soluble (NSS) and acid soluble (AS) collagens, and of eucollagens, from muscle, skin and tendon of bovines of different age. The relative proportions of the separated components were assessed spectrophotometrically.

The electrophoretograms indicated that the greater difficulties of extraction which distinguish AS from NSS collagen reflect a greater measure of cross-bonding between the α chains of the former.

They also indicated that there was a marked increase in the proportion of components having *intra*molecular cross bonds, in both NSS and AS collagens and in the three tissues studied, between the 5th and 9th month of gestation. In older animals there were also indications of an enhanced degree of *inter*molecular cross bonding.

Such changes help explain the increased toughness of the meat from older bovines despite a lower percentage of collagen.

Introduction

The solubility of collagen from bovine tissue has been found to decrease as the age of the animal increases. In general, a major proportion of the total collagen has become relatively insoluble by 1–2 years of age (Carmichael & Lawrie, 1967). The solubility of collagen is believed to depend on the degree of intramolecular, and possibly intermolecular, cross-bonding between the component α-chains of the tropocollagen molecule. Within a given tropocollagen molecule, a dimer (β component) is formed when two of the α chains are linked; and, when all three α chains are cross-bonded, a trimer (γ component) is produced (Altzelt, Hodge & Schmitt, 1961); Veis, Anesey & Cohen, 1960, 1961).

A further stage of cross-bonding occurs in collagen when α chains of different tropocollagen molecules become linked (Veis & Anesey, 1965). Because of the differences in molecular weight and charge arising from these differing degrees of cross-

Authors' address: Food Science Laboratories, University of Nottingham.

* Present address: Northwestern University, Chicago, Illinois, U.S.A.

linking, it seemed likely that the application of starch gel electrophoresis to collagen fractions of differing extractability would further elucidate the changes in the polymeric state of collagen with animal age. As a prerequisite to such a study the experimental conditions appropriate for the effective electrophoretic separation of monomer, dimer and trimer components had to be determined. Two preliminary communications on this topic have already been published (Carmichael & Lawrie, 1966; Carmichael, 1966).

Methods

(i) *Sample preparation*

Techniques for the preliminary extraction of non-collagenous protein as well as for the extraction of neutral salt soluble (NSS), acid soluble (AS) and mature collagen fractions were as described previously (Carmichael & Lawrie, 1967).

(ii) *Purification procedure*

Non-collagenous proteins were removed from the NSS and AS collagen extracts by precipitation with sodium chloride.

Purification of the NSS collagen extracts was accomplished by salting-out this collagen fraction with sodium chloride added to a final concentration of 20%. The precipitate was then dissolved in 0·2 M sodium chloride and an equal volume of 5 M sodium chloride added to the solution. The precipitate was removed by centrifugation and redissolved in 0·01 M acetic acid.

The AS collagen extracts were purified by precipitation with sodium chloride to a final concentration of 5% and the precipitate redissolved in 0·01 M acetic acid.

The mature collagen extracts were not further purified. It was found that the prolonged alkali pre-treatment used in the preparation of the mature collagen fractions effectively removed any non-collagenous proteins.

(iii) *Detection of non-collagenous protein*

The purity of the preparations of NSS and AS collagens was investigated by two independent methods.

(a) *Tyrosine determination.* Collagen has been found to contain a tyrosine level of approximately 0·5% (Lowther, 1963), whereas amino acid determination of muscular tissue indicates a tyrosine content of approximately 3·0%. Therefore, tissue extracts containing more than 1·0% tyrosine in the protein fraction were regarded as including non-collagenous protein.

The method used for tyrosine analysis was that of Ceriotti & Spandrio (1956). For some routine determinations the spectrophotometric assay of Cobbett, Kenchington & Ward (1962) was employed.

Protein concentration being related directly to the hydroxyproline content of the sample, this amino acid was determined by the method of Woessner (1960). In some

instances the nitrogen content was also determined, using the micro-Kjeldahl procedure.

(b) *Enzymic digestion.* Purified NSS and AS collagen samples were incubated with collagenase following the method of Seifter *et al.* (1964). Since the enzyme digests only collagen and gelatin, the absence of stainable components on subsequent electrophoresis was taken as an indication that the methods of preparation employed for the various collagen fractions were satisfactory.

(iv) *Thermal denaturation of collagen*

To permit electrophoretic separation of the several components in the solubilized collagens it was essential to destroy the hydrogen bonds responsible for the secondary structure of the NSS and AS collagens, and also in the low temperature extracted (LTE) and high temperature extracted (HTE) eucollagen. Samples were, therefore, thermally denatured by heating to 40°C for 5 min. According to Veis (1964), the primary structure, and covalent cross-bonding, between the α chains of tropocollagen is retained under these conditions.

(v) *Starch gel electrophoresis*

(a) *General.* Starch gel electrophoresis was carried out in a vertical plane with sample insertion as a free solution in sealed slots as described by Smithies (1955a, b; 1959a). The apparatus was constructed so that the gel could be cooled or warmed as required (Fig. 1). The gel block was prepared in two stages. The two end sections were

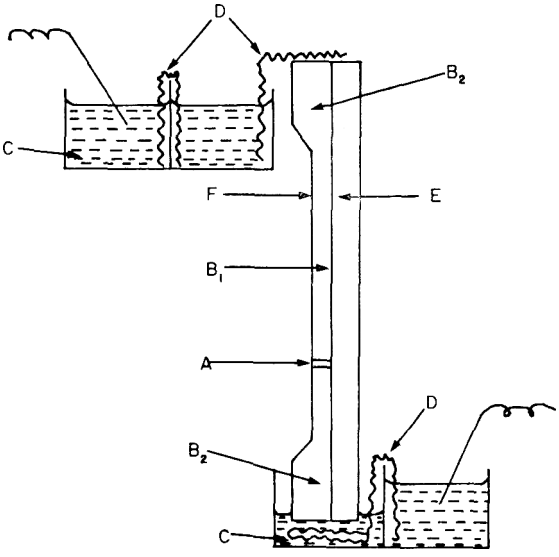

FIG. 1. Apparatus used for vertical starch gel electrophoresis. A = Sample slots; B_1= gel block (centre section;) B_2=gel block (end sections); C = electrode vessel; D=plastic foam bridge; E=thermo-regulatory compartment; F=plastic film.

poured first and allowed to set. Their inner edges were then trimmed off to leave a rectangular hollow into which the second gel was poured. Its dimensions were 12 cm wide, 18 cm long and 6 mm thick. The sample slots were formed in this centre section using a slot former (Smithies, 1959b) to carry ten slots 7 mm wide and 4 mm apart. In cases where a discontinuous buffer system was used the outer gel blocks incorporated the second buffer. Connection between the electrode vessels and the gel was maintained with sheets of plastic foam. Platinum electrodes were used.

(b) *Standardized operating conditions. NSS and AS collagens.* As a result of a series of ancillary experiments, it was found that the optimal separation of the components in NSS and AS collagens was achieved using a sodium acetate buffer (13 mM acetic acid; 17 mM sodium acetate; $I=0{\cdot}017$; pH 4·78) and starch concentration 15%. Before electrophoresis all samples were dialysed against this buffer.

Electrophoresis was carried out in a constant temperature room at 37°C for 9 hr (200 V, 30 mA).

Eucollagens. For eucollagen preparations it was found that optimal separation of the components was achieved using a discontinuous aluminium lactate buffer system, pH 3·0. The starch concentration was 15%.

Inner gel buffer compartment; aluminium lactate 2 mM; lactic acid 14·5 mM; urea 6 M.

Outer gel buffer compartments: glycine 0·1 mM; lactic acid 0·3 mM; urea 6 M.

Electrode vessels: glycine 0·1 mM; lactic acid 0·3 mM.

Before electrophoresis all samples were dialysed against 0·5 M sucrose with 6 M urea in 2 mM aluminium lactate–14·5 mM lactic acid buffer, pH 3·0.

Electrophoresis was carried out in ice-cooled apparatus for 9 hr (400 V, 30 mA falling to 20 mA).

(c) *Gel staining.* Composition of the stain employed was Nigrosine (water-soluble) 2% and Naphthalene Black 10B, 1%, in methanol–acetic acid–water, 4 : 1 : 5.

After electrophoresis the gel was cooled and sliced horizontally into four sheets so that two inner layers of 1·5 mm thickness were produced (the outer layers were discarded). The inner layers were immersed in the staining fluid for 20 min. The background stain was then removed by washing in several changes of solvent, over a period of 48 hr, and then in several changes of water over a further period of 8 hr.

(vi) *Determination of relative concentration of separated protein components*

The starch gel was rendered transparent, so that the concentration of each stained protein component could be measured spectrophotometrically. The most satisfactory procedure was based on that of Rubinstein, Owen & Larson (1960).

Carefully cleaned glass slides were dipped into hot 1·5% agar (containing 0·1% sodium azide as preservative) and dried at 100°C. The slides were then placed in a shallow dish and covered with a film of hot agar to a depth of 3 mm. After setting, the slides were cut and, after drying at 80°C, returned to the tray and covered with

hot agar to a depth of 7 mm. A section of suitable size was cut from the appropriate area of the starch gel, immersed briefly in hot agar to expel air bubbles, then placed on top of one of the prepared glass slides in the agar filled tray. The gel section was pressed firmly on to the slide surface, care being taken to exclude air bubbles.

After the agar in the tray had set, each slide with its adhering gel section was cut out and dried in a current of air at 40°C until transparent. The slides were then scanned in a spectrophotometer at 625 mμ. The readings of optical density were graphed.

Rubinstein *et al.* (1960) reported that the relationship between dye uptake and protein concentration was linear. This was verified using known quantities of bovine albumen.

The method of calculating peak area of Bush (1961) was employed. In this method it is assumed that each peak closely approximates to a normal distribution curve. With the complex mixture of components obtained, more sophisticated treatment was not justified.

Because of the difficulty of inserting an accurately known volume of sample into the starch gel slots, and in view of inter gel variability, the concentration of each component was not expressed in absolute terms but in relation to the α_2 chain component.

(vii) *Identification of separated components*

Since apparatus to distinguish α, β and γ components by sedimentation differences was not available the identity of the bands separated by electrophoresis had to be inferred by comparison with published data on mobility and amino acid composition and by enzymic digestion.

An electrophoretic pattern typical of those obtained in the present study is shown in Fig. 2. The work of Näntö, Maatela & Külonén (1963) suggested that the α_2

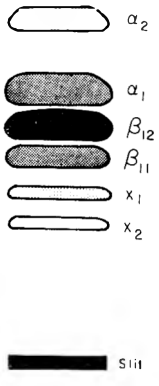

FIG. 2. Typical electrophoretogram of acid soluble collagen from bovine tissue.

component was the most mobile, followed by α_1, β_{12} and β_{11} (i.e. dimers comprising one α_1 and one α_2 chain, and two α_1 chains, respectively). Two still slower moving components 'X_1' and 'X_2' may represent other dimers (e.g. β_{23}) or, more possibly,

γ components. These designations have been given to the bands in Fig. 2. In some experiments a band was visible close to the origin. This is presumably a high molecular weight species and may be the so-called δ form (Veis & Anesey, 1965), i.e. a polymer of four α components involving intermolecular bonds.

Detailed amino acid analysis on cod fish skin collagen (Piez, 1965) has shown that the three α chains in the tropocollagen molecule from this tissue are different. In other vertebrate tissues, however, the tropocollagen molecule consists of two α_1 components and one α_2 component (Butler & Piez, 1967), although a minor variant of undetermined origin may be present in the α_1 component. Some evidence from electrophoretic heterogeneity of the α_1 band was obtained here which may well be ascribed to the presence of this minor variable.

Pronase, a proteolytic enzyme isolated from *Streptomyces griseus*, readily converts the β component of soluble collagen into the constituent α chains (Fujii, 1965). Incubation is carried out at 37°C for 48 hr. When the method was applied to AS collagen subsequent electrophoresis indicated that the bands designated β in Fig. 2 were no longer present.

Results

Before considering the effects of animal age on the electrophoretically distinct components of the various collagen fractions studied, some general aspects of the data may be noted. The optical density of stained starch gels (such as illustrated in Fig. 2), after qualitative assessment of 625 mμ by spectrophotometer, has been plotted against distance migrated in Fig. 3 for bovine tissues from the 5-month foetal stage. It is apparent that the patterns for NSS collagens from l. dorsi and skin are similar to one another but that they differ from that of tendon in having slower (and presumably more highly cross-linked) components. With respect to the patterns for AS collagens, on the other hand, there are resemblances between those for l. dorsi and tendon. Moreover, their components are considerably more differentiated, electrophoretically, than those from skin.

The concentrations of separated units (calculated from the plotted areas of optical density, as indicated above), relative to the α_2 monomer, are given for NSS collagens in Table 1 and for AS collagens in Table 2 derived from l. dorsi, skin and tendon of bovines of different ages and overall averages.

From Table 1 (and Fig. 3) it is clear that even in the 5-month foetus, some degree of intramolecular cross-linking of α chains in NSS collagen – as represented by the presence of β_{12} and β_{11} dimers – has already taken place in the three tissues studied. It is interesting to note, however, that this is markedly less in tendon than in skin and muscle. As the animal grows older, the proportion of dimer (and other intra- or intermolecularly linked forms, as represented by X_1 and X_2) increases. In the 9-month foetus, the degree of cross-linking appears to be greater in skin than in the other two tissues. On the other hand, there appears to be less cross-linking in the

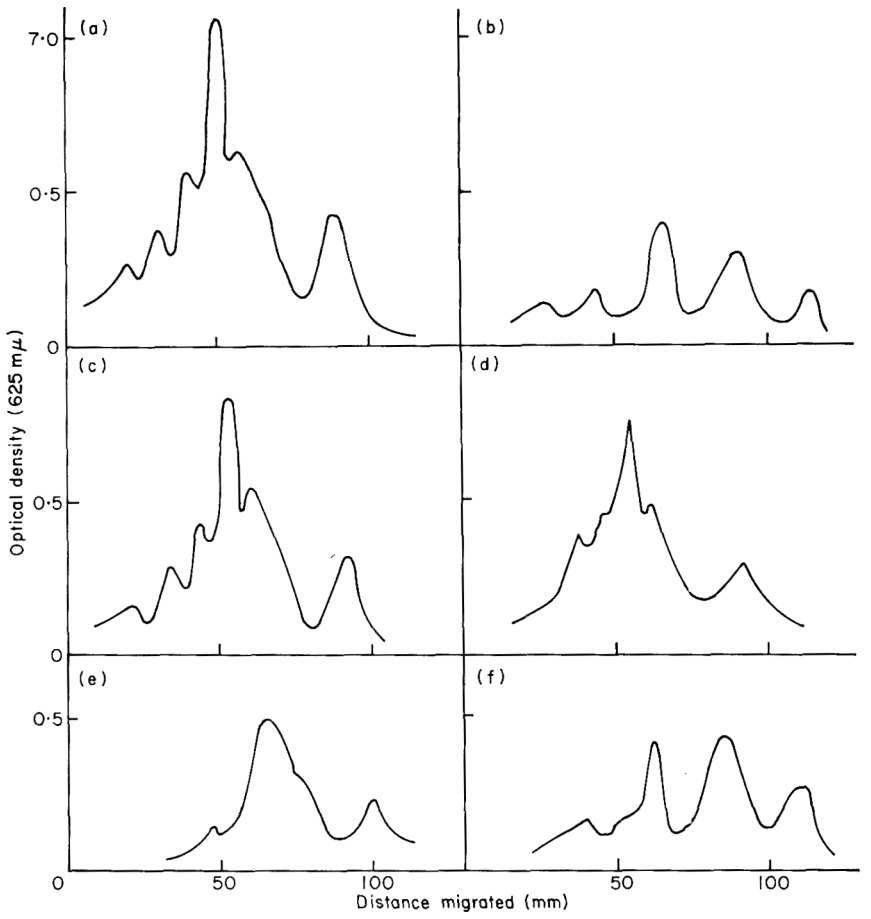

FIG. 3. Typical patterns obtained by photometric analysis of electrophoretograms of neutral salt soluble and acid soluble collagens from bovine l. dorsi, skin and tendon. Sample origin at zero. (a) Neutral salt soluble collagen—l. dorsi; (b) acid soluble collagen—l. dorsi; (c) neutral salt soluble collagen—skin; (d) acid soluble collagen—skin; (e) neutral salt soluble collagen—tendon; (f) acid soluble collagen—tendon.

skin of the 4-year-old bovine than in muscle (unfortunately data from tendons of 1- and 4-year-old animals were not available). Piez *et al.* (1963) have suggested that in vertebrate collagens the ratio of α_1 to α_2 is 2 : 1. The results obtained here, however, for bovine tendon did not always reproduce this ratio. Whether this is a true variation or an artifact caused by the method of preparation could not be determined.

The increased degree of cross-linking in skin with increasing animal age is also shown by the data on AS collagens in Table 2. Particularly noteworthy is the high proportion of 'X_2' in the 8-year-old animal. Data for the components in the AS collagens of muscle and tendon are more variable, there being a high proportion of

TABLE 1. Relative peak areas ($\alpha_2=1$) from the photometric analysis (625 mμ) of starch gel electrophoretograms of neutral salt soluble collagen from bovine tissues

Source of sample	Tissue	Relative peak area of electrophoretic components					
		α_2	α_1	β_{12}	β_{11}	X_1	X_2
Foetus	Muscle	1	2·0	1·4	0·5	0·5	–
(5-month)	Skin	1	1·7	1·6	1·1	0·8	–
	Tendon	1	4·3	0·4	–	–	–
Foetus	Muscle	1	1·5	1·7	1·8	0·8	0·4
(9-month)	Skin	–	–	–	–	–	–
	Tendon	1	1·8	1·0	0·4	–	–
Calf	Muscle	1	2·2	2·1	0·8	0·8	–
(6-month)	Skin	1	2·2	1·5	0·6	0·5	–
	Tendon	1	2·4	1·9	1·3	–	–
Heifer	Muscle	1	1·7	2·0	0·8	0·7	0·7
(1-year)	Skin	1	2·4	2·2	1·7	1·8	1·2
	Tendon	–	–	–	–	–	–
Steer	Muscle	1	2·2	3·0	1·2	0·6	0·4
(4-year)	Skin	1	2·8	1·1	0·3	0·3	1·4
	Tendon	–	–	–	–	–	–
Overall	Muscle	1	1·9	2·0	1·0	0·7	0·3
	Skin	1	2·3	1·6	0·9	0·8	0·7
	Tendon	1	2·8	1·1	0·6	0·7	–

the 'α_1' monomer in the tissues from the 1-year-old animal in comparison with younger and older bovines. Nevertheless, concomitantly, there is again evidence for increased cross-linking with increased animal age. It is clear that, as regards AS collagens, the degree of cross-linking in tendon is rather greater in the 5-month foetus than in muscle or skin—in contrast with the findings for NSS collagens.

A striking feature of the data as a whole is the enhanced degree of cross-linking in all three tissues, and in both NSS and AS collagens, between the 5th and 9th month of gestation.

For NSS collagen, without distinction of age, that for tendon has the least cross-linking; whereas for AS collagen it may be seen that cross-linking is greatest in skin.

In general, irrespective of age or tissue, the proportions of β_{11}, β_{12}, X_1 and X_2 are greater in AS than in NSS collagens.

The nature of the components separated from eucollagens has not been ascertained in the present study; but general features of electrophoretograms were observed. The low temperature extracted eucollagen fraction showed basically the same pattern

TABLE 2. Relative peak areas ($\alpha_2=1$) from the photometric analysis (625 mμ) of starch gel electrophoretograms of acid soluble collagen from bovine tissue

Source of sample	Tissue	Relative peak area of electrophoretic components					
		α_2	α_1	β_{12}	β_{11}	X_1	X_2
Foetus (5-month)	Muscle	1	1·9	1·6	0·6	0·6	–
	Skin	1	2·1	2·9	1·0	0·5	–
	Tendon	1	2·9	1·7	0·8	0·8	0·7
Foetus (9-month)	Muscle	1	1·1	0·6	1·2	1·4	–
	Skin	1	1·9	2·3	0·8	0·5	0·2
	Tendon	1	1·9	4·2	1·8	1·4	–
Calf (6-month)	Muscle	1	2·3	2·6	0·7	–	–
	Skin	1	1·5	2·8	1·4	1·7	0·3
	Tendon	1	2·4	2·6	0·8	0·3	–
Heifer (1-year)	Muscle	1	3·0	0·6	0·8	0·3	0·8
	Skin	1	2·4	3·4	1·9	1·7	0·5
	Tendon	1	5·2	3·7	2·6	2·0	–
Steer (4-year)	Muscle	–	–	–	–	–	–
	Skin	1	1·9	2·5	2·0	1·0	0·4
	Tendon	1	2·1	1·8	0·6	–	–
Cow (8-year)	Skin	1	1·7	1·9	1·9	1·8	3·3
Overall	Muscle	1	2·1	1·4	0·8	0·6	0·2
	Skin	1	1·9	2·6	1·5	1·2	0·8
	Tendon	1	2·9	2·8	1·3	0·9	0·1

(cf. Fig. 4) whether derived from muscle, skin or tendon. Moreover, it did not vary appreciably with the age of the animal. On the other hand, certain differences between tissues in the patterns for high temperature extracted eucollagen were noted. Thus l. dorsi had only three components, whereas there were about eight additional components in skin and tendon; and these had considerably greater mobility. There was some suggestion that the latter were more prevalent in the tissues of older animals.

Discussion

In the previous paper (Carmichael & Lawrie, 1967) it was shown that, with increasing animal age, changes occurred in the solubility of the various collagen fractions derived from bovine muscle (l. dorsi), skin and tendon. The proportion of the total collagen extractable in neutral salt solution or dilute acid diminished markedly by 1–2 years after birth.

The data presented here show that there is an increased formation of dimer or higher aggregates of the α chains with increased animal age. Veis *et al.* (1960) have concluded that the ready extractability of salt and dilute acid soluble forms of collagen from young tissue signifies that all fibrils in mature tissue are not equal in cross-linking density. Jackson & Bentley (1960) suggested that in developing connective tissue, at any given time, there is a continuous spectrum of collagen aggregates with varying degrees of cross-linkage; and Verzar (1964) commented that the maturation of tissue involves an increase in both intra- and intermolecular cross-links.

FIG. 4. Typical electrophoretogram of low temperature extracted eucollagen.

The extraction studies detailed in the first paper (Carmichael & Lawrie, 1967) revealed the decreased solubility of collagen in mature tissue with increased animal age. From the present data it may be deduced that this phenomenon can be attributed, at least in part, to an age-related increase in cross-linking between the polypeptide chains forming the collagen fibres.

The occurrence of components with a high electrophoretic mobility from the HTE eucollagen preparations may be explained in a similar manner. Thus, if it is accepted that the collagen from older animals possesses more extensive or stronger cross-linkages, then the presence of several cross-linkages would act to tie each peptide strand firmly on to the body of a fibre. Extraction of tissues from young animals would tend to release complete polypeptide strands by cleavage of the relatively few cross-linkages, as is evidenced by the easy extractability of salt and acid soluble fractions from tissues in this age group. In older, more highly cross-linked fibres, however, the number and strength of the inter- and intramolecular bonds is such that disruption of the fibrillar structure is achieved by rupture of peptide bonds along the polypeptide strand between cross-linkage points. This hypothesis is in accord with that advanced by Goll, Hoekstra & Bray (1964).

The increased formation of a cross-linked network of collagen fibrils as the animal ages is no doubt a basis for the superficial alteration in the tissue from a barely cohesive watery consistency characteristic of foetal tissue to the firm structure present

in mature animals. The electrophoretic changes in the more intractable eucollagen, interpreted as evidence of increased complexity in cross-linkage, may help to explain the increased toughness of the meat of older animals. This is despite the fact that the collagen is diluted, first by myofibrillar proteins and then by fat.

A surprising feature of the data is the finding that even in the tissues of the 5-month foetus some cross-linking of α chains has already taken place. With respect to neutral salt soluble collagen – though not with acid soluble collagen – this occurs earlier in muscle and skin than in tendon. It might have been expected that a tissue which is so predominantly composed of collagenous elements as the latter would have been the first to show the changes of maturation. Nevertheless, the electrophoretogram of neutral salt soluble collagen from the tendon of the 5-month foetal stage, indicating that monomer forms greatly preponderate, accords with the views of Mazurov & Orekhovitch (1960). It is significant that the neutral salt soluble collagens from lathyritic rats have α chains only. One aspect of the condition is clearly the failure of the α chains to cross-link (Martin *et al.*, 1961). Moreover, incubation of β gelatin with the lathyrogen, β-aminoproprionitrile, will break the cross-links, producing α components (Fessler & Bailey, 1966).

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The effect of post-mortem conditions on the extractability and adenosine triphosphatase activity of myofibrillar proteins of rabbit muscle

I. F. PENNY

Summary. 1. Washed myofibrils from rabbit muscle have been heated at pH values between 4·8 and 5·6 and temperatures between 35°C and 42°C. It has been found that, under these conditions, myofibrils lose their Ca^{2+} activated adenosine triphosphatase, their Mg^{2+} activated adenosine triphosphatase and also become less extractable in M KCl–30 mM sodium glycerophosphate, pH 6·2.

2. The reactions follow first-order kinetics and the rates are dependent on pH and temperature. The first order rate constants, enthalpies and entropies for the three reactions are sufficiently near each other to suggest that all three reactions are occurring simultaneously.

3. When a muscle is allowed to go into rigor at 37°C the extractability in M KCl–30 mM sodium glycerophosphate is reduced after 4 hr at 37°C when the pH of the muscle has reached 5·55. At the same time the Ca^{2+} adenosine triphosphatase activity falls but the Mg^{2+} adenosine triphosphatase does not. The latter is reduced by prolonging the period at 37°C to 6 hr.

4. It is suggested that there is present in muscle, undergoing rigor at 37°C, myosin which does not bind to actin and is readily denatured. When bound to actin, myosin in the myofibril is more resistant and denatures only after long exposure to a temperature of 37°C.

Introduction

The extractability of fibrillar proteins by salt solutions is a property which has been used, very widely, in studying changes which have occurred in fish and meat. The toughening which occurs in stored frozen fish, for instance, has been shown to be paralleled by loss in extractability of the fibrillar protein (Love, 1962). Connell (1962) suggested that these changes were due in part to denaturation and aggregation of myosin and to the formation of new actin–myosin links.

In most studies the extractability of the fibrillar proteins has been used to try to elucidate the changes occurring in the post-mortem period. The biochemical changes

Author's address: Agricultural Research Council, Meat Research Institute, Langford, Bristol.

which take place in muscle after death have been adequately reviewed by Bendall (1960). Briefly, it is the turnover of ATP which continues for some hours after death which is the most important reaction. This initiates the system of glycolysis, with the concomitant production of lactic acid and a lowering of pH of the muscle. As ATP is removed from the system the muscle loses its extensibility and becomes rigid due to the bonding of actin to myosin to form actomyosin. After about 24 hr the muscle softens and becomes limp but extensibility is not restored.

Recently, however, the importance of the temperature of the muscle and its pH during the first 12 hr post mortem has been realized. Normally in a muscle with a high glycogen content, the pH falls from a value of 7·0 to about 5·5 during the course of rigor but the rate of fall is such that the muscle will have cooled to well below body temperature (38°C) before the pH falls to its ultimate value. In certain cases, particularly in pig muscle, the rate of fall can often be very much faster so that the muscle will not have had time to cool below 38°C before the pH value has fallen below 6. Such conditions give rise to muscle which has been called 'white', 'watery' or PSE (pale, soft and exudative). This phenomenon has been reviewed by Bendall & Lawrie (1964) and Briskey (1964).

The extractability of the fibrillar protein from such muscle is less than 50% of that of meat allowed to go into rigor at lower temperatures, and it has been suggested by Bendall & Wismer-Pedersen (1962) that this arises from the deposition of denatured sarcoplasmic proteins on to the myofilaments of the muscle without necessarily involving the denaturation of the actomyosin itself. Sayre & Briskey (1963) also demonstrated that the loss of solubility of myofibrillar proteins was associated with the loss of solubility of sarcoplasmic proteins, the actual extent of the reduction being dependent on the post mortem conditions of temperature and pH. Scopes (1964) has shown that the protein creatine kinase is very labile and denatures readily under the environmental conditions which give rise to 'white' muscle.

However, the effect of potentially denaturing conditions of high temperature and low pH on isolated myofibril proteins had not been studied, and as a first attempt, the effect of temperature and pH on myosin was examined. The results (Penny, 1967) showed that myosin ATPase* was readily denatured under conditions which give rise to 'white' muscle, i.e. at pH values 5·3–6·0 and temperatures 33–42°C. It was also found that, between pH values 5·4 and 5·8 and temperatures 35° and 42°C, myosin would become insoluble in M potassium chloride, pH 6·2, but only after a lag phase during which the ATPase activity had been destroyed. These studies have now been extended to washed myofibrils, using the decrease in ATPase activity and in extractability in M potassium chloride to follow the extent of denaturation. The ATPase activity and extractability of myofibrils prepared from whole muscles, which have undergone rigor at 37°C have also been examined to discover if any denaturation of the myofibrillar protein had occurred.

* Adenosine triphosphatase.

Materials and Methods

ATP was obtained as the disodium salt from The Sigma Chemical Co. (St Louis, Mo., U.S.A.). All other reagents were Analar grade from British Drug Houses Ltd (Poole, Dorset). The water used was passed through a mixed resin bed (Elgastat, Elga Products Ltd, London).

Preparation of Myofibrils

The back and leg muscles from a newly killed rabbit were cooled in ice, minced and homogenized for 2 min in a top drive blender in 3 volumes of ice-cold 80 mM KCl–5 mM EGTA*–20 mM imidazole, adjusted with HCl to pH 7·2. The homogenate was centrifuged at 2000 ***g*** in a refrigerated centrifuge at 2°C for 10 min. The supernatant and the bottom layer of the sediment which contained mainly the connective tissue were discarded. The remainder of the sediment was suspended in a further 3 volumes of the homogenizing medium, and recentrifuged as above. Thereafter, the sediment was suspended in 0·1 M KCl and the centrifuging procedure described by Perry & Grey (1956) was followed. The final concentrated sediment of myofibrils was suspended in 30% glycerol and stored at −10°C. The myofibrils were recovered, when required, by centrifuging and washing with 0·1 M KCl. These preparations consisted of single myofibrils, the majority of which showed clear striations, i.e. A, I and Z bands, and an occasional H zone under a phase contrast microscope. However, the myofibrils were seldom straight, which indicates that the contracting effect of the endogenous ATP had not been completely eliminated. Myofibrils were also prepared from muscle, 24 hr post-mortem, by the above procedure with the omission of EGTA. In these preparations the myofibrils were always straight.

Denaturation of myofibrils

Myofibril suspensions in 0·1 M KCl containing approximately 30 mg protein/ml were diluted with an equal volume of 0·1 M sodium acetate buffer which had been adjusted with acetic acid to give pH values of 4·8–5·6. One-millilitre portions of these suspensions were pipetted into a series of tubes and heated in a constant temperature water bath (± 0·1°C). At regular time intervals a tube was withdrawn, and chilled in an ice bath. At the same time was added a chilled solution, the composition of which depended on the method to be used for determining denaturation as described below.

To measure the loss of Mg^{2+} activated ATPase activity of myofibrils, 3 ml of a mixture of 67 mM imidazole pH 7·2–6·7 mM $MgCl_2$–0·12 mM $CaCl_2$–148 mM KCl, were added to the 1 ml of denatured myofibrils. After the addition of 1 ml of 20 mM ATP (=4 mM, final concentration) to initiate the reaction, the total ionic strength of the above mixture was 0·15. To estimate the ATPase activity, the mixture was incubated at 20°C and the inorganic phosphate liberated was determined by Allen's

* Ethylene glycol bis(amino ethyl)tetraacetic acid.

(1940) method as described by Penny (1967). Under these conditions the liberation of inorganic phosphate (P_i) is linear with time (Bendall, 1961) and therefore the activity in μg atoms of P_i min was calculated from the slopes of the straight lines. Blank values of inorganic phosphorus were determined on samples in which the ATP or the myofibrils were omitted.

The Ca_2^+ activated ATPase was measured on the 1-ml suspensions of denatured myofibrils to which were added 3 ml of a mixture of 67 mM imidazole (pH 7·2)–13·3 mM $CaCl_2$–M KCl. The final ionic strength of this mixture with the addition of 1 ml of 20 mM ATP was 0·67. The ATPase was measured as for the Mg^{2+} ATPase.

The loss of solubility of fibrillar protein was determined by adding to the 1 ml of denatured myofibrils 4 ml of M KCl–30 mM sodium glycerophosphate buffer adjusted with HCl to pH 6·2. The mixture was shaken frequently and left overnight at +4°C. The undissolved portion of the myofibrils was removed by centrifuging at 35,000 ***g*** for 10 min and the soluble protein in the supernatant determined by measuring, on a Unicam SP500, the extinction at 280 mμ (corrected for light scattering by subtracting the extinction at 320 mμ). When standardized by measuring the nitrogen content of solutions by the Kjeldahl method, $E^{1\%}_{1cm}$ at 280–320 mμ = 7·80.

To measure the Ca^{2+} activated ATPase activity of the extracted protein, $CaCl_2$, imidazole and KCl were added to 3 ml of the centrifuged extract, to give the same final composition as above.

The effect of post-mortem conditions on myofibrillar proteins

One longissimus dorsi from a newly killed rabbit was divided in two. Myofibrils were prepared from one-half in the pre-rigor state (as above), the other half was held at room temperature (about 15°–18°C) for 24 hr before preparation of the myofibrils. The other longissimus dorsi muscle was wrapped in 'Saran' (Dow Chemical Co., Michigan, U.S.A.) and held at 37°C. Portions were removed at time intervals and then held at room temperature to complete a total post-mortem period of 24 hr. Myofibrils were then prepared from these samples. The appearance of these myofibrils under a phase contrast microscope was similar to that of normal post rigor myofibrils. A, I. and Z bands were clear and a considerable number also showed H-zones

The extractability of the myofibrils was determined by adding to 5 ml of the myofibril suspension, containing 30–40 mg protein/ml, 45 ml of M KCl–30 mM sodium glycerophosphate buffer adjusted with HCl to pH 6·2 and leaving the mixture overnight at + 4°C. The protein in the suspension and in the supernatant after centrifuging the suspension at 35,000 ***g*** for 15 min was measured by the Kjeldahl method, the nitrogen content of protein being taken as 16·7%.

The Ca^{2+} ATPase activity of the total suspension and the supernatant was determined by adding the appropriate amounts of $CaCl_2$, imidazole and ATP to give a mixture with the final composition of 0·75 M KCl–4 mM ATP–8 mM $CaCl_2$–20 mM imidazole buffer adjusted with HCl to pH 7·2. The incubation was carried out at

20°C. The specific activity of the total suspension will be referred to as Ca^{2+} $ATPase_1$.

Myofibrils were also extracted for 24 hr at 4°C with 0·1 M tetrasodium pyrophosphate–4mM $MgCl_2$–10 mM KH_2PO_4 adjusted with HCl to pH 7·0 which will be referred to in the text as 'pyrophosphate extractant'. This mixture, according to Corsi & Perry (1958), extracts mainly myosin from the myofibril. The protein contents of the suspension and centrifuged supernatant were determined. The Ca^{2+} ATPase activities were determined after the suspension and supernatant had been dialysed for 2 days against several changes of 0·9 M KCl.

The Ca^{2+} ATPase was also determined on whole myofibrils by adding to 1 ml of myofibrils, in 0·1 M KCl, 3 ml of a mixture of 148 mM KCl–13·3 mM $CaCl_2$–67 mM imidazole buffer, pH 7·2. The reaction was initiated by the addition of 1 ml of 20 mM ATP and carried out at 20°C. The specific activity will be referred to as Ca^{2+} $ATPase_2$. (Two ionic strengths were used to determine Ca^{2+} ATPase because $ATPase_1$ is most suitable for measuring extractable activity and $ATPase_2$ is more representative of the whole myofibril.)

Results

(A) *Treatment of washed myofibrils*

ATPase activity. It had previously been shown (Penny, 1967) that the loss of the ATPase activity of myosin, when heated in solution, was a first-order reaction. It has now been found that the heat denaturation of myofibrillar ATPase is also first order (Fig. 1). The myofibrils, however, are much more resistant to thermal deactivation and, as a result, the first-order constants are smaller than those found for myosin. At pH 5·4 and 37·5°C, the rate of loss of Ca^{2+} activated ATPase is $k = 1{\cdot}75 \times 10^{-4}$ sec $^{-1}$. The comparable constants for myosin and synthetic actomyosin (Penny, 1967), under the same conditions of pH and temperature, were $k = 1{\cdot}75 \times 10^{-2}$ sec^{-1} and $k = 3{\cdot}5 \times 10^{-3}$ sec^{-1}, respectively. Myofibrils could also withstand the effect of lower pH values at room temperature (about 15–18°C). The pH of a suspension of myofibrils could be lowered to 4·8 before any loss of ATPase activity was detected whereas with myosin the limit was pH 5·3.

To evaluate the effects of different temperatures and pH values on the ATPase systems of myofibrils, the logarithms of the first order constant for the rate of loss of activity (k) were plotted against pH. The straight lines obtained are shown in Fig. 2 for Mg^{2+} activated ATPase and in Fig. 3 for Ca^{2+} activated ATPase. In both cases a slope of approximately -2 was found. According to the theory first put forward by Steinhardt (1937) and since applied to the denaturation of many other proteins, the rate at which a protein denatures depends on the formation of ionized states which are thermolabile. The number of hydrogen ions involved in this process can be calculated from the slope of log k against pH, the negative value of which is equal to the number of hydrogen ions. It is not possible to say what the significance of this value of two hydrogen ions, found in these experiments, is with reference to the complex struc-

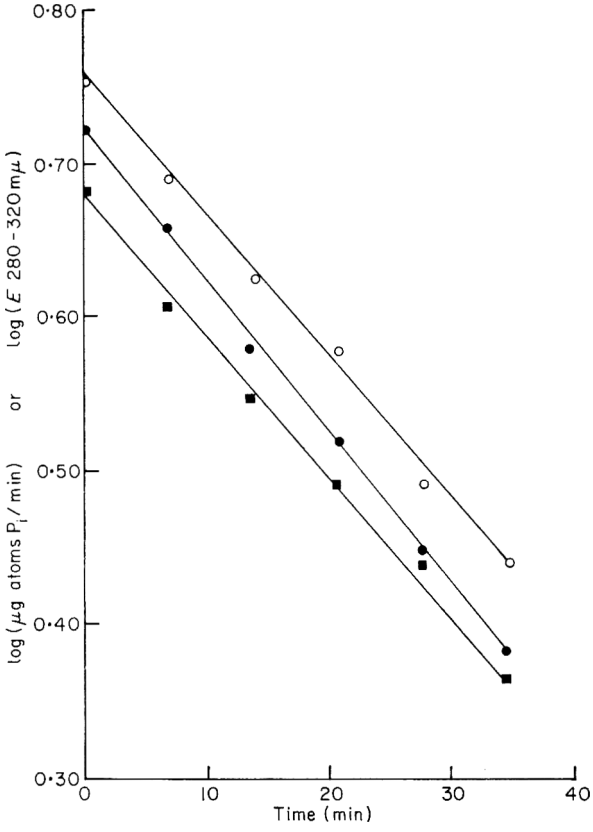

FIG. 1. First-order log plots for denaturation of myofibrils at 37·5°C and pH 5·25 as measured by: ■, Mg^{2+} ATPase activity (μg atoms P_i/min); ○, Ca^{2+} ATPase activity; and ●, extractability by M KCl–30 mM sodium glycerophosphate buffer, pH 6·2 (measured by extinction at 280–320 mμ).

ture of myofibrils, but it is obviously a somewhat different process from that found for myosin where only 1·3 hydrogen ions appear to be involved.

The results for the effect of temperature on the rate of denaturation were treated according to the integrated Arrhenius equation $\log k = -E/2{\cdot}3RT + \text{constant}$, and the plots are shown in Fig. 4. From the slopes of these lines the values of E, the energy of activation for the denaturation of the Mg^{2+} and Ca^{2+} ATPase, were found. The thermodynamic functions ΔH^*, the apparent enthalpy, and ΔS^*, the apparent entropy of activation, were calculated according to the Eyring theory of absolute reaction rates (Glasstone, 1948). The values found are given in Table 1. These show that the denaturation reactions for both Mg^{2+} and Ca^{2+} ATPase have very similar functions. It is, therefore, likely that the reaction which is occurring in the myofibrils has an identical effect on both enzyme systems.

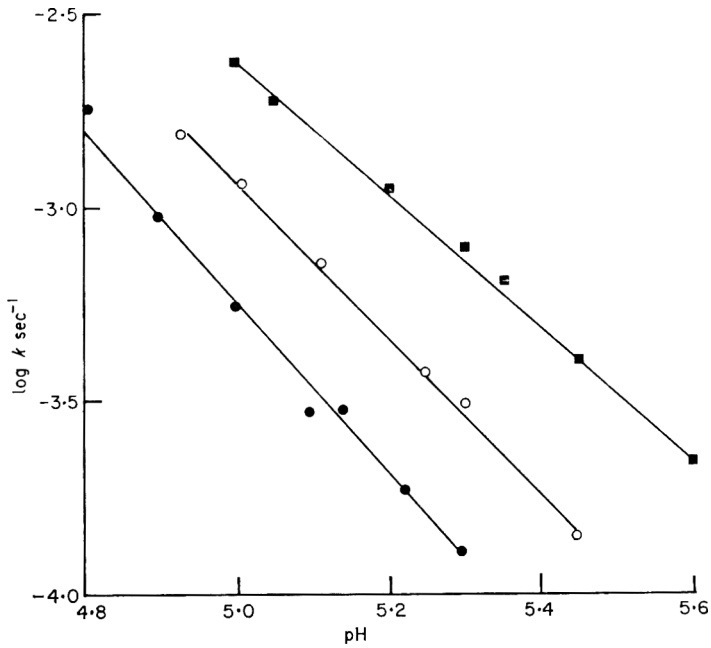

FIG. 2. Plot of log k sec^{-1} against pH for the denaturation of myofibrils, as measured by loss of Mg^{2+} ATPase activity at different temperatures, viz. ●, 35°C; ○, 37·5°C; ■, 40°C.

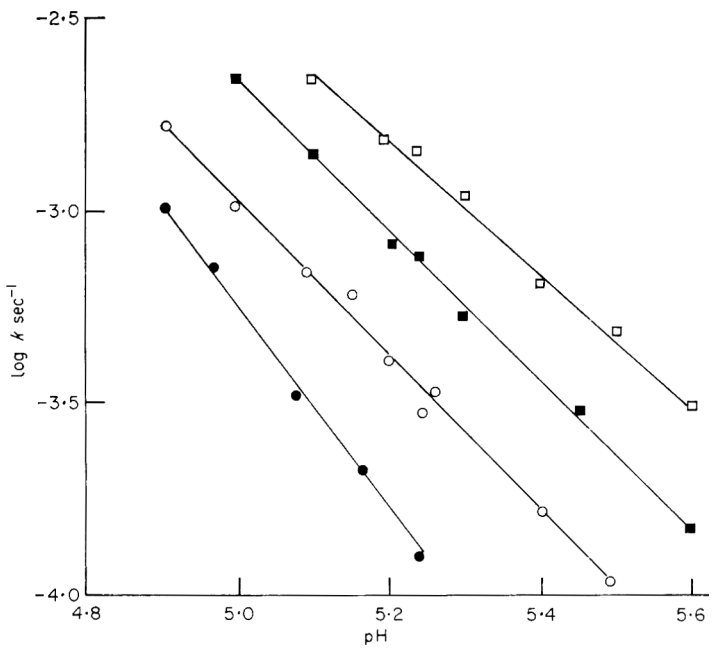

FIG. 3. Plot of log k sec^{-1} against pH for the denaturation of myofibrils as measured by loss of Ca^{2+} ATPase activity, at different temperatures, viz.: ●, 35°C; ○, 37·5°C; ■, 40°C; □, 42°C.

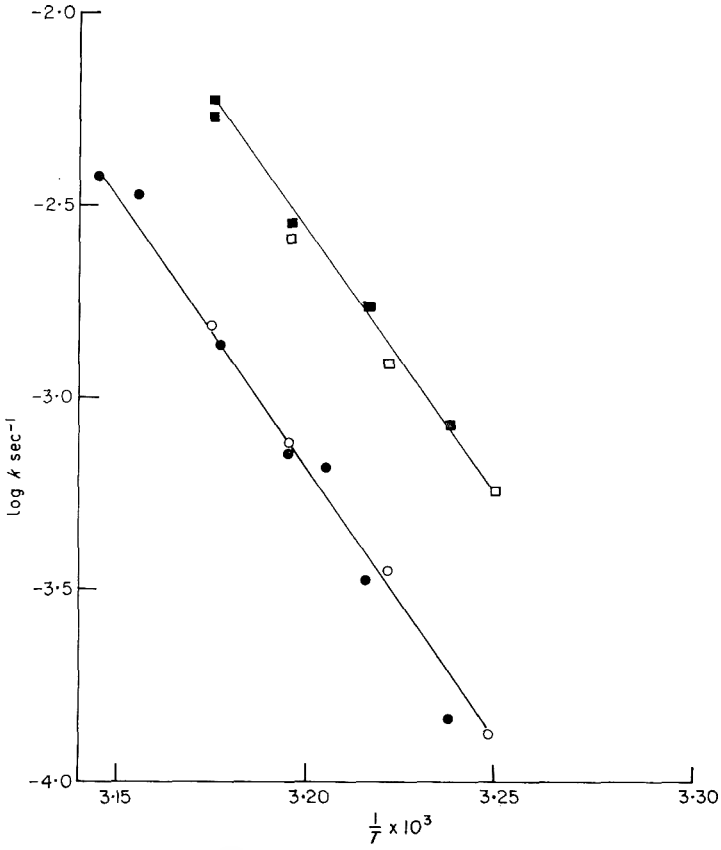

FIG. 4. Arrhenius plots of the effect of temperature on the rate of denaturation of myofibrils at pH 5·25 as measured by Ca^{2+} ATPase activity (●, ○) and at pH 5·0 as measured by Mg^{2+} ATPase activity (■, □). The open symbols are points taken from Figs. 2 and 3.

TABLE 1. The thermodynamic functions obtained for the rate of change in the properties of myofibrils heated at 37·5°C in 0·1 M KCl–0·1 M–sodium acetate buffer, pH 5·25, the change being measured by the loss in Mg^{2+} ATPase activity, the loss in Ca^{2+} ATPase activity and the loss in extractability by M KCl–30 mM sodium glycerophosphate buffer, pH 6·2

	First-order rate constant ($10^4 k$ sec^{-1})	ΔH^* (kcal/mole)	ΔS^* (cal/deg/mole)	log k/pH slope
Mg^{2+} ATPase	3·8	65·6	137	− 2·0
Ca^{2+} ATPase	3·5	68·7	147	− 1·98
Extractability (M KCl)	3·9	72·1	158	− 1·6

The values for log k/pH slope were obtained from Figs. 2, 3 and 5.

The common factor in the above observation is the myosin ATPase of the filaments, which in the case of Ca^{2+} activation is probably acting alone, whereas with Mg^{2+} present, its activity is modified by actin. It must be concluded, therefore, that it is primarily the myosin in the myofibril system which is being denatured. The values of ΔH^* and ΔS^*, however, are very much greater than those found for myosin. Therefore, it must be necessary to alter some feature of the organized myofibril structure, in addition to the structure of the heavy meromyosin 'head' of the myosin molecule which contains the enzyme sites, in order to inactivate the ATPase.

Extractability. It was found that, after heating myofibrils at different pH values at 37·5°C, they became less extractable in the M KCl–30 mM sodium glycerophosphate, pH 6·2, mixture. However, even after 24 hr at 37·5°C, a small fraction could still be extracted from the myofibrils. The extinction of this extract was about 15% of the initial 280–320 mμ value and the 280 mμ/260 mμ ratio was 0·90 compared with 1·40 for an extract of undenatured myofibrils. Thus this undenatured fraction contains large amounts of nucleotide and nucleic acid and is probably similar to that first found by Locker (1959) after the denaturation of myosin. To assess the rate of loss of extractability the extinction for the final extract, obtained after 24 hr heating, was subtracted from the other values. It was then found that the reaction followed first-order kinetics until about 70% of the protein had denatured and was no longer extractable. Thereafter the rate declined considerably. There was, however, no lag phase detected in initial stages of the reaction, which is in contrast to the results found with myosin.

The plot of the logarithm of the first-order constant obtained at different pH values at 37·5°C is shown in Fig. 5. The relationship was linear, as was found with the ATPase, but the slope was only $-1{\cdot}6$ instead of $-2{\cdot}0$.

The effect of temperature, at pH 5·2, was also determined and the results were treated according to the Arrhenius and Eyring equations. The calculated thermodynamic functions are shown in Table 1. A comparison with the results obtained for the inactivation of total ATPase show that a slightly higher enthalpy and entropy of activation are required to reduce the extractability of myofibrils than to inactivate the ATPase.

The ATPase activities of the solutions obtained above were also measured. It was found that the rate at which the amount of ATPase, extracted from the heated myofibrils diminished, followed a time course similar to that found for the loss of extractability; first-order kinetics were valid for the first 70% of the reaction and thereafter, the reaction was much slower.

The first-order rate constant for the loss of extractable ATPase was greater than that for the loss of protein extractability at pH values less than 5·3; the rates, for instance at pH 5·1, being $8{\cdot}9 \times 10^{-4}$ sec^{-1} and $7{\cdot}1 \times 10^{-4}$ sec^{-1}, respectively. The plot of the logarithms of the first-order constants against pH in Fig. 5 showed, however, that the slope was approximately $-2{\cdot}0$ and that at pH 5·4 the rates for the two reactions were the same.

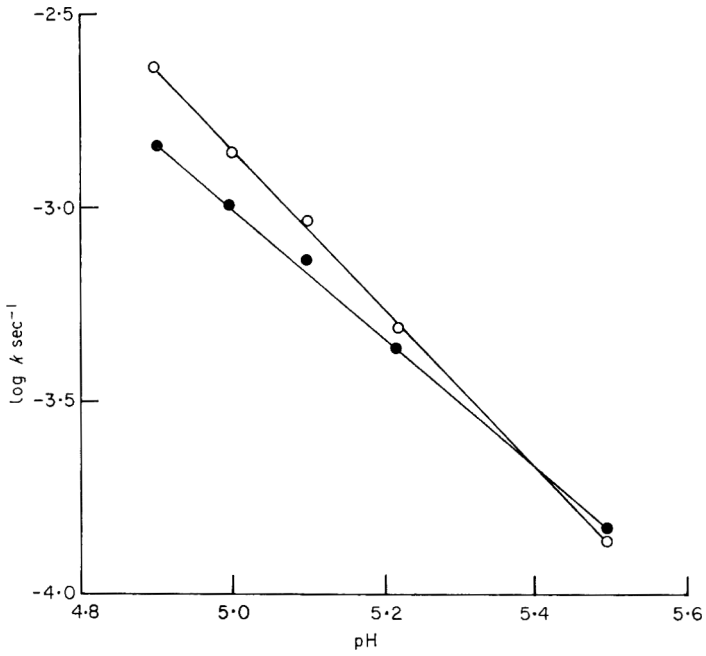

FIG. 5. Plot of log k sec^{-1} against pH for the denaturation of myofibrils at 37·5°C as measured by: ●, extractability in M KCl–30 mM sodium glycerophosphate buffer, pH 6·2; O, the loss of Ca^{2+} ATPase activity extracted by the above solution.

(B) *Treatment of intact muscle*

Extractability with M *KCl*–30 mM *sodium glycerophosphate, pH* 6·2. Myofibrils which has been prepared from muscles which had undergone the different post mortem conditions described above, were extracted with M KCl–30 mM sodium glycerophosphate, pH 6·2. The properties of the soluble fibrillar protein in the extract, after centrifuging at 35,000 ***g***, were compared with those of the total suspension including the insoluble residue. The results are shown in Table 2. The first significant result was the comparatively low solubility of 64% in the potassium chloride extract of myofibrils prepared from pre-rigor muscle. Several methods were used in an attempt to increase the solubility, such as prolonging the extraction time to 3 days at 4°C, extracting with constant stirring, and raising the pH of the myofibrils to 8·0 with NaOH so that they swelled before the addition of the extractant. By none of these methods was the solubility increased by more than 1 or 2%. Since it has been shown recently by Haga *et al.* (1966) that the addition of Ca^{2+} ions increased the extractability of F-actin from muscle, it seemed possible that the absence of Ca^{2+} ions from the pre-rigor preparations may have affected the solubility. However, the addition of 7 mM $CaCl_2$ to the extracting medium did not alter the amount soluble. The amount of protein extracted from muscle held 24 hr post-mortem increased to 70%.

TABLE 2. The effect of conditions of rigor on the extractability of myofibrillar proteins of longissimus dorsi muscles.

Conditions of rigor	pH	KCl extractability (%)	ATPase KCl solubility (%)	PP extractability (%)	ATPase PP solubility (%)
Pre-rigor	–	64 ± 1	72 ± 3	56 ± 5	97 ± 2
24 hr at 15°–18°C	–	70 ± 6	72 ± 7	70 ± 6	98 ± 2
2 hr at 37°C + 22 hr at 15°–18°C	6·32	70 ± 2	74 ± 8	62 ± 8	97 ± 2
3 hr at 37°C + 21 hr at 15°–18°C	5·95	69 ± 2	69 ± 8	62 ± 5	97 ± 2
4 hr at 37°C + 20 hr at 15°–18°C	5·75	38 ± 4	53 ± 3	5[illegible] ± 10	82 ± 12
6 hr at 37°C + 18 hr at 15°–18°C	5·60	28 ± 3	49 ± 9	3[illegible] ± 3	77 ± 4
8 hr at 37°C + 16 hr at 15°–18°C	5·7	23 ± 0	41 ± 10	24 ± 2	68 ± 3

The results show the averages, with the mean deviation, of three rabbits.

KCl extractability (%): The percentage of myofibrillar protein extracted by M KCl–30 mM sodium glycerophosphate, pH 6·2.

ATPase KCl solubility (%): The amount of total Ca^{2+} $ATPase_1$ activity soluble in the above solution.

PP extractability (%): The percentage of myofibrillar protein extracted by 0·1 M tetra sodium pyrophosphate–4 mM $MgCl_2$–10 mM KH_2PO_4, pH 7·0.

ATPase PP solubility (%): The amount of total Ca^{2+} $ATPase_1$ activity soluble in the above solution.

The pH was measured at 18°C at the time the muscle was removed from 37°C.

It is quite significant that the potassium chloride extractant dissolved only about 70% of the ATPase activity. In contrast, the pyrophosphate extractant, which extracts only about 50–60% of the protein from pre-rigor prepared myofibrils and somewhat more from the 24-hr post mortem samples, dissolved 95–100% of the ATPase activity, which agrees with the results of Corsi & Perry (1958). The incomplete extraction of the ATPase activity by the potassium chloride extractant suggests that some of the actomyosin, although not denatured, is held firmly within the myofibrillar structure. The pyrophosphate extractant on the other hand splits the bonds between myosin and actin more effectively and therefore the myosin is readily extracted.

The effect of holding muscle for different time intervals at 37°C are also shown in Table 2. It can be seen that during the period up to 3 hr at 37°C no significant change occurs in the extractability of the myofibrillar protein. However, after 4 hr at 37°C the extractability of the protein considerably decreased in both the potassium chloride and pyrophosphate extractants. It was at this stage also that the muscle first developed the characteristic pale and watery appearance. The amount of Ca^{2+} $ATPase_1$ activity extracted by both extractants also decreased. This is significant because it

implies that some of the myosin which is still capable of functioning as an ATPase is no longer being extracted by the pyrophosphate. When the muscle was held for a longer period at 37°C, the extractability decreased further.

The values of the pH of the muscle at the time of removal from 37°C given in Table 2 show that at the critical stage of 4 hr at 37°C the pH of the muscle was 5·75 at room temperature (18°C). However, from the results of Bendall & Wismer-Pedersen (1962) the pH of muscle is about 0·2 units lower at 37°C than at 18°C. Therefore, the actual pH of the muscle at the 4-hr period is about 5·55.

The effect of rigor at 37°C on the ATPase activities of myofibrils. The specific activities of the Ca^{2+} $ATPase_1$, the Ca^{2+} $ATPase_2$ and the Mg^{2+} ATPase are shown on Table 3.

TABLE 3. The effect of conditions of rigor on the Ca^{2+} ATPase and Mg^{2+} ATPase activity of myofibrils of longissimus dorsi muscles

Conditions of rigor	Ca^{2+} $ATPase_1$	Ca^{2+} $ATPase_2$	Mg^{2+} ATPase
Pre-rigor	0·092 ± 0·008	0·390 ± 0·02	0·304 ± 0·02
24 hr at 15°–18°C	0·095 ± 0·006	0·360 ± 0·03	0·295 ± 0·01
2 hr at 37°C + 22 hr at 15°–18°C	0·092 ± 0·004	0·353 ± 0·03	0·299 ± 0·02
3 hr at 37°C + 21 hr at 15°–18°C	0·092 ± 0·003	0·339 ± 0·03	0·297 ± 0·02
4 hr at 37°C + 20 hr at 15°–18°C	0·069 ± 0·008	0·272 ± 0·04	0·276 ± 0·02
6 hr at 37°C + 18 hr at 15°–18°C	0·050 ± 0·001	0·180 ± 0·03	0·182 ± 0·02
8 hr at 37°C + 16 hr at 15°–18°C	0·039 ± 0·008	0·154 ± 0·03	0·165 ± 0·03

Results are the averages, with mean deviation, of three rabbits and are expressed as μg atoms P_i/min/mg protein. Activities were determined at 20°C as follows:

Ca^{2+} $ATPase_1$: in 0·75 M KCl–4 mM ATP–8 mM $CaCl_2$–20 mM imidazole, pH 7·2.

Ca^{2+} $ATPase_2$: in 0·11 M KCl–4 mM ATP–8 mM $CaCl_2$–20 mM imidazole, pH 7·2.

Mg^{2+} ATPase: in 0·11 M KCl–4 mM ATP–mM $MgCl_2$–0·2 mM $CaCl_2$–20 mM imidazole, pH 7·2.

As in the case of extractability no significant changes were observed during the period up to 3 hr at 37°C. At the critical phase at 4 hr at 37°C, however, there were some anomalies in the results. The Ca^{2+} $ATPase_1$ activity fell to about 75% and Ca^{2+} $ATPase_2$ to about 70% of the original values. The Mg^{2+} ATPase, however, retained over 90% of its activity at this stage. After 6 hr at 37°C the activities of both Ca^{2+} ATPases and Mg^{2+} ATPase were considerably reduced.

Discussion

The three properties used to study the effect of pH and temperature on washed myofibrils were the Ca^{2+} ATPase, the Mg^{2+} ATPase and the extractability by M potassium

chloride. The results show quite conclusively that myofibrils are denatured by heating at 35°–42°C at pH values between 4·8 and 5·6. The data given in Table 1 indicate that the rates of deactivation of both ATPases and of the loss of extractability are very similar. Likewise the enthalpies and entropies of activation are about the same. It may be concluded, therefore, that all three properties are being altered simultaneously by the same reaction which is most likely to be the uncoiling of the myosin structure. In this respect, the results are very different from those obtained for heating solutions of myosin (Penny, 1967). In this case, the ATPase was deactivated first and then the solubility was reduced the latter process having much larger enthalpy and entropy of activation.

The data obtained for washed myofibrils can be used to interpret the effect of rigor at 37°C on the myofibrillar proteins of muscle. The critical stage, when the extractability of the myofibrils is reduced and there is some loss of Ca^{2+} ATPase activity, has been shown to occur between 3 and 4 hr, when the pH at 37°C had fallen to 5·55. From Figs. 3 and 5 by extrapolation to pH 5·55 it would be expected that after heating for 1 hr at 37°C at pH 5·55, the extractability of the washed myofibrils would be reduced by about 35% and the Ca^{2+} $ATPase_1$ activity by about 30%. The results in Tables 2 and 3 for the samples after 4 hr rigor at 37°C showed that, in comparison with normal post-rigor samples, the loss of extractability was about 50% but the loss in Ca^{2+} $ATPase_1$ was about 30%.

On this basis, it can be concluded that the main cause for the loss of ATPase activity was the denaturation of the fibrillar protein by the same mechanism obtaining in washed myofibrils. The greater loss in extractability in whole muscle as compared to washed myofibrils is probably due to a combination of fibrillar protein and denatured sarcoplasmic proteins with the formation of new links between the proteins as described by Bendall & Wismer-Pedersen (1962). This would explain why pyrophosphate can no longer extract all the Ca^{2+} $ATPase_1$ activity from heated muscle. It can be envisaged that the new links would be resistant to pyrophosphate and therefore the myosin involved would be held by the myofibrillar structure.

There is, however, another anomaly; namely that, in the 4-hr period, the Ca^{2+} ATPase activities have fallen but the Mg^{2+} ATPase has still at least 90% of its original activity. This would suggest that there is some unbound myosin in the myofibril of the muscle undergoing rigor at 37°C which contributes to the total Ca^{2+} ATPase. This would be expected on the sliding filament model of contraction (Huxley & Hanson, 1960) in cases where the H-zone is the middle of the sarcomere could still be detected. This myosin is more readily denatured and therefore the Ca^{2+} ATPase activity falls in the 4-hr period. The Mg^{2+} ATPase on the other hand is the enzyme associated with myosin bound to actin. This system, being more resistant to the effects of pH and temperature, denatures only after the period of 37°C has been extended beyond 4 hr.

In muscle undergoing rigor at 37°C, therefore, it is likely that there are three

systems which will denature: some sarcoplasmic proteins, as described by Bendall & Wismer-Pedersen (1962) and more exactly defined by Scopes (1964); unbound myosin; and myosin bound to actin. Of these, the latter requires the longest period at 37°C and/or the lowest pH before denaturation becomes apparent.

Acknowledgment

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Freeze-drying of beef

I. Theoretical freeze-drying rates of beef

R. BRALSFORD*

Summary. A theoretical model of freeze-drying is developed from which the dependence of drying rate and ice temperature on processing conditions can be calculated. The theory is based on the measured permeability and thermal conductivity of the completely dry material. When applied to beef steaks, the theory predicts that, at acceptable surface temperatures, the system is heat transfer limited and that the fastest sublimation rates should be obtained at high product surface temperatures combined with high cabinet pressures; if however the surface temperature is low (less than 20°C) the cabinet pressure should also be kept low. The probable effects of pre-drying treatments such as slow freezing and cooking are also discussed.

Introduction

Studies of the fundamentals of freeze-drying foods have tended to be of two kinds. Firstly, there have been studies of the purely mathematical type in which the transport properties of the food have been calculated in terms of its supposed physical structure. Secondly, there have been purely experimental studies in which the properties of the food have been inferred from the effects of altering the processing conditions. Both approaches have their limitations: the purely theoretical models are inevitably highly idealized and occasionally completely divorced from reality, whereas in the purely experimental approach, it is often difficult or impossible to separate properties of the food from those of the particular freeze-drier in use. Furthermore, the second method pre-supposes a simplicity in the relationship between cause and effect which the systems under study do not in fact possess. The present paper is concerned with the middle ground between these two extremes; it represents an attempt to predict the detailed drying behaviour of the food from the measured physical properties of the completely dry material. This paper deals with the measurement of permeability and thermal conductivity of beef and the development of a method by which these values may be used to compute the detailed theoretical drying behaviour. Part II (Bralsford, 1967) is concerned with a method for testing the predictions of the theory.

Author's address: Unilever Research Laboratory, Colworth House, Sharnbrook, Bedford.

* Present address: Unilever N.V., Burgemeester S'Jacobplein, Postbus 760, Rotterdam 2.

Materials and methods

Samples

The bulk of the permeability and thermal conductivity measurements were made using samples of cow beef topside, which, prior to drying, weighed between 30 and 50 g. The samples were in the form of circular discs or plugs. Most samples were dried raw after pre-freezing in about 15 min by contact with solid carbon dioxide but others were subjected to pre-drying treatments which included pressure cooking at 5 and 15 lb/in^2, and slow freezing over several days at -10°C.

Thermal conductivity

Two methods were used to measure thermal conductivity. The first was a dynamic method due to Mann & Forsyth (1956) in which an electrically heated probe was inserted into the material under test and its rate of rise of temperature was used to calculate the thermal conductivity of the material. In the second method, heat-flow transducers were placed on either side of the sample and used to measure the heat flowing through it from a heat source on one side, to a heat sink on the other. Thermocouples attached to fine copper grids measured the temperature difference across the sample. With the use of two transducers heat losses from the edges of the sample could be corrected for. In both methods the apparatus was enclosed in an evacuable enclosure through which water vapour or air could be flushed at any chosen pressure.

Although the dynamic method is theoretically unsound it was found to give results in good agreement with those obtained by the steady state method. Furthermore, it had the two advantages that it did not require samples of any particular geometry, nor did the temperature of any part of the sample differ from the mean by more than about 2°C during the course of the measurement.

Permeability

Samples up to 2 in. diameter and 1 in. thick were sealed into the sample holder, shown in Fig. 1, with the aid of Apiezon-compound Q. The sample and holder were

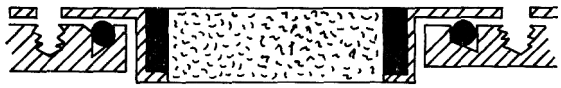

Fig. 1. Sample holder.

then sealed by way of a neoprene O-ring into a brass vacuum enclosure so as to divide the space within it into two parts. Air or water vapour was admitted to one part and pumped out of the other by a rotary vacuum pump *via* conventional refrigerated vapour traps. Total pressures on either side of the sample were measured with a matched pair of Pirani gauges or, in some later measurements, with a differential diaphragm gauge.

The latter instrument, the Atlas Microtorrmeter, had the advantage that pressure differences could be measured directly and since the calibration was independent of sample gas it could be used with mixtures of gases.

Measurement of mass transfer rate

The measurement of the permeability of freeze-dried foods, at the pressure employed in freeze-drying requires methods for measuring mass transfer rates of the order of 10^{-5} g.sec^{-1}. For permanent gases this problem is easily solved by allowing the exhaust gas from the vacuum pump to displace a pellet of water along a horizontal tube of known bore. Water vapour transfer rates can be measured by following the changing level of water contained in a narrow-bored glass tube as it evaporates into the permeability apparatus. In the latter case, however, it is necessary to compromise in the choice of the tube between one with a bore small enough to produce a rapidly changing level and one large enough to prevent excessive surface cooling and consequent reduction in vapour pressure. In practice this means watching the liquid level over a period of an hour or so in order to obtain reasonable accuracy. A better method for measuring water vapour transport rates is illustrated in Fig. 2. The glass cell

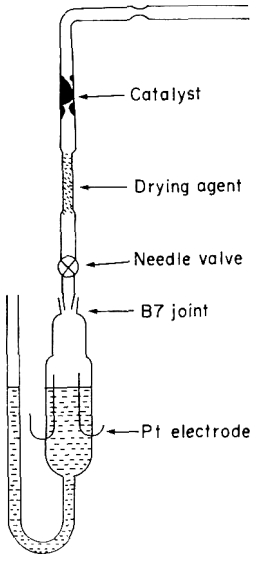

FIG. 2. Electrolytic water vapour generator.

contains about 2 ml of water and has platinum electrodes sealed through the walls. The electrodes are placed so as to project above the liquid surface and a current of a few milli-amperes passing between them electrolyses the water. The mixed oxygen and hydrogen gases pass through a needle valve, are dried and then come into contact with a palladium catalyst (L. Light & Co.) where they re-combine to form water vapour

which passes into the permeability apparatus. The needle valve is adjusted to balance the rate of release of gas against the rate of production by noting when the liquid level is steady in the side arm. Once set, the level tends to be self stabilizing; over-production of gas, for example forces liquid into the side arm thus reducing the effective electrode area and hence the electrolytic current.

Provided that the catalyst is 100% efficient, the electrolytic current is an absolute measure of the rate at which water vapour passes through the apparatus. A current of 100 mA produces about 10^{-5} g. sec^{-1} of water vapour. The volume of liquid in the cell is kept small so that it is quickly saturated with gas at the temperature of the cell; this is usually considerably above ambient because of ohmic heating.

Results and discussion

Thermal conductivity

Fig. 3 shows the result of thermal conductivity measurements on freeze-dried raw lean beef. The diagram shows the effect of changes in water vapour pressure and air pressure on the thermal conductivity along the fibres and also across the fibres. The results for air are in good agreement with those published by Harper (1962) and show the usual flattening out at high pressures. Results for water vapour do not seem to have been published previously but it is obvious from Fig. 3 that these are significantly different from those obtained with non-condensable gases. In particular, they show no flattening out over the range of pressures studied and the values lie increasingly far

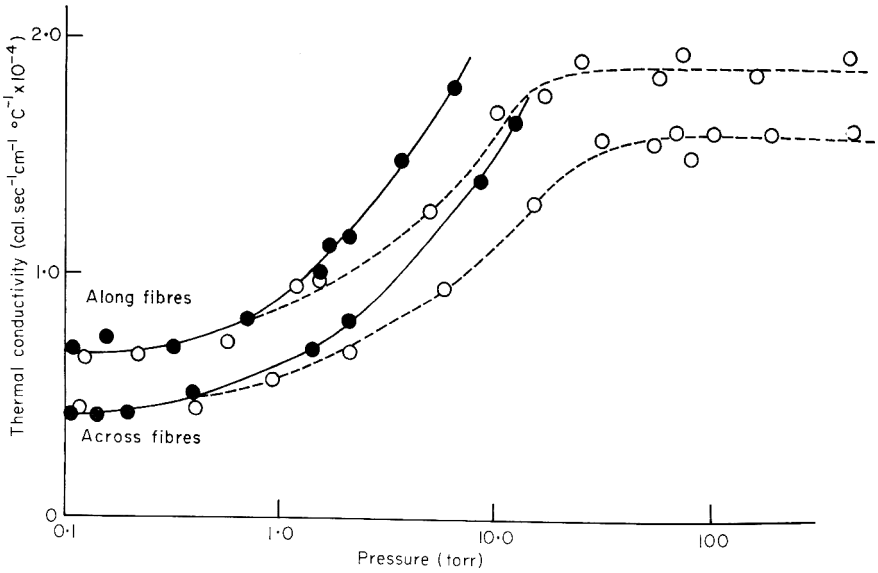

FIG. 3. Variation of thermal conductivity of freeze-dried lean beef with pressure. ○, Air; ●, water vapour.

above those for air. This is presumably because of increasing rehydration of the sample with increasing vapour pressure.

The various pre-drying treatments employed had no significant effect on the thermal conductivity parallel to the fibres but treatments leading to a more open structure, such as slow freezing or cooking, tended to reduce the conductivity in the perpendicular direction.

Permeability

In this work permeability (G) has been defined by the relation:

$$M = G\,(p_1 - p_2)\,A/x, \qquad (1)$$

where M is the mass flow rate, and p_1 and p_2 the pressures on the two sides of the sample which has a thickness x and area A. This is not the conventional definition but it has the advantage that it permits mass flow to be treated in a way analogous to heat flow.

Fig. 4 shows the results of permeability measurements made with raw freeze-dried lean beef where the flow was parallel to the meat fibres. The results for water vapour have not been continued to such high pressures as those for air because of the difficulty of measuring very high vapour mass transfer rates, but in both cases the permeability becomes independent of pressure in the region applicable to freeze-drying, i.e. 0–4·5 torr.

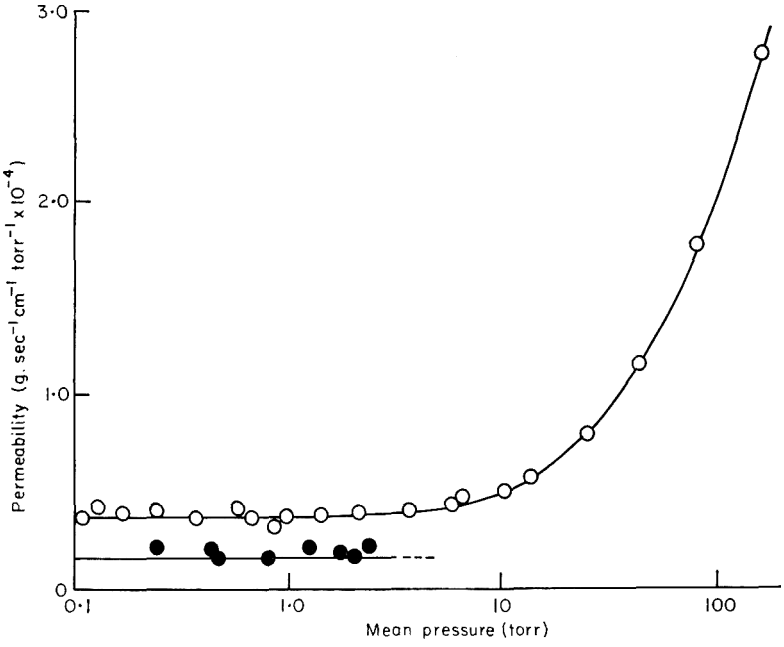

FIG. 4. Permeability of raw freeze-dried beef. Flow parallel to meat fibres: ●, air; ○, water vapour.

In general the permeability perpendicular to the fibres was found to be only about a third of the value parallel to the fibres. However, the permeability results tended to be much more variable between samples with the same nominal treatments than was the case with the thermal conductivity, presumably because of the much greater effect of any local irregularity in the structure of the sample. Table 1 summarizes the effects of pre-treatments on the permeability parallel to the fibres; the errors indicate the maximum deviation from the mean.

TABLE 1. Permeabilities of beef to water vapour at low pressure

	Mean permeability ($g.sec.^{-1}\ cm^{-1}\ torr^{-1} \times 10^{-5}$)	No. of samples
Cooked		
(1) 5 lb/in^2	1·5 ± 0·4	7
(2) 15 lb/in^2	1·9 ± 0·5	6
Raw		
(1) Quick frozen	1·4 ± 0·5	12
(2) Slow frozen	4·4 ± 1·5	8

The nature of the pre-treatment thus has a much greater effect on permeability than it does on thermal conductivity. Treatments leading to a more open structure, such as slow freezing, tend to increase the permeability.

The results presented here, which form the basis of the theoretical calculations, are in general similar to those published by Harper (1962) who has made measurements on a much wider range of foods. They do, however, differ in two important respects. Firstly, it is clear that hydration of the meat contributes significantly to the pressure dependence of the thermal conductivity, so that the behaviour in the presence of water vapour is quite different from the behaviour with other gases. Secondly, we find that the permeability of beef, and also root vegetables, tends towards a constant value at low pressure and does not pass through a minimum as reported by Harper. The behavour described here is the more readily understandable in terms of Knudson diffusion.

Calculations of theoretical drying behaviour

General considerations

The only two independent variables governing the freeze-drying process are the temperature and pressure at the food surface. Any useful theory of freeze-drying must describe the process in terms which use only these two parameters as variables. The aim of the calculation should be to predict the sublimation rate and the temperature of the ice core as functions of time, and also as functions of the geometry of the food piece. Furthermore, since we have very little knowledge concerning the microscopic processes involved in the freeze-drying, the only reasonable approach to the problem is to make

the maximum possible number of simplifying assumptions. These may be listed as follows:

(1) There is a sharp discontinuity in the moisture content at the ice front. In other words, we have taken the accepted model of freeze-drying in which the ice core and the porous layer are regarded as distinctly separate regions.

(2) The moisture content of the porous layer is in equilibrium with the water vapour flowing through it.

(3) The only resistance to the movement of water vapour within the food is that due to its passage through the labyrinth of pores in the dried layer. Any resistance due to cell walls is neglected.

On the basis of these assumptions, the thermal conductivity and permeability data may be applied directly to the calculation of heat and mass transfer rates. For simplicity, consider a piece of steak which is being dried from one side whose area (A) is large compared with the thickness of the piece (d). If the food piece has this form we may regard the cross-sectional area available for heat and mass transfer as independent of time.

When the porous layer has thickness x, the mass transfer rate (M) is given by:

$$M = G\,(p_i - p_s)\,A/x. \qquad (2)$$

Over the range of pressure applicable to freeze-drying, G is a constant independent of pressure, and is also assumed to be independent of temperature. The mass transfer rate is thus a function of only two variables, the pressure at the surface of the piece, p_s (chamber pressure) and the pressure at the ice surface, p_i. Now it follows from the considerations of kinetic theory that, except for very small porous layer thicknesses, the sublimation rate of ice evaporating into a vacuum is several orders of magnitude greater than that permitted by the permeability of the porous layer; consequently, the rate of production of vapour at the ice surface will always be sufficient to balance its rate of removal. It follows that the pressure at the ice surface, p_i in equation (2), is in fact the equilibrium vapour pressure of the ice, and this is uniquely related to ice temperature.

It is therefore possible to specify the sublimation rate in terms of ice temperature and chamber pressure only and Fig. 5(a) shows in a general way how the sublimation rate would vary with these two variables for a given porous layer thickness. The figure shows the expected result, that sublimation rate is greatest at low chamber pressure and at high ice temperature.

The corresponding heat transfer rate (Q) through the porous layer is given by:

$$Q = K\,(T_s - T_i)\,A/x, \qquad (3)$$

where K is the thermal conductivity, and T_s and T_i the surface and ice temperatures respectively. As we have already seen, the thermal conductivity term in equation (3) is a function of pressure. In order to calculate the heat transfer rate, therefore, the value of K must be chosen appropriately to the mean pressure in the porous layer $(p_i + p_s)/2$.

This can again be done quite generally and in terms of the same two variables (Fig. 5b).

The shape of the heat transfer curves requires some explanation. Imagine first of all that K is independent of pressure, then the heat transfer curve would simply be a straight line passing through T_s and having a slope proportional to $-K$ (broken line

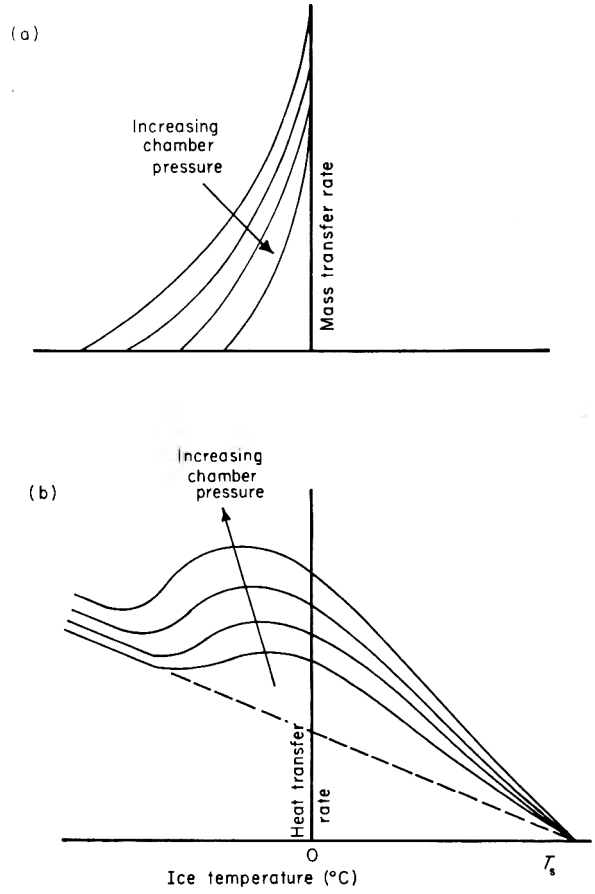

FIG. 5. Effect of chamber pressure on the transfer processes in the porous layer. (a) Mass transfer. (b) Heat transfer.

in Fig. 5b). The same line would describe all possible chamber pressures. However, since K increases with pressure, this straight line will be modified at chamber pressure $p_1, p_2, p_3, \ldots$, etc. Firstly, at very low ice temperatures the mean pressure in the porous layer will tend to $\frac{1}{2}p_1, \frac{1}{2}p_2, \ldots$, etc., with thermal conductivities $K_1, K_2, \ldots$ Hence, the single heat transfer line will be replaced by a set of straight lines whose gradients increase with increasing chamber pressures. All these lines when produced will pass

through the point on the temperature axis equal to the surface temperature. However, as the ice temperature increases so will the mean pressure in the porous layer, with corresponding increases in thermal conductivity. Each heat transfer curve will thus begin to deviate from linearity. In effect, every ordinate on the straight line must be multiplied by a factor which increases with ice temperature. All the heat transfer curves must nevertheless still pass through the point on the temperature axis equal to the surface temperature. We thus have the complete set of curves shown in Fig. 5(b). The chief point of interest in these generalized heat transfer curves is that it is possible for the heat transfer rate to be greater at high ice temperatures than at low ones, in spite of the smaller temperature gradient within the dried layer.

Heat and mass transfer are not of course independent quantities. They are related by the formula:

$$Q = L \times M, \tag{4}$$

where L is the latent heat of sublimation of ice. The value of L is known to vary only slightly over the range of 0° to −40°C. Hence from equations (2), (3) and (4), we obtain two simultaneous equations for the sublimation rate:

$$Mx/A = G(p_i - p_s), \tag{5}$$

$$Mx/A = -\frac{K}{L}(T_i - T_s) \tag{6}$$

If the surface conditions (p_s, T_s) are kept constant, inspection of these two equations shows that they can only be simultaneously satisfied if p_i and T_i are also independent of time. This is an important result, since it means that for steak-like food pieces, the ice temperature will remain constant throughout the drying process. This fact has been previously indicated by Harper (1962). The constancy of p_i and T_i also means that the right-hand sides of equations (5) and (6) do not involve time. We can, therefore, put $Mx/A = R$, which we term the *sublimation rate constant*. This constant enables us to define the sublimation rate for the complete process from beginning to end.

The most informative and convenient method of solving these two simultaneous rate equations is a graphical one. Equation (5) defines the rate permitted by permeability. Its general form has already been discussed. Equation (6) defines the rate permitted by the thermal conductivity. This function is identical with that plotted in Fig. 5(b), except that every ordinate must now be divided by the latent heat of sublimation. If these two sets of curves are plotted on the same graph, the intersection of any two rate curves having a common chamber pressure will define: (a) the ice temperature T_i, and (b) the sublimation rate constant R.

The process described in terms of sublimation rate constant

Once the rate constant has been obtained for a given set of surface conditions, the complete drying process can be calculated theoretically. For example, if a steak-like

food piece is dried from two sides, the actual sublimation rate at any time t is given by:

$$M = 2RA/x. \tag{7}$$

We also have:

$$M = 2A\,(\rho_0 - \rho)dx/dt, \tag{8}$$

where ρ_0 and ρ are the densities of the wet and dry parts of the food respectively.

Combining equations (7) and (8) and integrating gives:

$$t = (\rho_0 - \rho)\,x^2/2R, \tag{9}$$

showing the relationship between dried layer thickness and time.

The sublimation rate (M) is obtained as a function of time by combining equations (7) and (9), from which we obtain

$$M = A\,(2R\,(\rho_0 - \rho)/t)^{\frac{1}{2}}. \tag{10}$$

The total drying time is obtained by putting $x = d/2$ where d is the total thickness. Hence, from equation (9), the total drying time (T) is given by:

$$T = (\rho_0 - \rho)d^2/8R. \tag{11}$$

The same method can be applied to other geometric forms, e.g. cubes heated from one, two or three sides, and it is easily shown that the rate constant for these cases is closely related to that for steaks.

Beef

In Fig. 6 the two functions defining the sublimation rate constant for a sample of beef are plotted in the manner already indicated, i.e. the sublimation rates permitted by permeability and thermal conductivity are plotted as functions of ice temperature. The thermal conductivity data used in the calculation was that shown earlier in Fig. 3. The permeability constant was $1{\cdot}4 \times 10^{-5}$ g. sec^{-1} cm^{-1} torr^{-1}, i.e. a value appropriate to a dried sample of quickly-frozen, uncooked lean beef, with vapour flow parallel to the fibres. The surface temperature was taken as 60°C, which is the value generally accepted as the maximum that can be allowed if heat damage of the product is to be avoided. (The broken lines of the heat transfer curves have no real meaning since they represent conditions where $p_s > p_i$ and there can be no sublimation.)

The very fact that the two sets of curves intersect at all indicates at once that the sublimation rate is limited by the heat transfer in the porous layer. If permeability were the rate limiting factor, then the mass transfer curves would lie below the heat transfer curves; there would be no intersection at temperature below 0°C and the ice would always melt, whatever the chamber pressure.

The points of intersection of the rate curves show that increasing the chamber pressure not only results in higher ice temperatures but also increases the sublimation rate. This fact brings out the element of compromise inherent in freeze-drying; for the shortest drying times we must accept higher ice temperatures, and this leads to some

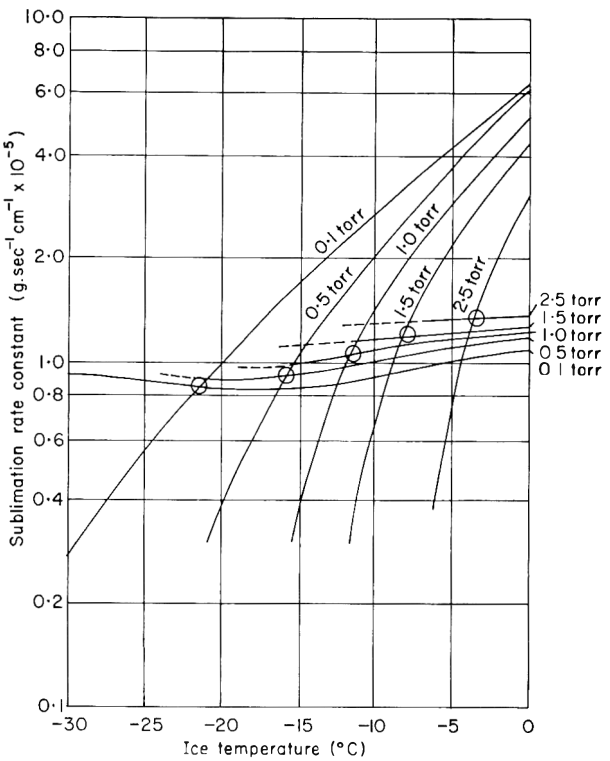

FIG. 6. Sublimation rate curves for lean beef (surface temperature 60°C).

loss of quality in the final product. If we accept that the maximum permissible ice temperature for acceptable beef products is about −10°C, and that the maximum permissible surface temperature is 60°C, the rate curves indicate that we should operate at a chamber pressure of about 1·25 torr.

When we consider surface temperatures other than 60°C, the situation changes. We can anticipate the probable effect of changing T_s if we look at the generalized heat transfer curves described earlier. We can distinguish two extreme cases. At low surface temperatures, all the heat transfer curves will intersect the temperature axis near 0°C, and they will inevitably have negative slopes in the freeee-drying region (Fig. 7a). At high surface temperatures, on the other hand, it is possible that they will have positive slopes (Fig. 7b). The points of intersection with the mass transfer curves will, therefore, give rate constants which decrease with increasing pressure when the surface temperature is low, but increase when the surface temperature is high. Fig. 8 was obtained for the specific case of beef by calculating heat transfer rate curves for a number of surface temperatures. This diagram confirms the fact already indicated that, at a surface temperature at 60°C, there is some slight advantage in working at high rather than low chamber pressure. But at a surface temperature of +100°C,

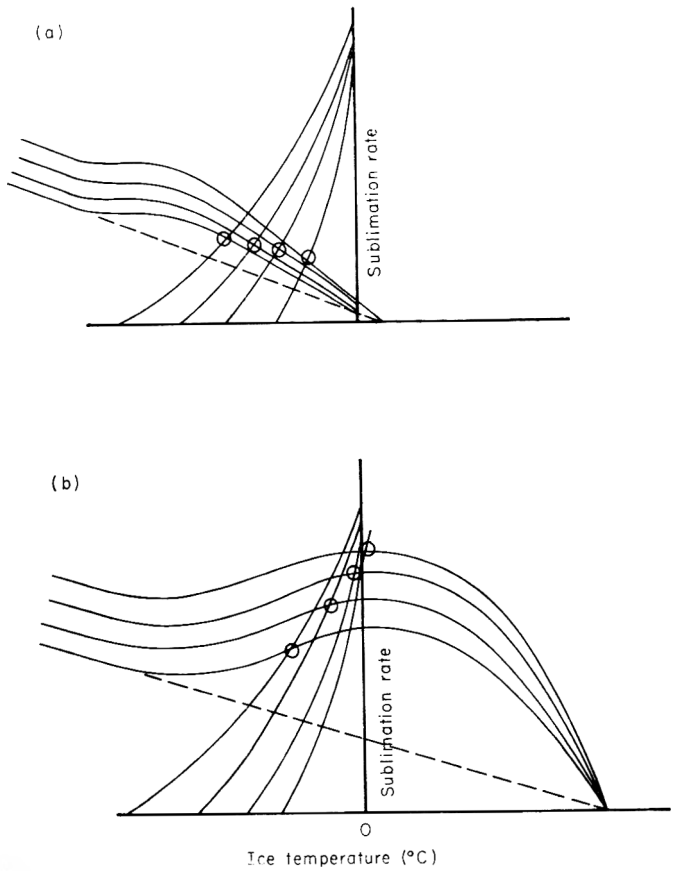

FIG. 7. Effect of surface temperature on the sublimation rate curves. (a) Low surface temperature. (b) High surface temperature.

the advantage is much greater; for example, changing the chamber pressure from 0 to 2 torr would increase the sublimation rate by about 80%, though admittedly with loss of quality. At a surface temperature below 20°C, on the other hand, the fastest sublimation rate is obtained at low pressures.

To determine the effect of pre-drying treatments on the rate curves, we must consider the effect of such treatments on the material's thermal conductivity and permeability. We have already seen that freezing-rate and cooking have a negligible effect on the thermal conductivity along the fibres. The heat transfer rate curves are, therefore, little affected by the pre-treatment. On the other hand, any pre-treatment which leads to a more open structure, such as slow freezing, increases the permeability by a factor of two or three. All the permeability rate curves will thus be shifted upwards in the rate curve diagram, and consequently their points of intersection with the heat

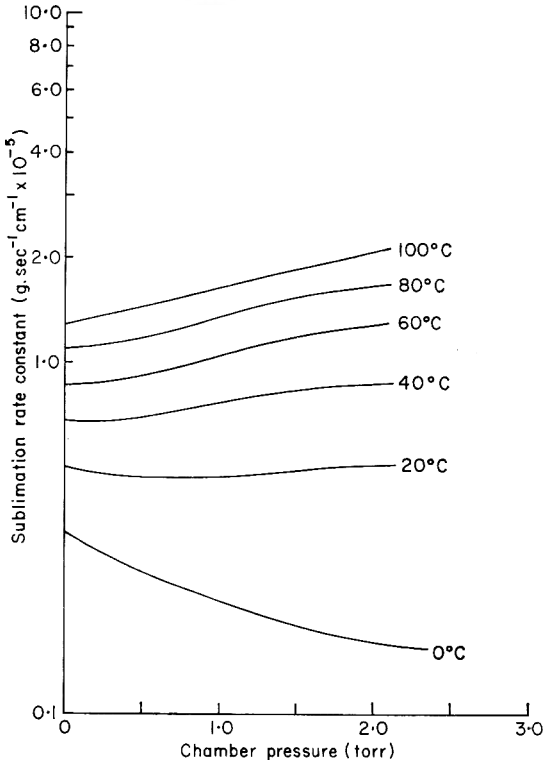

FIG. 8. Sublimation rate constant as function of chamber pressure at different surface temperatures.

transfer curves will be shifted towards lower ice temperatures (see Fig. 6). Increased permeability will, therefore, always result in a lower ice temperature but its effect on the sublimation rate will depend on the surface temperature. At low surface temperatures, where the heat transfer rate curves have a negative slope, increased permeability will increase the sublimation rate. At high surface temperatures, on the other hand, increasing the permeability of the material will decrease the sublimation rate (see Fig. 7).

Conclusions

It is possible by relatively simple methods to predict the freeze-drying behaviour of foods. Sublimation rate, total drying time, ice temperature and their dependence on processing conditions can be calculated by a theory which is based on measured physical properties of the dried material. The calculations suggest that for the case of beef the process is heat transfer limited and that the fastest drying conditions should be realized at high surface temperatures combined with high pressures, but if for any reason the surface temperature must be kept below 20°C, low cabinet pressures should be used.

The assumptions involved in these calculations are fairly drastic and it is impossible to justify many of them by argument. The justification of the theory must, therefore, depend on an experimental confirmation of its predictions; it is this which forms the second part of this paper.

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Freeze-drying of beef

II. A calorimetric method for comparing theoretical with actual drying rates (measurement of freeze-drying rates of beef)

R. BRALSFORD*

Summary. A calorimetric method is described by which some of the predictions of the theory described in Part I (Bralsford, 1967) are compared with the actual freeze-drying behaviour of beef. In particular, the method permits the continuous measurement of heat transfer into the sample as it dries. From this the sublimation rate at any time may be deduced and the end of sublimation is readily determined. For the most part the agreement between experiment and theory is good; the prediction that, at normal surface temperatures, the sublimation rate should increase with chamber pressure is confirmed. It is suggested that such discrepancies that do exist can be explained as the effects of liquid diffusion into the dried shell and that the consequences of this are that product quality must be expected to depend to some extent on the size of the food piece.

Introduction

In Part I (Bralsford, 1967), it was shown that two parameters of the freeze-drying system may be predicted theoretically. These are: (1) the temperature of the ice at the centre of the food piece, which should remain constant for steak-like pieces provided that the surface conditions remain unchanged, and (2) the actual sublimation rate.

To test the theory satisfactorily either, or preferably both, of these quantities should be measured experimentally. Inspection of the rate curve equations discussed in Part I (Bralsford, 1967), shows that the theory can be tested quite rigorously by a simple calorimetric method which avoids many of the difficulties associated with weighing the sample.

Theory of the method

Consider a sample whose area (A) is large compared with its thickness (d). Assume

Author's address: Unilever Research Laboratory, Colworth House, Sharnbrook, Bedford.

* Present address: Unilever N.V., Burgemeester S'Jacobplein, Postbus 760, Rotterdam 2.

that the sample is being dried from two sides, so that the thickness of the porous layer on each side is x at any time t. The total rate of heat flow into the sample (dQ/dt) is then given by:

$$\frac{dQ}{dt} = 2KA\ (T_s - T_i)/x, \tag{1}$$

where T_s and T_i are the surface and ice temperatures respectively. It should be remembered that, for the theory to be valid, T_i should be independent of time for a constant surface temperature and chamber pressure.

If each ice front recedes a distance dx in time dt, the quantity of ice sublimed will be $2(\rho_o - \rho)\ Adx$, where ρ_o and ρ are the densities of the wet and dried parts of the sample, respectively. The quantity of heat (dQ) required to sublime this quantity of ice is given by:

$$dQ = 2(\rho_o - \rho)\ AL\ dx, \tag{2}$$

where L = latent heat of sublimation.

From equations (1) and (2):

$$KA\ (T_s - T_i)\ dt = (\rho_o - \rho)\ ALx\ dx. \tag{3}$$

Integrating equation (3), the thickness of the dried layer at any time t is given by:

$$x^2 = 2K\ (T_s - T_i)\ t/(\rho_o - \rho)L, \tag{4}$$

or substituting in equation (1):

$$(dQ/dt)^2 = 2KA^2(T_s - T_i)\ (\rho_o - \rho)\ L/t. \tag{5}$$

This last equation shows us that a plot of $(dQ/dt)^{-2}$ against t should yield a straight line. Further, from this plot, dynamic values can be obtained for the following:

(1) The thermal conductivity of the porous layer: this can be obtained from the slope of the line, provided that we measure T_s and T_i. Comparison of this value with that measured for a completely dry sample will be a check on the validity of the basic assumptions.

(2) The rate constant: the theoretical sublimation rate M at a time when the porous layer has thickness x is given by:

$$M = 2RA/x,$$

where R is the calculated rate constant. The actual sublimation rate can be derived from the measured thermal conductivity and the temperature, so that:

$$M = 2K(T_s - T_i)\ A/Lx.$$

Thus, the theoretical rate constant R may be compared with the measured rate constant $K(T_s - T_i)/L$.

(3) Total drying time: The linear relationship, between $(dQ/dt)^{-2}$ and t will hold so long as ice remains in the sample. But at the end of sublimation, there should be a sharp drop in the heat flow to the sample, because the heat sink within it has disappeared. Ideally, we would expect the heat flow to fall to zero, but since it is generally

recognized that the end of sublimation is normally followed by a long period of desorption, this ideal will never be realized. Nevertheless, the end-point should be marked by a discontinuity in the heat flow curve from which we can obtain the total drying time.

Materials and methods

The variation of ice temperature with chamber pressure has been measured with the simple apparatus shown in Fig. 1. One of the plane faces of a thin cylindrical

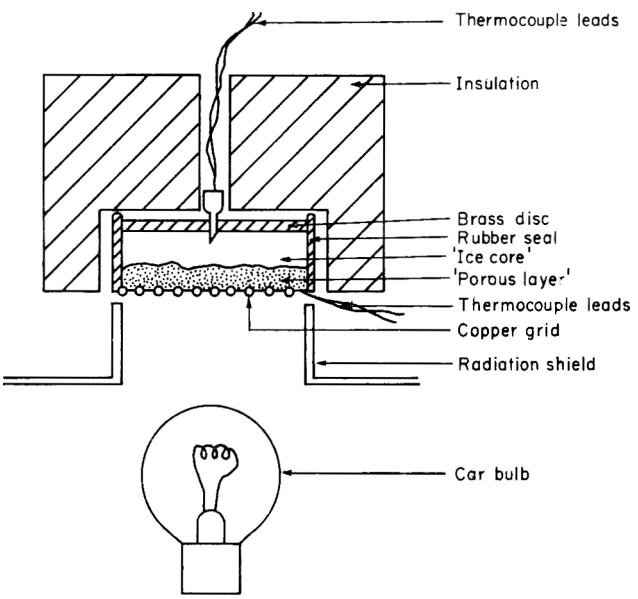

FIG. 1. Method of measuring variation of ice temperature with chamber pressure.

sample was heated so as to maintain a constant surface temperature. The remaining surfaces were sealed and carefully insulated. Consequently, as drying proceeded, the ice front receded uniformly from the open face into the sample. Thermocouples were used to measure surface and ice temperatures.

In several experiments, samples were dried at constant surface temperatures. For any given sample, the chamber pressure was held at a number of constant values and the ice temperature was measured when equilibrium was reached. The various chamber pressures were selected in random order. When the drying was completed, the sample was removed and permeability and thermal conductivity measurements made on it. From these measurements a theoretical ice temperature versus pressure relationship was calculated, in the manner already discussed in Part I (Bralsford, 1967).

The freeze-drying calorimeter is shown in Fig. 2. It consists essentially of a heavy

copper barrel (B) which may be closed at both ends and evacuated via the connection in the base (V). The barrel contains two close fitting copper pistons (P), each of which has a thin heat flow transducer (T) cemented to the centre of its inner surface. Both barrel and pistons are nickel-plated. The two transducers record the total heat flowing from the pistons through ribbed copper inserts (I) into the sample (S). The copper inserts allow sublimed vapour to escape, and each of them has a copper grid

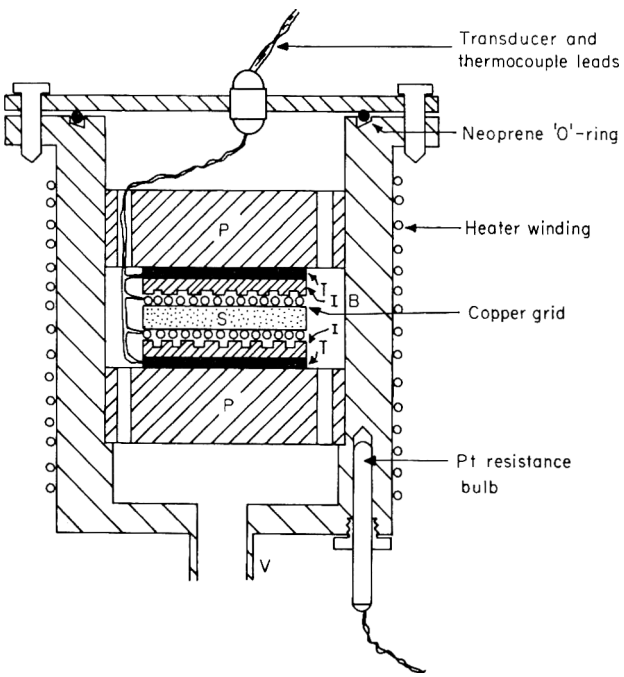

FIG. 2. Calorimeter for measuring heat transfer processes in freeze-drying.

soldered to the side in contact with the sample, to ensure a uniform surface temperature. Since the heat flowmeters are very thin, the temperature drop between the calorimeter block and the food surface is negligibly small, except in the very early stages of sublimation when the heat demand is high. A constant surface temperature can therefore be maintained by thermostating the calorimeter block at the required temperature. This is made easier by the calorimeter's large thermal capacity, which ensures that its temperature is little affected by the sample, whatever the heat demand. An Ether temperature controller, with a platinum resistance sensing element located in the barrel of the calorimeter, is used for temperature control purposes.

In the measurements under discussion, surface and ice temperatures were measured with fine thermocouples. Pressures were controlled at a pre-determined level by bleeding air into the vacuum pump, under which conditions, a negligible quantity of air diffused into the calorimeter. True cabinet pressures were measured with a

silicone-oil manometer, but for control purposes a Pirani gauge was used because of its faster response. The outputs from the flowmeters and thermocouples were fed to a four-channel potentiometric recorder.

All the samples for the calorimeter were taken from the centre of the same large piece of cooked cow-beef topside. They were sealed into polythene bags, and, after initial quick freezing, were stored at about $-10°C$ until required for drying. In all cases, samples were cut with the meat fibres perpendicular to the flat surfaces.

Each sample was weighed immediately before and immediately after drying. The difference in densities $(\rho_0 - \rho)$ was calculated from the weight loss and the initial volume of the sample, the latter being obtained by the water displacement method. This gave values for $(\rho_0 - \rho)$ of about 0·72 g/ml. When the density change was calculated using the final volume of the sample, values of $(\rho_0 - \rho)$ were unrealistically high, presumably because of shrinkage during drying. Inspection of the derivation of the heat flow equations will show that the former value is to be used, and that the existence of shrinkage requires no alteration of the basic equations.

Results and discussion

The continuous curves in Fig. 3 show the theoretical variation of ice temperature

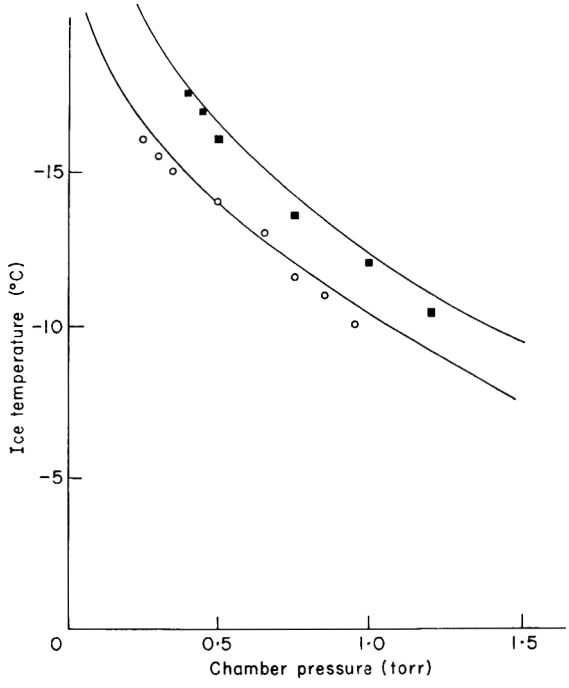

FIG. 3. Variation of ice temperature with chamber pressure for samples of beef (surface temperature 60°C). ○, Quick frozen; ●, slow frozen; —, theoretical.

with chamber pressure for two samples of beef, one quickly frozen and one slowly frozen. The experimental points on the graph, obtained with the apparatus shown in Fig. 1, represent the values actually measured. Considering the number of simplifying assumptions inherent in the theory, the agreement is remarkably good. However, the measured values of ice temperature do tend to be slightly higher than the theoretical ones.

Fig. 4 shows a recorder trace of the output from the freeze-drying calorimeter, i.e. the total heat flow into the sample, together with the surface and ice temperatures.

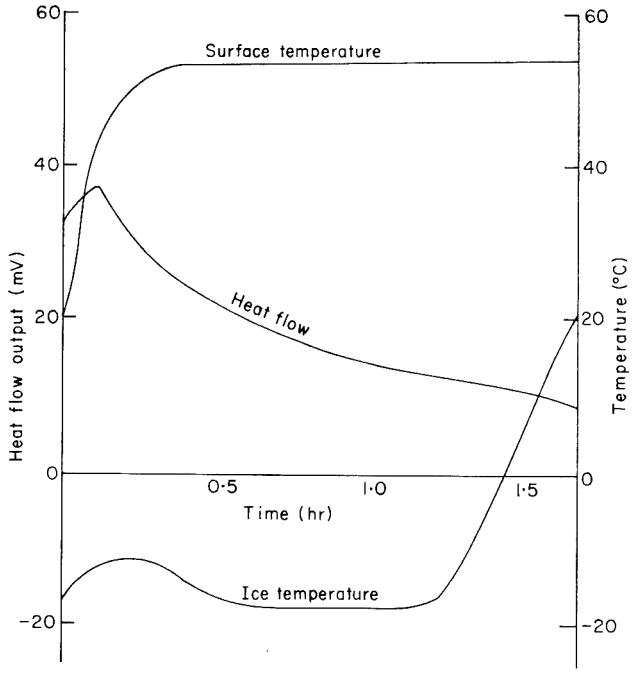

FIG. 4. Recorded trace of calorimeter outputs.

The surface temperature has reached its control value within about 15 min of pump down. The ice temperature rises slightly at first because of the stifling effect of the ribbed inserts during the initial high vapour removal rate, but thereafter remains reasonably constant as predicted by the theory.

Because of the finite warm-up time, the zero is in effect displaced by an amount t_0 and equation (5) must therefore be rewritten to take account of this:

$$(dQ/dt)^2 = 2KA^2(T_s - T_i)\ (\rho_0 - \rho)L/(t - t_0). \qquad (5a)$$

Fig. 5 shows a graph of $1/V^2$ plotted against t, where V is the flowmeter output (proportional to dQ/dt). This is linear, as expected, between the time that the surface temperature reaches its preset value and the time that sublimation ends. Extra-

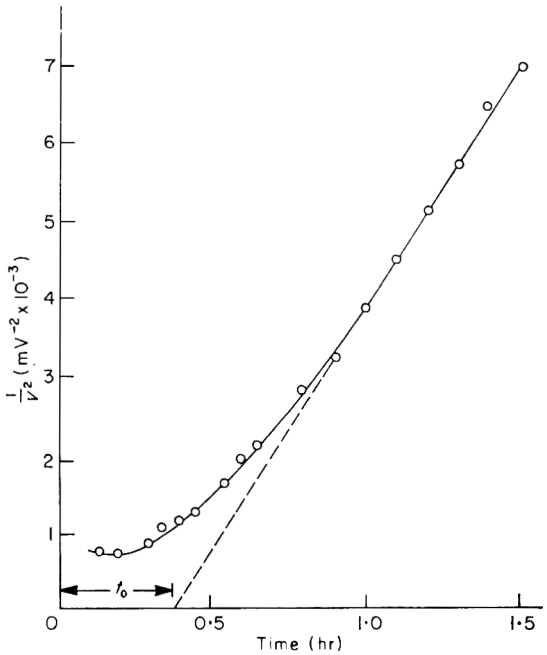

FIG. 5. $1/V^2$ plotted against time.

polation of the linear portion back to the time axis gives the zero correction t_0 required by equation (5a).

The end point is not easily detected in the straight forward presentation of Fig. 4, but when V is plotted against t on log–log scale (Fig. 6), the discontinuity in the curve is fairly sharp at low ice temperatures, although it becomes less so at higher temperatures. Equation (5) shows that the linear part of the log–log plot should have a slope of $-\frac{1}{2}$. In general this is not so, but adjustment of the time axis by an amount t_0 brings the slope close to its theoretical value.

Table 1 summarizes the results obtained for a series of samples dried under different pressure conditions but with the same surface temperature (60°C). Considering all the possible experimental errors involved, the agreement between the theoretical and experimental values of thermal conductivity are remarkably good. This shows that for beef the thermal conductivity measured during drying is virtually the same as that measured when the process is complete. There can be no doubt that the thermal conductivity increases with increased pressure in the porous layer.

The rate constants (R) also show good agreement, which is to be expected, since they depend directly on K, and only to a much smaller extent on the permeability of the porous layer.

Additional confidence in the results is gained from a further simple test. The area under the complete heat flow curve, when measured in appropriate units, should be

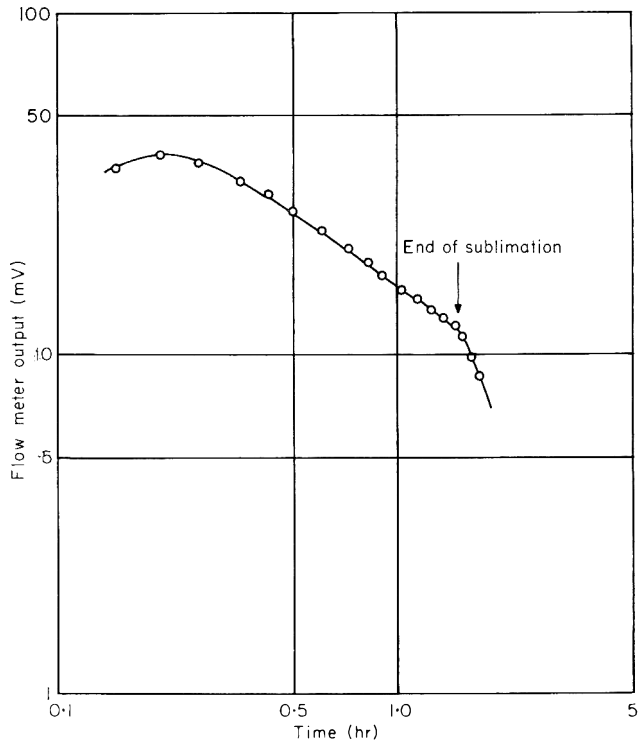

FIG. 6. log–log plot of heat flow against time.

TABLE 1. Comparison of theoretical and experimental drying parameters for cooked lean beef

Cabinet press (torr)	Ice temperature (°C)	Sample thickness (cm)	Density change (g cm^{-3})	Thermal conductivity (cal.sec.$^{-1}$cm^{-1} °C^{-1} × 10^{-5})		Rate constant (g.sec^{-1}cm^{-1} × 10^{-5})		Drying time (hr)	
				Post-drying	During drying	Calculated	Measured	Calculated	Measured
0·47	−17·5	0·70	0·70	8·10	8·0	0·90	0·84	1·35	1·15
0·64	−13·7	0·72	0·75	8·90	9·1	0·94	0·92	1·45	1·20
0·86	−11·5	0·68	0·71	9·70	9·4	1·01	0·92	1·15	1·15
0·98	−8·5	0·64	0·74	10·6	11·1	1·06	1·02	0·95	1·0
1·68	−10·5	0·68	0·76	11·1	11·9	1·24	1·16	1·0	0·85
1·80	−8·5	0·70	0·74	11·9	12·9	1·21	1·26	1·0	0·9
2·42	−7·5	0·68	0·72	13·1	12·4	1·35	1·15	0·85	1·2

equal to the product of the weight loss and the latent heat of sublimation of ice. Again the agreement is good.

If the experimental and calculated rate constants agree, then there is no reason to expect any discrepancy in the overall drying times. The agreement is reasonably good when the zero correction is applied, provided that the first deviation from linearity on the log–log plot is taken as an indication that sublimation has finished. At low temperatures there is no ambiguity; the break in the curve is quite sharp. But at higher pressures the change of slope is much more gradual. A possible explanation of this may lie in the higher equilibrium moisture content in the porous layer associated with the higher equilibrium moisture content in the porous layer associated with the higher pressure. The initial desorption rate will consequently be higher, and this will tend to mask the end of sublimation. There is however, an alternative explanation which seems more likely. At the higher ice temperature appreciable quantities of liquid phase water are present in the frozen core. In these circumstances it is probably an over-simplification to speak of a 'subliming ice front.' We must expect some liquid diffusion into the dried layer with the result that evaporation will now take place in a band of finite depth rather than at a sharply defined interface. The likely consequences of this will be most easily seen by reference to Fig. 7, which illustrates a

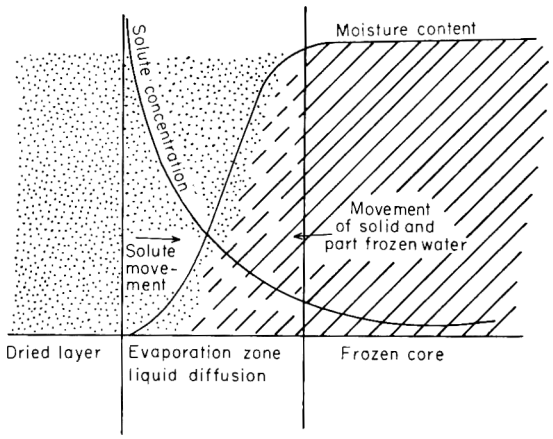

FIG. 7. Illustration of the concept of liquid diffusion in freeze-drying.

possible distribution of moisture in this region; the exact distribution is not important. If we imagine that the diffusion zone is fixed in space with the solid material moving through it, we can see that the solid will bring with it a mixture of ice and water, and as this passes through the diffusion zone, any solutes will be present in ever increasing concentration. The solutes will, therefore, tend to migrate against the water movement, and so doing, they will melt more ice, release more liquid-phase water, and tend to further broaden the diffusion zone. The chief limitation to this broaden-

ing process will be the continuous renewal of the interface by ice and water brought up by the solid.

In the early stages of drying, the rate of renewal of the interface will be comparatively high and the diffusion zone will be of limited width. The simple theory will therefore apply, and we need only recognize that 'x' no longer refers to a well defined dried-layer thickness, but is now the distance from the surface of the food to some point in an evaporation region of finite depth. However, as drying proceeds, the interface will be renewed less rapidly, and the diffusion zone will broaden until the process will eventually become one of vacuum drying rather than freeze-drying.

In support of the hypothesis outlined above is the observed fact that samples dried at higher pressures show a distinctly soft wet area if they are removed and sectioned shortly before the drying curve passes round the 'knee' on the log–log plot. Samples sectioned well before the knee have an ice core which is quite solid. In addition we would expect that our choice of the beginning of the knee as the point at which sublimation drying is presumed to have ceased, would give total drying times rather shorter than the theoretical ones, particularly at higher pressures. The results in Table 1 show evidence of this.

If the concept of liquid diffusion is substantially valid, the implication is that the quality of freeze-dried foods must be expected to depend on the size of the food piece as well as on the processing conditions. In other words, the process does not have the clear quality advantage over air-drying that it is commonly supposed to have for large food pieces.

Conclusions

From the substantially good agreement between the results presented here and the theoretical calculations presented in Part I (Bralsford, 1967) it is clear that the crude model which has been adopted describes the freeze-drying behaviour of beef reasonably well. It appears that beef behaves as though it were a fine-pored sponge soaked in water. Such discrepancies as do exist can be explained by making the model slightly more realistic, by supposing that the water in the sponge is replaced by a salt solution. The consequences of this are that product quality must be expected to depend to some extent on the size of the food piece; conditions which are adequate for small pieces may not be so for larger pieces.

On the question of optimum processing conditions it has been shown that increasing the chamber pressure always increases the thermal conductivity of the porous layer; at suitably high surface temperatures ($>20°C$) this increase is sufficient to produce a higher sublimation rate, in spite of the smaller temperature gradient within the porous layer.

The same model can be used to describe the freeze-drying behaviour of chicken, with similar success, but this cannnot be taken as indicating the general applicability of the theory. An identical approach to the problem of freeze-drying root vegetables

suggests that they too should behave in a very similar way to beef; in particular, these systems also should be heat transfer limited. However, this is manifestly not the case since it is quite easy to melt the ice core within a piece of carrot say, even with normal surface temperatures and the lowest cabinet pressures. The failure is hardly surprising; more surprising is the fact that this crude theory, which has been implied in many discussions of the rate-limiting factors in freeze-drying, works reasonably well for beef.

Acknowledgments

The author wishes to thank his colleagues of the Applied Physics Section at Colworth House for their valuable help and advice during the course of the work reported here

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Freeze-drying of cooked beef—some effects of freezing rate and freezing method

N. E. BENGTSSON

Summary. Results are given from comparative freeze-drying experiments studying the effects of freezing rate and of so-called thermal treatment on drying rate, water uptake and general quality of freeze-dried cooked beef. Unlike raw meat, cooked beef appeared to be little affected by freezing rate in connection with freeze-drying, except for markedly poorer appearance in the dried condition for the very slowly frozen product. Thermal treatment seemed to have no positive effect on drying rate or quality.

Introduction

In the freeze-drying of foods the length of the drying cycle is important to the economy of the operation, and the ability of the product to reconstitute fully and quickly is, likewise, important to convenience and quality in usage. Ice crystal size and location in the frozen product are generally considered important in both these respects. It is believed that rate of drying, as well as rate of water uptake, increases with the formation of larger intercellular ice crystals at slow freezing rates, resulting in larger channels for vapour and water transport. This opinion has been expressed in regard to foods in general by Kuprianoff (1962) and in regard to raw beef by Rolfe (1963) and Smithies (1962). Auerbach (1962) on the other hand found improved water uptake for very fast freezing rates of cooked meat, but did not investigate freezing rates below about 0·5 cm/hr.

Another possible means of improving drying rate and water uptake as well as quality in general was reported by Rey & Bastien (1962). For fruit juices he found that so-called thermal treatment, in principle consisting in conventional freezing followed by liquid nitrogen cooling to below −70°C and re-heating in order to achieve complete crystallization of freezable water, vastly improved freeze-drying rate and quality, and eliminated problems of incipient melting.

The primary objective of the present work has been to investigate whether very large intercellular ice crystals such as might be expected to form at very low freezing rates would lead to shorter drying time and quicker reconstitution, and what effects such treatment may have on other quality attributes. A secondary objective was to

Author's address: Swedish Institute for Food Preservation Research (SIK), Göteborg, Sweden.

look into whether 'thermal treatment' might possibly have a positive effect also on cooked meat, even though most of the freezable water might be expected to be frozen out already at conventional freezing temperatures. Measurements by Riedel (1957) have shown that for raw meat some slight amount of freezable water still remains unfrozen at −50°C. For cooked meat no such data were available.

Materials and methods

Lean meat of the longissimus dorsi muscle from 4–6-year-old cows, having an average fat content of 3%, was hung for 5–6 days. After trimming, the meat was cooked inside 15 × 7 cm stainless steel cylinders in water, with fibre direction parallel to the axis, to a final internal temperature of 75°C, cooled and sliced in 8mm slices transversely to the fibres.

Slices were frozen rapidly at 0·8 cm/hr freezing rate and very slowly at 0·06 cm/hr to a final temperature of −20°C. In experiments with 'thermal treatment' these freezing methods were combined with a secondary freeze by liquid nitrogen spray to a centre temperature of −70°C, followed by conditioning at −60°C before freeze-drying. The meat was freeze-dried to a final moisture level of about 2% in a pilot drier using radiant heat, the temperature at the surface and centre of the sample being measured by recording thermocouple. The chamber pressure and changes in weight (magneto-elastic load cell) were also recorded. The maximum temperatures for heating plates, product surface and ice front were 110°, 40° and −20°C, respectively. Chamber working pressure was maintained at 0·2 torr or less. The dried samples were nitrogen-packed at less than 1% 0_2 in gas-tight pouches of saran–cellophane–aluminium foil–polyethylene laminate and stored at room temperature up to 14 months.

Analytical methods

Residual moisture was determined by Karl Fisher titration or by drying in a vacuum oven, and oxygen headspace with a Beckman 777 oxygen analyser. Water uptake, or reconstitution, was determined by dipping a sample in water for 10 min while gradually warming from room temperature to serving temperature (70°C). The final weight was expressed as percent of original wet weight.

Water holding ability was determined by centrifuging according to Aitken, Casey & Penny (1962).

Organoleptic evaluation was carried out on the dried samples for colour and surface appearance, and on the reconstituted warm meat for taste, tenderness and juiciness by a six-member panel using a paired comparison technique with seven grade scale and evaluating statistically according to Scheffé (1952).

Results

The results of experiments with meat from six different animals comparing quick

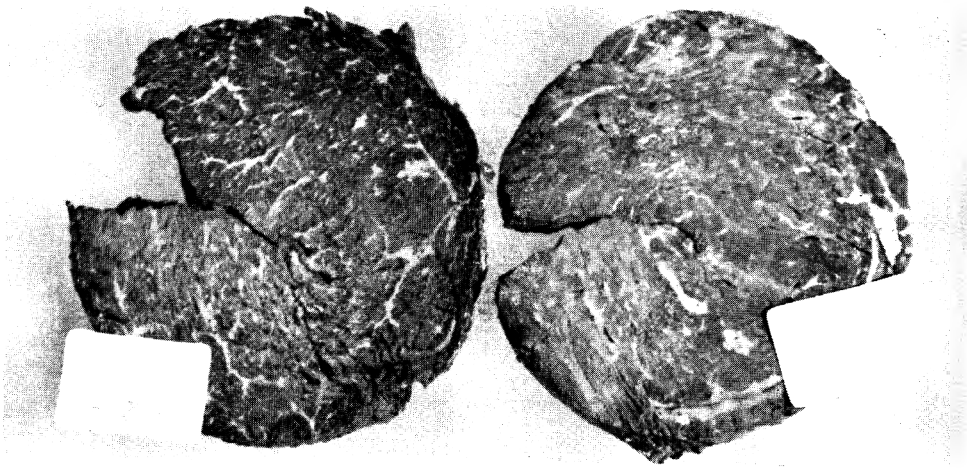

PLATE 1. Freeze-dried cooked beef. Freezing rate 0·06 cm/hr (left) and 0·8 cm hr (right).

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freezing and very slow freezing are summarized in Table 1 below.

TABLE 1. Very slow freezing compared to quick freezing for freeze-dried cooked beef (average values for six animals)

Evaluated property	Initial values		2–3 months storage		6–8 months storage		12–15 months storage	
	Quick freezing	Slow freezing	Quick freezing	Slow freezing	Quick freezing	Slow freezing	Quick freezing	Slow freezing
Drying time (hr)	6·5	6·2	–	–	–	–	–	–
Final moisture (%)	2·3	2·2	–	–	–	–	–	–
Headspace oxygen (%)	1·2	1·6	0·7	0·7	0·9	0·9	0·6	0·6
Reconstitution (% of original weight regained)	90·7	84·3	83·5	83·0	82·9	82·6	84·7	79·0
Centrifugation loss (%)	13·0	13·0	15·0	15·0	13·0	15·0	13·0	11·0

There was some tendency towards a very slight reduction in drying time for the slowly frozen meat and towards a slight advantage in water uptake for the quick frozen meat. The most striking difference between freezing rates was in product appearance, where the quick frozen was markedly brighter and fresher in colour and had a much smoother surface appearance (Plate 1).

However, this difference disappeared almost entirely after reconstitution and warming to eating temperature. In taste, tenderness and juiciness there was, initially, no noticeable difference. After 3 months storage, however, there was a tendency in favour of the quick frozen meat and after 6–8 months a slight, but statistically very significant, difference in that same direction. This difference again decreased after 15 months due to general deterioration of quality during storage at room temperature.

The results from experiments with a secondary freeze with liquid nitrogen spray (thermal treatment) are given in Table 2. They showed no promise in reducing drying time or improving quality by thermal treatment.

TABLE 2. Secondary freezing with liquid nitrogen in freeze-drying cooked beef (average values for four animals)

Evaluated property	After 1 week storage		After 2–3 months storage	
	Normal freezing	Thermal treatment	Normal freezing	Thermal treatment
Drying time (hr)	5·9	6·0	–	–
Final moisture (%)	2·1	1·8	–	–
Reconstitution (% of original weight regained)	88·5	88·4	31·7	85·3
Centrifugation loss (%)	18·2	18·1	15·0	15·9

Discussion

The magnitude of the difference in quality between freeze-dried cooked beef, that had been subjected to very slow freezing, and to quick freezing was perhaps surprisingly small considering the quality advantage that is often observed for quick-freezing in frozen foods, even if a significant difference in organoleptic quality developed in storage. The difference in surface colour of the dried product, on the other hand, should be quite noticeable to a potential consumer even if it did disappear on reconstitution. The advantage for slow freezing that might have been expected in drying rate and water uptake did not materialize. Judging from this study, therefore, little value can be expected from very slow freezing as a means of reducing drying cycles or improving water uptake for cooked meat. Intermediate freezing rates have not been studied in much detail but there seems little reason to expect other than intermediate quality compared to the rates investigated, especially when considering the work of Tuomy, Lechnir & Miller (1961) and Auerbach (1962) which indicated improvement in quality from further increases in freezing rates for cooked meat up to freezing with liquid nitrogen. It would appear then that cooked meat behaves differently in regard to freezing rate from raw meat, where there is some evidence in the literature of improved drying rate and water uptake with slow freezing. The experiments with thermal treatment with liquid nitrogen showed no particular promise with cooked meat, which may indicate that the level of unfrozen, freezable water is very low in cooked meat at temperatures around $-20°$ to $-30°$C. The lack of any dramatic response in quality of freeze-dried cooked beef to extremes of freezing conditions is perhaps to be traced back to the denaturated condition of the protein.

Conclusions

Freeze-drying experiments with cooked beef comparing rapid freezing at 0·8 cm/hr to slow freezing at 0·06 cm/hr showed no evidence of improved drying rate or water uptake as a result of larger ice crystals formed in slow freezing. The dried quick frozen product was clearly superior in appearance, even though this difference disappeared after reconstitution and heating. It also showed somewhat less storage deterioration in organoleptic qualities.

Thermal treatment in the hope of achieving more complete crystallization of water showed no positive effect on drying rate, water uptake or general quality.

Acknowledgments

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Relationship between meat quality and cooking losses

GY. GANTNER, L. KÖRMENDY, M. LOSONCZY AND F. LŐRINCZ

Summary. On the basis of visual assessment of exudative appearance, hams and shoulders were divided into two categories before curing: the 'normal' and the exudative ones. The 'normal' category included the DFD (dark, firm, dry) and the intermediate types having significantly lower average cooking loss. Since the two conditions are easily recognized, a preliminary selection is quite efficient. The efficiency of selection depends on the incidence of exudative meat to be processed. In the case of high incidence, selection may improve uniformity of pork products by decreasing cooking losses.

Introduction

Although the method of cooking has been recognized as a factor influencing the cooking losses of cured meat products, the quality of the raw meat also plays a definite role. In this respect one of the commonest deficiencies is the exudative character of the meat (Goutefongea, 1963; Briskey, 1964; Bendall & Lawrie, 1964). A considerable amount of information on pale, exudative muscle has been accumulated in recent years. Karmas & Thompson (1963) found a definite relationship between colour and percentage jelly of cured hams.

As is well known, there are two simple methods for determining the exudative character of meat. One of them is the measurement of pH 45 min after slaughter (Briskey & Wismer-Pedersen, 1961; Charpentier & Goutefongea, 1963). The other is the sensory colour test and assessment of general appearance 12–24 hr after slaughter

This paper deals with the relationship between exudative appearance and the cooking loss of cured meat products.

Experimental

Different muscles of the ham and shoulder differ in the degree of their watery appearance, though all muscles may affect, more or less, the cooking loss of the cured meat. It is also evident that, if scored subjectively, only those muscles may be taken into consideration which are on the surface of the dissected ham and shoulder. On the basis of Karmas & Thompson's (1963) observations, the gluteus medius was used for sensory tests in our investigations 24 hr after slaughter. The general appearance

Author's address: Hungarian Meat Research Institute, Budapest, IX, Gubacsi ut 6/b.

judged by colour and structure of the shoulders was also assessed. The watery structure is easily detectable with chilled meat by the pale colour and the lack in firmness. The surface of such hams is two-toned. The muscles next to the bone are darker, those farther from it (gluteus medius) are lighter. At the other extreme is the uniformly dark ham, with firm structure. Between these structures there are several intermediate types, those being, in fact, the most frequent.

In the course of our experiments the sensory test and selection took place immediately before curing. Two categories were distinguished: the exudative and the normal (non-exudative) pieces. The normal category included the DFD (dark, firm, dry) and the intermediate types. Both of these categories are well distinguishable by appearance and the rapid classification makes it possible to sort large numbers of hams and shoulders in a short time. The setting up of three or even four quality groups would make selection difficult from a practical point of view. The selected exudative and normal hams and shoulders were marked, then processed in the usual manner: pumping with curing brine, adding of cover brine, draining and canning without addition of phosphates (Lörincz, Kárpáti & Kökény, 1961). The cans were given a heat treatment in water at 78°C, until the centre reached a temperature of 69°C. Determination of the (gross) cooking loss was made after 10 days of cold storage.

$$\text{Percentage of cooking loss} = 100\left[1 - \frac{\text{Net weight after cooking}}{\text{Gross weight}}\right].$$

Results and discussion

With hams and shoulders so selected, two distinct distributions in cooking losses were obtained (Figs. 1 and 2). The distribution of exudative meat, as shown in Figs. 1 and 2, is shifted towards the greater losses.

Parameters of the distribution of shoulders are shown in Table 1.

The difference in means is highly significant. The standard deviation of exudative shoulders (in oval cans) differed significantly from 'normal' ones.

Essentially the same results were obtained with hams (Fig. 2) confirming the findings of Karmas & Thompson (1963) and of Wismer-Pedersen (1960). McLean & Kidney (1965) noted a good correlation between pH_{45} and percentage jelly in small cans; however, they did not evaluate the structure before curing. The average cooking loss was greater with exudative hams (the difference in means, 1·9%, is very highly significant); the standard deviation of the two distributions are, on the other hand, practically the same (Table 2).

The efficiency of grading depends upon the proportion of exudative joints in raw material to be processed. If the frequency of exudative joint is less than 15%, the efficiency of selection is low; if the frequency is 40% or more, the selection is significantly more efficient. The incidence of watery pork is highly variable (Bendall,

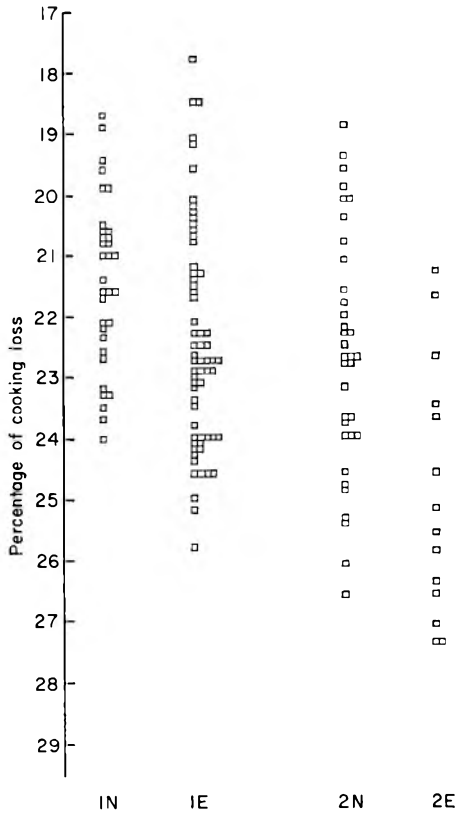

FIG 1

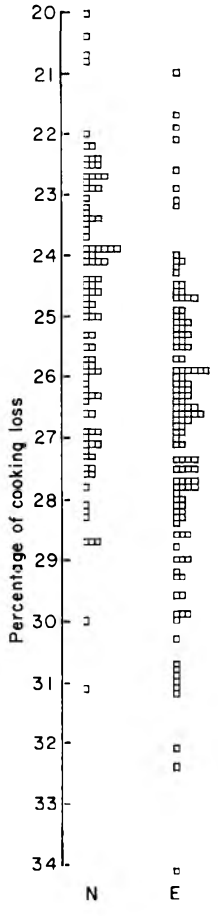

FIG. 2

FIG. 1. Distribution of cooking loss of shoulder in oblong (1) and in oval (2) cans. N=Normal; E=exudative.

FIG. 2. Distribution of cooking loss of ham in oval cans. N=Normal; E=exudative.

TABLE 1. Parameters of the distribution of the cooking loss of shoulders

Percentage of cooking loss	Normal	Exudative	Significant difference
Shoulders of 4·7–4·8 kg, in oblong cans			
$\bar{x}$	21·4	22·5	++
s	1·27	1·6	–
R	5·3	7·9	
n	34	62	
Above 22%	36·4	67·8	++
Above 23%	17·7	40·3	+
Above 24%	2·9	29·0	++
Shoulders of 4·1–4·2 kg, in oval cans			
$\bar{x}$	22·7	24·9	++
s	1·83	2·46	+
R	7·7	6·1	
n	36	14	
Above 24%	27·8	64·5	+
Above 25%	11·1	57·2	++
Above 26%	5·55	35·8	+

$\bar{x}$=Mean; s=standard deviation; R=range; n=number of observation (items); +=Significant; ++=highly significant; +++=very highly significant differences.

Table 2. Parameters of the distribution of the cooking loss of hams (5·6–5·7 kg, in oval cans)

Percentage of cooking loss	Normal	Exudative	Significant difference
$\bar{x}$	25·0	26·85	+++
s	2·21	2·05	–
R	11·9	13·1	
n	102	129	
Above 28%	8·8	27·1	+++
Above 29%	2·9	15·5	++
Above 30%	0·98	8·5	+

The symbols are the same as with Table 1.

Cuthbertson & Gatherum, 1965; Logtestijn, 1965; McLoughlin, 1965; Losonczy, 1967). Thus selection of meat before curing may be useful in decreasing cooking loss and improving uniformity of production.

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Observations on the inhibition of vegetative cells of *Clostridium sporogenes* by nitrite which has been autoclaved in a laboratory medium, discussed in the context of sub-lethally processed cured meats

J. A. PERIGO, E. WHITING AND T. E. BASHFORD

Summary. The ability of sodium nitrite incorporated in a laboratory medium to inhibit an inoculum of vegetative *Clostridium sporogenes* is greatly influenced by the temperature at which the nitrite containing medium is held prior to inoculation. At pH values of 6·0 and above, the inhibitory effect of a given concentration of nitrite is substantially enhanced when it is first autoclaved in the medium. It is believed that the nitrite, on heating, reacts with some component of the medium producing an unknown substance which is extremely inhibitory to the vegetative growth of *Cl. sporogenes*. The inhibitory activity of this unknown substance is very different from the inhibitory activity of sodium nitrite. The possible significance of these observations in relation to the stability of sublethally processed cured meats is discussed.

Introduction

Canned cured meat products are frequently subjected to a minimal heat treatment in order to maintain a satisfactory appearance and texture. This heat treatment is commonly so low that it would exert a barely significant lethal effect on certain spores considered in terms of their heat resistance in uncured media. Such sub-lethally processed cured meats, however, enjoy an excellent commercial and public health record; the majority of them are completely stable when stored for long periods at ambient temperatures.

A number of factors have been demonstrated to contribute to this stability. The relevant literature has been comprehensively reviewed by Spencer (1966) and Riemann (1963), and no extensive reappraisal will be attempted in this paper. Because the incidence of spores in the raw product is usually low (Gross, 1954) and because these spores may be much less heat resistant than similar spores grown in traditional laboratory media (Vinton, Martin & Gross, 1947), Riemann (1963) concludes that few

Authors' address: Research and Development Department, The Metal Box Co. Ltd, Kendal Avenue, London, W.3.

spores may survive the traditional cured meat heat process. Considering other evidence (Yesair & Cameron, 1942; Stumbo, Gross & Vinton, 1945; Silliker, Greenberg & Schack, 1958), Riemann (1963) also concludes that whilst the curing salts do not directly reduce the heat resistance of the spores, they do appear to inhibit the subsequent germination and outgrowth of those spores which survive the heat process. The surviving spores appear to have been damaged by the heat treatment and are consequently unable to germinate and grow in the presence of the curing salts. They will, however, germinate and grow if subcultured into salt-free media. Spencer (1966) concludes that in practice a stable product may be expected to result from a heat process of $F_0 = 0{\cdot}1$–$0{\cdot}4$ with a sodium chloride concentration of 3·5–4·0% in the water phase, an initial nitrite concentration of 75–150 ppm falling to a post process concentration of about 20 ppm and an incidence of less than 1 clostridial spore/g, but that this stability cannot be accounted for by any one of these factors in isolation and must be due to some interaction between the spores, the heat treatment and the curing salts.

Roberts & Ingram (1966) heated aerobic and anaerobic spores to various degrees, roughly from $F_0 = 0{\cdot}0015$ to $F_0 = 1{\cdot}5$, in the absence of curing salts and then transferred them to nutrient media containing these salts in various concentrations. In this way, they demonstrated that the more severe heat treatments are capable of rendering surviving spores considerably more sensitive to subsequent inhibition by salt concentrations of the order found in cured meats. These findings undoubtedly represent a major advance in understanding, illuminating a mechanism which may account for some of the anomalous results obtained by previous workers. Roberts & Ingram (1966) claim with justification that their findings for the first time point a clear way to a quantitatively satisfying explanation of the surprising effectiveness of modest heat treatments in preventing the development of bacterial spores in cured meat products; and yet these findings, especially concerning the role of nitrite, are not wholly satisfying.

Nitrite is a notoriously unstable salt, and this instability has, we believe, given rise to great confusion in the literature. Jensen & Hess (1941), when considering the stability of canned cured meat products, state: 'Nitrites react with protein when heated and are destroyed, thus leaving the meat in much the same state as fresh-cooked uncured meat'. The first half of this statement implies a reasonable assumption: it is common knowledge that a significant proportion of the inorganic nitrite added to these raw products is no longer detectable by the relevant analytical procedure after the product has been heated. This disappearance of inorganic nitrite is much greater when the nitrite is heated in meat than when it is heated to the same degree in water, and it is thus not unreasonable to assume that the meat proteins are reacting with and reducing the nitrite during the heat process. The second half of the statement, however, is not an essential nor even rational corollary to the first. It implies that the nitrite which disappears ceases to exert any inhibitory activity in the finished product. This assumption appears to be widely and uncritically accepted. Most authors appear

to be specifically concerned with the inhibitory activity of residual inorganic nitrite and many entirely evade the issue by adding unheated sterile filtered inorganic nitrite solutions to their previously heated experimental system.

In the experiments described in this paper we have directly challenged this assumption. Various quantities of nitrite have been heated at various temperatures and pH, in a basic medium which induces substantial nitrite disappearance. The ability of the resulting substrates to inhibit the vegetative growth of *Clostridium sporogenes* has then been determined by challenging each substrate with a standard, vigorously growing vegetative inoculum of this organism and observing whether this inoculum grows or is inhibited on subsequent incubation. In this way, we have investigated the interactions between nitrite, substrate, pH and processing temperature in terms of inhibitory activity without introducing the confounding interaction between the test organism and processing temperature.

Materials and methods

The basic medium used in this investigation was chosen because it supported vigorous growth of the test organism and also because substantial disappearance of nitrite resulted when this salt was autoclaved in the medium. Nitrite was determined by the absorptiometric estimation of the orange di-azo dye which is formed under standard conditions with sulphanilic acid and α naphthol.

The basic medium consisted of tryptone (Oxoid) 20 g, peptone (Oxoid) 10 g, Lab-lemco (Oxoid) 10 g, yeast extract (Oxoid) 5 g, NaCl (B.D.H. Analar) 5 g, K_2HPO_4 (B.D.H. Lab. Reagent) 2·5 g, glucose (B.D.H. Analar) 2 g, soluble starch (B.D.H. Analar) 1 g, sodium thioglycollate (B.D.H. Lab. Reagent) 1 g, bromocresolpurple (B.D.H.) 5 ml of a 0·4% solution, water 1000 ml. The pH of the medium was adjusted as required with HCl or NaOH (B.D.H. Analar) prior to the medium being dispensed in 20 ml quantities in 1-oz McCartney bottles. These bottles were closed with wadless polypropylene screw caps and then sterilized by autoclaving for 20 min at a steam pressure of 15 lb/in^2.

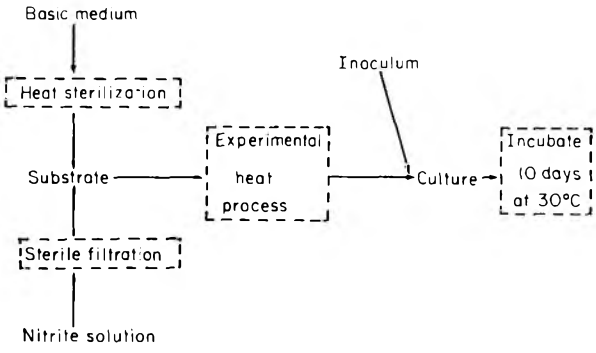
On completion of this sterilization and when the bottles had cooled, appropriate quantities of sterile filtered sodium nitrite (B.D.H. Analar) solutions were aseptically added. The concentration of the nitrite solutions used were such that the required dilutions could be obtained by adding not less than 0·05 ml and not more than 1·5 ml to each 1-oz bottle of medium.

The experimental heat processes were then accomplished by heating the McCartney bottles for 20 min at one of various temperatures. Temperatures up to 90°C were attained in a water bath, temperatures of 100°C in a steamer and temperatures above 100°C in an autoclave. With the object of simulating the canning process and in order to minimize the possible loss of volatile components, the McCartney bottle caps were tightly screwed down during the experimental heat process. In certain cases when the experimental heat process was a sterilizing process in its own right, the prior sterilization

of the basic medium and nitrite solution was omitted. Subsidiary experiments demonstrated that this omission did not significantly influence the final result.

The resulting bottles of substrate were subsequently inoculated with the test organism, putrefactive anerobe 3679, strain 8053 from the National Collection of Industrial Bacteria. Each McCartney bottle was inoculated with approximately 8×10^6 vigorously growing vegetative cells. The inoculum consisted of five drops from a 24-hr culture incubated in the basic medium at 30°C. After inoculation, each bottle of substrate is referred to as a culture.

The experimental procedure described above may be schematically represented as follows:



The work described in this paper may logically and conveniently be separated into three major experiments, although chronologically the work consisted of many smaller exercises performed over a period of approximately 1 year. The three major experiments were as follows:

(1) *The influence of pH on the inhibitory activity of unheated nitrite in the basic medium*

Various concentrations of nitrite incorporated in basic medium at various pH and not subjected to any experimental heat process were challenged with the test organism.

(2) *The influence of pH on the inhibitory activity of nitrite heated in the basic medium*

Various concentrations of nitrite were incorporated in the basic medium at various pH and heated for 20 min at 121°C prior to being challenged with the test organism.

(3) *The influence of heating temperature on the inhibitory activity of nitrite heated in the basic medium at constant pH*

Various concentrations of nitrite were incorporated in the basic medium at pH 6·0 and heated for 20 min at various temperatures prior to being challenged with the test organism.

Results and discussion

(1) *The influence of pH on the inhibitory activity of unheated nitrite in the basic medium*

The results of this experiment are set out as fractions in the body of Table 1. Each fraction refers to a unique substrate with a defined pH in the range pH 5·4–8·0 and with a defined nitrite content in the range 0–1500 ppm. The denominator of each fraction specifies the number of replicate cultures prepared at that locus, i.e. the number of bottles of unique substrate challenged with the test inoculum, and the numerator of each fraction specifies the number of these replicate cultures which failed to grow on subsequent incubation. These results are based on the preparation and observation of 1833 individual cultures.

At each pH a number of independent test inocula have been exposed to a nitrite treatment at various concentrations. At each of these nitrite levels a proportion r of the n inocula are observed to be inhibited. The interpretation of these quantal dose responses is facilitated if a model is established which relates the nitrite concentration x to the probability p of r cultures being inhibited out of n cultures tested. Such a model can be established if p is transformed so that the relationship between $y(p)$ and x is linear. The transformation most commonly employed to this end is the Probit transformation (Finney, 1952) where y is the standardized normal deviate exceeded with probability $1 - p$ (5 is commonly added in hand computation but this convention is not adopted here). Accordingly, probit regression lines have been fitted to the data in Table 1. Nitrite concentrations were expressed arithmetically as ppm and also logarithmically as $\log_{10}$ ppm. This analysis was accomplished with the aid of an ICT Orion computer using a maximum likelihood programme (Orion 25) developed by G. J. S. Ross of the Rothamsted Experimental Station. On completion of the analysis the following values are obtained at each pH level.

(i) The probability of inhibition corresponding to each experimental level of nitrite.

(ii) The 50% effective dose (ED_{50}), i.e. the concentration of nitrite most likely to inhibit 50% of the inocula.

(iii) The slope of the regression line.

(iv) Chi-squared, which provides an estimate of the probability of the deviations of observed responses from the computed regression line being due to chance variation alone.

On consideration of the results of this analysis it was immediately apparent that the best fit was obtained when the probit of inhibition was regressed on the logarithm of nitrite concentration. Thus the results of Experiment (1) are illustrated on this basis by the series of graphs in Fig. 1.

At each pH the computed regression is drawn as a solid line and the experimentally observed responses are plotted as open circles. It should be noted that the statistical weight carried by each point in Fig. 1 is dependent on the number of replicate cultures tested at that locus, which may be established by consulting the denominator of the

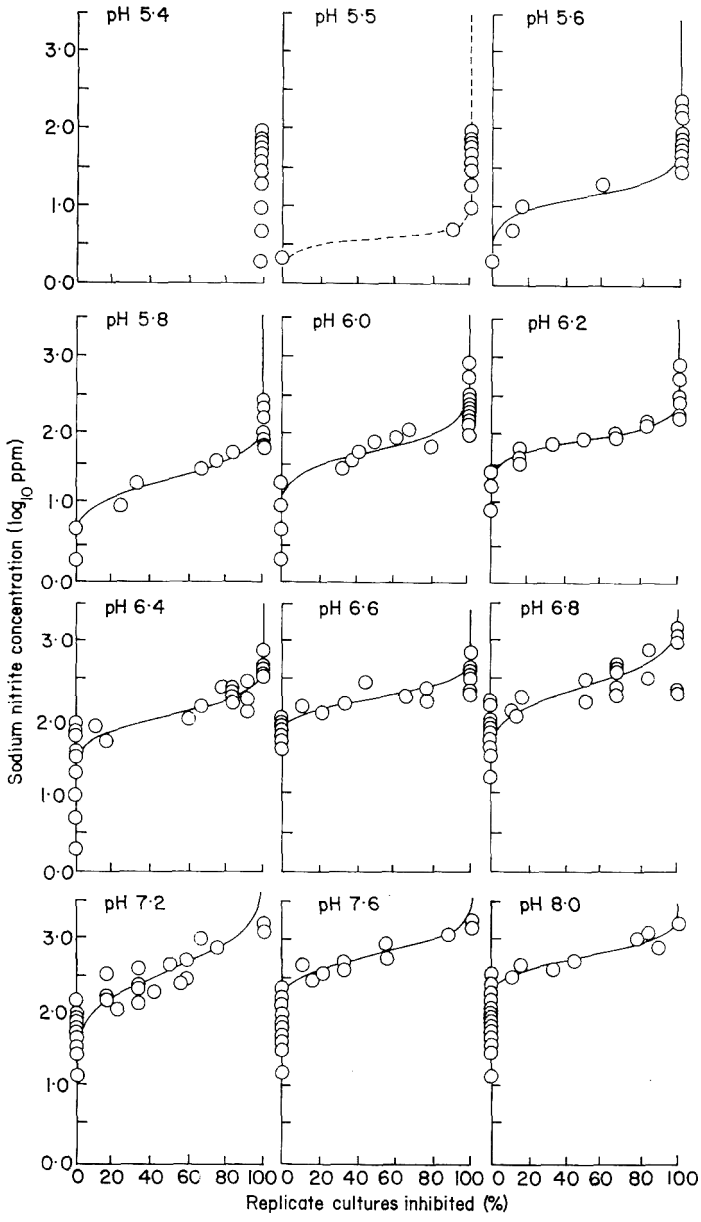


FIG. 1. The influence of unheated sodium nitrite concentration on the probability of vegetative cultures of *Cl. sporogenes* PA 3679 being inhibited at various pH values. Continuous curves established by probit analysis. The broken curve is a free-hand estimate.

TABLE 1. The influence of pH on the inhibitory activity of unheated sodium nitrite in the basic medium (No. of cultures inhibited/No. of cultures tested)

pH	Nitrite concentration (ppm)																														
	0	2	5	10	15	20	30	40	50	60	70	80	90	100	120	140	150	160	180	200	220	240	250	300	350	400	450	500	750	1000	1200	1500
5·4	1/9	9/9	9/9	9/9	–	9/9	9/9	9/9	9/9	9/9	9/9	9/9	–	9/9	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
5·5	0/10	0/9	9/10	10/10	–	10/10	9/9	10/10	9/9	10/10	9/9	10/10	–	10/10	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
5·6	0/12	0/9	1/9	2/12	–	7/12	12/12	12/12	12/12	12/12	12/12	12/12	–	12/12	–	–	3/3	–	–	3/3	–	–	3/3	–	–	–	–	–	–	–	–
5·8	0/12	0/9	0/9	3/12	–	4/12	8/12	9/12	10/12	12/12	9/9	12/12	–	12/12	–	–	3/3	–	–	3/3	–	–	3/3	–	–	–	–	–	–	–	–
6·0	0/16	0/3	0/4	0/7	–	0/10	3/9	5/13	5/12	8/10	3/6	8/13	3/3	11/16	9/9	9/9	3/3	6/6	6/6	9/9	3/3	3/3	6/6	6/6	–	–	–	3/3	3/3	–	–	–
6·2	0/9	–	–	0/3	–	0/6	0/6	1/6	1/6	1/6	1/3	3/6	2/3	6/9	5/6	5/6	3/3	3/3	3/3	6/6	–	–	6/6	3/3	–	–	–	3/3	3/3	–	–	–
6·4	0/15	0/3	0/3	0/3	–	0/3	0/3	0/9	2/12	0/6	0/6	1/9	0/3	9/15	11/12	8/12	–	10/12	11/12	10/12	5/6	5/6	7/9	11/12	3/3	3/3	3/3	9/9	6/6	–	–	–
6·6	0/9	–	–	–	–	–	–	0/3	0/9	0/3	0/3	0/3	0/3	0/9	2/9	1/9	–	3/9	7/9	6/9	3/3	3/3	7/9	4/9	3/3	3/3	3/3	9/9	6/6	–	–	–
6·8	0/15	–	–	–	0/3	–	0/3	0/3	0/6	0/3	0/3	0/6	0/3	2/15	1/9	0/6	0/6	3/6	1/6	8/12	3/3	2/2	6/9	6/12	5/6	6/9	4/6	8/12	10/12	9/9	6/6	6/6
7·2	0/15	–	–	–	0/3	–	0/3	0/3	0/6	0/3	0/3	0/6	0/3	0/15	2/9	2/6	0/6	1/6	1/6	5/12	1/3	1/3	5/9	7/12	1/6	3/9	3/6	7/12	9/12	6/9	6/6	6/6
7·6	0/12	–	–	–	0/3	–	0/3	0/3	0/3	0/3	0/3	0/3	0/3	0/12	0/3	–	0/9	–	–	0/9	–	–	1/6	2/9	2/6	1/9	2/6	5/9	5/9	8/9	6/6	6/6
8·0	0/12	–	–	–	0/3	–	0/3	0/3	0/3	0/3	0/3	0/3	0/3	0/12	0/3	–	0/9	–	–	0/9	–	–	0/6	1/9	0/6	3/9	1/6	4/9	8/9	7/9	5/6	6/6

corresponding fraction in Table 1. Because a logarithmic nitrite scale is used, the nitrite free controls cannot be represented in Fig. 1. As can be seen from Table 1, however, of a total of 146 nitrite-free controls inoculated, only one failed to grow and this occurred at pH 5·4. Because insufficient data were obtained at pH 5·4 and pH 5·5, the computer was unable to establish a regression line. A broken curve has been fitted by hand at pH 5·5 on the basis of the data available and taking into account the curves established at higher pH.

The unheated nitrite ED_{50} values obtained by this analysis are plotted against pH as open circles in Fig. 4. These values are computed from the experimental data in Table 1. On consideration of Fig. 4, it is at once apparent that the inhibitory effect of unheated nitrite is highly pH dependent. It is widely contended that the effective inhibitory agent in nitrite solutions is undissociated nitrous acid, and that on this basis a unit decrease in pH value would result in a ten-fold increase in the inhibitory effect of nitrite. Roberts & Ingram (1966) observed that for PA 3679 spores heated at 80°C for 20 min, the concentrations of nitrite needed to reduce the percentage survival to 1% over the range pH 6·5–7·5 were reasonably consistent with the 1 : 10 concentration ratio expected on the basis of the above-described theory. From Fig. 4 it will be seen that for vegetative cells of PA 3679, the ED_{50} values which we have observed are consistent with this theory over the range pH 5·8 (22 ppm) to pH 6·8 (250 ppm), but at pH values outside this range the relationship breaks down, the ED_{50} values being less than expected. The deviation from theory at pH values below pH 5·8 is probably due to the growth limiting effect of pH in its own right. The lower limit for vegetative growth of PA 3679 in the nitrite-free basic medium has been established to be between pH 5·3 and 5·4. The deviation from theory at pH values above pH 6·8 is not as easily explained, for although the upper pH growth limit has not been established, it is certainly higher than pH 8·0, which is the highest pH investigated in these experiments.

(2) *The influence of pH on the inhibitory activity of nitrite heated in the basic medium*

The results of this experiment are set out in Table 2 using the same conventions as were used for Table 1. These results are based on the preparation and observation of 784 individual cultures. On subjecting these data to probit analysis, it was again found that the best fit was obtained when the nitrite concentration was expressed logarithmically. These results are presented as a series of graphs in Fig. 2, using the same conventions as in Fig. 1.

At pH 5·5 the computed regression line was an extremely improbable fit. The probability of the deviation of the data from the computed curve being due to chance was less than 0·001 based on the calculated value of Chi-squared. Thus we are forced to conclude that substantial experimental error has occurred in this instance and have rejected the computed curve and replaced it with a free-hand estimate (broken curve). At pH 8·0 the data was not sufficient to enable a curve to be computed and again a

TABLE 2. The influence of pH on the inhibitory activity of sodium nitrite which has been heated for 20 min in the basic medium at 121°C (No. of cultures inhibited/No. of cultures tested)

pH	Nitrite concentration (ppm)													
	0	3	4	5	6	7	8	9	10	11	12	15	20	25
5·4	2/6	1/6	0/3	2/6	1/3	0/3	5/6	6/6	6/6	6/6	6/6	6/6	3/3	3/3
5·5	1/7	2/6	0/4	3/6	0/3	1/3	3/7	6/6	6/6	6/6	7/7	4/4	3/3	3/3
5·6	0/6	0/6	0/3	0/6	0/3	0/3	3/6	3/6	5/6	6/6	6/6	6/6	3/3	3/3
5·8	0/6	0/6	0/3	0/6	0/3	2/3	5/6	6/6	6/6	6/6	6/6	6/6	3/3	3/3
6·0	0/27	0/10	0/14	0/21	1/16	2/11	12/17	8/11	19/23	6/6	9/9	8/8	16/16	8/8
6·4	0/6	0/6	0/3	0/6	0/3	0/3	2/6	3/6	3/6	3/6	3/6	4/6	3/3	3/3
6·8	0/6	0/6	0/3	0/6	0/3	2/3	3/6	5/6	6/6	6/6	6/6	6/6	3/3	3/3
7·2	0/6	0/6	0/3	0/6	0/3	0/3	0/6	1/6	4/6	2/6	5/6	6/6	3/3	3/3
7·6	0/6	0/6	0/3	0/6	0/3	0/3	0/6	0/6	0/6	1/6	2/6	5/6	3/3	3/3
8·0	0/3	0/3	—	0/3	—	—	0/3	0/3	0/3	0/3	0/3	0/3	1/3	3/3

broken free-hand estimate is indicated. In both these cases the estimated curve is based on the data available and on the curves computed at other pH levels. Again the nitrite-free controls cannot be represented, but from Table 2 it is apparent that of seventy-nine such controls only three were inhibited, two at pH 5·4 and one at pH 5·5.

The ED_{50} values for heated nitrite are also plotted against pH as solid circles in Fig. 4. These ED_{50} values are computed from the data in Table 2. It is immediately apparent that the inhibitory effect of heated nitrite is very different from that of unheated nitrite, being only slightly pH dependent. The ED_{50} of heated nitrite rises from about 6 ppm at pH 5·5 to about 18 ppm at pH 8·0, whereas for unheated nitrite the corresponding ED_{50} values are approximately 3 ppm at pH 5·5 and 590 ppm at pH 8·0. Consequently, at higher pH values, nitrite which has been heated in the basic medium is very much more inhibitory than the same quantity of nitrite incorporated in the basic medium without heating. At pH 7·0 approximately 300 ppm of unheated nitrite are observed to inhibit 50% of the inocula, whereas 12 ppm nitrite

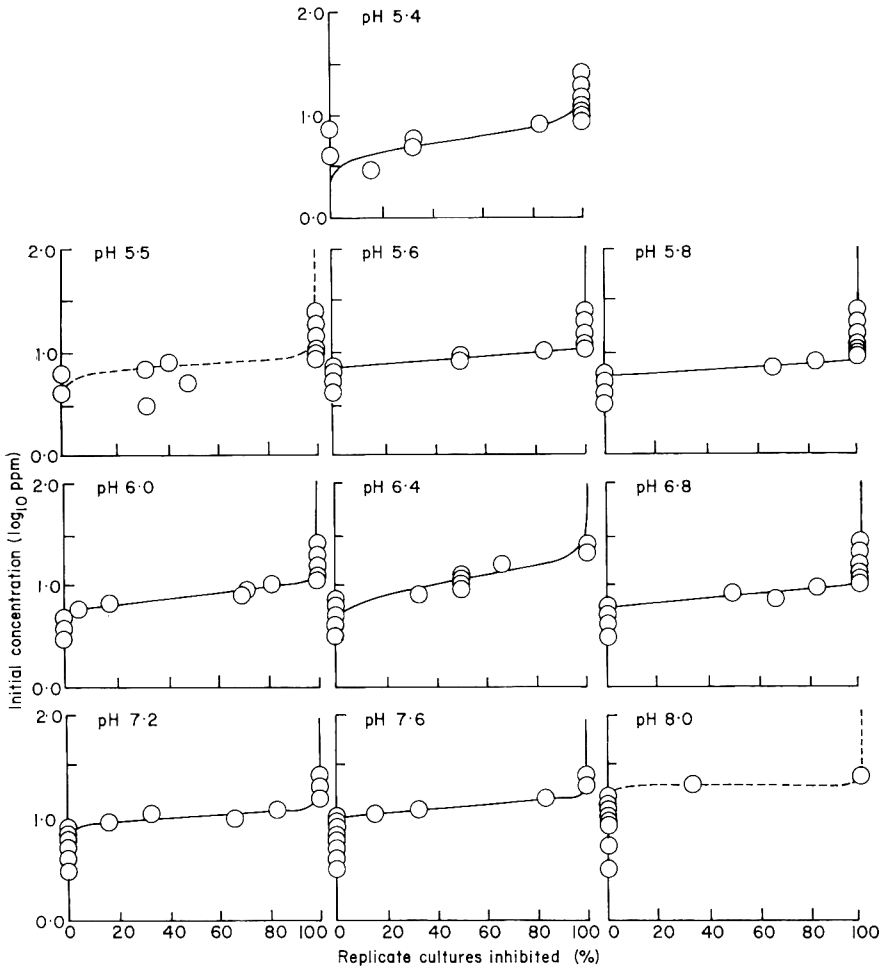


FIG. 2. The influence of sodium nitrite concentration, after heating in basic medium for 20 min at 121°C, on the probability of vegetative cultures of *Cl. sporogenes* PA 3679 being inhibited at various pH values. Continuous curves established by probit analysis. The broken curves are free-hand estimates.

are equally inhibitory after heating. At pH 6·0, which is more appropriate in the context of canned cured meats, the corresponding ED_{50} values are 52 ppm for unheated nitrite and 8 ppm for heated nitrite.

(3) *The influence of heating temperature on the inhibitory activity of nitrite in the basic medium at constant pH*

The results of this experiment are set out in Table 3 using the same conventions as before. The results are based on the preparation and observation of 1180 individual

TABLE 3. The influence of heating temperature on the inhibitory activity of sodium nitrite heated for 20 min in the basic medium at pH 6·0 (No. of cultures inhibited/No. of cultures tested)

Heating temperature (°C)	Nitrite concentration (ppm)																															
	0	1	2	3	4	5	6	7	8	9	10	11	12	14	15	16	18	20	25	30	35	40	45	50	55	60	65	70	75	80	90	100
Unheated	0/12	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	0/3	–	1/6	0/3	2/9	4/9	5/12	5/11	9/12	6/9	9/9	3/3	3/3	3/3	3/3
50°	0/9	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	1/6	0/3	3/6	0/3	4/6	2/6	3/9	4/9	4/9	3/3	4/6	3/3	3/3	3/3	3/3
60°	0/12	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	2/6	0/3	2/9	1/6	3/12	7/12	5/12	10/12	10/12	5/6	9/9	3/3	2/3	3/3	2/3
70°	0/12	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	0/6	0/3	3/6	0/3	3/12	3/12	6/12	6/12	12/12	5/6	9/9	3/3	3/3	3/3	3/3
80°	0/12	–	–	–	–	0/3	–	–	–	–	2/3	–	–	–	3/3	–	–	3/6	4/6	3/6	0/3	4/9	0/6	6/12	3/9	7/12	5/6	8/9	3/3	3/3	–	–
90°	0/14	–	–	–	–	0/3	–	–	–	–	2/3	–	–	–	3/3	–	–	3/6	3/3	6/9	0/3	9/12	7/9	13/15	11/12	15/15	11/12	15/15	3/3	3/3	–	–
95°	0/12	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	0/3	–	0/6	0/3	4/8	6/9	9/12	10/12	10/12	12/12	12/12	3/3	–	–	–
100°	0/6	–	0/3	–	0/3	1/3	0/3	–	1/3	–	3/6	–	–	–	5/6	–	–	6/6	6/6	6/6	–	3/3	–	–	–	–	–	–	–	–	–	–
109°	0/4	0/3	0/4	0/3	3/4	3/3	4/4	3/3	4/4	3/3	1/1	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
115°	0/7	–	0/4	0/3	0/4	0/6	3/7	2/3	4/4	3/3	4/4	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
121°	0/27	0/1	1/5	0/10	0/14	0/21	1/16	2/11	12/17	8/11	19/23	6/6	9/9	2/2	8/8	3/3	2/2	16/16	8/8	6/6	–	–	–	–	–	–	–	–	–	–	–	–
126°	0/4	–	0/1	–	0/4	0/3	0/3	0/3	0/4	–	0/4	–	1/4	3/4	–	4/4	1/1	1/1	–	–	–	–	–	–	–	–	–	–	–	–	–	–

cultures. Again, on probit analysis, the logarithm of nitrite concentration was found to give the best fit and the results are accordingly presented as a series of graphs in Fig. 3. The curves computed for 80° and 90°C were highly improbable on the basis of Chi-squared and have consequently been rejected and dashed free-hand estimates substituted. As will be seen from Table 3, none of the 131 nitrite-free controls was inhibited in this experiment.

The ED_{50} values for nitrite heated in the basic medium at various temperatures

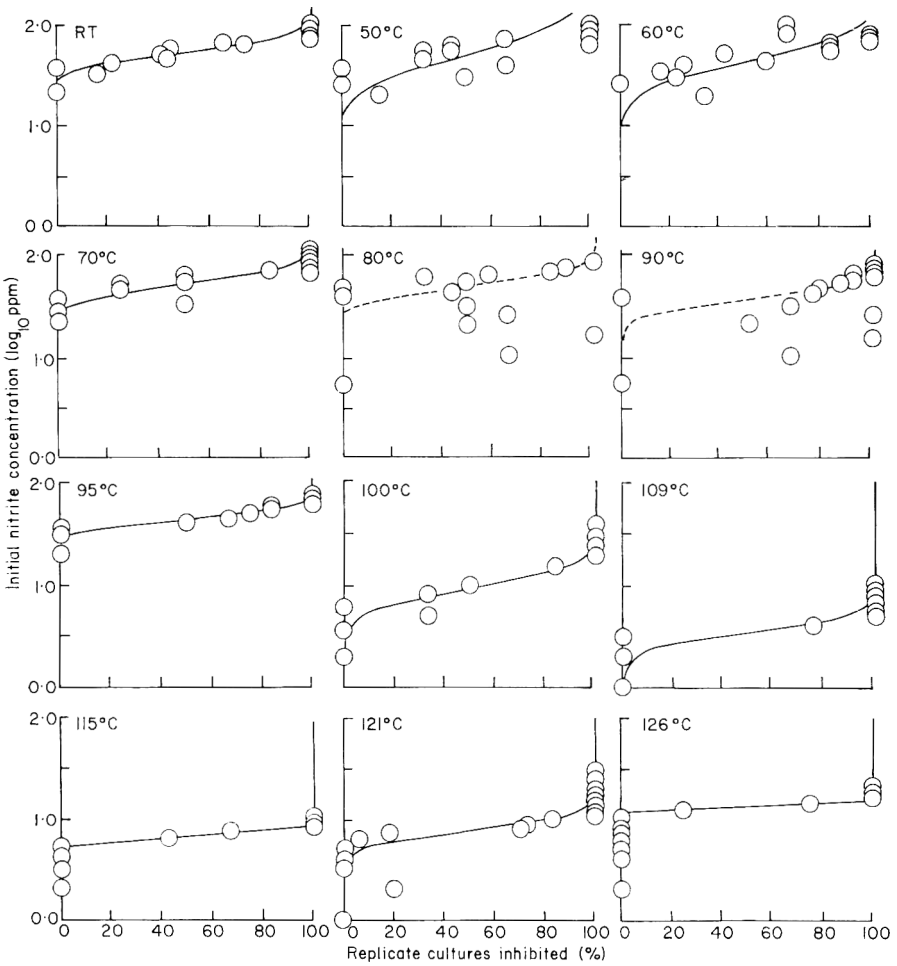


FIG. 3. The influence of sodium nitrite concentration, after heating in basic medium for 20 min at various temperatures, on the probability of vegetative cultures of *Cl. sporogenes* PA 3679 being inhibited at pH 6·0. Continuous curves established by probit analysis. The broken curves are free-hand estimates.

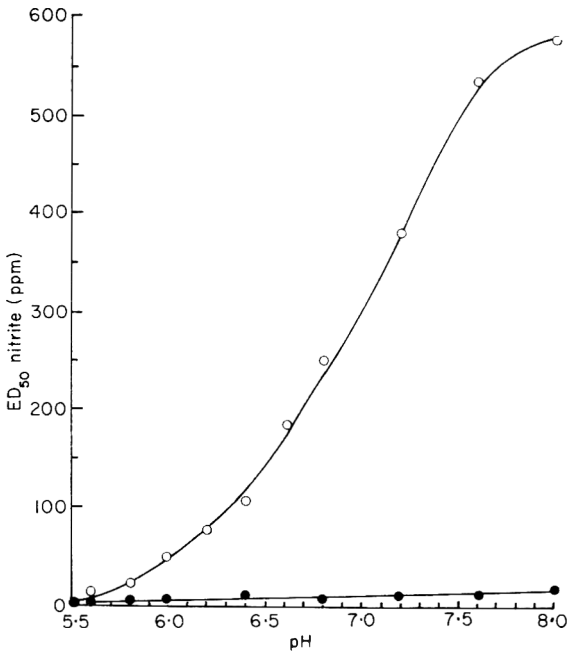


FIG. 4. The influence of pH on the ED_{50} values of nitrite incorporated in the basic medium without heating and of nitrite heated in the basic medium. ○, Unheated nitrite; ●, nitrite heated in the basic medium for 20 min at 121°C.

are plotted as open circles in Fig. 5. These ED_{50} values are computed from the data in Table 3. In each instance the heating time was 20 min and the substrate pH 6·0. The computed ED_{50} values corresponding to heating temperatures of 80° and 90°C are rejected as previously described and estimated values, represented by open squares, have been substituted in Fig. 5.

The ED_{50} value determined for the unheated nitrite controls in this experiment is 50 ppm. This value is in close agreement with the value of 52 ppm found for unheated nitrite at pH 6·0 in Experiment (1). As the heating temperature is increased from 50° to 95°C, the ED_{50} remains virtually constant and is very similar to that of unheated nitrite. As the temperature exceeds 100°C, however, the ED_{50} falls dramatically until at a temperature of 109°C it reaches a minimum value of 3·5 ppm. As the heating temperature is increased beyond 109°C the ED_{50} rises again, reaching 13 ppm at 126°C. It is of interest to note that in this experiment at 121°C the ED_{50} was found to be 7·6 ppm, which is very close to the ED_{50} value of 7·9 found at pH 6·0 in Experiment (2).

Thus it is apparent that the inhibitory activity of a given concentration of nitrite is increased if it is heated to an appropriate degree in the basic medium. This increase may be expressed quantitatively if the ED_{50} observed after 20 min heating at a par-

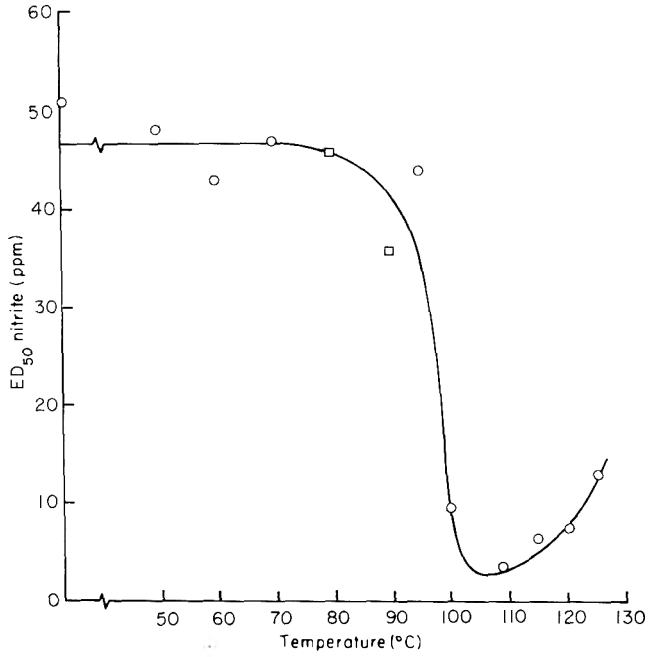


FIG. 5. The influence of heating temperature on the ED_{50} values of nitrite heated for 20 min in the basic medium at pH 6·0. ○, Values calculated by probit analysis; □, values transcribed from free-hand curves in Fig. 3.

ticular temperature is referred to the ED_{50} of unheated nitrite when an enhancement coefficient may be derived, i.e.:

$$\text{Enhancement coefficient} = ED_{50} \text{ unheated nitrite}/ED_{50} \text{ heated nitrite.}$$

In Fig. 6 the results of Experiment (3) are represented in terms of this enhancement coefficient. The graph is obtained by transposing the values represented by the curve in Fig. 5. It is apparent that the enhancement coefficient is very close to unity at heating temperatures between 50° and 90°C, i.e. the inhibitory activity of nitrite heated for 20 min at temperatures not exceeding 90°C is virtually identical with that of unheated nitrite. As the heating temperature is increased beyond 100°C, however, the enhancement coefficient increases dramatically until at about 109°C heated nitrite is more than fifteen times as inhibitory as unheated nitrite. As the heating temperature is increased beyond 109°C, the enhancement coefficient declines, but even at 126°C heated nitrite is still approximately three and a half times as inhibitory as the same concentration of unheated nitrite.

A subsidiary experiment was performed to establish the relationship between heating temperature and nitrite disappearance. Twelve aliquots each consisting of 20 ml basal medium at pH 6·0 and containing 50 ppm nitrite were dispensed into 1-oz

screw capped bottles. Eleven of these bottles were then heated for 20 min at one of the eleven temperatures investigated in Experiment (3). The remaining bottle served as an unheated control. Subsequently the residual nitrite concentration was determined in each bottle by the absorptiometric method previously described. The results of this

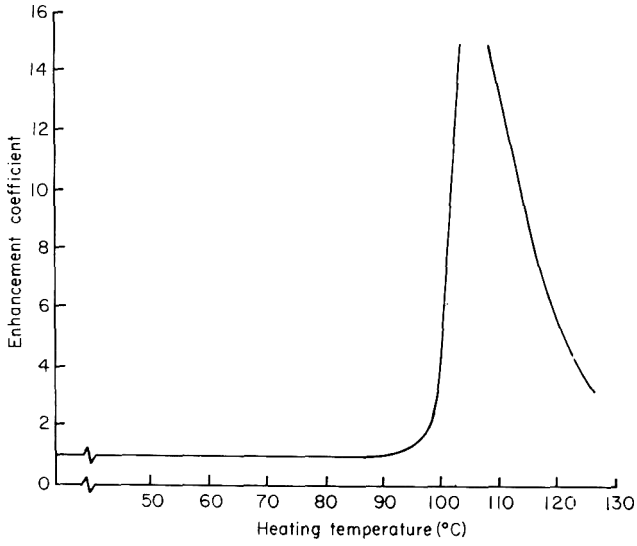


FIG. 6. The influence of heating temperature on the enhancement coefficient.

$$\text{Enhancement coefficient} = \frac{\text{ED}_{50} \text{ unheated nitrite at pH } 6{\cdot}0}{\text{ED}_{50} \text{ nitrite after 20 min heating at pH } 6{\cdot}0}.$$

investigation are illustrated in Fig. 7, in which percentage nitrite destroyed is plotted against heating temperature. It is apparent that when the initial nitrite concentration is 50 ppm at pH 6·0, perceptible nitrite destruction commences at a temperature of about 70°C when 2% (1 ppm) of the initial nitrite is destroyed in 20 min. As the heating temperature is increased the rate of nitrite disappearance increases in an exponential fashion, doubling with each 8°C rise of temperature, until at 115°C approximately 84% (42 ppm) of the initial nitrite is destroyed in 20 min. At higher temperatures the curve deviates from exponential; perhaps the reaction rate declines as the residual nitrite concentration approximates to zero, until at 126°C the curve is virtually horizontal. At this temperature some 93% (46·5 ppm) of the initial nitrite is destroyed in 20 min, the remaining 7% (3·5 ppm) apparently being very resistant to destruction.

If Figs. 6 and 7 are now compared, a correlation is apparent between the enhancement of nitrite inhibition and its disappearance in the analytical sense. At heating temperatures up to 90°C enhancement of inhibition is virtually absent (Fig. 6) and disappearance of nitrite is only slight (Fig. 7). As the heating temperature exceeds 100°C, however, marked enhancement of inhibition occurs (Fig. 6) and similarly nitrite disappearance becomes increasingly apparent.

The observations reported above are consistent with the hypothesis that, when nitrite is heated in the basic medium beyond a critical time and temperature, some reaction occurs which results in the substrate becoming more inhibitory to vegetative cells of PA 3679. This reaction might take one of two forms.

(1) The nitrite may react on heating with some component of the basic medium to yield an unknown substance which is actively inhibitory to the vegetative growth of PA 3679.

(2) The nitrite, on heating, may react with and destroy some essential nutrient in the basic medium, thereby rendering it incapable of supporting the vegetative growth of PA 3679.

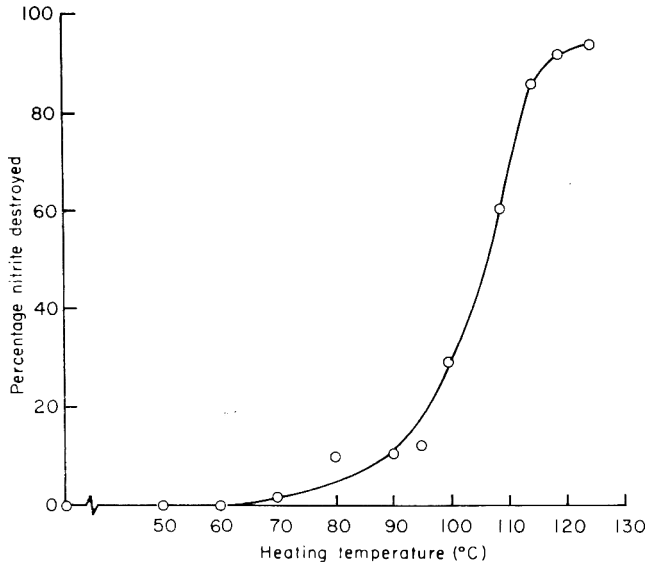


FIG. 7. The influence of heating temperature on the proportion of nitrite which disappears when a 50 ppm solution of nitrite in basic medium is heated for 20 min at pH 6·0.

Of these two alternatives, the first is considered to be the more probable on the following grounds. Inhibitory substrate cannot be rendered non-inhibitory simply by re-fortification with any of the basic medium components, but only by dilution to a degree consistent with the original concentration of autoclaved nitrite involved. If, for example, basic medium is autoclaved for 20 min at 121°C with 30 ppm nitrite, which is four times the ED_{50} under these process conditions, it can only be rendered non-inhibitory (supporting the growth of more than 50% of the inocula) by the addition of at least three volumes of sterile nitrite-free basic medium.

Moreover, the inhibition of nitrite is progressively enhanced as the heating temperature is increased from 90°C to about 105°C, but this enhancement declines as the

heating temperature is raised above 110°C (Fig. 6). This decline at high temperatures is consistent with the theory that an unknown actively inhibitory agent is formed when nitrite and basic medium are heated to a temperature in excess of 90°C and that this unknown agent is itself destroyed at temperatures exceeding 110°C. The decline of inhibitory activity at high temperature is not so easily reconciled with the alternative theory of essential nutrient destruction.

Conclusions

(1) *The inhibitory activity of unheated nitrite*

In Experiment (1) it was observed that when nitrite was incorporated, without heating, in the basic medium at each of the pH levels investigated, the probability of survival of an inoculum of 8×10^6 vegetative PA 3679 cells, when expressed in units of probit, was directly proportional to the log of the nitrite concentration. The slope of the computed regression at pH values of 5·6, 5·8, 6·0, 6·2, 6·4, 6·6, 6·8, 7·2 and 8·0 was 4·5, 3·5, 3·8, 5·3, 4·0, 5·3, 3·0, 2·4, 4·3 and 4·8, respectively. These values of slope do not appear to vary systematically with changing pH and, therefore, an average value of 4·1 is assumed for the true slope of the regression line over the pH range investigated. Thus we conclude that at any pH in the range 5·6–8·0, a ten-fold (one log cycle) change of unheated nitrite concentration will result in a change in the probability of inhibition equivalent to 4·1 units of probit.

Assuming a common slope of 4·1 and accepting the upper curve in Fig. 4 as the best estimate of variation of ED_{50} with changing pH, a synoptic figure may be derived which relates the probability of inhibition to the nitrite concentration at any pH in the range pH 5·6–8·0. In Fig. 8, which has been derived on this basis, nitrite concentration is represented on a log–linear ordinate and the percentage probability of inhibition is represented on a probit–linear abscissa. Fig. 8 is, of course, restricted specifically to the probability of inhibition of an actively growing inoculum of 8×10^6 vegetative cells. If smaller inocula or single cells are considered, then the probability of inhibition at any defined pH and nitrite concentration will be increased accordingly.

As noted previously, the inhibitory effect of unheated nitrite is highly pH dependent. As the pH is increased over the range pH 5·8–6·8, a ten-fold increase in inhibitory activity is apparent, which is consistent with the assumption that the effective inhibitory agent is undissociated nitrous acid. At pH values outside this range, however, the situation becomes more involved.

(2) *The enhancement of the inhibitory activity of nitrite which has been heated in the basic medium*

When nitrite is heated in the basic medium for 20 min at temperatures above 90°C a decline occurs in the concentration of nitrite which is detectable by the relevant analytical procedure and at the same time the inhibitory effect of the substrate is increased. The nitrite apparently reacts on heating with some component of the

basic medium to yield an unknown substance which is actively inhibitory to the vegetative growth of PA 3679. The rate at which this unknown substance is produced is maximal at a temperature of about 110°C. At temperatures exceeding 110°C the unknown substance appears to breakdown or react in such a way that its inhibitory activity declines.

Any quantitative consideration of the inhibitory activity of this unknown substance is complicated because its concentration cannot be determined. It may be that after a defined heat process in basic medium at constant pH, the concentration of unknown

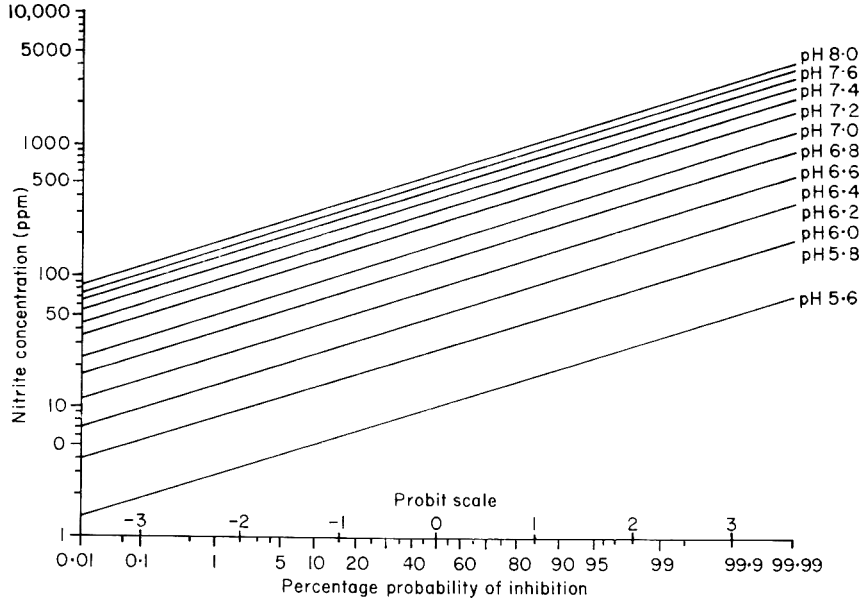


FIG. 8. The influence of unheated sodium nitrite concentration on the probability of an inoculum of 8×10^6 vegetative cells of *Cl. sporogenes* PA 3679 being inhibited in basic medium at various pH values.

which is formed is proportional to the concentration of nitrite initially present. If this is the case, then an examination of the relationship between initial nitrite concentration and post process inhibitory activity may reflect the relationship between the resulting concentration of unknown and its inhibitory effect.

In Experiment (2) it was observed that when nitrite was heated in the basic medium for 20 min at 121°C, at each pH the probability of survival of the test inoculum when expressed in units of probit was directly proportional to the log of the initial nitrite concentration. The slope of the computed regression at pH values of 5·4, 5·6, 5·8, 6·0, 6·4, 6·8, 7·2 and 7·6 was 6·5, 16·7, 22·7, 12·3, 5·8, 15·1, 14·8 and 16·6, respectively.

These values of slope do not appear to vary systematically with pH but are somewhat inconsistent, varying from 5·8 to 22·7. In the circumstances, the average of 13·8 is

the best estimate which can be made of the true slope of the regression line over the pH range investigated but this average can only be accepted with extreme caution. If the lower curve in Fig. 4 is accepted as the best estimate of variation of ED_{50} with pH for nitrite heated in basic medium at 121°C for 20 min, and assuming a slope of 13·8, a synoptic figure may be derived which relates the probability of inhibition to the heated nitrite concentrations. In Fig. 9, which has been derived on this basis, heated nitrite concentration is represented on a log–linear ordinate and the percentage probability of inhibition is represented on a probit linear abscissa. As postulated above,

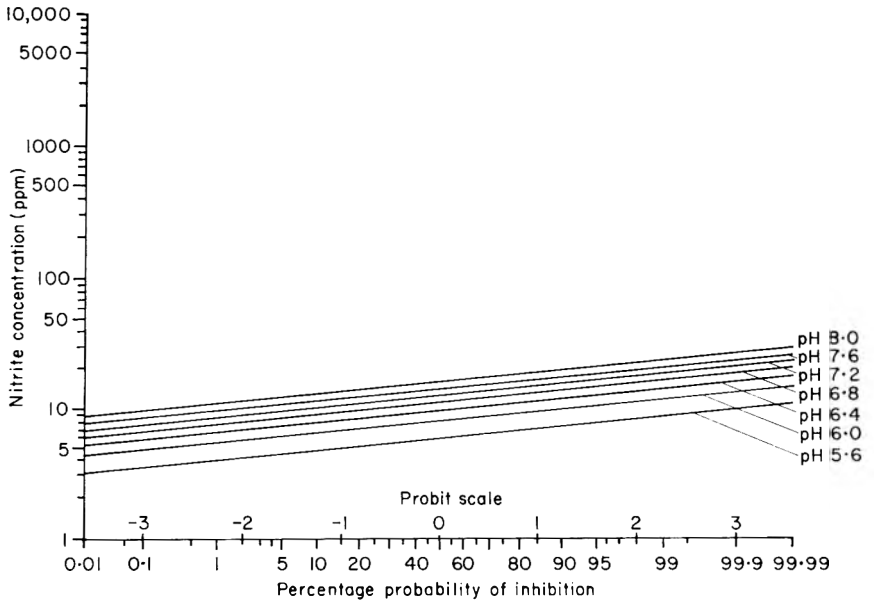


FIG. 9. The influence of sodium nitrite concentration, after heating in basic medium for 20 min at 121°C, on the probability of an inoculum of 8×10^6 vegetative cells of *Cl. sporogenes* PA 3679, being inhibited at various pH values.

it is possible that each value of initial nitrite concentration on the ordinate of Fig. 9 may correspond to some specific but indeterminate concentration of unknown inhibitor. Fig. 9, like Fig. 8, is specifically applicable only to an inoculum of 8×10^6 vegetative PA 3679 cells. Because of the doubt as to the slope of the regression lines however, Fig. 9 cannot be accepted with the same degree of confidence as Fig. 8.

Thus, in summary, our thesis is that when nitrite is sufficiently heated in the basic medium, it is involved in a reaction resulting in the production of some unknown inhibitory substance. This unknown substance differs from inorganic nitrite in three important respects. First, its inhibitory activity is only slightly pH dependent, whereas the activity of inorganic nitrite is highly pH dependent (Fig. 4). Secondly, it elicits

a much less variable response from the test inoculum. For example, as can be seen from Figs. 8 and 9, to increase the probability of inhibition from 5 to 95% (i.e. 3·3 probit units) necessitates a 1·7-fold (0·24 log cycle) increase of heated nitrite concentration, whereas to obtain the same increase in the probability of inhibition using unheated nitrite necessitates a 6·3-fold (0·8 log cycle) increase of concentration. Thirdly, it is apparently an extremely potent inhibitor. For example, sufficient unknown inhibitor is produced by autoclaving only 3·5 ppm nitrite for 20 min in basic medium at 109°C to inhibit the test inoculum in 50% of trials. Moreover, it is unlikely that more than a small proportion of this initial 3·5 ppm of nitrite may be usefully employed in producing the unknown inhibitor.

Turning now to the practical implications of this thesis: whilst it may have been demonstrated in this investigation that an unknown inhibitory agent is formed when nitrite is heat processed in a synthetic laboratory medium, it has not to date been established that the same agent is formed when nitrite is processed in commercially canned cured meat products. It is known, however, that substantial disappearance of nitrite does occur during the processing of these products and it seems not improbable that, in some circumstances at least, part of this nitrite may be involved in reactions leading to the production of such an inhibitory agent. If this is the case, then two interesting points arise. First, the commercial heat process must be considered not only in terms of its microbial lethal effect, but also in terms of its nitrite degrading effect. Secondly, Roberts & Ingram (1966) conclude that small variations in pH may markedly affect the stability of cured meats. This conclusion is probably justified in the case of commodities such as large canned hams and ox tongues in which the temperature of a substantial part of the pack may not exceed 90°C during processing and in which the effective inhibitory agent may consequently be the pH dependent undissociated nitrous acid. The conclusion may not be justified, however, in the case of smaller ham, tongue and pork luncheon meat packs, where substantially higher centre temperatures are attained and in which the effective inhibitor may be the unknown pH independent agent.

It would be naïve to suppose that the mechanism described above could wholly account for the remarkable stability of sublethally processed cured meats, but it may well play an important complementary role to the spore sensitizing mechanism described by Roberts & Ingram (1966). Certainly there is sufficient justification for the mounting of a serious attempt at isolating and identifying this agent in the synthetic system and searching for it in commercial products.

Acknowledgments

The authors wish to acknowledge the expert technical assistance of P. Mouncey, Mrs H. R. Simmonds and J. E. Wysocki, and to thank R. R. Armstrong for his advice on the statistical aspects of this work and on the use of the Rothamsted probit analysis

programme, which was made available to the Metal Box Company by kind permission of Dr Frank Yates, C.B.E., Head of the Department of Statistics at Rothamsted, Mr D. H. Rees providing copies of the appropriate tapes. We also thank H. W. Gearing and the staff of the Metal Box Company Finance Department for the provision of computer facilities.

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Book Review

Chemical Additives in Food. Ed. by E. W. L. Goodwin.
London: J. & A. Churchill Ltd. Pp. 128. 20s.

When the sins of the food manufacturer or the food technologist come up for popular discussion, there is hardly a subject on which our domestic sages delight to dwell more intimidatingly than on the horrors of the addition of the so-called 'chemicals' to foods. Formerly it was the devastating effect of eating tinned food of the appalling results that followed the pasteurization of milk or the devitalizing effect on food of the use on farms of 'chemical' fertilizers that excited their displeasure. Why chemicals should have such a low rating in domestic or pop-journalistic assessment is not easy to discover. Did not Paracelsus as early as 1526 point out that even the human body is 'a Conglomeration of Chymical Matters'? But unto each age its own bogies; they mostly get laid, or any modicum of danger extracted from them by legislation, as knowledge filters down. Nevertheless, it might be well worth while to have the introductory chapter of the present book, in which J. G. Davis puts in clear perspective what the addition of chemicals to foods really means, reprinted in some popular journal for the sake of the apprehensive.

The book itself, short but useful, consists of seven papers given at a symposium held at Coventry Technical College in 1965. These papers deal with food preservation, the biological evaluation of a proposed new additive, anti-oxidants, food packaging aspects, flavours and colours of foods. Public health considerations and legislative measures are given due weight throughout. The paper by J. McL. Philp on the practical steps actually taken by a team of food technologists to evaluate a new emulsifier shows what an extensive and thorough examination is given today to any potential additive before it is either rejected or deemed suitable for use in the food industry.

From the paper by E. M. Verbeek it is interesting to learn that flavour (one of the most important food characteristics from the consumer standpoint, and one of the most elusive scientifically because it usually depends on a mixture of substances, some of which are chemically unstable, present in very small amounts) is now undergoing thorough examination from the positive development angle and not merely from the analytical. It may be that quite new flavours may, in the not too distant future, adorn the table of the average household as well as that of the epicure. As regards food colours, it is now well known how much they can enhance (or depress) flavour appreciation as well as improve the general attractiveness and even digestibility of foods. Care is nevertheless needed in the use of synthetic colours, some of which, pleasing though they may be to the eye, have pharmacological effects of a less pleasant nature. The permitted list, as R. P. Winston points out in a chapter dealing mainly with control, is in most of the developed countries, short. There is room for more

properly evaluated, synthetic colours stable under modern food-processing techniques.

In sum, there is sufficient of an up-to-date approach in nearly all of the papers collected in this book to make it a recommended purchase for any of those interested in the problems of modern food technology except perhaps for the alarmist who would probably prefer not to be debunked.

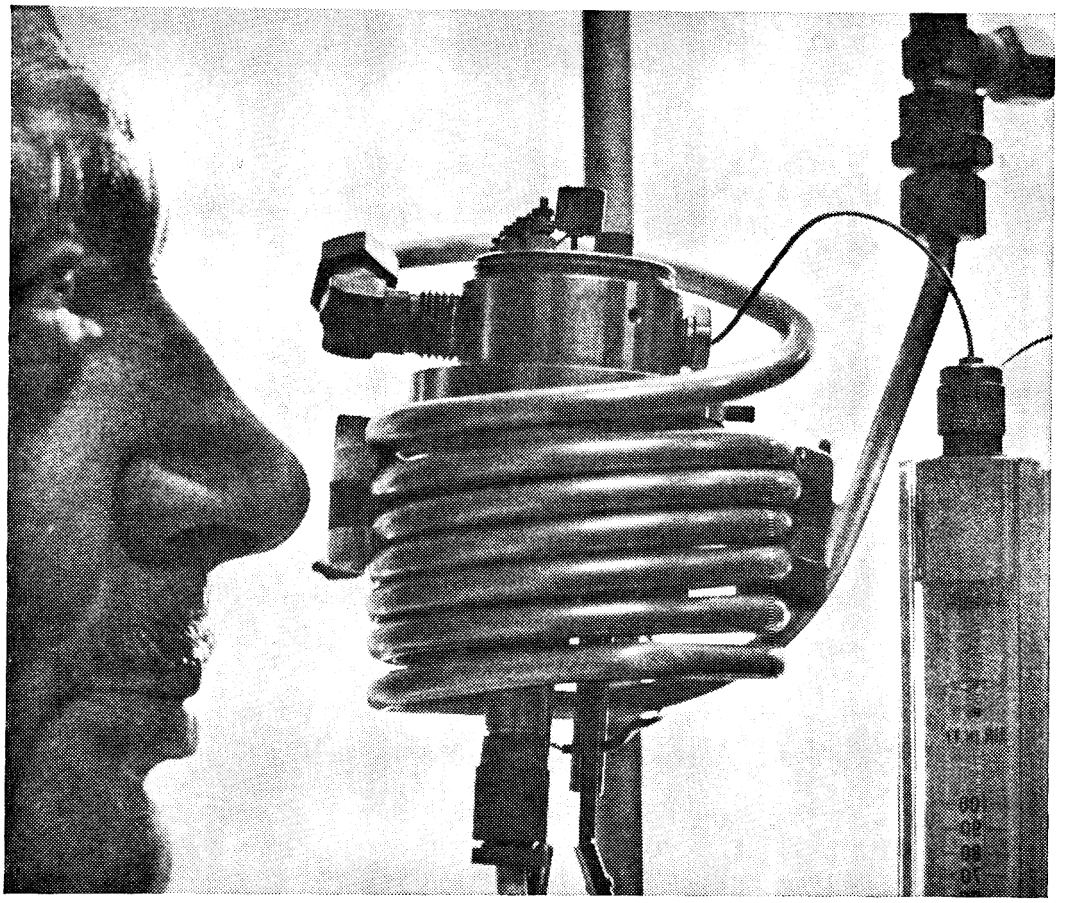
H. D. KAY

Errata

CAMERON, A.G. (1967) An assessment of the potential application of the method of attenuated total reflectance (ATR) infrared qualitative analysis to food materials. *J. Fd Technol.* **2**, 223–232.

On page 227 the caption to Fig. 3 should read:
FIG. 3. ATR spectra of: (a) water (—) and milk (- - -) at 40°, (b) butter at 40°, and (c) cheddar cheese (left) and casein as a methylene iodide mull (right) both at 45°.

On page 230 the caption to Fig. 4 should read:
FIG. 4. (a) ATR spectra of 50% solutions of fructose (left), glucose (centre) and sucrose (right) at 40°; (b) ATR spectrum of condensed sweetened full cream milk at 40°; and (c) IR transmission spectrum (KBr disc) of full cream dried milk (left) and corresponding ATR (right) spectrum at 40°.



Nose...meets...automatic nose

As any dog will tell you, the human nose is a sensitive but imprecise instrument. In other words your nose can pick up a 'scent' but not discriminate between scents, as a bloodhound can.

But even a prize bloodhound would not be able to equal the degree of discrimination of the apparatus in the picture. Called a gas chromatograph, it is used by Metal Box Packaging Research to determine the characteristics of aromas. Gas chromatography is just one of many research tools used by Metal Box to control the quality of packaging.

Physicists, chemists, biologists, and food technologists are also engaged in developing new forms of Metal Box packaging—not just in metal but in plastics and paper too.

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Leading manufacturers of packaging in metal, paper and plastics.

Light engineering and marketing organisation with 38 factories in Britain, 32 overseas.

Research team of over 300 continuously engaged in projects involving food technology, physics, chemistry and microbiology.

A youthful and vigorous industry with tremendous potential for the future.

MB There's more to Metal Box than metal boxes

– they're leaders in research into metal, paper and plastic packaging

MB 10-105/D/1

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Meat Science and Technology Number

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Abbreviations. Abbreviations for some of the commoner units are given below. The abbreviation for the plural of a unit is the same as that for the singular unless confusion is likely to arise.

gram(s)	g	second(s)	sec
kilogram(s)	kg	cubic millimetre(s)	mm^3
milligram(s)		millimetre(s)	mm
(10^{-3} g)	mg	centimetre(s)	cm
microgram(s)		litre(s)	l
(10^{-6} g)	µg	millilitre(s)	ml
nanogram(s)		pound(s)	lb
(10^{-9} g)	ng	gallon(s)	gal
hour(s)	hr	milliequivalent	mEq
minute(s)	min	R_F values	R_F

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