Volume 9 Number 1 March 1974

Journal of Food Technology

Published for the Institute of Food Science and Technology (U.K.) by Blackwell Scientific Publications
Oxford London Edinburgh Melbourne

JOURNAL OF FOOD TECHNOLOGY

Institute of Food Science and Technology (U.K.)

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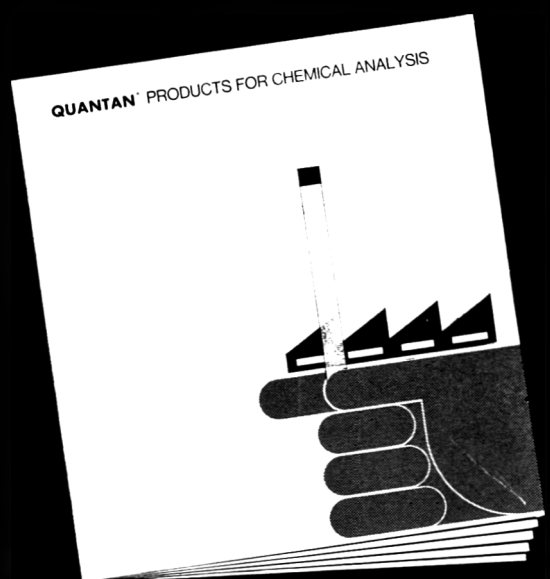

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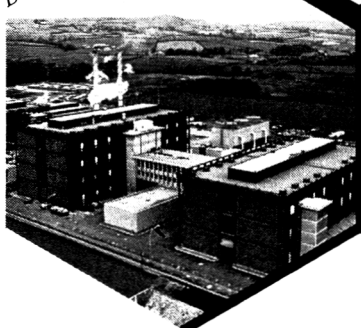

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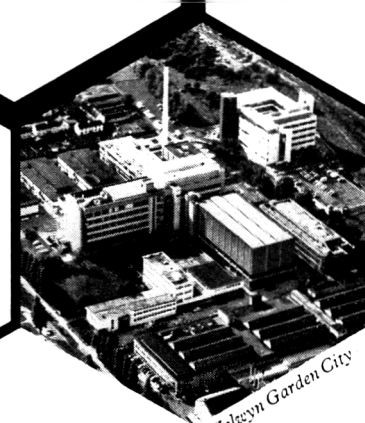

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J. Fd Technol. (1974) **9**, 1–12.

Taste adaptation: the case of the wandering zero

M. O'MAHONY

Summary

Some recent developments in taste psychophysics are reviewed. The zero (level of adaptation) for taste appears to vary with salivary content, which in turn varies with physiological changes and any residual taste stimulus that has recently been placed in the mouth. The effect of the interstimulus procedure, whether mouthrinsing or expectoration, on the amount of residual stimulus is discussed and is seen to be important in determining thresholds and intensity measures. The effect of language and criterion on thresholds is also noted. Some psychophysical methods of solving these problems are discussed in relation to food tasting.

The study of taste assessment and psychophysics is spread, rather unfortunately, over a wide range of disciplines. This means that a long time may elapse before research in one field reaches another and the history of taste research is, in some ways, the history of non communication. This brief review attempts to describe a few important findings and concepts which are new in the field of academic psychophysics and are worth communicating to those in the field of Food Science, involved in taste measurement.

Taste adaptation

Taste adaptation has been known for a long time and for an introduction to its effects, readers are referred to the excellent monograph by Amerine, Pangborn & Roessler (1965). Suffice it to say that if the taste receptors start signalling to the brain the presence of a constant stimulus, the message becomes attenuated; this can be regarded as a shift in the zero for taste. To the taster, the attenuation is perceived as a gradual reduction in taste intensity often resulting in the taste actually vanishing (complete adaptation). This attenuation or adaptation appears to be due to a specific inhibition of the taste receptor sites rather than the exhaustion of some receptive substance in the cell. It can be thought of as the volume switch being gradually turned down for the taste apparatus.

Furthermore, different types of receptor sites on the taste membrane appear to undergo the process of adaptation independently. Taste psychophysicists use this to

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investigate whether adaptation to one stimulus affects the taste intensity of another stimulus and hence whether they share common receptor sites. This is the whole study of cross-adaptation (Bogart, 1970; McBurney & Lucas, 1966; Meiselman, 1968a, b, c; Smith & McBurney, 1967, 1969). In one interesting study, McBurney & Shick (1971) adapted subjects to a variety of stimuli and noted the effects of this on the taste of water. For example, after adaptation to urea, water tends to taste salty or sweet and after adaptation to $MgSO_4$, sweet.

For the purposes of this review, one particular stimulus will be dealt with, NaCl, because it is with this stimulus that a lot of recent research has been concerned.

Salt taste adaptation and salivary salt

Early work by Hahn (1936) showed that adaptation to NaCl raised the NaCl threshold while Bekesy (1960) used a semiautomatic gustometer to show that adaptation caused a constant shift in threshold. McBurney & Pfaffmann (1963) demonstrated that the NaCl threshold remained a few millimolar above the concentration of sodium in the saliva, in the same way that it remained a few millimolar above the NaCl concentration of an adapting solution, when a constant flow procedure was used to fix the adaptation level of the receptors before applying taste stimuli.

This means that salt thresholds measured *in situ* (in the mouth) will vary with salt concentration of the saliva resting in the mouth, to which the taste buds are normally adapted. If changes in concentration of salivary salt are not too rapid, complete adaptation will render the taster unaware of them.

The salt concentration of the saliva resting in the mouth can vary in two ways. First, the concentration of the salt in the saliva secreted into the mouth by the salivary glands is subject to constant fluctuation. Such fluctuations are gradual and the taste buds remain completely adapted.

The second source of variation of salt levels in saliva is potentially far greater; this is the presence of small untasted quantities of salt that may appear in the mouth, during a taste experiment, as residual amounts left over from prior tastings. Such residuals are hopefully removed from the mouth by the interstimulus procedure, usually expectoration or rinsing, employed during the experiment. There is, however, no research to support any of these interstimulus procedures used, although the amount of residual left in the mouth, and hence the level of adaptation of the taste receptors, will be determined to a great extent by the procedure employed. It is also possible that dilution due to mouthrinsing may reduce the salt concentration in the mouth below that secreted by the salivary glands. The effects of residuals and mouthrinses will be dealt with in the next section.

The literature regarding the factors affecting the variation of salt concentration in the saliva secreted by the salivary glands and the factors affecting the variation of salt threshold is full of methodological difficulties and contradictions but, in general,

the notion that an increase in salivary salt results in an increase in salt threshold is supported. Any effects due to interstimulus procedures stay consistent within a given study and so do not interfere greatly.

Salivary sodium secreted by the salivary glands varies with salivary flow rate and salt and drug intake (Rapoport, Evans & Wong, 1961), duration of stimulation (Shannon & Prigmore, 1969a; O'Mahony, 1973e), diurnal (Prader *et al.*, 1955) day to day (Shannon & Prigmore, 1959b) and menstrual changes (Chesley & Hellman, 1957), adrenal cortical changes (Frawley & Thorn, 1951), heart failure (White, Gordon, Leiter, 1950), various pathological states (Becks, 1928) and exercise (Chesley & Hellman, 1957). This presents a formidable number of variables that could influence thresholds.

Yensen (1958, 1959a, b) showed that salt thresholds varied diurnally and with salt intake in the same manner as salivary salt. On the other hand, thresholds do not appear to vary with flow rate changes, brought about by the wearing of dentures (Kapur, Collister & Fischer, 1967a, b) nor do they vary with adrenal cortical changes (Henkin, 1967) in the manner expected from salivary changes. However, in these last two cases, thresholds were measured by pair comparison with water and it is possible that subjects distinguished the pairs by the subadapting taste of water rather than the supraadapting taste of salt.

Reports on the salivary salt variation with altitude are conflicting (Barer & Iakovleva, 1962; Williams, 1961) and no clear trends are indicated for thresholds (Finkelstein & Pippitt, 1958; Maga & Lorenz, 1972). Most evidence is also against sex variations in salivary salt concentration and thresholds (Anders, 1956; Shannon & Prigmore, 1958; Cooper, Bilash & Zubek, 1959) while the same is true for age variation although there is evidence that salt levels and thresholds increase after approximately 40 years of age (Becks, 1928; Grad, 1954). So, although there is a great deal of research still needed in the area, it does seem that salivary salt variations may play a part in determining thresholds.

It now remains to examine some of the recent work on the effects on taste thresholds of salt residuals remaining in the mouth from prior tastings, and other effects that depend on the interstimulus procedure.

Salt residuals from prior tastings and interstimulus procedures

There is a great variety of recommendations for the choice of interstimulus procedure to be used. Some authors advocate mouthrinses between tastings (Dahlberg & Penczek, 1941) while others do not, claiming that they reduce reliability (Bekesy, 1966) or that they introduce a new stimulus (Meiselman & Dzendolet, 1967). Meiselman & Dzendolet (1967) and Crocker (1945) argue that saliva is the best washing medium while Dallenbach & Dallenbach (1943) believe that deleterious effects of mouthrinsing are greater than any adaptation changes that occur without them.

When mouthrinsing is not used, fixed rest intervals have varied from 1 hr (Heymans, 1899) to 25 sec (Brown, 1914). When mouthrinsing is used, tapwater is the recommended choice (Bornstein, 1940) while several authors prefer it to be lukewarm (Crocker, 1945; Lorant & Lorant, 1948). The number of rinses is generally low, although up to five have been specified (Bruvold & Gaffey, 1965). Other authors allow subjects to judge for themselves how many mouthrinses are required (Gregson, 1966) while Cameron (1947) recommends that not only the number of rinses, but also whether to use them, should be left to the discretion of the subject; this would appear to introduce many uncontrolled variables.

Clearly the level of salt residuals and the level of adaptation of the taste receptors must depend on the interstimulus interval employed. But despite all the recommendations for various interstimulus procedures, it is only recently that these procedures have been systematically investigated.

O'Mahony (1972a) investigated how long residuals from a 15 sec 1 M NaCl mouthrinse remained in the mouth, using flame photometry to analyse the saliva at given intervals after expectoration of the salt rinse. LiCl was mixed with the mouthrinse as a label for the exogenous NaCl so that it would not be confused with NaCl excreted from the salivary glands.

After half an hour of constant expectoration, every 15 sec, eight of nine subjects still had residual exogenous NaCl in the saliva. However, as these residuals were of the of the order of 1 mM concentration, it may be asked whether they were 'significant'. A definition of significance, in this context, may be made by asking whether residual NaCl from the molar mouthrinse could have been tasted with the taste receptors adapted to salivary concentrations present before the molar mouthrinse. From McBurney & Pfaffmann's (1963) and O'Mahony's (1972c) results, a concentration of 5 mM above the adapting level can be easily tasted and so residuals from the molar mouthrinse greater than 5 mM may be regarded as significant.

Armed with this definition of significance, it may be said that 'significant' residuals remained in the saliva for 7–17 min (mean 10·4 min) after the molar rinse with rapid expectoration. This may not be half an hour but it is considerably longer than common interstimulus intervals.

Similarly, if the mouth was rinsed once with tapwater for 15 sec after the molar salt rinse, 'significant' residuals stayed in the mouth, for eight subjects, for 6–10 min. Five such rinses cleared residuals for some subjects while for others they remained 7–8 min.

If instead of expectorating rapidly, seven subjects merely rinsed out their mouthes repeatedly with tapwater, about five rinses were required to remove 'significant' residuals, although residuals detectable by flame photometry sometimes remained for fifty rinses.

One of the main results of this work was that there were great individual differences in the persistence of residuals so that the use of a mean number of rinses or mean expectoration period would not be satisfactory for all subjects. Differences were probably

due to variations in the efficiency of expectoration and rinsing, which in turn depends on the amount of rinse used, salivary flow rate, vigour of oral movements and size of the oral cavity.

Furthermore, rapid and complete adaptation to high residual levels made it impossible for subjects to judge for themselves when the mouth was free of significant residuals. This means that there is no advantage in allowing subjects to decide for themselves the number of mouthrinses needed (O'Mahony, 1974a).

Residual effects on salt taste sensitivity

Certainly it could be seen that the common interstimulus procedures did not clear the mouth of salt residuals; it was to be expected then, that repetitive tasting using only common interstimulus procedures, would result in drifts in sensitivity, the amount of drift depending on the efficiency of the interstimulus procedure in clearing the residuals from the mouth.

O'Mahony (1972b) studied such sensitivity drifts with five subjects who tasted 10ml samples of NaCl solution in ten successive ascending series. An ascending series means simply that the solutions were presented in order of increasing concentration. Solutions ranged in concentration 10–1000 mM and subjects were required to indicate whether the solutions were above the detection threshold. Classically, the 'detection threshold' is the lowest concentration at which a subject can distinguish the presence of salt but not necessarily by its salty quality; the 'recognition threshold' at which a salty taste is recognized occurs at a higher salt concentration. Three interstimulus procedures were used: 15 sec rest, 15 sec tapwater mouthrinse and 2 min rest; the mode results for five subjects are given in Table 1. The '+' sign indicates that the solution concentration

TABLE 1. Mode identification records for five subjects using different interstimulus procedures

NaCl concentration (mM)	Interstimulus procedure																													
	15 sec rest										2 min rest										Mouthrinse									
	Series number										Series number										Series number									
	1	2	3	4	5	6	7	8	9	10	1	2	3	4	5	6	7	8	9	10	1	2	3	4	5	6	7	8	9	10
1000	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
800	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
600	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
400	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
200	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
150	+	+	+	+	+	+	+	+	0	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
100	+	0	0	0	0	0	0	+	0	0	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
50	+	0	0	0	0	0	0	0	0	0	+	+	0	0	0	0	0	0	0	0	+	+	+	+	+	+	+	+	+	+
10	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	+	0	0	0	0	0	0	0	0	0

was above the detection threshold while '0' indicates that it was not. In all three conditions it can be seen from the table that the detection threshold rose in later series with repetitive tasting, the mouthrinsing interstimulus procedure being the most efficient at reducing the drift and the 15 sec rest the least efficient.

These were, however, concentrated solutions which would leave considerable residuals; threshold measurements of sensitivity involve less concentrated solutions which may not leave residuals large enough to cause a drift. This was tested in the same way with nine subjects using repetitive ascending series presentation of distilled water and solutions of NaCl of concentration ranging 1–80 mM, concentrations more commonly used in threshold measurement. Two interstimulus procedures were used: 15 sec rest and 15 sec tapwater mouthrinse. The resulting mode detection thresholds with successive tastings are given in the similar Table 2.

TABLE 2. Mode identification records for nine subjects using different interstimulus procedures

NaCl concentration (mM)	Interstimulus procedure: 15 sec rest, Series number										Interstimulus procedure: Mouthrinse, Series number									
	1	2	3	4	5	6	7	8	9	10	1	2	3	4	5	6	7	8	9	10
80	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
60	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
40	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
20	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
15	+	+	+	+	+	+	+	0	0	+	+	+	+	+	+	+	+	+	+	+
10	+	+	+	+	+	0	0	0	0	0	+	+	+	+	+	+	+	+	+	+
5	+	0	0	0	0	0	0	0	0	0	+	+	0	+	+	+	0	0	0	+
1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Distilled water	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0

Again, it can be seen that the drift in sensitivity was reduced by the mouthrinse. The no mouthrinse mode values show irregular drift, which does not reflect the more regular patterns noted in individual subjects. It can also be seen that on average the mouthrinsing interstimulus procedure maintains a lower level of adaptation; mouthrinsing is not only more efficient at removing residuals from the mouth but may also dilute the excreted saliva (O'Mahony, 1971). It is expected, then, that threshold measurements would be lower if mouthrinses were used between tastings.

O'Mahony (1973b), using twenty subjects noted lower mean detection thresholds (8·6 mM *v.* 15·4 mM) when mouthrinses were used between tastings. The same was seen for recognition thresholds.

The literature also supports these findings. Comparisons can be made between authors

for a given psychophysical procedure; here the ascending series procedure is chosen. Dallenbach & Dallenbach (1943) using three subjects found lower detection thresholds with mouthrinsing (5·1, 6·8 mM) than without (38·8, 39·6, 47·4 mM). These higher values without mouthrinsing are also found in other studies: Mefferd & Wieland (1968), using three subjects, obtained values of 29·5, 27·3 and 22·8 mM; Fabian & Blum (1943), using fifteen subjects, found a mean detection threshold of 11 mM while Richter & MacLean (1939), with twenty-four subjects, found a mean value of 8·0 mM. The lower value in the latter study was probably due to easier detection caused by larger stimulus quantities. Finkelstein & Pippitt's (1958) low mean mouthrinsing detection thresholds (mean 7·4, no units quoted), for ten subjects, appear comparable to Dallenbach & Dallenbach's (1943).

The same is found for ascending series measures of recognition thresholds. Studies which do not use a mouthrinsing interstimulus procedure (Fabian & Blum, 1943; Mefferd & Wieland, 1968; Mosel & Kantrowitz, 1952; Richter & MacLean, 1939) tend to yield higher thresholds than those that do (Finkelstein & Pippitt, 1958; Maga & Lorenz, 1972).

The ascending series method is not the only procedure for which mouthrinses yield lower detection thresholds; for instance, another common procedure for measuring thresholds is the Method of Constant Stimuli. With this procedure, stimuli of varying concentration are presented repeatedly, in random order, to the subject. Higher concentrations are detected all the time while lower concentrations are detected none of the time. The concentration that is interpolated as being detected 50% of the time is called the threshold. Using this procedure, O'Mahony (1973a) obtained lower thresholds with mouthrinsing than without, after a molar salt mouthrinse. Similarly Richter & MacLean's Choice Methods Nos. 1 & 2 (Richter & MacLean, 1939) are methods of measuring thresholds which involve pair comparison with water and the many water tastings involved act like mouthrinses and yield lower thresholds.

So it can be seen that NaCl threshold values are largely determined by the level of NaCl in the saliva; this depends on the salivary gland function and even more on residuals from prior tastings. Threshold measures monitor the level of salt in saliva rather than the sensitivity of the taste receptors *per se*.

Salt taste intensity measurements

Another area of psychophysical measurements in which the effects of residuals can be detected is the general field of intensity scaling. A common procedure used for salt taste intensity scaling is the Method of Direct Magnitude Estimation (Stevens, 1957). In this procedure a standard solution is tasted by the subject and given the intensity of say, 10. Comparison solutions, some weaker some stronger, are then tasted by the subject who estimates their intensity on a scale where the standard has a value of 10, expressing perceived strength in terms of multiples or fractions. Plotting the logarithm

of the intensity estimate against the logarithm of the solution concentration traditionally yields a straight line. This is expressed as Stevens' Power Law:

$$\log I = n \log C + A$$

where

I = perceived intensity,

C = solution concentration

and

'n' and 'A' are constants.

Such a plot is called a power function and its slope, 'n', is called the exponent. Psychologists are particularly interested in the exponents of such intensity functions and how they vary between the senses.

If mouthrinses are taken between tastings the level of residuals will be kept fairly low throughout the experiment. If they are not taken, the average level of residuals will be higher during the experiment; this in turn means a higher level of adaptation (a high zero) which will yield lower intensity estimates for the comparison solutions. If the intensity function is a power function, the lower intensity estimates will cause the function to have an increased slope (exponent) due to the non linear nature of the logarithmic axes.

Hence, a consideration of adaptation to residuals would predict that intensity functions constructed, using a no mouthrinsing procedure, would yield higher exponents than for a mouthrinsing procedure.

O'Mahony (1973c) tested this prediction with five subjects, using a 200 mM NaCl solution as the standard and six salt concentrations, each presented ten times, ranging from 50 mM to 1·5 M, as the comparisons. Table 3 gives the exponents for all five subjects in two conditions: first, sipping solutions at 15 sec intervals with expectoration (the no mouthrinsing condition) and second, with a 15 sec tapwater mouthrinse between the tastings (the mouthrinsing condition). It can be seen that all mouthrinsing exponents were lower, confirming the prediction.

TABLE 3. Power function exponents obtained using the method of direct magnitude estimation

Subject	With mouthrinsing	No mouthrinsing
1	0·45	0·57
2	0·63	1·20
3	1·30	1·64
4	0·92	1·59
5	1·63	1·87
Pooled data	0·80	1·08

Stevens (1969) had previously investigated the effect of mouthrinsing *v.* no mouthrinsing on exponents and found no difference. But this study can be criticized in its unrelated samples design, the smaller number of presentations of comparison solutions and the bad spacing of its comparison concentrations. The latter meant that points on his graph were bunched together and a great weighting was given to the estimates for one concentration in the determination of the exponent. Stevens acknowledged the faults in his design and urged further experimentation. A further study by O'Mahony (1973f) followed Steven's procedure by using fewer presentations of the comparison solutions and pooling the results from ten subjects, but did not follow Stevens by using a more even spacing of comparison solutions. In this study the expected lower exponent (0·88) was obtained for the mouthrinsing condition than for no mouthrinsing (0·98).

The same picture can be seen by examining the literature for Direct Magnitude Intensity Scaling for the NaCl taste. The more thorough the rinsing procedure the lower the exponent. Bruvold & Gaffey's (1965) thorough mouthrinsing procedure (up to five rinses) yielded an exponent of 0·92 while Jones & Marcus's (1961) and McFadden, Barr & Young's (1971) single rinsing procedure yielded slightly higher exponents (1·05 and 0·93–1·34). Meiselman's (1968c, 1971) less thorough 2 min rest procedure yielded even higher exponents (1·43 and 1·35). Steven's (1969) single mouthrinse procedure yielded a higher exponent (1·4) than expected; however, higher concentration solutions were used in the study compensating for the more thorough interstimulus procedure. Ekman (1961) presented each comparison after a standard, with no rest interval, and obtained the highest exponent of all, 1·59. Thus the exponent can be seen to decrease as the interstimulus procedure becomes more thorough.

An interesting point arises from Ekman's (1961) version of the Method of Direct Magnitude Estimation. Instead of presenting the standard once before all the comparisons, it was presented alternately with the comparisons. This procedure allows constant recalibration of the subject's scale with adaptation changes and should minimize drift effects. Ekman's short interstimulus interval overcame any recalibration compensation but given say, two procedures, rinsing and no rinsing, a constant recalibration should reduce the discrepancy between the two. O'Mahony & Wingate (1973) found that this was certainly the case. The difference between exponents for rinsing *v.* no mouthrinsing was certainly reduced using an Ekman type repeated standards procedure rather than a Stevens single standard presentation procedure.

By considering an average residual level during intensity scaling, any comparison solutions below this level will tend to be zero rated for intensity as they would be below the level of adaptation. This should produce a cut-off effect causing S-shaped deviations from the power function. Again, this is found to be the case (O'Mahony, 1973d).

Hence, for intensity scaling, one of the most common gustatory psychophysical activities after threshold measurement, adaptation to residuals can be seen to determine, to a great extent, not only the resulting exponents but also the deviations from the predicted functions.

Flow procedures

The main trouble with residuals from prior tastings is that they vary a great deal so that any estimation of their amount using a mean value will only be satisfactory for a few subjects. Constant monitoring (by flame photometry) is inconvenient for sodium stimuli and for other stimuli is often technically impossible. The answer to the problem depends on the aims of the psychophysical experiment.

If the aim is an academic one, namely the investigation of the taste receptor mechanism with as little interference from the environment of the oral cavity as possible, then a flow procedure is a useful alternative. Such a procedure uses an apparatus (Andersson *et al.*, 1971; O'Mahony, 1974b) which provides a constant flow over the tongue of a stimulus of a known concentration, thus fixing the level of adaptation. After adaptation to this flow, the stimulus required for study can be presented.

A development of this technique has been used to get over a further problem in taste measurement. Salt taste thresholds, like other taste thresholds, are described by the recognition threshold where the salt taste appears and the detection threshold, where the salt can be perceived as present although the taste is not yet salty; it is perceived as a 'tingle' or sometimes as a sweet taste on the tongue. However, recent research into the qualitative description of subadapting salt tastes (O'Mahony, 1973f; O'Mahony & Godman, 1973a, b) shows that below the recognition threshold there are many more sensations than merely 'detection' or 'tastelessness', any of which could be taken as the criterion for detection. In the absence of a satisfactory language to describe these sensations there is no control on the criterion for detection used by various subjects, rendering intersubject comparison difficult. This problem can be overcome by using criterion free measures of sensitivity, namely the Signal Detection Measures (Green & Swets, 1966; McNicol, 1972). They have not been used very much for gustatory measurements, but have recently been applied, along with a flow procedure, for measuring sensitivity to salt (O'Mahony, 1972c). So powerful is this combination of criterion free and flow procedures that subjects can use them to distinguish between a single and double distilled water stimulus (O'Mahony, 1972d).

Food research

However useful flow procedures may be for purely academic research, they are impracticable for food assessment. In this case, the aim, very often, is to sample food flavours in the oral environment. Workers should then be aware of residuals building up in the mouth and interpret their results accordingly. Comparison of blocks of trials may monitor any sensitivity drifts while it may be possible under ideal circumstances to carry out the standard tests in such a way that stimuli to be compared are tested under as similar adapting conditions as possible.

To be sure, the psychophysical effects discussed here have involved a simple ionic stimulus, but there is every reason to expect this effect to generalize to other stimuli,

as long as small amounts are able to remain unperceived in the mouth. Sensitivity drifts due to adaptation to residuals may well hold the explanatory key to 'taste fatigue'. At least such drifts should not be ignored and the choice of an interstimulus procedure should not be taken lightly. The choice of interstimulus procedure has all too often been made without any supporting experimental evidence and not only has the choice of procedure been taken lightly, but the procedure chosen has often not been reported.

It is hoped, then, that these recent findings in the field of academic taste testing, which play such a great part in the determination of psychophysical results may be heeded by those in the field of food assessment and that variables such as the interstimulus procedure may be given the attention they deserve.

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(*Received* 13 *June* 1973)

J. Fd Technol. (1974) **9**, 13–20.

Effect of structure disrupting treatments on volatile release from freeze-dried maltose

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Summary

Solutions of 1% 1-propanol and 20% maltose in water were freeze-dried under conditions resulting in retention of 69·5% of the propanol. The freeze-dried solutions were exposed to vapours of several organic compounds which varied in molecular size and polarity. Those vapours which were adsorbed in significant amounts caused a partial release of the entrapped propanol.

Heating the freeze-dried solutions in vacuum at temperatures of 37°C, 50°C, 65°C, and 82°C had no effect, but at 100°C a partial release of the propanol was achieved.

The results support the previously postulated microregion theory of volatile retention in freeze-dried carbohydrate solutions.

Introduction

Retention of volatiles in freeze-dried foods is controlled by interactions of the volatiles with non-volatile solids and water during freezing, drying, and storage. Flink & Karel (1970a) studied volatile retention in freeze-dried carbohydrate solutions, and postulated that crystallization of water during freezing results in the formation of microregions containing highly concentrated solutions of carbohydrates and volatiles. As the local moisture content within these regions decreases, first due to freezing and then to sublimation, there occur associations between the molecules of solute. In the case of carbohydrates these associations are caused by hydrogen bonds formed between hydroxyl groups of carbohydrate molecules (Flink & Karel, 1972; Karel & Flink, 1973). We have recently observed (Chirife, Karel & Flink, 1973) that molecular associations entrapping volatiles within microregions seem to occur also in polar polymers containing no hydroxyl group.

The structure of the microregions and the permeability of these regions to water and to organic vapours depends strongly on local water content. As this content decreases,

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the ease of loss of organic volatiles decreases until at some critical moisture level there is no further loss (Flink & Karel, 1970a, b). Exposure of freeze-dried carbohydrate solutions containing entrapped volatiles to water vapour shows the following pattern: at low humidities there is no volatile loss even after evacuation for prolonged periods; at higher humidities a rapid volatile loss occurs, until a new level of retention is reached, which is again stable unless the humidity is increased further.

It was determined that the critical point for initiation of volatile loss corresponds to sorption of water to levels above the calculated B.E.T. monolayer value (Flink & Karel, 1972). Below this level water is sorbed on those hydroxyl groups of the carbohydrates which do not participate in the structure-forming hydrogen bonds. Adsorption of water in amounts below the monolayer value does not therefore disrupt microregion structure, and the volatile retention is not diminished. At moisture contents above this level, however, the sorbed water competes for hydroxyl groups involved in structure forming, the microregion structure is disrupted, and a volatile loss occurs. Humidification resulted in a new level of retention, as long as the original structure of the freeze-dried materials was not destroyed by an amount of water sufficient to cause either dissolution or crystallization.

These observations confirmed the existence of a microstructure which undergoes partial collapse upon humidification to a level above the monolayer value, this partial collapse becoming complete only upon dissolution or crystallization (Flink & Karel, 1972).

The microstructure developed in freeze-dried carbohydrates and responsible for volatile retention can be disrupted by influences other than sorption of water. Other polar molecules capable of structure disruption should release entrapped volatiles, and so should high temperatures by causing thermal disruption of structure.

Flink (1969) studied the extraction of freeze-dried carbohydrates containing entrapped volatiles with different solvents, including water, methanol, ethanol, propanol, butanol, and acetone. He observed trends which indicated that size and polarity of the solvent molecules influenced the removal of retained volatiles from the dry solid, and that these differences were related to the ability of the solvent to disrupt microregion structure.

The present paper is devoted to a study of microregion disruption and volatile release by sorption of polar vapours, and by heating to elevated temperatures.

Materials and methods

Retention of 1-propanol in freeze-dried carbohydrates was studied in a model system consisting of maltose, ^{14}C-labelled 1-propanol, and water (20 : 1 : 79 by weight). The model system was prepared by dissolving maltose in water and adding propanol. Five millilitres of the solution were pipetted into 50-ml Erlenmeyer flasks frozen in liquid nitrogen, and then freeze-dried for 48 hr at ambient temperature and at a pressure of

less than 0·1 torr in a Virtis model 10 MRTR freeze drier. 1-Propanol was mixed with ^{14}C-labelled 1-propanol to give the desired specific radioactivity. The radioactive propanol was obtained from International Chemical and Nuclear Corporation, Irving, California.

In several experiments the freeze-dried solutions were exposed to vapours of various liquids maintained at constant temperatures and vapour activities. The liquids included water, methanol, ethanol, formic acid, acetic anhydride, ethyl ether, benzene, and aniline. All materials used in this study were of reagent grade and were not further purified prior to use.

The control of vapour pressures at saturation levels required only temperature control; where the activities were to be maintained at levels below saturation, we used binary mixtures. Saturated salt solutions were used in the case of water, since activities of these solutions are readily available from literature. Vapour pressures below saturation were obtained for methanol and ethanol by preparing mixtures with the relatively non-volatile diethyl phthalate. Activity of alcohol vapours over such mixture was known from literature (Dornte, 1929); in the case of formic acid, a mixture of 69 g of 90·9% formic acid with 46 g of glycerol was used (molar fraction of formic acid 0·615). The deviation of the activity from the ideal value was not determined in this case. The activity of water in this mixture was estimated to be well below the levels producing volatile release (Flink & Karel, 1972).

Sorption of the various vapours was accomplished by placing flasks of the freeze-dried model system containing no propanol in vacuum desiccators over the appropriate vapour-generating liquid or mixture. The amount of vapour sorbed at each vapour activity was determined by change in weight. Since in each case only one vapour was sorbed, change in weight gave the amount of sorption directly.

Analysis of 1-propanol

The 1-propanol content was determined by measuring the radioactivity of the samples with a liquid scintillation counter.

The dried samples of maltose were dissolved in water (to 10% solution); 1 ml of this solution was added to 10 ml of water-miscible scintillator (2,5-diphenyloxazole 1 g, naphthalene 100 g, dioxane to 1000 ml) in the counting vial, and the resulting solution was counted with a Beckman LS-230 liquid scintillation counter.

Results and discussion

Maltose was chosen as the carbohydrate system to be investigated and ^{14}C-labelled 1-propanol was the volatile.

Like other sugars, maltose upon freeze drying gives an amorphous cake which recrystallizes upon humidification (Guilbot & Drapron, 1969). The results of humidification of maltose at 75% relative humidity are presented in Fig. 1. Humidification causes the

moisture content to increase to approximately 25% (dry basis) and then to fall, indicating recrystallization. The crystallization of amorphous sugars upon addition of sufficient water to break some hydrogen bonds and allow the sugar molecules sufficient mobility to form the more stable crystalline state, and the attendant release of water, are well-documented in literature (White & Cakebread, 1966; Makower & Dye, 1956; Flink & Karel, 1972). The humidification had a pronounced effect on retention of entrapped 1-propanol. After freeze drying and before humidification the amorphous cake of maltose contained 3·47 g 1-propanol/100 g maltose. As the water content of the maltose increased towards 25%, the propanol content was decreasing slowly towards a new value of about 50% of the original. Then, as recrystallization began, propanol was lost rapidly; the final retention was very low. The rate of propanol loss during this period paralleled the progress of crystallization. The high rate of propanol loss is a consequence of the extensive disruption of the carbohydrate-carbohydrate bonds present in the amorphous cake. The results obtained with maltose thus are very similar to those found by Flink & Karel (1972) in crystallization of lactose after exposure to 61% relative humidity.

In Fig. 2 we present results obtained when the propanol-containing freeze-dried maltose was exposed to vapours of several organic compounds. Saturated vapour of ethyl ether caused no release of propanol at either 25°C or 37°C. Ethanol at an activity of 0·75 also failed to produce any loss of propanol at 25°C. With both ethanol and ether the lack of propanol release was associated with negligible sorption of the two vapours in the amorphous maltose. Sorption of ether was below detectability limits, and the sorption of ethanol was about 0·55% on dry basis.

Acetic anhydride was adsorbed to a slightly greater extent than ethanol (1·2% dry basis): this sorption resulted in a small but significant amount of released 1-propanol.

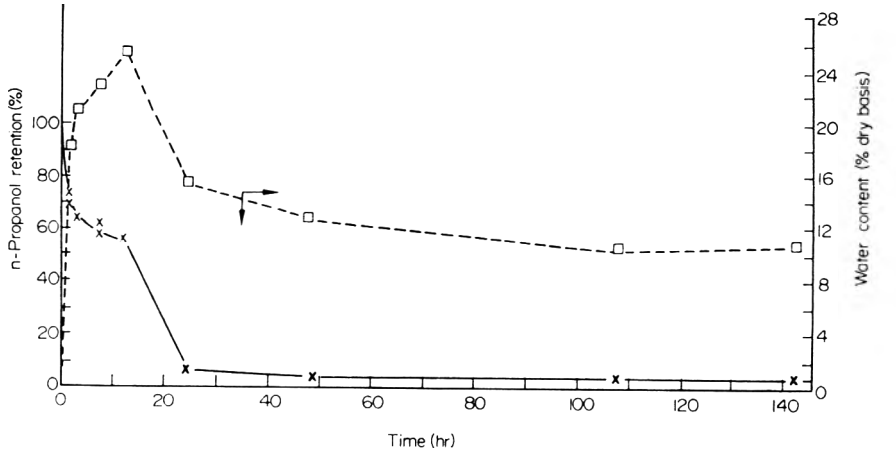

FIG. 1. Adsorption of water (dashed lines) and retention of 1-propanol (solid lines) in freeze-dried maltose humidified to 75% relative humidity at 25°C.

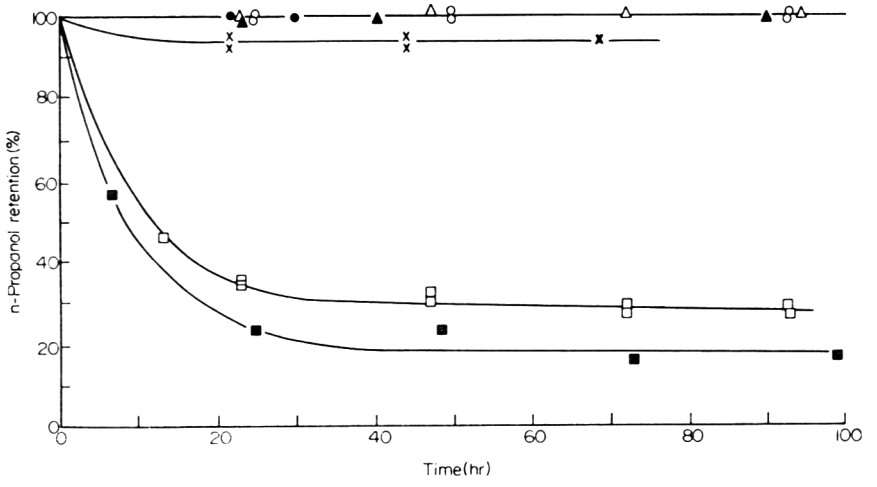

FIG. 2. Loss of 1-propanol from freeze-dried maltose exposed to several organic vapours. ○, Ethyl ether, saturated vapour, 25°C; ●, ethyl ether, saturated vapour, 37°C; △, methanol, vapour activity 0·17, 25°C; ▲, ethanol, vapour activity 0·75, 25°C; ×, acetic anhydride, saturated vapour, 25°C; □, methanol, vapour activity 0·75, 25°C; ■, formic acid, vapour over solution with a mole fraction of 0·615, 25°C.

Figure 2 also shows results obtained by exposing the freeze-dried maltose to formic acid over a ternary mixture of formic acid, water, and glycerol (mole fraction of formic acid = 0·615). The total sorption amounted to approximately 32 g/100 g maltose, and resulted in disruption of maltose cake structure, as evidenced by substantial loss of the entrapped propanol. A rapid initial loss of the entrapped volatile occurred during the first day of exposure to formic acid vapour, followed by an approach to a new retention level.

Similar behaviour was observed with methanol vapour at an activity of 0·75, but methanol at an activity of 0·17 had no effect.

The sorption of methanol at an activity of 0·17 was 1·42% (dry basis), and 14·4% (dry basis) at an activity of 0·75.

The ability of methanol to strongly interact with carbohydrates is well-documented in literature: it is significant that methanol can act as a solvent for a number of carbohydrates (Moyle, 1972). The structure-disrupting effect of methanol is similar to that observed with water. In Fig. 3 we compare the effects of water vapour and of methanol vapour at selected activities. At an activity of 0·61 water sorption was 15·0%; at an activity of 0·75 it reached 25·5% before decreasing again due to crystallization. At an activity of 0·75, methanol sorbed was 14·4%, and at an activity of 0·98 it reached 26·4%, then decreased to 18·2%.

As expected from the considerations of both size and polarity, water is a more effective disruptor of maltose structure than methanol. Methanol at an activity of

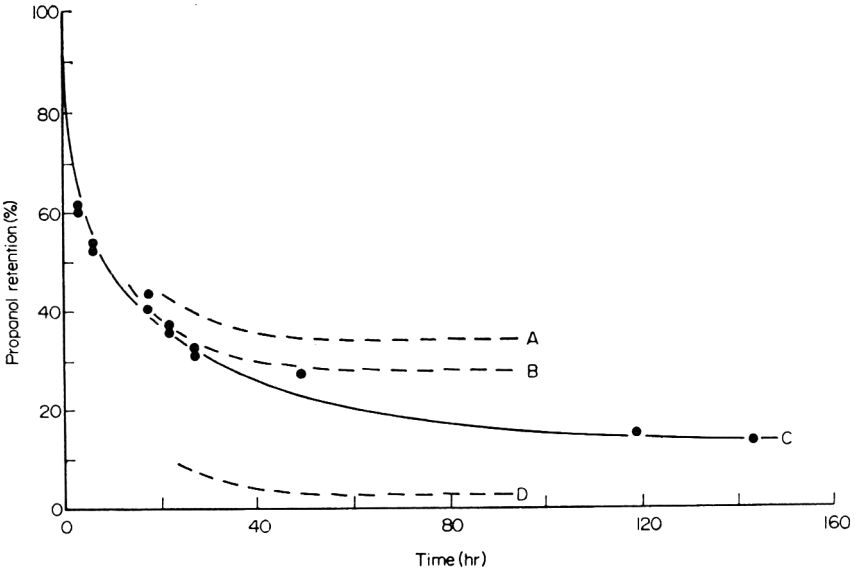

FIG. 3. Effect of water and of methanol on retention of propanol in freeze-dried maltose at 25°C. (A) Water, vapour activity 0·61 (2-propanol; Flink & Karel, 1972). (B) Methanol, vapour activity 0·75 (1-propanol; present study). (C) Methanol, vapour activity 0·98 (1-propanol, present study). (D) Water, vapour activity 0·75 (1-propanol; present study).

0·98 releases less propanol than water at an activity of 0·75; and at an activity of 0·61 water releases slightly more 2-propanol (Flink & Karel, 1972) than methanol at an activity of 0·75 releases 1-propanol.

In a recent review of non-aqueous solvent for carbohydrate, Moyle (1972) noted that the ability of various compounds to act as solvents depends not only on polarity, but also on molecular size, both factors being involved in the ability to disrupt the crystal lattice of the carbohydrates, which is similar to disruption of the structure in micro-regions containing entrapped volatile in freeze-dried maltose.

To demonstrate further the separate effects of polarity and size we exposed freeze-dried maltose containing entrapped propanol to saturated vapours of benzene and aniline. Benzene was not sorbed and produced no release of propanol; aniline, however, was sorbed to the extent of approximately 10%, producing a substantial release of the entrapped propanol (Fig. 4). The shape of the release curve is again consistent with a partial disruption of the retaining structure in the maltose.

In a series of studies on release of entrapped volatiles from freeze-dried carbohydrates, we especially noted the coincidence of structural changes observable visually at the onset of volatile release. These changes have been described (Flink & Karel, 1972) as follows: 'Whenever loss of volatile occurred, it was accompanied by a visible change in the dry cake. The resulting structure can be best described as a thick, viscous, glassy, amorphous mass.' Such structure changes are also capable of being produced

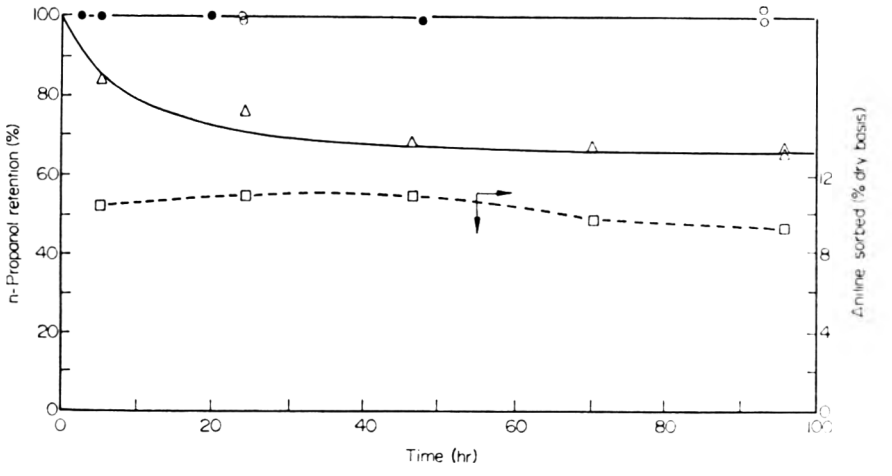

FIG. 4. Retention of 1-propanol in freeze-dried maltose exposed to saturated vapours of benzene and aniline. Sorption of aniline is shown by a dashed line; benzene was not sorbed in measurable amounts. ○, Benzene, 25°C; ●, benzene, 37°C; △, aniline, 25°C; □, sorption of aniline, 25°C.

by exposure to elevated temperatures: often phase transitions occur over a narrow temperature range, below which there is no effect. These transitions occur in crystalline forms (Hodge, Rendleman & Nelson, 1972), but can also be produced in amorphous structures (Mackenzie, 1966).

Freeze-dried samples of maltose-1-propanol were placed in desiccators over anhydrous calcium sulphate and activated charcoal and evacuated at 37°C, 50°C, 65°C, 82°C, and 100°C. The experiments at 37°C and 50°C were conducted in constant temperature rooms. For the higher temperatures a desiccator was partially filled with purified sand as a heat transmission medium, and was placed inside an oven with temperature control. When the steady state at the desired temperature was reached ($\pm$ 1·5°C), the samples were buried in the sand and the desiccator was evacuated.

In Fig. 5 we show that between 37°C and 82°C there is no significant loss of volatile. However, when the temperature is raised to 100°C a rapid volatile loss occurs, and the 1-propanol content asymptotically approaches a new level of retention. This loss was associated with observable changes in the structure of the freeze-dried maltose.

These results offer positive support to the postulated mechanism of retention (Flink & Karel, 1970a, b) The microregion structure consists of hydrogen-bonded carbohydrate molecules; thermal energy from heating will cause disruption only when it is above some energy level. Therefore, as proved experimentally, volatile retention remains constant when the sample is heated at temperatures at which no structural changes occur.

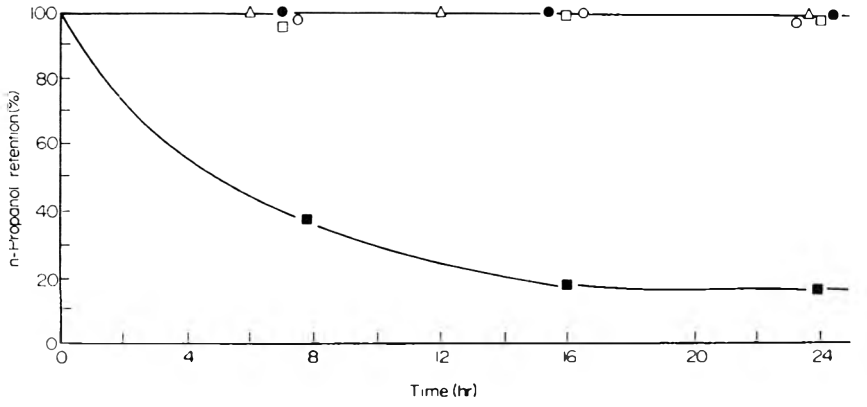

FIG. 5. Retention of 1-propanol in freeze-dried maltose heated at several temperatures. △, 37C°; ○, 50°C; ●, 65°C;□, 82°C; ■,100°C.

Acknowledgments

The authors acknowledge the support of the Universidad de Buenos Aires and Facultad de Ciencias Exactas y Naturales in form of a travel grant to Dr Chirife. This study was also supported in part by Contract No. 9-12485 from the Manned Spacecraft Center, NASA, Houston. The authors are grateful to Professor James Flink for his valuable discussions of the present work.

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(*Received* 8 *May* 1973)

J. Fd Technol. (1974) 9, 21–29.

Dietary enzymic hydrolysates of protein with reduced bitterness

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Summary

It is thought that a pre-digested dietary protein could have applications for patients with digestive disorders, and especially for the condition of cystic fibrosis. Enzymic hydrolysis, compared with acid hydrolysis, has the advantage of retaining the nutritional value of the protein, but is often associated with the occurrence of a bitter peptide which makes the product unpalatable. Eggwhite and casein have been investigated as protein substrates and treated with endopeptidases and pig's kidney tissue as a source of exopeptidases; hydrolysates relatively free of bitterness and containing small peptides and over 50% free amino acids have been obtained.

Introduction

Cystic fibrosis is a genetic disease manifested by a deficiency in the secretion of pancreatic enzymes into the small intestine, and by the presence of large quantities of thick and sticky mucus in the lumen of the respiratory and digestive tracts; both these abnormalities contribute to reduced metabolism of ingested fats and protein and the lowered digestive function of these patients is a matter of considerable concern. In order that children inheriting the cystic fibrosis syndrome should survive to adulthood it is essential that they absorb an adequate supply of amino acids for tissue synthesis, and for the formation of antibodies to combat the respiratory infections to which they are especially prone.

Winitz *et al.* (1965) have shown that full health can be maintained on a completely synthetic diet composed of amino acids, glucose, essential fatty acids, minerals and vitamins. There are various medical disorders involving impaired digestion, including cystic fibrosis, where oral administration of a pre-digested protein could prove advantageous but the cost of a synthetic diet makes it impractical. Therefore, it was considered that a hydrolysed protein product might be of value.

Acid hydrolysis of protein has the disadvantage that some of the amino acids are totally, or partially, destroyed. Enzymic hydrolysis causes no destruction or racemiza-

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tion of the amino acids and the nutritional quality of the original protein is retained. However, there are two disadvantages associated with enzymic digestion of protein: the hydrolysis rarely goes to completion and this type of hydrolysate contains an unpleasant bitter-tasting peptide (Carr, Loughheed & Baker, 1956). The former criticism may not be serious since any incompletely digested protein will be in the form of small peptides and these may still be of nutritional value, even to patients with impaired digestion. It is of greater importance that the product should be organoleptically acceptable.

The present investigation has been undertaken to formulate an enzymically hydrolysed protein with a high content of free amino acids and the minimal amount of bitterness. In the first instance eggwhite was chosen as the substrate because of the high Biological Value of this protein. On account of the frothing and other physical properties of albumen which made it difficult to work with, and because of the high cost of this raw material, alternative substrates have been investigated. Although casein has a lower Biological Value, it is still a nutritionally valuable protein and is cheap; casein has been employed in most of these studies.

Endopeptidases split the protein molecule primarily into peptides, and exopeptidases remove amino acids singly from the ends of the peptide molecules. Digestion of casein with the endopeptidase trypsin has been shown to result in the formation of one or more bitter peptides for which various amino acid compositions and molecular sizes have been reported (Gordon & Speck, 1965; Matoba, Hayashi & Hata, 1970; Sullivan & Jago, 1972). By investigating alternative endopeptidases and incorporating hydrolysis with exopeptidases it was hoped that any bitter peptides which were initially released would be broken down subsequently. A hydrolysate of high free amino acid content and reduced bitterness would then be obtained.

Experimental

Experimental details of the procedures which have been used are given in Fig. 1. The natural pH of aqueous solutions of dried eggwhite was 5·4 and no adjustment was made before the first hydrolysis with ficin or papain; the casein suspensions were more acid and were adjusted to pH 6·5 before the initial enzymic hydrolysis. All enzymic treatments were carried out at 37–40°. The enzyme systems for the second stage of hydrolysis, after adjustment to pH 8·0, are activated by manganese and 7·5 ml 0·2 M $MnCl_2$ aqueous solution per 100 g substrate protein was added.

At the end of the digestion procedure the hydrolysate was boiled to inactivate the enzymes and to coagulate any undigested protein, cooled, and centrifuged to remove any solid material. The recovery value for the digest was the nitrogen content of the supernatant expressed as a percentage of the nitrogen content of the initial protein. No allowance was made for the nitrogen content of any enzyme compounds which were added but this never accounted for more than 4·4% of the total nitrogen.

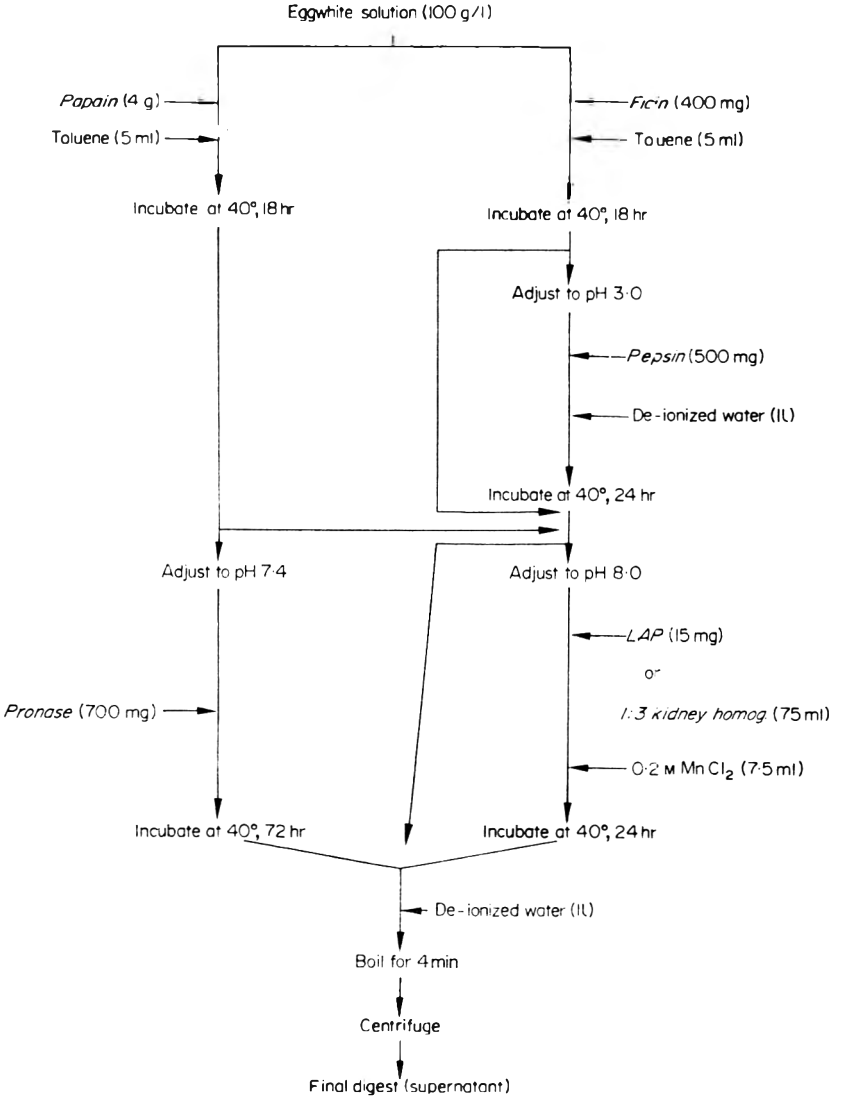

FIG. 1. Flow diagram for different enzymic treatments.

The degree of hydrolysis in the digest, i.e. a measure of the free amino acid content, was assessed by either, or both, of two methods:

(a) Formol titration according to Melnick & Oser (1949).

(b) Dialysis of 100 ml digest in Visking tubing suspended in 500 ml deionized water for 24 hr, after which the nitrogen content of the dialysate was determined and expressed as a percentage of the nitrogen content of 100 ml digest. (This arbitrarily chosen procedure for measuring the free amino acid content was subsequently

shown to give good agreement with the more refined, but lengthier, technique of gel filtration. Extending the time for dialysis resulted in small peptides also passing into the dialysate.)

It is generally necessary to include a preservative during the period of enzymic hydrolysis; for the initial laboratory investigations 5% ethanol or 0·5% toluene was added. These preservatives were removed during the final heating, the toluene and water at this concentration forming an azeotropic mixture with a lowered boiling point of 83°. Additional preservative was added to the centrifuged digest if it was required to be stored in the liquid state or, alternatively, the digest was freeze-dried or spray-dried. The choice of an acceptable preservative for a product destined for oral consumption, and presenting no hazard during large scale production, required a separate investigation.

Results

Eggwhite as substrate

(1) *Ficin/pepsin hydrolysis.* The procedure was carried out according to the flow diagram in Fig. 1 and the results are included in Table 1. Adjusting the pH of the final digest to 6·0 did not overcome the very bitter flavour and only served to increase the ion content. Stirring during incubation did not noticeably increase the level of dialysable nitrogen in the digest. A series of trials confirmed that the levels chosen for ficin and pepsin, and their respective incubation periods, gave the optimum conditions for hydrolysis.

TABLE 1. Analyses of eggwhite digests

	% Recovery	% Dialysable nitrogen		Flavour
		Based on digest N	Based on original N	
Ficin/pepsin	99·4	21·0	20·9	Very bitter
Ficin/LAP	50·1	27·9	14·0	Not bitter
Ficin/pepsin/LAP	57·0	39·5	22·5	Slightly bitter
Ficin/kidney	44·7	32·4	14·6	Not bitter
Ficin/pepsin/kidney	56·8	55·1	31·3	Not bitter
Papain/kidney	60·6	38·7	23·5	Not bitter
Papain/pronase	85·5	62·0	53·0	Slightly bitter

The low content (21%) of free amino acids and the bitter taste were to be expected since both ficin and pepsin are endopeptidases, and it was necessary to include an exopeptidase to increase the degree of hydrolysis. Leucine aminopeptidase (LAP) was chosen.

(2) *Ficin/LAP and ficin/pepsin/LAP hydrolysis.* A crude preparation of LAP was prepared in the laboratory from pig's kidney (Spackman, Smith & Brown, 1955), but its activity (Binkley & Torres, 1960) fell off rapidly, despite storing at low temperature. Therefore, a commercial preparation was tested at a level of 15 mg LAP/100 g eggwhite, plus the addition of manganese to activate the enzyme, Fig. 1.

The results for these two hydrolysates are given in Table 1 and show an increase in the percentage of free amino acids in the final digests, but this apparent improvement was negated by the reduced recovery of nitrogenous material; the LAP hydrolysis required adjustment of the ficin and ficin/pepsin digests to pH 8·0 which caused the larger peptides to come out of solution and their removal at the final centrifuging. The ficin/LAP digest was free of bitterness, presumably because there was insufficient breakdown of the protein to release the bitter peptides. The further treatment of the ficin/pepsin digest with LAP showed some improvement in reducing the original intense bitterness.

(3) *Ficin/kidney and ficin/pepsin/kidney hydrolysis.* As LAP is expensive to obtain commercially and difficult to isolate, the effect of adding the original source of the enzyme to the digest was considered, bearing in mind that the wide variety of other proteolytic enzymes present in pig's kidney would improve hydrolysis. In particular, prolidase would be added along with LAP and enhance the degree of hydrolysis.

A 1 : 3 aqueous homogenate of the cortex of fresh pig's kidney was added to the endopeptidase digests at a level of 4% kidney nitrogen to substrate nitrogen, Fig. 1; manganese was also added. No further hydrolysis was obtained by increasing the level of kidney tissue. The properties of the ficin/kidney digest did not differ significantly from those of the ficin/LAP digest; the ficin/pepsin/kidney digests were relatively free of bitterness and contained over 50% free amino acids, Table 1.

(4) *Papain/kidney hydrolysis.* Attention was turned to improving the initial endopeptidase hydrolysis to increase the number of peptides available for the exopeptidases in the kidney homogenate. Papain, which is a less specific endopeptidase (Hill & Schmidt, 1962) than ficin, and has a faster rate of hydrolysis than pepsin, was tested in place of these two enzymes.

The recovery from the papain/kidney digest was somewhat higher (60·6%) than that from the ficin/pepsin/kidney digest (56·8%), reflecting a smaller proportion of large peptides because of the more extensive hydrolysis by papain compared with the ficin/pepsin system. The percentage dialysable nitrogen was lower (Table 1), however, indicating that fewer free amino acids were released.

Although the desired increase in free amino acids was not achieved, this digest had the advantage of a very low ion concentration because of the minimal pH adjustments, a shorter incubation period, and an acceptable flavour.

(5) *Papain/pronase hydrolysis.* Pronase, a broad-spectrum protease of microbial origin (*Streptomyces griseus*), was tested as a possible alternative to the mammalian proteolytic enzymes of kidney. Treatment with this enzyme had the disadvantage of requiring 72 hr

incubation for optimum hydrolysis, Fig. 1. The content of free amino acids (62%) in the papain/pronase digest was the highest of all the enzyme systems tested but, even so, a bitter taste was still detectable. The cost, in time and in the price of the commercial enzyme preparation, together with the undesirable flavour, meant that this enzymic treatment was unlikely to have a practical application.

Casein as substrate

During the enzymic hydrolysis of eggwhite it was necessary to work with dilute solutions to avoid the formation of a gel; if the final digest was eventually to be dried to powder form a large volume of water would have to be removed, thereby adding to the cost of the product. Therefore, an alternative protein substrate, namely, casein was chosen in order to work under more concentrated conditions. Some of the enzyme treatments reported for eggwhite were repeated with Glaxo casein C: 100 g casein was suspended in 1600 ml water and there was no further dilution during the period of enzymic hydrolysis. The results are summarized in Table 2 and show that casein was more susceptible to hydrolysis than eggwhite treated under comparable enzymic conditions. The ficin/pepsin/kidney and papain/kidney digests of casein were also relatively free of the bitter flavour, the latter being preferable because of the minimal pH adjustment and requiring less incubation time.

Wet casein precipitated from liquid pasteurized skim milk, or from commercially cultured buttermilk, was also investigated as a protein substrate. The casein was precipitated at pH 4·5 with 2 N HCl or a 20% citric acid solution (the buttermilk required

TABLE 2. Analyses of casein digests

	% Recovery	% Dialysable nitrogen		Flavour
		Based on digest N	Based on original N	
Casein powder				
Ficin/pepsin	70·5	47·9	33·8	Very bitter
Ficin/pepsin/kidney	93·7	53·8	50·4	Not bitter
Papain/kidney	99·9	50·7	50·6	Not bitter
Papain/kidney digests				
Skim milk casein	78·4	53·8	—	not bitter
Skim milk casein—boiled	76·5	52·8	—	Slightly bitter
Buttermilk casein	82·9	58·7	—	Not bitter
Buttermilk casein—boiled	85·5	54·4	—	Slightly bitter

very little pH adjustment), well washed to remove the lactose, and re-suspended in water to a volume approximately three-quarters that of the initial sample of milk, and subjected to papain/kidney hydrolysis; in some instances the washed casein was boiled to give a firmer curd. The results are given in Table 2. The recovery values for the skim milk indicate that most, or all, of the casein has been utilized; for the buttermilk, nitrogen recovery values higher than the theoretical casein content (even after subtracting 4% for the added kidney tissue) were obtained which may be associated with changes in the milk during the preliminary culturing treatment. Although the flavour of different batches of any one type of digest tended to vary slightly, there was an overall impression that the digests prepared from precipitated casein which was not subjected to boiling were relatively free of the bitter factor; some bitterness usually occurred in the digests prepared from the boiled casein curd. There was no significant difference in the degree of hydrolysis obtained from the two forms of precipitated casein.

Discussion

Dvorak (1968) has reported total hydrolysis of beef serum albumin to free amino acids after enzymic digestion with purified papain, LAP and prolidase. His biochemical analyses were performed on milligram quantities of substrate and the larger scale studies during the present investigation have shown that a maximum of 50–60% liberation of free amino acids was obtainable with a variety of enzyme systems and taking eggwhite and casein as the sources of protein. However, it is hoped to show in future trials that human volunteers suffering from impaired digestion of protein can benefit from this type of product comprised of amino acids and small peptides.

Hydrolysis of eggwhite and casein with the ficin/pepsin system yield only 20·9% and 33·8%, respectively, of the original substrate as free amino acids; these digests were strongly acid to taste, as well as containing a very bitter flavour. Complete recovery was obtained when eggwhite was subjected to treatment with ficin/pepsin but under similar conditions 30% of the initial casein was removed after boiling and centrifuging. Therefore, the endopeptidases hydrolysed eggwhite to medium-sized, acid-soluble peptides with some free amino acids whereas casein yielded approximately equal amounts of large coaguable peptides, smaller soluble peptides, and free amino acids. This observation showed that the enzymic hydrolysis was not random but depended on specific sites in the molecules of the two proteins and/or their physical structure.

The inability of LAP to hydrolyse the peptides resulting from endopeptidase digestion, plus the adjustment to pH 8·0, were responsible for the low recovery values of 50·1% from the ficin/LAP digest (even the bitter peptide was not released) and 57·0% from the ficin/pepsin/LAP digest of eggwhite. These findings illustrated the specificity of LAP as an exopeptidase.

The replacement of LAP by kidney homogenate did not increase the alkali-soluble

constituents obtained from eggwhite, as shown by recovery values of 44·7% and 56·8% for ficin/kidney and ficin/pepsin/kidney digests, respectively. However, the presence of additional enzymes in the kidney increased the hydrolysis from 22·5% free amino acids in the ficin/pepsin/LAP digest to 31·3% in the ficin/pepsin/kidney digest based on original nitrogen. The role of pepsin in releasing more terminal groups by endopeptidase activity is noticeable when ficin/kidney and ficin/pepsin/kidney digests of eggwhite are compared.

Similarly, with casein, the addition of kidney to the ficin/pepsin system raised the degree of hydrolysis from 33·8% to 50·4% dialysable nitrogen based on original nitrogen. The recovery was also considerably increased as a result of the hydrolysis of large peptides by kidney enzymes which had not been the case with eggwhite.

The papain/kidney system, when compared with the ficin/pepsin/kidney procedure, showed a slightly higher recovery value with both eggwhite and casein indicating an increase in the amount of smaller soluble peptides at the expense of some of the larger ones which are removed by centrifuging. With casein, the liberation of free amino acids in the papain/kidney digest remained the same as in the ficin/pepsin/kidney digest; with eggwhite, the degree of hydrolysis with papain/kidney treatment was noticeably lower, again indicating the importance of amino acid sequence in the molecule in determining the efficiency of hydrolysis of different enzyme systems for different proteins.

The principal feature of this investigation was the discovery that within the spectrum of the proteolytic enzymes in pig's kidney there is a system capable of hydrolysing the peptide responsible for the bitter flavour (B.P. 1338936). Although the complex of microbial proteases, defined as pronase, resulted in a higher degree of hydrolysis than that obtained with kidney enzymes the bitter peptide appeared to persist. Substitution of LAP for kidney tissue did not prevent the occurrence of the bitter factor so that alternative exopeptidases present in kidney tissue, but not in *Streptomyces*, must be responsible for the hydrolysis of this peptide.

There were indications that the physical form of the substrate influenced the extent to which the bitter peptide was hydrolysed during enzymic digestion. Acid casein powder and the soft casein curd precipitated from liquid fat-free milk provided the least bitter digests, whereas the precipitated casein which had been boiled to give a firmer curd tended to retain some bitterness when treated under identical conditions. A cursory examination of casein in the form of sodium caseinate as a possible substrate also yielded a bitter tasting product; similarly, precipitating casein from reconstituted dried skim milk powder presented handling problems and gave a slightly bitter tasting digest.

As a result of this survey of enzyme systems and substrates on a laboratory scale, it was concluded that casein powder subjected to papain and pig kidney hydrolysis was likely to prove the most promising technique for further development. This procedure was considered to be economically feasible, to require only a maximum of 48 hr for incubation, to involve minimal pH adjustments thereby keeping the salt content at a low level, and to yield almost complete recovery of the original protein substrate.

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(*Received* 27 *April* 1973)

J. Fd Technol. (1974) **9**, 31–41.

Evaluation of methods affecting mutton tenderness

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Summary

Much of the myofibrillar toughness which occurs in some muscles in mutton carcasses, when they are hung from the Achilles tendon and conditioned at 0–1°C, can be avoided by conditioning at 7–8°C. Fewer muscles are significantly affected by changes in conditioning temperature when carcasses are hung from the pelvis. A modified version of the 'pelvic hanging method' increased sarcomere lengths in the forequarter muscles and provided a more compact carcass without losing the benefits of pelvic hanging. A drug treatment, which successfully prevented struggling at slaughter, increased toughness in those muscles not restrained from cold-shortening by the skeletal framework.

Although inoculated *Pseudomonas* spp. and *E. coli* organisms normally decreased on exposed areas of the carcasses at both conditioning temperatures, there was an increase in pseudomonas organisms on exposed areas of the brisket conditioned at 7–8°C.

Introduction

Since Locker (1960) first showed that muscular shortening during the rigor process was related to toughness there have been many papers describing the effect of this shortening on toughness. Locker & Hagyard (1963) found that the extent of pre-rigor shortening was dependent upon temperature and that shortening was minimal at about 15–16°C and greatest at 0–1°C. However conditioning carcasses at about 15°C may be hazardous from a bacteriological standpoint since such temperatures are above the minimum growth temperatures of many food poisoning organisms (Michener & Elliott, 1964). It has been reported that holding meat at elevated temperatures, even in gas impermeable bags, is a potentially dangerous practice and temperatures should be reduced below 7°C to completely inhibit the growth of salmonellae and *E. coli* on meat (Shaw & Nicol, 1969). Reducing the humidity reduces bacterial growth (Michener & Elliott, 1964), but increases moisture losses and hence carcass weight loss.

Until recently meat processors had to consider whether they should (a) condition carcasses at temperatures of 15–16°C, in order to reduce myofibrillar toughening, and

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accept the moisture and weight losses concomitant with the low humidities needed to inhibit bacterial growth or (b) inhibit bacterial growth by conditioning and storing at temperatures below 7°C and accept any increased toughness. However, it has been shown for beef (Hostetler *et al.*, 1972; Bouton *et al.*, 1973) and for mutton (Quarrier, Carpenter & Smith, 1972; Bouton & Harris, 1972a) that hanging carcasses, pre-rigor, from the aitch bone or pelvis, rather than from the Achilles tendon, could significantly improve the tenderness of some of the more economically important muscles.

Although the effects of conditioning temperature, hanging method and ageing are known they have not previously been compared on one group of animals. In the present paper the effectiveness of ageing, pre-rigor conditioning and/or hanging treatments has been determined by evaluating the tenderness of muscles from mutton carcasses conditioned at 0–1°C or 7–8°C and hung in different ways. Tenderness was assessed using shear force, sarcomere length, and adhesion measurements. As an additional treatment a drug, etorphine, was used to prevent struggling at slaughter.

Materials and methods

Animals, and their pre- and post-slaughter treatments

Thirty-two Merino X wethers, about 3 years old, with a mean carcass weight of 23·3 (s.e. ±0·4) kg were used. Animals were slaughtered in groups of eight. They were stunned with a captive-bolt pistol and blood samples were collected at exsanguination for plasma glucose determinations, using the method of Hugget & Nixon (1957).

Animals were assigned at random to four basic treatments. The four treatments were three carcass hanging methods (one of which was Achilles tendon hanging, which was regarded as a control) and a pre-slaughter injection of etorphine. Animals treated with etorphine were injected intramuscularly with 0·3 ml of 'Large Animal Immobilon*'. This preparation is a mixture of etorphine and acepromazine (a tranquilizer). Each ml contains 2·45 mg etorphine hydrochloride (0·225% etorphine base), 10 mg of acepromazine maleate BPC and 0–1% chlorocresol, in saline. Injected animals collapsed with 4·4 (s.e. ±0·5) min and were slaughtered 6·8 (s.e. ±0·4) min after injection.

The three carcass shapes obtained using the three different hanging methods are illustrated in Plate 1. The carcasses from the etorphine-treated animals and from the controls were hung from the Achilles tendon. The other two hanging treatments were pelvic hanging and a modification of this method in which both fore and hind legs were tied back. The compactness of carcasses subjected to this modified pelvic hanging method is readily apparent from Plate 1. (The carcass hung originally from the Achilles tendon has been re-hung from the pelvis so that each pelvis is on about

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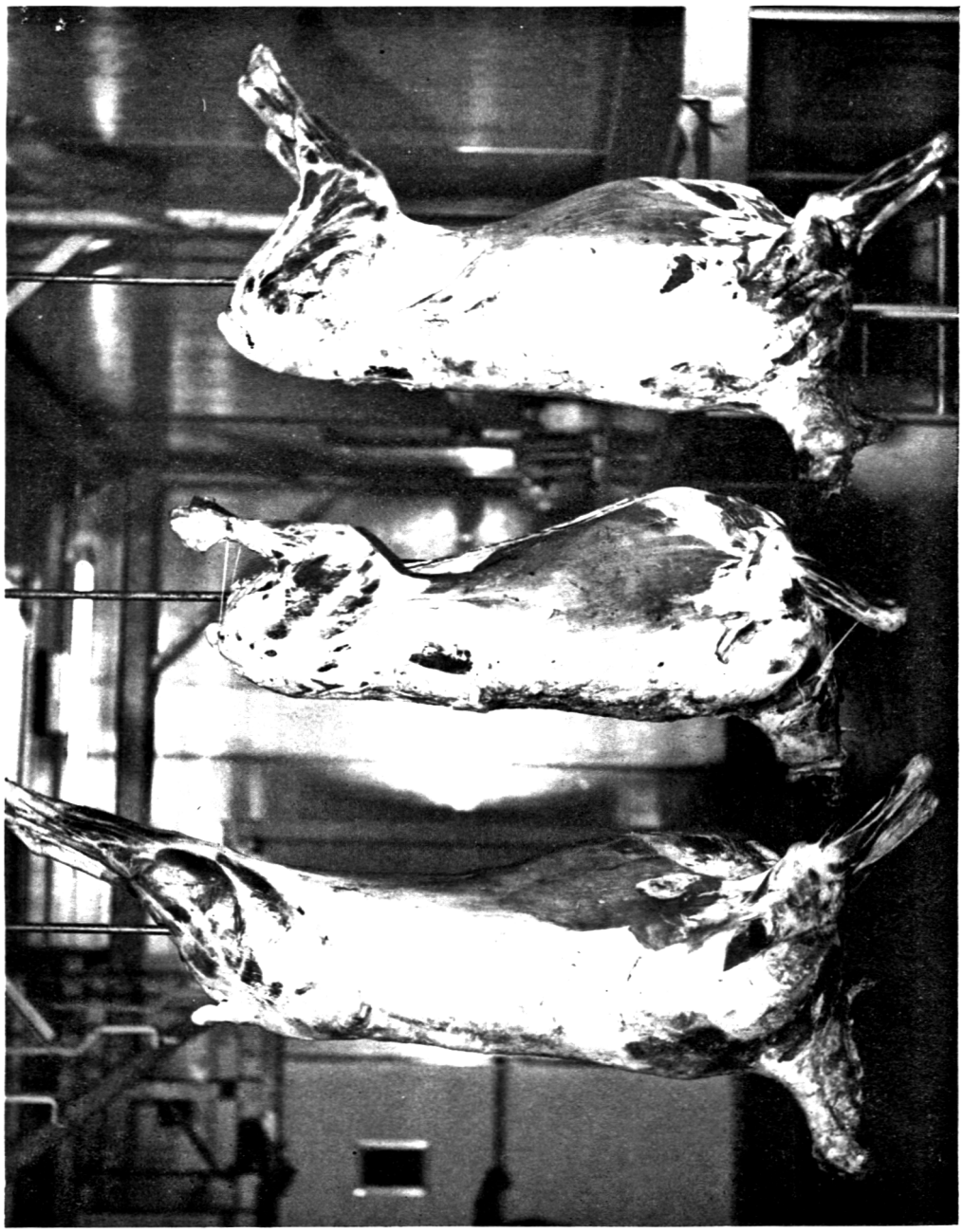

PLATE 1. The different shapes of sheep carcasses produced by the three hanging methods described in the text.

(*Facing p.* 32)

the same level.) Carcasses subjected to the four basic treatments were conditioned at two temperatures, 0–1 or 7–8°C, for 1 day.

Chiller conditions and carcass temperatures

Carcasses were chilled in rooms maintained at 0–1°C or 7–8°C. Thermocouples were used to monitor temperatures at (a) the surface of each carcass, 3 cm laterally from the backbone at the level of the last rib, and (b) at the same site, but at the centre of the longissimus dorsi muscle on the opposite side of the carcass. Air flow rate in the 0–1°C chiller was 120–160 m/min and in the 7° chiller 30–50 m/min. The humidity of the rooms was monitored using a humidity probe (Carseldine, 1972). The drying power of these environments was estimated by determining weight loss from six full gelatin plates, 9 cm in diameter, weighed before and after 24 hr exposure in the rooms (Scott & Vickery, 1939).

Assessment of bacterial growth

A strain of *E. coli* was grown in nutrient broth at 20°C for 48 hr, and a strain of a psychrophilic *Pseudomonas* species, originally isolated from beef stored at 0–2°C was grown in nutrient broth at 0–2°C for 14 days. Equal quantities of broth from these cultures were mixed together and diluted 100-fold in 0·1% peptone solution and swabbed liberally, using cotton wool, over the rump, mid-line and brisket on the freshly dressed sheep carcasses. This gave an initial count on the carcasses of 10^4–10^5/cm^2 for each organism. Half of each inoculated area was covered with thin polyethylene film (0·0015 in thick), to retard moisture loss after initial sampling, and the other half was left exposed. Two pieces each 5 cm^2 in area were cut from each inoculated surface and blended together in 90 ml of 0·1% peptone solution. A sub-sample containing 0·1 ml of the blend, or appropriate ten-fold dilutions of the blend, were spread evenly over the surface of tryptone-glucose-yeast extract agar plates. Six plates were used for each dilution of which two plates were incubated at 0–2°C for 21 days, two at 20°C for 4 days and two at 37°C for 24 hr. The *E. coli* grew at 37°C and 20°C, but not at 0–2°C and the psychrophilic pseudomonas grew at 0–2°C and 20°C, but not at 37°C. It was possible to differentiate *E. coli* and *Pseudomonas* at 20°C by colonial morphology and make separate counts of each. Using the three incubation temperatures, cross checks could be made on the accuracy of the counts obtained for each organism.

After the 24 hr conditioning period during which the carcasses were held at either 0–1°C or 7–8°C another two samples were taken from each inoculated area, one from the exposed portion and a second from the covered area. These samples were cut from the surface and treated in the same way as before. Altogether, twelve carcasses were inoculated and tested, six at each temperature. These carcasses were always tested in pairs, i.e. one carcass was held at each conditioning temperature during each run.

Muscle treatment

Muscles were dissected from both sides of the carcasses after the conditioning period. Muscles from one side were cooked that day whereas muscles from the other side were aged in evacuated gas-impermeable plastic bags for a further 3 weeks at 0–1°C before cooking. The muscles used were semimembranosus (SM), adductor (A), biceps femoris (BF), semitendinosus (ST), vastus lateralis (VL), rectus femoris (RF), gluteus medius (GM), longissimus dorsi (LD), psoas major (PM), triceps brachii (TB), supraspinatus (SS) and infraspinatus (IS). The pH of the LD from all animals was measured at room temperature, before cooking, using a Philips digital pH meter (PW 9408) with a Philips combined electrode (C 64/1).

Sarcomere length measurements

The lengths of the sarcomeres of the twelve muscles (raw) taken from each carcass were measured using a light diffraction method. A He-Ne laser was used as a light source (Rome, 1967). Unfixed fibre bundles, between microscope slides, were placed in the beam of the laser, the diffraction pattern measured and sarcomere lengths calculated. The measurements of four samples from different locations within each muscle were averaged to give the mean sarcomere lengths.

Cooking methods

Muscles were cooked for 90 min in polyethylene bags, tightly clipped around the samples and totally immersed in water baths maintained at $75 \pm \frac{1}{2}$°C. The method has been described previously (Bouton, Harris & Shorthose, 1971). The cooked samples (in their own juices) were cooled in cold running water then stored overnight at 0–1°C before preparing samples for the various objective measurements.

Mechanical measurements

Shear force measurements were carried out on all muscles except the PM and IS muscles. The shear device used is based on the apparatus described by Bratzler (1932). It has been shown (Bouton & Harris, 1972b, c) that these shear force measurements mainly reflect differences in myofibrillar toughness. Connective tissue strength of the SM muscle was assessed by measurements of adhesion between the meat fibres using the method described by Bouton & Harris (1972a, b, c).

Statistical analysis of results

The eight different treatments (three hanging and one drug treatment each at two conditioning temperatures) were allotted to the animals at random and replicated four times. The eight main treatments were split into sub-groups viz. aged and non-aged. The two ageing treatments were applied to the two sides of each carcass. Analysis of variance was used to calculate the appropriate error to be used for comparing treatment effects.

Results

Plasma glucose concentrations at slaughter

Etorphine-injected animals have significantly ($P < 0{\cdot}05$) greater mean plasma glucose concentration, 98 mg/100 ml, than that of the other, non-injected, animals, 82 mg/100 ml. It has been found that sheep injected with physiological saline had plasma glucose concentrations greater, by about this amount, than non-injected controls (Shorthose, unpublished data). The etorphine injection was used solely to prevent kicking at slaughter. The action of etorphine *per se* was not under study so no saline-injected controls were used. It was, thus, not possible to determine if this increase in glucose concentration was due to the stress of the injection or to effects of the injected drugs.

Chiller conditions, rates of carcass cooling and carcass weight loss

Humidity varied within and between 'runs' in the two chillers, but mean humidities in both the 7–8°C chiller and in the 0–1°C chiller were in the range 88–90%. The drying powers (Scott & Vickery, 1939) in the two chillers were 90·5 (s.e. ±6·4) and 22·4 (s.e. ±1·6) mg $H_2O/cm^2/hr$ for the 7–8°C and 0–1°C chillers respectively.

Carcasses conditioned at 7–8°C lost 2·44% of hot carcass weight in 24 hr while those conditioned at 0–1°C lost 1·97% in the same time. In carcasses chilled at 0–1°C the centre temperature of the LD (at the last rib) reached 16°C in 157 (s.e. ±6) min, 8°C in 250 (s.e. ±9) min and 1°C in 540 (s.e. ±15) min. Similarly the LD centre temperature in carcasses chilled at 7–8°C reached 16°C in 225 (s.e. ±8) min and 8°C in 467 (s.e. ±20) min. The times quoted are times from slaughter and not from the time the carcasses entered the chillers. Measurements of pH taken on the raw meat just prior to cooking show that muscle pH values were in the range 5·4–5·8.

Sarcomere length measurements

Mean sarcomere lengths for the twelve muscles conditioned at 0–1°C are listed in Table 1. Sarcomere lengths for those muscles from the carcasses conditioned at 7–8°C, for which the shear force values are most affected by chilling temperature, are listed in parentheses. The muscles have been considered, in general, as being in three main groups viz. A, B and C. The Group A muscles (SM, BF, GM and LD), from carcasses hung from the Achilles tendon and conditioned at 0–1°C have sarcomere lengths of less than 1·8 μm. Group B muscles (VL, A) contracted less and have sarcomere lengths in the range 1·8–2·0 μm. Group C muscles (ST, RF, TB and SS) generally have sarcomere lengths appreciably greater than 2·0 μm. The IS and PM muscles are not included in these basic groups because no shear force measurements were carried out on them.

Pelvic suspension significantly increases sarcomere lengths of BF, GM, SM, VL, A and ST muscles, decreases sarcomere lengths in the PM, SS and IS muscles and did not significantly affect the RF and TB muscles. The LD muscles from the 'pelvic

TABLE 1. Mean sarcomere length values (μm) for mutton muscles from carcasses subjected to various hanging and drug treatments and conditioned at 0–1°C

Muscle		Hanging treatment*			
		Achilles tendon	Achilles tendon + drug	Pelvic	Modified pelvic
BF	Group A	1·75(1·88)	1·58(1·87)	2·75	2·93
GM	Group A	1·76(1·75)	1·65(1·80)	2·40	2·40
LD	Group A	1·70(1·81)	1·72(1·85)	1·79(2·02)	1·91(1·96)
SM	Group A	1·77(1·89)	1·67(1·88)	2·67	2·65
VL	Group B	1·89(2·00)	2·00(1·98)	2·62	3·21
A	Group B	1·87(1·99)	1·95(1·88)	2·61	2·96
RF	Group C	2·20	2·22	2·37	2·99
ST	Group C	2·40	2·39	2·79	2·00
TB	Group C	2·04	2·04	2·03	3·43
SS	Group C	2·10	2·23	1·98	2·69
IS		2·07	2·07	1·88	2·66
PM		3·24	3·09	2·57	2·33

LSD (5%) Hanging treatments 0·21.
LSD (5%) Temperature treatment 0·11.
LSD (5%) For effect of drug treatment (Achilles tendon only) 0·11.
* Figures in parentheses are values obtained for muscles from carcasses conditioned at 7–8°C.

hung' carcasses show significantly ($P < 0{\cdot}05$) increased sarcomere lengths compared with carcasses hung by the Achilles tendon. The modification of the pelvic suspension method produces further significant increases in the sarcomere lengths of the VL, A and RF muscles as well as increasing sarcomere lengths in the forequarter muscles (TB, SS and IS). Sarcomere lengths of the ST and PM muscles are, however, significantly reduced compared to all the other treatments.

Most muscles from the carcasses conditioned at 7–8°C have slightly, but significantly, longer sarcomeres than the same muscles from carcasses conditioned at 0–1°C. The mean sarcomere length of Group A muscles from carcasses of etorphine-treated animals (1·66 μm) are significantly ($P < 0{\cdot}05$) shorter than those from carcasses of the untreated animals (1·75 μm) which were also hung from the Achilles tendon. Taylor, Chrystall & Rhodes (1972) used pre-slaughter injections of $MgSO_4$ to avoid the struggling normally occurring at slaughter and found that sarcomere lengths in cooked lamb LD muscles were significantly shorter in treated animals than in non-injected controls.

Shear force measurements

Mean shear force values obtained for the ten muscles used from each animal are shown in Table 2. The muscles are considered in the three groups mentioned earlier viz.

Group A, Group B and Group C, which have been shown from sarcomere length measurements (Table 1) to represent muscles with respectively, large, small and no pre-rigor contraction effects if still attached to the carcass during conditioning.

The shear force values for those Group A and B muscles conditioned at 0–1°C and taken from carcasses hung from the Achilles tendon are significantly ($P < 0{\cdot}001$) greater than for those taken from carcasses hung from the pelvis. Conditioning at 7–8°C reduces the difference in shear force values between the muscles from carcasses hung from Achilles tendon or from the pelvis to non-significance although mean shear force values still tend to be greater for muscles from the carcasses hung by the Achilles tendons.

TABLE 2. Mean shear force values (kg) obtained for sheep muscles which have been subjected to various pre- and post-slaughter treatments

Muscle group and treatment	Conditioning treatment in °C	Hanging treatment			
		Achilles tendon	Achilles tendon + drug	Pelvic	Modified pelvic
Group A (SM, BF, GM, LD)					
Non-aged	0	8·90	11·82	5·10	4·90
	7	5·54	5·33	4·28	4·24
Aged	0	5·21	6·30	3·56	3·96
	7	3·50	3·29	3·37	3·24
Group B (VL, A)					
Non-aged	0	6·95	8·03	5·02	4·92
	7	5·06	5·03	4·58	4·70
Aged	0	3·20	3·78	3·50	4·06
	7	3·46	3·05	3·66	3·48
Group C (ST, RF, TB, SS)					
Non-aged	0	5·85	5·80	5·64	5·54
	7	5·23	5·10	5·17	5·09
Aged	0	3·64	3·59	3·12	3·59
	7	3·30	3·41	3·25	3·38

LSD (5%)	Group A	Group B	Group C
Hanging treatment	2·41	1·23	0·88
Conditioning treatment	1·71	0·87	0·62
Ageing treatment	0·80	0·49	0·28

No significant effects on the shear force values of Group C muscles due to carcass hanging method or to conditioning temperature are found. Ageing significantly ($P < 0{\cdot}001$) reduces the shear force values in all three muscle groups. The large differences, attributable to carcass hanging method, found in shear force values between Group A and B muscles conditioned at 0–1°C, are much smaller after ageing has reduced myofibrillar toughness.

The Group A muscles from animals treated with etorphine have significantly greater shear force values than those obtained for the muscles from the untreated animals also hung from the Achilles tendon and conditioned at 0–1°C. No significant differences have been found in the cooling rates of these carcasses, but the sarcomere lengths (shown in Table 1) are shorter in these muscles from the drug-treated animals. The reasons for this effect of the drug treatment on shear force and sarcomere length values could possibly be associated with the effects on the rate of post mortem glycolysis of pre-slaughter stress, the drug itself or both of these factors. Elucidation is considered to be beyond the scope of this experiment. Taylor *et al.* (1972) also found increased toughness in LD muscles from $MgSO_4$ injected lambs compared with non-injected controls. They considered that the slower rate of post mortem glycolysis produced by the $MgSO_4$ allowed maximal cold-shortening.

Adhesion measurements

Adhesion measurements of connective tissue strength of the SM gave mean values of 0·66, 0·59, 0·37 and 0·37 kg/cm^2 (LSD, $P = 0{\cdot}05$, $= 0{\cdot}12$) for Achilles tendon, Achilles tendon + drug, pelvic and modified pelvic suspension methods respectively. Both Achilles tendon suspension methods produce significantly greater adhesion values than either of the pelvic suspension methods. This result confirms other results (Bouton *et al.*, 1973; Bouton & Harris, 1972a) which showed that stretching significantly reduced connective tissue strength.

Bacteriological changes

The changes which occur in the numbers of inoculated *E. coli* and psychrophilic *Pseudomonas* during 24 hr periods at the two conditioning temperatures, are summarized in Table 3. The results obtained in each of six paired tests are similar and are averaged. Significant ($P < 0{\cdot}001$) decreases in the numbers of *E. coli* usually occur on exposed areas of the carcasses at both conditioning temperatures. On covered areas, however, there is little change at 0–1°C but some significant increases occur at the higher conditioning temperature.

With the exception of the brisket area, the numbers of *Pseudomonas* organisms normally decrease on the exposed areas of the carcasses at both conditioning temperatures. On the brisket, there is almost no change in the numbers of these spoilage organisms at the lower temperature but a substantial increase occurs at 7–8°C. The covered areas

TABLE 3. Average $\log_{10}$ change* in inoculated *E.coli* and *Pseudomonas* during 24 hr conditioning at two temperatures

Organism	Conditioning temperature of carcass (°C)	Area of carcass sampled	Average $\log_{10}$ change/cm²	
			Exposed area	Covered area
E. coli	0–1°	Hind leg	−0·89	+0·19
		Mid line	−0·60	−0·16
		Brisket	−0·25	−0·20
	7–8°	Hind leg	−1·10	+0·55
		Mid line	−0·82	+0·51
		Brisket	−0·26	+0·20
Pseudomonas	0–1°	Hind leg	−0·78	+0·85
		Mid line	−0·48	+0·75
		Brisket	−0·08	+0·27
	7–8°	Hind leg	−0·55	+1·71
		Mid line	−0·56	+1·79
		Brisket	+0·91	+1·56

* Mean of six tests.

show an increase in *Pseudomonas* organisms at both conditioning temperatures with quite large increases occurring at the higher temperature.

Compared with the other sampled areas, the brisket tends to give anomalous results. There are smaller decreases on exposed areas and, strangely, smaller increases on covered areas. This same pattern is discernible with both types of inoculated organisms but is more pronounced with *Pseudomonas*. The phenomenon is probably associated with faster heat loss and hence lower moisture loss from the thin brisket area (Scott & Vickery, 1939). Consequently, sufficient water may be retained in even the exposed surface tissues at this site to enable some growth of psychrophilic bacteria to take place during conditioning at 7–8°C and to prevent death by desiccation at 1–0°C.

Discussions and conclusions

The primary objective of the experiments reported in this paper is to compare the relative merits of conditioning at 0–1 or 7–8°C, different carcass suspension methods, and ageing from the aspects of both tenderness and bacterial growth. It is shown that under certain chiller conditions, such as those occurring when surfaces remain moist due to contact between carcasses, there could be some growth of undesirable food poisoning and spoilage organisms within 24 hr, even on carcasses chilled at 0–1°C. Of the

hanging, conditioning and ageing treatments considered, four yield comparable tenderness results for the Group A and B muscles (SM, BF, GM, LD, VL and A). These treatments are (a) pelvic hanging and conditioning at 0–1°C for 1 day, (b) pelvic hanging and conditioning at 7–8°C for 1 day, (c) Achilles tendon hanging and conditioning at 7–8°C and (d) Achilles tendon hanging, conditioning at 0–1°C followed by ageing at 0–1°C for 3 weeks. Of these treatments both the pelvic hanging methods, at 0–1°C, appear to offer the best combination of tenderness and bacteriological safety coupled with minimum delay before marketing and/or eating.

The drug treatment prevents struggling at slaughter. Group A muscles from the drug-treated animals are more susceptible to cold shortening and yield the shortest sarcomeres and highest shear force values obtained for any of the treatments involving conditioning at 0–1°C. When carcasses are conditioned at 7–8°C, where cold shortening is less, shear force values of the Group A muscles from the drug treated animals are not significantly different from the values obtained for the other treatments. The results from the drug treatment are included as an example of a pre-slaughter treatment which increases toughness (for reasons which are not altogether clear) although only in those muscles which are not prevented from cold shortening by the skeletal framework.

Group C muscles from Achilles tendon hung carcasses (or Group A, B and C muscles from pelvic hung carcasses) are clearly least affected by the pre-slaughter treatment and by post-slaughter conditioning temperature. These muscles thus appear to be the best choice for experiments where processing variables could obscure any treatment effects.

Acknowledgments

This work was supported in part by funds from the Australian Meat Research Committee.

We acknowledge Mr D. W. Roberts, at whose suggestion the modification to the 'pelvic hanging' method was tried and the expert technical assistance of Miss Robin Beard and Mr Bob Dickinson.

We wish to thank Reckitt and Colman, Pharmaceutical Division, for their generous gift of the 'large Animal Immobilon' used in this work.

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Release of lysosomal enzymes during post mortem conditioning and their relationship to tenderness

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Summary

Samples of the M. longissimus dorsi were taken from beef carcasses 1 hr, 24 hr, 5 days, 7 days and 14 days post mortem. Tenderness (shear force), the amount of protein and hydroxyproline in filtered muscle homogenates, the activity of β-glucuronidase in muscle fractions and the electron histochemical determination of acid phosphatase were measured at these post mortem times.

Shear force was a maximum at 24 hr and decreased thereafter to a value less than that of the 1 hr samples by 14 days. The amount of protein and hydroxyproline in filtered muscle homogenates was a minimum at 24 hr and increased to a maximum at 14 days. The specific activity of free β-glucuronidase (a lysosomal enzyme) increased with post mortem ageing, while the specific activity of bound (sedimentable) β-glucuronidase decreased with post mortem ageing, suggesting that this enzyme is released during the conditioning period. Electron histochemical determination of acid phosphatase activity showed the activity to be localized in specific areas around the I-band at early post mortem times: the activity was more dispersed throughout the cell at later post mortem times.

Introduction

Lysosomes contain several acid hydrolases within the lysosomal membrane (Hirsch & Cohn, 1964; Tappel, 1966). Under certain conditions such as low pH, physical disruption and treatment with detergents the lysosomal membrane is rendered labile. This releases the lysosomal enzymes into the surrounding milieu (Weissman, 1964). Lysosomal enzymes have been identified in many tissues (Strauss, 1967); but some authors have attributed lysosomal enzymes found in muscle tissue to the presence of macrophages and other reticulo-endothelial cells (Tappel, 1966; Kohn, 1969). Recent evidence (Canonico & Bird, 1970; Bird, 1971) however, has indicated that lysosomes

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are present within muscle cells *per se* as well as within reticulo-endothelial cells found in muscle tissue. The integrity of the lysosomal membrane and the activity of lysosomal enzymes in post mortem muscle is of interest, since the acid hydrolases present in lysosomes could be responsible, at least partly, for the post mortem breakdown of proteins and other components in muscle, which is concomitant with tenderization of the meat during conditioning.

Materials and methods

Sampling procedures

Two Holstein steers (12–18 months old) provided the material. Following slaughter according to normal procedures a sample 5 cm in length was removed from M. longissimus dorsi (left hand side), from the region of the thirteenth rib, 1 hr after death. The carcasses were then chilled for 24 hr at 2°C, when a second similar sample was removed from the same muscle in the area 10 cm posterior to the first sampling location. This was done to ensure that fibres which had been cut when the 1 hr sample was taken, would not be included in the 24 hr sample. Subsequent samples were taken immediately posterior to the 24 hr sample after holding at 2°C for 5, 7 and 14 days post mortem. After each sample was taken from the carcass it was trimmed of fat and connective tissue and the exposed surface was removed to reduce microbial contamination. After trimming, each sample was immediately prepared for analysis as outlined in the following sections.

Tenderness measurement

The medial halves of the 5 cm long L. dorsi muscle samples were placed in Cryovac bags and cooked in boiling water for 45 min. Samples were allowed to cool to room temperature and strips of 1 cm^2 cross-section were cut, parallel with the fibres, from the cooled muscles. A shear was made across the muscle fibres to evaluate tenderness. The force required to shear the muscle strips was measured using a Wolodkewitsch Universal testing instrument and utilizing the small shear jaws and the 15 kg resistance spring. Six to ten shear values were taken for each sample.

Homogenization and fractionation of muscle

Centrifugal fractionation was accomplished by a modification of the methods of Canonico & Bird (1969, 1970). Portions of each sample were minced and homogenized for 50 sec at full speed in an M.S.E. homogenizer. The homogenizing solution contained 0·25 M sucrose and 0·02 M KCl. The ratio of muscle to homogenizing solution was 1 : 5 w/v. The homogenate was then adjusted to pH 7·3 with 0·1 N KOH and filtered through two layers of cheese cloth. The residue left after filtration was prepared for electron microscopy and the filtrate was rehomogenized in a motor-driven Uni-Flo teflon pestle homogenizer at an air speed of 800 rev/min.

The final homogenate was centrifuged at 700 ***g*** for 10 min in an M.S.E. Superspeed-70 to obtain a nuclear fraction (N). The supernatant from the nuclear fraction was then centrifuged at 22,000 ***g*** for 10 min to obtain a mitochondrial fraction (M). The mitochondrial supernatant was then centrifuged at 105,000 ***g*** for 120 min to obtain a microsomal fraction (R) and an unsedimentable fraction (U) (the final supernatant). The pellets obtained after all centrifugations were resuspended in the homogenization solution and the pH was readjusted to pH 7·3 with 0·1 N KOH. Analysis for enzyme activity was made for all fractions immediately after the final centrifugation. The protein concentration of each fraction was measured by the method of Lowry *et al.* (1951) using bovine serum albumin as a standard. The hydroxyproline concentration of the filtered homogenate was measured according to the method described by Goll, Bray & Hoekstra (1963) in order to determine the amount of collagen fragments, if any, that had passed through the cheese cloth after homogenization.

The β-glucuronidase activity of the centrifugal fractions was measured by the methods described by Canonico & Bird (1969, 1970). A 0·2 ml aliquot of each fraction was added to 0·7 ml of 0·16 M acetate buffer (pH 5·0) and 0·1 ml of 0·01 M phenolpthaleinomono-β-glucuronic acid. The assay mixture was then incubated for 10 hr at 37°C. After incubation, assays were terminated by adding 3 ml of a glycine buffer (pH 10·7) which contained 0·133 M glycine, 0·067 NaCl and 0·083 M Na_2CO_3. The mixture was centrifuged at 3800 ***g*** for 10 min and the absorbance of the supernatant was read at 540 nm against a tissue fraction blank.

Electron histochemical localization of acid phosphatase

Portions of muscle samples were cut into strips 3 mm square in cross-section and about 4 cm long with muscle fibres on the long axis of the sample. The ends of these muscle strips were immobilized to prevent contraction during fixing. Muscle strips were fixed for 4 hr in a 2·5% glutaraldehyde solution buffered with 0·014 M NaH_2PO_4 and 0·082 M Na_2HPO_4. The fixing solution had a pH of 7·4 and contained 0·086 M NaCl. The glutaraldehyde fixed tissues were rinsed in 7·5% sucrose, frozen and cut at 50 μm on a freezing microtome. The sections were collected in sucrose solution and then incubated in acid phosphatase media for 20 min at 37°C according to the method described by Barka (1967). After incubation, sections were rinsed in distilled water, in 2% acetic acid and in water again before postfixing in osmium tetroxide for 1 hr as described by Sjöstrand (1967). Fixation and dehydration were accomplished at room temperature. Samples were dehydrated in ethanol, washed in two changes of propylene oxide, embedded in Epon 812 (Luft, 1961) and sectioned on a Reichert OmU2 ultramicrotome. Sections were mounted on uncoated copper grids and stained in uranyl acetate (Sjöstrand, 1967). Electron micrographs were obtained with a Japan Electron Optics Laboratory Co. Ltd. JEM-6C electron microscope operated at an accelerating voltage of 80 kV and recorded on Ilford N-50 photographic plates.

Results and discussion

A plot of shear values (tenderness) against time post mortem is presented in Fig. 1. The nature of the tenderness changes found in this study agree with those generally reported in the literature. It is interesting to note, however, that the tenderness after 7 days of conditioning is almost equal to that pre-rigor (1 hr post mortem); and that after 14 days of conditioning, the muscles were as tender as before rigor. Thus, in this study, the tenderizing effects of conditioning eliminated the toughness which had

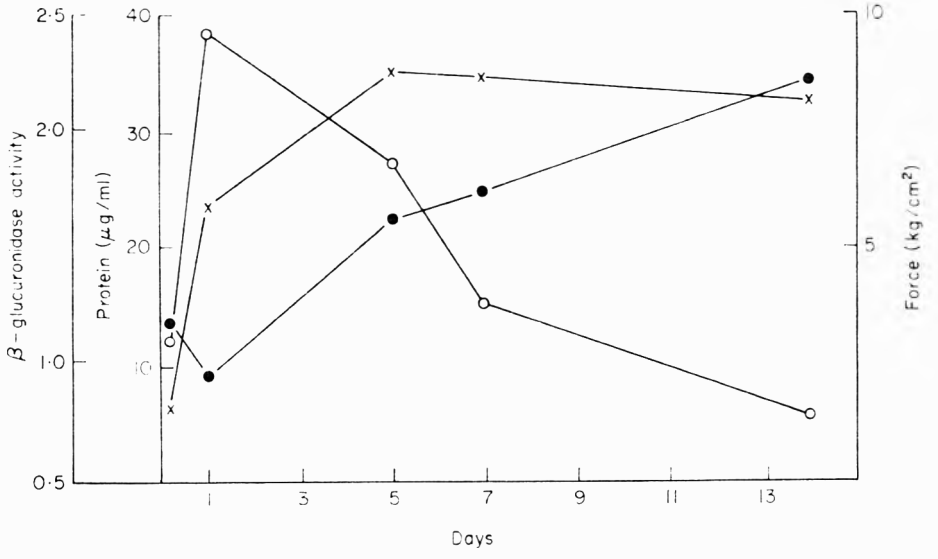

FIG. 1. Cooked muscle (bovine L. dorsi) shear force (○), the amount of protein present in filtrates from muscle homogenates (●) and β-glucuronidase activity in the latter (×) against days post mortem. Shear force is given as maximum reading (kg/cm²) obtained with the Wolodkewitsch instrument, protein as μg/ml of homogenate and enzyme activity in arbitrary units. (Plot represents mean of at least six determinations in each case.)

characteristically developed in the muscle during rigor mortis. The toughness associated with rigor is generally considered to be due to the degree of interdigitation (Locker, 1960) and cross-bonding of actin and myosin filaments, but at the present time there is no adequate explanation for the long recognized tenderizing which occurs during the subsequent conditioning period (Lehmann, 1907).

Davey & Gilbert (1968) and Penny (1970) have shown that myofibrillar proteins are more soluble in high ionic strength (0·6 M KCl) solutions after conditioning; but Penny (1970) found only a small increase in the solubility of the two proteins, actin and α-actinin, at low ionic strength (5 mM tris). A large increase in soluble protein, in the present study, would not be expected because of the low ionic strength (20 mM KCl)

used. Thus, the increase in protein found in these fractions probably reflects a lower resistance of the muscle to homogenization. The increase in susceptibility to homogenization which was observed during the conditioning period could be due to loss of integrity of the connective tissue matrix or of the muscle cells, or both.

By comparing the graphs in Fig. 1, it can be seen that the amount of protein present in the homogenate after filtration is in close agreement with the tenderness of the muscle. Thus, although more work is needed in this area, resistance to homogenization as shown in this study could be used as a measure of tenderness. A measure of tenderness such as this would be useful because it could be applied to raw samples of meat.

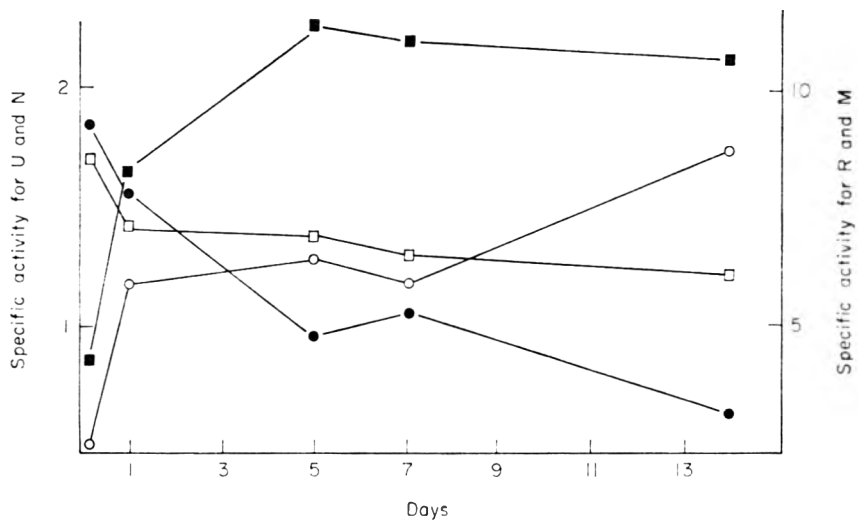

FIG. 2. β-glucuronidase activity, against time post mortem, for centrifugal fractions of bovine L. dorsi. Enzyme activity given in arbitrary units for the fractions studied. ■, Unsedimented (U); ●, mitochondrial (M); ○, microsomal (R); □, nuclear (N).

A plot of the β-glucuronidase specific activity in the nuclear (N), mitochondrial (M), microsomal (R) and unsedimentable fractions (U) against time post mortem is presented in Fig. 2. There was an increase in the specific activity of the R and U fractions and a decrease in the specific activity of the N and M fractions over the conditioning period. This could be explained if larger (or more intact) particles contained the enzyme early post mortem and these were disrupted during conditioning. This would allow the enzyme associated with these particles to be free in the medium (U fraction) if the enzyme was not bound to a membrane. On the other hand if the enzyme remained bound to the ruptured membrane its activity would become associated with the cell fraction having a slower rate of sedimentation (R). To determine the integrity of the membranes, Triton X-100, which is known to rupture them (Canonico & Bird, 1969), was added to each fraction, to a concentration of 0·02%, before incubation. Triton X-100 increased the

activity that was present in the N and M fractions by 35% and 25% respectively, while the activity that was present in the R fractions was virtually unaffected and the activity that was present in the U fraction was decreased by about 10%. Canonico & Bird (1969) also found a decrease in free β-glucuronidase activity with Triton X-100. Thus, the results of the present study show that the lysosomal β-glucuronidase of beef muscle is, at least partially, bounded by some sort of membrane and that post mortem conditioning of beef muscle allows this enzyme to become free. A plot of free β-glucuronidase activity (U fractions) against time post mortem shows that increased tenderness was accompanied by an increase in free β-glucuronidase activity (Fig. 1).

Although the increase in tenderness over the conditioning period could be due to the action of cathepsins, and other enzymes that are usually released concomitantly (Ono, 1970; 1971; Valin, 1970; Canonico & Bird, 1970), β-glucuronidase itself could affect the tenderness of muscle tissue by hydrolysing glucose-galactose moities that are present in collagen (Blumenfield *et al.*, 1963; Butler & Cunningham, 1966) and the mucopolysaccharide protein complexes of the connective tissue matrix (Partridge, 1948; Lowther, Toole & Herrington, 1970). Roden (1965) has shown that the connection of protein to polysaccharide, in the connective tissue matrix, partially consists of a β-glucuronidic disaccharide linkage and that this linkage is cleaved by β-glucuronidase. Also McIntosh (1966, 1967) has characterized a mucopolysaccharide protein complex of bovine muscle and has shown an increase in the solubilization of this complex after conditioning.

Canonico & Bird (1970) have demonstrated that there are two distinct species of lysosomes in rat muscle homogenates. One species originates from muscle cells themselves and the other species from macrophages and other connective tissue cells. Thus, if lysosomes of connective tissue cells were ruptured and the enzymes released into the connective tissue matrix during conditioning, they would presumably have an affect on the integrity of this matrix. In order to determine if the integrity of the connective tissue was affected during conditioning, the amount of collagen which could pass through cheese cloth after homogenization was measured by determining the amount of hydroxyproline in the filtrate. The amount of hydroxyproline increased from 4·44 μg/ml at 1 hr post mortem to 10·63 μg/ml at 14 days post mortem, indicating that collagen fragments were probably smaller at 14 days post mortem.

An electron micrograph showing the localization of acid phosphatase activity of muscle tissue at 1 hr post mortem is shown in Plate 1. As can be seen from the micrograph, the acid phosphatase activity at 1 hr post mortem (black granules marked by arrows) is primarily localized in the areas around the I band. Although no definite membrane can be seen surrounding the areas of enzyme activity, this activity seems to be rather specific and is not scattered throughout the sarcoplasm. However, some muscle fibres appeared to contain much less activity than others. Previous reports of the amount and distribution of acid phosphatase in bovine muscle were not found in the literature. However, Dutson, Pearson & Fennell (1971) and Dutson *et al.* (1971) have shown by histochemical methods using the light microscope that different amounts of this enzyme

PLATE 1. Electron micrograph showing acid phosphatase activity in skeletal muscle at 1 hr post mortem. Black deposits (indicated by arrows) correspond with areas of enzyme activity. 'Z' indicates Z lines. × 22,500.

(*Facing p.* 48)

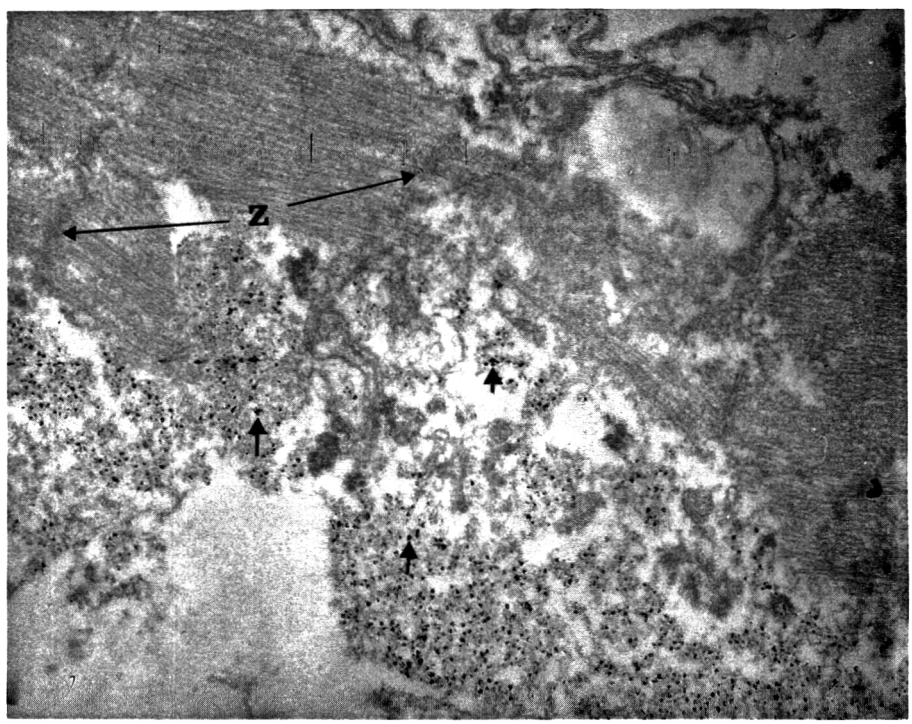

PLATE 2. Electron micrograph showing acid phosphatase activity in skeletal muscle at 5 days post mortem. Black deposits (indicated by arrows) correspond with enzyme activity. 'Z' indicates Z lines. ×22,500.

PLATE 3. Electron micrograph of muscle tissue at 1 hr post mortem showing mitochondria (M) and sarcoplasmic reticulum (SR). ×25,500.

PLATE 4. Electron micrograph of muscle tissue at 5 days post mortem showing mitochondria (M) and remnants of sarcoplasmic reticulum (SR). ×25,500.

are present in the different fibre types in porcine muscle. Similar differences in beef muscle could account for the present findings.

An electron micrograph of the location of muscle acid phosphatase at 5 days post mortem is shown in Plate 2. In this plate the sites of acid phosphatase activity coincide with areas of protein; however, there appears to be no specific localization of activity similar to that found in 1 hr samples. Thus, by 5 days post mortem, the pattern of acid phosphatase activity appears to be changed from localized areas around the I band to more nonspecific locations throughout the sarcoplasm. Although only samples at 1 hr and 5 days post mortem were analysed, it appears that post mortem ageing disrupts localized areas of acid phosphatase activity, causing the enzyme to spread widely in the sarcoplasm. The appearance of mitochondria and sarcoplasmic reticulum at 5 days post mortem was also disrupted when compared to 1 hr samples (Plates 3 and 4), indicating a general disorganization of intracellular membranes during conditioning. These results agree with those showing an increase in free β-glucuronidase activity, and a loss of integrity of the sedimentable particles containing the β-glucuronidase activity, during conditioning.

Acknowledgments

This work, including a postdoctoral fellowship awarded to one of us (T.R.D.) was supported by a grant from the J. Sainsbury Centenary Fund, which is gratefully acknowledged. The authors wish to thank Mr S. Grudgings for his technical assistance.

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(*Received* 2 *June* 1973)

J. Fd Technol. (1974) **9**, 51–58.

The mechanism of cold-induced shortening in beef muscle

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Summary

When pre-rigor beef muscle is chilled, it undergoes a slow contracture from the coupling of mechanical and chemical events within the myofibrils. Cold-shortening occurs through a thirty- to forty-fold increase in the concentration of ionic calcium in the myofibrillar region as the temperature of pre-rigor muscle is reduced from 15°C to 0°C.

Introduction

Although muscular contraction is normally triggered by a nervous impulse, artificial stimuli such as those induced by drugs, various electrolytes, increased pressure and high temperature also produce contraction (Hoyle, 1957). Chilling is one such stimulus and produces a contracture in smooth muscles (Smith, 1952; Guttman & Ross, 1958), and in the striated muscles of both the bovine and ovine species (Locker & Hagyard, 1963; Marsh & Leet, 1966). Cold shortenings commence with variable delays of up to 3 hr post mortem and develop until rigor onset at 16–24 hr, by which time shortenings of up to 60% of the initial muscle length may occur. As cold shortening precedes that of rigor by many hours, the two physiological events are quite different and unassociated phenomena.

Cold shortening in the muscles from major meat-producing animals is a matter of considerable commercial significance, since shortenings in the muscle fibre are associated with a very pronounced toughening of the cooked meat (Marsh & Leet, 1966).

In studies of the biochemical basis of cold shortening, Cassens & Newbold (1967) found that rigor mortis onset is hastened with the fall of pre-rigor muscle temperature from 5°C to 1°C. As expected, glycolytic rates and adenosinetriphosphate hydrolysis were also found to be speeded up (Newbold & Scopes, 1967). These studies show that cold shortening is coupled to adenosinetriphosphate hydrolysis, the most likely explanation being that this is potentiated by Ca^{2+}.

The present study shows that cold shortening can be explained adequately in terms of

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such a build-up of Ca^{2+} in the myofibrils, through discharge from the sarcoplasmic reticulum.

Methods

Processing of muscles

Sternomandibularis muscles from Angus steers 1½–4 years old were trimmed for experimental treatment within 60 min of slaughter, and were cut into longitudinal strips at least 15 cm × 5 cm × 5 cm. Some strips were placed horizontally and unrestrained at their excised length. Others were stretched in a vertical position with loads of 40 g/cm². The two sets—unstretched and loaded—were stored in constant-temperature rooms in small, air-tight chambers containing a moist nitrogen atmosphere. Half of each set was stored at 15°C to enter rigor mortis, and the others at 0°C to cold shorten before undergoing the changes of rigor. Moist nitrogen of the required temperature was passed through the chambers at approximately ten replacement volumes an hour. Temperature equilibrations to 15°C and 0°C, measured by thermocouple probes inserted into test strips, were achieved in approximately 30 min and 45 min respectively.

Small portions of muscle were cut from the unrestrained and loaded strips at time intervals over a 24 hr period. Special care was taken to prevent unwanted stretching or shortening of the unsampled material. For this reason, complete sections (approximately 1·5 cm long) were cut at each sampling from the top end of the loaded muscle strips, after transfer of the supporting clip to a lower level. By this procedure, the unsampled portions of the strips remained constantly loaded throughout the experiment.

Tissue extracts were prepared by homogenizing the weighed muscle samples (1–1·5 g) with perchloric acid (10 ml, 0·5 M) to denature protein which was removed by centrifugation. The clear supernatants (5 ml) were neutralized to pH 4·5–6·0 with NaOH and made to 10 ml with water. The method of Lohmann, as modified by Bendall (1951), was used to determine the concentrations of acid-labile phosphate in these neutralized solutions. Since acid-labile phosphate is largely derived from ATP in muscle tissue (Bendall & Davey, 1957), it is assumed to be equivalent to this nucleotide for the purpose of the present study.

Fibre-piece preparation

Pre-rigor muscle (not more than 60 min post mortem) was coarsely minced and immediately homogenized for 15 sec in a Waring Blender with 1 volume of a chilled solution (8–10°C) of KCl (0·14 M)—imidazole-hydrochloride buffer (0·03 M, pH 7·0). Microscopical examination showed that the preparations contained a few myofibrils, many fibre pieces with apparently intact sarcolemma and occasional fibre bundles. Samples (20 g) of the pre-rigor fibre pieces were transferred within 5 min of homogenization to thin-walled test tubes, and in experiments testing calcium effects 5 ml of Ca-EGTA buffer (25 mM, pH 7·0) was stirred into the slurries. All the slurries were then

incubated for 24 hr, half in an ice bath at 0°C, the remainder in a water bath at 15°C. Temperature equilibration, measured by thermocouple probes in test samples was achieved in 15–20 min.

Calcium buffers

Ca-EGTA buffers giving the required Ca^{2+} were prepared according to Weber & Herz (1963), by making use of the relationship that at pH 7·0

$$[Ca^{2+}]\,[EGTA^{4-}]\,[EGTA^{3-}]\,[EGTA^{2-}]/[Ca\text{-}EGTA] = 1{\cdot}9\times10^{-7}$$

(A. Weber, personal communication).

Settled volume

The fibre-piece preparations were stored for 24 hr at either 0°C (0°C fibre pieces) or 15°C (15°C fibre pieces) to enter rigor mortis. They were then brought to room temperature (22–24°C) for determining settling characteristics of the material. This was achieved by suspending the fibre pieces (20 g) in a graduated cylinder (internal diameter 2·8 cm) to a volume of 100 ml with imidazole buffer (0·02 M, pH 7·0) containing KCl (0·15 M) and settling them under gravity. The degree of shortening in the fibre pieces determined settling rate. In an unshortened state they were flexible, tangling with their neighbours to form a self-supporting matrix that was slow to settle. In a highly shortened state they were straight and rigid, did not form an entwined matrix and therefore settled more rapidly. The fibre pieces settled as a definite layer with an easily measured time course.

Microscopical examination

Micrographs were obtained of unshortened and shortened fibre pieces from preparations that had been stored for 24 hr at 15°C and 0°C respectively. Small samples of the preparations suitably diluted with KCl (0·16 M) were held in a droplet on a glass slide and viewed under a planachromat objective.

Isolated myofibrils in the preparations were viewed by phase-contrast microscopy, at a magnification of 2000 times.

Results

As a guide to the sources of energy for cold shortening, a comparison has been made of post-mortem changes in ATP in muscle strips, brought either to internal temperatures of 15°C or 0°C. Since very little external work is done by a muscle free to shorten horizontally, the chemical changes are too small to identify accurately (cf. Davies, 1963). For this reason, they have been estimated in muscle strips doing work by cold shortening against an external load.

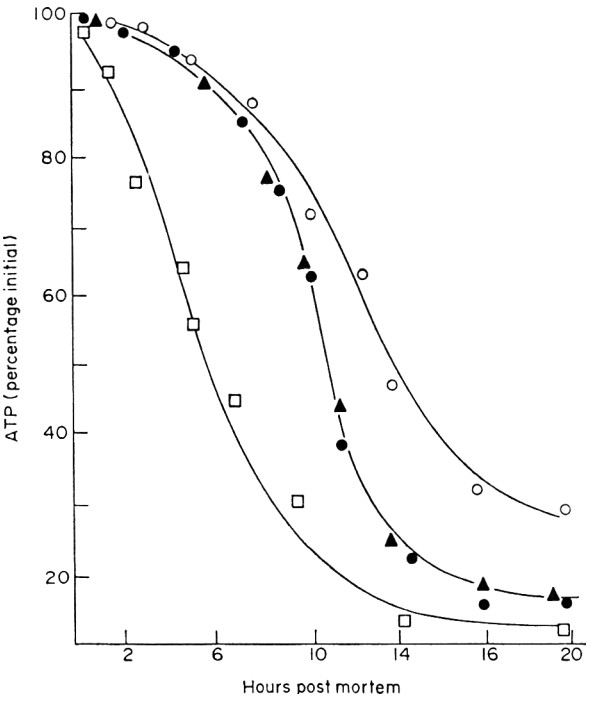

FIG. 1. The time-courses of ATP disappearance at 0°C and 15°C in strips of bovine sternomandibularis muscles, either unloaded or loaded (40 g/cm²). ATP content (%initial). ○, 0°C, unloaded; □, 0°C, loaded; ▲, 15°C, unloaded; ●, 15°C, loaded.

Figure 1 illustrates the effect of loading on the disappearance of ATP from muscles chilled either to 0°C or 15°C.

In muscles cold shortening without a load (Fig. 1) the pattern of ATP disappearance at 0°C was characteristic of normal rigor onset, with a rapid phase occurring some 6–10 hr post mortem after a period of slow decline. ATP disappearance in muscle doing work in cold shortening against an external load (40 g/cm²) at 0°C followed a much more rapid course, declining from the moment of load application to 20–30% of the initial value in 6 hr. During this period an unsampled muscle strip had shortened under load by 58% of the horizontal resting length.

Figure 1 also shows that since cold shortening does not occur at 15°C, loading had no effect on ATP disappearance. These results demonstrate that cold shortening, like physiological contraction, is due to the coupling of mechanical and chemical events within the myofibrils.

Plate 1 shows that brief homogenization of bovine sternomandibularis muscle produces a suspension of single fibre pieces up to 1 mm in length. During storage for 24 hr at 0°C these become rigid, and through cold shortening, on average 40% thicker (Plate 1a). At 15°C they remained unshortened and flexible (Plate 1b).

PLATE 1. Photomicrographs of fibre pieces from bovine sternomandibularis muscle. Shortened (a) through storage for 24 hr at 0°C. Unshortened (b) through storage for 24 hr at 15°C. × 40.

(*Facing p.* 54)

Single myofibrils occasionally encountered in such suspensions, and also disorganized fibre pieces with adjacent myofibrils partially broken away from each other, did not contract at 0°C, but retained their unshortened sarcomere patterns throughout the storage period.

These observations limit the possible sites of cold action in the tissue. Since the fibre pieces cold shorten, while single myofibrils do not, the cold is having its effect on one or more of the membranous components, the sarcolemma, the transverse tubular system, and the sarcoplasmic reticulum of the highly ordered fibre pieces.

The pronounced effect of low temperatures in producing shortened fibre pieces should also be reflected in the gravity-settling characteristics of their suspensions.

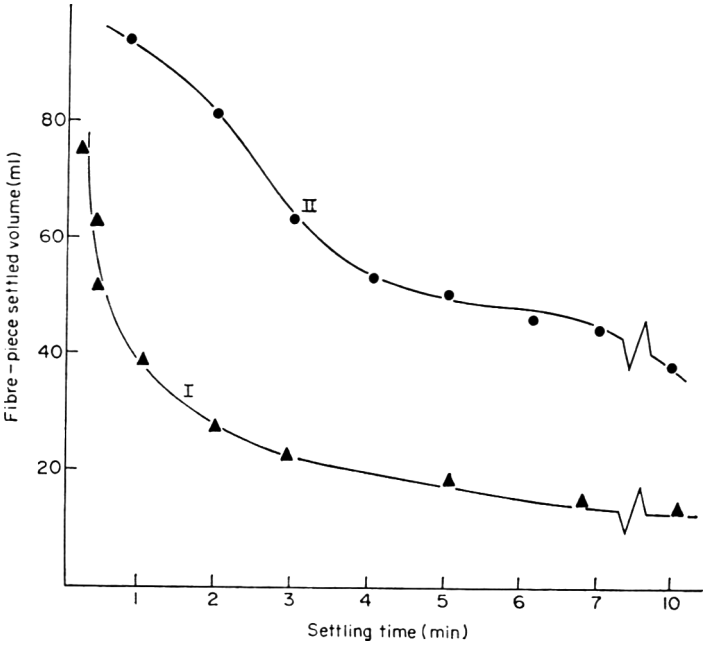

FIG. 2. The time-courses of gravity settling of fibre-piece suspensions. Curve II, fibre pieces unshortened through storage at 15°C. Curve I, fibre pieces shortened by approximately 40% through storage at 0°C.

This is shown in Fig. 2, which compares the time courses of settling suspensions held at 15°C and 0°C. The shortened fibre pieces of the 0°C suspension settled more rapidly (Curve I) than those held at 15°C (Curve II). Thus after 3 min the 0°C fibre pieces had settled by 96% of the 10 min value, while the 15°C fibre pieces had settled by less than 40% of the 10 min value.

That ionic calcium is the ultimate trigger for cold contracture can be inferred from Fig. 3, which demonstrates the effect of controlling $[Ca^{2+}]$ on the time course of settling of the 0°C fibre pieces.

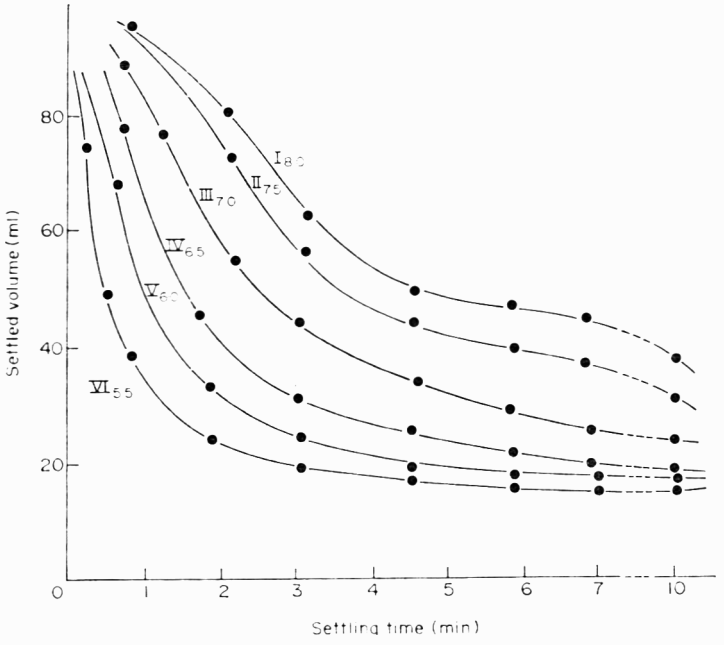

FIG. 3. The time-courses of gravity settling (Curves I–VI) of 0°C fibre pieces incubated in Ca-EGTA buffers over the range pCa 8·0–5·5. By controlling Ca^{2+} at high pCa values (pCa 7·9–9·0) the fibre pieces do not cold shorten and settle slowly. The number beside each curve is the pCa value at which the fibre pieces were incubated.

This is achieved with Ca-EGTA buffers. In their presence the settling curves of the 15°C fibre pieces are virtually the same as the 0°C material. This is illustrated in Fig. 4, which can be used to determine roughly the $[Ca^{2+}]$ causing cold shortening in the fibre pieces by comparing the 3 min-settled volumes induced by cold with those brought about by buffered calcium. The 3 min volume in the 15°-suspension, with no calcium buffer, was equivalent to a pCa value of 7·9–8·0. In the 0°C suspension which had undergone shortening, the volume was equivalent to a pCa value of 6·4–6·5. Isolated myofibrils in the suspensions did not shorten until the $[Ca^{2+}]$ was slightly higher at pCa 6·2–6·3.

Thus in the 15°C fibre pieces the $[Ca^{2+}]$ in the region of the myofibrils is so low (pCa 7·9–8·0) that shortening does not occur. In the 0°C fibre pieces the $[Ca^{2+}]$ has increased thirty- to forty-fold to pCa 6·4–6·5, and the fibre pieces cold shorten.

Discussion

The build-up of Ca^{2+} sufficient to cause cold shortening results from an upset in the complicated ionic balance across the muscle-cell membranes.

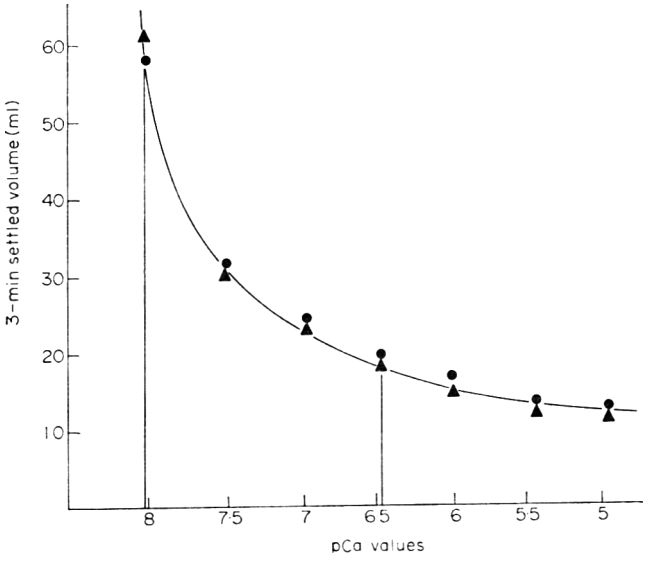

FIG. 4. The relationship between the settling characteristics (3 min-settled volume) and the pCa values of fibre-piece suspensions buffered with respect to Ca^{2+}, and stored for 24 hr at both 0°C (●) and 15°C (▲). The 3 min-settled volumes of suspensions of fibre-pieces *unbuffered* with respect to Ca^{2+} were 19 ml when stored at 0°C, and 58 ml when stored at 15°C. Thus the concentrations of free calcium in these suspensions—indicated by the vertical lines—were equivalent to pCa values of 6·5 approximately for the suspension stored at 0°C and 8·0 approximately for the 15°C suspension.

Lowered temperature reduces substantially the activity of the pump sucking Ca^{2+} from the myofibrillar domain into the sarcoplasmic reticulum (Martonosi & Feretos, 1964). Chilling therefore is likely to increase the steady-state concentration of intracellular calcium by creating a new balance between the release and adsorption of this ion.

An alternative reason for such marked calcium build-up can be found in the properties of the phospholipids of the cell membrane. A characteristic of these is their ability to undergo very distinct temperature-dependent phase transitions, often producing membranes of changed pore size and electrical properties (Gurd, 1963; Benedetti & Emmilot, 1965; Caputo, 1968; Luzzati *et al.*, 1968). With an increased porosity, pumping would be less able to stem the flow of Ca^{2+} from the leaky reticulum. This would lead to a build-up of steady state Ca^{2+} in the myofibrils to a level where shortening occurs.

The effect of cold is by no means a generally encountered phenomenon, either amongst species, or even amongst muscles from a single animal (Locker & Hagyard, 1963; Davey, unpublished). Thus rabbit semitendinosus muscle undergoes cold shortening, while the psoas and longissimus dorsi muscles do not; nor do the sartorius and toe muscles of the frog. To add to this rather confusing pattern of distribution, maxi-

mum power development during cold shortening for the specific bovine muscle studied (sternomandibularis) declines markedly with mean muscle cross-section (Davey, unpublished).

Such variable reaction to cold shows that low temperature is not sufficient in itself to produce shortening, although the underlying changes triggering a mechanical response in some muscles probably happens to a greater or lesser degree in all muscles. The build-up of Ca^{2+} in the myofibrils to a level inducing contraction probably depends upon the degree to which the muscle sarcoplasmic reticulum has been developed. It is less inclined to occur in fast-acting muscles rich in the calcium-absorbing reticulum (Revel, 1964) than in slow-acting muscles where the reticulum is not so abundant (Hasselbach, 1964).

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(*Received* 5 *June* 1973)

J. Fd Technol. (1974) **9**, 59–68.

On the nature of the haematin-protein bonding in cooked meat

D. A. LEDWARD

Summary

Heat, urea or suitable modification of the reactive proteins can all serve to bring about the transfer of haematin from metmyoglobin (Mb) to any one of several of the other proteins found in meat. The complexes formed are all spectrally similar to those found in cooked meat and are assumed to be the same. Studies on the effect of pH and chemical modification of selected amino acid residues in the reactive proteins suggests that these haemoproteins are predominantly haematin di-imadazole complexes. The effect of selected amino acids on the spectra of Mb in 6 M urea also support the view that in cooked meat the haemoproteins are mainly di-imadazole complexes, the imadazole residues being supplied by the histidine groups of the bound protein.

Introduction

It has recently been suggested (Ledward, 1971) that the haemoproteins present in cooked meat are complexes of haematin and any of several of the denatured proteins present in cooked meat. It is well established that the haematin iron in cooked meat is in the ferric state (Tappel, 1957; Tarladgis, 1962) and analysis of the ESR spectra of cooked meat indicates that the ferric ion is in a low spin inducing environment (Ledward, 1971), i.e. it possesses only one unpaired *d* electron (Hollacher, 1966). This suggests that, as well as the four N atoms of the porphyrin ring system, at least one of the other two ligands attached to the ferric ion is either a N atom of an amino group or a S atom of a methionine or cysteine residue (Brill & Williams, 1961). The sixth ligand could also be from a low spin inducing protein side chain (i.e. a N or S atom) or possibly water (Ledward, 1971).

As the marketing of precooked and partially cooked meat products is becoming increasingly important it is desirable to understand the nature of the haematin complexes present as they may catalyse such processes as lipid oxidation and so decrease storage life (Tarladgis, 1962). The previous study (Ledward, 1971) showed that the haemoproteins present in cooked meat were spectrally similar to those obtained on heating aqueous muscle extracts or mixtures of myoglobin and bovine serum albumin

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at pH 6. If serum albumin and myoglobin were allowed to stand in 5·5 M urea at pH 6 spectrally similar complexes were also formed. These protein mixtures, containing haematin—BSA complexes, were used as models to describe the possible nature of the haemoproteins present in cooked meat. The present work was carried out to gain further insight into the haematin-protein bonding in these cooked meat haemoproteins.

Materials and methods

Aqueous muscle extracts and solutions of bovine serum albumin (BSA) and horse heart metmyoglobin (Mb) were prepared as described previously (Ledward, 1971).

In an attempt to determine the amino acid residues bonded to the haematin iron either BSA or the proteins in aqueous muscle extracts were modified. The S containing groups of cysteine and methionine were modified by reaction with 0·2 M bromoacetate for 6 hr at 22°C and pH 6·8, in both acetate buffer and 8 M urea (Banaszak *et al.*, 1963). The guanidine groups of arginine and the ϵ-amino groups of lysine were modified by reaction with 3% glyoxal for 48 hr at pH 9·2 and 22°C (Nakaya, Horinishi & Shibata, 1967).

After reaction all the modified proteins were dialysed against distilled water at 4°C. For comparison, duplicate protein solutions were treated similarly except that no modifying reagent was added.

To further elucidate the reactive groups in the proteins the spectra of solutions of Mb (1 mg.ml^{-1}) and selected amino acids (6 mg.ml^{-1}) were studied as a function of urea concentration and pH. pH was adjusted by the addition of NaOH or HCl.

Chromatography

To prepare the BSA-haematin complex(es) solutions of Mb (2·0 mg.ml^{-1}) and BSA ($\sim$15 mg.ml^{-1}) were made up in 5·5 M urea at either pH 6·0 (0·05 M phosphate) or pH 8·9 (0·05 M borate). After 24 hr the solutions were fractionated directly on Sephadex G-100 (bed volume $\sim$ 130 ml height 40 cm and flow rate $\sim$ 30 ml. hr^{-1}), the eluant being either phosphate (pH 6·0) or borate (pH 8·9). Some of the solutions were dialysed against urea free buffers prior to fractionation. Fractions were collected at 8 min intervals and the absorbance determined in 1 cm cuvettes at 280 nm (all proteins) and 410 nm (haemoproteins). The same column and collecting procedure was used to separate the soluble haematin-protein complexes present in the modified aqueous muscle extracts.

Spectral analysis

Absorption spectra of haematin containing solutions were recorded on a Unicam SP 1800 spectrophotometer at a scan rate of 1 nm per sec. In certain cases it was necessary to dilute the solution with the appropriate solvent to record the Soret ($\sim$400 nm) absorption peak.

Results

Studies on the BSA-haematin complex

Chromatograms of the dialysed BSA-Mb solutions, at both pH 6·0 and 8·9, were the same as found previously (Ledward, 1971), i.e. the haematin-BSA complexes eluted (as two peaks) just after the void volume, V_0, while renatured monomeric Mb and apomyoglobin eluted at about 2·4 V_0. In some cases the urea solutions of Mb-BSA were placed directly on the column and the resultant chromatograms were the same as obtained from solutions that had been dialysed free of urea.

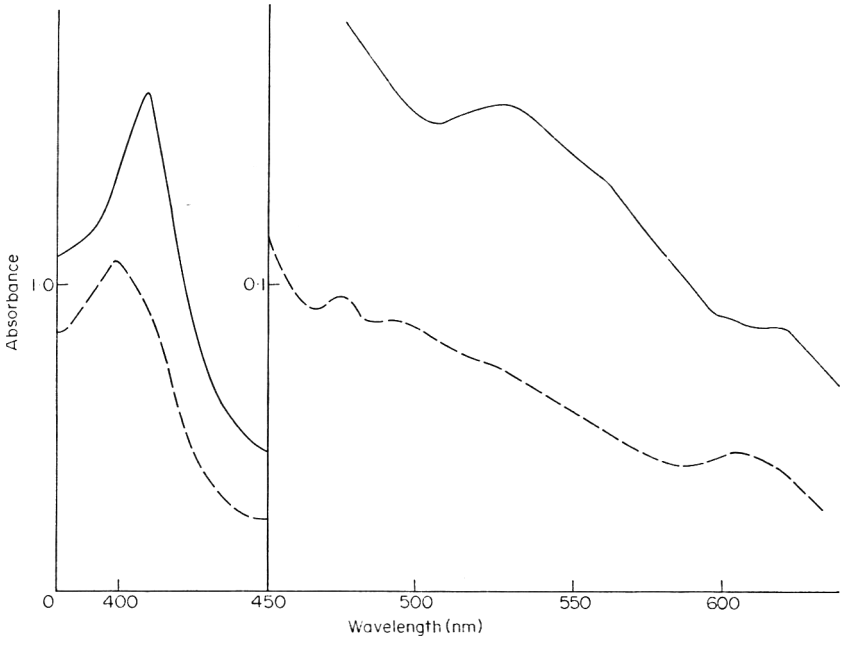

FIG. 1. Absorption spectra of the BSA-haematin complex at pH 6·0 (———) and pH 10·0 (– – –).

The spectra of the BSA-haematin complexes were not affected by the method of preparation, i.e. whether the urea was removed via dialysis or by fractionation on the column. The spectra of these complexes, which were independent of pH in the range 6·0–8·9, had absorption peaks at 410, ~535 and shoulders at ~580 and 620 nm typical of cooked meat (Ledward, 1971). However, on raising the pH further a marked spectral change occurred (Fig. 1) in that the Soret peak moved to shorter wavelengths and peaks appeared at ~480 nm and 605 nm whilst the peak at ~530 nm and the shoulder at ~620 nm disappeared. The pK value for this transition, found by plotting the absorption maximum of the Soret peak against pH, was 9·1 ± 0·3 (Fig. 2).

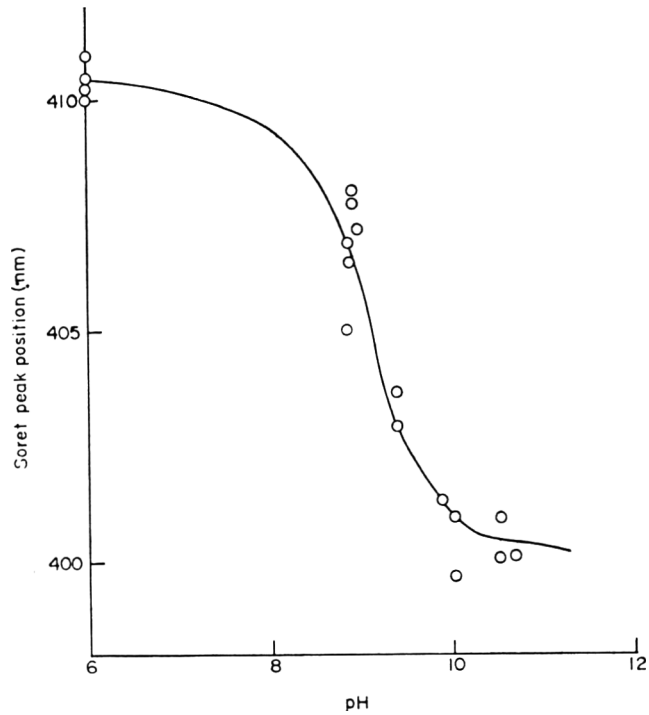

FIG. 2. The position of the Soret absorption maximum for the BSA-haematin complex as a function of pH.

Studies on the S-containing amino acids

When the cysteine and methionine residues in BSA were modified by reaction with bromoacetate there was no significant change in the amount of Mb precipitated from solution on heating solutions of Mb + BSA. Thus, on heating Mb (1·4 mg.ml^{-1}) with BSA (10·0 mg.ml^{-1}) at 68°C and pH 6·8 for 2 hr, 24·5% Mb was lost from the solution containing the unmodified protein and 26·2% from that containing the modified BSA. If the modification was performed in 8 M urea then the percentages lost were 27·4 and 28·6% respectively. No loss of Mb occurred in solutions containing no BSA at 68°C and pH 6·8.

In attempts to further elucidate the role, if any, of the S-containing amino acids in cooked meat haemoprotein the spectral properties of solutions of Mb (1 mg.ml^{-1}) and excess S-compound (6 mg.ml^{-1}) were studied. β-mercaptoethanol gives a complex in which an S atom is thought to bond as the sixth ligand to the ferric ion, the fifth ligand being an imadazole bound histidine residue of apomyoglobin (Peisach & Blumberg, 1971). The spectrum of this complex was not typical of cooked meat as it yielded absorption peaks with maxima at 425, 545 and 580 nm. Cysteine gave a spectrum typical of a mixture of sulphmyoglobin (Nicholls, 1968), oxymyoglobin and

metmyoglobin which on oxidation with ferricyanide did not yield a cooked meat haemoprotein spectrum. On increasing urea concentration the spectrum became more typical of Mb in urea. Methionine gave no indication of reaction with Mb under all conditions studied.

Studies on the basic amino acids

Other possible low spin inducing ligands are the imadazole group of histidine and/or the amino group of lysine and/or the guanidino group of arginine. Hence an aqueous muscle extract containing 3% glyoxal was adjusted to pH 9·2 and left at 20°C for 48 hr when most of the available ϵ-amino and guanadine, but not the imadazole, groups would react (Nakaya *et al.*, 1967). After dialysis against distilled water the spectrum of the muscle extract displayed absorption maxima at 410 and ~535 nm and shoulders at ~580 and 620 nm typical of cooked meat (Fig. 3) and, upon heating at 65°C and pH 6·2, remained unchanged with only slight precipitation, although marked precipitation occurred in the untreated extract at pH 6·2 and 68°C. However marked precipitation occurred on heating the modified extract of pH < 5 to 65°C.

In the modified muscle extract the spectrum was independent of pH over the whole range studied (6·0–10·5).

When glyoxal and Mb were mixed at pH 6·0 or 9·2 no formation of a cooked meat haemoprotein was evident. Chromatography of the modified muscle extract at pH 6·2 verified that the observed spectrum was due to haematin being bound to other proteins and not direct reaction between glyoxal and Mb (Fig. 4). The spectrum of the proteins

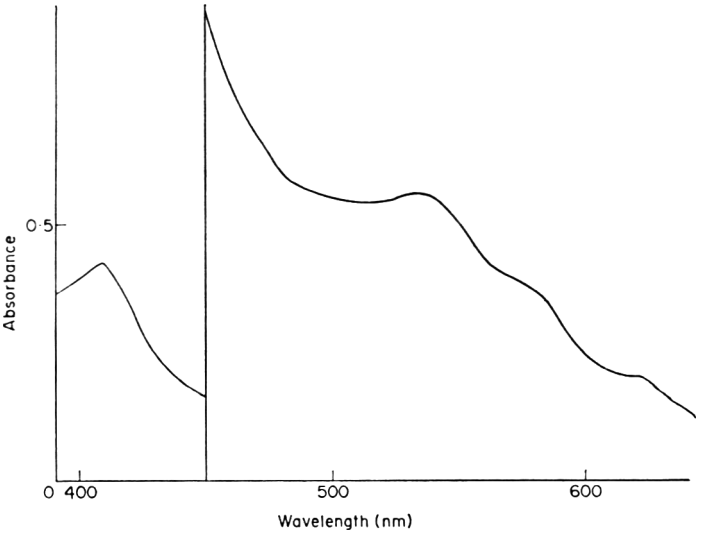

FIG. 3. Absorption spectra of an aqueous muscle extract, at pH 6·2, after reaction with 3% glyoxal for 48 hr at pH 9·2 and 20°C. The solution was diluted in the ratio indicated to record the Soret region.

in the initial haematin positive peak was typical of cooked meat haemoprotein. There was no evidence of haematin transfer in aqueous muscle extracts undergoing the same experimental procedure, but without addition of glyoxal. However, these solutions yielded precipitates of cooked meat haemoprotein on heating (Ledward, 1971).

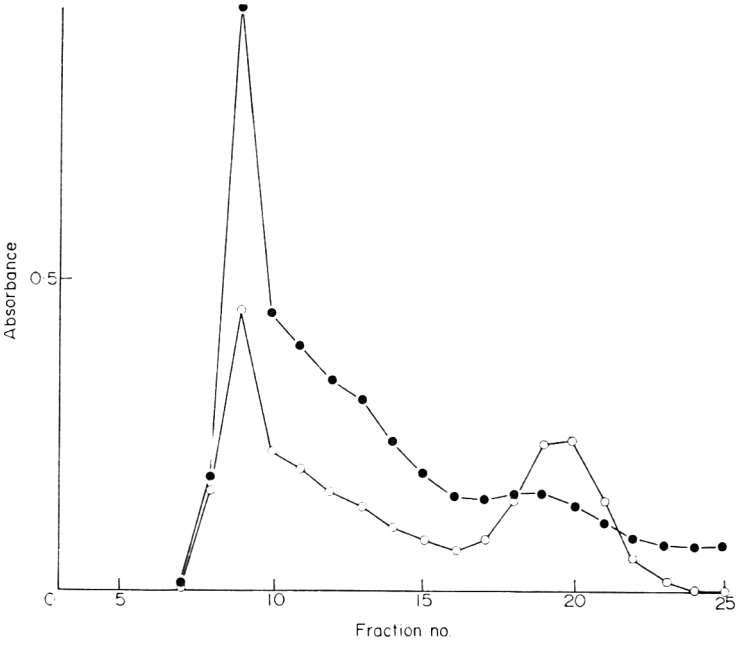

FIG. 4. Chromatogram obtained on fractionating 4 ml of the solution described in Fig. 3 on Sephadex G-100 at 20°C (bed volume ~130 ml, height 40 cm and flow rate ~30 ml hr^{-1}). Fractions were collected at 8 min intervals and the absorbances determined at 280 nm (●) and 410 nm (○).

To gain further insight into the role of the basic amino acids in the formation of cooked meat haemoprotein the spectral properties of Mb (1 mg.ml^{-1}) and selected amino acids (6 mg.ml^{-1}) at pH 6·0 and different urea concentrations were investigated. Except for cysteine no reaction was apparent at urea concentrations of < 5 M. The spectra of Mb plus different amino acids in 6 M urea at pH 6·5 are shown in Fig. 5. It is seen that histidine, lysine and arginine all yielded complexes that with absorption peaks at ~410 and ~535 nm and shoulders at ~580 and 620 nm were spectrally similar to cooked meat haemoprotein, but that methionine did not. With increasing pH all the spectra became characteristic of Mb in 6 M urea, i.e. there was no evidence of reaction between Mb and the different amino acids at pH ⩾ 10 and, as with Mb in urea, the Soret peak shifted from ~400 nm to about 392 nm at pH 10 (Fig. 6). In Fig. 6 the position of the Soret maxima is plotted as a function of pH for all the different reaction mixtures and, if as seems probable, decreased Soret absorption, occurring at shorter

wavelengths corresponds to decreased haematin-amino acid interaction then it is seen that the histidine complex was apparently more stable (pk = 9·2 ± 0·2) towards alkali than the others (pk about 8·5). The fact that the ~535 nm peak was evident at higher pH values for the histidine complex than for the lysine and arginine complexes also suggested that the histidine complex was more stable towards alkali.

This complex formation was, in all cases, reversible as upon removal of urea Mb was reformed.

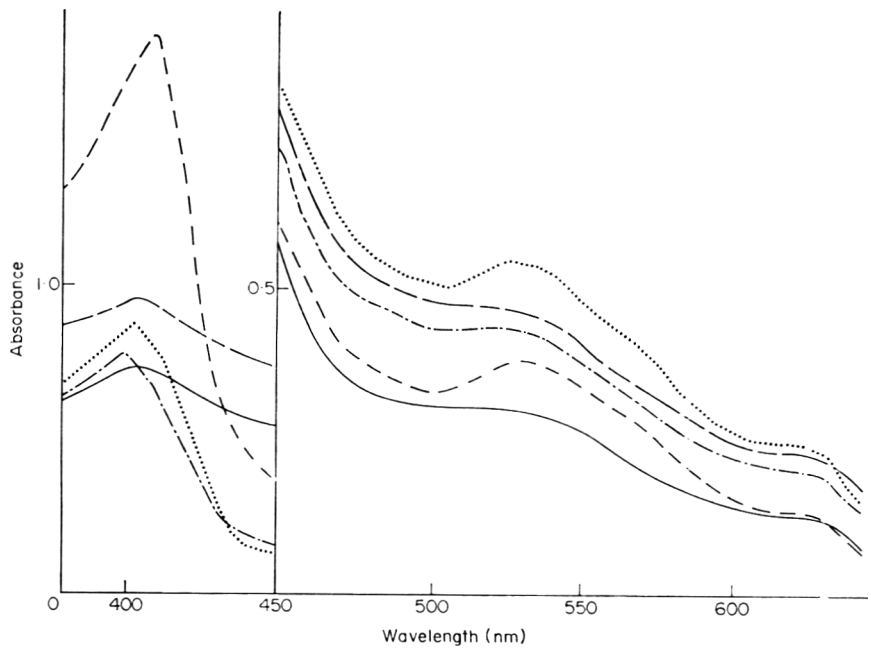

FIG. 5. Absorption spectra of Mb (———); Mb + methionine (— - — - —); Mb + histidine (– – – –); Mb + lysine (–·–·–) and Mb + arginine (· · · ·) in 6 M urea at pH 6·5. In all cases the Mb concentration was 1 mg.ml^{-1} and the amino acid concentration was 6 mg.ml^{-1}. The spectra are arbitrarily displaced on the absorbance axis to aid clarification. Soret peaks determined on diluted solutions.

Discussion

The high pH spectrum shown in Fig. 1 is typical of high spin ferric haemoproteins (Brill & Williams, 1961) and thus is unlikely to correspond to the ionization of a water molecule as this involves the conversion of a high spin inducing ligand (H_2O) to a low spin inducing ligand (OH^-). This suggests that water is not one of the ligands in cooked meat haemoprotein and that both ligands are supplied by the bound protein (BSA). This was borne out by the observation that in the glyoxal treated muscle extracts, which were spectrally similar to cooked meat and to the low pH form of the BSA

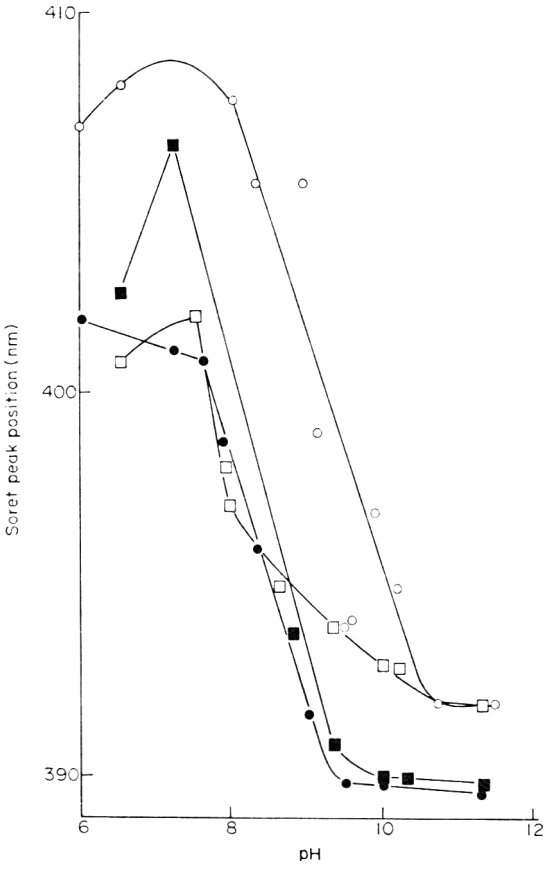

FIG. 6. The position of the Soret absorption maximum in 6 M urea for Mb (○); Mb + arginine (●); Mb + lysine (□) and Mb + histidine (■) as a function of pH.

haematin complex (Figs. 1 and 3), the spectra were independent of pH (6·0–10·5) although ferric bound water in haemoproteins normally ionizes at pH 7–9 (George & Hanania, 1952). The pH dependent spectral change observed in the BSA-haematin complex (Fig. 1) may be related to the ionization of either the lysine or arginine residues, which cannot ionize in the glyoxal treated complex, leading to a molecular rearrangement around the haematin and concomitant change of ligand(s).

ESR spectra of the denaturation products of haemoglobin suggest that in this case both ligands to the haematin are supplied by the denatured globin (Peisach & Blumberg, 1971). Thus it is reasonable that other denatured proteins (e.g. BSA) with greater affinity for haematin (Ledward, 1971) should supply both ligands in cooked meat haemoproteins.

As no significant difference was found in the ability of BSA and bromoacetate treated BSA to precipitate Mb at 68°C it is unlikely that the S containing amino acids, methio-

nine and cysteine, are involved in the formation of cooked meat haemoprotein. This conclusion was also suggested by the apparent lack of reaction between Mb and methionine under all conditions (e.g. Fig. 5) and the formation of non-cooked meat type complexes between Mb and β-mercaptoethanol or cysteine.

In the glyoxal treated samples, in which the lysine and arginine residues were modified, haematin transfer from Mb to the other proteins readily occurred even at room temperature (Figs. 3 and 4) whereas heat, or urea, was necessary for the reaction to occur between Mb and the unmodified proteins. However it is probable that the mechanism of haematin transfer was the same in both cases except that the decreased positive charge on the modified proteins enables the reactions to occur at room temperature while heat, or urea, is necessary to allow reaction between the unmodified proteins and the haematin of myoglobin. The decreased positive charge on the proteins in the modified extract was evident from the shift of the isoelectric point, as indicated by protein aggregation and precipitation, to lower pH.

As modification of the reactive groups of cysteine, methionine, lysine and arginine failed to prevent the formation of cooked meat haemoprotein(s) it is probable that the reactive ligand in the haematin transfer reaction is the imadazole residue of histidine. This conclusion is also indicated by the apparent stability of the histidine-haematin complex formed under similar conditions to those employed to form the soluble BSA-haematin complex, i.e. 5–6 M urea at near neutral pH (Figs 5 and 6). In this complex the haematin was presumably present as a di-imadazole complex, with a histidine residue from the globin and/or free histidine residues, supplying the imadazole ligands. The differences observed between the different amino acids argue against the α-amino group being the bound ligand. These results, in conjugation with those obtained with the modified proteins, suggest that cooked meat haemoproteins may also be mainly, but not necessarily 100%, di-imadazole complexes, the ligands being supplied by any of the denatured proteins present in cooked meat. The fact that certain proteins have more affinity for haematin than others may be related to the spatial arrangement of the histidine residues in the protein in conjugation with its ability to form stabilizing porphyrin-protein interactions via salt-linkages, and hydrogen and hydrophobic bonds. These factors could account for the higher affinity of haematin for BSA than for apomyoglobin even though apomyoglobin has a greater number of histidine residues per unit weight. The lack of such stabilizing bonds would account for the limited stability, towards both alkali and removal of urea, of the histidine-Mb complex.

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(*Received* 25 *June* 1973)

J. Fd Technol. (1974) **9**, 69–78.

Utilization of edible protein from meat industry by-products and waste

I. Factors influencing the extractability of protein from bovine and ovine stomach and lungs

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Summary

Data concerning the effect of pH, salt concentration and temperature on the extractability of protein from meat waste tissues are presented. The tissues investigated so far include bovine and ovine stomach and lungs. It appears that native proteins can be recovered in quantity from these tissues at room temperature over a wide range of pH values using solvents of low salt concentration (e.g. 0–0·01 M NaCl). Protein isolates have been characterized and compositional differences detected using polyacrylamide gel electrophoresis incorporating sodium dodecyl sulphate (SDS). This technique demonstrated marked differences in the electrophoretical patterns of proteins from lung and stomach tissues, the subunit molecular weights of which ranged from 13,500 to 87,000 for lung protein and from 13,500 to 145,000 for stomach protein.

Introduction

There has been an interest for some time in increasing the production of conventional plant and animal proteins and in the development of new sources of food proteins. The severe shortage of protein of high biological value in developing countries and the high cost of meat in those which are more fortunate economically has directed much work on the possibility of fabricating protein-rich foods from plant sources in palatable form. Artificial meat-like products which have controlled texture, flavour and nutritive value have been marketed and can substitute directly for meat or economically extend the bulk, and help the texture of conventional meat products (Sjostrom, 1963).

In view of the frequent drawbacks of low acceptability, absence of organoleptic quality and high cost of meat-like products prefabricated from vegetable or bacterial sources, it seems highly desirable to reassess the potential for making edible foods from the substantial amount of slaughterhouse protein which is currently wasted. Various

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by-products of the meat industry are high in high quality protein (Olson, 1970) but, being not directly edible, mainly create pollution problems.

It has been suggested recently that a considerable amount of meatworks protein now fed to animals could be processed rather differently for inclusion in human foods (Vickery, 1968). Studies concerning these aims have in the main pursued solvent extraction of fat and dehydration as a means of protein recovery. Examples of work of this type are in the production of meat protein concentrate (Levin, 1970) and in the removal of meat residues from meat and bone meal by carbon tetrachloride flotation (Nash & Mathews, 1971). Although protein concentrates may be produced in this way, the processes cause denaturation of the proteins with subsequent loss of functionality and decrease in nutritive value (Meinke, Rahman & Mattil, 1972).

If proteins from meat waste are to be fabricated into palatable forms for consumption, it appeared useful to investigate the possibility of extracting the proteins in a form which retains their solubility and nutritive value. The solubility characteristics from stomach and lung tissues at various pH's, salt concentrations and temperatures have been investigated in this study.

Materials and methods

Selection and preparation of raw materials

Offals were obtained fresh from the slaughterhouse and comprised bovine lung, bovine rumen, bovine reticulum, bovine omasum, bovine abomasum, sheep lung, and sheep stomach. After thorough washing and scraping of stomach mucous linings, the tissues were minced.

Assessment of protein extractability

An experimental design similar to that described by Meinke *et al.* (1972) was used. The effect of pH on protein extractability was determined by homogenizing 2 g portions of the minced tissue in 60 ml of aqueous extracting medium. The total volume of extractant comprised the water contained in the tissue, the volume of acid (HCl) or base (NaOH) added to create the desired pH and the water added to make a final volume of 60 ml. The suspensions were allowed to stand for 2 hr at room temperature (21°C) with continuous agitation.

The effect of salt concentration over the range of pH values was determined in a similar manner on minced sheep lung and stomach tissues. Differing ionic strengths were achieved by incorporating 0·01 M, 0·1 M, 0·25 M and 1 M sodium chloride and 0·05 M and 0·5 M calcium chloride respectively into the extracting medium, the pH of which was adjusted as before.

Temperature and time factors were investigated at neutral pH. Temperatures were varied between 0°C and 50°C and times between zero and 3 hr.

In each case, the suspensions were clarified by centrifugation for 15 min at 900 ***g***. Total protein ($N \times 6{\cdot}25$) was determined by the microkjeldahl method on 2 ml aliquots of the clear supernatants and the percentage protein solubilized calculated on the basis of the 60 ml volume of extractant.

The total protein content of each tissue was again determined by the microkjeldahl method.

Preparation of protein samples for electrophoresis

Proteins were extracted from defatted sheep lung and stomach tissues at pH 7, 9 and 11 respectively. The samples were purified by ammonium sulphate precipitation, dialysed for 48 hr and freeze dried. Seven milligrams of dried protein was incubated overnight at 25–35°C in electrophoresis buffer containing 1% SDS and 1% β-mercaptoethanol. Sucrose (10% w/v) was added to all samples which were then retained for electrophoresis.

Polyacrylamide gel electrophoresis

A thin-layer vertical slab polyacrylamide gel was employed similar to that used by Parsons & Lawrie (1972). The apparatus has recently been described in detail by Roberts (1972). The buffer system was continuous and consisted of 0·025 M sodium barbitone and 0·1% SDS, the pH being adjusted to 8·5 with HCl. Electrophoresis was carried out using an 8% acrylamide gel, overlayered with a 3% starter gel. The space above the 3% gel was filled with a solution of 0·1% SDS and 0·1% β-mercaptoethanol in stock buffer, sample slots being formed by inserting lengths of rubber tubing into the gel. About 40 μl of sample was applied to each slot. Electrophoresis was initially carried out at 10 mA for 30 min. The remainder of electrophoresis was carried out for 2 hr at 50 mA falling to 35 mA. Gels were stained in 0·2% naphthalene black dissolved in methanol/acetic acid solution and cleared in several changes of methanol/acetic acid solution.

Molecular weight determination

It has been found that an inverse linear relationship exists between the relative migration distances and the logarithm of molecular weights of protein subunits released by SDS treatment (Shapiro, Vinuela & Maizel, 1967). This serves as a rapid and simple estimation of proteins and polypeptides.

Employing the electrophoretical system previously described, a calibration graph of mobility *v.* log-molecular weight was constructed using the following proteins and enzymes: cytochrome C (11,700), lysozyme (14,300), ribonuclease A (15,800), myoglobin (16,890), trypsin (23,300), rennin (34,000), aldolase (40,000), 3 phosphoglycerate kinase (48,500), catalase (57,500), albumin (69,000) and phosphorylase B (92,500).

These marker proteins were run under exactly the same electrophoretical conditions

and gel dimensions as were the unknown lung and rumen proteins to enable comparisons to be drawn. One milligram of each marker protein was dissolved in 1 ml of SDS/mercaptoethanol solution and incubated as before. Mobility was taken as the distance moved by the protein band divided by the total vertical length of the 8% gel.

Laser densitometry

The thin layer polyacrylamide electrophoretograms were scanned using the technique of laser beam densitometry described by Parsons *et al.* (1969).

Results

Protein extractability

The total protein contents of tissues studied during the course of the work are listed in Table 1. The protein solubility profiles of Fig. 1 illustrate the effect of pH on the extractability of proteins from bovine tissues at room temperature. The curves show a minimum protein solubility in the pH range 4·5–5·0. In this pH range 12–34% of the protein was extracted. On both the acidic and basic sides of this pH range protein solubility increased. The curves obtained for sheep tissues (Fig. 2) follow a similar pattern. Figure 3 represents graphically data obtained in this study related to the influence of sodium chloride on the solubility of protein from sheep lung. The graphs show a decrease in protein extracted at high and low pH values with increasing salt concentrations, whilst an increase in protein solubility occurs at the minimum solubility point (pH 4·5). A 1 M sodium chloride solution greatly inhibited the extraction of proteins at high and low pH values. Depression of extractability was caused to an even greater extent by calcium chloride.

TABLE 1. Protein contents of meat waste tissues

Tissue	% Protein
Bovine	
Lung	15·9–17·2
Rumen	10·2–13·1
Reticulum	9·2–10·1
Omasum	8·5– 9·5
Abomasum	7·1– 9·0
Sheep	
Lung	15·5–16·5
Stomach	11·6–14·2
Pig	
Lung	13·6–14·8
Stomach	13·5–14·4
Blood plasma	7·5

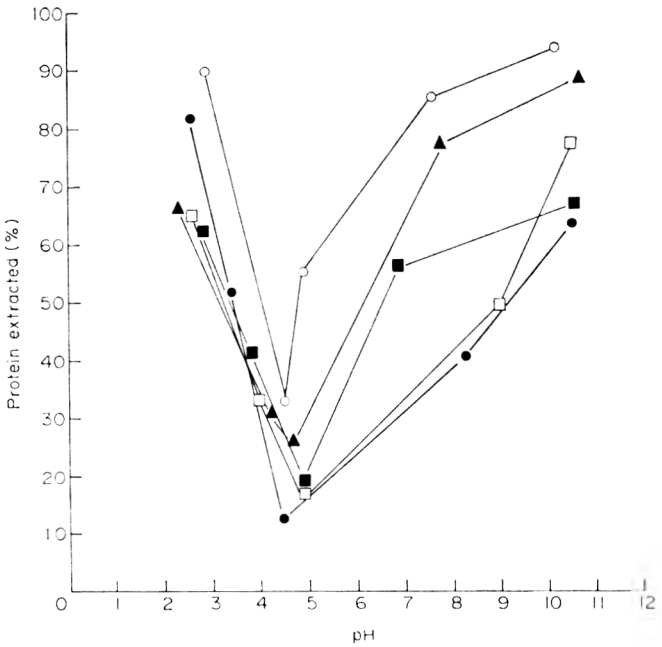


FIG. 1. The effect of pH on the extractability of protein from bovine tissues at room temperature. ■, Lung; □, rumen; ●, reticulum; ▲, omasum; ○, abomasum.

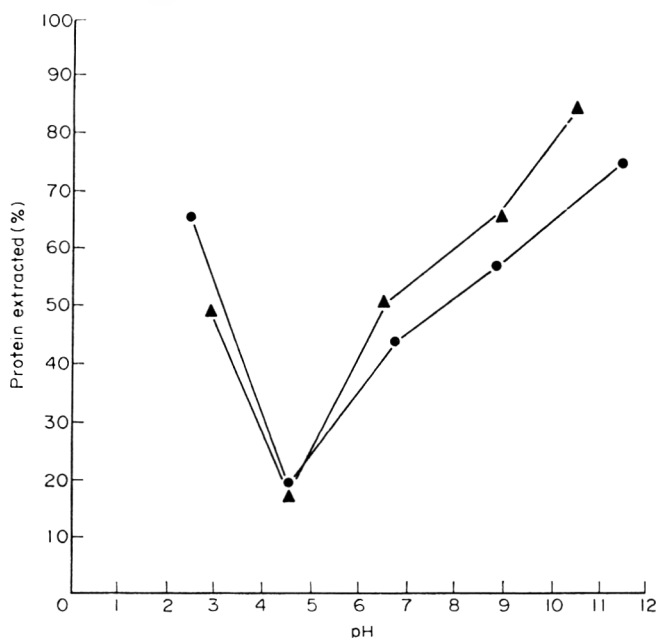


FIG. 2. The effect of pH on the extractability of protein from sheep tissues at room temperature. ▲, Lung; ●, stomach.

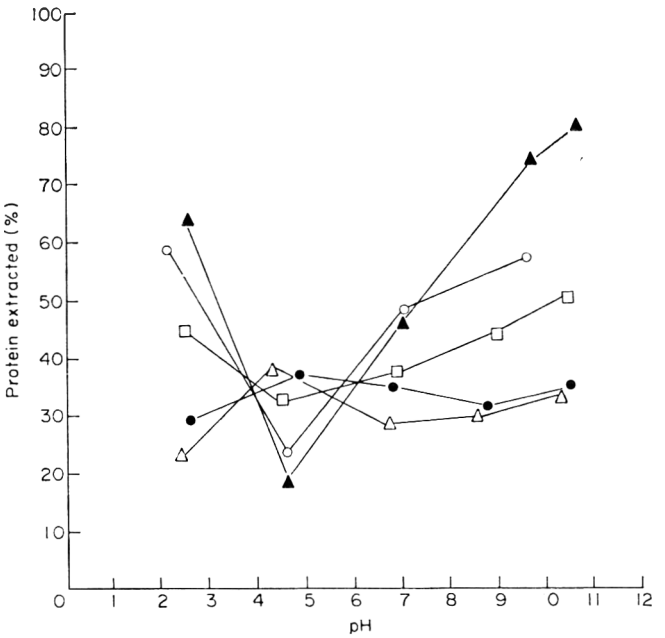


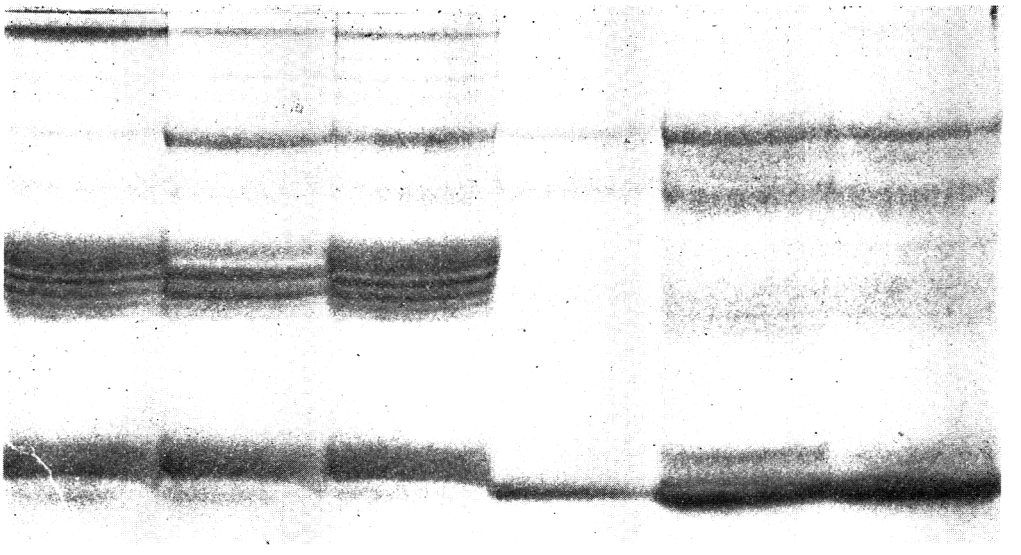
FIG. 3. The variation in the extractability of protein from sheep lung with pH as affected by salt concentration (room temperature). ▲, NaCl absent; ○, 0·01 M NaCl; □, 0·1 M NaCl; ●, 0·25 M NaCl; △, 1 M NaCl.

The extractability of sheep stomach and lung proteins at neutral pH gradually increased as the temperature of extraction was raised to 25°C and 30°C respectively. For lung, protein solubility increased from 51% at 0°C to 60% at 30°C and, for stomach, 42% of the protein was solubilized at 0°C rising to 48% at 25°C. Above thesetempe ratures, solubilities decreased.

Data obtained for the effect of time on protein extractability showed that the greatest degree of solubilization occurred within the first hour of suspension in the extracting medium, after homogenizing for 1 min. After 1 hr, the amount of protein in solution appeared to remain constant.

Polyacrylamide gel electrophoresis

Electrophoresis incorporating SDS and β-mercaptoethanol with sodium barbitone buffer (pH 8·5) was satisfactory in separating protein components extracted from sheep lung and stomach tissues. Typical electrophoretograms for six protein extracts are shown in Plate 1. Using the same technique, a straight line plot of log molecular weight *v.* mobility of marker proteins was obtained. The calibration graph is shown in Fig. 4. The molecular weights of the unknown components in the electrophoretograms were estimated from the calibration curve and are listed in Table 2.



(a) (b) (c) (d) (e) (f)

PLATE 1. Polyacrylamide electrophoretograms in SDS of proteins extractable from sheep tissues at alkaline pH's. (a) p H10·7 extract from stomach, (b) pH 6·8 extract from stomach, (c) pH 8·9 extract from stomach, (d) pH 10·6 extract from lung, (e) pH 6·9 extract from lung, (f) pH 8·8 extract from lung. Sample origin at the top.

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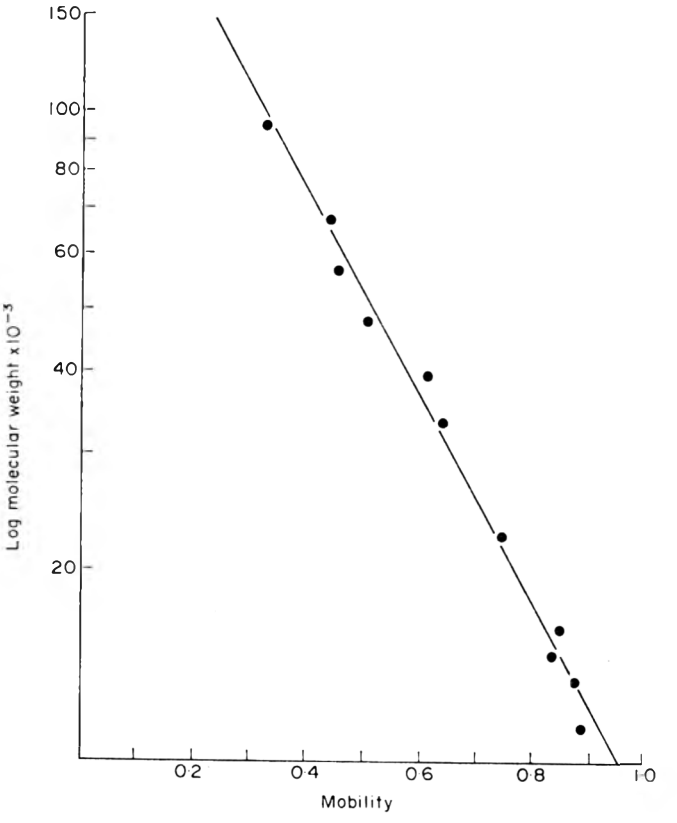

FIG. 4. Plot of the logarithm of the molecular weights of marker proteins treated with SDS versus their mobilities on an 8% polyacrylamide gel.

Electrophoretic patterns from the different tissue proteins showed marked differences on visual examination, the densitometric traces demonstrating this even more clearly. Figure 5a and d are densitometric traces of protein extracted from sheep stomach and lung respectively, both at neutral pH. Among the more obvious differences was the fact that the stomach protein possessed several components of high molecular weight, in the region 105,000–145,000, which were absent in the lung protein. More bands were discernible in the rumen protein electrophoretograms (fifteen components) than in those for lung (eleven components). A most noticeable feature from the lung protein patterns was the presence of a fast-migrating component of molecular weight 13,500, giving a very distinct peak on densitometric scanning. This component also appears to be present in the rumen protein but to a much lesser extent.

The densitometer traces indicate little difference due to the pH of extraction on the general electrophoretic pattern, the full range of components appearing in each case. For example, Fig. 5a–c shows the scans obtained for proteins extracted from sheep stomach at pH 6·9, 8·9 and 10·7 respectively. The most notable pH effect concerns a

TABLE 2. Molecular weights of protein subunits extracted from sheep lung and stomach estimated by SDS-polyacrylamide gel electrophoresis

Lung		Stomach	
Component no. [see Fig. 5d]	Estimated mol. wt	Component no. [see Fig. 5a]	Estimated mol. wt
1	87,000	1	145,000
2	79,000	2	132,000
3	71,000	3	118,000
4	69,000	4	105,000
5	51,000	5	87,000
6	46,000	6	64,000
7	35,500	7	45,000
8	32,500	8	41,500
9	17,000	9	39,300
10	15,000	10	36,000
11	13,500	11	34,500
		12	27,500
		13	23,000
		14	15,500
		15	13,500

protein subunit of molecular weight 87,000 which appears to decrease in concentration as the pH of extraction is increased from 6·9 to 10·7.

Discussion

The solubility profiles determined in this study may serve as a useful guide to the amount of soluble protein recoverable from various raw materials. It is interesting to note that using basic extractants there appear to be wide variations in the amount of protein in solution. For example, at pH 8, the levels of the proteins of bovine rumen, lung and omasum in solution were 42, 60 and 78% of the total protein respectively. Also, although bovine omasum and abomasum have relatively low protein contents, the proteins have the greatest solubility. In fact, it appears that more than 90% of the proteins of omasum and abomasum can be extracted at pH 10. Thus, the proteins from each of these raw materials should be considered as unique and possessing inherent functional properties not necessarily similar to those from any of the others.

Estimations of the amount of protein which could be recovered fairly easily as a curd or protein isolate can also be made using the data of Fig. 1. For instance, essentially 70% of the protein of rumen was soluble at pH 3·5 or 10. The solubility curve also showed that about 20% of the protein was soluble at pH 4·7. Therefore, by extracting the protein at pH 3·5 or 10 and then adjusting the extracts to pH 4·7, a curd containing

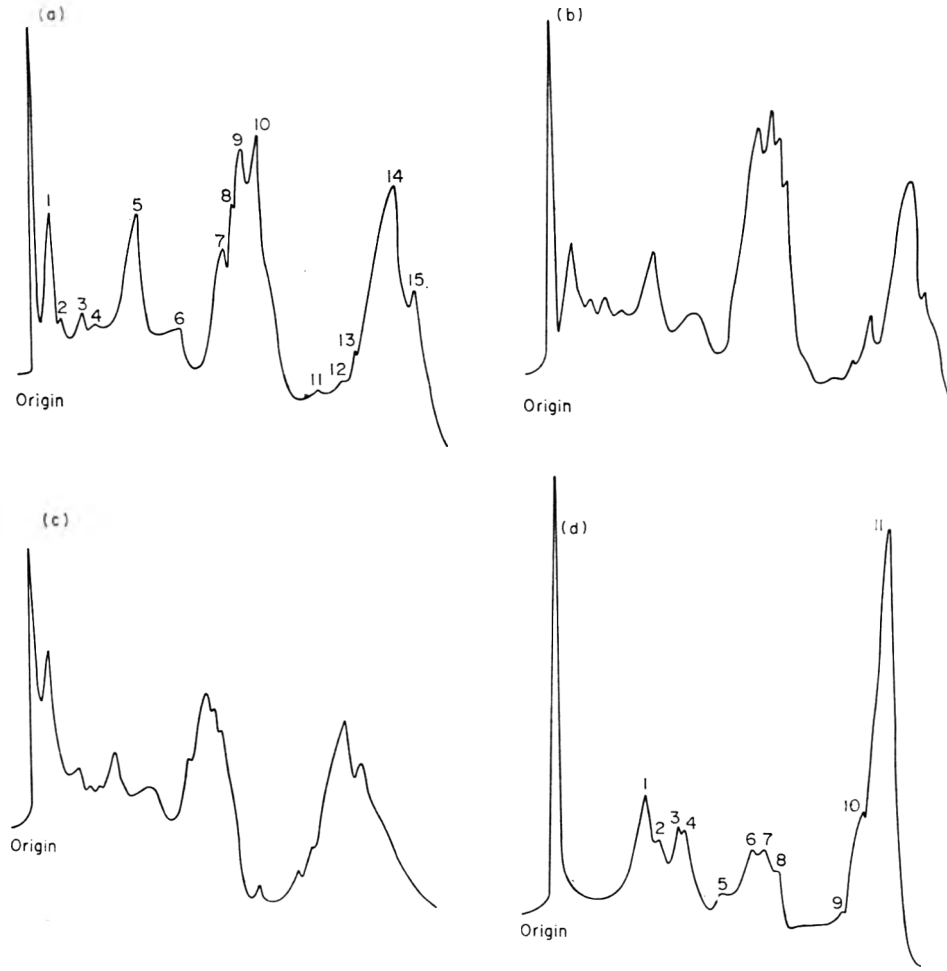

FIG. 5. Densitometric tracings (by laser beam) of polyacrylamide electrophoretograms in SDS of proteins extractable from (a) sheep stomach at pH 6·9, (b) sheep stomach at pH 8·9, (c) sheep stomach at pH 10·7, (d) sheep lung at pH 6·8. The estimated molecular weights of the proteins represented by the numbered peaks are listed in Table 2.

the major portion of the protein of rumen should be obtained. Another observation was the obvious depression of solubility at high and low pH by increasing salt concentrations so that in general it would appear that the proteins of these tissues are most economically extracted at low ionic strength. This has also been demonstrated in studies concerning the recovery of proteins from many plant and fish sources (Mattil, 1971).

The electrophoretograms showed clear differences in the properties of protein extracts recovered from lung and stomach tissues. The different protein components of these extracts may not only give rise to nutritional variations but also differences in texture-forming properties. This warrants further investigation. However, SDS-polyacrylamide

gel electrophoresis is a useful preliminary technique for obviating compositional differences in protein extracts.

Proteins isolated from meat waste tissues by procedures of this kind would retain their functional characteristics and nutritional merit. They could then be further processed into palatable textured food systems or used as binders and emulsifiers in existing foods whilst concomitantly increasing their nutritive value. An important aspect is the fact that these protein isolates would be in a suitable form for conversion into fibrous meat analogues.

Naturally, the development of products of this kind will require detailed assessment of their hygienic and microbiological implications. Thus the extent to which material derived from unthrifty animals could be utilized would need to be established. It is hoped to consider such assessment at a later stage in this series of investigations.

Acknowledgments

This work was supported by a grant from the Meat and Livestock Commission which is gratefully acknowledged.

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(*Received* 1 *July* 1973)

J. Fd Technol. (1974) **9**, 79–86.

Baking performance of hen's egg yolk plasma before and after induced aggregation

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Summary

Yolk plasma has been examined before and after (a) freeze-thaw gelation and (b) heat-setting at 62·5°C for aggregation states by gel filtration and electron microscopy and for its performance in standard Madeira cake baking. Frozen and thawed plasma consists predominantly of large aggregated species of lipoprotein particles. Heat set plasma has a lower degree of aggregation than frozen and thawed samples. It is richer in aggregated species than untreated plasma. The aggregation state is not readily reversed by egg white and/or sucrose The degree of aggregation does not appear to affect the baking performance of plasma. Implications of this finding are discussed in terms of possible relationship of lipoprotein size distribution to baking function.

Introduction

A recent study has demonstrated that granule-free yolk plasma derived from hens' infertile eggs is the principal determinant of yolk functions during the baking of Madeira cakes (Kamat *et al.*, 1973). Yolk plasma consists predominantly of a low density lipoprotein (LDL) component which accounts for 86% of plasma solids. The basic physical structure of LDL is now well established (Cook & Martin, 1969; Kamat *et al.*, 1972). It is a sphere of triglyceride oil surrounded by a monomolecular layer of phospholipids and proteins. Diameters of LDL range from 15 to 45 nm (Nichols, Forte & Coggiola, 1969). Although the interrelation between LDL structure and baking performance is complex, it appears that macromolecular integrity of yolk lipoproteins is necessary to retain cake quality (Kamat *et al.*, 1973).

An important function that is demanded of yolk in this recipe is the emulsification of fat. The mechanism by which yolk fulfils this function is however not clear. Recent evidence (Chang, Powrie & Fennema, 1972) suggests that in the case of mayonnaise, yolk lipoprotein particles stabilize oil droplets. It is therefore conceivable that the fat in Madeira recipe can similarly be stabilized by LDL particles. Indeed, a simple theoretical model for such a mechanism has already been proposed (Kamat *et al.*,

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1973). Within the confines of this model, Madeira fat surface area is approximately $1{\cdot}12 \times 10^7$ cm^2, whereas yolk lipoprotein surface area is 16×10^7 cm^2. It is further postulated that yolk lipoproteins emulsify fat by forming a monolayer around fat droplets. If this is indeed the case, then the surface area of yolk lipoproteins becomes a critical requirement for emulsification of fat. This requirement is examined in the present investigation as follows. The baking performance of yolk plasma is compared before and after substantial reduction of available LDL area achieved by aggregation induced either by (i) heat-setting or by (ii) freeze-thaw gelation.

Materials and methods

Preparation of yolk plasma

Eggs were purchased from a local supermarket. Liquid yolk was collected from each egg according to Powrie, Little & Lopez (1963). Granule-free plasma was separated by ultracentrifugation of yolk at 60,000 ***g*** for 18 hr at 4°C. The solids content of plasma thus obtained was approximately 50%. It was stored in closed plastic bottles under nitrogen at 4°C until used. Separate 1 ml aliquots were subjected to (a) heating for $\frac{1}{2}$ hr at temperatures between 25° and 65°C and (b) three cycles of freezing at −18°C overnight and thawing at +25°C. Setting or gelation was judged visually using complete inhibition of flow as the end point. Aggregation state was monitored by gel-filtration and electron microscopy. Some heat-set and gelled samples were dispersed with egg-white and/or sucrose in buffer at the same concentration as in a cake batter and examined by gel-filtration.

Gel-filtration

Estimates of aggregate size of LDL in yolk plasma were made using Biogel A 50M agarose (100–200 mesh, Biorad Laboratories) supported in an adjustable Pharmacia SR 25 column equilibrated to 20 cm pressure of Tris buffer (0·05 M containing 2 nm EDTA, 0·02% NaN_3), pH 8 at +15°C. All samples were compared with control plasma at 10% solids concentration in the equilibrating buffer. Frozen and thawed samples were resuspended in the buffer containing 1 or 2% glucose and run with glucose-buffer solution. Fractions were collected automatically and the eluant was monitored at 280 nm on an LKB Uvicord II. Accurate elution volumes were deduced from fraction weights and the recorded profiles.

The column void volume (V_0) was indicated by the elution volume of diluted cow's milk. Total volume (V_t) was indicated by the elution volume of 2% NaI in the equilibrating buffer. Calculation of (V_1) the internal gel volume, and determination of (K_{av}), the penetration coefficient (a measure of the extent of penetration of the solute into the internal gel volume), were made according to Partrick *et al.* (1972). Size estimates of yolk plasma components were obtained by comparison of the sample elution volumes with observed elution volumes of reference standards, e.g. globulin,

thyroglobulin, porcine plasma LDL, and Tomato bushy stunt virus (a gift from Dr B. Kassanis, Rothampsted Experimental Station, Harpenden). The latter two were chosen because their Stoke's radii are comparable (Margolis, 1967) to those of egg yolk LDL particles as examined by electron microscopy (Nichols *et al.*, 1969).

Electron microscopy

Dilute suspensions of control and treated yolk plasma made in the Tris EDTA buffer (vide gel-filtration) by gentle homogenization, were examined by negative staining with phosphotungstic acid adjusted to pH 7. Micrographs were taken at 20,000 (low) and 100,000 (high) magnification.

Cake trials

Control and aggregated yolk plasma were compared with whole liquid yolk for their baking performance in the standard Madeira recipe. Aggregated samples of yolk plasma were prepared by either (i) heat-setting at 62·5°C for 1½ hr or (ii) gelled by three cycles of freezing overnight at −18°C and thawing at +25°C.

The basic ingredients of this recipe were as follows.

Chlorinated cake flour	710 g
Castor sugar	600 g
Margarine	600 g
Eggs	600 g

Order of mixing

Stage 1: cream sugar and fat (Hobart Mixer, 10 min, speed 2).

Stage 2: add 216 g of control yolk or control yolk plasma or aggregated yolk plasma +384 g of white (Hobart Mixer, 5 min, speed 2).

Stage 3: add flour (Hobart Mixer, 2 min, speed 1).

The following specific volumes were measured after each addition:

VAC—volume after creaming;
VAE—volume after egg addition;
FBV—final batter volume.

The baking time was 80–90 min at 177°C. The finished cakes were examined externally for crust appearance and internally for texture and softness of the crumb, and their cake specific volumes (CSV) were measured.

Results

Gel-filtration

The elution profiles of control and heat treated yolk plasma are shown in Fig. 1. The control plasma has three components each differing in distribution of molecular

size. I, eluting at the void volume, is probably aggregated LDL. II, is an intermediate component representing predominantly non-aggregated LDL. III is most likely a mixture of non-aggregated LDL + livetins (chick serum proteins). The major component is II representing 50–60% of the sample weight. From its elution behaviour, it has a mean diameter of 24 nm, with distribution range between 15 and 23 nm. These dimensions are in good agreement with those reported for LDL by Nichols *et al.* (1969).

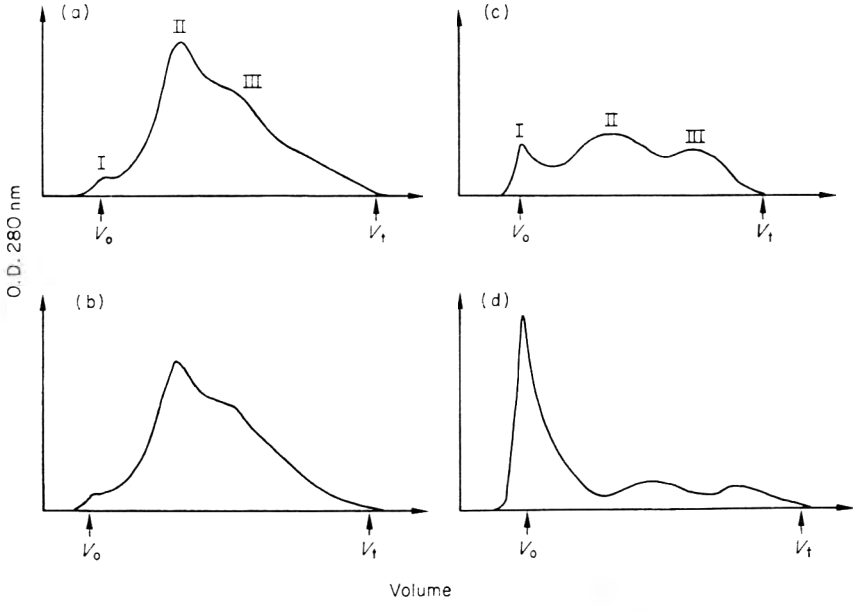

FIG. 1. Gel-filtration elution pattern of untreated yolk plasma at room and higher temperatures. (a) Control; (b) 55°C, 0·5 hr; (c) 60°C, 0·5 hr; (d) 62·5°C, 0·5 hr.

The elution profile does not show any dramatic change until after exposure of yolk plasma to 60°C, when aggregation of LDL begins to cause an increase in the intensity of peak I eluting at the void volume. At 62·5°C, the plasma sets to a firm gel. The profile of a suspension of this gel demonstrates that the intermediate size species has virtually been replaced by aggregates of larger sizes, exceeding 50 nm in diameter. The profile is not reversed when the set plasma is dispersed with egg white and sucrose and allowed to stand for 2 hr. Preliminary observation, however, indicates that dilution and extended storage of heat-set plasma bring about a significant deaggregation of the material.

Exposure of yolk plasma to freeze-thaw cycles produced visible gelation and massive aggregation (*vide* electron microscopy). The material when suspended however, was difficult to manipulate. Initial gel filtration profiles were anomalous in that they revealed only the non-aggregated fraction. It was noted that part of the material remained at the point of application and was strongly bound to the gel, a property exhibited by many aromatic compounds (Bretthauer & Golichowski, 1967). When 1–2%

glucose was added to the equilibrating buffer and the sample resuspended in the same, reasonable elution profiles were obtained. Figure 2a is the elution profile of this material. Most of the LDL type structures are aggregated and are eluted at the void volume. The small peak occurring at the elution volume corresponding to a mean diameter around 5 nm is mainly due to livetins. The results suggest that livetins do not participate significantly in the gelation process. Furthermore, dispersion of the frozen and thawed

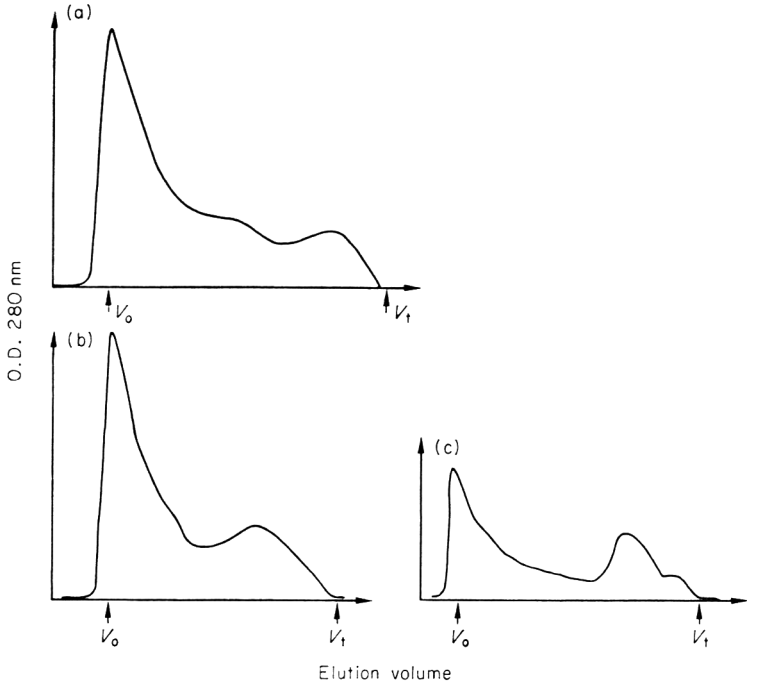

FIG. 2. Gel-filtration elution pattern of a suspension of yolk plasma gelled by freeze-thawing before (a) and after dispersion (b) with egg white and sucrose and (c) with egg white alone.

plasma with cake ingredients viz. the egg white + sucrose (Fig. 2b) or egg white alone (Fig. 2c) at the same concentration as in the recipe, did not reverse the process of aggregation during a period of 1 or 2 hr. Dilution and extended storage of the frozen and thawed material did not reveal any change in the profile. The results demonstrate that aggregation induced by freeze-thaw gelation is irreversible or at least very slowly reversible as compared to that produced by heat-setting at 62·5°C.

Electron microscopy

Plate 1 is a typical low magnification electron micrograph of yolk plasma. Both single particles and aggregated species are visible, the former however are preponderant. The single particles show a diameter distribution between 10 and 70 nm with a peak around

30 nm. The aggregated species vary in diameter between 0·25 and 0·75 μm. At high magnification (Plate 2), the interior of the aggregate species is clearly seen as consisting of clusters of numerous single particles. Some 70 nm particles are formed by cross-linking of two small particles, although single particles as large as 0·1 μm in diameter can also be seen. Whether the latter represent coalescence or are native to yolk plasma is not known. It is possible that preparative treatment, e.g. drying on the grid may produce artefacts arising from non-native coalescence or aggregation.

At low magnification (Plate 3) the ultra-structure of heat-set plasma indicates (i) a significant increase in the size range of aggregated species 0·2–0·6 μm in diameter and (ii) distinctly visible clustering of single particles (25–40 nm diameter) on a scale greater than that observed in the case of control plasma. At high magnification, numerous profiles of single particles 0·1–0·25 μm and aggregates 0·6–0·7 μm can be seen. The proportion of 30 nm diameter particles however seems to be greater than can be reconciled with gel-filtration elution pattern of the material (Fig. 1a). This may be because of the large dilution involved in the preparation of samples for electron microscopy.

Compared with the control and heat-set plasma, the frozen and thawed material shows a much greater increase in the overall aggregation state (Plates 5 and 6). At low magnification (Plate 5) 0·25–0·75 μm single particles are numerous. In addition, massive aggregates 3·5–5 μm in size consisting of cross-linked single species are seen. An enlarged view of this crosslinked network is shown in Plate 6. The cross-linked particles with holes in the aggregated give a sponge like appearance to the material. The frozen and thawed plasma exhibits a degree of aggregation which is greater than that of control plasma by about one to two orders of magnitude.

Cake trials

Results of the Madeira cake trial are presented in Table 1. They demonstrate that prior gelation of yolk plasma by freeze thawing or prior heat-setting at 62·5°C do not affect the baking performance of the plasma in this recipe. In fact, the performance is comparable with that of unfrozen yolk plasma or whole liquid yolk. The finished cakes

TABLE 1. Maderia cake trial data—weight for weight replaced of yolk by control or aggregated yolk plasma

	Fraction	VAC*	VAE*	FBV*	CSV*	Comments
1	Control whole yolk	2·0	1·90	1·53	3·15	Control slightly firmer
2	Control yolk plasma	2·0	1·86	1·53	2·95	All cakes acceptable
3	Heat set yolk plasma	1·90	1·90	1·56	3·10	Similar in texture,
4	Frozen and thawed yolk plasma	2·0	1·95	1·59	3·13	colour and taste

* See text for details.

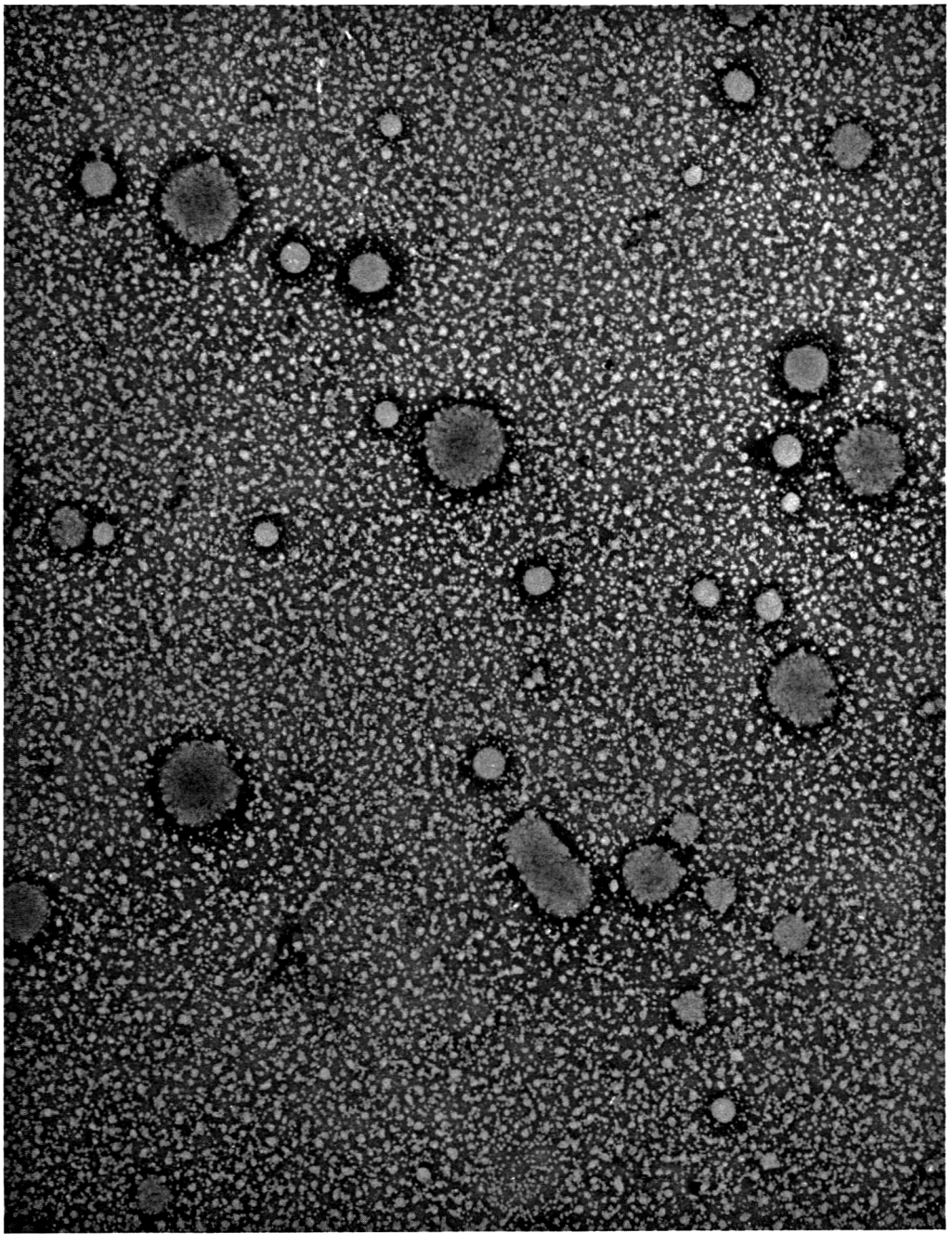

PLATE 1. Electron micrograph of a dilute solution of untreated yolk plasma. × 14,000.

(*Facing p.* 84)

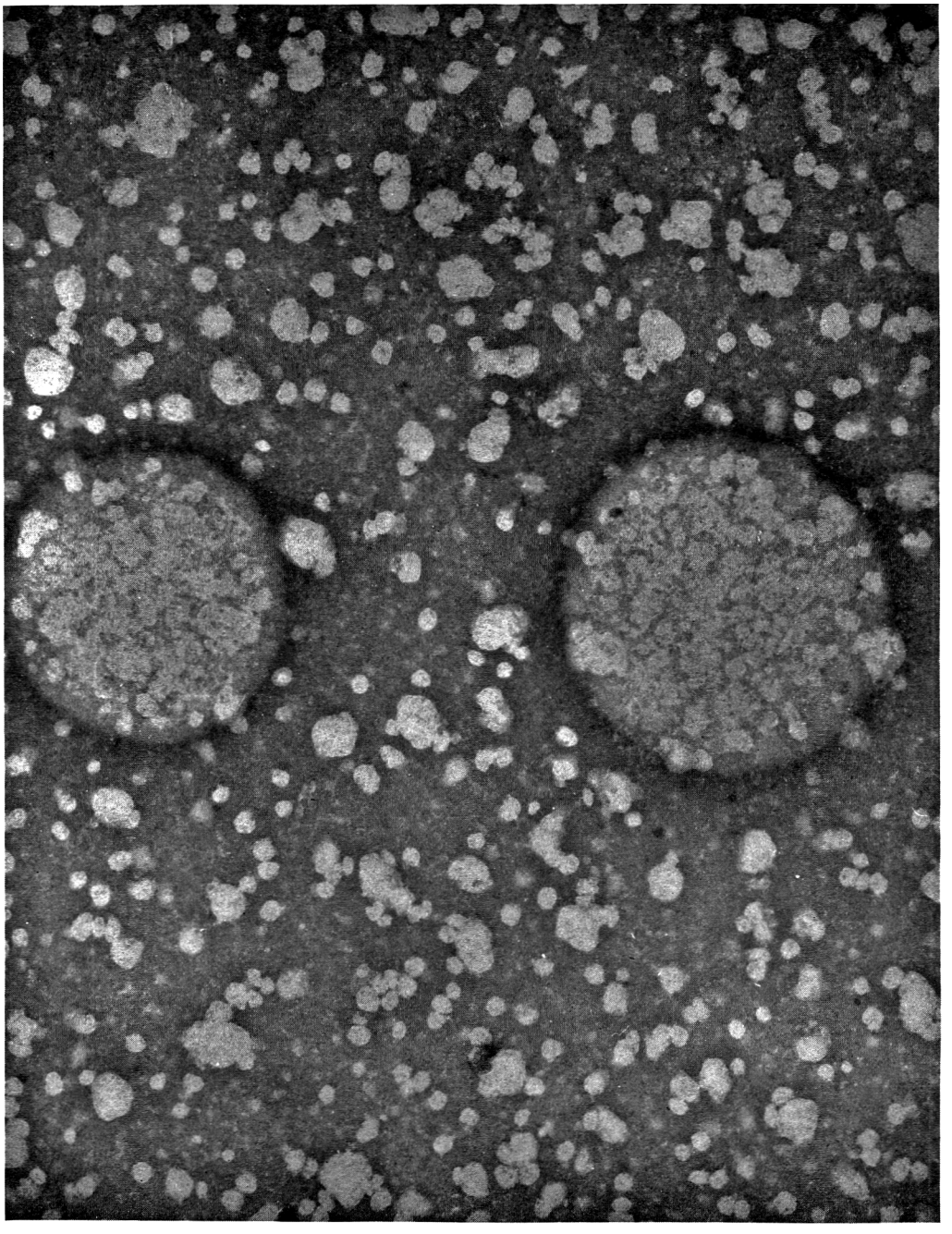

PLATE 2. Electron micrograph of a dilute solution of untreated yolk plasma. × 70,000.

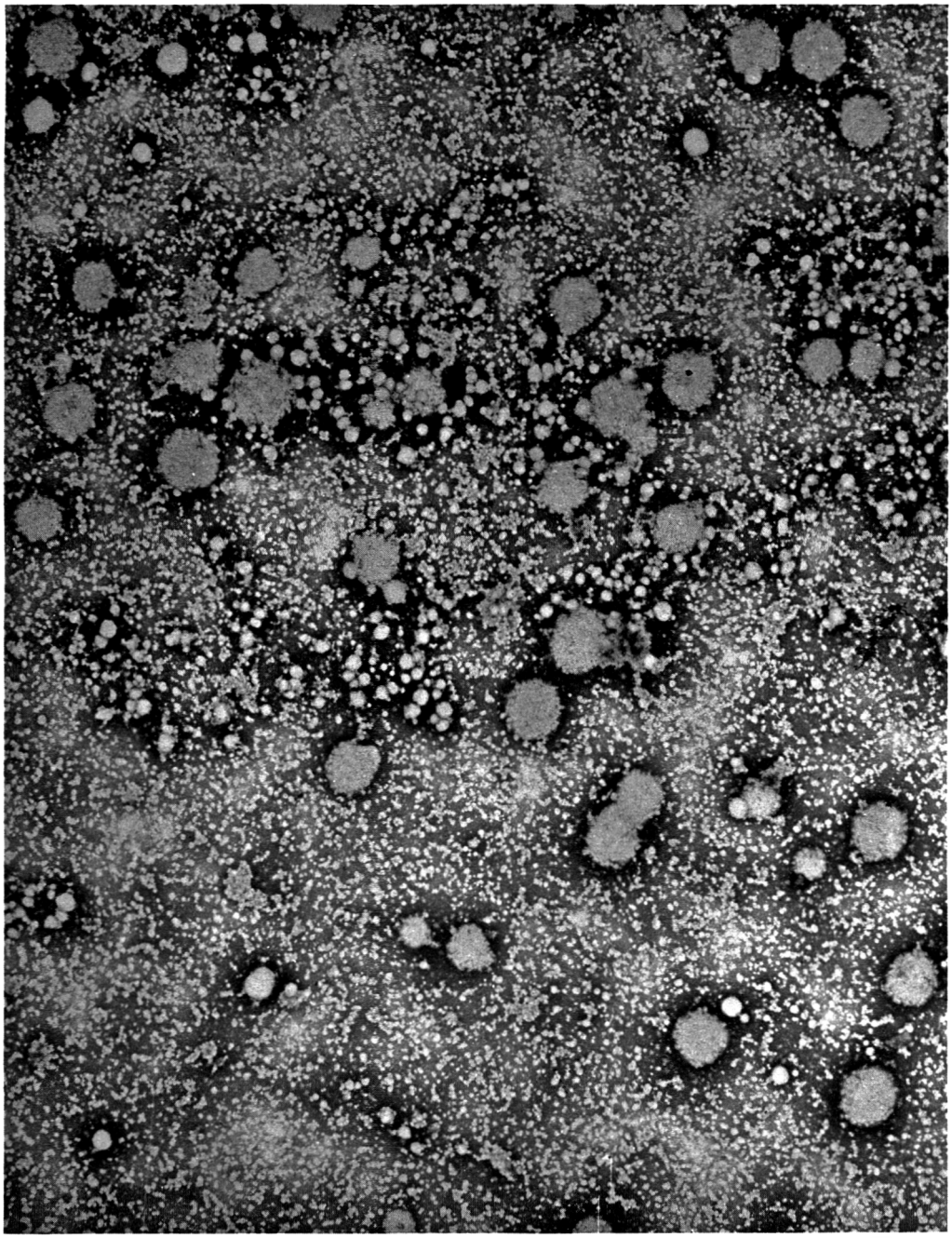

PLATE 3. Electron micrograph of a dilute suspension of heat-set yolk plasma. × 14,000.

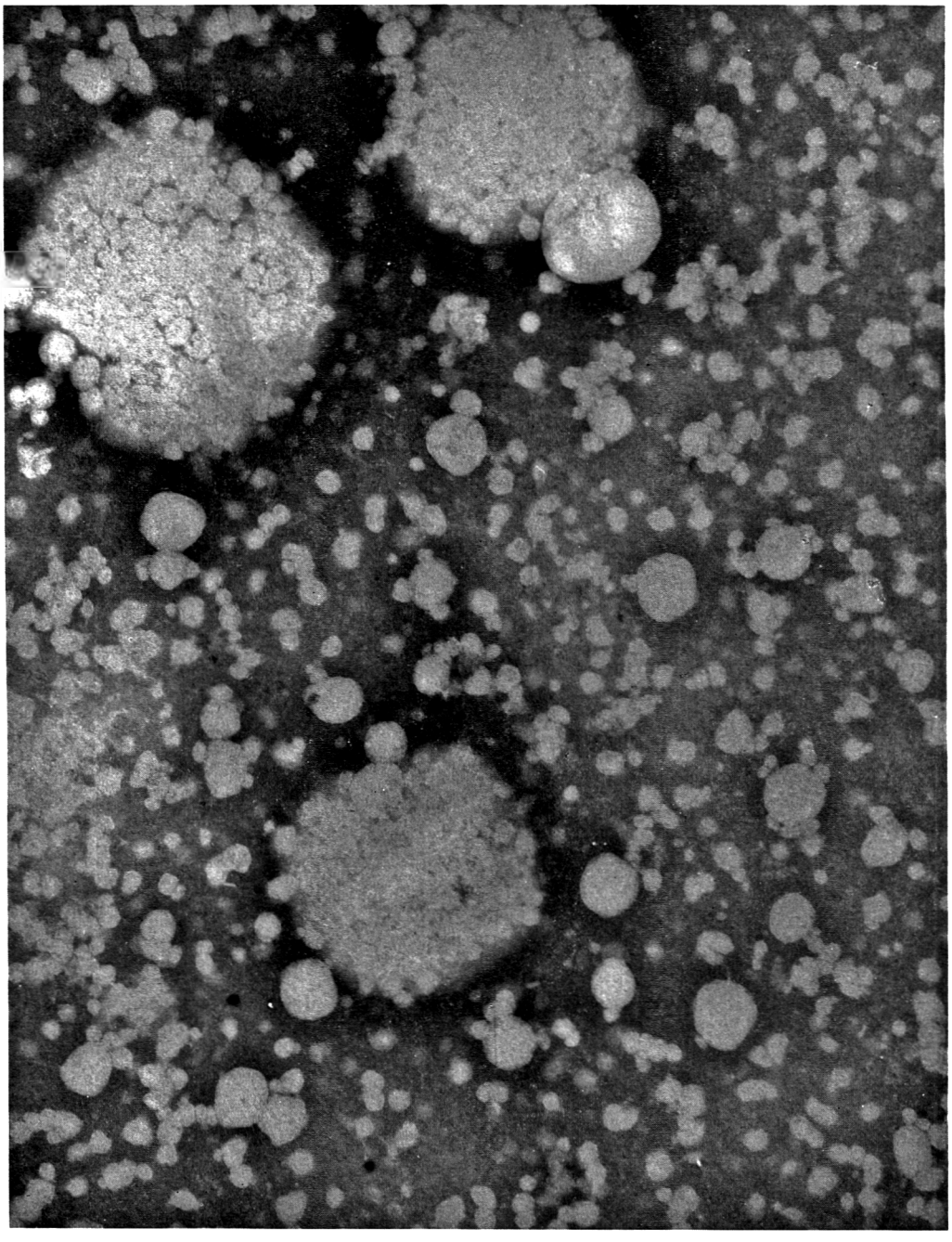

PLATE 4. Electron micrograph of a dilute suspension of heat-set yolk plasma. × 70,000.

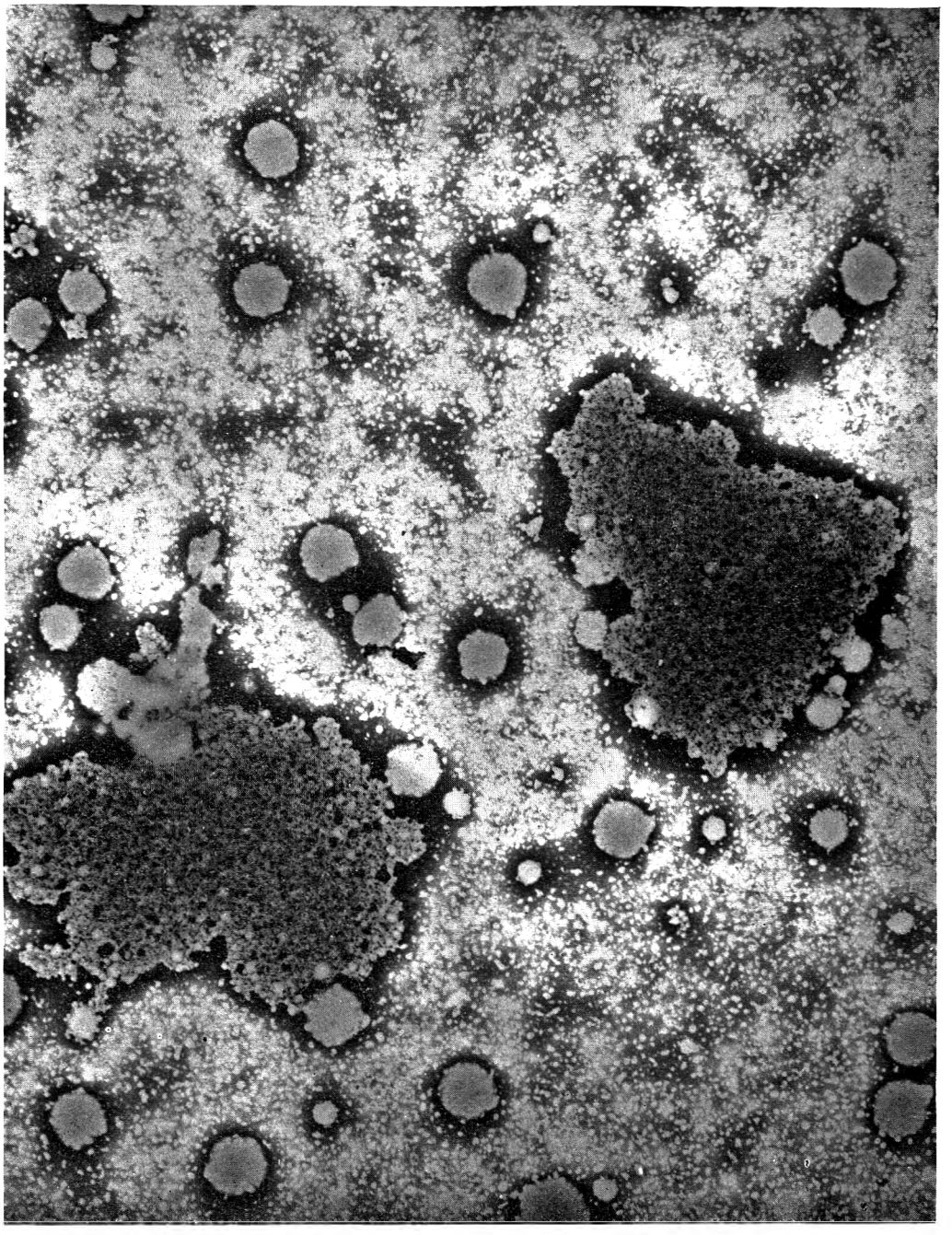

PLATE 5. Electron micrograph of a dilute homogenate of frozen and thawed yolk plasma. × 14,000.

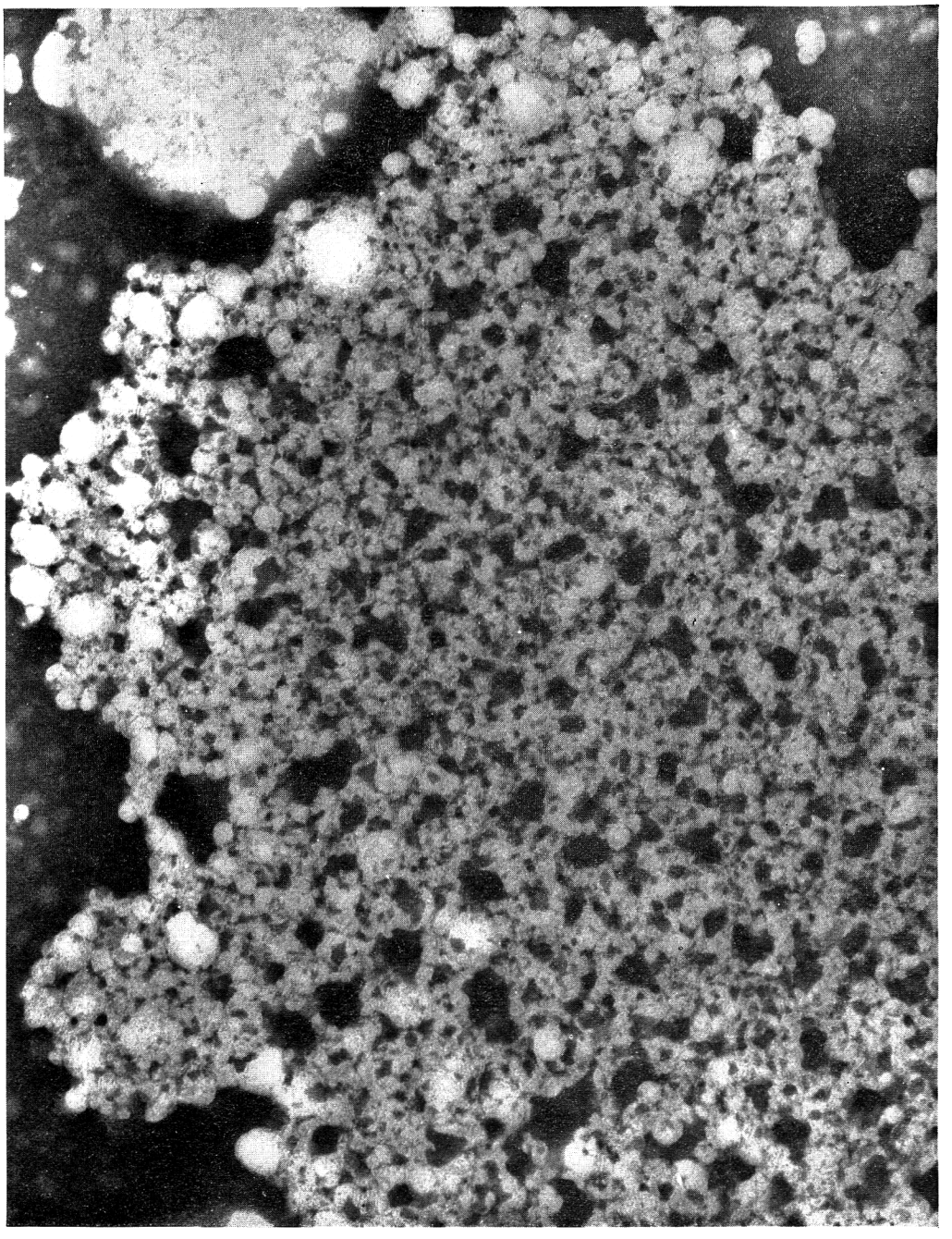

PLATE 6. An enlarged view of the large aggregate seen in Plate 5. × 70,000.

could not be distinguished from one another by a blind test. This is consistent with the early observations of Kamat *et al.* (1973) that frozen and thawed whole yolk gave Madeira cakes comparable with those obtained with unfrozen liquid yolk.

Discussion

Many lipoprotein systems exhibit aggregation phenomenon which is dependent on various factors like pH, ionic strength and concentration (Mauldin & Fisher, 1970; Wallach, Kamat & Gail, 1966). In the present study, all samples of control and treated yolk plasma were examined under identical conditions of these factors by gel filtration and electron microscopy. It is therefore reasonable to conclude that the observed aggregation states are directly comparable. We assume that these can be extrapolated to yolk plasma at 50% solids used for cake trials.

The foregoing results demonstrate that the degree of aggregation is in the following order: frozen and thawed plasma > heat-set plasma > control plasma. The gel filtration elution profiles of control and treated samples are in good agreement with the corresponding electron micrographs. The observed aggregation is predominantly due to cross-linking of LDL particles and is probably associated with the LDL protein moieties as suggested by other workers (Mahadevan, Satyanarayan & Kumar, 1969; Kumar & Mahadevan, 1970). Massive aggregation in frozen and thawed samples is consistent with the reported pseudoplastic behaviour and increased viscosity of yolk plasma treated similarly (Chang, 1970).

The aggregated samples give Madeira cakes which are virtually indistinguishable from those obtained with control plasma or whole liquid yolk. This indicates that the total surface area of lipoprotein particles is not critical for optimum yolk function in the Madeira recipe. It is possible that in the total environment of cake-making these particles may deaggregate and may still cover the fat surface as a monolayer. However, two of the batter ingredients, egg white and sucrose do not bring about a detectable deaggregation of the aggregated samples. If deaggregation is not occurring either at the batter stage or during subsequent baking, then it is unnecessary to invoke surface-coverage of fat by a monolayer of lipoproteins. It is possible that the surface of fat droplets may be covered by irregular arrays of lipoprotein multilayers.

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J. Fd Technol. (1974) **9**, 87–93.

Hydration and gelation of modified potato starches

W. G. CHILTON AND R. COLLISON

Summary

The gelation characteristics of natural potato starch and two modified starches have been compared with their water sorption isotherms determined at 25°C. The pregelatinized starch gels more readily than the natural starch, but has a lower water sorption capacity below 90% relative humidity.

The cross-linked starch gels less readily than the natural starch on heating but has a similar water sorption isotherm.

Introduction

Amongst the various modified starches which are widely used in the food industry are cross-linked and pregelatinized starches. The starting material in most cases is granular maize starch or potato starch.

Cross-linking can be brought about by a number of chemical agents including phosphorus oxychloride. Cross-linked starches have better heat-resisting properties than the parent starch and are used in products such as canned pie fillings. They also have an increased resistance to acid hydrolysis and are useful as thickeners in acid foods such as salad cream.

Pregelatinized starch is prepared by heating an aqueous starch slurry on a drum drier, which gelatinizes and subsequently dehydrates the starch all within a few seconds. The resulting material is able to reform a gel with cold water or milk, without any need for heating. Pregelatinized starches are used as thickening or gelling agents when heating is to be avoided, for example in instant puddings and pie fillings.

This paper describes the effects of cross-linking and pregelatinization of potato starch on gelation and water sorption characteristics.

Materials and methods

Materials

Three starches were used, one a typical potato starch and two modified starches derived from the same potato starch.

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Cross-linking was carried out using phosphorus oxychloride (2 cm^3 per 1000 g starch) at room temperature.

The pregelatinized starch was prepared on a drum drier from a slurry containing 40 g potato starch to 60 g water.

Brabender viscograph

A standard Brabender Viscograph (type 800 400) was used. Pastes of 5% and 8% starch in water were heated from 25°C to 90°C at 1·5 degrees per minute, held at 90°C for 20 min and subsequently cooled at 1·5 degrees per min. The paste consistency was recorded continuously in arbitrary Brabender units.

Sorption experiments

One gram samples of starch, previously dried in a vacuum oven at 110°C for 16 hr, were allowed to equilibrate in open weighing bottles over a variety of saturated salt solutions in sealed glass jars. The salt solutions were selected to give a range of relative humidities (Kaye & Laby, 1966). The jars were kept in a thermostatically controlled oven at 25°C ($\pm$ 0·4°C), and the starch samples weighed daily to 0·1 mg until equilibrium was reached.

Results

Viscograph curves

The viscograph curves for the three starches at 5% concentration are shown in Fig. 1. With natural potato starch, the viscosity remains almost zero until gelation starts at about 50°C, and then increases rapidly until reaching a peak of 1100 Brabender units at 75°C. On further heating, the viscosity decreases due to thermal degradation of the starch paste. This is followed by a viscosity rise on cooling, due to starch set-back.

The curves for the modified starches are very different. The pregelatinized starch forms a viscous paste within a few minutes of mixing with cold water, and this is reflected in the viscograph curve. However, the continuous heating and stirring produced in the instrument rapidly breaks down the paste to such an extent, that even after cooling a satisfactory gel is not reformed.

The cross-linked starch at the same concentration (5%) gels at about 60°C as was evident from microscopic examination, but this is not reflected in the viscosity which remains very low throughout heating and subsequent cooling. A higher concentration of cross-linked starch is necessary to produce a measurable viscosity (see Fig. 2 for 8% suspension), where an almost linear curve illustrates its thermal and mechanical stability.

Sorption isotherms

The water sorption isotherms for all these starches show the same general characteristics, and all are sigmoidal in shape (Fig. 3). Whereas the cross-linked starch is not

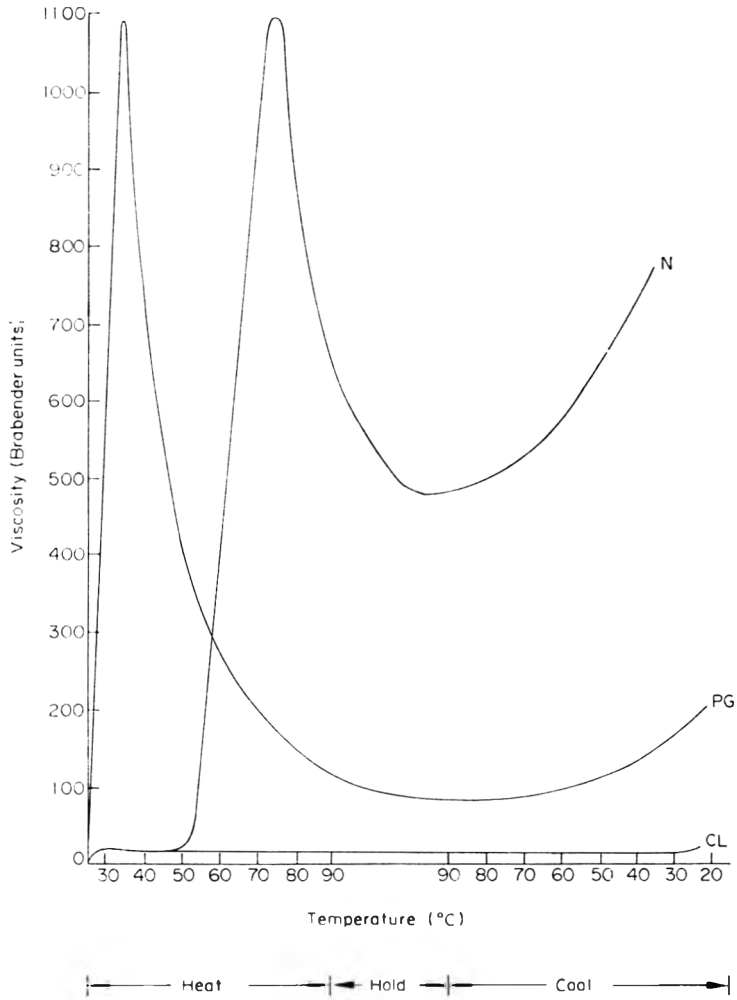


FIG. 1. Viscograph curves of 5% pastes of pregelatinized (PG), cross-linked (CL) and natural potato starch (N).

very different from the natural starch, the pregelatinized starch absorbs significantly less water at relative humidities up to 90%, but more water at higher humidities.

Sorption kinetics

The rates of water uptake followed a similar pattern for all three starches. Figure 4 shows the results for natural potato starch.

The time required to reach equilibrium is greater at the higher relative humidities, for example 3 or 4 days at 30% relative humidity compared with 15 days at 96% relative humidity. This is contrary to the usual pattern for fibres such as wool and

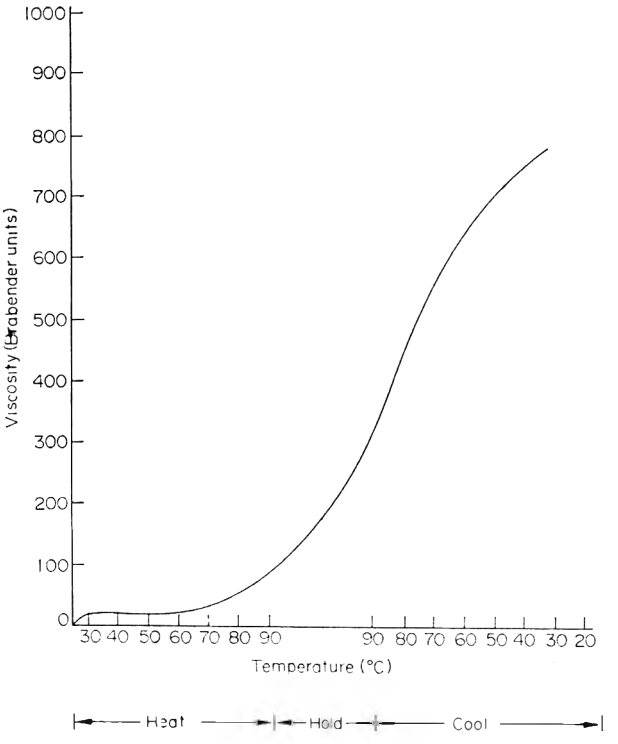


FIG. 2. Viscograph curve of 8% cross-linked starch.

regenerated cellulose (Crank, 1960) where equilibria are reached more quickly at the higher relative humidities.

It is suggested that with starch, the maximum uptake of moisture at high humidities requires time to allow for some molecular rearrangement within the granule, whereas at lower humidities the equilibria moisture can be absorbed with less structural change.

Discussion

(a) *Comparison of pregelatinized and natural starch*

The pregelatinized starch forms a gel much more easily than the natural potato starch. At high humidities, above 90% relative humidity, it also absorbs more water from the atmosphere, but at lower humidities its moisture uptake is lower than that of the parent potato starch.

Natural potato starch exists in the form of granules which contain branched (amylopectin) and unbranched (amylose) molecules. These are hydrogen bonded to form regions of crystalline material interspersed with amorphous regions of non-crystalline material. Many polymer chemists hold the view that there is not a sharp division

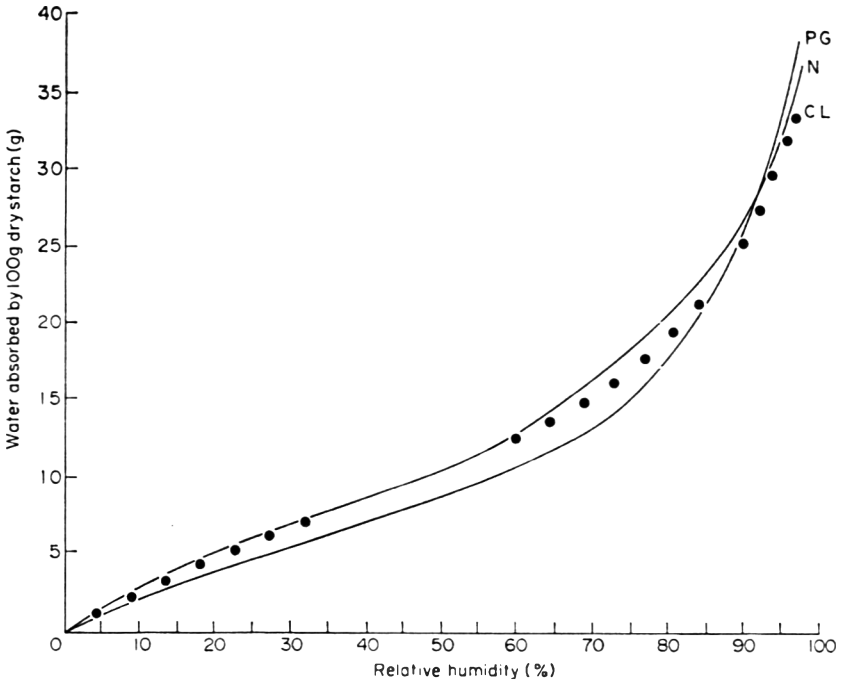


FIG. 3. Sorption isotherms of pregelatinized (PG), cross-linked (CL), and natural potato potato starch (N) at 25°C.

between crystalline and amorphous regions, but a spectrum of areas of varying degrees of order.

On gelation, some of the bonding within the granule is broken down (Leach, McCowan & Schoch, 1959). This allows the granules to swell and some of the starch molecules to disperse into solution. The pattern of gelation varies according to the botanical source of the starch, and is a reflection of the strength of bonding between starch molecules. Despite variations in the detailed pattern of behaviour, most starches start the gelation process at about 50°C.

The manufacture of pregelatinized starch involves gelation and disruption of the granules, followed immediately by rapid dehydration which allows little time for the starch molecules to reorientate themselves. As a result the internal bonding is weaker than in the original potato starch, and pregelatinized starch will readily reform a gel on mixing with cold water

The absorption of water from the atmosphere on to starch granules has been extensively studied, and the evidence strongly suggests that water molecules are distributed at sites throughout the granule and are not restricted to the surface (Hellman & Melvin, 1950). In this respect starch resembles other polymers including wool and cellulose (Cassie, 1945).

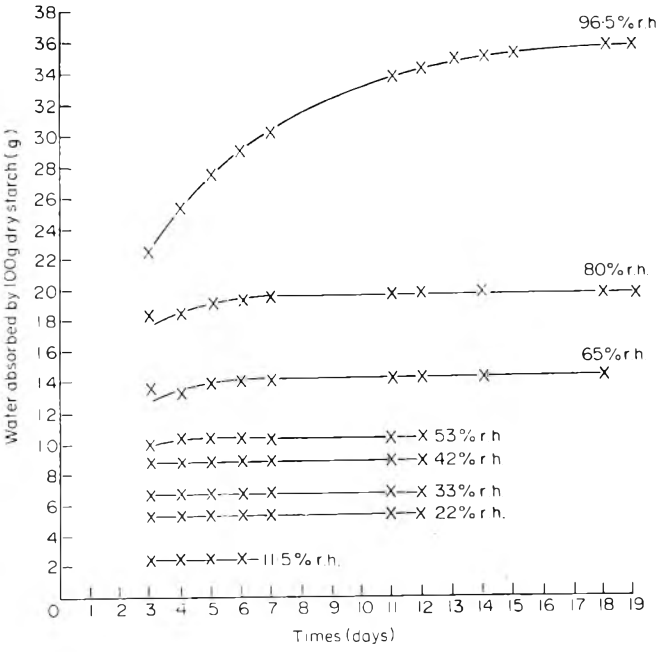


FIG. 4. The effect of relative humidity on the rate of water sorption by potato starch at 25°C.

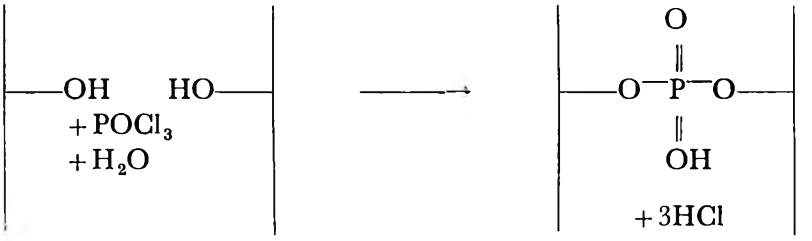
The nature of the water binding depends upon the amount of water present. Evidence from X-ray diffraction (Volman *et al.*, 1960) and from heats of dehydration (Collison & Dickson, 1971) suggests that the most heavily bound water (up to about 15%) is arranged so that each water molecule is hydrogen bonded on to two hydroxyl groups, either on adjacent starch molecules or on the same molecule. In order to form this structure, the starch chains must be arranged in a precise manner to bind the water.

In general, the extent of water sorption on to polymers at lower relative humidities depends on the availability of low energy sorption sites on the polymer. In the case of starch, these sites occur where starch-water-starch hydrogen bonds can form. The disarrangement of starch molecules during the manufacture of pregelatinized starch may well reduce its ability to combine with water in this manner, and this would explain why pregelatinized starch absorbs less water at lower relative humidities.

At high relative humidities the water is absorbed as multilayers of which only the first layer is directly linked to the polymer. Under these conditions, the amount of water sorbed will be restricted by the constraining effect of the crystallite regions, which limit the distortion which the granules are able to withstand on taking up water. Consequently the pregelatinized starch with its weaker internal bonding will be able to absorb more water than the potato starch.

(b) *The effect of cross-linking*

Cross-linking is brought about by the action of phosphorus oxychloride on dry starch, but as water is normally added in the food preparation stage the overall reaction may be written as follows.



Microscopic examination indicates that cross-linking does not damage the granule, but the chemical linking of adjacent starch molecules does prevent molecular dispersion on gelation. These effects of cross-linking are reflected by the properties of the starch in water sorption and gelation; the water sorption isotherm for the parent and cross-linked starches are very similar, whereas a lower viscosity paste is produced by the cross-linked product. Cross-linking also confers thermal and mechanical stability on the potato starch as shown by the continually rising viscosity in Fig. 2. By comparison the prolonged heating and stirring produced in the Viscograph breaks down natural potato starch gels which have relatively poor thermal stability.

Acknowledgments

We would like to thank AVEBE (Holland) for kindly providing the starch samples, and Mr R. M. Kordowicz for experimental assistance.

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(*Received* 11 *June* 1973)

J. Fd Technol. (1974) **9**, 95–103.

Quality of home frozen vegetables

I. Effects of blanching and/or cooling in various solutions on organoleptic assessments and Vitamin C content

MARGARET A. HUDSON, VALERIE J. SHARPLES, ELIZABETH PICKFORD AND MARGARET LEACH

Summary

Home frozen vegetables are normally blanched in water and cooled in iced water before freezing. In this survey, the effects of common household additives to blanching and/or cooling water were studied. Runner beans, peas, perpetual spinach and brussels sprouts were assessed by a tasting panel after being blanched and/or cooled in one of the following solutions, 1·2% NaCl, 2·0% NaCl, 1·2% sucrose, 0·25% sodium carbonate or 0·25% sodium bicarbonate, and stored at −18°C. The appearance of sprouts was slightly improved by 1·2% NaCl and 1·2% sucrose blanching liquids. Flavour of runner beans was significantly increased by the use of 1·2% salt, but the 2% levels significantly lowered scores for appearance and texture. Sugar significantly lowered texture scores for beans, but improved those for peas. Quality of spinach was not significantly affected by any treatment. Carbonate and bicarbonate treatments tended to have an adverse effect on texture and appearance.

Vitamin C analyses for thawed frozen sprouts blanched in 1·2% NaCl or 1·2% sucrose showed that the treatments did not lower the ascorbic acid content.

Introduction

It is widely accepted that if the pH of cooking or blanching water of vegetables is increased there is a softening of tissues, less conversion of chlorophyll to the olive brown pigment phaeophytin and a greater loss of ascorbic acid. Less is known about flavour changes, but Sweeney & Martin (1961) report a loss of flavour in frozen green vegetables blanched in buffers of pH greater than 7. Generally alkaline blanches are not recommended even though they may improve colour.

The use of sodium chloride in blanching water has sometimes been recommended, but Stoll (1970) reported loss of quality when kidney beans were blanched in salt before

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freezing. Woyke & Szaniawska (1969) found that 2% salt in blanching water had a stabilizing effect on Vitamin C and a favourable effect on the organoleptic properties of cauliflower and broccoli stored for 3 and 6 months at freezer temperatures. Tressler & Evers (1957) mention that hot sugar and acid solutions have been recommended for blanching but are not commonly used. Weinmann & Stührk (1941), working on dried vegetables, found that 0·5 or 1·0% NaCl added to blanching water reduced loss of dry matter and mineral substances, though this was an unimportant loss when gain in weight from NaCl was taken into account. Salt has been reported to affect texture and Lenz, Ramo & Weckel (1966) found that it decreased toughness of canned peas when added to the blanching water. A few books on home freezing recommend that vegetables should be packaged in brine to make them more tender, but Joslyn & Cruess (1929) found no marked differences between controls and treatments after packaging with and without brine, and our own exploratory results (Hudson & Sharples, 1970) also indicated no advantage from the use of this method.

As so little work had been done on the effects of additives to blanching water of frozen vegetables, this work was undertaken in an attempt to gain a fuller insight into the effects of common household substances on organoleptic changes in home frozen vegetables after storage at −18°C for periods of up to 12 months. The following treatments were used:

(1) addition of 1·2% sodium chloride* to blanching and/or cooling water;
(2) addition of 2·0% sodium chloride to blanching water;
(3) addition of 1·2% sucrose to blanching and/or cooling water;
(4) addition of 0·25% sodium carbonate or bicarbonate to blanching water.

In addition losses of Vitamin C were determined in thawed uncooked sprouts which had been blanched in untreated water or in water to which NaCl or sucrose had been added.

Materials and methods

Materials

Freshly picked runner beans (Goliath), peas (Onward) and sprouts (Thor) were purchased from local growers. Spinach (Perpetual) was grown at the Research Station. All material was in a good condition, and to make it representative of home use there was some latitude in the maturity of the vegetables. The peas tended to be starchy, as is sometimes the case when surplus material in the garden is used for home freezing.

Organoleptic assessments (*Table* 2)

Material was prepared in the normal way for freezing (M.A.F.F. Bulletin 21, 1971), i.e. spinach leaves were deribbed and washed, peas were shelled, beans were strung

* 1·2% (1 level teaspoon/pint water) is the salt concentration normally used in the home for cooking vegetables.

and cut into 2 cm diagonal slices, and the outer leaves of sprouts were removed. The prepared material was divided into 333 g samples, each of which was loosely tied in butter muslin and plunged for 3 min into 5120 ml boiling water in an aluminium saucepan, then cooled for 3 min in 3413 ml cold (2°C) water. Tap water (Bristol Waterworks, Barrow Gurney Reservoir) with a total hardness as $CaCO_3$ of 200 ppm was used throughout, apart from some experiments with sprouts where glass distilled water was used for blanching and cooling, but not for cooking. After drainage, material was packaged in polythene bags in waxed cartons, frozen at −18°C and stored at this temperature. Replicate material of beans and peas was tasted after 3, 6 and 9 months, and replicate samples of spinach after 5 months. In the case of brussels sprouts sets of material were tasted after 0·5, 3, 6 and 12 months.

Material was cooked from frozen in 284 ml tap water to which 1·2% sodium chloride was always added. In an additional treatment, commonly used in the home, 1·2% sucrose was added to the cooking water of peas. The water was brought to the boil in a covered aluminium saucepan, the vegetables were added and the lid was kept on during cooking. Peas were cooked 4 min, beans 7 min, sprouts 8·5 min and spinach 9 min from the time the frozen block was immersed in the boiling water.

Up to six different treatments of the same vegetable were served at any one time to the tasting panel, which consisted of eight to sixteen experienced tasters. Equal halves of each treatment were placed into two containers, one of which was used for assessments of colour and appearance, and the other for evaluations of texture and flavour. Marking was on a 1–7 hedonic (personal preference) scale, 7 being excellent, 6 good, 5 above average, 4 average, 3 below average, 2 poor, 1 objectionable.

Analyses of variance were made for individual storage periods and for the combined tasting sessions, but data in the tables have been restricted to the latter analyses, since only two instances were found where there were significant differences between means for different storage periods.

Vitamin C determination (*Table* 3)

One hundred grams of sprouts (Thor) in butter muslin were blanched 3 min in 1 l tap or distilled water, with or without 1·2% sodium chloride or 1·2% sucrose, cooled in 568 ml cold tap or distilled water, packed in polythene bags in cartons, and frozen and stored at −18°C. Material was tested in triplicate after 1, 6 and 12 months' storage.

The uncooked frozen material, thawed overnight at 2°C, was homogenized for 3 min in 160 ml of 6% metaphosphoric acid, and the filtrate obtained was diluted to give an acid concentration of 2%. Ascorbic acid + reductone-like compounds (AS + R) and dehydroascorbic acid + dehydroreductone-like substances (DHA + DHR) were assayed by the dichloroindophenol/homocysteine thiolactone method of Timberlake & Bridle (1968). Values for R and DHR were also obtained, but as negative values were sometimes obtained for DHR, separate values for the interfering substances have not been given. As no notable changes in the levels of Vitamin C occurred during the 12 month

storage period results, expressed as mg/100 g fresh material, are for the means of three storage periods.

Results and discussion

pH values of blanching solutions

Mean pH values for the blanching liquor before boiling and after being brought to the boil and immediately cooled are given in Table 1. The solutions were made up in aluminium saucepans, similar to those used for the blanching work.

TABLE 1. pH values of blanching solutions

Solution	Before boiling	After bringing to the boil
Plain tap water	7·6	8·0
1·2% NaCl in tap water	7·4	7·8
2·0% NaCl in tap water	7·4	7·8
1·2% sucrose in tap water	7·6	7·8
0·25% Na_2CO_3 in tap water	10·2	10·0
0·25% $NaHCO_3$ in tap water	8·1	8·1
Distilled water*	5·3	5·9
1·2% NaC. in distilled water*	5·2	5·6
1·2% sucrose in distilled water*	5·5	5·9

* Distilled water values were obtained using a ground glass sleeve reference electrode and a screened glass electrode. Despite these precautions an upward drift of pH was still detectable, and the values are only approximate.

1·2% *sodium chloride treatments*

In all vegetables there was a suggestion that 1·2% salt improved flavour, but the increase in mean scores was only significant in the case of runner beans blanched or cooled in the solution ($P=0·05$). Beans had a significantly worse flavour ($P=0·05$) after the longest (9 month) storage period. Salt treatments had no significant effect on texture or colour scores, but significantly improved appearance of sprouts blanched in distilled water ($P=0·01$). There was the indication that colour was improved in sprouts blanched in salt distilled water and that after both tap and distilled salt water blanches an initial improvement in colour occurred which was lost by the end of a year's storage. This may explain why no significant colour improvements were detected in salt blanched material which was first assessed after 3 months storage. (Subsequent papers in this series show that salt added to blanching water reduces the conversion of chlorophyll to phaeophytin.)

Vitamin C levels of sprouts were not adversely affected by the addition of 1·2% salt to the blanching water.

2% *sodium chloride treatments*

Two per cent salt added to blanching water caused softening and sliminess in runner beans and significantly ($P=0·01$) lowered scores for texture and appearance; there was an indication that flavour was also worse. Other vegetables were not affected, though results suggested that the 2% salt blanch marginally improved quality of spinach and sprouts.

1·2% *sucrose treatments*

In the case of sprouts blanched in 1·2% sucrose tap water there was an apparent increase of all mean scores, but this was significant only for appearance ($P=0·001$). The additive had no adverse effect on the vitamin C concentration of the uncooked vegetables. Sugar had a favourable effect on the texture of peas when added to the blanching ($P=0·001$) or cooling ($P=0·01$) water or to both ($P=0·01$), and apparently reduced mealiness. Peas treated with sugars had a significantly better texture ($P=0·05$) after 3 months than after 6 or 9 months storage. Texture was also improved ($P=0·05$) by the well established practice of cooking water blanched frozen peas in sugared water but this method was significantly less effective ($P=0·05$) than blanching in sugar and cooking in salted water without sugar.

Beans were the only vegetables tested which were adversely affected by the sucrose treatments. Mean scores were generally lower and a significant fall in texture quality was detected after the addition of sucrose to blanching ($P=0·01$) or cooling ($P=0·05$) water.

0·25% *sodium bicarbonate and sodium carbonate solutions*

Bristol tap water is very alkaline, and the addition of 0·25% sodium bicarbonate scarcely changes the pH (Table 1), but 0·25% sodium carbonate increases the pH to 10·2. High pH's are known to favour chlorophyll retention and to affect texture adversely, and in these experiments a significantly better colour was observed in beans blanched in carbonate ($P=0·01$). Texture was not significantly affected but bicarbonate added to the blanching water of peas significantly lowered scores for appearance ($P=0·01$) and there were indications that the appearance and texture of all vegetables blanched in the two solutions were worsened. Flavour of beans blanched in carbonate was significantly lowered ($P=0·05$).

Conclusions

The addition of 0·25% sodium bicarbonate or sodium carbonate to blanching water produced poorer overall quality of cooked frozen vegetables than conventional plain

TABLE 2. Organoleptic assessments (scoring scale 1–7) of cooked vegetables blanched and/or cooled in various solutions and stored at −18°C

	Blanching water	Cooling water	Beans (tap water)		Peas (tap water)		Spinach (tap water)		Sprouts (tap water)		Sprouts (distilled water)	
			Mean score*	s.e.	Mean score*	s.e.	Mean score*	s.e.	Mean score*	s.e.	Mean score*	s.e.
Appearance	—	—	4·3a	0·20	3·7a	0·15	3·9a	0·13	3·2a	0·22	3·6a	0·22
	1·2% NaCl	—	4·2a		3·8a		3·6a		3·6a		4·6b	
	—	1·2% NaCl	4·2a		3·6a		3·9a		—		—	
	1·2% NaCl	1·2% NaCl	4·1a		3·9a		3·6a		—		—	
Colour	—	—	4·1a	0·11	3·9a	0·09	3·9a	0·14	4·0a	0·22	3·3a	0·22
	1·2% NaCl	—	4·2a		4·1a		3·9a		4·1a		3·9a	
	—	1·2% NaCl	4·0a		4·0a		4·1a		—		—	
	1·2% NaCl	1·2% NaCl	4·1a		4·0a		4·1a		—		—	
Texture	—	—	4·0a	0·15	3·6a	0·15	4·2a	0·15	3·5a	0·12	3·7a	0·12
	1·2% NaCl	—	3·8a		3·5a		4·2a		3·6a		3·6a	
	—	1·2% NaCl	4·1a		3·6a		4·2a		—		—	
	1·2% NaCl	1·2% NaCl	3·9a		3·7a		4·0a		—		—	
Flavour	—	—	3·7a	0·11	3·6a	0·10	3·9a	0·21	3·4a	0·16	3·4a	0·16
	1·2% NaCl	—	4·1b		3·7a		4·2a		3·9a		3·5a	
	—	1·2% NaCl	4·1b		3·8a		4·7a		—		—	
	1·2% NaCl	1·2% NaCl	3·9ab		3·8a		4·1a		—		—	
Appearance	—	—	4·4a	0·16	3·7a	0·15	3·9a	0·14	3·9a	0·31	—	—
	2·0% NaCl	—	3·6b		3·5a		4·2a		4·0a		—	
Colour	—	—	4·3a	0·10	3·9a	0·09	4·4a	0·13	3·7a	0·28	—	—
	2·0% NaCl	—	4·2a		3·9a		4·7a		4·0a		—	
Texture	—	—	4·4a	0·11	3·6a	0·15	4·1a	0·22	3·9a	0·15	—	—
	2·0% NaCl	—	3·9b		3·5a		4·3a		3·8a		—	
Flavour	—	—	4·0a	0·15	3·6a	0·10	4·1a	0·20	3·5a	0·18	—	—
	2·0% NaCl	—	3·7a		3·9a		4·1a		3·8a		—	

Appearance	—	—	4·4a	0·16	3·7a	0·15	3·9a	0·14	3·2a	0·22	3·6a	0·22
	1·2% sucrose	—	4·4a		3·8a		4·1a		4·5b		3·8a	
	—	1·2% sucrose	4·3a		3·6a		3·9a		—		—	
	1·2% sucrose	1·2% sucrose	4·4a		3·6a		3·9a		—		—	
	—	—	—		3·8a†		—		—		—	
Colour	—	—	4·3a	0·10	3·9a	0·08	4·4a	0·13	4·0a	0·22	3·3a	0·22
	1·2% sucrose	—	4·1a†		4·0a		4·7a		4·2a		3·0a	
	—	1·2% sucrose	4·3a		4·2a		4·3a		—		—	
	1·2% sucrose	1·2% sucrose	4·2a		3·9a		4·4a		—		—	
	—	—	—		4·1a†		—		—		—	
Texture	—	—	4·4c	0·11	3·4a	0·06	4·1a	0·22	3·5a	0·12	3·7a	0·12
	1·2% sucrose	—	3·9a		3·9d		4·2a		3·6a		3·7a	
	—	1·2% sucrose	4·0ab		3·7cd		4·1a		—		—	
	1·2% sucrose	1·2% sucrose	4·0abc		3·6bc		4·0a		—		—	
	—	—	—		3·6bc†		—		—		—	
Flavour	—	—	4·0a	0·15	3·6a	0·12	4·1a	0·20	3·4a	0·16	3·4a	0·16
	1·2% sucrose	—	3·8a		4·0a		4·2a		3·8a		3·4a	
	—	1·2% sucrose	4·0a		3·8a		3·7a		—		—	
	1·2% sucrose	1·2% sucrose	3·9a		3·8a		3·9a		—		—	
	—	—	—		3·9a†		—		—		—	
Appearance	—	—	4·3a	0·20	3·7b	0·15	3·9a	0·23	—	—	—	—
	0·25% $NaHCO_3$	—	3·6a		2·8a		3·8a		—		—	
	0·25% Na_2CO_3	—	3·6a		—		3·8a		—		—	
Colour	—	—	4·1a	0·11	3·9a	0·09	3·9a	0·17	—	—	—	—
	0·25% $NaHCO_3$	—	4·4ab		3·6a		4·0a		—		—	
	0·25% Na_2CO_3	—	4·7b		—		4·0a		—		—	
Texture	—	—	4·0a	0·15	3·6a	0·15	4·2a	0·25	—	—	—	—
	0·25% $NaHCO_3$	—	3·7a		3·5a		4·1a		—		—	
	0·25% Na_2CO_3	—	3·4a		—		3·8a		—		—	
Flavour	—	—	3·7b	0·11	3·6a	0·10	3·9a	0·27	—	—	—	—
	0·25% $NaHCO_3$	—	3·5ab		3·7a		4·2a		—		—	
	0·25% Na_2CO_3	—	3·3a		—		3·6a		—		—	

* Mean scores followed by the same letter do not differ significantly from one another (at $P=0·05$).

† 1·2% sucrose added to cooking water.

TABLE 3. Vitamin C content of thawed frozen sprouts blanched with and without additives

Blanching water	Additive	Ascorbic and dehydroascorbic acids + R + DHR (mg/100 g)	Ascorbic acid + R (mg/100 g)	Dehydroascorbic acid + DHR (mg/100 g)
Tap	—	109·8	98·4	11·4
	1·2% NaCl	108·7	101·1	7·6
	1·2% sucrose	103·1	102·1	1·0
Glass distilled	—	101·1	94·5	6·6
	1·2% NaCl	107·7	100·3	7·4
	1·2% sucrose	109·6	102·1	7·5

R = reductone-like compounds.
DHR = dehydroreductone-like substances.

water blanching treatments and is not to be recommended. The use of sodium chloride or sucrose in blanching and/or cooling water sometimes produced marginally better quality, which varied according to the vegetable in question. The additions of these two common household substances to blanching water of sprouts had no adverse effect on vitamin C content, and thus it seems that there is no objection to their use for scalding.

The quality (flavour) of runner beans was improved by adding 1·2% sodium chloride to the blanching or cooling water, but the use of higher salt concentrations or the use of 1·2% salt in both the blanching and cooling water were less favourable (2% salt in the blanching water significantly lowered scores for appearance and texture). The use of sucrose solutions for blanching or cooling also had an adverse effect on texture and is not to be recommended for this vegetable.

Quality of peas was not affected by salt treatments, but the texture was significantly improved after addition of sugar to blanching and/or cooling water. The addition of sugar to blanching water appeared to produce better results than the conventional treatment of water blanching and cooking in sugar.

Spinach was not affected significantly by any of the treatments, but seemed to benefit by the addition of 2% sodium chloride to the blanching water.

Quality of brussels sprouts was marginally improved by the use of 1·2% sucrose or 1·2% NaCl in the blanching water.

From these results it can be concluded that somewhat better results can be obtained in the home by the addition of 1·2% sodium chloride to the blanching water of runner beans and sprouts and by the addition of 1·2% sucrose to the blanching water of peas and sprouts. Two per cent salt solutions can be used with advantage for blanching perpetual spinach, but in the case of runner beans this level is too high to produce good results.

Acknowledgments

This work was financed by the Ministry of Agriculture, Fisheries and Food. Miss M. Holgate and the Statistics Section are thanked for statistical guidance and the Tasting Panel for its participation in the work.

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(*Received* 11 *May* 1973)

J. Fd Technol. (1974) **9**, 105–114.

Quality of home frozen vegetables

II. Effects of blanching and/or cooling in various solutions on conversion of chlorophyll

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Summary

The previous paper in this series covered organoleptic and Vitamin C assessments of vegetables blanched and/or cooled in various salt and sugar solutions, and stored at −18°C for up to 12 months. This paper deals with the effects of these additives on percentage conversion of chlorophyll to phaeophytin after blanching or after storage. Dietrich's method (1958) was used for the assays.

Results indicated that a number of salts (NaCl, KCl, Na_2SO_4, K_2SO_4) added to blanching water had little effect on pH, but could cause a considerable reduction in the amount of chlorophyll conversion in spinach and sprouts. Levels of NaCl above 1·2% in tap blanching water and the addition of 1·2% NaCl to the cooling water were less effective than the addition of 1·2% salt to the blanching water. During serial blanching in 1·2% NaCl tap water, chlorophyll retention was highest after the first blanch. In peas and runner beans 1·2% NaCl added to tap blanching water caused little reduction in chlorophyll conversion, but during 9 months storage the beneficial effect of the salt was maintained in the uncooked material. 0·25% sodium carbonate had the greatest effect in increasing pH and in reducing chlorophyll conversion. 0·25% sodium bicarbonate reduced conversion to the same extent as 1·2% NaCl, but induced an increase in pH. Using distilled water, levels of conversion were much higher, and the 1·2% NaCl treatments had a greater effect. Sucrose was not effective in preventing conversion.

Introduction

It is well known that chlorophyll is more easily converted to its olive-brown degradation product phaeophytin at low pH's than under more alkaline conditions (Mackinney & Weast, 1940; Schneider, 1967; Sweeney & Martin, 1961), and it is normally assumed that loss of colour during cooking is due to release of cellular acids which have a deleterious effect on the chlorophyll. Many attempts have been made to improve colour of frozen vegetables by blanching in alkaline solutions (Kanitz, 1941; Schneider, 1967; Sweeney & Martin, 1961). Sweeney & Martin (1961) found pH changes between

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6 and 7 to be crucial in the retention of chlorophyll; after blanching in buffers above pH 7 little colour improvement was obtained. These authors found, as did Eheart & Gott (1965), that chlorophyll *a* was more easily degraded than chlorophyll *b* and that chlorophyll retention was closely linked with the pH of the cell sap.

Bengtsson & Bosund (1969) blanched peas in tap water and in concentrated blanching water and obtained the same flavour for the two treatments, but a poorer coloured product when concentrated blanching water was used. They attributed this to the lower pH of the concentrated blanching water.

Many workers have suggested that pH is not the only factor influencing conversion of chlorophyll. Eheart & Gott (1965) proposed that the rate of H^+ ion transport or the degree of chloroplast rupture might affect conversion, and Fleischman (1969) considered that for spinach a sodium phosphate buffer improved colour, not only by raising pH and increasing the amount of chlorophyll to chlorophyllide conversion, but also by binding chlorophyll and chlorophyllide to the chloroplast matrix, thus making them less vulnerable to the attack of acids.

It is generally agreed that most chlorophyll degradation occurs during the cooking, rather than the blanching process, but there is confusion in the literature as to how much chlorophyll degradation occurs during storage at $-18°C$, the average running temperature for the domestic freezer. Dietrich *et al.* (1960) found the rate of chlorophyll conversion to increase exponentially with temperature and found that after 9 months storage at $-18°C$ a further 8% and 2% conversion occurred in bush beans (Dietrich *et al.*, 1957) and spinach (Dietrich *et al.*, 1960) respectively. Wagenknecht, Lee & Boyle (1952) found no chlorophyll loss in peas stored at $-18°C$, and Eheart (1967) found significant increases in chlorophyll *a* and total chlorophyll when frozen broccoli was stored for 6 months, but by the end of 12 months percentage chlorophyll retention had fallen to below its original level.

In the present investigations a study of the effects of salts and sugars, added to blanching and/or cooling water, on chlorophyll retention before freezing and after storage at $-18°C$ was made. The effects of these substances on organoleptic assessments and Vitamin C of various green vegetables has previously been reported (Hudson *et al.*, 1974). Weinmann & Stühr k (1941) found that 0·5 and 1·0% levels of salt in blanching water improved colour of frozen white cabbage, but had no effect on red cabbage; the salt was also found to prevent minerals being lost during blanching. Eheart (1969) soaked broccoli for 24 hr in 2·5% NaCl solution before water blanching and obtained extremely low chlorophyll retention.

Materials and methods

Materials

Spinach (Perpetual) and some of the brussels sprouts (Thor) were grown at Long Ashton. Peas (Onward), beans, (Achievement) and a few brussels sprouts were

purchased from local growers. For processing, spinach leaves were deribbed and washed, peas were shelled and beans were strung and chunked. Only the outer green leaflets of sprouts were used.

Methods

Throughout the investigations blanching was carried out by placing 130 g of fresh material into a butter muslin bag which was immersed in 2100 ml of untreated or treated boiling water for 3 min. Material was then removed from the boiling water, cooled for 3 min in 1400 ml cold (2°C) untreated or treated water and drained. Unless otherwise stated tap water, with a total hardness as $CaCO_3$ of 200 ppm and a pH of 7·8–8·3, from Barrow Gurney reservoir was used. All blanching was carried out using small aluminium saucepans, which were loosely covered with lids. For serial blanching, the volume of the liquid was adjusted to 2100 ml with untreated water after each successive scald. Material was packaged in polythene bags inside waxed cartons and frozen and stored at −18°C.

Apart from a few experiments where spinach and runner beans were cooked for 6 and 10 min respectively in aluminium saucepans with lids, all chlorophyll/phaeophytin analyses were made on uncooked material.

Throughout the experiments the method of Dietrich (1958) has been used for determinations of percentage conversion of chlorophyll to phaeophytin. Assays were carried out on both freshly blanched and on frozen material, which was placed in a refrigerator at 2°C the night before the determinations. Three replicates were generally used for each test.

Results

pH values of blanching solutions

Values for pH of the pure solutions made up in aluminium saucepans before boiling, after the solution reached boiling point, and after 3 min boiling are given in Table 1.

TABLE 1. Approximate pH values of blanching solutions (A) before boiling, (B) after being brought to boil and (C) after being boiled for 3 min in aluminium saucepans

Solution	Tap water			Distilled water		
	A	B	C	A	B	C
Plain water	7·6	8·0	8·4	5·3	5·9	7·7
1·2% NaCl	7·4	7·8	8·8	5·2	5·6	7·1
2·0% NaCl	7·4	7·8	8·9	5·2	6·0	7·1
1·53% KCl	7·6	7·8	8·9	5·5	6·2	7·6
1·46% Na_2SO_4	7·8	8·1	9·3	5·8	6·3	7·7
1·79% K_2SO_4	7·6	8·0	9·2	5·5	6·2	7·3
0·25% Na_2CO_3	10·2	10·0	10·0	10·2	10·2	10·2
0·25% $NaHCO_3$	8·1	8·6	9·5	8·3	8·8	9·6
1·2% sucrose	7·6	7·8	8·4	5·5	6·4	7·6

The values are approximate only, due to the difficulty in standardizing the amount of carbon dioxide given off during boiling, and due to the upward drift in pH readings which occurred especially with the unbuffered distilled water solutions.

The figures serve to show that, except in the case of Na_2CO_3 solutions, there is during 3 min boiling, an increase in pH, probably due to release of carbon dioxide and, to a lesser extent, to solubilization of aluminium. 0·25% Na_2CO_3 in both tap and distilled water increased the pH to about 10·0 and $NaHCO_3$ raised the pH's of tap and distilled water by approximately 0·5 and 3·0 units respectively. The other salts and sucrose had little effect on pH.

Changes in chlorophyll/phaeophytin ratios after blanching in increasing concentrations of NaCl

From Table 2 it is seen that chlorophyll conversion was reduced by the addition of 1·2% salt to blanching water. This applied especially in the case of spinach, where conversion could be reduced by 6% in salted tap water and by about 12% in salted

TABLE 2. Percentage conversion of chlorophyll to phaeophytin for leaves blanched in plain or salted water

NaCl concn (%)	Sprouts		Spinach	
	Fresh* tap‡	Stored† tap‡	Fresh* tap‡	Fresh* distilled§
0	10·5	16·1	8·5	25·4
0·6	10·3	13·8		16·7
1·2	9·6	11·3	2·6	14·9
1·6	9·5	11·6		
2·0	13·2	13·1	3·3	13·7
3·0				13·2

* Freshly harvested.
† Material stored for one night in a humid atmosphere at 2°C.
‡ Barrow Gurney tap water.
§ Glass distilled water.

distilled water. Salt induced reduction was less marked in the case of sprouts, and was higher for sprouts stored overnight at 2°C than for fresh material. In the case of both spinach and sprouts blanched in tap water, reduction in conversion was always greatest when 1·2% levels of salt were used; higher levels had less effect. However, when spinach was blanched in distilled water, chlorophyll protection increased as the salt levels were raised, and maximum protection was found with 3·0% NaCl, the highest level of salt tested.

Changes in chlorophyll/phaeophytin ratios during serial blanching in tap water, in 1·2% salt water and in 1·2% sucrose

From Table 3 it is seen that when six successive batches of spinach are blanched in the same tap water, chlorophyll conversion is slightly lowered in the second and third blanches, but increases above the level of the first scald in the fourth, fifth and sixth

TABLE 3. Percentage conversion of chlorophyll to phaeophytin after serial blanching of freshly harvested spinach in tap water, in 1·2% salt tap water and in 1·2% sucrose tap water

Blanch	Tap water	1·2% salt tap water	1·2% sucrose tap water
1	7·4	2·8	8·5
2	6·8	4·1	9·1
3	6·9	3·3	8·6
4	9·0	4·7	11·4
5	8·2	5·5	10·4
6	9·2	6·3	11·2

blanches, indicating that a critical level of solutes is reached in the water during the third blanch. After the addition of 1·2% salt to tap blanching water, conversion of chlorophyll to phaeophytin increases during successive blanches; by the sixth blanch the level of conversion is still lower than that in the tap water blanches.

1·2% sucrose has no effect in preventing chlorophyll conversion during blanching, and the levels of conversion slightly exceed those found for plain tap water.

Changes in chlorophyll/phaeophytin ratios after blanching in various salt solutions

As sodium chloride had been found to influence percentage conversion of chlorophyll during blanching, the effects of other salts were tested to see whether the Na^+ or Cl^- played any particular role in preventing conversion. Amounts of the salts chosen were such that the weight of the ion in question was the same as that in 1·2% NaCl, i.e. 1·53% KCl, 1·79% K_2SO_4 and 1·46% Na_2SO_4 were used. Table 1 gives the pH values of the respective solutions.

Spinach (Perpetual) and sprouts (Thor) were tested using tap and distilled water and mean values are given in Table 4. All the salts used reduced percentage conversion, and there were only small differences between the effects of the different salts. In the case of tap water treatments, sodium sulphate always had the greatest effect in preventing conversion.

The results confirmed those given in Table 2, i.e. that there was two to three times as much conversion in distilled water as in tap water, and that the percentage salt induced protection was always greater for the distilled water treatments.

TABLE 4. Percentage conversion of chlorophyll to phaeophytin for leaves blanched in tap anddistilled water to which salts were addec

Additive	Sprouts*		Spinach†	
	Tap	Distilled	Tap	Distilled
—	17·3	32·2	7·3	18·8
1·2% NaCl	10·1	23·9	5·1	13·7
1·53% KCl	11·9	22·6	5·6	12·5
1·46% Na_2SO_4	8·8	20·6	3·4	14·7
1·79% K_2SO_4	12·2	21·5	5·6	13·0

* Shop-purchased material.
† Freshly harvested.

Changes in chlorophyll/phaeophytin ratios after blanching and/or cooling in NaCl, sucrose, $NaHCO_3$ and Na_2CO_3 solutions and storing for 0, 3, 6 and *9 months at 18°C*

These investigations complement the organoleptic assessments carried out in Part I of this series of papers (Hudson *et al.*, 1974). Results (Table 5) show that chlorophyll conversion in peas and runner beans is little affected by the use of salt in pre-freezing treatments. Conversion is apparently greatest with the sugar treatments. Both sodium bicarbonate and carbonate are slightly effective in preventing conversion.

During storage, there is a gradual increase in the percentage conversion values. There is no evidence to suspect that any particular treatments are especially effective in reducing the conversion that occurs during storage. For peas, least conversion during storage occurred for the 1·2% NaCl blanched material, and in the case of the beans the two carbonate treatments were most effective in preventing conversion. After 9 months storage, the lowest overall conversions are for blanches in 1·2% salt (peas) and 0·25% sodium carbonate (beans); no carbonate treatments were carried out for peas.

After 9 months it is seen that chlorophyll conversion in unblanched tissues had occurred twice as rapidly in peas as in beans. In fact, the conversion appeared to be so slow in beans that even after 9 months storage, conversion in unblanched material was only 8% compared with 16–22% in the blanched samples. After 6 months the percentage conversions in blanched and unblanched peas were similar.

In the case of both beans and spinach, the addition of NaCl to cooling water had relatively little effect in preventing conversion, and for both vegetables the most effective stabilizing medium was a blanch in Na_2CO_3.

Chlorophyll conversion after cooking in tap water and salted tap water

Fresh spinach (Perpetual) and runner beans (Achievement) were cooked for 6 and 10 min respectively in unsalted and salted (1·2%) tap water. Cooking in salted tap

water reduced conversion in spinach from 30% to 22·2%. In the case of beans, the salt water reduced conversion from 40% to 36·2%.

TABLE 5. Percentage conversion of chlorophyll to phaeophytin before and after storage at −18°C in unblanched vegetables and in uncooked vegetables blanched and/or cooled in various tap water solutions

Vegetable	Additive in blanching water	Additive in cooling water	Months storage at −18°C			
			0*	3	6	9
Peas† (unblanched)	—	—	0	3·5	10·2	14·6
Peas† (blanched)	—	—	8·4	10·9	12·5	12·3
	1·2% NaCl	—	8·0	8·7	9·1	10·5
	—	1·2% NaCl	8·0	9·5	11·5	12·0
	1·2% NaCl	1·2% NaCl	6·4	8·8	9·2	10·7
	2·0% NaCl	—	7·0	8·4	10·0	10·9
	1·2% Sucrose	—	9·0	11·0	12·3	12·9
	—	1·2% Sucrose	8·7	11·0	12·4	14·0
	1·2% Sucrose	1·2% Sucrose	8·8	10·4	13·9	13·3
	0·25% $NaHCO_3$	—	7·3	9·4	10·1	11·4
Beans† (unblanched)	—	—	0	2·1	5·6	8·0
Beans† (blanched)	—	—	15·0	18·1	20·6	21·4
	1·2% NaCl	—	14·0	17·3	18·9	19·6
	—	1·2% NaCl	15·1	18·2	20·8	21·4
	1·2% NaCl	1·2% NaCl	13·8	17·9	19·4	19·7
	2·0% NaCl	—	14·3	16·7	17·5	17·6
	1·2% Sugar	—	16·5	20·2	21·2	22·2
	—	1·2% Sugar	15·5	18·7	19·9	21·6
	1·2% Sugar	1·2% Sugar	16·3	19·2	20·4	21·7
	0·25% $NaHCO_3$	—	14·1	15·1	16·2	16·4
	0·25% Na_2CO_3	—	13·6	13·7	14·9	16·0
Spinach‡ (blanched)	—	—	8·5			
	1·2% NaCl	—	2·6			
	—	1·2% NaCl	6·0			
	1·2% NaCl	1·2% NaCl	3·0			
	2·0% NaCl	—	3·3			
	0·25% $NaHCO_3$	—	3·1			
	0·25% Na_2CO_3	—	1·0			

* Assayed for chlorophyll/phaeophytin conversion directly after blanching.

† Harvested 24 hr before blanching.

‡ Freshly harvested.

Discussion

Results obtained indicated that the amount of chlorophyll conversion during blanching is dependent not only upon the pH of the solution, but also upon the composition and concentration of the blanching medium and upon the condition of the material. Low concentrations of salts (e.g. 1·2% NaCl) in blanching water scarcely affected pH and reduced chlorophyll conversion in the leafy vegetables used, but had less effect on bulky tissues where fewer cells come into contact with the blanching medium. As salts had a greater effect when added to blanching than to cooling water, it is thought that the heat causes some liquidation of the fat membranes and allows the ions to reach the chloroplasts. Non-ionic additives do not have the same protective effect.

Data indicated that the level of ionic substances in blanching water was of importance for maximum retention of chlorophyll. With hard water, the most beneficial levels of added NaCl lay in the region of 1·2%; higher levels were not as effective. With distilled water, and presumably soft water, this level can be exceeded with advantage. As expected more conversion was found when purer, more acidic water was used for the blanching medium; results showed clearly that salt was far more effective in preventing conversion when added to the blanching liquor of this water.

Sodium bicarbonate and carbonate blanching solutions prevent conversion primarily, presumably, due to their alkaline properties, but considering the results, increase of ionic concentration may play a role.

The amount of chlorophyll conversion and the degree of salt induced chlorophyll protection were found to be related to the condition of the material used. Tables 2 and 4 show three states of freshness of sprouts, i.e. freshly picked, 1-day-old and more than 1-day-old (bought material) and for these three ages addition of 1·2% salt to tap water increased chlorophyll retention during blanching by 1, 5 and 7% respectively.

Sweeney & Martin (1961) analysed chlorophyll changes in uncooked blanched frozen vegetables and found chlorophyll losses in spinach, peas, green beans and sprouts to be 10, 10·2, 23·4 and 31% respectively. Our results for similar vegetables, but different cultivars, showed 8, 8, 15 and 10–14% conversion respectively. Sweeney & Martin found the pH of the four vegetables to be 6·8, 7·0, 6·2 and 6·3 respectively and concluded that the higher cellular pH was responsible for lower conversion values. As our conversion levels for beans and sprouts also exceeded those found in spinach and peas, the same theory probably accounts for the differences in chlorophyll retention of the materials which we used.

Possibly the conflicting results on the occurrence of chlorophyll conversion in blanched vegetables stored at −18°C is due to the fact that some analyses (Dietrich *et al.*, 1957, 1960) were made on raw material, whilst others (Eheart, 1967) were made on the cooked product. Our findings (Table 5) that chlorophyll degradation does occur at −18°C, support those of Dietrich; the harmonizing results may be due to the similar techniques used and to analysis of raw, rather than cooked, materials.

The storage data presented in this paper are for peas and runner beans, which were the two vegetables for which salt blanching had little effect on chlorophyll conversion. It is thus not surprising that in the previous paper, significant sensory colour differences between controls and treatments were not found for peas, and that for runner beans they occurred only when the blanching medium was 0·25% Na_2CO_3, a treatment which significantly improved colour. This treatment was the most effective in protecting chlorophyll during the storage of the beans, and after each of the three storage periods the conversion values were about 5% lower than for the controls. This is an indication that if significant sensory colour changes are to be detected, then percentage chlorophyll conversion differences must be 5% or more, at least in runner beans, and probably in peas.

Sensory assessments for colour of sprouts, mentioned in the previous paper, suggested that after blanching in 1·2% salt there is marked colour improvement for the first few weeks of storage, but gradually the colour scores for both treatments and controls become equal. However, chlorophyll results on uncooked frozen material (Table 5) indicated that salt induced protection of chlorophyll is maintained throughout storage. Further work is needed to show whether salt induced protection is maintained after cooking.

Conclusions

Alkali blanching treatments to improve colour are not recommended, owing to undesirable flavour changes, softening of the tissues and increased instability of ascorbic acid. The results suggest that salt added to blanching water can reduce chlorophyll degradation, especially of leafy vegetables, without noticeably affecting the pH. Salt induced colour improvements in leafy vegetables are likely to be greatest with short storage periods, such as are experienced with home freezing practices. Further, it seems that salt blanching will have its greatest value in soft water areas. In hard water areas, 1·2% salt gives good results for some vegetables, but in soft water regions the concentration could be increased to 2%. If the salt concentration is too great, then advantages of colour improvement are lost. Sugar blanching does not induce increased chlorophyll retention.

Acknowledgment

This work was financed by the Ministry of Agriculture, Fisheries and Food.

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(*Received* 11 *May* 1973)

J. Fd Technol. (1974) **9**, 115–118.

Technical note: Thermal perception in the mouth and lips

J. O'CONNOR, V. STEVENSON AND A. W. HOLMES

We have been interested in thermal perception in the labial regions and buccal cavity. In the catering industry much food is sold 'hot', but this is a subjective and relative quality. The experience of 'hotness' is quite distinct from that of 'warmness' which in turn is distinct from 'coldness'. Even so within each zone of thermal experience considerable discrimination is possible. In this note the ability of a group of subjects to perceive temperature differences is reported.

The nature and extent of research carried out in the field of temperature sensitivity is limited and often contradictory; Kling & Riggs (1972) have discussed the evolution of the theory of thermal reception and perception. The sensations of coldness and warmness are generally held to be due to stimulation of separate receptors: the so-called warm and cold fibres. For example Zotterman (1953) studied the impulse frequencies of cold and warm fibres in the tongues of cats. Zotterman's results are shown in Fig. 1.

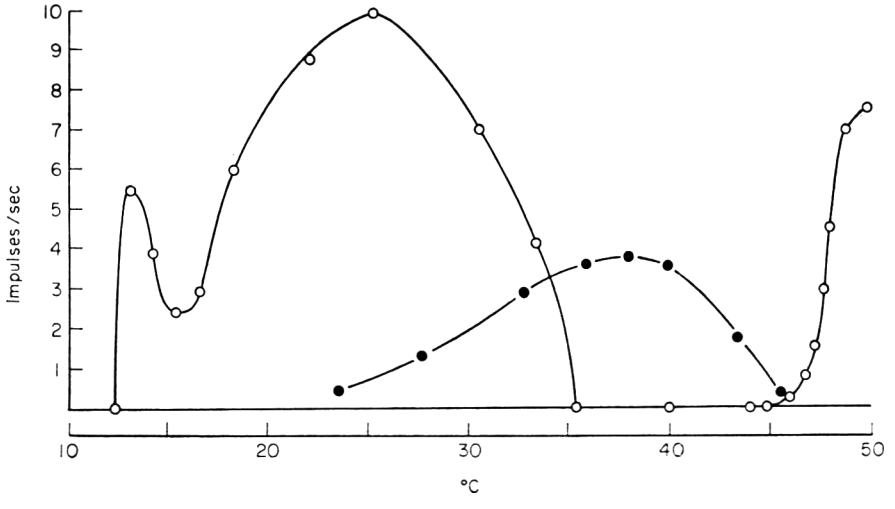

FIG. 1. Graph showing the frequency of the steady discharge of a single cold fibre (open circles) and of a single warm fibre (filled circles) when the receptors were exposed to constant temperatures within the range 10–50°C (after Zotterman, 1953). The vertical axis is in impulses per second and the horizontal axis is in degrees Centigrade.

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Hilgard (1962) notes that the sensation of hotness is due to stimulation of warm and cold fibres simultaneously and cold fibres have two thresholds of activation. This is clearly seen in Zotterman's graph.

It has long been recognized that temperature influences taste threshold values and flavour perception (Amerine, Pangbourn & Roessler, 1965). In order to exclude any taste or odour responses which could have distorted results, the medium chosen for the present study was water.

The method used to test discriminatory ability was based on the triangle difference test. Each subject was presented with two separate trios of insulated cups of water under code. The cups were of the commercial expanded polymer type. In each trio the water in one cup was either 5°C above or below the temperature of water in the other two cups. Fourteen steps of temperature were employed ranging from 5°C to 75°C.

Ten subjects were used for each temperature step; a total of 100 subjects was used, the majority of whom were university students. Groups were selected informally. Each subject was given an instruction sheet before entering the test area and precautions were taken against communication between those who had taken the test and those awaiting it. The temperature of the water in the cups was measured continuously by means of thermocouples and adjusted as necessary. At high temperature sipping rather than swallowing was advised. The results are given in Table 1 and in the form of a histogram (Fig. 2) which shows the percentage of subjects who correctly identified the odd sample in both trios. Interesting variations are seen in the perception of temperature difference across the temperature range. It appears that in the step 5°C to 10°C discriminatory ability is low, but between 10°C and 15°C it is high. Perception of

TABLE 1. Analysis of results

Temperature range (°C)	Nos correct (both tests)	Nos correct (one test)	Incorrect
5–10	6	5	9
10–15	20	0	0
15–20	14	3	3
20–25	12	4	4
25–30	18	1	1
30–35	20		0
35–40	8	5	7
40–45	12	3	5
45–50	12	4	4
50–55	10	4	6
55–60	10	4	6
60–65	10	4	6
65–70	6	5	9
70–75	6	4	10

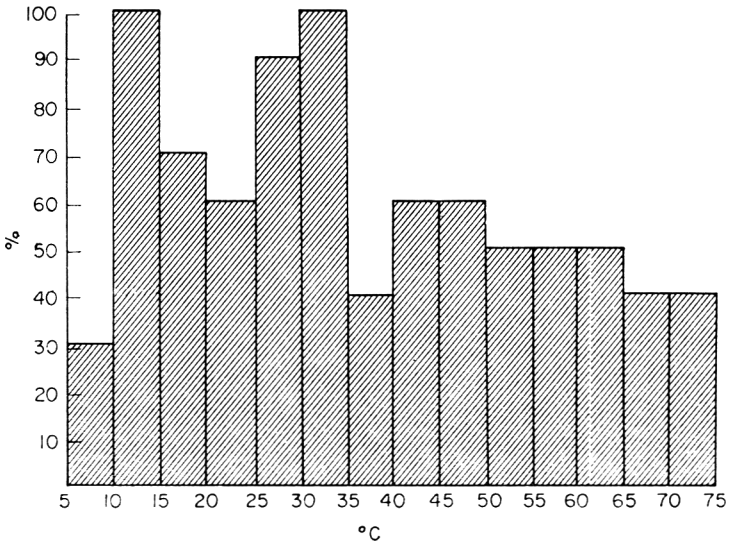

FIG. 2. Histogram showing variation of temperature sensitivity with temperature. The vertical axis shows the percentage of correct decisions made by subjects. The horizontal axis is in degrees Centigrade.

difference appears less acute between 15°C and 25°C, but seems more acute near the temperature of the body. At temperatures close to and higher than the body differences are less readily noted and above 60°C the sensations of hotness and pain swamp the sensitivity towards difference.

TABLE 2. Significances of responses in different temperature ranges

Temperature °C	Correct	Incorrect
(a) Comparison of 5–10°C with 10–15°C		
5–10	11	9
10–15	20	0
	$\chi_c^2 = 9$ $P < 0{\cdot}005$	
(b) Comparison of 15–25°C with 10–15 or 25–35°C		
15–25	33	7
10–15+25–35	59	1
	$\chi_c^2 = 6{\cdot}2$ $P = 0{\cdot}005$	
(c) Comparison of 10–35°C with 35–75°C		
10–35	92	8
35–75	107	53
	$\chi_c^2 = 21$ $P \ll 0{\cdot}005$	

In the cat cold fibres are activated at about 12°C and this may explain our subjects' inability to differentiate between 5°C and 10°C. The cold fibres cease to respond at 35°C at which point there is a fall in our subjects' ability, who are probably depending on warm fibre signals only. At 45°C the cold fibres of the cat are reactivated and our subjects' ability rose in a similar way. In the light of the apparent similarity between the two sets of data, the significance of that produced in the present experiments has been examined statistically. Results from temperatures, which, on the basis of the cat tests, might be expected to show differences are compared in Table 2. The significance of the differences between the temperature ranges was established by the χ^2 test for 2×2 contingency tables (Fisher & Yates, 1943).

Further studies in this area would be welcomed, particularly those directed towards finding acceptable ranges of temperature for different fluids and foodstuffs with application to catering operation.

Acknowledgments

This study was carried out in the Department of Hotel and Catering Management at the University of Surrey. Statistical treatment of the results was by Dr E. H. Steiner.

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(*Received* 4 *June* 1973)

J. Fd Technol. (1974) **9**, 119–122.

Technical note: Changes in the velocity of ultrasound in meat during freezing

C. A. MILES AND C. L. CUTTING

Methods of estimating the proportion of ice in an aqueous mixture such as a food-stuff have hitherto been chiefly either dilatometric (Moran, 1930) of calorimetric (Plank, 1925; Riedel, 1957). The velocity of ultrasound, which is greater in ice than it is in water, has not previously been measured in frozen meat and is now shown to provide a quantitative estimate of the ice content of frozen meat. Further, acoustic velocity could be used to estimate the mean specific enthalpy of partially frozen meat, which is a physical parameter that is important to refrigeration engineers.

The group velocity of pulses of ultrasound through a comminuted sample of lean beef was determined by a direct measurement of the pulse transmission time. An ultrasonic flaw detector was used in conjunction with a time interval meter, basically a 10 MHz clock. The excitation pulse derived from the flaw detector started the clock after a known delay of a few μsec. This pulse was applied to a 20 mm diameter 2·5 MHz crystal of lead titanate/zirconate which emitted an ultrasonic pulse into the sample. After a few centimetres of travel the pulse was received by a similar transducer mounted in opposition to the transmitter and the received signal amplified and applied to the time interval meter to stop the clock. In this way the pulse transit time could be measured with an uncertainty $\pm 0{\cdot}1$ μsec after allowing for the 'zero error' (Krautkrämer & Krautkrämer, 1969) by an initial experiment with water. The temperature of the sample was controlled to within $\pm 0{\cdot}03$°C of any pre-set temperature in the range -25°C to $+20$°C.

The tissue, which was M. longissimus thoracis excised from a beef side at the eleventh, twelfth and thirteenth rib positions, was chemically analysed by a standard method (S.A.C., in preparation) to reveal a composition of: 6·8% extractable fat, 70·2% water. The comminuted sample was vacuum packaged, before ultrasonic measurements were made, to remove the air trapped during blending.

The results are presented in Fig. 1. The velocity through the unfrozen meat changed only slightly with temperature, roughly 0·12%/°C over the range 0–10°C. At the onset of freezing the velocity of sound increased abruptly and rapidly with reducing temperature. By -5°C, at which temperature foods such as meat are generally regarded as substantially frozen (Reay, Banks & Cutting, 1950), the velocity of sound had increased by 70% of its unfrozen value. As the temperature was reduced further the rate of change

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in velocity with temperature declined progressively. At −20°C the velocity was roughly double that at 0°C. In Fig. 1 the reciprocal of the velocity squared is plotted as a function of temperature to reveal the similar form of this curve to the ice content data of Fleming (1969) obtained by calorimetry.

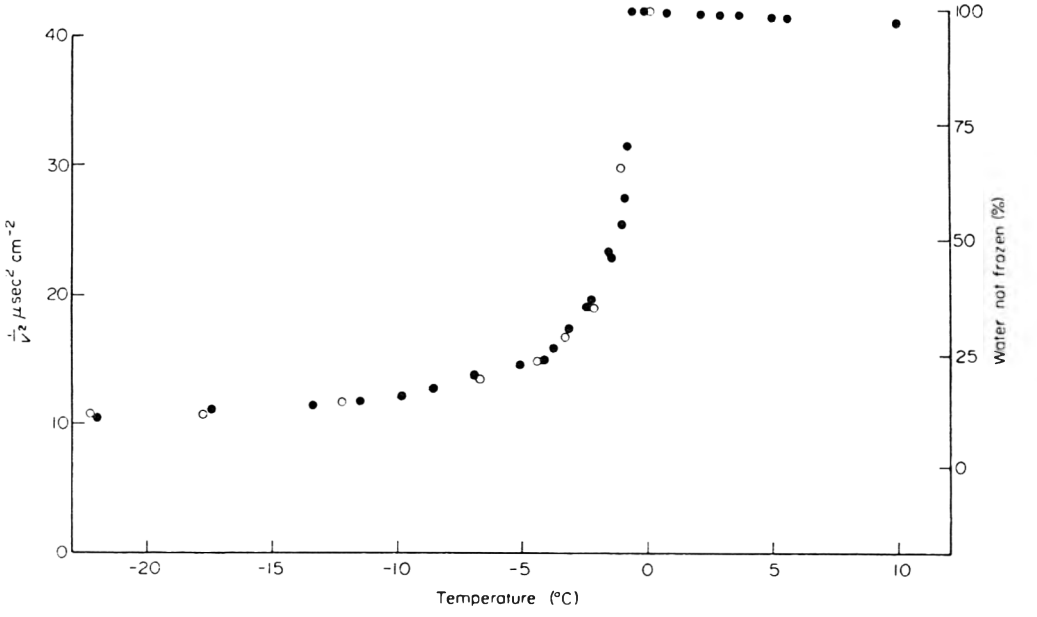

FIG. 1. Comparison of the velocity of ultrasound (V) (●) in beef with calorimetric estimates of unfrozen water (○) in veal (Fleming, 1969).

The specific enthalpy H at a given temperature θ is the quantity of heat required to raise the temperature of unit mass of material from a predetermined temperature (here −40°C) to θ. The mean specific enthalpy $\overline{H}$ of a partially frozen mass of meat thus determines the uniform temperature the meat would attain at equilibrium if stored adiabatically. Several measurements of the enthalpy of lean meat as a function of temperature are available (Riedel, 1957; Fleming, 1969) and when these are plotted against the reciprocal of the velocity of sound at the same temperature a roughly linear relationship is obtained (Fig. 2). This relation: $H=(B/V)+C$ might be useful industrially as the following calculation shows.

Consider a slab of partially frozen meat bounded by two parallel planes, $x=0$, and $x=l$, between which the non-uniform temperature distribution is a function of x only. The mean specific enthalpy is given by

$$\overline{H}=\frac{\int_0^l H\rho dx}{\int_0^l \rho dx}=\frac{\int_0^l \left[\frac{B}{V}+C\right]\rho dx}{\int_0^l \rho dx}$$

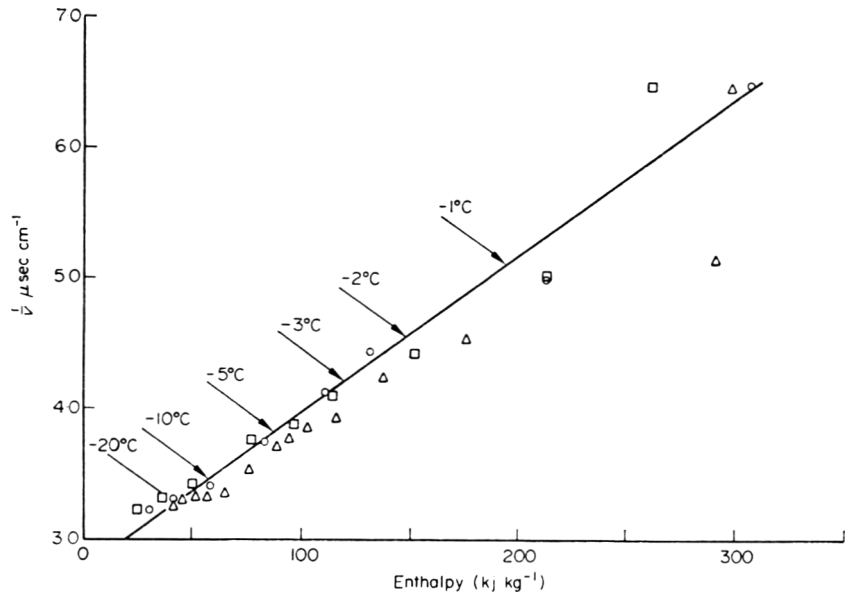

FIG. 2. Relationship between the reciprocal of the velocity of ultrasound (V) and the specific enthalpy of lean meat. △, Beef: 74% water, less than 4% fat (Riedel, 1957). ○, Veal: 77·5% water, 4·4% fat (Fleming, 1969). □, Lamb: 64·9% water, 11·7% fat (Fleming, 1969).

where ρ and V are the density and velocity of sound respectively. While H and V vary by factors of 10 and 2 respectively over the range -10°C to 0°C, ρ varies by 10% only (Jason & Jowitt, 1969), so that to a first approximation the integration on the right-hand side may be performed by taking the mean density outside the integral.

If this is done the solution is

$$\overline{H} = B\left(\frac{\overline{1}}{V}\right) + C \qquad (1)$$

where $(\overline{1}/V)$ is the transmission time divided by the distance travelled, a quantity that could be measured directly.

Equation (1) shows that Fig. 2 may be used to determine the mean specific enthalpy of a partially frozen block of meat from velocity of sound data. If $(\overline{1}/V)$ is 5·1 μsec cm^{-1} then the block would equilibrate under adiabatic conditions to -1°C, if 3·8 μsec/cm then the temperature would be -5°C, and if 3·4 μsec/cm -10°C etc.

This relation was derived from velocity measurements of a homogeneous and isotropic sample of minced muscle. Other experiments (Miles, unpublished) have shown that in a frozen homogeneous mince of meat composed of 25% fat, the velocity of sound at -10°C is 13% smaller than a similar mince with 5% fat. It has also been shown that frozen muscle is acoustically anisotropic, the velocity of sound perpendicular to the

fibres being about 6% lower than that parallel to the fibres. It is therefore necessary to investigate the effects of inhomogeneity, fat content and anisotropy on relation (1) before the technique could be used industrially.

Acknowledgments

Thanks are due to Mr D. Shore for carrying out the experiments.

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(*Received* 11 *June* 1973)

J. Fd Technol. (1974) 9, 123–124.

Technical note: The destruction of thiamine during chapati baking

A. B. CHAUDRI AND H. G. MULLER

Various types of chapati are eaten as a staple food by an estimated 500 million people who depend on them *int. al.* for their thiamine intake (Chaudhri & Muller, 1970). It is well known that on baking of cereal products some thiamine may be destroyed and in bread the loss may be as high as 25% (Coppock, Carpenter & Knight, 1956; Maleki & Daghir, 1967). In chapati, losses from nil (Chawdhry, 1968) to 73% (Hashmi, Ullah & Ahmed, 1954) have been reported.

The loss of thiamine depends primarily on the conditions of the baking test and the thickness of the product. The following standard test was developed in this laboratory: the Farinograph is adjusted to 25°C and 300 g of flour placed into the bowl. Three per cent less water than that found in the standard absorption test (A.A.C.C., 1962) is added, and the dough mixed for 4 min on fast speed. The dough is then removed and allowed to rest at room temperature for 1 hr under a perspex cover. One hundred gram dough pieces are then scaled off, rolled manually and each flattened into discs of 15 cm diameter using a rolling pin and a wooden board on which a circle of the correct diameter has been drawn. One point five grams of the original flour is used for dusting each dough piece.

The chapati is then baked on a 20·3 cm diameter hotplate (Belling 17) for 4 min at various heat controlled settings (standard setting 1·5). After 1 min baking the chapati is turned over and baked for 1 min on the other side. It is then turned every 30 sec until the 4 min are up.

During baking the temperature was recorded every 30 sec, using a Doran thermocouple potentiometer (Doran Instruments Co. Ltd, Stroud, Glos.), and a previously standardized Constantan thermocouple. This was placed between the hotplate and the chapati. A graph of temperature against time for each heat controlled setting was plotted and the time averaged temperature calculated by dividing the unit area under the curve up to each baking time, by the time of baking.

Thiamine was estimated using the A.A.C.C. thiochrome method (No. 86–80). The regression line (Fig. 1) shows a significant correlation ($r = -0{\cdot}70$) between thiamine destruction and time averaged temperature. At a time averaged temperature of 257·5°C. thiamine loss increased from 18·3% to 62·1% when chapati thickness was reduced by half.

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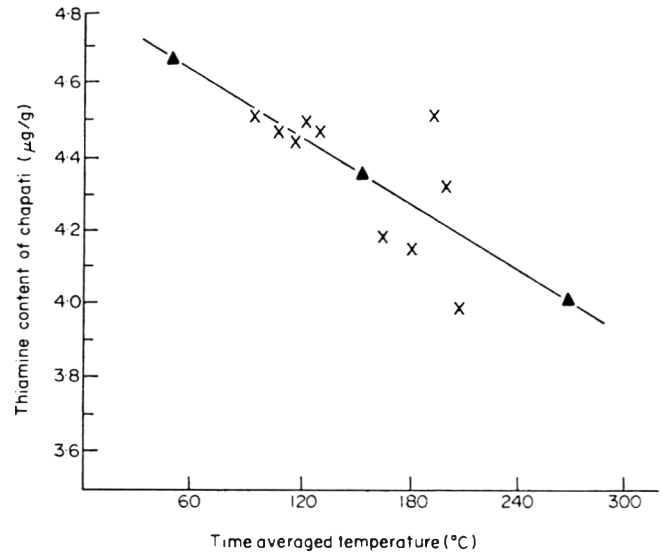

FIG. 1. Time averaged temperature versus thiamine content. (Each point represents the mean of three to five readings.)

Thick chapatis tend to be eaten in rural areas, thinner ones in the cities. It would appear that with the thinner type, excessive thiamine losses may occur. This is an important aspect of Asian nutrition which is receiving further attention in this laboratory.

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Book Reviews

Handbook of Food Additives. Ed. by T. E. FURIA.

Cleveland, Ohio: Chemical Rubber Co., 1973. 2nd edition. Pp. xi × 918. £18.25.

The second edition of the *Handbook of Food Additives* incorporates fresh information on the properties and uses of many food additives resulting from recent developments in food technology, as well as changes in the regulatory status of food additives which have been imposed by the FDA since the book was first published in 1968. The new edition, which contains over 200 pages more than the first volume, is a mine of technical but readable information concerning the nature, properties, sources, uses and applications of direct food additives. It certainly fulfils the editor's intention of keeping abreast with developments in food technology, and, in spite of a considerable price rise, is well worth the money spent.

Certain updating changes have been made to the chapters on enzymes, vitamins and amino acids, antimicrobial food additives, antioxidants, sequestrants, starch, surface active agents, flavour potentiators and colour additives in food (now the fourteenth chapter instead of the first). Since the future use of saccharin hangs in the balance, and new artificial sweeteners are far from approved for food use, the original chapter on non-nutritive sweeteners, which contains a great deal of useful information on properties, sensory testing and food applications of saccharin as well as the now prohibited cyclamates, has been re-issued in this edition. The chapters dealing with acidulants, gums and polyhydric alcohols contain new material accompanied by numerous additional references, and the tables indicating the characteristics and applications of natural and synthetic flavouring materials have been considerably enlarged. Additives which have recently been added to the approved list, including the colouring matter F D & C Red No. 40, xanthan gum and microcrystalline cellulose, are described in the relevant chapters.

Part I of the book concludes with a completely fresh chapter on phosphates in food processing. The classification, characteristics, toxicology and functions of phosphates, and their applications in numerous classes of food products, including dairy, egg, fruit and vegetable, meat, seafood and processed protein products, gums, gels, fats and oils, are covered in this excellent review which contains over 1000 references, documented at the end of the chapter, and covers 163 pages.

Part II of the book, in which the regulatory status of each direct food additive in the US is set out, has been enlarged, particularly with the addition to the GRAS list of over 100 new flavouring materials, and clarified, since information on alternative names or sources of additives, FEMA number, reference to the relevant regulation and legal limitations of usage is now presented in tabular form. The list is undoubtedly of value

for reference purposes, provided it is borne in mind that food additives are constantly under review and revision and alteration of the regulations are liable to occur fairly frequently.

D. W. FLOWERDEW

Progress in Industrial Microbiology Volume 12. Ed. by D. J. D. HOCKENHULL. London and Edinburgh: Churchill Livingstone, 1973. Pp. vi + 242. £6.50.

This volume continues in the format of earlier volumes in the series by covering a diverse range of topics of interest to applied microbiologists. Not all the articles are of direct relevance to food technologists, but with the increasing utilization of fermentation procedures in the food industry many of the topics may be of interest to some food scientists and technologists.

The first article (C. S. Hesseltine and W. C. Haynes) is concerned with 'Sources and management of microorganisms for the development of a fermentation industry'. This is essential reading for anyone concerned with maintenance of a culture collection, no matter how small. Sources of organisms, the locations of the major collections of industrially useful microorganisms, problems of maintaining stable industrial cultures and regulations regarding the deposit of cultures for patent purposes are but a few examples of the valuable information included in this article.

In an excellent article on 'Water relations and heat resistance of microorganisms' Janet E. L. Corry discusses the concept of water activity and its effect on the growth and survival of microorganisms. Of particular interest to the food microbiologist is the collation of data from a wide variety of sources on the limiting a_w values for growth of microorganisms. The observed effects of solute concentration and of reduced water activity levels on the heat resistance of microorganisms in both laboratory heating menstrua and in foods indicates that a more complex situation exists than has hitherto-fore been realized.

For workers in the field of fermentation technology and possibly for food technologists interested in the high speed blending of food ingredients, the article by M. J. Hall and co-authors will be of interest. This paper considers the physical chemistry of foam formation in fermentation systems and the use of both chemical and mechanical systems for foam control. The effects of defoaming procedures on microbial metabolism, oxygen transfer, etc. are described for a number of fermentation systems and the necessity for use of 'permitted' chemical agents for use in food fermentations is stressed.

In a very interesting article on yeast cytology and metabolism, H. Suomalainen and co-authors discuss the chemistry, biochemistry and morphology of yeast cell walls and the cytoplasmic membrane. Factors affecting the growth of yeasts are considered from a biochemical viewpoint. Of interest to the fermentation microbiologist and technologist is an article on 'Genetics of *Penicillium chrysogenum*' by C. Ball.

The omission of addresses for some authors is perhaps unfortunate but with this

exception the book is well produced, clearly printed with few typographic errors and well indexed. The book is recommended for the industrial microbiologist and the fermentation technologist in the food and allied areas.

B. JARVIS

Books received

Edible Coatings and Soluble Packaging. By R. DANIELS.
New Jersey: Noyes Data Corporation, 1973. Pp. VIII + 360. $36.00.

This Food Technology Review No. 3 discusses special properties of materials which play a dual role as package and food component.

Chronic Cassava Toxicity. Ed. by B. NESTLE and R. MACINTYRE.
Ottawa: International Development Research Centre, 1973. Pp. 162. Can. $5.00.

Proceedings of an interdisciplinary workshop held in London in 1973.

Food for Peace. By ROBERT G. STANLEY.
New York: Gordon and Breach, 1973. Pp. XII + 267. $6.00.

This book documents some recent aid programmes in an attempt to produce alternatives for the future.

Second Book on Food and Nutrition. By D. MATHEWS and D. WELLS.
London: The Flour Advisory Bureau, 1973. Pp. X + 242. £1·80.

A general elementary background to Food Science useful for schools.

JOURNAL OF FOOD TECHNOLOGY: NOTICE TO CONTRIBUTORS

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References. Only papers closely related to the author's work should be included; exhaustive lists should be avoided. References should be made by giving the author's surname, with the year of publication in parentheses. When reference is made to a work by three authors all names should be given when cited for the first time, and thereafter only the first name, adding *et al.*, e.g. Smith *et al.* (1958). The '*et al.*' form should always be used for works by four or more authors. If several papers by the same author and from the same year are cited, a, b, c, etc., should be put after the year of publication, e.g. Smith *et al.* (1958a). All references should be brought together at the end of the paper in alphabetical order. References to articles and papers should mention (a) name(s) of the author(s); (b) year of publication in parentheses; (c) title of journal, underlined, abbreviated according to the *World List of Scientific Publications*, 4th edn and supplements; (d) volume number; number of first page of article. References to books and monographs should include (a) name(s) and initials of author(s) or editor(s); year of publication in parentheses; (b) title, underlined; (c) edition; (d) page referred to; (e) publisher; (f) place.

Standard usage. The *Concise Oxford English Dictionary* is used as a reference for all spelling and hyphenation. Verbs which contain the suffix ize (ise) and their derivatives should be spelt with the z. Statistics and measurements should always be given in figures, i.e. 10 min, 20 hr, 5 ml, except where the number begins the sentence. When the number does *not* refer to a unit of measurement, it is spelt out except where the number is greater than one hundred.

Abbreviations. Abbreviations for some commoner units are given below. The abbreviation for the plural of a unit is the same as that for the singular. Wherever possible the metric SI units should be used unless they conflict with generally accepted current practice. Conversion factors to SI units are shown where appropriate.

SI UNITS

gram	g	Joule	J
kilogram	kg $= 10^3$ g	Newton	N
milligram	mg $= 10^{-3}$ g	Watt	W
metre	m	Centigrade	°C
millimetre	mm $= 10^{-3}$ m	hour	hr
micrometre	μm $= 10^{-6}$ m	minute	min
nanometre	nm $= 10^{-9}$ m	second	sec
litre	l $= 10^{-3}$ m^3		

NON SI UNITS

inch	in	= 25·4 mm
foot	ft	= 0·3048 m
square inch	in^2	= 645·16 mm^2
square foot	ft^2	= 0·092903 m^2
cubic inch	in^3	= 1·63871 × 10^4 mm^3
cubic foot	ft^3	= 0·028317 m^3
gallon	gal	= 4·5461 l
pound	lb	= 0·453592 kg
pound/cubic inch	lb in^{-3}	= 2·76799 × 10^4 kg m^{-3}
dyne		= 10^{-5} N
Calorie (15°C)	cal	= 4·1855 J
British Thermal Unit	BTU	= 1055·06 J
Horsepower	HP	= 745·700 W
Fahrenheit	°F	= 9/5 T°C + 32

Figures. In the text these should be given Arabic numbers, e.g. Fig. 3. They should be marked on the backs with the name(s) of the author(s) and the title of the paper. Where there is any possible doubt as to the orientation of a figure the top should be marked with an arrow. Each figure must bear a reference number corresponding to a similar number in the text. Photographs and photomicrographs should be unmounted glossy prints and should not be retouched. Line diagrams should be on separate sheets; they should be drawn with black Indian ink on white paper and should be about four times the area of the final reproduction. Lines and lettering should be of sufficient thickness and size to stand reduction to one-half or one-third. Whenever possible, the originals of line diagrams, prepared as described above, should be submitted and not photographs. The legends of all the figures should be typed together on a single sheet of paper headed 'Legends to Figures'.

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Journal of Food Technology
Volume 9 Number 1 March 1974

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Printed by Adlard and Son Ltd, Bartholomew Press, Dorking