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Foot-and-mouth disease, its relation to meat and meat processing

P. C. B. ROBERTS

Summary

The survival of foot-and-mouth disease virus (FMDV) in meat and meat products is briefly reviewed.

An evaluation of established food processes in relation to viral inactivation is presented, together with a discussion on the application of novel processes. The potential for basing process evaluation on a non-restricted, but essentially similar virus, is also discussed.

Introduction

Foot-and-mouth disease (FMD) has been the subject of active research in the United Kingdom since the early part of this century. Although legislative measures for its control extend back exactly 100 years, the disease still presents an ever present threat to nations with heavily vested interests in the raising of stock and is an acute embarrassment to international trade in meat and meat products.

Despite the lack of an immediate solution, it is to be expected that the immunological and prophylactic approaches, being pursued in laboratories throughout the world, will ultimately eradicate the disease. It is possible, as has been suggested by Goldblith (1963), that, in conjunction with the virologist and veterinarian, the food scientist and technologist may make a valuable contribution in providing an interim solution to the problem of disease dissemination by trade in meat and meat products.

It is hoped that this present survey will serve to introduce the subject to many who, perhaps, have not previously considered it, and also, to discuss some approaches that may prove worthy of investigation.

Survival of FMDV in meat and meat products

The survival of FMDV in animal products has long been recognized. Roux *et al.* (1921) reported that samples of defibrinated blood from infected cattle, kept in a tube, at between −1°C and −2°C, were infective after 6 months. Stockman *et al.* (1927) recorded survival of virus in the carcases of infected guinea-pigs stored at 2–7°C. They

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found that blood in unbled carcases remained infective for 35–46 days, and that virus could be detected in bone marrow for up to 96 days. Similar observations were made on the carcases of cattle and pigs.

Bacon carcases stored at freezing or chilling temperatures, or those treated by wet or dry salting processes contained infective virus within the bone marrow for at least 42 days. Virus was recovered from the bone marrow of frozen beef carcases (−9 to −13°C) after 76 days, and preparations of crushed bone fed to pigs transmitted the disease.

With the realization of the potential for dissemination of FMD by meat trading, Andrews *et al.* (1931), continued research into virus survival under conditions closely simulating those of the imported meat trade. Carcases were maintained at chill temperatures and the offals frozen. Virus survival periods of up to 33 days in tongues and cheek, and 80 days in bone marrow and blood clots were recorded. Hides removed, salted and stored at ambient temperature, remained infective for 46 days. These workers also noted the effect of pH on survival of virus in epithelial preparations; the optimum for survival proved to be pH 7·4.

Hof (1933) was able to demonstrate that the irregular recovery of virus from muscular tissue was associated with the hydrogen ion concentration, tissue having a low ultimate pH proving the least infective. Prolonged survival of the virus in muscle tissue is only likely if the pH is above 6·2 (Henderson & Brooksby, 1948).

Henderson & Brooksby, using an improved method for titrating virus (Henderson, 1945) which entailed simultaneous multiple inoculation of the bovine tongue, investigated the effects of the 'quick-freezing' of beef on virus survival. The observations reported here were confined to boned quarters rapidly frozen pre-rigor. They concluded that 'quick-freezing' suspends the development of acidity in musculature, and provided the frozen state is maintained, virus infectivity is prolonged indefinitely. The effect of pH was fully established; those tissues exhibiting a high ultimate pH, namely, liver, kidney, rumen, lymph node and blood, were the most likely to harbour active virus in the chilled or frozen state.

Cottral, Cox & Baldwin (1960) drew further attention to virus survival in lymph nodes, bone marrow and blood clots. These same workers in 1961 inoculated Hereford steers with Vallée A type virus and under anaesthesia removed the right prescapular lymph nodes 20 hr post inoculation. Though the steers appeared normal, and no clinical signs of foot-and-mouth disease were apparent until 24 hr post inoculation, the lymph nodes were shown to have high virus titres; a similar observation was made after regression of disease symptoms.

Virus was demonstrated in bone marrow stored at 1°C 194 days post slaughter and also in haemal nodes; the latter are small, and widely distributed throughout the carcase, and would prove even more difficult to remove during trimming than lymph nodes.

Scott, Cottral & Gailiunas (1965) isolated virus from the pituitary gland and, in lower titres, from regions of the central nervous system in early clinical and convalescent

stages of the disease. Even though gross cutaneous lesions are usually found only in the pedal area of bovine skin, Gailiunas & Cottral (1966) found high viral titres in dermal and epidermal tissue at thirteen different body sites, persisting, in some steers, for 5 days after the cessation of viremia.

The implications of the presence of active virus in animals exhibiting no symptoms of foot-and-mouth disease are readily apparent.

In a series of experiments organized by the Argentine-United States Joint Commission on Foot-and-Mouth Disease (Anon, 1966) the survival of virus in lymph nodes from susceptible Argentine cattle and from cattle immunized by repeated vaccination was compared. Vaccination markedly reduced the chance of recovering virus from lymph nodes at the time of slaughter, of cattle exposed to virus 32 hr previously.

Dry salt cured meat, prepared from both control and inoculated cattle, and containing intact prescapular and prefemoral lymph nodes was examined for the presence of active virus after holding for 31–38 days in cold storage. Virus was not recovered from the cured lymph nodes of any of the vaccinated cattle (i.e. 0/42) but was recovered from 4/15 of the unvaccinated controls.

Inactivation of FMDV in foodstuffs

Little is known of the survival of viruses in processed foods (Heidelbaugh & Giron, 1969). What is becoming increasingly apparent, however, is that food processes designed to eradicate or control bacterial and fungal contaminants, cannot be assumed to inactivate food-borne viruses. Considerable caution should be exercised in the interpretation of results from inactivation studies in the early literature. It is as well to bear in mind that, apart from lack of recourse to the more sophisticated techniques and equipment of today, it is only within the last 2 decades that a fundamental knowledge of viral structure has been established.

Thermal treatment:

Zeller & Wederman (1930) demonstrated that FMDV in contaminated milk could be inactivated by pasteurization for 15 min at 60–63°C; legislation in Germany, applicable to milk from infected farms, was based on this observation. Galloway (1931) found infected milk proved virulent after exposure in capillary tubes to 60–65°C for 2·5 sec but not 5 sec. Andrews *et al.* (1937) directed attention to the study of thermal inactivation of FMDV in whole organs. Using calves' thymus glands (sweet-breads), the temperature curves for the interior of the organs were followed for a variety of cooking procedures. It was apparent that in prescribing boiling for a fixed and limited period for the purpose of viral destruction, it was also necessary to indicate a minimum period to be observed in raising the temperature to boiling point.

The thermal inactivation studies of Dimopoullos *et al.* (1959) were particularly thorough, and the methods used well documented. Infected tissue suspensions, prepared

from bovine tongue epithelium, were heated at temperatures from 56°C to 85°C for periods varying up to 24 hr. Infectivity for cattle was demonstrable when large doses of suspensions that had been heated for 4 hr at 85°C, were inoculated by several routes. Heating at 56°C for 24 hr did not inactivate the virus or affect the complement-fixing activity of the antigen. Infectivity was often detected in animals inoculated with dilutions of the test material, though not in those given undiluted material.

Heidelbaugh & Graves (1968) packed four infected bovine lymph nodes separately into the centre of 4 × 4 × 6 in cans of ground beef, and subjected these to the equivalent of commercial canned beef pasteurization, i.e. a maximum centre temperature of 61°C. The treated lymph nodes were homogenized and injected into a large number of mice and one steer; no infections resulted. The contrast with the observations of Dimopoullos *et al.* (1959) was speculatively attributed to the existence of an optimal temperature range for FMDV destruction *in situ*.

A similar observation on a pasteurization process was made by Savi *et al.* (1965). Hams prepared from FMD infected pigs, and subsequently pasteurized, were shown to be non-infective on titreing in tryptinized calf kidney cells. The titre method in this instance is not as critical as extract inoculation in the original species. One novel form of heat treatment to which reference has been found is that of induction or dielectric heating (Zarotschenzeff, 1944a, b). Treatment of meat, contained between parallel copper electrodes, for 1–2 min at frequencies between 2 and 100 megacycles per sec was claimed to result in no colour or structural changes in the meat, and the elimination of FMDV. No experimental evidence relating to virus survival was submitted with the claim.

The theoretical aspects of high temperature inactivation of viruses have only recently been studied. The work of Bachrach (1959, 1961, 1964, 1965), Brown & Wild (1966) and Ahl (1967) relates to FMDV.

Chemical treatment

Curing as a means of inactivating FMDV has been investigated by many workers. Stockman & Minett (1927) found that both dry and wet salt curing of infective bacon carcases failed to inactivate virus in bone marrow. Bovine lymph nodes, from the carcases of infected donor cattle, stored in salt-cured muscle tissue in wooden barrels at 1°C still proved infective after 50 days (Cottrall, Cox & Baldwin, 1960). A statistical analysis of a number of curing processes applied to FMDV in suspension between pH 6·5 and 7·3 indicated that 20% NaCl was the only salt concentration tested that significantly reduced infectivity over 33 days at 4°C (Heidelbaugh & Graves, 1968). The results obtained by the same researchers for the introduction of citric acid into salt cures suggest the modified cure might be applicable to processing meat for the destruction of infectivity.

The resistance of FMDV, situated in hides, to chemical treatment (Gailiunas & Cottral, 1967) indicates that similar resistance may be encountered in other dense tissues.

Acidification with lactic acid has been practised in some countries in an attempt to reduce superficial contamination of carcases by FMDV (Brooksby, 1958; Niggli, 1956). *The Code of Federal Regulations* (Anon, 1958), stipulates that animal products from endemic FMD areas may not be imported into the United States without prior subjection to heat treatment. It allows, however, in cases where the active fraction of certain glands and tissues is destroyed by heat, adjustment of the pH to 3·8 or 13·7. A patent specification (Turner, 1968) has been published whereby meat infected with FMDV is comminuted in order to mix muscle and lymphatic tissue, and then acidified to pH 2·5, thereby destroying the virus. The survival of FMDV in 'Sangwinit', a Polish foodstuff made by the acidification of fresh blood has been reported, and attributed to the use of partially coagulated blood (Janowski *et al.*, 1967). Products from coagulum-free blood had zero viral titre.

The use of nitrous oxide to render meat virus free has also been patented (Harper, 1962). Comminuted raw meat is subjected to nitrous oxide in the absence of oxygen for 24–72 hr at 35°C.

Irradiation

Irradiation as a means of inactivating FMDV in foodstuffs has been investigated by Baldelli (Baldelli, 1966; Baldelli *et al.*, 1964, 1965). The blood and bone marrow of infected pigs required a minimum of 1·75 Mrad whilst 1·5 Mrad was necessary to inactivate virus in lymph nodes. Dehydrated virus proved even more difficult to inactivate.

Discussion

International trade in meat and meat products

Any reference to meat importation into this country presents political problems and is liable to raise adverse comment from those who advocate isolation. It is hoped that by being briefly objective, it may be possible to judge whether self sufficiency in meat production remains a logical argument or an economic impossibility.

Import trade origins

The policy of allowing animals to achieve full maturity, which supplied the needs of a small population in the first half of the 19th Century, could not meet the demands of a rapidly increasing urbanized society in the second half of the century. The problem in South America and Australasia at the same time, was the exact opposite. Sheep were valued for their wool and cattle were worth little more than their hides. New Zealand had thirty-eight sheep per head of population in 1871 and surplus animals sold for a shilling or sixpence (Harrison, 1963).

Heavy sheep losses in the United Kingdom during the winter of 1860–61, followed by a marked prevalence of pleuropneumonia in cattle, which devastated herds, aggravated

the shortage of meat. In response to the situation, Australia pioneered the canned-meat trade and by 1880 was exporting 16,000,000 pounds of canned beef annually to Britain.

The introduction of refrigeration in the summer of 1876 enabled carcase meat to be imported from America; the trade developed rapidly and between 31 June 1876 and 31 March 1877, 250,000 cwt of chilled beef were imported from the U.S.A. (Ewart, 1878). In 1880 the first cargo of beef and mutton from Australia was landed at London Docks. Trade with South America, which, until 1898 had consisted essentially of live cattle and sheep imports, ceased abruptly in 1900 following outbreaks of FMD. The small established trade in chilled meat expanded and frozen meat was shipped in increasing quantities from 1901 onwards.

Present trading level

By 1938 imported meat exceeded home killed meat in the ratio of 9:8. This was the peak for meat imports. Home production was increased dramatically during the Second World War and is now maintained at approximately three quarters of the total consumption. Imports of mutton and lamb exceed home production by some 5%, whilst beef imports represent only 25% of that consumed.

Future developments

As in all areas of commerce, specialization provides the key to the development of the meat trade.

The continued encroachment on agricultural land by urbanization in this country acts in opposition to further increasing meat production. Stock raising policies, dependent on supplementation of rations by the importation of cereal crops, and the consequential redirection of such crops from primary human nutrition, are in the long term doomed to failure. The exacerbation of world protein deficiency by a wasteful process of conversion, to supply the 'steak hungry' affluent nations, is hardly likely to be tolerated by those bordering on starvation and malnutrition.

The foregoing statement is not intended to convey, however, that the ruminant is defunct. Far from being outmoded, cattle and sheep have an invaluable part to play in supplying high quality protein from raw materials which, at the present time, they are best equipped to process.

It is difficult to find anything other than emotional argument in favour of the crowded pastures of Devon and Hereford when comparison is made with the relatively unpopulated areas of the pampas, the outback, and the plains of Canterbury.

The growing demand by the retail butchery trade for chilled and frozen boneless cuts is evidence of the rapid acceptance of an innovation by a particular sector of the population. With this in mind, one can speculate that the introduction of meat, prepacked and processed so as to eliminate FMDV, would in turn find a ready market, despite what might well be a radical change of appearance from the raw product.

Evaluation of established food processes

It has yet to be established whether the highly infectious nature of FMDV allows for an arbitrary viral titre for food products to be envisaged below which the product might be deemed 'safe'. Ideally to be designated 'safe', the product should not only contain no active virus, but no free infective RNA should be present.

Thermal treatment, designed to ensure commercial sterility, represents the only established food process which has not been implicated or suspected in the transmission of FMD. Thermal pasteurization applied to canned meat products, though not implicated in a specific transmission of FMD, cannot be considered wholly safe; there exists the possibility of virus complexing with other proteins (Hansen & Holm, 1950; Wehmeyer, 1957) thereby enhancing its thermal stability. The formation of such a complex possibly explains the recent unexpected observation of Heidelbaugh & Giron (1969). They found that polio virus, though considered as relatively labile when subjected to laboratory lyophilization, survived freeze dehydration when inoculated in a variety of foods. From a practical standpoint, the chance of any canned, boneless, thermally treated product transmitting FMD is very small indeed. The major draw-back for canned products is the high cost.

Consideration of the thermal treatment of meats other than in cans is particularly relevant at the present time. There is a considerable demand in the U.K. for offals, not met by home production, both for human consumption and processing by the pet-food industry. As from 1 October 1969, import of offals from countries where FMD is endemic was limited to 'processed offals'. There is however, some confusion over the interpretation of 'processed', and a lack of published evidence for the efficacy of thermal treatments designed to inactivate FMDV. A directive from the Ministry of Agriculture, Fisheries and Food (17 October 1969) advises that a minimum centre temperature of 70°C held for not less than 30 min, or alternatively 80°C held for 15 min would be acceptable, provided the product appears 'wholly cooked' and all risks of re-contamination are avoided.

Chemical treatments must be viewed with more suspicion. To ensure total inactivation of virus, the treatment must reach the infective agent in its every location. Attention has been drawn to the possibility of active virus being harboured in blood clots and dense tissue, where it is effectively protected from inactivation. Though acidification of comminuted meat with lactic acid has been suggested, the resultant product might have little value both in terms of texture and taste; it has recently been shown that high concentrations of lactic acid in particular, have an undesirable effect on the eating quality of meat (Dryden, Marchello & Ray, 1969). The same applies to any curing process modified by acid inclusion.

Curing, as practised, has little effect with regard to FMDV inactivation. The inclusion of bone and minor lymph nodes in cured pork products provides a potential source of active virus which might lead to outbreaks of FMD.

The use of any chemical agent to inactivate FMDV is most unlikely. The effective

viricides react with protein and would thus not only entail very high dose rates to be effective, but would present toxicological problems. It is interesting to reflect that ethylene oxide was at one time considered as a possible sterilizing agent for food purposes (Ginsberg & Wilson, 1950).

The inability to penetrate dense proteinaceous material dispenses with any consideration of non-ionizing radiation. Ionizing radiation, by virtue of the exceedingly high dose rates that are necessary to inactivate FMDV located in carcase material, raises organoleptic, nutritional and possibly toxicological problems greater than those which have resulted in condemnation of the process in other contexts.

The application of novel processes

At this juncture it is possible to speculate as to what novel processes, if any, might be applied to the inactivation of FMDV in foodstuffs. It is worth noting that, although this survey has concerned itself with a specific viral entity, the remarkable resistance of FMDV implies that any success in its inactivation has wider connotations relating to micro-organisms in general.

The observation of Smith & Krueger (1952) that vibriophage, when heated for a short time, becomes permanently sensitive to cold shock is of interest. The very simplicity of such a process recommends the experiment being repeated substituting FMDV for phage; no reference to such a treatment of FMDV has been found in the literature.

Attention has been drawn to the thermal stabilization of viruses by increased pressure (Johnson, Baylor & Frazer, 1948). It is postulated that reduced pressure is likely to have an opposite effect, namely thermal sensitization. Heating virus-contaminated foodstuffs under continuous vacuum or the application of one or more rapid reductions of pressure to heated foodstuffs, might well induce disruption of the capsid material.

Another possible means of increasing thermal sensitization would be the simultaneous treatment of a foodstuff with heat and ultrasonic energy. Though the ultrasonic energy would be considerably damped by proteinaceous foodstuffs, disruption of protective aggregates might be achieved. Ultrasonic treatment has been claimed to tenderize meat (Hagen, 1961; Lachman, Ozolins & Walker, 1962) and this could represent an additional benefit.

Goldblith (1963) has previously suggested that a combined thermal and irradiation treatment might provide a means of overcoming the radiation resistance of FMDV, but to date, no investigation along these lines appears to have been published

Although some attention was focussed by Zarotschenzeff (1944a, b) on the dielectric heating of meat as a means of inactivating FMDV, and the technique has been established for the defrosting of meat and fish (Bengtsson, 1962), there are no relevant references to FMDV inactivation by this means.

The availability of high power microwave generators, and the growing use of microwave heating in the food industry, suggests that rapid and uniform heating of meat to inactivate FMDV by this means should be investigated. By rapid elevation to high

temperatures, gross structural changes are minimized and high vitamin retention levels are claimed (Decareau, 1961). In the context of FMDV inactivation, studies are needed to determine whether, using microwave heating, a situation might arise whereby environmental RNase is inactivated prior to capsid disruption, with infective free RNA resulting. There is a possibility that the selectivity of microwave energy for free water might be employed advantageously for viral inactivation, by manipulation of the temperature and state of a foodstuff prior to heating.

Microwaves have been successfully used in sterilizing food after packaging in plastic pouches (Anon, 1963) and such a process might find a ready application to meat products. It is envisaged that any successful process for the inactivation of FMDV in meat and meat products will necessitate the treatment of relatively small samples (e.g. individual portions). Sliced meat, having a high surface area to volume ratio, would be subject to considerable weight losses on processing. Such losses might well be reduced by using a laser beam to 'slice' the meat, as the highly localized and intense heat would effectively seal the cut surface. An added advantage of laser 'slicing' would be the aseptic nature of the process.

It is considered that chemical processing has little to offer in inactivating FMDV within foodstuffs. The main objections may be summarized as: toxicological, uncertainty of penetration and adverse effect on flavour, the latter being particularly relevant to acidification.

Process evaluation based on a non-restricted virus

The stated aim of this survey was to promote interest in FMDV and its relation to foodstuffs, in doing so however there is a self-evident stumbling block. The highly contagious nature of FMD precludes experimentation with the virus anywhere other than at establishments where elaborate controls are exercised, and precautions adopted, to prevent the escape of virus. In this country work with FMDV has been restricted to the Animal Virus Research Institute, Pirbright, since 1939.

It would be of considerable value if there were a similar non-restricted virus that could be substituted for FMDV in the preliminary evaluation of any process designed to inactivate FMDV in foodstuffs.

It is suggested that the virus best suited for such a purpose is that of avian encephalomyelitis (Epidemic Tremor). A member of the picornaviruses, the avian encephalomyelitis virus is comparable with FMDV in almost every respect, more especially in having the same morphology, chemical constitution and resistance to inactivation by organic solvents, freezing, dehydration and heating (Andrewes & Pereira, 1967).

Avian encephalomyelitis virus is readily cultivated in fertile chicken eggs and the disease affects growing chicks, causing characteristic and easily recognized symptoms of ataxia, followed by tremors of the head and later, somnolence and death. A further practical advantage is that neutralizing antibodies of avian encephalomyelitis can be titrated in tissue culture using established techniques.

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Relation between pH and tenderness in cooked muscle

CHRISTINE L. MILES AND R. A. LAWRIE

Summary

Shear-force and pH were determined in corresponding cooked pre- and post-rigor samples of *l. dorsi* muscles from normal and adrenalized rabbits. The relative tenderness of pre-rigor meat was associated with a high pH; but this parameter was apparently affected by other unidentified factors.

Introduction

As empirical observation long ago indicated, meat cooked before the onset of *rigor mortis* is relatively tender, whereas that cooked immediately after *rigor mortis* (and before the tenderizing effects of conditioning) is relatively tough. With the recent recognition that shortening of muscles during the onset of *rigor mortis* is an important factor contributing to toughness in meat (Locker & Hagyard, 1963; Marsh, 1964), it might have seemed that the tenderness of pre-rigor meat was due to its non-shortened condition. As Marsh & Carse (1968) showed, however, shortening is particularly severe in meat cooked pre-rigor (i.e. when it is free to shorten). More expectedly, the marked shortening of muscle which has been frozen pre-rigor and thawed ('thaw-rigor') before cooking *is* associated with severe toughness in the meat (Marsh & Leet, 1966). Since a high pH had been shown to favour tenderness in meat (Howard & Lawrie, 1956), it seemed possible that the tenderness of meat when cooked pre-rigor, might reflect a high pH, caused by heat inactivation of the glycolytic enzymes, and thus stoppage of glycolysis, the effect of which might predominate over the tendency to toughness due to shortening. The present experiments were undertaken to consider this possibility and to compare the effect with that of high pH achieved by preslaughter glycogen depletion.

Materials and methods

Rabbits (six) were relaxed for 15 min pre-slaughter by myanesin (300 mg/kg intraperitoneally): others (four) were injected in addition with adrenalin (0·5 mg/kg subcutaneously), at 4 hr pre-slaughter, to deplete muscle glycogen reserves.

L. dorsi muscles were removed within a few minutes of death. Paired samples (ca.

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5 g) were then cut sequentially (proceeding from lumbar towards thoracic regions) at the same anatomical level from right and left rabbit *l. dorsi* muscles, at hourly intervals after death, the earliest samples being examined at about 10 min *post mortem*. The muscles concerned were kept moist at room temperature ($\sim$ 15°C) during the sampling period.

One sample of each pair was plunged into boiling water and cooked (at the boiling point) for 40 min ('pre-rigor'): the other was allowed to go into *rigor mortis* at 0°C and similarly cooked 48 hr later ('post-rigor').

A block (2 cm $\times$ 1 cm $\times$ 1cm) was cut from each sample by suitably mounted parallel scalpel blades and the shear-force (in kg) of the sample (at 1 cm thickness) determined using a Wolodkewich Universal tenderometer. The pH of the samples (approx. 1 g homogenized in 10 ml distilled water) was subsequently measured by glass electrode.

To study the effect of 'thaw-rigor', another series of comparisons was made, at hourly intervals *post mortem*, of pH and tenderness of paired samples from right and left *l. dorsi* (four rabbits) one member being cooked immediately (as above), the other being frozen in liquid nitrogen, thawed at room temperature and then cooked. With certain of these 'thaw-rigor' samples the shear-force was beyond the capacity of the instrument to record (with the accessories available) even at 0·5 cm thickness.

To obviate the effect of animal maturity the rabbits chosen were of approximately the same weight (4 kg).

Results

It was found that samples of *l. dorsi*, excised and cooked as soon as possible after slaughter had a high pH, near the *in vivo* values, and required a relatively low shear-force. It was thus again confirmed that cooked pre-rigor muscle is tender. In addition it was found that the cooking of such pre-rigor muscle arrested *post mortem* glycolysis. Samples taken at hourly intervals thereafter showed that the pH was arrested at progressively lower values and that there was a concomitant increase in the force necessary to shear the cooked muscle. On the other hand the paired samples, in which *post mortem* glycolysis was allowed to reach completion (post-rigor) before cooking, had a uniformly low pH – and a uniformly low tenderness. Typical data on these time-related changes are shown in Fig. 1.

Mean values for pH and shear-force in respect of the *l. dorsi* samples excised and cooked ca. 10 min *post mortem* (i.e. those showing the greatest pre-rigor effect), and in respect of the paired samples cooked post-rigor, are given in Table 1.

The high pH and low shear-force of the pre-rigor samples in comparison with the corresponding post-rigor samples is evident. Also shown in Table 1 are mean pre-rigor and post-rigor values for these early samples from the adrenalized rabbits. The high pH in both pre- and post-rigor cooked samples induced by the drug was clearly associated with a greater degree of tenderness than in samples from the untreated rabbits (Group I).

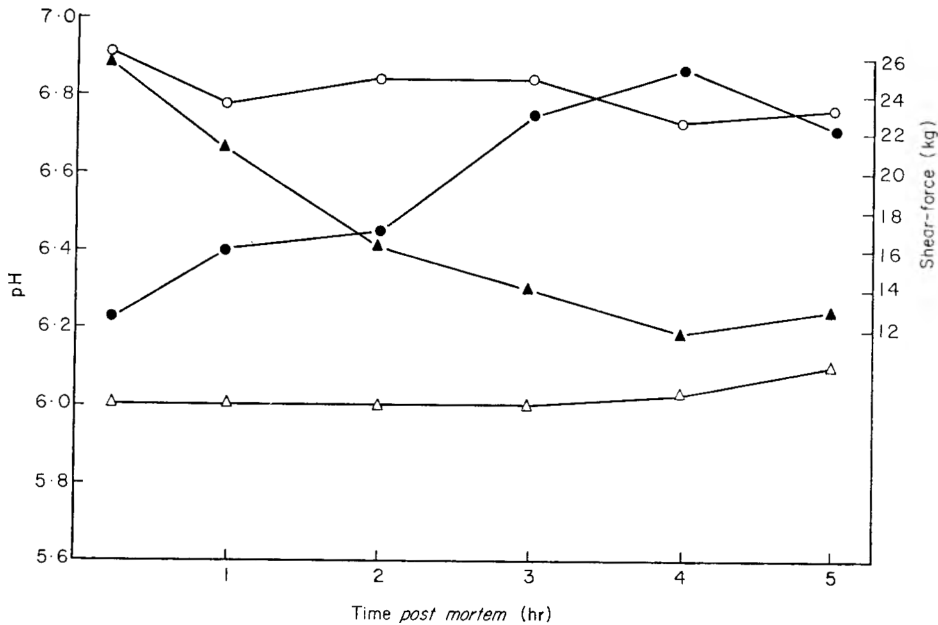

FIG. 1. Typical curve showing relationship between time post mortem, pH and shear-force in rabbit *l. dorsi* after cooking. ● = force to shear, ▲ = pH, in samples cooked immediately, O = force to shear, △ = pH, in paired samples cooked after 24 hr storage.

The samples which were frozen pre-rigor then thawed to induce 'thaw-rigor' before cooking had a markedly lower pH and lower tenderness than control material (Table 1). This depression of pH caused by 'thaw-rigor' conditions decreased as time from death increased, the pH approaching the level of normal samples cooked post-rigor. Tenderness concomitantly *increased.* Typical values, illustrating this feature, are given

TABLE 1. Comparison of pre-rigor and post-rigor pH and shear values for cooked, paired samples of rabbit *l. dorsi*

Group	No. of rabbits	pH		Shear-force (kg)	
		Pre-rigor	Post-rigor	Pre-rigor	Post-rigor
I. Normal	6	6·68 ± 0·06	5·97 ± 0·05	12·0 ± 0·3	24·2 ± 0·6
II. Adrenalized	4	7·12 ± 0·07	6·78 ± 0·04	10·3 ± 0·4	17·7 ± 1·1
III. 'Thaw-rigor'	4	6·55 ± 0·04	5·50 ± 0·04*	10·8 ± 1·1	⩾27

*Frozen, pre-rigor, and thawed before cooking ('thaw-rigor').

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in Table 2. There is some suggestion that freezing *per se* may have had a slight tenderizing effect.

When data on pH and shear-force for the various samples are plotted (Fig. 2) there is seen to be a definite relationship between these parameters, a high pH being associated with low shear-force and vice versa. The data from the adrenalin-treated rabbits, however, seem to belong to a different population since, for a given shear-force, the pH in the adrenalized group is greater.

A check of the effect of cooking *per se* indicated that it had produced an elevation of 0·33 ± 0·01 pH units above that of the corresponding raw sample, a response which is usual (cf. Bendall, 1946).

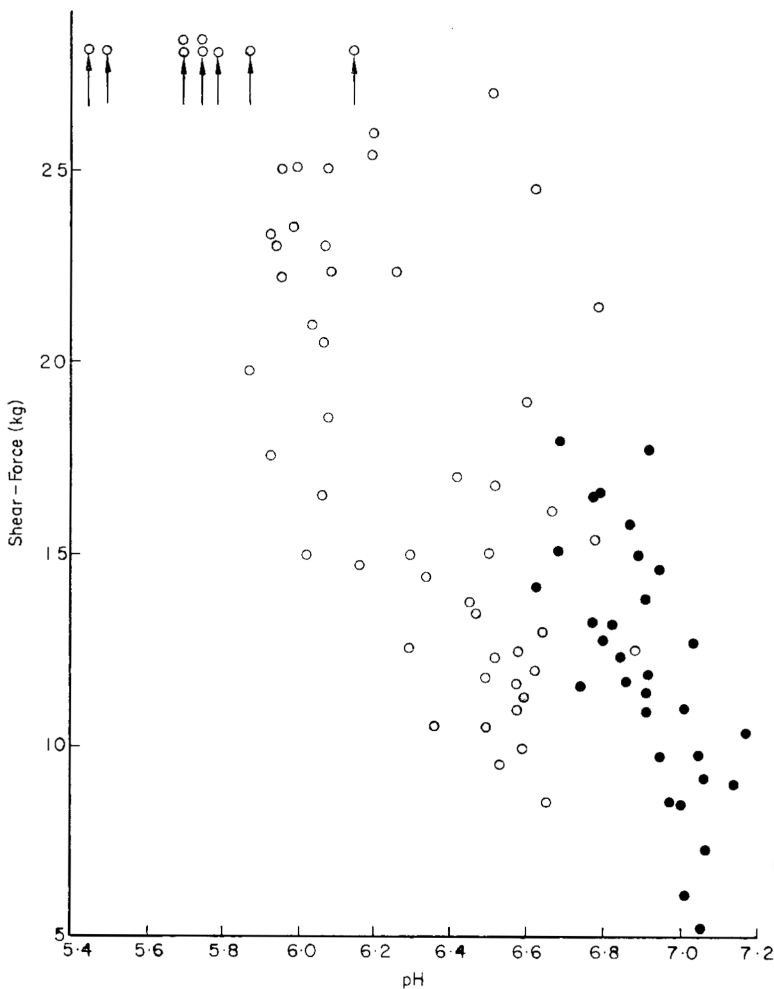

Fig. 2. Relationship between pH and shear-force in cooked samples of rabbit *l. dorsi*. Closed circles denote data from adrenalized rabbits. Arrows denote shear-force values greater than 27 kg.

TABLE 2. pH and shear values for samples of rabbit *l. dorsi* at various times *post mortem.*

	Cooked		Frozen, thawed, cooked ('Thaw-rigor')	
Time *post-mortem*	pH	Shear-force (kg)	pH	Shear-force (kg)
10 min.	6·59	11·0	5·42	⩾27
1 hr	6·36	10·5	5·66	24·5
3 hr	6·03	15·0	5·92	10·0

Discussion

As judged by the evidence from rabbit *l. dorsi*, it appears that the long recognized tenderness of meat when cooked pre-rigor can be associated with a high pH (achieved by heat inactivation of the glycolytic enzymes). Moreover, for the pH range 5·4 – 7·2, there is a direct proportionality between pH and tenderness in this muscle. This contrasts with the situation in beef and lamb, where a minimum value for tenderness is found between pH 5·6 – 6·0, according to the muscle being studied (Howard & Lawrie, 1956: Bouton & Shorthose, 1969).

That pH *per se* cannot be entirely responsible for the degree of tenderness observed, even within a given muscle when neither the content nor the nature of the connective tissue are contributory factors, is suggested by the data from the adrenalized rabbits. These indicate that the very high pH levels achieved by the use of the drug were not reflected by a correspondingly great increment in tenderness. At the other extreme of meat pH marked toughness was detected in the 'thaw-rigor' samples. In the latter condition the severe shortening, which is known to be largely responsible for the toughness (Locker & Hagyard, 1963: Marsh & Leet, 1966), is not offset by an elevated pH. Indeed the pH, in the present work, was found to be lower than control samples, suggesting that the 'thaw-rigor' conditions had potentiated the breakdown of muscle glycogen.

A high pH in muscle is known not only to enhance water holding capacity (Empey, 1933) but also to enhance water content (Lawrie, Pomeroy & Cuthbertson, 1963). It must be presumed that these circumstances mitigate against those interactions of the muscle proteins to which toughness is due. It is thus of interest that Marsh & Carse (1969) should have reported recently that if meat is cooked pre-rigor under conditions when the muscles are restrained from shortening, it becomes *tougher*. It may be that the cooking of meat pre-rigor, when so extended, accentuates the water loss which causes isodimensional shrinkage of sarcomeres in cooking (Giles, 1969), notwithstanding the high pH; whereas this is perhaps more readily resisted by the lower surface : volume ratio of the cooked pre-rigor meat when free to shorten.

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The effect of muscle excision before the onset of rigor mortis on the palatability of beef

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Summary

A study has been made to determine if organoleptically and microbiologically acceptable beef could be produced by pre-rigor excision followed by rigor setting and short-term aging of bovine muscle. Six carcasses (three steers, two young bulls and one old bull) were subjected to each of three treatments: (1) the control side of each carcass was chilled at 9°C for 24 hr; (2) one sample (PRE-24) from each of five sites on the other side of each carcass was removed pre-rigor and stored at 15°C for 24 hr; (3) one sample (PRE-48) from each of five sites was stored at 15°C for 48 hr.

Although the PRE-24 samples of the middle of the biceps femoris, anterior longissimus, and posterior longissimus were generally assessed to be similar to their controls in tenderness and other organoleptic properties a further 24 hr storage at 15°C resulted in a significant tenderization of the PRE-48 samples from these locations relative to their controls ($P < 0{\cdot}05$). There was no treatment effect on the semimembranosus, whereas the semitendinosus toughened after being excised pre-rigor. Mean bacterial numbers were held within the range 10^2–10^5/cm^2 muscle surface after 48 hr storage at 15°C in a sealed, gas-impermeable bag.

The investigation shows that a procedure for the pre-rigor excision of bovine muscle can produce organoleptically acceptable beef of a satisfactory microbiological standard.

Introduction

Traditionally, in the processing of beef, primal cuts are removed from the carcass sides after the musculature has entered rigor mortis during chilling. Chilling has been considered an essential part of the process since it limits the proliferation of aerobic surface bacteria and of anaerobic bacteria associated with bone taint in deep tissue (Callow & Ingram, 1955). The chilling of pre-rigor excised bovine muscle produces shortening

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which increases as the temperature of the muscle is lowered from 15°C. Minimum shortening in isolated fresh beef muscles occurs in the temperature region of 14–19°C. At higher temperatures (35–37°C) shortening coincides with rigor mortis, but at lower temperatures (0–10°C) shortening begins rapidly and usually immediately (Locker & Hagyard, 1963; Cassens & Newbold, 1967). Cold-induced shortening can also occur in muscle *in situ* (Marsh, Woodhams & Leet, 1968). Furthermore, chilling pre-rigor is not the only way of producing shortened muscle; considerable passive shortening can also result from the postural position if beef sides are hung by their back legs during processing in the traditional manner (Herring, Cassens & Briskey, 1965).

That muscle can enter rigor mortis in a shortened state is of considerable importance to the meat processor since such shortening produces toughness in meat (Locker & Hagyard, 1963; Marsh & Leet, 1966), and consequently an important aspect of the processing of beef should be the prevention of muscle shortening. With this in mind, a re-examination has been made of the traditional method of beef processing. It was conducted to determine if organoleptically and microbiologically acceptable beef could be produced by pre-rigor excision followed by rigor setting and short-term aging of bovine muscle. Since it was possible that pre-rigor excision could influence quality to different extents depending on animal maturity and choice of muscle, several maturities and muscles were included in this study.

Experimental

Meat source

Meat was obtained from Angus steers (approximately 1½ years old), young Angus Bulls (approximately 2 years old), and an old Angus bull (more than 5 years old). The following wholesale cuts were used in the study: cube roll (anterior longissimus at the tenth and eleventh thoracic vertebrae); strip loin (posterior longissimus at the third and fourth lumbar vertebrae); outside round (middle of the semimembranosus); and eye round (middle of the semitendinosus). The parentheses indicate the anatomical location of final sample. The pre-rigor boneless wholesale cuts were removed from one side of each steer carcass within 2 hr of slaughter. (Approximately 1 kg samples were excised pre-rigor from each of the bull carcasses.) Each wholesale cut was divided into two portions so that each portion contained a part of one of the above mentioned muscles. Each portion was placed into a gas-impermeable bag which was then evacuated and sealed. The cuts excised pre-rigor were stored at 15°C (air flow 300 ft/min). The muscle temperature was lowered to 15°C within 10 hr. One portion (PRE-24) of each cut was removed from the 15°C room at 24 hr *post mortem*, the other (PRE-48) at 48 hr *post mortem*. All samples were trimmed, rebagged, and frozen immediately at −14°C (air flow 600 ft/min). Control cuts were removed from the other sides of the same carcasses after chilling for 24 hr at 9°C (air flow 50 ft/min). The control sides were cut into boneless wholesale cuts at 24 hr *post mortem*. The trimmed muscles were

packed in gas-impermeable bags, which were then evacuated, sealed, and placed in the blast freezer.

To determine the time-course of aging in beef, both sternomandibularis muscles were removed from each of six Angus steers (approximately 1½ years old) within 2 hr of slaughter. Each muscle was trimmed and cut transversely into half samples, which were supported horizontally on thin plastic film at 15°C (relative humidity 100%). At intervals of 24 hr, samples from each animal were transferred from 15°C to −14°C for freezing.

Microbiological evaluations

Bacterial samples were taken by swabbing 5 cm^2 areas on the surface of the carcasses and boneless cuts. Plating-out on appropriate dilutions was carried out in plate-count agar (DIFCO). Triplicate plates of each dilution were incubated at 37°C and 25°C for 3 and 5 days respectively. The control sides were sampled in various positions before and after chilling. Boneless meat cuts (cube roll, strip loin, inside round, eye round and outside round) from the same side of each carcass were swabbed after excision. The same cuts, excised pre-rigor from each of the experimental sides, were sampled immediately after excision (PRE-0) and after storage at 15°C for 24 hr (PRE-24) and 48 hr (PRE-48).

Sample evaluations

Sections, approximately 3 cm thick, were cut from each muscle portion in the frozen state, and were placed in individual weighted plastic bags in a water bath at 80°C for 40 min. After cooking, the samples were cooled immediately in an ice-water bath. Triplicate shear force values were obtained on precisely-measured sections of the chilled, cooked samples (Davey & Gilbert, 1969). The characteristics of tenderness, juiciness, texture, and general acceptability were determined on 1½ cm cubes of each sample by a trained taste panel using a 9 point scale (Marsh, Woodhams & Leet, 1966).

Results

Shear-force values

Fig. 1 shows the relationship between the shear-force values of the cooked meat and the *post mortem* storage times for sternomandibularis muscles. Maximum aging was achieved in 72 hr at 15°C while the mean shear-force value decreased 15 units between 24 and 48 hr. In further study, *post mortem* storage was set at 48 hr since bacterial growth was excessive at longer periods.

Fig. 2 shows the mean shear-force values for each muscle of the six animals considered in the main body of the experiment. Shear-force values for the control and the PRE-24 samples within each of the muscles, biceps femoris, semimembranosus, anterior longissimus, and posterior longissimus are at similar levels. With the exception of the semimembranosus, the same muscles became significantly ($P < 0{\cdot}05$) more tender than the controls

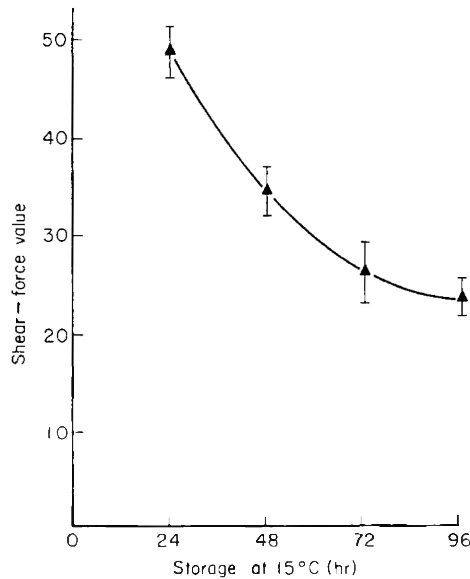

FIG. 1. The effect of storage at 15°C of sternomandibularis muscles from six steers on shear-force values. Vertical lines indicate ± SEM.

after an additional 24 hr of incubation at 15°C (PRE-48). The semitendinosus had significantly ($P < 0{\cdot}05$) higher shear-force values at both the PRE-24 and the PRE-48 treatments than the controls.

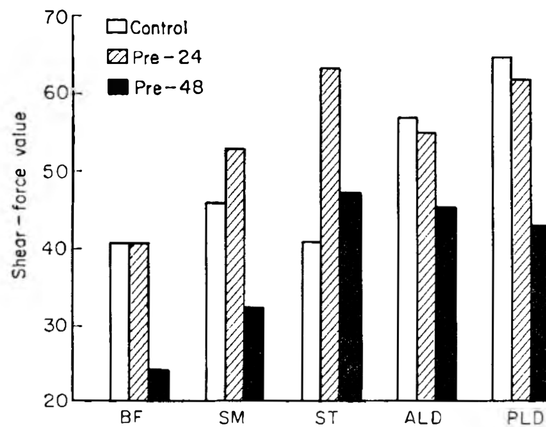

FIG. 2. The effect of pre-rigor excision on shear-force values of several muscles from all ($n = 6$) animals considered in this study. BF = biceps femoris, SM = semimembranosus, ST = semitendinosus, ALD = anterior longissimus, PLD = posterior longissimus.

Taste panel assessments

Taste panel assessments of tenderness largely support the tenderometer evaluations. The results of taste assessments of the muscles of the three steers examined are shown in

TABLE 1. Taste Panel Scores

	Muscles sampled				
	Biceps femoris	Semi-membranosus	Semi-tendinosus	Anterior longissimus	Posterior longissimus
Tenderness					
Control	4·2*	4·5	4·4	5·4	4·7
PRE-24	4·8	4·7	3·6	5·3	5·6†
PRE-48	5·4†	4·7	4·2	5·9	6·5
Juiciness					
Control	5·4	5·7	5·5	5·7	5·7
PRE-24	5·8	5·6	6·0	5·8	6·6‡
PRE-48	5·9	5·9	5·9	5·6	5·7
Texture					
Control	3·9	4·2	4·7	5·2	4·5
PRE-24	4·1	5·3†	3·7	5·1	5·2
PRE-48	4·5	4·9†	4·3	5·8	5·8‡
General acceptibility					
Control	4·4	5·0	5·3	5·8	5·2
PRE-24	5·2†	4·9	4·3	5·8	6·1
PRE-48	5·5†	4·9	5·0	6·3†	6·6†

*Mean taste panel scores from three steers. A score of 1 is the most undesirable and a score of 9 is the most desirable.

†Differences from control significant at 5% level.

‡Differences from control significant at 1% level.

Table 1. There were no significant ($P < 0·05$) differences in tenderness between the control and either the PRE-24 or the PRE-48 samples within the semimembranosus, semitendinosus and the anterior longissimus muscles. On the other hand, the biceps receiving the PRE-48 treatment was significantly ($P < 0·05$) more tender than the control, whereas the posterior longissimus was more tender at both the PRE-24 and the PRE-48 treatments. General acceptability was also significantly improved ($P < 0·05$) by pre-rigor excision in both the PRE-24 and the PRE-48 treatments of the biceps femoris and in the PRE-48 treatments of the anterior and posterior longissimus.

Microbiological assessments

The results of the microbiological analyses of the carcass surfaces are shown in Table 2.

TABLE 2. Carcass surface bacterial counts

	Site of sample				
	Buttock	Aitch-bone	Flank	Fore-shank	Neck
Plates incubated at 37°C					
Control before chilling	2·84* ±0·30	2·97 ±0·20	2·95 ±0·26	2·36 ±0·48	3·79 ±0·31
Control after chilling 24 hr	2·22 ±0·26	2·89 ±0·18	2·11 ±0·17	2·31 ±0·40	2·99 ±0·28
Plates incubated at 25°C					
Control before chilling	3·13 ±0·34	2·84 ±0·21	3·18 ±0·21	2·41 ±0·52	4·01 ±0·36
Control after chilling 24 hr	2·98 ±0·18	3·19 ±0·14	2·67 ±0·17	2·82 ±0·19	3·13 ±0·39

*Values are means of logarithms of bacteria per cm^2 surface from three steers ± SEM.

As expected, the area of the neck had the highest level of contamination (Scott & Vickery, 1939). Most of the carcasses sampled had mean bacterial numbers of less than $10^3/cm^2$ of the carcass surface. The results of the microbiological analyses of the various wholesale cuts are shown in Table 3. Mean bacterial numbers on the cuts from the control half of the carcasses were generally less than $10^3/cm^2$. The pre-rigor excised muscles showed surface counts of a similar or slightly higher level. Only in the strip loin did the mean bacterial level rise above $10^4/cm^2$ after 48 hr at 15°C.

Discussion

The present results show that muscles, excised from beef carcasses pre-rigor and maintained at 15°C for 24 hr to ensure that the muscles are fully in rigor, are usually as tender as muscles which are excised from carcasses after chilling for 24 hr. Extension of the period of 15°C storage for a further 24 hr (PRE-48) produces a considerable aging in muscles such as the biceps femoris, anterior longissimus, and posterior longissimus. The PRE-24 and the PRE-48 semitendinosus samples were tougher than the control. Considerable shortening, which may be enhanced by a high collagen content (Herring, Cassens & Briskey, 1967), was observed on pre-rigor excision of the semitendinosus. Toughening of the semitendinosus was most notable in the large bull carcass where the heavy fore-quarter placed a considerable strain on certain muscles of the hind-quarter.

TABLE 3. Surface bacterial counts of wholesale meat cuts from three steers

Treatment	Cut sampled				
	Cube roll	Strip loin	Inside round	Eye round	Outside round
Plates incubated at 37°C					
Control	2·66* ±0·16	3·03 ±0·29	2·91 ±0·22	2·11 ±0·44	2·35 ±0·30
PRE-0	2·70 ±0·25	3·42 ±0·54	3·06 ±0·50	1·87 ±0·27	1·90 ±0·43
PRE-24	3·17 ±0·24	3·65 ±0·51	2·94 ±0·49	2·13 ±0·43	3·32 ±0·36
PRE-48	3·91 ±0·34	4·39 ±0·88	3·54 ±0·78	2·52 ±0·22	3·24 ±0·20
Plates incubated at 25°C					
Control	2·62 ±0·12	3·42 ±0·43	2·97 ±0·15	2·08 ±0·45	2·15 ±0·52
PRE-0	2·77 ±0·21	3·66 ±0·53	3·39 ±0·51	1·93 ±0·39	2.18 ±0·47
PRE-24	3·28 ±0·29	3·83 ±0·47	3·05 ±0·52	2·29 ±0·56	3·04 ±0·67
PRE-48	3·94 ±0·40	4·45 ±0·87	3·51 ±0·80	2·52 ±0·26	3·41 ±0·42

*Values are means of logarithms of bacteria per cm^2 surface of wholesale beef cuts from three steers ± SEM.

However, the biceps femoris is passively shortened on the vertically suspended beef side (Herring *et al.*, 1965) so that pre-rigor excision apparently produces a lengthening of the muscle with accompanying tenderness advantages.

Although the effect of pre-rigor excision on beef tenderness was the main aspect of this study, taste panel assessments show that other palatability characteristics of juiciness, texture, and general acceptability can be preserved on storage for 48 hr at 15°C in evacuated and sealed bags. Furthermore, microbiological spoilage is satisfactorily controlled during the prolonged storage at 15°C.

The metabolism of pre-rigor muscles packaged in evacuated, sealed bags of low gas permeability would be expected not only to use up the oxygen remaining, but also to

produce carbon dioxide which is itself inhibitory to the growth of spoilage bacteria (Haines, 1933; Scott, 1938; Clark & Lentz, 1969). As a result of these conditions, the mean bacterial numbers have been held to within the range 10^2–10^5/cm^2 muscle surface. This is less than the value of 10^6/cm^2, usually accepted as the limit of allowable micro-organisms.

Although this is a preliminary report, it does show that organoleptically acceptable meat of a satisfactory microbiological standard can be produced by pre-rigor excision of prime cuts from beef carcasses. Such a procedure, if proved practicable, could produce considerable saving in by-passing the conventional beef chiller while ensuring a standard product.

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Rigor tensions and gaping in cod muscle

J. R. BURT, N. R. JONES*, A.S. McGILL AND G. D. STROUD

Summary

The effects of temperature on the breaking stress and rigor tension of cod muscle fibres are described and related to the occurrence of gaping in fillets consequent on high temperature rigor mortis. This type of gaping is due to a combination of the greater rigor tensions generated at higher temperatures and the lowering of the inherent strength of the tissues; these effects proceed to such an extent that at elevated temperatures the muscle pulls itself to pieces. The magnitudes of the rigor tensions produced at a given temperature are similar whether or not the system is rendered anoxic.

The results obtained are discussed in the light of published observations on the temperature dependence of cod fillet shrinkage and on the behaviour of mammalian muscle in similar situations. Attempts are also made to relate the size of the tensions that develop to the concentration at rigor, or changes in concentration between death and rigor, of lactate and adenosine triphosphate (ATP).

Introduction

The problem of gaping, or separation of adjacent flakes, in fillets cut from thawed frozen fish is one that has during recent years been recognized as being of importance to the fish processing industry (Banks, 1962; Jones, 1964, 1965, 1969; Love, 1968; Love & Robertson, 1968; Burt *et al.*, 1969) and the origins and causes of this quality defect are now being investigated systematically. Love and his co-workers have already identified and described many of the factors involved in the production of gaping and in varying the extent of its occurrence (Love, 1968; Love & Robertson, 1968; Love, Lavéty & Steel, 1969; Love & Haq, 1970) but one of the first postulated explanations (Banks, 1962), in which proteolysis of the sheets of connective tissue or myocommata is regarded as probably causing a weakening of the bond between adjacent blocks of muscle or myotomes, has not so far been demonstrated experimentally. Jones (1964) stated that at temperatures above 17°C the onset of rigor mortis in cod muscle on the bone is accompanied by contractions so strong that the connective structure, perhaps

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itself thermally weakened, fails and gaping ensues. The work described in this paper was started largely in an attempt to put this latter statement on a quantitative basis.

That muscles not only grow rigid but can also shorten in rigor mortis has been known since at least 1833 and this knowledge has obvious implications in the field of forensic science (Forster, 1963) as well as in that of food technology. Fish fillets that have been cut from the skeleton before the onset of rigor mortis are known to contract; the rate of shrinkage, the extent of such contractions and the severity of associated changes in quality being governed by factors such as temperature, pH, *ante mortem* exercise, *post mortem* handling, freezing, frozen storage and thawing (Dyer & Fraser, 1961; Love, 1962; Buttkus, 1963; Jones, 1964, 1965, 1969; Bykov, 1967). Were these contractions to be opposed, as occurs while the fillet is still attached to the skeleton, it is to be expected that they would manifest themselves in the form of an increase in tension within the musculature and that this tension should be measurable. In fact, Buttkus (1963) did succeed in demonstrating the increases in tension that develop at 20°C, during the pre rigor phase, in strips of red and white muscle of lingcod (*Ophiodon elongatus*). Similar studies of tension development in mammalian muscle systems have since been published (Busch, Parrish & Goll, 1967; Galloway & Goll, 1967; Jungk *et al.*, 1967) but in these latter instances the effects of variations in the environmental temperature were also investigated.

The results of the studies described in this paper include the effects of temperature on the breaking stress of cod muscle fibres and of muscle-connective tissue systems as well as the effects of temperature and anoxia on the development of rigor tension in cod muscle fibres.

Materials and Methods

The fish used for this study were all aquarium-maintained cod (*Gadus morhua* L.) which had been feeding for at least 6 weeks after capture by trawl in the North Sea off Aberdeen. Immediately prior to use a fish would be captured and killed by stunning with a minimum of struggling, eviscerated and removed without delay to a low temperature room (1°C) where subsequent manipulations would be carried out.

Muscle and muscle-connective tissue preparations

These were obtained from the belly flaps where it was noticed that the inter-myocommatal distances were greatest. Small strips of white muscle, with the myofibrils running along the length of them, were cut from this region and mounted between jaws of platinum foil using Eastman 910 adhesive. Care was taken to stimulate the muscle as little as possible during these manipulations. Two types of mount were prepared: one in which muscle fibres only were visible between the jaws (Fig. 1a) and another in which a piece of connective tissue sheet was present approximately mid-way between the jaws (Fig.

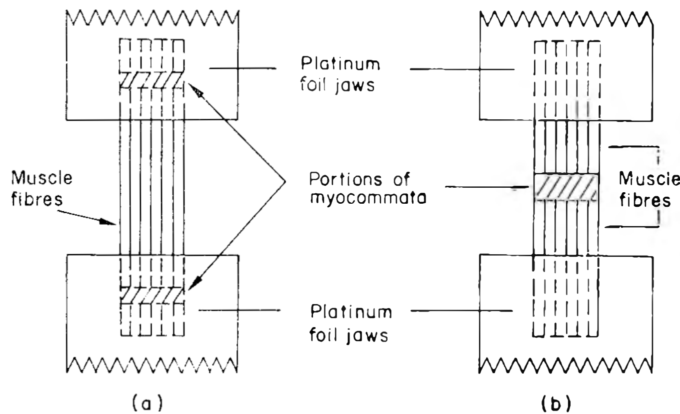

FIG. 1. Schematic representation of (a) muscle-only and (b) muscle-connective tissue preparations used.

lb). Depending on the size of the fish taken, these muscle preparations varied in length from 5 to 15 mm and in weight from 10 to 25 mg. Immediately after mounting, the preparations were placed in a humid, constant temperature environment. Each fish would be used to provide between six and ten of each type of these. The initial resting length (unloaded) of tissue between the jaws was measured for each preparation as soon as it was mounted.

Measurements of breaking stress —1

All the pieces of mounted tissue, as prepared above, from any one fish were allowed to contract freely at a set temperature (between 0°C and 30°C). Some short time before these samples were expected to set in rigor mortis one of the muscle-only samples was suspended from a clamp, still in the humid temperature controlled environment, a lightweight hook fixed to the lower pair of jaws and weights added to this hook until the muscle was stretched back to its original length. This procedure was repeated at intervals with each of the other muscle-only samples in turn until it was found that one of them had gone into full rigor and, being no longer extensible, broke. The force required to extend the immediately preceding sample to its original length was noted and this value was used to calculate the breaking stress of the tissue. The muscle strip was then cut off flush with the edge of the jaws and weighed. Assuming the specific gravity of cod flesh to be 1·05 between 0°C and 30°C (cf. Jason, 1958), and as the length of the sample was known, the breaking stress of the muscle going into rigor at a given temperature could be calculated. Each time a muscle-only sample was used, one of the muscle-connective tissue ones was taken, loaded till it broke and a note taken of where the break occurred.

Isometric measurements of rigor tension

An electro-magnetic force balance* was modified to take the muscle preparations and hold them in a humid, constant temperature chamber (Fig. 2). This instrument holds the muscle strip at constant length: any tendency to contract is automatically

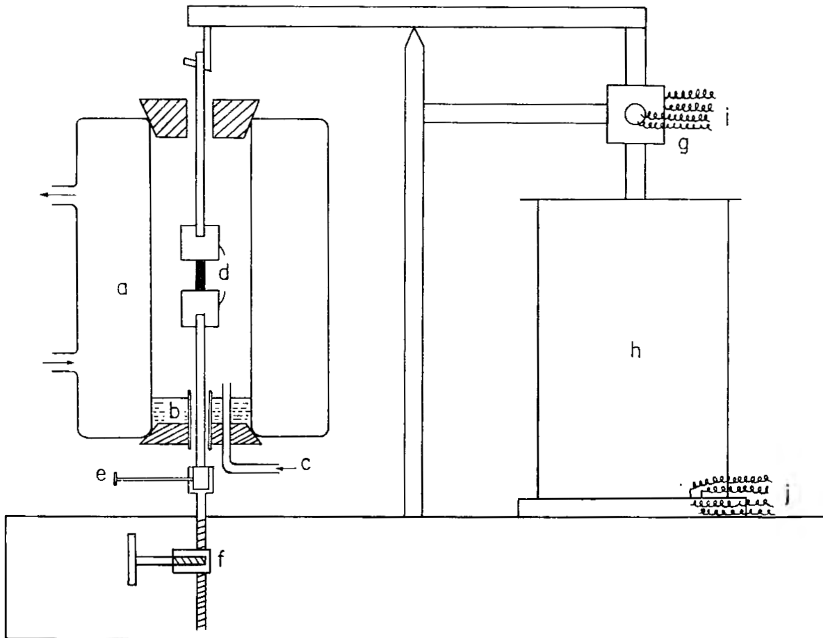

Fig. 2. Schematic diagram of modified automatic balance (Not to scale). a, circulating-water thermal jacket; b, water; c, gas flush; d, mounted muscle preparation; e, locking screw; f, adjustment screw; g, photo-electric null point sensor; h, electromagnetic coil; j, leads to and from control unit.

countered by an increase in the current supplied to the coil of the electro-magnet and the apparatus is thereby maintained in balance all the time. The power fed to the coil is monitored continuously by the control unit which also supplies a signal to a potentiometric recorder. The complete assembly was calibrated so that a full-scale deflection on the recorder was equivalent to an increase in force of 196·2 mN generated within the muscle. Some sample recorder traces are illustrated in Fig. 3 showing the time-course of force development at three different temperatures for muscle preparations. The dotted portions of the traces were made while the muscle was not under tension and the vertical distances between the coresponding points 'a' and 'b' are proportional to the tensions which developed. Knowing the weight, length and specific gravity of the samples the rigor tensions can then be calculated. In the examples shown these were 2·1 N/cm^2 for the 17°C sample and 7·7 N/cm^2 for the 25°C one. The 30°C sample broke at a tension value of 14·5 N/cm^2.

*Designed by Dr A. C. Jason and Mr A. Lees of Torry Research Station and made in the Station's workshops.

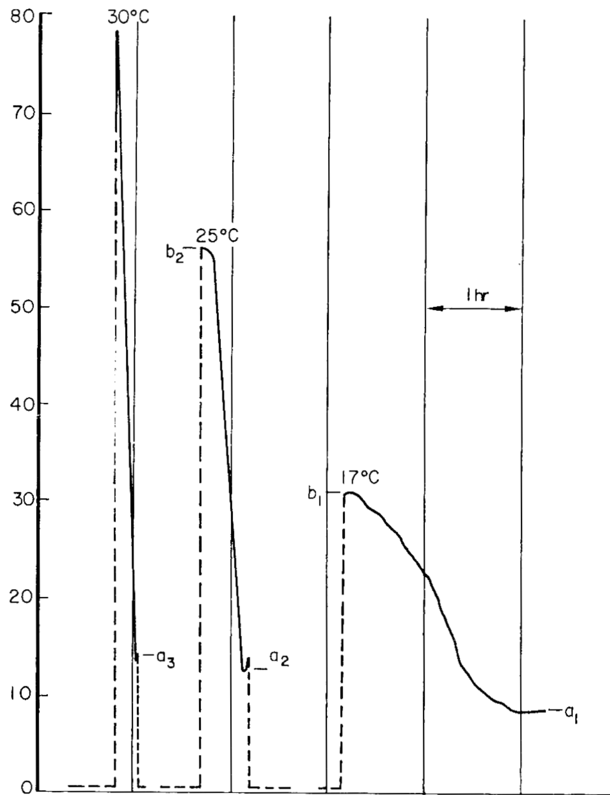

FIG. 3. Development of rigor tension in cod white muscle fibres.

For the determination of rigor tensions, muscle-only samples were again used. Of all those prepared from a single fish at any one time, one was used immediately in the apparatus at the experimental temperature while the others were kept unloaded and in a humid atmosphere at 0°C until they could be used; the higher the experimental temperature, the more samples could be used from each fish.

The rigor tension developed in cod muscle at several temperatures in the range 0-30°C was determined for three environmental conditions: still air and flowing oxygen-free nitrogen or air. The atmosphere in each case was kept saturated with water vapour throughout. As soon as the samples used in the still-air experiments had been weighed, they were extracted for subsequent analysis for ATP and lactate.

Measurements of breaking stress —2

A few determinations of breaking stress at rigor were also made using the automatic balance. Muscle-only samples were allowed to set in rigor while being held isometric in the balance assembly so that the rigor force was recorded. Using the adjustment screw,

the muscle preparation was then pulled gently downwards through about 3 mm thus throwing the instrument off balance. As the arm of the balance was automatically pulled back to its equilibrium point, the piece of in-rigor muscle was stretched till it broke. The recorder trace showed the point at which the break occurred as well as the force required to produce the break. Breaking stress was calculated as above and the results are plotted along with those obtained from the earlier breaking stress experiments (Fig. 4).

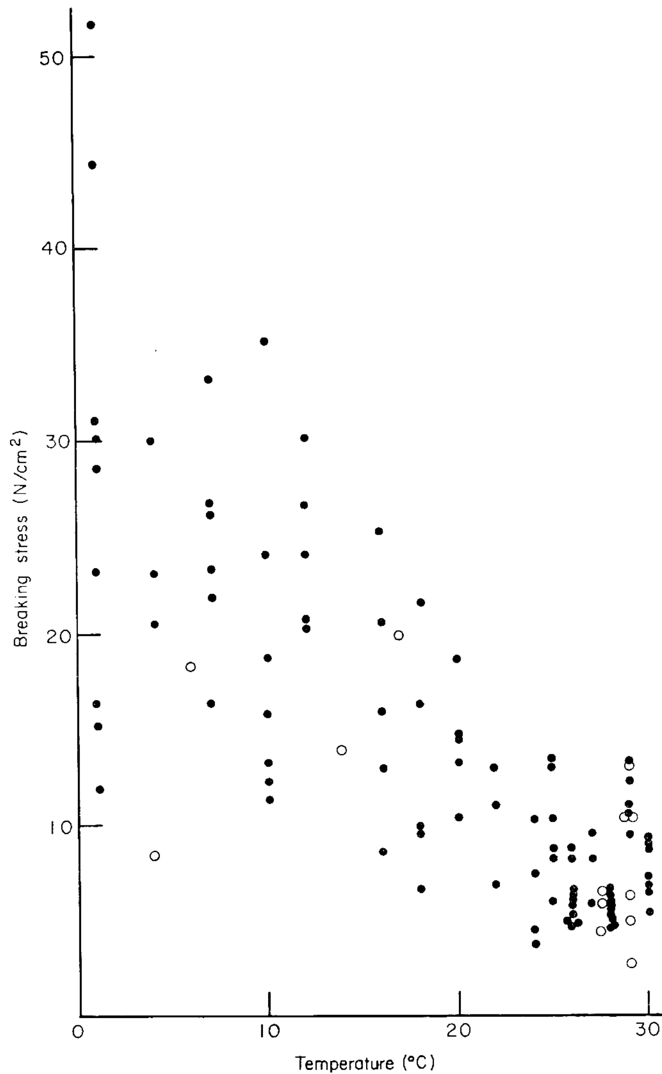

FIG. 4. Breaking stress of cod white muscle at different temperatures. ● = from loading experiments; ○ = from experiments using the automatic balance.

Analysis of ATP and lactate concentrations in muscle samples

After the isometric tension samples were in full rigor, they were cut from the mount and weighed. They were then homogenized immediately for 2 min in a microhomogenizer with a Teflon grinder with 1 ml of 0·6 N chilled perchloric acid. The precipitated protein was removed by filtration through sintered glass, 0·2 ml of 0·15 M tris buffer (pH 7·6) was added to the filtrate which was then neutralized with 0·86 N potassium hydroxide. Similar extracts were made from portions of muscle, again from the belly flaps, taken immediately after the death of the fish and also at the times when each mount was placed in the electro-magnetic balance for starting rigor tension measurements. ATP analyses were carried out by the method of Lamprecht & Trautschold (1963) and lactate ones by that of Hohorst (1963).

Results and discussion

The effect of temperature on the breaking stress of cod muscle fibres at or just before the onset of full rigor is shown in Fig. 4. The values determined in the experiments with muscle that had set in rigor under tension fall largely within the scatter of those obtained from muscle going into rigor while contracting freely. The values plotted in the former case consist of the total stress registered on the recorder; i.e. a rigor tension component plus the additional stress required to break the muscle preparation. It is interesting to note that these values are roughly comparable with those found necessary to break the myotome-myocomma interface at various temperatures: 12·8 N/cm^2 over the range

TABLE I. Percentage occurrence of breaks at the myotome–myocomma interface

	Up to and including rigor		At rigor mortis	
Temperature (°C)	No. of observations	(%)	No. of observations	(%)
1	19	2	6	8
5	6	2	2	0
7	26	20	5	19
12	8	3	3	14
16	24	25	4	28
18	24	55	5	30
22	9	67	3	68
25	24	0	5	0
30	38	68	12	58

10–25°C and thence falling to 8·8 N/cm^2 at 30°C and to 0·6 N/cm^2 at 40°C (Love, 1968). Unfortunately this comparison cannot be taken too far since Love's values were obtained at a constant time (3 hr) *post mortem* when, depending on temperature, the specimens used would be in differing rigor states while the results reported here were obtained at different times *post mortem* but with samples that were all in equivalent rigor states. A further difference between the two sets of experiments lies in the material used. The preparations used to obtain these values did not contain any myocommal material (Fig. 1a) while Love's did. However, the position where the breaks occur when a band of connective tissue is present in the preparation (Fig. 1b) was determined for samples taken at the point of full rigor and also at various times up to that point. An analysis of this data is presented in Table 1 in terms of how frequently the break was observed to occur at the myotome-myocomma interface for each experimental temperature. It is apparent that the relative strengths of the muscle fibres and the connective tissue change with temperature. At the lower temperatures studied it is the muscle that is more prone to break, while with increasing temperature, it is in the connective tissue (or at the boundary between it and the muscle fibres) where the breaks become more likely. The reasons behind the anomalous values obtained at 25°C are not known.

The development of tension in fish muscle as it enters rigor mortis under isometric conditions is shown, for three temperatures, in Fig. 3. These traces illustrate both the more rapid development in tension and the higher tensions that arise with increases in temperature. In addition, the sharp peak on the 30°C trace indicates just when the sample broke before it set in rigor. The levelling off at the tops of the traces obtained at the two lower temperatures shows where these muscle samples set in rigor while the sharp reductions (dotted lines) indicate where these samples were dismantled for analysis.

Those samples that were left under tension in the balance assembly beyond the point of rigor onset demonstrated that the rigor tension value was maintained for a time equivalent to the rigor period. These findings contrast with the observations made by Buttkus (1963) on red and white muscle strips from lingcod and by Jungk *et al.* (1967) on rabbit and beef muscle strips in which the rigor tension began to decrease as soon as it reached a maximum.

The rigor tensions that developed in cod white muscle fibres at different temperatures from 0°C to 30°C in still air are plotted against temperature in Fig. 5. The tensions found at temperatures up to 17°C are remarkably constant on average while a marked increase is apparent from that temperature upwards. At the higher temperatures (25–30°C) an increasing number of the samples broke before the muscle fibres set in rigor mortis and values obtained from such experiments have not been plotted. Only data from experiments in which flat maxima were reached on the recorder traces have been used.

Since cod fillets contract less vigorously at chill temperatures than they do at elevated ones (Dyer & Fraser, 1961) it was not surprising to find a direct relationship betweeen

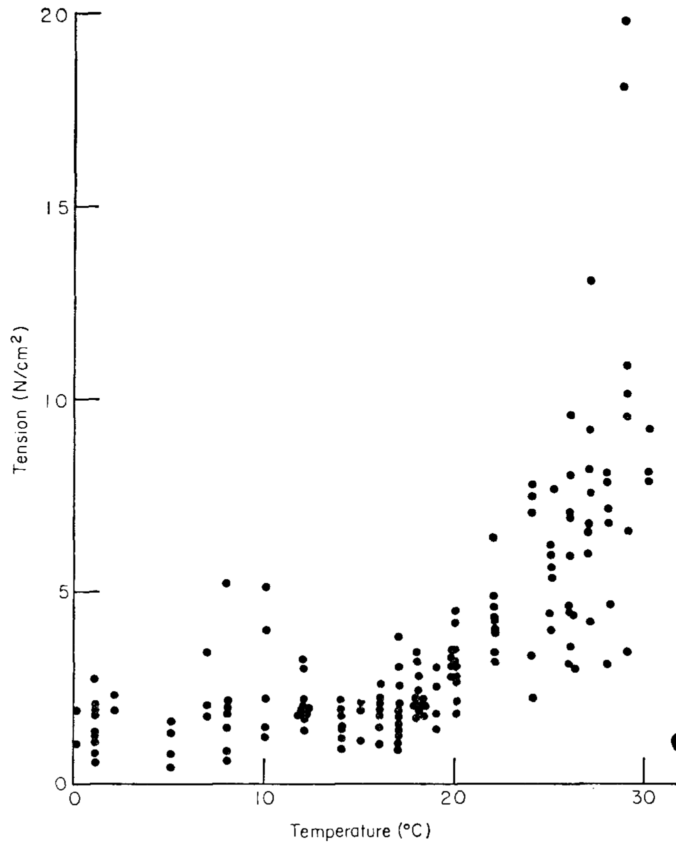

FIG. 5. Rigor tension of cod white muscle at different temperatures in still air.

tension and temperature throughout the temperature range studied. This contrasts with the behaviour of beef (Busch *et al.*, 1967) and pork (Galloway & Goll, 1967) muscle strips which produce higher tensions at 2°C than they do at 16°, 25° and 37°C, but is similar to that of rabbit ones (Jungk *et al.*, 1967). Busch *et al.* (1967) have speculated that this large tension development at 2°C very likely originates from the same events as those which lead to the cold shortening which was first described by Locker & Hagyard (1963) in bovine muscle and later by Cook & Langsworth (1966) and by Galloway & Goll (1967) in ovine and porcine muscle respectively. Rabbit muscle is again more similar to that of cod than to that of the other mammals studied in not producing a cold shortening effect (Locker & Hagyard, 1963).

The discontinuity in the tension-temperature curve around 17°C is strongly reminiscent of sharp changes in some other properties of cod muscle at this same temperature and could be indicative of a common, underlying biochemical or physico-chemical causal mechanism. Dyer & Fraser (1961) described the different rates of shrinkage

which cod fillets exhibit below and above 16°C or so and also the marked increases in drip which are found above this temperature. The nature of the rigor process in gutted cod also appears to change at 17°C (Jones *et al.*, 1965) to such an extent that above this temperature muscle blocks become compacted and tough and there is considerable fluid loss. Furthermore, an Arrhenius plot of the time taken by cod to enter rigor mortis also showed a sharp discontinuity in the region of 17°C. This type of discontinuity is not unique to fish however. Similar biphasic Arrhenius plots, with breaks around 16°C, have been reported for the action of rabbit muscle myosin on inosine triphosphate and on ATP in the presence of dinitrophenol (Levy, Sharon & Koshland, 1959; Levy *et al.*, 1962), attributable to a change in conformation of the enzyme-substrate complex.

The results of the final set of experiments that were carried out are summarized in Fig. 6 which gives the relationship between temperature and the rigor tensions which develop in flowing air and in flowing oxygen-free nitrogen. It is noticeable that the

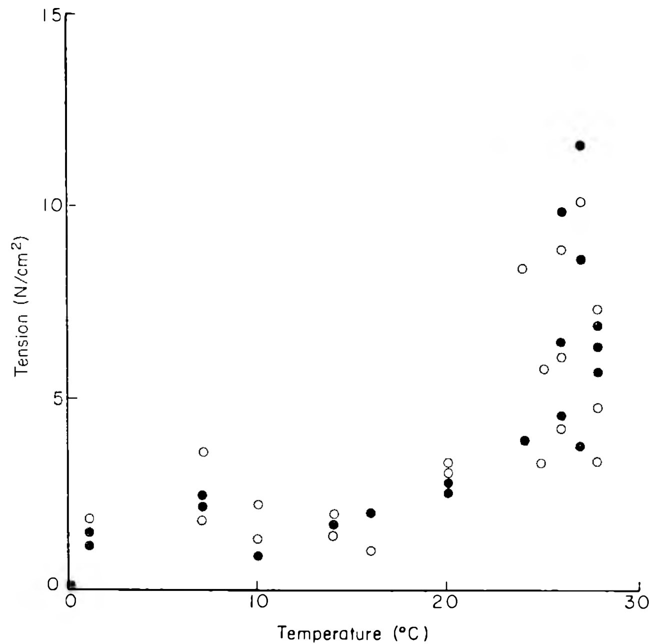

Fig. 6. Rigor tension of cod white muscle at different temperatures in a flowing atmosphere. ○ = air; ● = oxygen-free nitrogen.

nature of the environmental atmosphere did not make any difference to these tensions and that the results are remarkably similar to those obtained in still air (Fig. 5). This is to be expected in view of the essentially anaerobic nature of the metabolism in white muscle and the latter's almost complete dependence on the Embden-Meyerhof pathway for energy production. By contrast, Buttkus (1963) found that the rate of rigor contraction of lingcod red muscle is affected by the oxygen concentration in the surrounding

atmosphere; an increase in oxygen concentration above that present in air inhibiting rigor contraction.

While every precaution was taken to prevent drying of the muscle samples during tension development, the possibility of dehydration occurring was not lost sight of. In fact, Galloway & Goll (1967) ascribed much of the small amount of shortening they observed in porcine muscle strips held at 25° and 37°C to dehydration. However, it is felt for two reasons that this did not occur in the present study. Firstly, unless it were fully saturated with water vapour, a flowing atmosphere would be expected to be a more effective drying agent than a stationary one and virtually identical results were obtained under these two sets of conditions (Figs 5 and 6). Secondly, test samples of muscle of similar dimensions did not show any weight loss as a result of holding in the constant-temperature chamber for 24 hr.

Earlier reports from this laboratory (Jones *et al.*, 1965; Burt & Stroud, 1966) have discussed the discrepancies which exist between observations made on cod muscle during the immediate *post mortem* period and mammalian muscle rigor mortis theory. The analytical results obtained during the study reported here have agreed with our earlier findings in that neither lactate nor ATP levels correlate simply with the onset of rigor. Correlations between rigor tension or temperature and the following metabolite parameters were also tested for but none were found to be of any significance: lactate or ATP concentration at rigor and change in lactate or ATP concentration between death and rigor.

That the change in lactate concentration between death and the onset of rigor mortis does not show any correlation with the rigor tension which develops is particularly disappointing in view of the findings that fish in good condition gape more than those in poor condition (Love, 1968). This was attributed to the strength of pull produced during the rigor contraction of muscle becoming weaker as the condition of fish deteriorates and it might have been expected that higher tensions would result from greater metabolite turnovers. However, not all the glycogen broken down in cod muscle appears as lactate: a large fraction is cleaved hydrolytically to form glucose and maltose (Burt, 1966) and a small, temperature-dependent one is shunted aside to form L-α-glycerophosphate. It would thus appear extremely difficult to unravel the relative importances to total gaping of pH lowering in reducing tissue strength (Love & Haq, 1970) and of higher energy production in increasing the force of contraction.

When Figs 4 and 5 are superposed a cross-over is obvious in the region of 20–25°C. The conclusion that can be drawn from this is that, as the temperature in this region rises, an increasing proportion of muscle fibres produces sufficient force to overcome the inherent strength of the tissues. Unfortunately, figures do not appear to be available for the tension that is produced during normal contraction in fish muscle for comparison with these rigor tensions, but Jungk *et al.* (1967) and Bendall (1969) have quoted values of about 30 N/cm^2 and of 13 to 21 N/cm^2 for the maximum strengths of tetanic

contraction in mammalian and frog muscles respectively when they are operating at their normal lengths. Jungk *et al.* also quote a value of about 1 N/cm^2 for the rigor tension. The rigor values obtained here range from 1 to 20 N/cm^2 depending on temperature and, particularly at the higher temperatures, are therefore quite substantial.

To conclude, it has been demonstrated that the immediate cause of the gaping found in fillets cut from cod that have been accelerated into rigor mortis by high temperatures is due to a combination of the greater rigor tensions generated at higher temperatures and the lowering of the inherent strength of the tissues; these effects proceed to such an extent that at temperatures from about 20°C upwards the muscle literally tears itself apart.

Acknowledgments

Acknowledgment is made of the help received from Dr A. C. Jason and Mr G. R. Peters in discussing some of the physical aspects of this work which was carried out as part of the programme of the Ministry of Technology.

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The rapid determination of fat in chocolate and related products using low resolution nuclear magnetic resonance

P. H. WIGGALL, A. D. INCE AND ELIZABETH WALKER

Summary

The short-term and long-term stability of the Newport NMR Quantity Analyser when used for cocoa butter under various instrumental conditions has been investigated, and the optimum operational conditions selected. Following calibration of the instrument, techniques were developed for the rapid determination of fat in milk and plain chocolate, milk chocolate paste and crumb, cocoa liquor and powder, and the results compared with those of conventional extraction methods. The NMR results were higher, and products with relatively high moisture content required pre-drying, but after applying standard corrections the agreement with the extraction methods was generally good. Precision and accuracy of the corrected NMR method as used in the laboratory was considered to be satisfactory for control purposes, and by using a temperature compensation technique in the factory, a rapid off-line process control technique has been developed.

Introduction

A very rapid method of determining the fat content of chocolate and related products was required to aid process control. Of the techniques available, low-resolution nuclear magnetic resonance (NMR) appeared to be the most promising, especially since it was non-destructive and could possibly be developed into an in-line system. The potential of the technique was subsequently confirmed by a feasibility study carried out by Varian Associates (Oref, 1965). Since then, work has continued in this country using equipment manufactured by Newport Instruments Limited, and developments have now reached the stage where instrumentation and a simple technique are available with accuracy and precision acceptable for control purposes.

The principle of low-resolution NMR has previously been described (Harlan, 1964; Simpson, 1968) but briefly, the technique depends on the fact that the resonance of hydrogen nuclei in the solid state is weak and very broad, whereas nuclei in the liquid state exhibit a strong narrow resonance. The low-resolution spectrometer is

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designed to ignore the resonance due to nuclei in the solid state, and to measure the remaining narrow absorption which is proportional to the number in the liquid phase. Thus, unlike hydrogen nuclei present in water and liquid fat, those in substances such as solid sugar, cellulose or solid fat do not give a detectable NMR signal. This ability to differentiate between these two phases has been utilized for the determination of water in wheat (Miller & Kaslow, 1963), coal (Ladner, 1964); fat in seeds (Conway & Earle, 1963), maize (Alexander *et al.*, 1967), soybeans (Collins *et al.*, 1967); and the liquid/solid content of fats (Chapman, Richards & Yorke, 1960; Ferren & Morse, 1963; Pohle, Taylor & Gregory, 1965; Bosin & Marmor, 1968).

Description of equipment

The equipment, shown in Plate 1, consists of an electronic console connected to a magnet assembly, a digital voltmeter (DVM), a supplementary modulation (SM) unit and a sample temperature controller. The latter blows hot air at controlled temperatures varying from ambient to 100°C through the magnet assembly so that during NMR measurement samples can be maintained at any predetermined temperature.

A 2·7 MHz oscillator supplies radiofrequency (RF) energy to an RF coil placed in the gap of the magnet providing a homogeneous field of 640 gauss. Modulation coils on the magnet sweep the field through the proton resonance value about sixty times every second. The energy absorbed from the RF coil by the proton resonances is detected, amplified and displayed on an oscilloscope, the latter being used to ensure correct RF tuning. The energy loss, which is proportional to the area under the absorption peak, is quantitatively indicated on a panel meter. The signal amplitude depends on the number of protons in the liquid phase of the sample, the RF level and the amount of amplification applied by the audio frequency (AF) gain control. The latter is adjusted for each substance to give the optimum meter reading. Greater precision of measurement is obtained by electronic integration of the area for fixed periods of time, followed by digital display. Three integration periods are available, 8·2 sec, 33 sec and 2 min 11 sec and, under ideal conditions, integration over the longer periods improves precision in the ratio of 1:2:4. An automatic loss control circuit compensates within limits for any variation in conductive losses between samples of the same substance.

With certain substances having long relaxation times, RF saturation causes signal variations. When the power of the RF field is large the protons rapidly reach the excited state and, because of the long relaxation time, the signal level decreases. Such saturation can be reduced by using a low RF level, but then precision is poorer because of the low signal/noise ratio. Newport Instruments Limited have shown that the long term variations due to saturation effects may often be prevented by superimposing a supplementary magnetic field on the modulated main magnetic field.

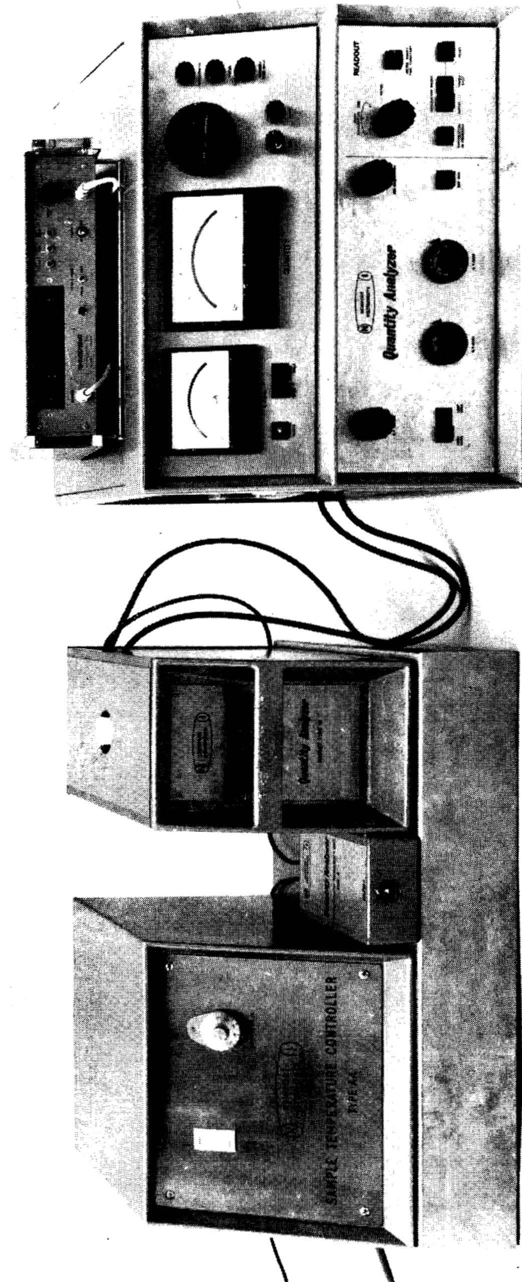

PLATE 1. Newport Quantity Analyser with ancillary equipment: supplementary modulation unit, digital voltmeter and sample temperature controller.

Experimental

Samples

The initial experiments were carried out with filtered samples of dairy butter oil and pressed cocoa butter, these being the major fats in use in the chocolate industry. The chocolate products were usually taken from normal production, but some chocolate samples were specially prepared. Cocoa powders were examined in the condition received from the plant, but the milk chocolate crumb samples were finely milled to facilitate extraction of fat by the conventional methods of fat determination. In the case of milk and plain chocolate, milk chocolate paste (unrefined mixture of milled crumb and fat) and cocoa liquor, the samples were initially moulded to ensure homogeneity, but some paste samples were tested immediately after removal from the plant.

During the course of the work, experiments were carried out to evaluate long-term stability of the equipment, for which it was preferable to use a substance not subject to RF saturation. Preliminary work had shown that cocoa butter was unacceptable for this reason, and Newport Instruments Limited suggested the use of silicone rubber, or water relaxed with manganous chloride. Silicone rubber was preferred because of its greater suitability for factory use. Although a solid, the polymer structure confers liquid-like properties to this substance.

Thermal treatment of NMR samples

Equilibrated samples: A 100 ml glass Nessler cylinder was filled with sample to a depth no greater than 2 inches, equivalent to a sample volume of 40 ml, and the sample weight determined to an accuracy of 10 mg. It was essential that the 2 inches depth was not exceeded, otherwise the sample would not be completely within the homogeneous part of the magnetic field, and variable results would be obtained. The sample tube was equilibrated for 1 hr by insertion into a drilled aluminium block immersed in a water bath at the appropriate temperature, usually 50°C although some tests were carried out at 45, 40 and 35°C.

Unequilibrated samples: Some milk chocolate paste samples were completely melted in a warm oven, stirred thoroughly, and then a 40 ml portion transferred to the sample tube, weighed and the sample temperature rapidly determined with a thermistor thermometer. The tube was preheated to prevent cooling of the sample and the formation of a large temperature gradient. In other experiments carried out in the factory, paste samples were transferred to the tubes immediately after removal from the plant. In this case, the tubes were made of P.T.F.E. to avoid the use of glass in production areas.

NMR method of fat determination

The AF gain, RF level and DVM integration period were suitably set, and the sample temperature controller adjusted to the temperature of the sample. The sample tube was positioned in the magnet assembly, the RF tuning adjusted, the integration period started, and the DVM reading noted at the end of the cycle.

Conventional methods of fat determination

Soxhlet method: 3 g of sample were extracted overnight using 40/60 petroleum ether.

Mojonnier method: 1 g of sample was dispersed in warm, alcoholic ammonia solution contained in a Mojonnier flask, and extracted twice with 1:1 petroleum ether/diethyl ether solvent. The two ethereal portions were separated from the aqueous residues by centrifugation and decanted into an open dish. After evaporation of the ether, the dish was dried for 15 min at 135°C, 20 torr before weighing. This method was preferred to other chemical pretreatment methods because of its rapidity and convenience.

Methods of moisture determination

Brabender Oven: 10 g of sample was dried for 1 hr at 105°C using a Brabender oven – an air-ventilated oven with positions for ten sample dishes and incorporating weighing facilities for direct reading of moisture content.

Vacuum Oven: 2 g of sample were spread over a dried filter paper contained in a dish, and dried overnight at 80°C, 20 torr.

Results and discussion

Optimum instrument conditions

Using 15 g of cocoa butter, previously equilibrated at 50°C, replicate DVM readings were taken with RF levels of 100, 200, 300, 400 and 500 μA, and using the three integration periods. To give greatest sensitivity, the AF gain was set so that almost maximum DVM readings were obtained. A setting of 370 was found to be appropriate with the instrument and conditions used. Readings were taken without the use of SM, and also with the SM unit switched on. In the absence of SM precision was poor and there was statistical evidence of a cycling effect in the readings. The cycling effect, attributed to RF saturation, was subsequently confirmed by further experiments in which the output of the panel meter was continuously recorded for about 30 min. When SM was used precision was improved about three-fold, which was consistent with the occurrence of RF saturation. Table 1 shows the coefficients of variation for replication of DVM readings at full-scale deflection (2 v) using various instrumental conditions. The improvement in precision with longer integration was generally of the order expected, and greatest precision was obtained at 300 μA, which was confirmed by the continuous recordings obtained from the panel meter.

The optimum conditions, subsequently used in further work, were believed to be the use of SM, an AF gain of 370 and an RF level of 300μA. It was thought that an acceptable compromise between precision and rapidity would be obtained if the mean of five readings using the 33 sec integration period was determined. Spurious results would be immediately obvious if five readings were taken, but for factory use by semi-skilled personnel it would be easier and less prone to arithmetical error if one reading at the 2 min 11 sec integration period were taken.

TABLE 1. Coefficients of variation of DVM readings using various instrumental conditions

RF level (μA)	Integration period (sec)		
	8·2	33	131
200	0·47	0·28	0·17
300	0·37	0·27	0·14
400	0·75	0·38	0·19

Long-term instrument stability

The DVM readings for a silicone rubber standard and 15 g of cocoa butter were determined daily using the selected conditions. Analysis of variance of the results showed that there was a significant difference between the readings from day to day, the 95% confidence limits for the mean DVM reading from day to day being ±0·010 and ±0·011 v for the rubber standard and cocoa butter respectively. The variation in the cocoa butter readings appeared to be caused by instrument drift rather than a variation in the effects of RF saturation. To allow for the slight drift, the DVM readings obtained each day were corrected by a factor based on the rubber standard reading. However, in routine use it would be preferable to adjust the AF gain daily so that a reading within a specified range was always obtained with the rubber standard.

Calibration

The daily standardization of the instrument with cocoa butter was, in fact, a calibration, but it was desirable to determine the linearity of the relationship between DVM reading and weight of cocoa butter. A series of experiments was carried out on different days in which the mean DVM reading at 50°C of twenty-four portions of cocoa butter of different weight within the range 5–15 g were determined. Statistical analysis produced the equation

$$W = 7{\cdot}868\,R$$

where W = weight of cocoa butter, g.

R = DVM reading, v.

The residual error was 0·053 g. Similar tests carried out with dairy butter oil produced the equation

$$W = 8{\cdot}22\,R.$$

Clearly, the presence of milk fat in a sample would theoretically cause low results if the cocoa butter equation was used to calculate fat content. A further thirty-nine determinations were carried out with the same cocoa butter portions after equilibration at temperatures between 50° and 35°C. Multiple regression analysis gave the equation

$$W = 7{\cdot}863\,R + 0{\cdot}036\,R\,(T{-}50)$$

where T = temperature of sample, °C.

The correlation was very highly significant and the residual error was 0·055 g.

For samples equilibrated at 50°C (i.e. the 50°C NMR method), the weight of fat contained in the sample was calculated using the cocoa butter linear regression equation, but for samples tested at other temperatures, the temperature compensated equation was used.

Milk chocolate paste, milk and plain chocolate

A series of 50°C NMR determinations were carried out with fifteen chocolate pastes, eight milk and seventeen plain chocolates. Five milk and six plain chocolate samples were specially prepared so that there was either a gradation in fat content with constant viscosity, or the viscosity varied whilst the fat content was constant. The sample weights required to fill the sample tubes to the 40 ml mark were about 30 g for the paste, and 45 g for the chocolate.

A comparison of these results, and those obtained by conventional extraction methods, are summarized statistically in Table 2, which shows the means and standard deviations of the differences between the methods. The standard deviation for replication of the Soxhlet, Mojonnier and NMR methods were 0·05, 0·20 and 0·17% fat respectively.

The NMR method gave the highest results, but after deducting the mean difference between the NMR and Mojonnier methods from the NMR results, the agreement between these two methods was reasonable for all three products, bearing in mind the precision of the methods. Apart from the bias, the NMR method agreed well with the Soxhlet method for plain chocolate, but for milk chocolate and paste the agreement was less satisfactory and the NMR method gave exceptionally high results. With the samples examined, the NMR results did not appear to be affected by variations in viscosity or moisture content, as determined using the vacuum oven, but a constant contribution to the signal by the water present may have caused the positive error. The presence of milk fat in the milk chocolate and paste was ignored since the fat composition was almost constant, and a simple subtractive correction could be applied to the results to correlate the NMR method with the conventional methods.

TABLE 2. Differences (% fat) between methods for plain chocolate, milk chocolate and paste

Product	Difference					
	NMR–Mojonnier		NMR–Soxhlet		Mojonnier–Soxhlet	
	MD	SD	MD	SD	MD	SD
Plain chocolate	0·67	0·25	0·45	0·13	— 0·22	0·27
Milk chocolate	0·70	0·15	0·83	0·29	0·13	0·25
Milk chocolate paste	0·65	0·25	0·88	0·34	0·23	0·31

MD= Mean difference and SD = Standard deviation.

It is well known that 'fat-locking' occurs in milk chocolate crumb, and that simple extraction methods, e.g. Soxhlet, do not remove fat as efficiently as extraction methods using a chemical pretreatment, e.g. Mojonnier. Thus, assuming the latter method gives accurate results for both milk and plain chocolate, comparison of the mean (Mojonnier–Soxhlet) differences for the three products indicates that the Soxhlet method reported results for the plain chocolate which were, on average, about 0·4% fat higher than those obtained for samples of the same total fat content, but made from milk chocolate crumb. This strongly suggested that the crumb fat locking is carried through to the milk chocolate, and that the Soxhlet method also reports low results for this product.

Subsequent experiments in which the residues from Soxhlet extractions were extracted again by the Mojonnier procedure confirmed such fat-locking; 0·05% fat was obtained from a plain chocolate residue and a mean of 0·30% fat was obtained from four milk chocolate residues.

Milk chocolate crumb

Twenty-four samples were tested by the 50°C NMR method, both before and after drying in the Brabender oven. The sample weights varied between 20 and 25 g. To evaluate more fully the effect of moisture on the NMR results, six samples were also equilibrated at various humidities and the 50°C NMR determinations repeated.

The moisture content of the samples as received from the plant was sufficiently high to cause positive errors in the NMR method but, after drying, the mean (NMR–Mojonnier) difference reduced to 0·12%. The standard deviation of individual differences was 0·20% fat, and the standard deviation for replication of the NMR method for crumb was 0·14% fat. Fig. 1 graphically presents the relationship between moisture content and error of the NMR method, error being calculated as the difference between NMR results (dry basis) for the undried and dried samples. There

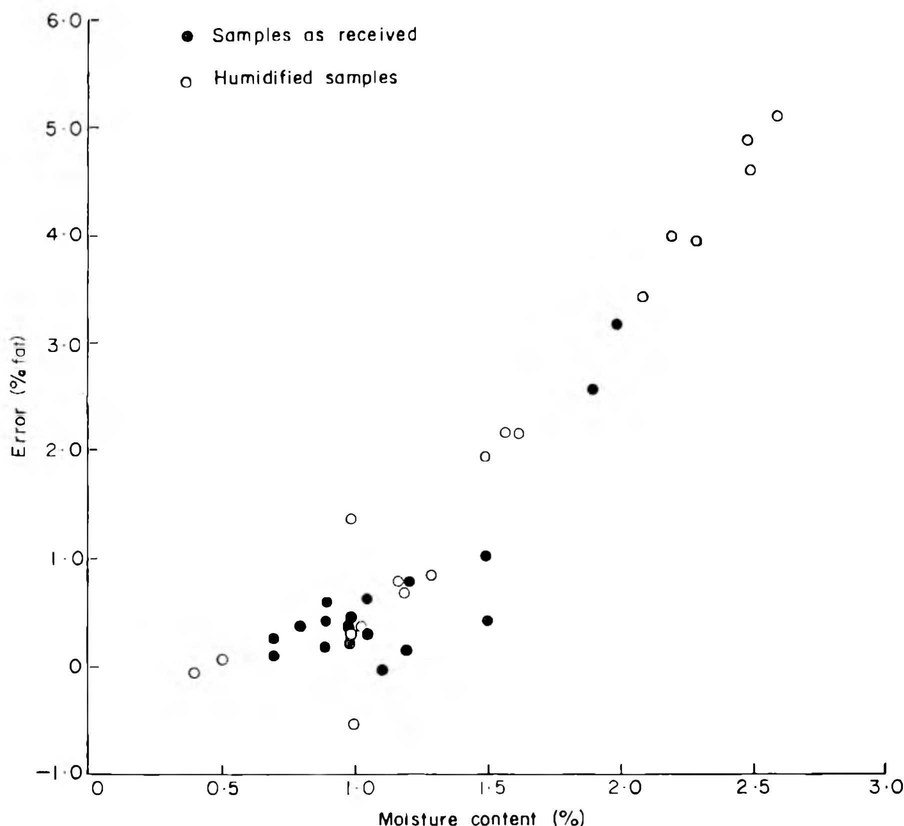

FIG. 1. Relationship between moisture content of milk chocolate crumb samples and the accuracy of the NMR method.

appeared to be a threshold level of about 0·7% moisture below which moisture would not interfere with the NMR method. Since bound water does not give an NMR signal, this threshold might be the onset of water binding.

Cocoa powder

Twelve samples of cocoa powder, as received and after drying in the Brabender oven, were tested by the 50°C NMR method. Samples of normal moisture content, 3–5%, gave grossly high results, the error being as great as 3·5% apparent fat. As expected, drying improved the accuracy of the method, the (NMR–Soxhlet) differences having a mean of 0·67% fat and a standard deviation of 0·28%.

Cocoa liquor

Eleven samples, as received from the plant, were tested by the 50°C NMR method, but the moisture content range, 0·3%, was too small to determine if variations in

moisture significantly affected the NMR method. The (NMR–Soxhlet) differences were distributed with a mean of 1·17% fat and a standard deviation of 0·18%.

Process control

The 50°C NMR method was more rapid and labour-saving than conventional extraction methods, but nevertheless, the time delay due to the equilibration period reduced the effectiveness of the technique for process control purposes. Having calibrated the instrument with cocoa butter for samples at temperatures between 35° and 50°C, fifteen NMR determinations were carried out in the laboratory with unequilibrated milk chocolate paste samples, and the fat content calculated using the temperature compensated equation. There was no significant difference in accuracy between the two NMR methods, the differences between the temperature compensated results and the corresponding 50°C NMR results being within the range ±0·24% fat.

A further twenty-five determinations were carried out in the factory immediately after removing the paste samples from the process stream. Initially, the method did not allow a time delay between filling the sample tube and measurement of the NMR signal, but results using this technique were usually low. However, when the tube was allowed to stand for 5 min in the aluminium block immersed in the 50°C water bath before testing, the results were of the same accuracy as the laboratory determinations, i.e. within ±0·25% of the 50°C NMR results. The samples were taken immediately after mixing the cold chocolate crumb powder with hot fat, the resulting mixture apparently having a temperature considerably above the melting point of cocoa butter. It was feasible that the crumb powder was cooler, and a delay was necessary before measuring the NMR signal to allow the crumb fat to melt completely.

Conclusions

By following a standardized technique total fat can be determined within the accuracy and precision required for control purposes in chocolate and related products by using low resolution nuclear magnetic resonance. The main factors affecting the method are moisture content and temperature. The samples preferably should be dry, but certain products can tolerate small quantities of water. Cold samples must be equilibrated to a temperature above the melting point of cocoa butter to ensure complete liquefaction of fat, but for dry products of consistent temperature and containing no solid fat, a temperature compensated technique is applicable which is suitable for very rapid off-line process control in the factory. With further development this equipment could possibly be the basis of an in-line system.

Acknowledgments

The authors are indebted to Mr A. Turner and Mr T. L. Harris for recognizing the potential of the technique for chocolate products and Miss S. Scadding for the

statistical interpretation of the results. We also thank the staff of Newport Instruments Limited for their frequent advice, and British Food Manufacturing Industries Research Association for helpful discussions.

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Heat treatment of flour as an alternative to chlorination

JUDITH V. RUSSO AND C. A. DOE

Summary

Experimental work leading to the discovery that heat treatment of air classified turbo ground flours to a temperature of 120°C produces flours with vastly improved high ratio sponge and cake baking properties is described. The starch in the heat treated flour is found to be resistant to swelling in DMSO solutions – a fact which can be utilized to monitor the heat treatment process. The mechanism of the improving action is still obscure.

Introduction

Chlorination of cake flours has been practised in this country and the U.S.A. for nearly 40 years (Montzheimer, 1931; Smith, 1931). The normal level of treatment is from 5 to 10 oz chlorine per sack (280 lb). The practical effect of chlorination apart from a bleaching of flour pigments, is to produce a flour which permits the mass production of cakes and sponges with high levels of sugar and water in proportion to the flour. High ratio cakes and sponges made from untreated flour are characterized by a 'collapsed' cellular structure and a pudding-like texture. The production of a satisfactory structure in cake or sponge is dependent on achieving a delicate balance between the flowing of the aerated batter and expansion of the air bubbles which occur during the first part of baking and the subsequent setting of the cell wall components. This balance is upset when the sugar content is increased and is restored using a chlorinated flour. The exact mechanism of chlorination is still incompletely understood but some of its practical effects have been elucidated in this study and have enabled us to produce a flour with similar properties to chlorinated flour but without using a chemical additive (J. Lyons & Co. Ltd, British Patent 1,110,711).

Toxicological evidence on the effects of chlorination is sparse and the British Food Standards Committee in 1960 and FAO/WHO in 1967 both reported that their information on chlorinated flour was insufficient to evaluate the position. The Flour Milling and Baking Research Association are currently studying the problem using feeding trials on rats but their results are still inconclusive.

In view of our possible eventual entry into the Common Market (chlorination of cake flours is not allowed in E.E.C. countries) and because of the possibility of recom-

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mendations by the Food Standards Committee to ban chlorination, the production of a cake flour free from chemical additives is of particular importance.

Experimental

Lay ake (used as a standard test for quality of flour or gluten/starch mixtures)

		(g)
A	Whole egg	438
	Water	130·5
	Sugar	507
	Solid cake colour	0·2
	Vanilla flavour	2·0
	Glucose syrup (41°B)	11·25
B	Phosphate baking powder	15
	Flour	460
	Full fat soya flour	18·75

Ingredients A were first mixed for 3 min in a 3 qt Hobart bowl. Ingredients B were then added during 2¼ min, scraped down, mixed for 15 sec, scraped down and mixed for 5 sec. The mix was poured into a Morton pressure mixer and beaten for 1½ min at 20 psi.

Six and a half ounces of batter were piped into recessed baking tins of 7½ inches diameter, and baked for about 15 min at 400°F.

When starch and dried gluten were being tested to replace flour 405 g starch and 55 g dried gluten were used. In tests where the effect of dried albumen was being studied the egg solids were reduced by one eighth and replaced by dried albumen.

Water was added to bring the total water back to normal.

Madeira cake

		(g)
A	Flour	450
	Full fat soya flour	60
	Salt	11·25
	Phosphate baking powder	15
	Skim milk powder	15
B	Flour	190
	Compound fat	225

C	67% Sugar syrup	915
	Frozen whole egg	315
	Water	60
	Cake colour solution	3·15

Ingredients A were sieved together; ingredients C were blended together. Using a Hobart mixer with 10 qt bowl and spade attachment ingredients B were mixed to a paste on speed one, then aerated on speed three to an aerated mix weight of 58·2–61·8 g per 100 ml. Ingredients C were added during 30 sec mixing on speed one. After a further 15 sec mixing on speed two, ingredients A were added during 2 min mixing on speed one. The whole mixture was mixed for a further 2 min on speed two. Nine oz of batter were scaled in 4½ inches diam. tins and baked for 1 hr at 350°F.

In tests where the effect of dried albumen was being studied the egg solids were reduced by one quarter and replaced by albumen solids.

High ratio cakes

		(g)
A	High ratio fat	425
	High ratio flour	568
	Castor sugar	681
	Phosphate baking powder	28
	Skimmed milk powder	74
	Salt	14
B	Frozen whole egg	482
	Water	308

Ingredients A were blended for 2 min on speed one on a Hobart mixer using a 10 qt bowl and spade attachment. The egg and water were blended and one third was added whilst mixing on slow speed. The mixture was then mixed for 1 min on speed two. The remaining liquid was added during ½ min. Then the mixture was mixed for a further 4 min on speed two. Thirteen oz batter were scaled in 5¼ inches diam. tins and baked for 65 min at 350°F.

Miniature 'half-bake' oven

This is illustrated in Fig. 1. The oven which was heated over a gas flame had a cavity in it which was just large enough to hold a recessed baking tin which had been cut in half. The front of the tin was sealed with a glass observer plate. This enabled the formation of the structure of the cake at the centre to be observed under conditions closely approaching those occurring during normal baking, i.e. the central area was the last place to receive heat. The surface of the glass was etched with a scale to enable height measurements to be taken during the baking of the cakes. The curves obtained by plotting cake height against time are termed cake growth curves.

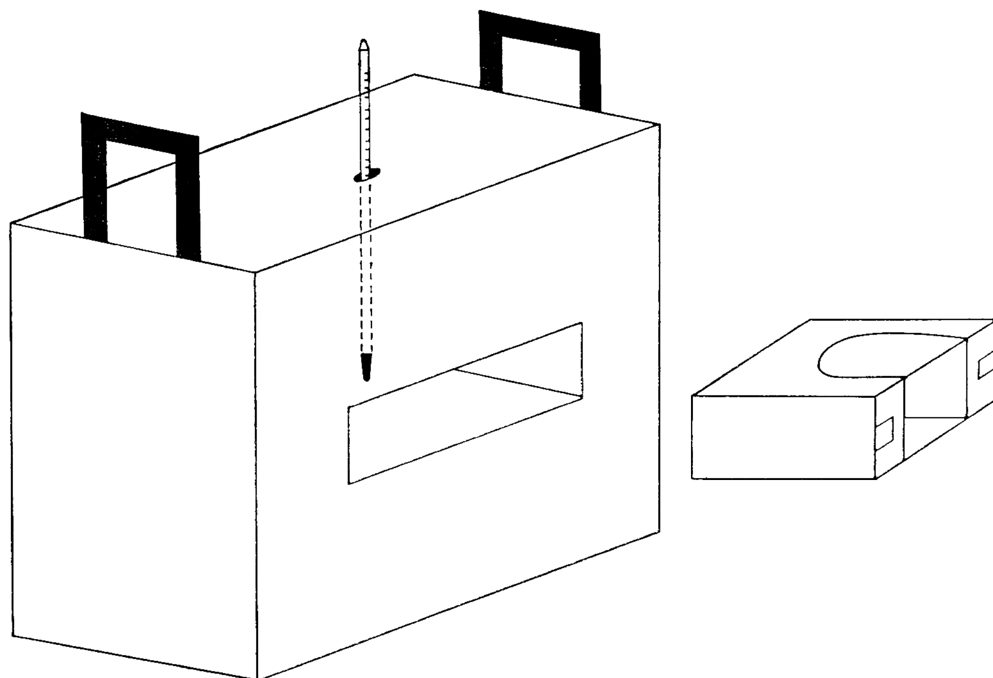

FIG. 1. Miniature 'half-bake' oven.

'Slurry' amylograph measurements

Flour 250 g or starch 230 g
Sugar 250 g
Phosphate/citric acid buffer pH 7·0 250 ml
(Buffer 353 ml 0·1 M citric acid + 1647 ml 0·2 M Na_2HPO_4)

The flour, sugar and buffer solution were mixed together to form a slurry then poured into the bowl of the Brabender amylograph which was programmed on a rising temperature scale at 1·5°C per minute starting at 20°C. Initially the viscosity fell, then began to rise rapidly. At 80°C the sensitivity of the instrument was reduced by suspending a 900 g weight from the pulley wheel at the back of the instrument. When the viscosity had increased sufficiently the curve started to rise again. The temperature at which the first and second rise of the curve occurred were noted.

Method of heat treating flour or starch

The rotating drum used for heat treatment in the laboratory is illustrated in Fig. 2. Flour or starch (3 lb) was placed in the drum which was rotated by an electric motor over two bunsen burners fitted with spreaders. The lid of the drum was fitted with iron rods to improve the tumbling action within the drum. Moisture was allowed to escape through the central perforated hollow tube on which the drum rotates.

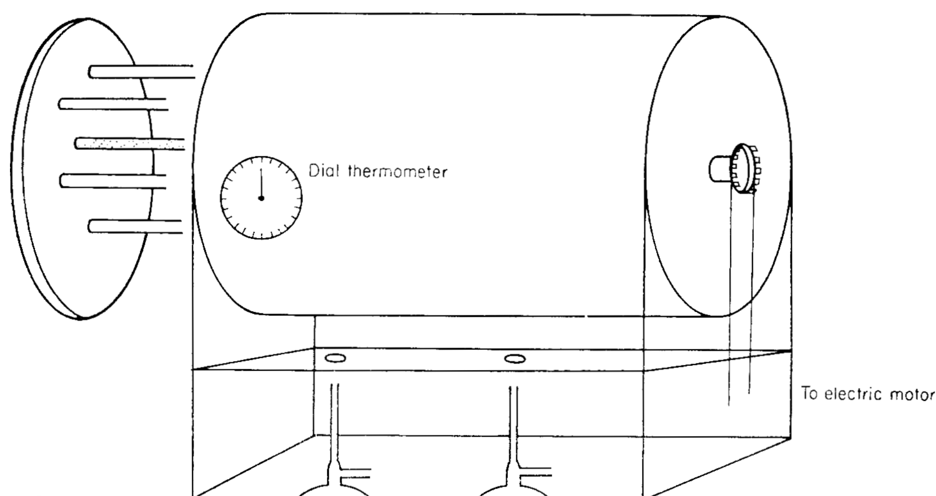

FIG. 2. Laboratory heat treatment apparatus.

The flour or starch was brought up to the required temperature as indicated on the dial thermometer then it was emptied out and allowed to cool and rehumidify in shallow trays at room temperature.

In the semi-commercial test the flour was heat treated in a Balfour Scott Reitz thermascrew at a feed rate of 4–5 cwt/hour as described in British Patent 1,110,711.

Method of cake photography

The technique was based on that of Buddemeyer & Gottl (1963). The cake was cut in half in the usual manner for assessing the grain and texture then a slice of thickness approx $\frac{1}{8}$ inch was cut. In some cases cutting was facilitated if the cake was frozen overnight and cut when frozen. The thin slice was placed on a sheet of perspex. This was placed in the negative carrier of the photographic enlarger and the enlarger adjusted to give an image on the base board of the same size as the original.

The light which passed through the cake section fell directly onto a sheet of No. 2 bromide paper and the paper developed in high contrast developer. The result was a negative photograph. The method is quick and gives an accurate record of the grain of the cake.

Photographs of layer and madeira cakes are shown in Plates 1 and 2.

Dimethyl sulphoxide (DMSO) swelling method for measuring the effect of heat treatment

Flour or starch (0·5 ± 0·05 g) was weighed into a 15 ml conical shaped graduated centrifuge tube (graduation 0·5 ml and/or 0·1 ml). DMSO solution (5 ml of 80% w/w) kept at 25°C was added to flour in portions of 1 ml, 2 ml and 2 ml and mixed to a smooth paste with a glass rod after each addition.

The tubes were then stoppered and placed in a water bath at 25°C for 2 hr. Samples were mixed every 30 min.

After 2 hr the tubes were placed in iced water for 5 min to quench the swelling eaction then allowed to warm up for 5 min before centrifuging. The centrifuge used was a MSE bench model. Speed was increased to top No. 7 setting (3000 rpm) in 15 sec. Centrifuging was carried out for 8 min. The time taken for the centrifuge to stop after switching off was 60–80 sec. The volumes of the total and of the bottom layer were recorded and the % sedimentation volume was calculated.

$$\%\ \text{Sedimentation volume} = \frac{\text{vol. of bottom layer}}{\text{vol. of total}} \times 100$$

The results were then expressed in terms of an internal standard as:

$$\%\ \text{Sedimentation volume normalized} = \frac{\%\ \text{sedimentation volume of heat treated sample} \times 100}{\%\ \text{sedimentation volume of untreated sample}}$$

Method of measuring protein solubility

Flour (1 g) was blended into 75 ml solvent (acetic acid (0·1 M)–Urea (3 M)) and the mixture was homogenized for 2 min (MSE homogenizer). The suspension was transferred to a beaker and kept at 20°C with intermittent stirring for 1 to 2 hr. It was then stirred vigorously and 5 g samples transferred to 10 ml polypropylene centrifuge tubes. After centrifuging (30 min at 140,000 × **g**) approx 3 ml of clear supernatant solution was pipetted off and the absorbance measured in 1 cm cell against solvent at 280, 320 and 360 nm.

% protein w/v in the solution is given by

$$(E_{280} - (2E_{320} - E_{360})) \times 0{\cdot}152$$

(This factor was obtained by calibrating the method against protein determined by the Kjeldahl method).

Then the percentage of the total flour protein extracted by the solvent is given by:

$$\frac{(E_{280} - (2E_{320} - E_{360})) \times 11{\cdot}40 \times 100}{\%\ \text{Protein in flour}}$$

Results

Comparison of the pairs of samples in Table 1 show that an important effect of chlorination is a lowering of temperature of pasting of starch and an associated increase in

viscosity at any given temperature. The effect of this during cake baking is shown by the cake growth curves in Fig. 3. These show that there is an early setting of cake structure (7 min) in the case of the chlorinated sample, whereas in the case of the untreated

TABLE 1. Effect of chlorination on 'slurry amylograph'

Product	Minimum viscosity (Brabender units)	Temp. at first viscosity rise (°C)	Viscosity at 80°C (Brabender units)	Temp. at second viscosity rise (reduced sensitivity) (°C)
Untreated high ratio flour A	90	73	160	86
Laboratory chlorinated flour A	120	72	380	83·5
Untreated cake flour B	100	72·5	380	84·5
Laboratory chlorinated flour B	150	69·5	950	80
Commercial starch	20	79	30	86·5
Laboratory chlorinated commercial starch	40	75·5	140	84·5

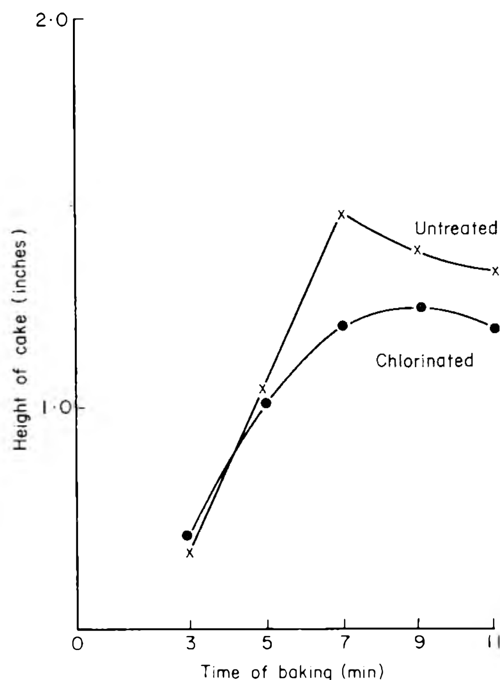

FIG. 3. Cake growth curves.

sample there is merely a flow followed by collapse of structure during the baking period.

Layer cake baking tests using dried gluten and commercial starch to replace flour showed two distinctive features: (1) the mere separation of the gluten from the starch obviated the 'collapsed' structure in the sponge although the crumb texture was too friable and (2) the condition of the dried gluten was of no importance – equally good results being obtained whether the gluten was freeze-dried, chlorinated or autoclaved. On the other hand chlorinated starch and untreated dried gluten produced sponges and sponge growth curves almost identical with those produced by chlorinated flour.

Since all the effects of chlorination so far studied and reported by other workers (Chamberlain, 1962; Sollars, 1958, 1961; Cornford, 1961) highlight the importance of changes brought about in the starch, work was concentrated on altering the starch by means other than chlorination.

Study of the literature showed that Katz (1939) found that starch heated to 140°C began to lose its crystalline character and more recent work by Schierbaum & Taufel (1962) indicated that dehydration of starch by heating at temperatures from 70° to 200°C to a moisture content of less than 5% led to irreversible lowering of the gelatinization temperature.

This work suggested that heating starch to temperatures higher than 100°C may have an improving effect. In baking tests using heat treated starch and dried gluten the optimum heat treatment temperature was found to be 120°C.

Scrutiny of Table 2 shows that the pasting temperature of the starch fell with increasing temperature of heat treatment from 150°C onwards but the decreases in temperature were not as great as those noticed on chlorination.

Following the improved baking performance of starch obtained by heat treatment the method was applied to complete untreated cake flours. Using a conventionally roller milled cake flour excessive 'balling-up' occurred on heat treatment to 120°C and the baking properties were unsatisfactory. However when the method was applied to a flour which had been turbo-milled and air classified and in which the starch was well separated from the protein a slight lowering in pasting temperature on the slurry amylograph was detected (see Flour C, Table 2), and the layer cake baking performance matched or almost matched that of a chlorinated flour. It can be seen from Table 2 that the pasting temperatures were not as low as those obtained in a chlorinated flour; this possibly explains the fact that in the baking test the crumb of the cake made with the heat treated flour was somewhat too friable. This friability was corrected by bringing down the overall setting temperature of the batter by replacing a proportion of the eggs solids by dried albumen solids. In the layer cake recipe the optimum formulation was found to be one in which one eighth egg solids were replaced by albumen solids. (It was shown that the addition of albumen to a recipe using untreated flour did not radically improve performance.)

Table 3 illustrates the effect of variation of heat treatment times and temperatures

TABLE 2. Effect of heat treatment on 'slurry amylograph' characteristics

Product	Minimum viscosity (Brabender units)	Temp. at first viscosity rise (°C)	Viscosity at 80°C (Brabender units)	Temp. at second viscosity rise (reduced sensitivity) (°C)
Commercial starch	20	80	20	87
Commercial starch heated to 100°C	20	80	20	87·5
Commercial starch heated to 140°C	20	79	30	87
Commercial starch heated to 150°C	20	80	30	86·5
Commercial starch heated to 160°C	30	78·5	60	85·5
Commercial starch heated to 180°C	40	77·5	70	85·5
Untreated air classified flour C	125	78·5	170	84·5
Flour C heated to 120°C	160	78·5	230	83·5
Laboratory chlorinated flour C	190	74	330	82·5

TABLE 3. Effect of variation of heat treatment on flour baking properties

Treatment	Moisture ex drum (%)	Comments on layer cake baking test
100°C (instantaneously)	9·2	Pasty eating quality
100°C (held ½ hr)	8·0	Pasty eating quality
105°C (instantaneously)	9·8	Pasty eating quality
105°C (held ½ hr)	7·8	Pasty eating quality
110°C (instantaneously)	8·7	Passable, slightly pasty
110°C (held ½ hr)	6·9	Reasonably good, better than above
115°C (instantaneously)	6·9	Slightly tough coarse grain
115°C (held ½ hr)	6·4	Satisfactory
120°C (instantaneously)	6·6	Satisfactory
120°C (held ½ hr)	5·5	Satisfactory
125°C (instantaneously)	6·4	Good eating quality, slightly coarse grain
125°C (held ½ hr)	4·9	Passable, slightly tough
130°C (instantaneously)	5·8	Satisfactory
130°C (held ½ hr)	4·0	Dry and poor flavour
135°C (instantaneously)	5·1	Somewhat dry, borderline passable
140°C (instantaneously)	4·3	Dry and poor flavour

on the layer cake baking properties of an air classified turbo-ground flour. The layer cake recipe with albumen level as stated above was used.

Table 3 confirms that 120°C is the optimum heat treatment temperature and that the time of holding at this temperature is not critical. The table shows that in all cases of satisfactory baking performance the moisture level of the flour was reduced to below 7%. Moreover it was found that heating flour to 120°C without loss of moisture produced a less satisfactory cake. Too high a treatment temperature has a deleterious effect on baking texture and flavour.

Plates 1 and 2 demonstrate layer cakes and madeira cakes baked with untreated, heat treated and chlorinated flour. In the case of the layer cake made with heat treated flour one eighth of the egg solids were replaced by albumen solids and in the case of the madeira cake one quarter of the egg solids were replaced by albumen solids.

Semi-commercial heat treatment of flour enabled factory scale baking trials to be made. In these trials it was found that in general the flour could be used satisfactorily without reformulation in sponge goods but in some fat containing sponge and cake recipes it was necessary to introduce a proportion of dried albumen. The amount

TABLE 4. Solubility of protein in 0·1 M acetic acid and 3 M urea

Sample	Heat treatment (°C)	Protein extracted (%)
Flour No. 1	Untreated	71·4
	100	70·7
	120	64·1
	140	52·5
Flour No. 2	Untreated	71
	100	64
	115	52
	120	51
	125	48
	130	46
	135	50
	140	48
Flour No. 3	Untreated	73
	100	65
	115	65
	120	54
	125	51
	130	52
	135	47
	140	47

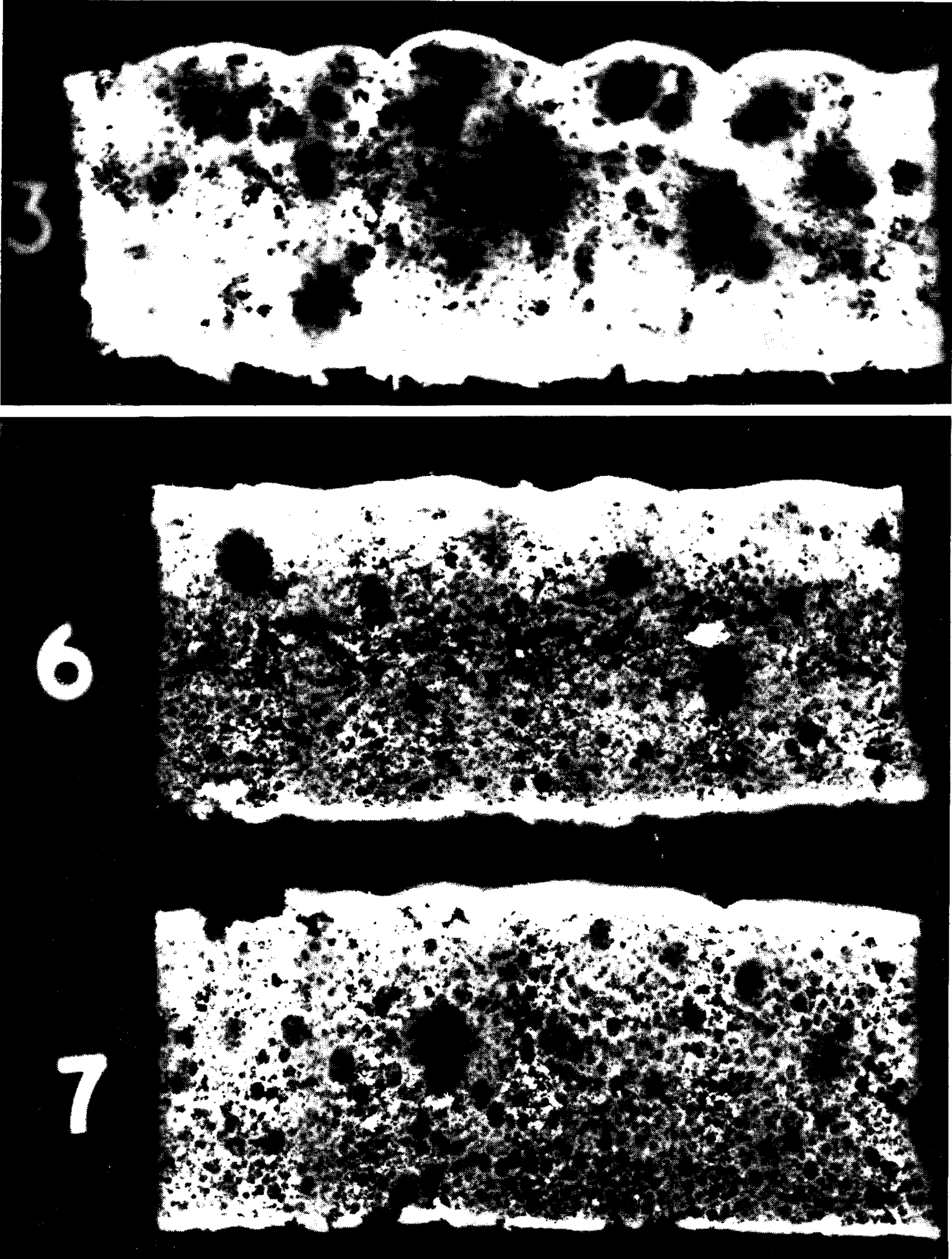

PLATE 1. Layer cakes. 3, Untreated flour; 6, Heat-treated flour; 7, Chlorinated flour.

(*Facing p.* 372)

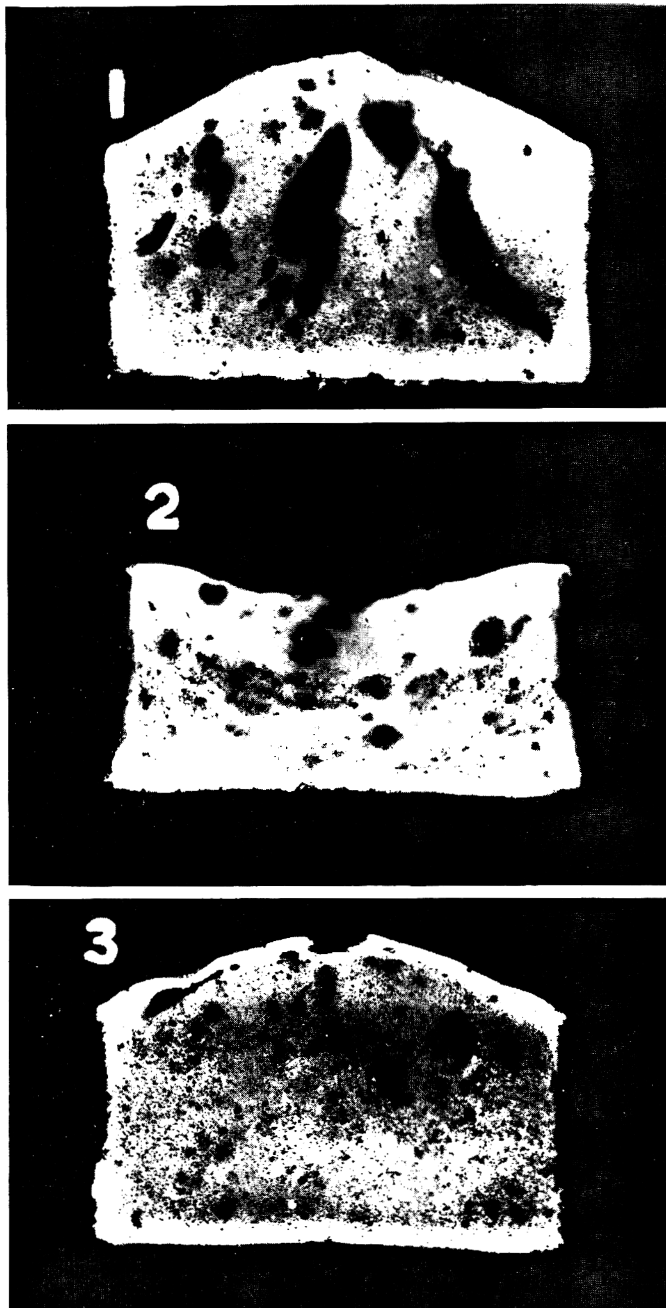

PLATE 2. Madeira cakes. 1, Chlorinated flour; 2, Untreated flour; 3, Heat treated flour.

(*Facing plate* 3)

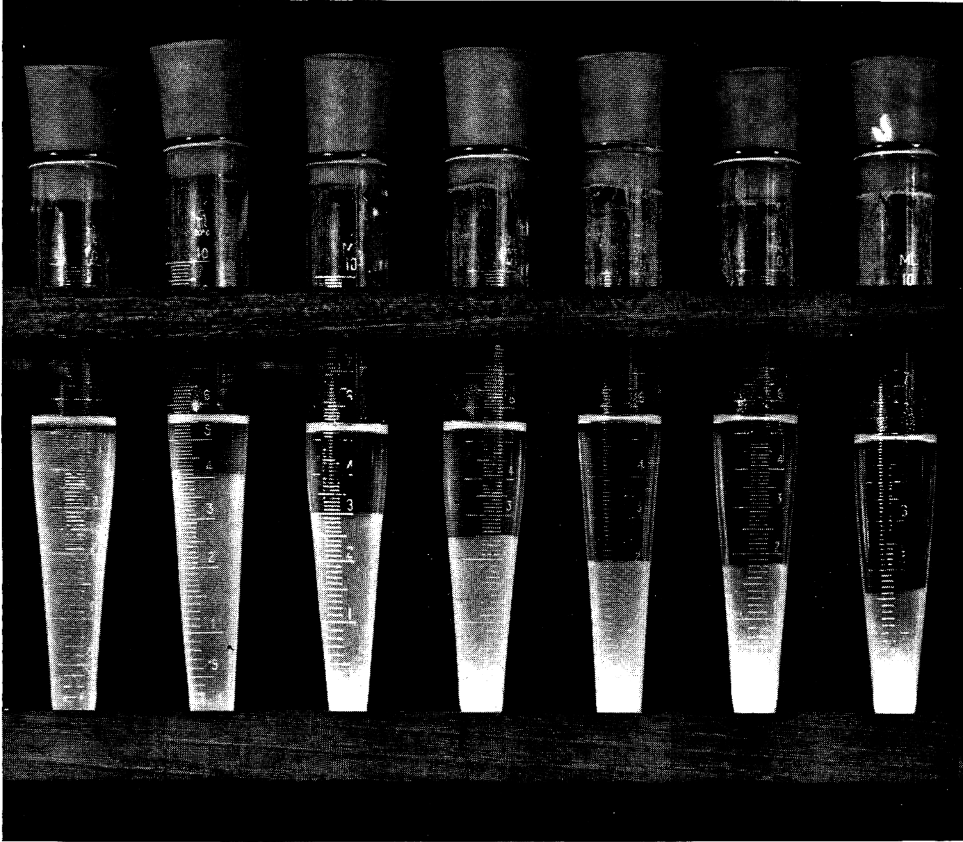

PLATE 3. Sedimentation volumes after centrifugation following swelling in DMSO. Left to right: untreated, 80°, 100°, 110°, 120°, 130° and 140°C heat treated flour.

(*Facing plate* 2)

required varied with the recipe used. High protein fruit cake flour was also successfully heat treated in this series cf experiments.

In order to investigate chemical changes brought about by the heat treatment process both the protein and the starch were studied. The effect of heat treatment on solubility of protein was investigated and it was found that the most sensitive solvent system was 0·1 M acetic acid and 3 M urea. Results of solubility determinations are given in Table 4.

In every case heat treatment of the flour led in general to a progressive reduction in protein solubility but the extent of reduction varied with the particular flour being studied. One general point that was deduced from these determinations was that a protein extractability of 64% or less was a strong indication that a flour had been heat treated.

Changes brought about in the starch were studied by examining the effect of 80% w/w DMSO solutions on heat treated flours. From microscopical examination it was found that the starch granules of heat treated flours were resistant to swelling in this solvent. It was found that no distortion of the starch granules occurred until the time of contact approached 120 min, whereas with untreated flour distortion commenced at 45 min. From these findings a differential swelling test which could be used to monitor the heat treatment process was evolved. Results of this test are shown in Table 5 and Plate 3.

TABLE 5. Swelling tests on heat treated flours and starches

	Sedimentation % (normalized)				
Sample treatment	Flour X	Flour Y	Starch A	Starch B	Starch C
Untreated	100	100	100	100	100
Heat treated to 100°C	85		–	–	
Heat treated to 120°C	70·2	76·0	68·5	66·5	100
Heat treated to 130°C	71·5	–	68·5	–	100
Heat treated to 140°C	71·5	76·7	–	65·5	98

The sedimentation value was found to be sensitive to the moisture content of the sample; the value falls with reduction in moisture content and rises with increase in moisture content. For meaningful results it is therefore necessary to examine comparative samples at the same moisture content. The precision of the method is increased by incorporating an internal standard of an untreated sample and expressing the results 'normalized'.

It can be seen from Table 5 that every sample except starch C decreased in sedimentation volume on heat treatment and that for samples heat treated within the range 120–140°C the normalized sedimentation had a value of the order of 70%. Starch C had a low initial sedimentation volume and heat treatment brought about no further

change. This starch sample was 5 years old and may have undergone a slow oxidation. Another interesting feature of this starch was that its baking properties were satisfactory without heat treatment and heat treatment brought about no change.

Discussion

The mechanism of the change of properties brought about by heat treatment of flour is still obscure and is forming the subject of continuing investigation. Susceptibility to attack by α-amylase is not affected by heat treatment of flour or starch and scanning electron microscope studies do not reveal any differences in superficial structure of starch granules. The differential swelling in 80% DMSO is a most interesting phenomenon and further studies are being made of the extent to which this solvent leaches amylose from the treated and untreated granules. The possibility that the heat treatment may be an oxidation process is also being investigated.

Acknowledgments

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The bacteriology of 'Scampi' (*Nephrops norvegicus*). I. Preliminary bacteriological, chemical and sensory studies

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Summary

A bacteriological, chemical and sensory study has been made of iced and un-iced scampi (*Nephrops norvegicus*) stored at an ambient temperature of 2·2°C. After 8–10 days storage, the scampi became inedible due to the presence of strong, ammoniacal, sour odours and flavours. The bacterial counts, of iced and un-iced scampi, both of which were made at 20°C and 37°C, rose sharply after the fourth day reaching values of 10^6/g at 20°C and 10^4/g at 37°C at the end of the storage period. The initial bacterial flora, consisting mainly of coryneforms (80%), gradually changed during storage until it finally consisted mainly of *Achromobacter* species (70%). The total volatile base and trimethylamine content of the flesh also increased considerably during storage, reaching values of approximately 70 and 20 mg N/100 g flesh after 10–12 days; initial values were about 20 and 0·5 mg N/100 g flesh, respectively.

Introduction

Over the past decade, fishing for *Nephrops norvegicus*, often commonly known as the Norway lobster, the Dublin Bay Prawn, or 'scampi', has been steadily increasing in importance in the United Kingdom. A total of 161,000 cwt (8184 tons) were landed in the UK in 1968 – 19,000 cwt (1000 tons) in England and Wales and 142,000 cwt (7200 tons) in Scotland – yielding a total sum of £1,900,000. In Scotland alone, the catch has been increasing steadily from 57,464 cwt (2900 tons) in 1961 valued at £379,000, to 142,000 cwt (7200 tons) in 1968 worth £1,622,000. The average price per cwt has risen from 131/10d in 1961 to 228/- in 1968. *Nephrops* landings are fifth in importance by value, after cod, haddock, whiting and herring.

The fishery

Nephrops are caught throughout the year all round Scotland, particularly in the Moray Firth, the Firths of Clyde and Forth, the Minches and off the Orkneys. English

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catches are seasonal, about half being landed at North Shields from grounds off the Northumberland coast. Irish boats fish between the Isle of Man and Ireland.

In open water the animals are generally caught by a light trawl, but close inshore, creels normally are used, particularly where, because of rocks, such a trawl cannot be operated, as in the Skye–Kyle area.

Nephrops caught in creels are landed whole and without ice. Those caught by trawl are usually headed at sea and the tails boxed and merely covered with a layer of ice. The time interval between catching and landing is a matter of a few hours for inshore vessels and 20–48 hr for the seine netters, but in extreme cases the delay may be up to 5 or 6 days. There are no time-temperature records available for *Nephrops* between catching and landing.

Before satisfactory processing of *Nephrops* can begin, they require to be conditioned so that the flesh can be removed readily from the shell. The traditional method is to store the *Nephrops* in ice, usually for 2–4 days, during which time autolytic and/or bacterial enzymes destroy or loosen the membrane binding the flesh to the shell. This method, although effective, allows unnecessary spoilage to take place both by the naturally occurring enzymes and bacteria and by the micro-organisms introduced by the ice. An equally efficient and more hygienic method of conditioning is to freeze the *Nephrops* as soon as possible after catching. The flesh is then readily removed from the shell after thawing.

It would be desirable, therefore, to have some knowledge of the factors affecting the keeping quality of *Nephrops* after catching which would lead to improved handling and storage on ship and shore. *A priori*, it was assumed that of the conditions normally operating after catching, bacterial spoilage would be an important factor. Consequently, a quantitative and qualitative study was undertaken of the bacterial flora of the fresh scampi and of the flora during subsequent storage at chill or ice temperature. Since sensory examination of the spoiling scampi indicated that bases, such as ammonia and lower amines were present, chemical analysis of the types and amounts of bases in the flesh was also carried out. These studies, together with a sensory description of the changes occurring during storage and spoilage, could lead to the development of objective bacteriological or chemical indices of spoilage as has been attempted for fish, such as cod and haddock.

Apart from an advisory leaflet by Early (1967) no studies of this kind have so far been published in the U.K. Work has been published on species of prawns of the genera *Penaeus, Metapenaeus* and *Parapenaeopsis* in India (Govindan, 1962; Jacob *et al.*, 1962; Lekshmy, Govindan & Pillai, 1962; Pillai, Sastri & Nayar, 1961; Velankar & Govindan, 1959; Velankar *et al.*, 1961) with regard to chemical examination and bacterial loads of the iced and un-iced prawn, but no detailed bacterial survey of the flora was made. Velankar & Govindan (1959) found that the bacterial flora of the iced prawns (*Penaeus indicus*) consisted mainly of Gram negative achromogenic rods and cocco-bacilli and, to a lesser extent, of orange and yellow pigmented rods and micro-

cocci, whereas Williams, Rees & Campbell (1952) working on fresh shrimp of the same genus (*Penaeus setiferus*), but of different location, found that the bacterial flora on the shell was the same as that of other marine products and that the intestinal flora contained equal numbers of representatives of the genera *Bacterium*, *Achromobacter* and *Micrococcus*.

Materials and methods

6·4 kg of freshly-landed, headless scampi (tails) were collected from each of the ports of Lossiemouth, Buckie and Peterhead, only samples from the last named port being iced at sea, and were taken to the laboratory in insulated containers, chilled by ice held in sealed packs to avoid contamination of the scampi by any bacteria in the ice. On arrival at the laboratory a sample was immediately taken for bacteriological, chemical and sensory analysis and the remainder stored unshelled with or without ice in a chill room at 2·2°C, further samples being taken at 2, 4, 6, 8 and 10 or 12 days storage until they were termed inedible by the taste panel.

Sensory examination

Approximately twelve *Nephrops* were taken at each examination. They were first examined unshelled in the raw state. They were then shelled and cooked by steaming in a closed casserole for 30 min and examined by at least five members of a taste panel expert in the sensory examination of a variety of species of fish. The panel members were asked to describe the characteristic odours and texture of the unshelled scampi and the raw scampi flesh, the odour, texture and flavour of the cooked flesh and to state whether they would accept or reject the material.

Bacteriological methods

Bacterial counts

200 g of the peeled, deveined, scampi flesh were washed in running tap water then aseptically homogenized in 400 ml sterile M/15 phosphate buffer of pH 7·0 containing 0·1% peptone. Thereafter, serial decimal dilutions were made in the same diluent and duplicate surface aerobic counts made on Oxoid CM 3 agar plates using the modified Miles & Misra (1938) technique of Appleman, Bain & Shewan (1964). 0·1 ml of each dilution was pipetted in drops on to the surface of petri plates containing solidified 'CM 3 Oxoid' nutrient agar. Duplicate plates were incubated at 20°C for 5 days and 37°C for 3 days.

Qualitative analysis

One hundred colonies were picked at random, using randomizing tables, from the count plates incubated at 20°C at selected stages of storage. In all, 1200 colonies were

examined and identified to the genus level using the determinative schemes of Hendrie, Hodgkiss & Shewan (1964) and Hendrie, Mitchell & Shewan (1968). A limited study was similarly made of the bacterial flora of the ice used for storage to determine whether it had any effect on the flora of iced scampi.

Chemical examination

Total volatile bases (TVB)

The total volatile base content of the tissue was estimated in an extract by homogenising 50 g of scampi flesh in 150 ml of 5% trichloroacetic acid (TCA), filtering, steam distilling the bases from a sample of the filtrate into standard N/100 HCl (using a Hoskins apparatus) and back titrating with N/100 NaOH.

The TVB value is expressed as mg N per 100 g of flesh.

Trimethylamine-nitrogen (TMA-N)

A further sample of the TCA extract prepared above was used to determine the TMA content, using an automated procedure (Burt & Murray, 1964) and a modification of Dyer's method (1959).

pH value

10 g of the tissue were homogenized in 40 ml of distilled water and the pH value of the homogenate determined using a glass electrode pH meter.

Moisture content

10 g of the tissue were distilled in toluene using the apparatus of Dean & Stark.

Results and discussion

Sensory assessment

The taste panel data which were essentially the same for all three batches of scampi are collected together in Table 1. It was not possible from these data alone and the limited number of samples, to devise a score sheet as has been done for numerous fish and fish products, but, in general there is agreement with the provisional descriptions drawn up by Early (1967).

The results show that in the fresh *Nephrops* there is a neutral or seawater-like odour; a sweet, strong, shellfish flavour; firm, translucent flesh and a hard, deep pink shell. During storage, e.g. after about 10 days in ice, these characteristics give place to sour, ammoniacal odours and flavours; soft, opaque, grey/white flesh and a soft, paler pink shell, often accompanied by 'black spot'. The latter phenomenon has already been widely recognized in spoiling shrimps and prawns and is due to a melanin producing

TABLE 1. Taste panel comments on fresh and ice stored nephrops

Days storage	Characteristics of the shell	Characteristics of the uncooked flesh	Characteristics after cooking of flesh		
			Odour	Flavour	Texture
Fresh					
1	Neutral to seaweedy odour, hard to touch; pink to orange colour	Neutral to fishy odour; translucent appearance, firm to touch	Neutral to fishy	Sweet, strong shellfishy, meaty	Firm, some softness, chewy, meaty, elastic
2	Neutral odour, hard, pink to orange colour	Shellfishy to fishy; translucent, firm	Neutral, slightly seaweedy, shellfishy or fishy, meaty	Somewhat less sweet, meaty and tart	Firm, chewy, elastic, meaty
3	Softer to touch, pink/orange	Neutral odour, translucent, softer but still firm	Faint shellfishy, boiled clothes, meaty	Sweet	Firm
4	Softer shell, neutral odour, pink/orange	Neutral odour, some opacity, translucent to white	Slightly stale, neutral odour	Slightly sweet to sweet	Firm but some softness, chewy
6	Neutral, slightly shellfishy, firm, pink	Shellfishy odour, musty, firm, white	Metallic, boiled clothes, slightly musty, neutral, slightly ammoniacal	Neutral to bitter; slightly sour, meaty, some sweetness	Firm, chewy, rubbery to soft
7	Slight ammoniacal odour, pink with blackening of tail	Slight ammoniacal odour, grey-white appearance	Slight ammonia	Sweet	Firm
8	Slight ammoniacal odour, sweaty sour, soft, pale pink blackening of tail	Bready, slight to strong ammoniacal, slight H_2S, white to grey, opaque, soft	Ammoniacal, sour milk, boiled clothes	Neutral to quite sweet, slightly sour, meaty	Firm to soft and mushy, rubbery

TABLE 1—*continued*

Days storage	Characteristics of the shell	Characteristics of the uncooked flesh	Characteristics after cooking of flesh		
			Odour	Flavour	Texture
9	Ammoniacal, faint pink, extensive black discoloration	Strong ammoniacal, soft, opaque	Ammoniacal, boiled clothes, slightly faecal, acetic acid	Sour, sweet, bitter	Firm to rubbery and soft
10*	Ammoniacal, pale pink, greening to extensive blackening	Grey, opaque soft, musty, slippery ammoniacal	Ammoniacal, boiled clothes, faecal	Sour, soapy, bitter, some ammoniacal and musty	Soft to firm, rubbery, and gritty
12*	Ammoniacal, pale pink	Ammoniacal, white	Strong ammonia, sweaty	Musty, sour	Rubbery

* Inedible.

enzyme system. If the presence of these sour, ammoniacal odours and flavours are indicative of spoilage, then, compared with fish such as cod, scampi flesh spoils at a much faster rate.

As will be seen from Table 8, there is some uptake of moisture during the first six days of storage. This may account in some way for the softer, mushier texture in the stored scampi (see Table 1).

Bacteriological results

Bacterial counts

Tables 2 and 3 show that bacterial counts at both 20°C and 37°C increase only after the third to sixth days of storage, and at 20°C reach 10^6/g after 7–10 days when the tissue is rejected by the taste panel. At 37°C the counts are always lower by one log value than those at 20°C for comparable storage times. In general, there was little difference between the counts of the iced and un-iced scampi; these figures are one or two log values below those for white fish, such as cod and haddock, during storage in ice (Shewan, 1961).

TABLE 2. Bacterial count at 20° C/g tissue

Port of origin	Days storage					
	1	2	4	6	8	10
Lossiemouth						
Un-iced	$2\cdot3 \times 10^{4}$	$9\cdot6 \times 10^{4}$	$9\cdot6 \times 10^{4}$	$5\cdot4 \times 10^{5}$	$1\cdot05 \times 10^{6}$	Discarded
Iced		$9\cdot3 \times 10^{4}$	$4\cdot8 \times 10^{4}$	$2\cdot16 \times 10^{5}$	$1\cdot13 \times 10^{6}$	$>10^{6}$
Peterhead	$4\cdot6 \times 10^{4}$	No data	$3\cdot3 \times 10^{5}$*	$1\cdot08 \times 10^{6}$†	$> 10^{6}$	
Buckie	$7\cdot2 \times 10^{3}$	$0\cdot5 \times 10^{3}$	$3\cdot5 \times 10^{4}$	$6\cdot42 \times 10^{4}$	$4\cdot32 \times 10^{5}$	$2\cdot25 \times 10^{6}$

* Day 3. † Day 7.

Flora

It will be observed from Table 4, that in the fresh scampi the 'coryneform' group comprises the bulk (81%) of the flora, the remainder belonging to the *Achromobacter*, *Pseudomonas*, *Micrococcus* and *Cytophaga* genera. These results are somewhat similar to other unpublished data at Torry Research Station.

However, Table 4 shows that during storage the 'coryneforms' gradually decrease in importance, until, by the tenth day, they comprise only 19% of the flora of the iced *Nephrops* and by the eighth day, 27% of the un-iced. They are replaced as the predominant species by the *Achromobacter* group, which increases from 11% in the fresh scampi, to over 70% in both the iced and un-iced, after 8–10 days storage.

TABLE 3. Bacterial count at 37° C/g tissue

Port of origin	Days storage					
	1	2	4	6	8	10
Lossiemouth						
Un-iced	$3\cdot3 \times 10^{3}$	$4\cdot9 \times 10^{3}$	$6\cdot2 \times 10^{3}$	$1\cdot5 \times 10^{4}$	$2\cdot6 \times 10^{4}$	Discarded
Iced		$1\cdot5 \times 10^{4}$	$6\cdot6 \times 10^{3}$	$1\cdot2 \times 10^{4}$	$3\cdot7 \times 10^{4}$	$4\cdot5 \times 10^{4}$
Peterhead	$4\cdot7 \times 10^{3}$	$1\cdot5 \times 10^{4}$	$7\cdot5 \times 10^{4}$*	$2\cdot6 \times 10^{4}$†	$4\cdot2 \times 10^{4}$	$1\cdot77 \times 10^{5}$ ‡
Buckie	$5\cdot8 \times 10^{3}$	$9\cdot5 \times 10^{3}$	$4\cdot5 \times 10^{3}$	$3\cdot0 \times 10^{4}$	$7\cdot8 \times 10^{3}$	$1\cdot65 \times 10^{4}$

* Day 3. † Day 7. ‡ Day 9.

TABLE 4. Bacterial flora of *Nephrops* flesh and ice used for storage (percentage per sample)

Days storage	Cory-ne-forms	*Achro-mobac-ter* sp.	*Pseudomonas* sp.				*Flavo-bacter-ium* sp.	*Cyto-phaga* sp.	*Entero-bacter-* sp.	*Aero-monas formi-cans*	*Bacter-ium-anitra-tum*	*Micro-coccus* sp.
			GpI	GpII	GpIII	GpIV						
Lossiemouth												
2 Fresh*	81	11	1			2		2				
Iced	34	32	8	13	1	3	2			2		5
Un-iced	72	10		4	7	2		3				2
4 Iced	79	9	3				1					8
Un-iced	73	25		2								
6 Iced	33	58	3		1	1		3				1
Un-iced	23	73		2		1			1			
8 Iced	13	72							1		14	
Un-iced	27	70						1	1			1
10 Iced	19	70	4	3	1			1				2
Un-iced	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT
Ice	70	12					4					14

NT = Not tested. * 3% Unclassified.

These results are significantly different from those occurring in most species of marine white fish for which data are available (Shewan, 1961). It is quite evident that, for North Sea fish species at least, the 'coryneforms' comprise a much lower percentage in fresh fish (10%) and during subsequent storage are almost completely eliminated. The *Pseudomonas* group, on the other hand, although accounting for 40–50% of the original flora, increase steadily during storage and by the tenth to twelfth day constitute 60–90% of the flora, the remainder consisting mainly of *Achromobacter* species. In one further experiment (not reported in Table 4) using iced scampi from the Buckie area and examining fewer organisms, the same pattern of change in flora was observed.

The predominance of the *Achromobacter* species in the spoilage flora of *Nephrops* was, therefore, quite an unexpected finding, in the light of previous experience with fish stored under comparable conditions. However, it should be stressed that the data presented here are relatively few and the conclusions may well have to be modified when more data become available.

Chemical

It seems that from the bacteriological results, the spoilage pattern, in chemical terms,

TABLE 5. TVB mg N/100 g *Nephrops* muscle

Port of origin	Days storage 1	2	4	6	8	10	12
Lossiemouth							
Un-iced	23·4	27·2	33·6	71·4	100·9		
Iced	23·4	26·2	15·7	12·2	46·4	73·6	
Peterhead		27·2	34·9		67·2	68·8*	
Buckie	14·7	21·4	35·8	37·4	47·0	61·4	98·2

*Day 9.

could be quite different from that occurring in marine fish. Examination of Tables 5 and 6 shows that the pattern of production of both TVB and TMA is, indeed, somewhat different from that in haddock and cod, for example. On the tenth day of storage in ice, the values of TMA and TVB for the latter species are about 5 and 15 mg N/100 g flesh, respectively, whereas in the iced scampi, after the same storage period, the TMA and TVB values are 8·0–22·0 mg N/100 g and 61·0–74·0 mg N/100 g, respectively.

TABLE 6. TMA mg N/100 g *Nephrops* muscle

Port of origin	Method used	Days storage 1	2	4	6	8	10	12
Lossiemouth								
Un-iced	Automatic	0·6	0·8	1·9	9·6	27·2		
	Dyer	0·4	0·9	2·1	10·1	24·5		
Iced	Automatic	0·6	0·6	0·7	1·5	10·2	12·4	
	Dyer	0·4	1·9	1·1	1·9	9·9	13·2	
Peterhead	Automatic			1·0*	12·4†	14·8		
	Dyer		1·7	6·5*	15·4†	17·8		
Buckie	Automatic	0·2	0·4	0·4	2·2	16·8	8·8	24·8
	Dyer	1·3	1·4	1·8	4·7	14·7	21·7	23·3

* Day 3. † Day 7.

The large amounts of bases produced are also evidenced by the pH of the flesh during storage, which rises from about 6·7 to about 8·0 (Table 7). It was observed during these experiments that the iced scampi generally became unacceptable to the taste panel when the pH of the flesh reached 7·8.

TABLE 7. pH of the *Nephrops* flesh

Port of origin	Days storage					
	1	2	4	6	8	10
Lossiemouth						
Un-iced	6·7	6·5	6·9	7·3	7·3	
Iced	6·7	6·5	6·4	7·1	7·8	7·9
Peterhead		6·5	7·2*	7·5†	8·0	
Buckie	6·9	7·0	7·2	7·7	8·1	8·1

* Day 3. † Day 7.

TABLE 8. Percentage moisture content of the *Nephrops* flesh

Port of origin	Days storage				
	2	4	6	8	10
Lossiemouth					
Un-iced	75	82	81	75	
Iced	71	81	84	83	
Buckie	68	71	73	75	74

Limited experiments made with pure cultures of the bacteria present in spoiling scampi flesh inoculated onto sterile cod muscle, failed to produce any of the characteristic odours present in either spoiling fish or scampi flesh. However, heavy inoculation of some of the same pure cultures onto defrosted scampi flesh produced the characteristic odours of spoiled scampi as described by the taste panel in the normal spoilage runs. The controls did not show the same advanced spoilage characteristics as the inoculated samples at the same time of examination.

Preliminary studies, of fresh and spoiled scampi tissue extracts using thin layer chromatography have shown that, in addition to TMA, dimethylamine (DMA) is also being produced.

It seems evident, therefore, that the pattern of spoilage of scampi flesh during storage, whether iced or un-iced, appears to be somewhat different to that of fish, such as cod and haddock.

It is possible that, as for the *Penaeus*, *Metapenaeus* and *Parapenaeopsis* prawn species, the TMA, TVB and possibly the DMA contents of *Nephrops norvegicus* could be useful as objective indices of spoilage during storage, although a considerable amount of data is still required to confirm this.

Acknowledgment

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A study of some factors affecting the methylene blue test and the effect of freezing on the bacterial content of ice cream

JEAN ALEXANDER AND J. ROTHWELL

Summary

The effect of *E. coli* and *B. cereus* on methylene blue reduction time in ice cream was studied. A general correlation was established between reduction time and numbers of organisms. Lower numbers of *B. cereus* than *E. coli* were required to bring about reduction in the same times.

Considerable destruction of bacteria was observed during freezing in vertical and soft serve freezers accompanied by an increase in reduction time. This was shown to occur very rapidly after a critical point of ice crystal formation was reached, irrespective of temperature. Increased concentration of total solids did not increase this destruction and bacterial destruction was attributed to mechanical damage.

Introduction

Statutory regulations and requirements for the bacteriological quality of ice cream are in existence in many countries. These usually take the form of a maximum permitted total count (e.g. in Switzerland 25,000, in France 300,000 organisms per g) together with a maximum count of coliform organisms.

In the United Kingdom the maintenance of an adequate bacteriological standard is carried out by strict observance of the heat treatment regulations, and careful inspection of licensing of premises where ice cream is produced, stored and sold. Except in Scotland the main test used is a modified form of the methylene blue test for milk.

This test was introduced as a 'provisional' or advisory test following the report (1947) of a sub-committee of the Public Health Laboratory Service. Although the report included results of comparisons between the methylene blue grade, plate counts and numbers of coliforms present, little or no systematic investigation appears to have been made of the effect of specific organisms in sterile or near sterile ice cream and ice cream mix either then or since.

The present investigations were therefore carried out to attempt to study the following:

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1. The correlation between methylene blue grading and the *E. coli* type I. and *B. cereus* content.
2. Comparisons between the methylene blue test in ice cream and in ice cream mix.
3. The effect of the freezing process on the bacterial content of ice cream.
4. The effects of the pre-incubation period on the test.
5. Effects of ingredients on the test, with special reference to the sweetening agent.

Materials and methods

Organisms

These were *Escherichia coli* type I (indol +ve; methyl red +ve V.P. −ve; citrate −ve) obtained from the National Institute for Research in Dairying, Shinfield, and *Bacillus cereus* obtained from the Microbiology Department of Reading University. Cultures were maintained on tryptone glucose yeast agar, and fresh slopes were prepared monthly. Working cultures were prepared as required by inoculation from the stock cultures into nutrient broth.

Colony count determinations

These were carried out as required, for *E. coli* type I using MacConkey agar No. 3. and ¼ strength Ringers solution as the diluent. For *B. cereus* tryptone glucose yeast agar was used, with 0·85% saline of normal strength as the diluent, as ¼ strength Ringers gave results which did not agree between dilutions.

Methylene blue test

This was carried out as described in the Report (1947) in the *Monthly Bulletin of the Ministry of Health.* Where *B. cereus* was used a 'blue control' tube was prepared by mixing 8 ml Ringer's solution, 2 ml ice cream, and adding bromo-cresol green to match the initial colour of the methylene blue tubes before pre-incubation.

Ice Cream

The following types of mix were employed as convenient:

(a) Sterile canned mix, 7–8% fat, obtained from commercial sources.

(b) Complete cold mix powders 8–9% fat, which required reconstitution with cold water, and

(c) A virtually sterile mix of 7% fat content made from the basic ingredients by careful preparation and heat treatment.

Ice cream freezers

(a) Vertical batch brine refrigerated
(b) Horizontal pressure batch
(c) Continuous Flo-creematic
(d) Carpigianni soft-serve

Experiments and results

1. *Effect of the number of E. coli organisms present in ice cream mix and the methylene blue reduction time*

Varying numbers of *E. coli* type I organisms were inoculated into sterile mixes and the methylene blue test carried out. Numbers of organisms present were determined in the original sample. Fig. 1 shows the number of organisms in the initial mix plotted

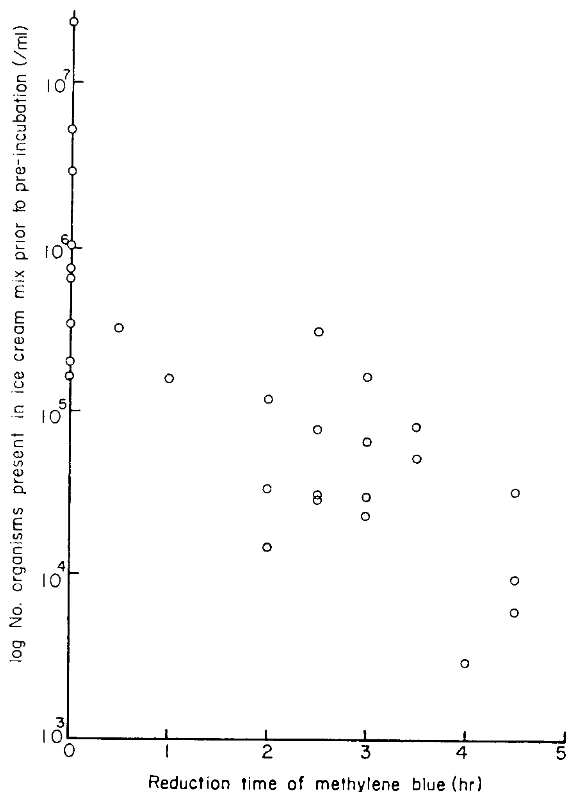

FIG. 1. Effect of the number *E. coli.* type I bacteria in ice cream mix, on the time taken to reduce methylene blue.

against the methylene blue reduction time, indicating that an increase in the number of organisms present initially gave more rapid reduction times. Ice cream mix containing about 1 million organisms per ml might be expected to give Grade 4, while Grade 1 or 2 would be expected if about 10,000 organisms per ml were present.

2. *Effect of the number of E. coli organisms present in ice cream and the methylene blue reduction time*

Sterile mix was frozen in a sterilized vertical type freezer, and hardened at $-15°C$ for 24 hr. Samples were then allowed to melt at room temperature and inoculated as in

(1). Mixes prior to inoculation were all Grade 1 in the methylene blue test, and coliforms were absent from 0·1 ml. In similar experiments ice cream prepared from basis ingredients was also used. Fig. 2 shows the number of *E. coli* type I organisms present

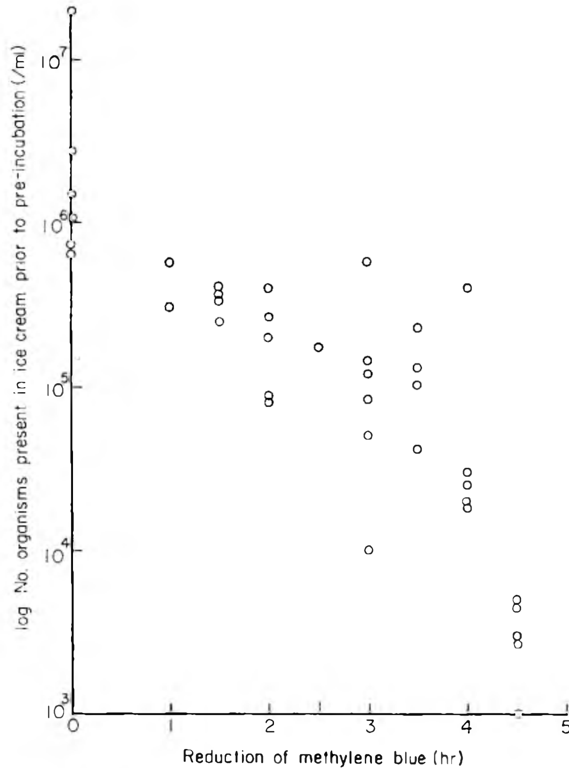

Fig. 2. Effect of the number *E. coli* type I bacteria in ice cream on the time taken to reduce methylene blue.

plotted against the time to reduce methylene blue. It will be seen that a correlation appears to exist between the number of organisms and the reduction times.

3. *Effect of the number of B. cereus in ice cream mix and in ice cream and the methylene blue reduction time*

Experiments were carried out as in (1) and (2), but using *B. cereus* instead of *E. coli*. Results are shown in Figs 3 and 4 and it will be seen fewer *B. cereus* organisms than *E. coli* were required to reduce the methylene blue and the same reduction time was obtained with fewer *B. cereus* organisms in ice cream than in ice cream mix. This may have

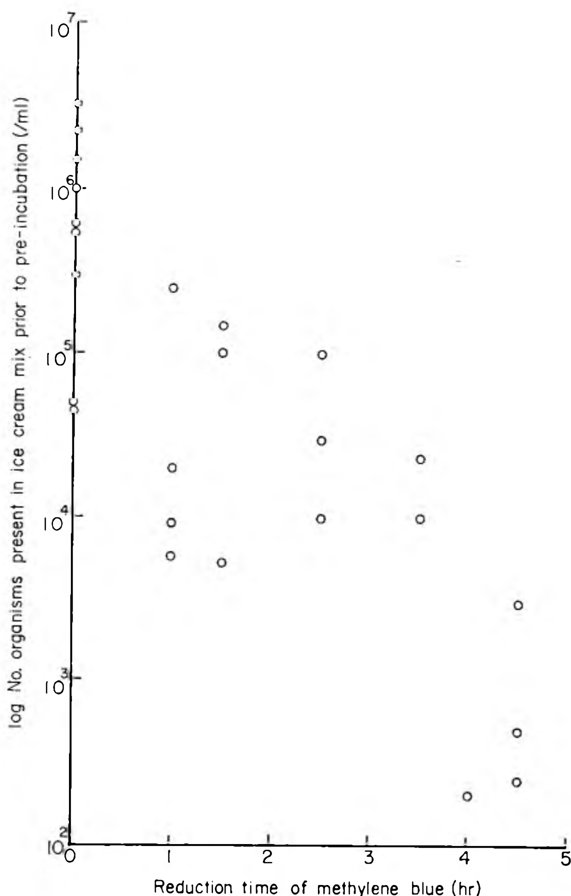

FIG. 3. Relationship between the number of *B. cereus* organisms in ice cream mix and and the time taken to reduce methylene blue.

been caused by the fact that *B. cereus* is aerobic and may grow better in ice cream than in mix due to the higher oxygen content of ice cream.

4. *The effect of the pre-incubation period on the bacterial content and methylene blue grade of ice cream.*

Sterile ice cream mix was frozen in a carefully sterilized vertical freezer, and samples of the frozen ice cream were hardened in sterile honey jars for 24 hr at −22°C. The samples were then allowed to thaw at room temperature for 1 hr before 10 ml quantities were pipetted into a series of sterile test tubes. These tubes were then inoculated with between 1×10^{-1} and 1×10^{-4} ml of a 24 hr broth culture of *E. coli* type I. The methylene blue grade of each sample was determined, and the number of organisms in each sample before and after the pre-incubation period of the test were estimated. Table 1 shows the results of a series of test tube inoculations. It will be seen that a correlation

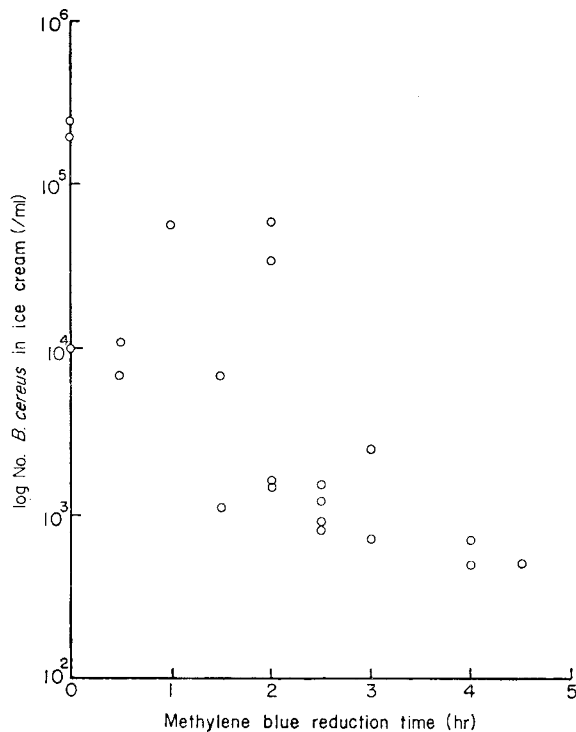

FIG. 4. Relationship between the number of *B. cereus* organisms in ice cream on the methylene blue reduction time.

appears to exist between the numbers of organisms present initially and the methylene blue grade, the pre-incubation period appeared to cause an increase in the number of organisms present and that the rate of this increase appeared to depend on the initial number of organisms present. This rate of increase was lower and the greater was the number of organisms present, until a final constant value was reached.

5. *The effect of the freezing process on the bacterial content of ice cream, and on the methylene blue reduction time.*

An attempt to discover what effect the amount of air introduced into ice cream during the freezing process and on the methylene blue grade was not successful. After inoculation of a mix with an *E. coli* type I culture it was frozen in a vertical freezer, samples taken at regular intervals and tested. After an initial apparent increase in numbers of organisms there occurred decreases depending on the length of time of freezing. This could not be related to overrun and therefore the effect of the whole freezing process on the bacterial content was subjected to careful investigation.

One gallon quantities of carefully prepared mixes were inoculated with 10 ml of a 24 hr broth culture of *E. coli* type I. The mix was then frozen in a vertical freezer.

TABLE 1. The effect of the number of *E. coli* type I organisms present initially in ice cream on the time to reduce methylene blue and on the count after pre-incubation

Initial colony count per ml	Colony count per ml after pre-incubation	Methylene blue reduction time (hr)	Grade
$1{\cdot}5 \times 10^6$	$2{\cdot}35 \times 10^8$	0	4
$1{\cdot}5 \times 10^6$	$6{\cdot}0 \times 10^8$	0	4
$1{\cdot}1 \times 10^6$	$3{\cdot}35 \times 10^8$	0	4
$7{\cdot}35 \times 10^5$	$2{\cdot}9 \times 10^8$	0	4
$6{\cdot}55 \times 10^5$	$3{\cdot}1 \times 10^8$	0	4
$4{\cdot}0 \times 10^5$	$3{\cdot}05 \times 10^8$	$1\frac{1}{2}$	3
$8{\cdot}1 \times 10^4$	$2{\cdot}15 \times 10^8$	2	3
$8{\cdot}95 \times 10^4$	$1{\cdot}65 \times 10^7$	2	3
$1{\cdot}0 \times 10^4$	$6{\cdot}1 \times 10^6$	3	2
$2{\cdot}7 \times 10^3$	$1{\cdot}0 \times 10^7$	>4	1
$2{\cdot}0 \times 10^2$	$1{\cdot}0 \times 10^6$	>4	1

Mix samples were taken prior to freezing, and then every 5 min during the freezing process. These samples were placed in sterile honey jars and hardened at -22°C for 24 hr before being tested. The number of coliforms present in each sample was determined.

This investigation was repeated using a soft-serve freezer, a horizontal batch freezer and a Flo-creematic continuous freezer. The results of typical experiments are shown in Fig. 5. It will be seen that the rates of destruction of bacteria in the vertical, horizontal batch and soft-serve freezers were similar. The initial rate of destruction in the continuous freezer was greatest, but after the initial freezing period the numbers surviving fluctuated considerably as more inoculated mix flowed through the freezer. The rate of destruction in the vertical varied with the temperature of the brine refrigerant.

Table 2 shows the typical effect of the freezing process on the methylene blue grade and number of surviving organisms, and it will be seen that as freezing proceeded the methylene blue time increased from $1\frac{1}{2}$ to > 4 hr, and counts dropped from $6{\cdot}5 \times 10^5$ to $6{\cdot}3 \times 10^4$ coliform organisms per ml.

Fig. 6 shows that, using a soft-serve freezer, when mixes inoculated with several different organisms were treated as described above the same pattern of destruction of micro-organisms occurred. *E. coli* type I showed the greatest resistance in these experiments, which also included *E. coli* type II, *A. aerogenes* type II, *B. cereus* and *Strep. faecalis*.

Aeration alone, however, without freezing did not cause any reduction in the numbers of organisms in an inoculated ice cream mix, as shown in Table 3.

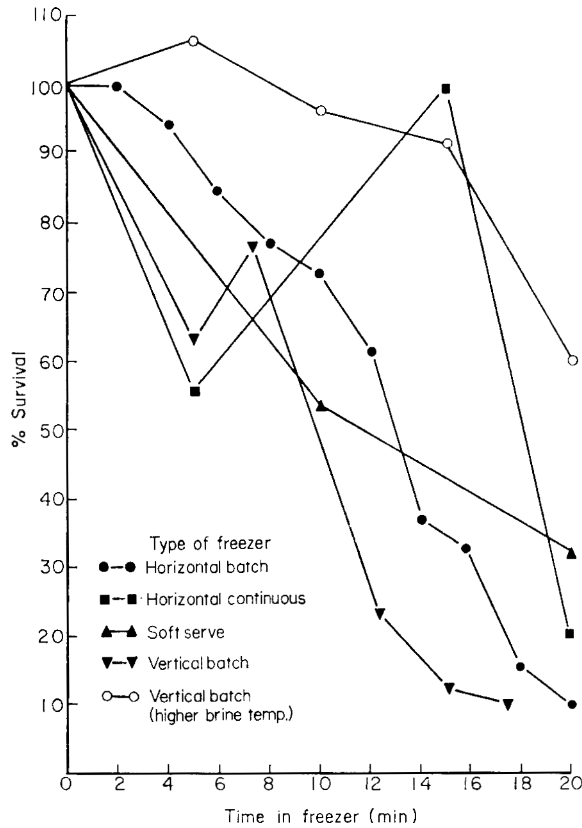

Fig. 5. Effect of freezing ice cream mixes in different freezers, on the bacterial content.

Reduction of numbers did not occur unless the mix was being refrigerated and aerated (agitated) at the same time. Fig. 7, which plots the percentage survival of *E. coli* type I organisms in an ice cream mix against the mix temperature shows that the maximum destruction occurred at temperatures below about 26°F. At this temperature the maximum formation of ice would be expected.

6. *The effect of variations in total solids content of an ice cream mix on the freezing of the mix and rate of destruction of the organisms*

Ice cream mixes were prepared containing 7% fat, 12% sugar, and 0·5% combined emulsifier stabilizer, and in which the total solids contents was varied from 25% to 45% by increasing milk solids-not-fat content from 5·5% to 25%. After processing in the usual way these mixes were frozen after inoculation with *E. coli* type I cultures. Other mixes were frozen without inoculation with bacteria.

The effect of increasing the total solids content on the freezing of the ice cream is shown in Fig. 8. It will be seen that these curves exhibit the usual freezing curve shape,

TABLE 2. The effect of the freezing process on the number of coliform organisms and the methylene blue grade of ice cream mix (Vertical freezer)

Time sampled (min)	Temperature (°F)	Coliform count (org/ml)	% survival	Methylene blue reduction time (hr)
0	40	$6{\cdot}5 \times 10^5$	100	1·5
2	35	$6{\cdot}5 \times 10^5$	100	2
4	34	$6{\cdot}1 \times 10^5$	94	2
6	28·5	$5{\cdot}5 \times 10^5$	85	3
8	26	5×10^5	77	3
10	25·5	$4{\cdot}7 \times 10^5$	72	3
12	25·5	4×10^5	61·5	3
14	24·5	$2{\cdot}4 \times 10^5$	37	4
16	23·5	$2{\cdot}1 \times 10^5$	32	4
18	22	1×10^5	15	4
20	21	$6{\cdot}3 \times 10^4$	10	4

and, as is to be expected, as total solids content was increased the mixes froze at correspondingly lower temperature, and more rapidly.

Figs 9 and 10 show that the destruction of bacteria appeared to occur at a similar rate in all mixes, but the temperature at which it occurred varied, with lower temperatures being required for mixes with higher total solids. This appeared to coincide with the reduction in the freezing points of those mixes. The data for 45% total solids in Figs 9 and 10 relate to separate experiments.

TABLE 3. The effect of aeration alone of an ice cream mix on the number of organisms and on the methylene blue reduction time

Time sampled (min)	Coliform count (orgs/ml)	Reduction time (hr)
0	$2{\cdot}5 \times 10^5$	1·5
10	$3{\cdot}0 \times 10^5$	1·5
30	$3{\cdot}5 \times 10^5$	1·5
60	$4{\cdot}4 \times 10^5$	1·5
120	$3{\cdot}6 \times 10^5$	1·5

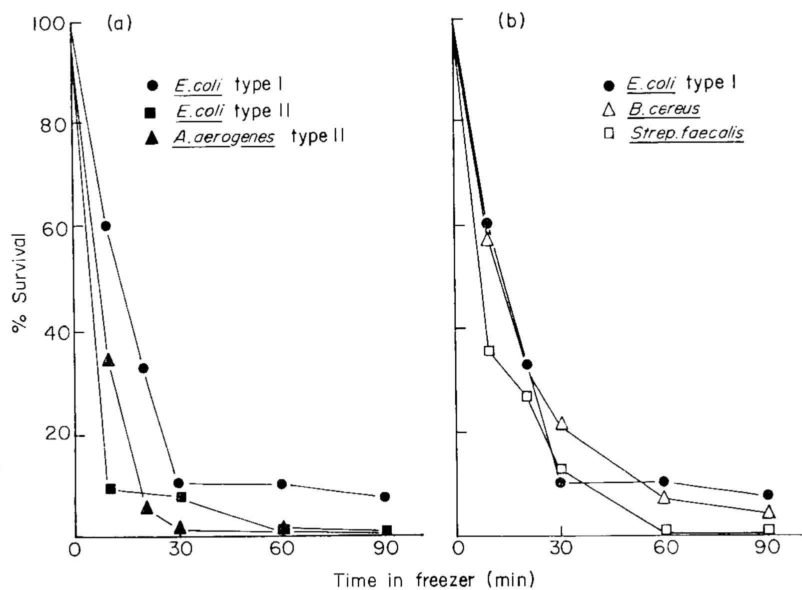

FIG. 6. Survival of bacteria during freezing in a Carpigiani Soft Serve Freezer.

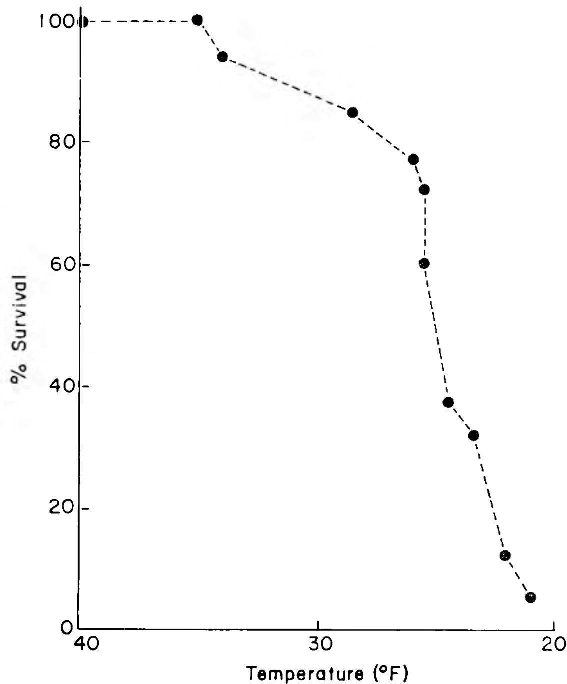

FIG. 7. Effect of the freezing process on the number of coliform organisms in ice cream mix.

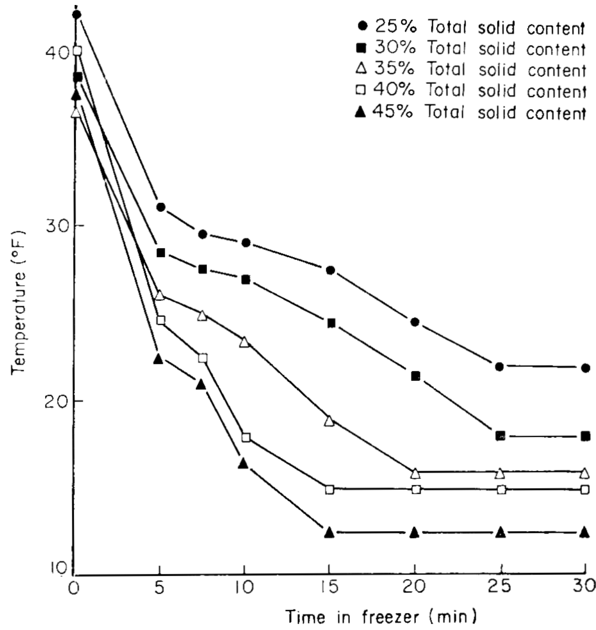

FIG. 8. Effect of the total solids content of ice cream mix on the temperature of freezing.

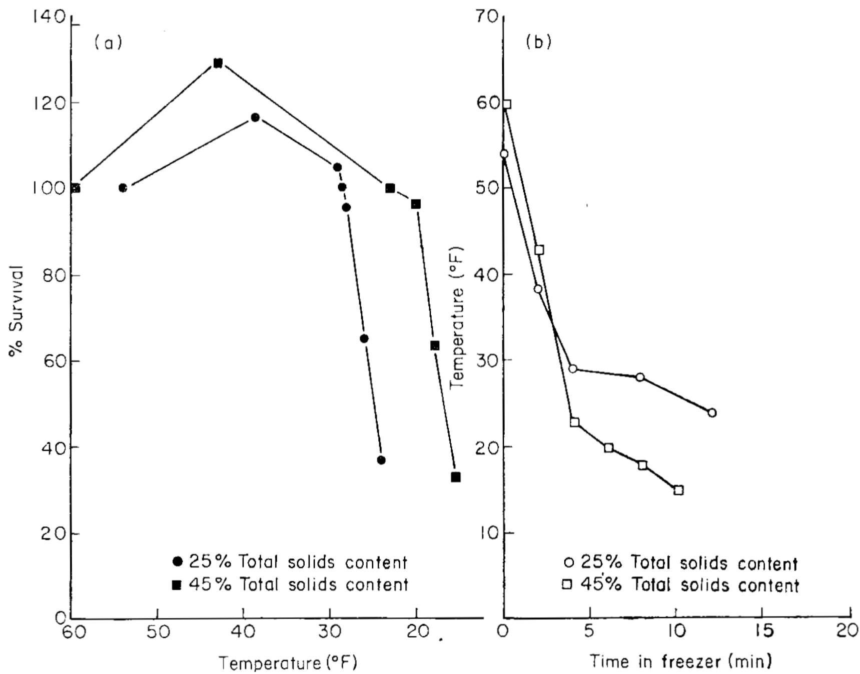

FIG. 9. The rate of bacterial destruction (a) and the rate of freezing (b) of ice cream mixes of different total solids content.

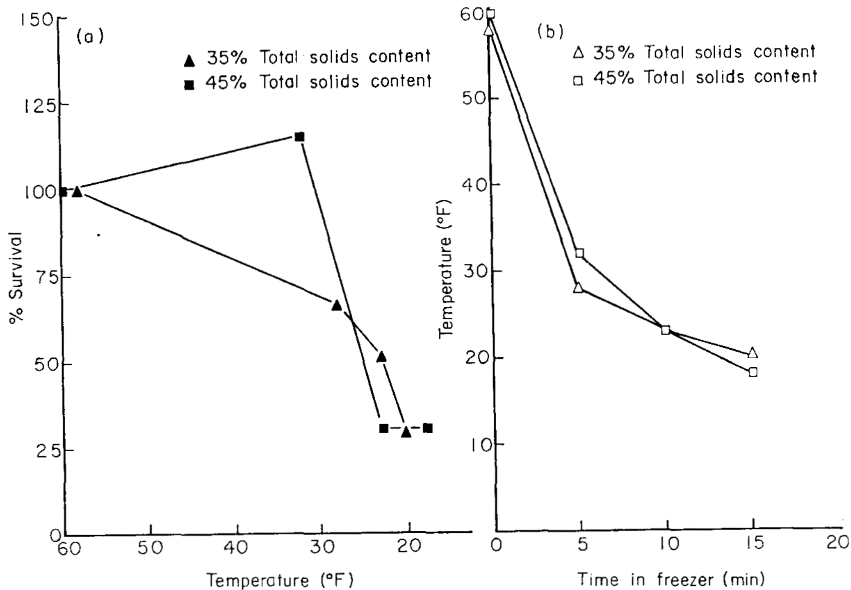

Fig. 10. The rate of bacterial destruction (a) and the rate of freezing (b) of ice cream mixes of different total solids content.

7. The effect of the freezing process on the numbers of organisms present in ice cream mixes containing varying percentages of dextrose.

Mixes containing 7% fat, 11·4% milk solids-not-fat and 0·5% emulsifier/stabilizer were made in which the sucrose was replaced by varying quantities of dextrose as shown in Table 4.

Table 4. Sugar/dextrose mixtures used in ice cream mixes

Mix	Control	1	2	3	4
Sucrose (%)	12	9	6	3	—
Dextrose (%)	—	3	6	9	12

On freezing these mixes, and other similar series of mixes which were inoculated with *E. coli* type I and *B. cereus*, the effect of increasing dextrose content, with its associated lowering of the freezing temperature of the ice cream, was shown to delay the sudden increase in the rate of bacterial reduction. This is shown in Figs 11, 12 and 13.

This delay in the destruction of the organisms was presumably due to the delay in the commencement of ice crystal formation which occurred with increasing dextrose content, as destruction of bacteria appeared to occur irrespective of temperature, but simultaneously with ice crystal formation.

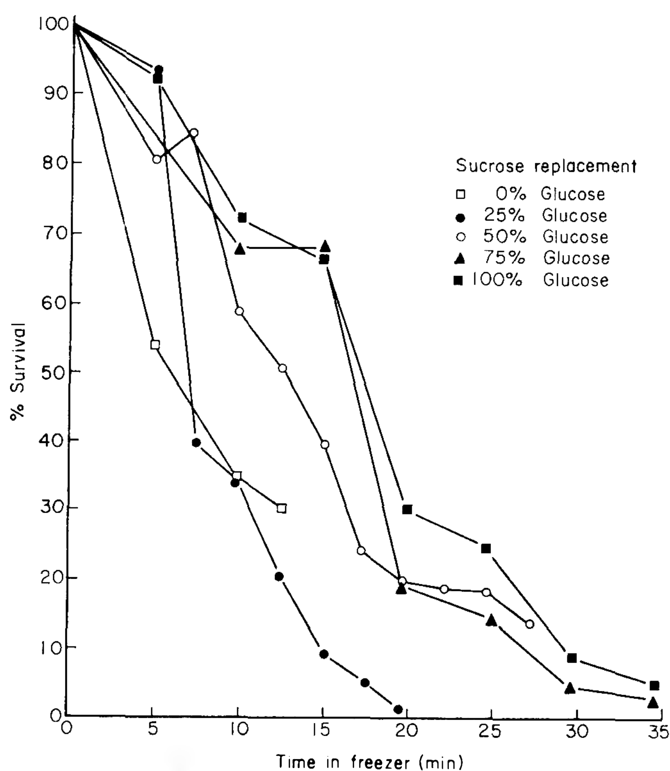

FIG. 11. % Survival of *E. coli* type I in ice cream mixes containing various percentages of glucose, during freezing in the Vertical Freezer.

Discussion and conclusions

Experiments 1–5 show that the presence of *E. coli* type I and *B. cereus* affected the rate of methylene blue reduction, and approximate numbers can be given for this (see Table 5).

TABLE 5. Approximate numbers of organisms compared with methylene blue gradings in ice cream

Organism	Numbers per ml	Methylene blue grade
E. coli type I	0 < 10,000	1
	> 10,000 < 75,000	2
	> 75,000 < 100,000	3
	> 100,000	4
B. cereus	0 < 500	1
	> 500 < 1500	2
	> 1500 < 50,000	3
	> 50,000	4

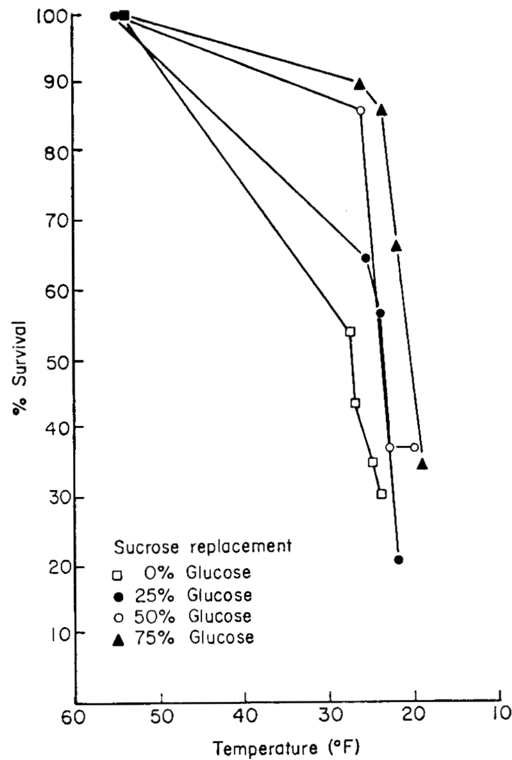

FIG. 12. Effect of the temperature of ice cream during freezing on the bacterial content.

It will be seen that considerably fewer *B. cereus* organisms than *E. coli* are required to give corresponding reductions. *E. coli* should not normally be present in ice cream as it is a heat treated product. If it is found this must mean either faulty heat-treatment or post-heat treatment contamination. *B. cereus* spores, however, may be present in the original raw materials and could survive the heat treatment. If they are the cause of poor methylene blue gradings the control tube should also be decolourized.

The remaining experiments showed in many cases a most remarkable drop in the numbers of organisms which survived combined freezing and agitation during the freezing process. Mazur (1966) showed that freezing under quiescent conditions does in most cases cause damage to bacterial cells which ultimately results in death. Mechanisms may include intracellular freezing of water, and cell dehydration caused by increased concentration of solutes inside and outside the cell by ice formation in a cell suspension. The survival of organisms under these conditions was usually much greater than the survival under the conditions described in this paper.

In ice cream manufacture it was generally thought that freezing a mix reduces the number of bacteria present, despite the fact that there are substances present such as sugar and fat which might exert a protective effect.

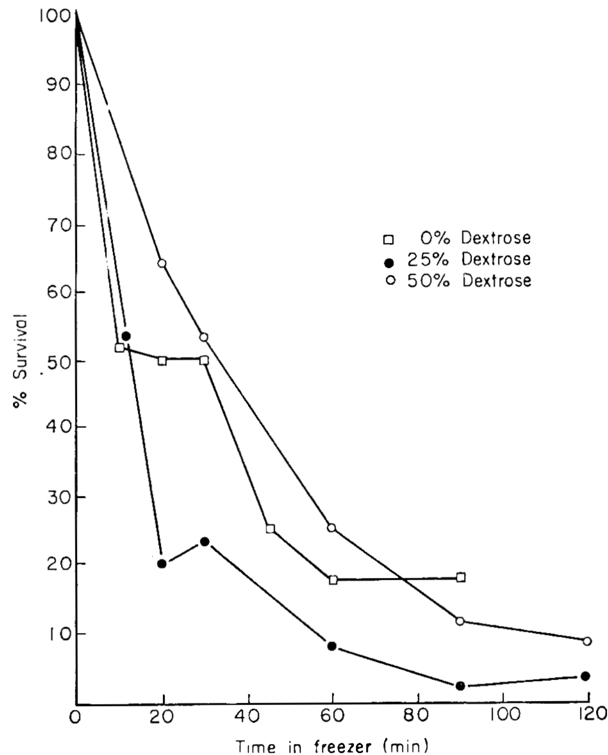

FIG. 13. Effect of various percentages of dextrose on the survival of *B. cereus* in ice cream mix.

Hammer & Goss (1917) studied the influence of freezing, hardening and storage on the bacterial content of ice cream, and found an apparent increase in bacterial numbers had occurred during freezing which they attributed to dissociation of bacterial clumps.

Fay & Olson (1924) and Ellenberger (1919) also found an increase in numbers during freezing. Fabian & Cromley (1923) who examined forty-one samples of ice cream mix and ice cream, before and after freezing, found an increase to have occurred in 46%, and a decrease in 36·6% of the samples during freezing. They attributed the increases to breakdown of bacterial clumps, and considered if the mix had no clumps as it entered the freezer there should be a decrease due to the unfavourable temperatures of freezing.

Foley & Sheuring (1965a) found that initial increases in plate counts occurred during the pre-crystallization period in a conventional type of soft serve freezer, which they attributed to the usual dispersion of clumps and chains of bacteria. A reduction in counts occurred during the period of ice crystal formation. These authors also studied the rate of destruction during freezing (Foley & Sheuring, 1965b) using several different

organisms. With normal freezing the rate of destruction climbed rapidly to a maximum between 5 and 7½ min from commencement of freezing, then decreased. When the freezing rate was reduced by half the rate of destruction showed a much more gradual increase reaching a maximum 17½–20 min after the start of freezing.

The factors which influenced the lethal rates of bacteria during freezing in a soft serve freezer were also studied by them (Foley & Sheuring, 1965c). They showed that agitation during freezing had a pronounced effect, and the rate of destruction was also influenced by mix pH and type of organism. Variations in overrun did not appear to have any effect.

It is felt that the destruction described in this paper is similarly associated with ice crystal formation since the temperature at which freezing occurred coincided with the point at which destruction began. In mixes with high total solids there was no indication that the destruction rate increased with the total solids. Destruction was only found to occur when ice crystals were present, and the freezing mix was being agitated—the conditions found to be necessary by Foley & Sheuring as mentioned. The destruction under the conditions reported here would appear to have been caused by mechanical damage to the bacterial cells by grinding with ice crystals.

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Analysis of the active principle in the biological insecticide *Bacillus thuringiensis* Berliner

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Summary

A detailed analysis of the toxic principle of the biological insecticide, the crystals of *B. thuringiensis,* is given. Almost all (96·1%) of the nitrogen in the crystals, but only about 85% of the weight, is accounted for by amino acids. The imino acid, proline, is absent. A non-nitrogenous fraction, accounting for 12% of the dry weight of the crystals, has been separated by dissolving the polypeptide part in dilute alkali. The non-nitrogenous fraction is largely carbohydrate, and the crystals are probably composed of glycoprotein.

The insolubility of the crystals cannot be fully explained by reference solely to the amino acid composition, and it is suggested that the carbohydrate moiety makes a contribution to the insolubility.

Introduction

Cultures of *Bacillus thuringiensis* and related bacteria are used as the active principle in some biological insecticides. The products are added to flour and other food products stored in bulk, where they are toxic when ingested by the larvae of flour moths and other lepidopterous pests. Insecticides of this type have a greater specificity than wholly chemical insecticides and *B. thuringiensis* is also known to be without effect on humans and other vertebrates consuming treated foodstuffs. Nevertheless a major drawback to more widespread use of this type of biological insecticide is that the methods of manufacture cause viable bacterial spores to be included in the product.

It is characteristic of all bacteria in the *B. thuringiensis* group that a parasporal inclusion is formed at sporulation. The toxicity of the products is due entirely to the presence of the inclusions, thus making the presence of spores an unnecessary hazard. The aim of this investigation was to obtain a more complete understanding of the composition and structure of the inclusions so that progress may be made towards synthesis of the toxic principle free from contamination by spores or cell fragments.

Analytical studies of the inclusions (now usually referred to as crystals) have been

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extremely limited and knowledge of their composition is far from complete. The crystals are believed to be proteinaceous since their ultra-violet absorption spectrum, both in the solid state and in solution in alkali, resembles that of a protein (Hannay & Fitz-James, 1955; Lecadet, 1965) and the crystals are known to include amino acid residues. A qualitative amino acid analysis of the crystals was made, by means of paper chromatography of a 12 hr acid hydrolysate, soon after the first isolation of the crystals (Hannay & Fitz-James, 1955). Some confirmation of this analysis was obtained from a quantitative paper chromatographic analysis of an acid hydrolysate of the crystals of *B. sotto*, an organism closely related to *B. thuringiensis* (Angus, 1956).

Further analyses of *B. thuringiensis* crystals have substantially confirmed the earlier results obtained by paper chromatography. Acid hydrolysates were analysed (Lecadet, 1965; Holmes & Monro, 1965) by ion-exchange chromatography, a technique capable of greater accuracy than paper chromatography. However, in neither case did allowance appear to have been made for factors such as the destruction of amino acids during hydrolysis, so that it is not easy to assess the results quantitatively. Furthermore, none of the analyses yet published complies with the four criteria which Tristram & Smith (1963) consider essential in a satisfactory amino-acid analysis.

The total nitrogen content of the crystals has been variously reported as 16·5% (Monro, 1959; Holmes & Monro, 1965), 15·6–16·2% (Lecadet, 1966) and 17·5% (Hannay & Fitz-James, 1955). These figures may be presumed to be on a dry, ash-free basis, though in no case is this stated. In view of the discrepancies in total nitrogen contents shown by different analyses, and in the absence of a fully quantitative amino acid analysis, the achievement of the latter for the crystals of a well-defined strain of *B. thuringiensis* was considered to be of prime importance. Without such an amino acid analysis the question of whether the crystals are entirely polypeptide in nature or whether they include non-polypeptide material cannot be adequately considered, nor can further work on the chemical structure of the crystals be usefully carried out. Previous workers do not appear to have investigated the possibility of the crystals containing non-peptide material. In order to obtain a reliable and complete analysis of the proteinaceous component of the crystals a careful amino acid analysis has been undertaken on the semi-micro scale. This scale had to be adopted, despite the drawback of some small loss of precision, as a total of only 150 mg of crystals had been isolated and purified (Bateson, 1966) for this and for other analyses.

Materials and methods

Isolation of crystals prior to analysis

The crystals were isolated from the organism commonly known as *B. thuringiensis* Berliner but more correctly known, according to the most recent classification of crystalliferous bacteria (Norris, 1964) as a Group I organism, strain TM (isolated by Mattes). *B. thuringiensis* is not a fastidious organism and was readily cultivated on

nutrient agar by incubating at 30°C for 5 days after inoculation. The crude harvest was washed free from vegetative cells and cell debris as described by Angus (1956), yielding a concentrated suspension consisting entirely of crystals and spores. Crystals were isolated from the suspension by an adaptation of the method of Bateson (1965) which was devised by Rogers (1967). The crystal preparation contained less than 1% by weight of non-crystal material, the extraneous matter being entirely spores (Bateson, 1966).

Hydrolysis of crystals

Four hydrolysates were prepared, with hydrolysis times of 20, 45, 72 and 142 hr respectively. Four samples of approximately 6 mg each were hydrolysed by means of 4·0 ml of approximately 6 N HCl (constant boiling HCl, having been distilled three times in glass apparatus) under vacuum in a sealed tube. The tubes were immersed in an oil bath held at 105°C ± 0·2°. After hydrolysis the solution was diluted to 25 ml and the HCl was removed from 5 ml aliquots of the hydrolysates by repeated evaporation under vacuum over P_2O_5 and NaOH. The dried hydrolysates were stored at 2°C over P_2O_5 until required for analysis.

Amino acid analysis

The method of analysis of the hydrolysates by ion-exchange chromatography was that of Moore & Stein (1951), as modified by Eastoe (1955). The semi-micro procedure of Eastoe (1961) was adopted in detail except for slight variations mentioned below. Prior to the analysis of hydrolysates the semi-micro apparatus was fully calibrated using mixtures of chromatographically homogeneous amino acids (obtained from British Drug Houses Ltd, Poole, England). For analysis a solution equivalent to about 1 mg dry, ash-free hydrolysed crystals was applied to the ion-exchange column and for calibration 0·25 ml of a solution containing about 1 mole of each amino acid per ml was applied.

Threonine and serine

The ion-exchange resin (Dowex 50 W × 8, mesh 200–400, batch 3786) was unable to resolve threonine and serine under the conditions described by Eastoe (1955) and these amino acids were determined separately. If 5% (v/v) of methanol was incorporated in the pH 3·42 citrate buffer and the temperature of the water-jacket of the ion-exchange column was suddenly lowered to 15°C as soon as aspartic acid began to be eluted the resolution was found to be satisfactory (Bidmead & Ley, 1958). A separate analysis incorporating these changes in the elution conditions enabled threonine and serine to be determined.

Cysteine and cystine

Cysteine and cystine were oxidized to cysteic acid by treatment of the crystals with

performic acid prior to hydrolysis. Cysteic acid is more amenable to determination by ion-exchange chromatography than cysteine and cystine.

Performic acid was prepared by mixing 9 vols. formic acid (A.R.) with 1 vol. 30% hydrogen peroxide (A.R.) and allowing the mixture to stand at room temperature for 1 hr. About 10 mg of crystals were oxidized by treatment with 2 ml of performic acid solution at 4°C for 16 hr. Performic acid was removed by evaporating the solution at 65°C, adding 1 ml of water and re-evaporating to dryness at 65°C. The oxidized crystals were then hydrolysed in the usual way. Cysteic acid may be determined simultaneously with the other amino acids (Bidmead & Ley, 1958) but in this instance separate hydrolysates were prepared and analysed. A solution equivalent to about 5 mg of dry, ash-free crystals was applied to the ion-exchange column.

Tryptophan

The method of Spies & Chambers (1948, 1949) for the determination of tryptophan in solid protein was adopted. Spies and Chambers described the optimum reaction conditions for a variety of proteins and also laid down generalized conditions applicable to proteins with largely unknown properties. Because of the known insolubility of the crystals the initial reaction, namely condensation of tryptophan in the protein with p-dimethylaminobenzaldehyde, was allowed to proceed for 16 hr at 25°C ± 0·2°. About 3 mg of crystals were used in each replicate determination.

Total nitrogen

The semi-micro procedure described by Heaps (1964) was used.

Ash

5 mg samples of the crystals were ignited at 550°C for 2 hr in pre-ignited platinum crucibles, after a preliminary charring in a bunsen flame.

Treatment of crystals with alkali

The polypeptide part of the crystals was dissolved, and the non-polypeptide part obtained as a residue by treating the crystals with alkali. About 20 mg of crystals were mixed with 50 ml of 0·1 N NaOH solution and the whole continuously stirred for 16 hr. The residue was centrifuged off and washed until free of NaOH before being dried in readiness for qualitative analysis.

Infra-red spectroscopy

1 mg of the alkali-insoluble fraction of the crystals was dispersed in a translucent disc of 200 mg of KBr. The infra-red absorption spectrum of the fraction was determined and recorded using a Unicam S.P.200 Infra-red Spectrophotometer.

Molisch test for carbohydrate

1 mg of the alkali-insoluble fraction was dissolved and hydrolysed in a few drops of 70% H_2SO_4 for 4 hr. The solution was diluted to about 1 ml (i.e. approximately 2 N H_2SO_4) and tested for carbohydrates as described by Dische (1962).

Paper chromatography

1 mg of the alkali-insoluble fraction was hydrolysed to monosaccharides by dissolving it in a few drops of 70% H_2SO_4 for 2 hr, diluting to about 2 N H_2SO_4 and heating the solution to 100°C for a further 3 hr. The cooled solution was de-ionized by passage through a column of 'de-acidite' FF–IP anion exchange resin (carbonate form) and evaporated almost to dryness in a rotary evaporator. The resulting solution was examined, together with sugar solutions of known composition, by descending chromatography on Whatman No. 1 filter paper (Barrett & Northcote, 1965). The solvent was ethyl acetate : pyridine : water (8 : 2 : 1, by volume). The position of the sugars after chromatography was revealed by dipping the paper in a 3% (w/v) solution of p-anisidine hydrochloride in absolute alcohol and heating at 100°C for 15–20 min. This reagent reveals hexoses as yellow-brown spots and pentoses as mauve spots. Duplicate chromatograms were examined for the presence of amino sugars by treatment with a 2% (w/v) solution of ninhydrin in butanol : acetic acid (20 : 1, by volume) followed by heating at 100°C for 20 min.

Results

Amino acid analysis of B. thuringiensis crystals

The results are set out in Tables 1 and 2. Table 1 includes data published by other workers, and the figures of Holmes & Monro (1965) and Monro (1959) shown in column (a) have been recalculated from the original, where they were expressed unconventionally in terms of the protein recovered from the analysis instead of in terms of protein analysed.

In calculating the amount of each amino acid present in the crystals from the data obtained by analysis of individual hydrolysates (Table 1) certain guiding principles were followed but were not adhered to rigidly. It is well known that different amino acids are released and/or destroyed at different rates during acid hydrolysis of a protein, but the rates depend to such an extent on the amino acid sequence that every protein must be considered individually. In this case a change in the content of an amino acid from one hydrolysis time to the next of more than 5% has generally been regarded as representing a real change in composition and a change of less than 5% as simply falling within the experimental error of the method. Such a system is not ideal because in the case of a very scarce amino acid a very small change in amount could represent more than 5%. Nevertheless, the amino acids present were divided into four groups on this basis, as follows:

1. Those which are totally released from the protein after 20 hr hydrolysis or less and have not begun to be decomposed after 142 hr, namely arginine, glutamic acid, glycine, histidine and ½-cystine (i.e. cysteic acid).

2. Those which are totally released after 20 hr or less and begin to be decomposed thereafter, namely alanine, aspartic acid (in which the first signs of decomposition were observed after 142 hr), lysine, serine (decomposition commenced immediately) and methionine (decomposition began immediately and methionine was absent from the 45 hr and subsequent hydrolysates). The preferred values given in the tables, have been obtained from plots of the amount found in each hydrolysate versus the time of hydrolysis. With serine and lysine, for example, the plots (shallow curves) were extrapolated back to zero time to obtain the preferred values (Bateson, 1966).

3. Those which reach a maximum after 45 hr or 72 hr and then begin to be destroyed, namely isoleucine, valine (which have a plateau at 45 hr and 72 hr), leucine (which reaches a maximum at 72 hr) and tyrosine (maximum at 45 hr).

4. Compounds which appear progressively from 20–142 hr. Ammonia is the only member of this group. The bulk of the ammonia observed is amide ammonia, which is completely released after 20 hr (as are aspartic and glutamic acids) but ammonia increases with time due to its formation from unstable amino acids during acid hydrolysis.

Total nitrogen

The total nitrogen content of the dry, ash-free crystals is 14·88%.

Alkali-insoluble fraction

This strongly hydrophilic fraction was found to account for 12% of the dry weight of the crystals. It contains no nitrogen, and qualitative analysis indicates that the fraction is largely, if not wholly, carbohydrate. The infra-red absorption spectrum resembles that of cellulose, though it is not identical and the fraction gives a positive reaction to the Molisch test. Paper chromatography of a hydrolysate shows that it comprises the hexoses glucose and mannose and the pentoses arabinose and xylose. Although quantitative chromatography was not performed it was possible to deduce from the qualitative chromatogram that glucose is far more abundant than any of the other sugars.

In view of the absence of nitrogen from the fraction it was not surprising to find also an absence of amino sugars upon examination of a separate chromatogram. This is an important observation in relation to the nitrogen recovery calculated for the amino acid analysis—see Discussion.

Discussion and conclusions

Tristram & Smith (1963) listed four criteria which must be satisfied by a satisfactory amino acid analysis, and which it is considered the analysis described here satisfies.

1. 'The ion-exchange apparatus must be carefully calibrated.' Before hydrolysates were analysed it was confirmed that synthetic mixtures of amino acids could be resolved and estimated satisfactorily. The preliminary calibration not only facilitated the subsequent evaluation of chromatogram peaks, but it also assisted in the resolution of a difficulty concerning proline. Table 1 shows that other workers have found considerable amounts of proline in the crystals but that it is completely absent according to the present analysis. During calibration proline was resolved as a small, but separate, peak between glutamic acid and glycine, but when hydrolysates were analysed no

TABLE 1. Amino acid analysis of *B. thuringiensis* crystals. The Table also includes the analyses of (a) Monro (1959) and Holmes & Monro (1965), (b) Lecadet (1965)—see text

Amino acid	g Amino acid residue per 100 g dry, ash-free protein.						
Hydrolysis	20 hr	45 hr	72 hr	142 hr	Derived amount in crystals	(a)	(b)
Alanine	4·18	4·09	4·11	3·71	4·13	3·02	3·80
Arginine	6·65	6·73	6·41	6·90	6·76	6·86	7·81
Aspartic acid	9·79	9·38	9·68	9·08	9·62	10·02	12·57
Cystine ($\frac{1}{2}$-cyS)*	ND	1·07	1·05	ND	1·06	0·15	1·35
Glutamic acid	12·49	12·54	12·58	12·38	12·54	10·54	12.0
Glycine	2·75	2·79	2·99	2·78	2·74	3·15	4·25
Histidine	2·72	2·26	2·25	2·17	2·25	1·82	2·65
iso-Leucine	4·00	4·77	4·73	4·40	4·75	5·00	4·4
Leucine	7·51	7·81	8·07	7·41	8·07	7·14	7·65
Lysine	4·09	3·26	3·15	3·00	3·46	3·09	3·4
Methionine	0·33	0	0	0	0·33	1.00	1·53
Phenylalanine	4·60	5·18	5·14	4·78	5·56	5·79	6·77
Proline	0	0	0	0	0	3·68	4·37
Serine	4·55	4·45	4·21	3·25	4·62	4·35	4·37
Threonine	4·21	4·39	3·96	3·80	4·30	4·34	5·76
Tryptophan	ND	ND	ND	ND	2·02	2·24	2·05
Tyrosine	5·01	5·72	5·48	4·37	5·72	5·22	6·62
Valine	3·92	4·86	4·82	4·20	4·84	4·57	5·0
Ammonia	2·04	1·95	2·15	2·32	1·52	1·94	ND
Total					83·9	83·9	96·35

ND: not determined.

*cysteine + cystine determined as cysteic acid.

such peak was observed. The possibility that proline had for some reason failed to be resolved from glutamic acid was considered. When the extinction of the fractions eluted from the ion-exchange column was measured (after development of the fractions

with ninhydrin reagent) at 440 nm in addition to 570 nm a small peak (440 nm) was recorded coinciding with the glutamic acid peak (570 nm). It was shown conclusively, however, that the small peak was no larger than could be entirely accounted for by the presence of glutamic acid alone. By observing the extinction at 570 nm and 440 nm of solutions known to contain glutamic acid alone, or mixtures of glutamic acid and proline in varying proportions, it was shown that the addition of proline to a glutamic acid solution, at a level of 5% of the molar concentration of glutamic acid, could be satisfactorily detected. On this evidence, proline is not present in the crystals. The conformation of the polypeptide chains, therefore, is in no way affected by pyrrolidine rings, as with some proteins.

2. 'The weight of the hydrolysate applied to the column should be compared with the total weight of the amino acids recovered from the column. Also the nitrogen content of the hydrolysate should be determined analytically and compared with the calculated nitrogen content of the recovered amino acids.' The former comparison serves to check the purity of the sample hydrolysed (i.e. whether or not the sample is 100% polypeptide material), the latter is a check on the experimental precision of the technique.

The nitrogen recovered from the analysis may be calculated from Table 1 as 14·29 g per 100 g dry, ash-free crystals analysed (cf. total nitrogen of the crystals by analysis: 14·88%), which represents a nitrogen recovery of 96·1%. This figure falls within the error of ±5% inherent in the semi-micro technique (Eastoe, 1961) and confirms the precision of the analysis. It may also be seen from Table 1 that a weight recovery of 83·9% was obtained from the analysis. The possibility of losses of amino acids during analysis may be disregarded because of the satisfactory nitrogen recovery, so the low weight recovery must be due to the presence of non-nitrogenous material in the sample of crystals analysed. The method by which the crystals were analysed strongly suggests that the 12% of carbohydrate found is an integral part of the crystals and not an artifact introduced during the isolation. The only possible carbohydrate-containing artifact would be bacterial spores, which are known to account for less than 1% of the weight of the preparation. The alkali-insoluble carbohydrate fraction, unlike spores, is amorphous microscopically. However, spores are known to resist cold alkali even to the extent of retaining viability.

The total nitrogen content of the crystals is somewhat low for a protein, though not beyond the bounds of possibility. The authenticity of the nitrogen content was confirmed by calibration of the whole semi-micro Kjeldahl procedure. When expressed in terms of the polypeptide fraction of the crystals the total nitrogen content becomes 16·9%.

3. 'Acid hydrolysis should be carried out for periods of 20, 40, 70 and 140 hr and the results averaged or extrapolated to provide the best results for amino acids which are either labile or difficult to hydrolyse.' Except for slight variations in the precise times of hydrolysis this condition has been fulfilled.

4. 'Independent analyses should be carried out for tryptophan, cysteine/cystine and amide nitrogen.' This criterion is met with the exception of the amide nitrogen, which was estimated from ion-exchange chromatography data. Provided allowance is made for the conversion of amino acid nitrogen to ammonia, amide nitrogen may be estimated satsifactorily in this way (Eastoe & Courts, 1963). In view of the scarcity of crystals a separate analysis was not performed.

Other workers have published amino acid analyses of the crystals of crystalliferous bacteria but there is reason to question the accuracy and precision of these analyses. The earliest quantitative analysis, of the crystals of *B. sotto* by paper chromatography of an acid hydrolysate, produced a weight recovery of 91·6%, and a nitrogen recovery of 82·3% may be calculated (Angus, 1956). The latter figure indicates a lack of precision. Amide nitrogen was not determined but only a small part of the missing nitrogen could be in this form. Furthermore, in any precise analysis the nitrogen recovery must equal or exceed the weight recovery, though the omission of amide nitrogen may account for some of the reversal in this case.

Two more recent analyses of the crystals of *B. thuringiensis* by ion-exchange chromatography of acid hydrolysates have been made. Apart from the question of precision, single hydrolysates only were analysed in each case, with hydrolysis times of 24 hr. (Monro, 1959; Holmes & Monro, 1965) and 18 hr (Lecadet, 1965). In the former papers the results were expressed as g amino acid residue per 100 g protein recovered from the analysis instead of in the more conventional manner of g amino acid residue per 100 g protein analysed. A recalculation of the published data (see Table 1) reveals a weight recovery of 83·9% and a nitrogen recovery of 89·9%, which illustrates a lack of precision concealed by the original expression of the data. This is not surprising, since Monro (1959) stated that 'discrepancies between the analyses of two samples from the same preparation are just as great as the discrepancies between samples from different preparations. It is, therefore, clear that the errors which arise during analysis were greater than those arising through variation in preparations used for analysis.'

In the latter paper (Lecadet, 1965) a weight recovery of 96·4% and a nitrogen recovery of 93·7 – 97·3% are calculable (total nitrogen was recorded as 15·6 – 16·2%). Amide nitrogen was not recorded and it is impossible to speculate satisfactorily on the probable precision of the analysis.

The presence in the crystals of a substantial amount of carbohydrate has not previously been reported. Indeed, Lecadet (1965) claimed the complete absence of sugars from crystals tested by the orcinol method (Francois, Marschall & Neuberger, 1962) and Holmes & Monro (1965) found only 0·5% carbohydrate, which may possibly have been due to the presence of spores. There are two reasons why the presence of carbohydrate may have remained unnoticed hitherto. Firstly, the careful comparison between weight and nitrogen recoveries, which provided the first clue to the existence of the carbohydrate, has not been made before. Secondly, solutions of the crystals have

almost always been prepared from a suspension of crystals and spores so that spores could be isolated simultaneously. In this way the insoluble carbohydrate fraction will always have been hidden by spores, making its recovery impossible. The present analysis suggests that the crystals may be composed of glycoprotein. On the evidence at present available it is not possible to be absolutely certain that the carbohydrate is covalently bonded to the protein and the possibility that the crystals are composed of two separate components, carbohydrate and protein, cannot be ignored. Nevertheless the conditions needed to secure a separation – prolonged treatment with cold alkali – are thought to involve the breaking of covalent bonds. Similar procedures are used to effect the separation of carbohydrate and protein in several well studied mucoproteins of animal origin and it seems likely that the crystals may also be composed of carbohydrate–polypeptide complexes.

The most outstanding property of the crystals, which has never been satisfactorily explained, is their complete insolubility below a pH of about 12·2 (Hannay and Fitz-James, 1955). Amino acids which frequently confer insolubility on proteins are cysteine and cystine, which were collectively determined here as ½-cystine. The keratins, in which the frequency of occurrence of ½-cystine residues usually ranges from 70–171 residues/1000 residues, owe their insolubility to these amino acids (Ward & Lundgren, 1954). A frequency as low as 24 residues/1000 residues may occur in certain insoluble keratins, but in the crystals the frequency is much lower, namely 12·7 residues/1000 residues. However, the degree of solubility shown by a protein is not necessarily directly related to its ½-cystine content. For instance, insulin has 66 ½-cystine residues/1000 residues and is completely soluble. The different solubilities of keratins and insulin may be explained if the polypeptide chains of keratin are extensively cross-linked by disulphide bridges whereas those of insulin are not. The frequency of occurrence of ½-cystine residues can be less important than their spacing in the polypeptide chain (Ward & Lundgren, 1954). The evidence presented in relation to the keratins, which are fibrous proteins, may not apply to a consideration of the crystal protein which, incidentally, has a mean residue weight (113·6) resembling that of globular proteins in general (115). The frequency of occurence of ½-cystine in the crystals nevertheless appears by comparison with other proteins to be too low to account completely for the insolubility. The use of thioglycollic acid lowers the pH required to dissolve the crystals of *B. alesti* by about 0·5 pH unit (Young & Fitz-James, 1959), which suggests that disulphide bridges make a small contribution to the insolubility.

Hydrogen bonding between polypeptide chains is a factor which can be involved in protein insolubility, e.g., it contributes to the insolubility of collagen, but such interaction does not necessarily lead to insolubility. The possible extent of hydrogen bonding in any particular protein may be assessed from the content of polar amino-acids, namely non-amidized aspartic and glutamic acids (with a combined frequency of 105 residues/1000 residues) and arginine, histidine and lysine, is shown in Table 2. None of the polar amino acids occurs noticeably more frequently than the rest of the

TABLE 2. Amino acid analysis of *B. thuringiensis* crystals

	Mole amino acid residues per 100,000 g dry, ash-free protein	Residues per 1000 amino acid residues
Alanine	58·1	70·9
Arginine	43·3	52·8
Aspartic acid	83·6	101·9
Cystine ($\frac{1}{2}$-cyS)*	10·4	12·7
Glutamic acid	97·1	118·4
Glycine	48·0	58·5
Histidine	16·3	19·9
iso-Leucine	42·0	51·2
Leucine	71·3	87·0
Lysine	27·0	32·9
Methionine	2·5	3·0
Phenylalanine	35·1	42·8
Serine	53·1	64·8
Threonine	42·5	51·8
Tryptophan	10·9	13·3
Tryosine	35·1	42·8
Valine	48·8	59·5
Ammonia	94·9	115·7

*cysteine + cystine determined as cysteic acid.

amino acids but there is in any case little difference to be detected between the frequency of polar amino acids in soluble and insoluble proteins. Further support is given to the lack of importance of hydrogen bonding in the insolubility of the crystals by studies of *B. alesti* crystals. Treatment with a concentrated solution of urea did not render the crystals more soluble (Young & Fitz-James, 1959).

The iso-electric point of a protein is also related to its content of polar amino acids. The iso-electric point of the crystals has been reported as around pH 5 (Hannay & Fitz-James, 1955) and Lecadet (1965) has ascribed the somewhat low iso-electric point to the high content of non-amidized aspartic and glutamic acids, which determine the balance between acidic and basic amino acids, rather than the total acid content. If pK values for amino acids combined in proteins are assumed (Fox & Foster, 1957) an iso-electric point around pH 5·5 may be calculated from the data in Table 2. Individual pK values may vary considerably but the calculated iso-electric point will not vary by more than ± 1 pH unit even if extreme pK values are assumed.

In some cases the insolubility of proteins is unconnected with the polypeptide parts of the proteins but depends on some other material conjugated with the polypeptide.

For instance, collagen is believed to owe its insolubility in part to some 2–3% of carbohydrate associated with it. In the absence of any conclusive evidence from the amino acid composition it is suggested that the carbohydrates may be contributing to the insolubility of the crystals from *B. thuringiensis*.

Although carbohydrate may be important for the insolubility of the crystals it does not appear to be essential for their toxicity. Fitz-James, Toumanoff & Young (1958) used an alkaline extract of crystals and spores, after dialysis to remove the alkali, and found it toxic to susceptible insects. This extract, on the basis of our observations, would be free from carbohydrate and contain only polypeptides. Some scission of the polypeptide chains will inevitably have occurred during the alkaline treatment, yet the toxic activity had been retained. This suggests that, by analogy with enzymes, the conformation of only a small region of the polypeptide chain has to be maintained intact. The detailed nature of this region is still unknown.

The role of the carbohydrate, both in the structure of the intact crystals and in the assimilation of the toxic factor in the gut of insects, also remains to be elucidated.

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J. Fd Technol. (1970) 5, 417–421.

Evaluation of electrolytic tinplates for juice containers

M. LANDAU AND C. H. MANNHEIM

Summary

The corrosion resistance of three ordinary and two K-type makes of tinplate was investigated, with grapefruit juice as test medium. Four criteria, namely the Alloy Tin Couple, Iron Solution, Pickle Lag Values, and dissolved tin after storage at 25° and 35°C, were used. K Plate proved significantly superior to ordinary plate with identical nominal coating weights but from different sources. The ATC test was found to compare best with dissolved-tin values in a grapefruit juice test pack. It was also shown that this test is suitable for corrosivity evaluation of different media.

Electron-microscopic examination showed a more continuous tin-iron alloy layer, with smaller crystals, in the K Plate.

Introduction

Evaluation of the suitability of conventional and new types of tinplate for existing and new products, as well as revision of container specifications, are regularly called for.

The main function of the container is to protect the contents over a maximum period under prevailing storage conditions, without impairing their quality or being affected by them. The most reliable, though time-consuming, evaluation technique is the test-pack method. In the case of cans, this refers to the time required for first failure, 50% failure or vacuum loss or to the time required to reach a predetermined dissolved tin level (such as 250 ppm).

Several rapid methods have been proposed to evaluate the corrosion resistance of tin plates. These include the Pickle-Lag (PL) test (Anon, 1960a), the Iron Solution Value (ISV) (Anon, 1960b) with various modifications, the Alloy Tin Couple Current (ATC) (Kamm & Willey, 1961a; b, Carter & Butler, 1961), and continuous in-can potential measurements as indicator of dissolved tin (Reznik & Mannheim, 1956). Electron-microscopic examinations have also been used in evaluating new tinplates (Kamm & Krickl, 1965). All these techniques have brought about new and more corrosion-resistant materials such as K plate (Kamm & Krickl, 1965), whose characteristics are PL less than 10 sec, ISV less than 20 μg iron, and average ATC less than 0·05 $\mu A/cm^2$

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The object of this study was to compare different methods (or their modifications) and a grapefruit juice test-pack, with the rate of tin dissolution as shelf-life indicator. Grapefruit juice was used because previous investigators chose it as a good representative for a group of products. Another object was evaluation of various makes of electrolytic tinplate, with the same nominal coating, but from different sources with two coatings weights of the same K Plate.

Materials and methods

The *PL test* consists of determining the time required for hydrogen to evolve from a tin-free plate sample immersed in 6 N HCl at 90°C.

In these experiments, the samples (8 × 65 mm) were cleaned and the tin coating stripped as outlined in the *Canco Technical Service Bulletin* (Anon, 1960a). Determination of the time was based on measuring the change in pressure in a 125 ml closed vessel by means of a pressure transducer connected to a Honeywell Electronic 19 recorder. This modification was found to be more sensitive than that used previously (Anon, 1960a).

The modified *ISV test* is based on determining the dissolved iron under standard conditions. While previously the whole sample was used, a new technique has now been introduced, involving a small exposed segment of the tin–iron alloy.

The experimental procedure was again as described in the *Canco Technical Service Bulletin* (Anon., 1960b), except that the tin coating was stripped chemically after cleaning, as for the ATC test (see below).

The *ATC test* consists of measuring the current flowing between a plate sample (with the tin coating stripped down to the alloy layer) and a pure tin electrode of considerably larger area. The test cell used, a 25 litre oblong glass container with a perspex lid, permitted simultaneous testing of sixteen samples.

A new sample holder was designed (Fig. 1) in which the sample (2·42 cm^2) was fixed by means of an O ring. All rubber fittings were boiled in 10% NaOH for 30 min prior to use. Pure tin-foil electrodes (Fisher Co.), cleaned cathodically in 5% Na_2CO_3, were used.

Reconstituted commercial grapefruit juice (10°Brix) served as test medium. The juice was deaerated in a Herbot Vacuum deaerator homogenizer and the vacuum broken with nitrogen and then 190 mg/l $SnCl_2 \cdot 2H_2O$ were added to the juice, as well as 0·5 g/l potassium sorbate as preservative. All measurements were made at 25°C (± 1°).

Samples were cleaned with acetone and then cathodically in 0·5% Na_2CO_3, using a 10 volt potential, for about 2 sec. The tin coating was stripped in a solution of 2% KIO_3 in 2% NaOH (Butler & Carter, 1963).

Measurements were carried out as described by Carter & Butler (1961), the current being determined indirectly through the potential between sample and electrode, using an external resistance.

Tin coating weight was measured electrolytically by the method described by Kunze & Willey, (1954) using a tin stripper built in this laboratory (Mannheim & Hamburger, 1970). Dissolved tin was determined by digesting sample followed by iodometric titration (National Canners Association, 1968). Electron Microscopic photographs were made according to the method described by Ebben & Lawson (1963).

Ordinary A2 cans were taken at random from three different local can makers. All cans had a nominal 1·00 lb/base, electrolytic tin coating. K plate was received from J. J. Carnaud & Forges de Basse-Indre. Two types of plate were received, 1·00 and 0·75 lb/base box.

Experimental

Five different types of A2 cans (Table 1) were tested for all criteria described above, filled with grapefruit juice at 90°C, sealed, cooled and stored at 25° and 35°C. Contents were checked periodically, in triplicate, for dissolved tin content.

In order to evaluate the suitability of the ATC test for measuring the corrosivity of different media, parallel tests were carried out on juice with admixtures of 10 ppm SO_2 and 15 ppm, Cu^{++}. Results given are averages of thirty six determinations.

Results and discussions

Results summarized in Table 1 show clearly that K plate has a higher corrosion resistance than ordinary electrolytic tin plate. The dissolved tin content in the 1·00 lb/bb K cans was 111 ppm after 10 months storage at 35°C, compared with 142–166 ppm in their ordinary 1·00 lb/bb counterparts. Even the juice in the 0·75 lb/bb K cans showed a lower tin content compared with the same juice in 1·00 lb/bb ordinary cans. Similar results were obtained at 25°C.

TABLE 1. Results of tests on five makes of tinplate.

No.	Type	Source of supply	Nominal coating (lb/bb)	Actual tin coating (lb/bb)	Alloy layer (lb/bb)	ATC (μAm/cm²)	ISV (μg)	Pickle lag (sec)	Dissolved tin after 10 months at 25°C (ppm)	Dissolved tin after 10 months at 35°C (ppm)
1	Ordinary	A	1·00	0·994	0·072	0·177	29	9·25	128	166
2	Ordinary	B	1·00	0·974	0·081	0·118	32	14·5	106	160
3	Ordinary	C	1·00	0·980	0·083	0·092	25	11·5	99	142
4	K	D	0·75	0·758	0·114	0·046	12	3·5	92	125
5	K	D	1·00	1·096	0·098	0·054	7·5	5·4	86	111

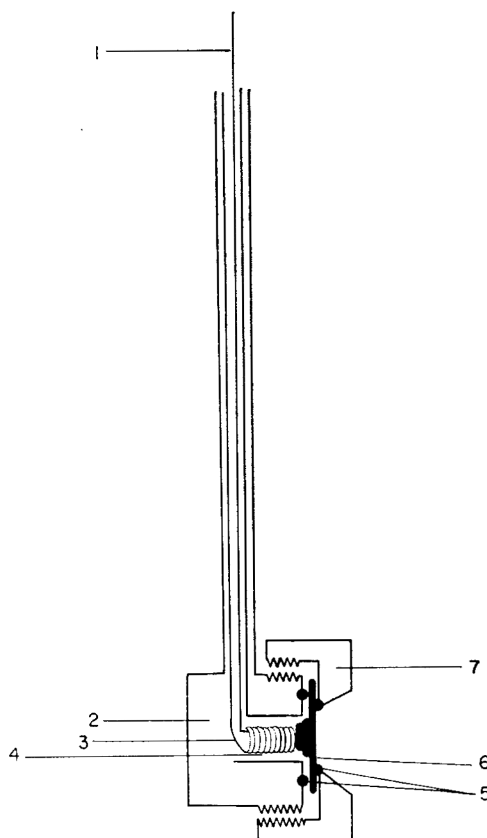


FIG. 1. Sample holder. 1, connecting wire; 2, Perspex body; 3, copper rod; 4, spring; 5, O rings; 6, sample; 7, Perspex cover.

Significant differences were also found in the PL, ISV and ATC tests. ATC values for K Plate were about the same as those stipulated for it by U.S. specifications (0·05 μA/cm²), while those for the ordinary plate ranged from two to five times that limit. Since the ATC and dissolved-tin values showed the same trend, the former is judged suitable for grading tin plate with respect to corrosion resistance. By contrast, as no correlation could be found between the ISV and PL values and the tin results, they cannot be regarded as suitable for this purpose.

In these experiments, the 0·75 lb/bb K cans were superior to the 1·00 lb/bb regular cans by all criteria; this is attributable to the thicker alloy layer in the former—0·115 as against 0·072—0·083 lb/bb.

Electron-microscopic pictures (Plate 1a) show a much more continuous alloy layer in K plate. Plate 1b shows the difference in crystal size, those of K plate being smaller and more regular.

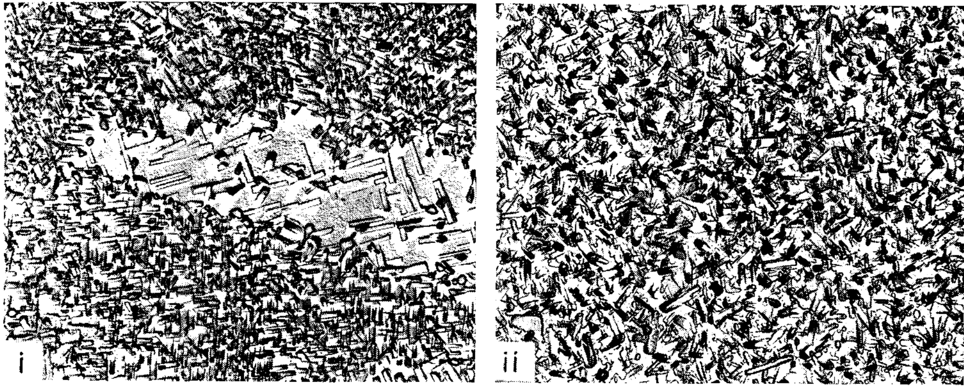
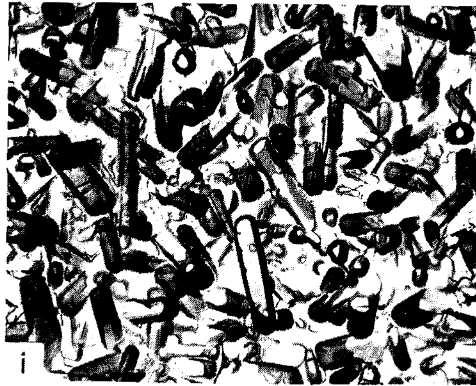
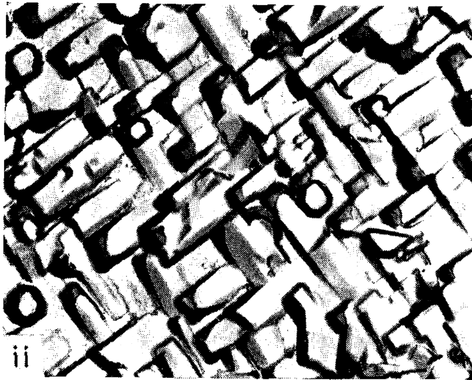


PLATE 1a.





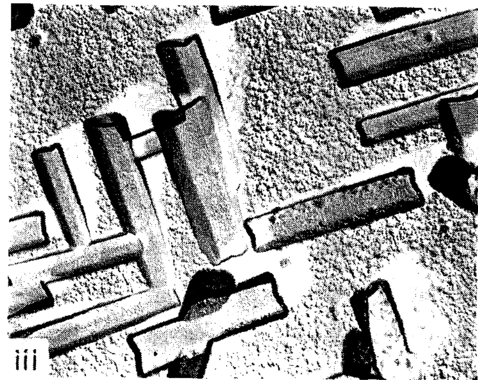


PLATE 1b.

PLATE 1a. Electron microscope photographs of typical sections of the tin-iron layer of 1·00 lb/bb tinplates × 6000, (i) Regular plate, (ii) K plates.
PLATE 1b. Electron microscope photographs of the tin-iron layer of 1·00 lb/bb tinplates × 25,000, (i) K plate; (ii) regular plate, continuous section; (iii) regular plate, discontinuous section.

(*Facing p.* 420)

ATC data for the admixed juices are summarized in Table 2. Results, based on thirty six determinations for each type, indicate that despite a high scatter, differentiation between K and ordinary plate is possible; they also confirm the suitability of the ATC test for comparative corrosivity evaluation. Further work along these lines is strongly recommended.

TABLE 2. ATC values for five makes of tinplate in pure and admixed grapefruit juice

No.	Type	Nominal coating (lb/bb)	ATC	(μAm/cm^2)	
			None*	10 ppm SO_2*	15 ppm Cu^{++}*
1	Ordinary	1·00	0·177	0·400–0·500	0·194–0·250
2	Ordinary	1·00	0·118	0·388–0·412	0·131–0·188
3	Ordinary	1·00	0·092	0·362–0·412	0·112–0·160
4	K	0·75	0·046	0·256–0·323	0·062–0·125
5	K	1·00	0·054	0·250–0·323	0·069–0·112

*Admixture.

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J. Fd Technol. (1970) 5, 423–426

Book Reviews

Analytical Methods Used in Sugar Refining. Ed. by R. W. PLEWS.
London: Elsevier Publishing Company Ltd, 1970. Pp. 234 + viii. £5 10s.

The analytical procedures of the sugar industry often consist of highly specialized techniques developed and bequeathed orally by generations of refinery chemists or locked away in confidential copies of standard factory control handbooks. Most of the classical textbooks of sugar analysis are now out of print.

The publication of the methods of sugar analysis used by Tate & Lyle Refineries Limited is therefore all the more welcome and Mr Plews and his associates are to be congratulated on presenting these methods in such a readable form. Several of the methods are those recommended by I.C.U.M.S.A. but this publication does not merely repeat the stark details of the approved I.C.U.M.S.A. procedure; the principle and mechanism of each method are described in an introduction and the text notes possible snags and includes analytical hints which can only have been acquired by painstaking familiarity with the techniques.

The book does not provide a general description of analytical methods used in sugar refining; it describes those methods found most suitable for the particular circumstances at four refineries of the Tate & Lyle Group and consequently, for some parameters, only a single analytical method is described even though there may be available other methods which could be more suitable under different operating conditions. For some parameters, particularly determination of water, a variety of methods are described and discussed.

Apart from description of methods in general use in the refineries, there is a comprehensive coverage of methods for determination of inorganic non-sugars and trace elements, and of microbiological examination of sugars. The varied analytical tasks undertaken in the chemical control of sugar factories are further illustrated by chapters on analysis of charcoal, of water and of fuel with a concluding chapter on determination of brewer's extract.

The whole book is well produced and illustrated with comprehensive subject and reagent indexes. It should become a standard item in the library of all sugar analysts and it is to be hoped that this venture will encourage other sugar manufacturers to publish their standard analytical handbook.

J. F. T. OLDFIELD

Margarine—An Economic Social and Scientific History 1869–1969. Ed. by J. H. VAN STUYVENBERG.
Liverpool: Liverpool University Press, 1970. Pp. xxiii + 342. 84s.

This book was written to commemorate the centenary of the invention of margarine. To produce such a volume requires a dedicated editor and a team of enthusiasts, each

an expert in his own field of endeavour. Since the book is essentially a review of an industry and its many ramifications, the topics to be dealt with must be carefully and deliberately chosen. When these criteria are fulfilled it is not surprising that a book of considerable merit and importance emerges.

After a short biographical note on Mège Mouriés, the founder of the industry, one is led into a balanced review of the social and economic aspects of the cause of the emergence of the industry and of the economic structure and changes which occurred, and which are still occurring perhaps at an accelerated rate, in relation to its subsequent development. This chapter leads on naturally to one concerned with the raw materials used in margarine manufacture. Here is described not only the production and procurement of the fats and oils used, but also the structure and organization of production and marketing of these raw materials. Further the role of government in influencing, either directly or indirectly, the raw materials situation is noted. For this type of book it is perhaps rather surprising to note that the technology and production of margarine, the raison d'etre of the present volume, is dealt with in only 36 pages.

The most interesting and important sections of the book, at least as far as the reviewer is concerned, are those dealing with nutritional and dietary aspects, and research. The components of margarine are elegantly dealt with in the former, within the framework of a general survey of the scope of the nutritional sciences as we know them today. The section on research is dealt with in a similar way, the major developments in the broad field of fat research being described and their relevance to margarine technology and science discussed. Thus among the topics reviewed are the selectivity of catalysts, the most recent development in flavour research, and the role of polyunsaturated acids. With regard to the latter, it is interesting to note that conclusions arrived at in this chapter are not entirely in agreement with those recorded in the preceding chapter.

Marketing is adequately dealt with in the penultimate chapter, while in the final chapter aspects of government intervention are discussed. By and large such interventions have sought to protect the dairy industry of the respective countries.

It is difficult to say for whom this book was primarily written or for whom it will have the greatest appeal. It is obviously not written for the specialist social historian, the economist, the nutritionist, nor the technologist and/or research worker in the field of oils and fats. All can however learn much from this book, as indeed will the enquiring layman. The attention of the specialist is directed to suggestions for further reading at the end of each chapter. It is eminently readable and is remarkably free from typographical errors, the most obvious being the formula for benzoic acid, p. 175. It deserves a much wider publicity than it would get in a purely scientific or technological library.

M. L. MEARA

Evaluation of Novel Protein Products. Proceedings of the International Biological Programme (IBP) and Wenner-Gren Central Symposium, Stockholm, 1968. Ed. by A. E. BENDER, R. KIHLBERG, B. LÖFQVIST & L. MUNCK.
Oxford: Pergamon Press Ltd, 1970. Pp. 390 + viii. £7.

Reading a book such as this, draws one's attention quite forcibly to the rapid progress of knowledge in this important field of 'Novel Protein Products'. It contains an account of the proceedings of an International Symposium held in Stockholm in September 1968, at which a wide range of topics were covered by world experts. At the time the information presented was the latest thinking, but the lapse of two years has removed much of the impact. The problems the organizers have with the lecturers, and more so with the printers, are appreciated but with more speed the value of the publication would be greater.

Even so this book still provides a reasonable survey of the field, covering in the seven sessions topics as diverse as economics and radio-immuno assays in some forty papers, and should be essential reading for those who will be concerned with this work.

The first session on the world food problems suffers from too much generalization and although the individual titles (such as Food Habits and Taboos) are potentially exciting one is left with vague statements.

The session on novel protein sources was more satisfactory and provides a useful summary of information on a wide variety of sources. It contains, for instance, a most useful evaluation of the potential of animal husbandry proteins, an area which tends to be overlooked by many because of arguments on conversion ratios being against it. However the authors draw attention to the fact that animal proteins can be produced on marginal land which is useless for any other form of food production. Nutritional evaluation covers two sections and occupies over one-third of the book, paying particular attention to the availability as distinct from the total content of individual amino acids. Techniques, varying from animal experiments through microbiology to enzymic methods are considered. In one paper, however, the microbiological method is advocated for total amino acids—an interesting full turn of the circle of analytical techniques. The main virtue is claimed to be the lack of complicated and expensive equipment. The technique now appears to be capable of results which are comparable with those of ion exchange techniques—at least for some amino acids—but the tedious work involved makes one wonder if it will find general acceptance.

The toxicity section never really gets to grips with the problems. Oser's paper is a general survey of toxicological techniques, while Nilsson discusses Botulism, Staph. enterotoxin, etc. and only provides general information. Strangely a paper on the functional evaluation of proteins in food systems is included in this section, but here again the generality detracts from the value.

The sixth session devoted to processing and storage is more valuable in drawing attention to the variation in protein value which can be brought about by the normal

treatments to which the raw material is subjected. This may not be serious for most people but for the people who have a marginal diet it can be critical.

The final session is an erudite discussion of the role scientists are playing in solving the world food problem.

A. W. Holmes

Books Received

Home grown Cereals Authority Progress Report on Research and Development 1969/70.

Meat Product Manufacture. By E. Karmas.
U.S.A.: Noyes Data Corporation, 1970. Pp. viii + 273. $35.

Fruit Juice Technology. By M. Gutterson.
U.S.A.: Noyes Data Corporation, 1970. Pp. vii + 206. $35.

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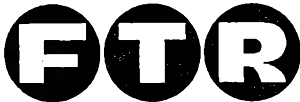

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gram(s)	g	second(s)	sec
kilogram(s)	kg	cubic millimetre(s)	mm^3
milligram(s) (10^{-3} g)	mg	millimetre(s)	mm
		centimetre(s)	cm
microgram(s) (10^{-6} g)	μg	litre(s)	l
		millilitre(s)	ml
nanogram(s) (10^{-9} g)	ng	pound(s)	lb
		gallon(s)	gal
hour(s)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

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