Chemical changes associated with microbial growth on meat stored at chill temperatures

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INTRODUCTION

The presence and/or growth of microbes on meat are major factors determining its safety and shelf-life. While growth of organisms of primary concern from a public health point of view can be effectively controlled by strict adherence to recommended chill storage temperatures, shelf-life/acceptability cannot be prolonged unduly in this way. Chill storage simply selects for organisms able to grow at these low, but above freezing, temperatures, the majority in the case of meat being bacteria. These so-called psychrotrophic bacteria are able to grow at or near to 0°C but grow optimally at normal ambient temperatures. Enormous effort has been expended over the years on enumerating, classifying and developing identification schemes for these organisms, with significant advances being made in the last 5 years in the case of the pseudomonads, which typically dominate the flora of meat stored in air (Shaw & Latty, 1982; 1984; Molin & Ternström, 1982), and of the lactic acid bacteria which dominate in vacuum packs (Shaw & Harding, 1984; 1985). On the other hand, comparatively little attention has been paid to the metabolic activities of these organisms, despite the fact that they are major causes of the loss of some desirable properties of meat e.g. its colour, and of the appearance of undesirable ones such as off-odour and off-flavour. Together these processes constitute spoilage. The relative importance of the changes may vary from product to product, with conditions of storage and intended use etc. It is the purpose of this contribution to describe the chemical changes associated with bacterial growth on meat stored at chill temperatures, and in particular those involved in off-odour development. The possible value of the changes as indices of spoilage/shelf life will also be discussed.

MEAT COMPOSITION

Meat is a complex substrate containing an array of small, molecular weight compounds in addition to protein and lipid, the approximate composition of a typical post-rigor muscle being as follows:

	% (w/v)		% (w/v)		% (w/v)
Protein	20	Nicotinamide nucleotides	0.3	Anserine/carnosine	0.3
Lipid	3	Glycogen	up to 0.1	Water	75
Lactic acid	up to 0.9	Monosaccharides	up to 0.3	ATP degradation	0.3
Amino acids	0.4	(mainly glucose; includes		products (mainly	
Creatine	0.5	sugar phosphates)		IMP, inosine)	

Although small in comparison with those of protein and lipid, the concentrations of the small molecular weight compounds are all sufficient to support massive microbial numbers but the concentrations of lactic acid, glucose and glycogen can all vary enormously. Prolonged pre-slaughter stress, e.g. cold, fright, can deplete the animal's muscle glycogen reserves, the source of glucose and lactic acid, leading in severe cases to the so-called 'dark-cutting' (dark, firm, dry; DFD) condition. As we shall see later this condition, characterized chemically by low glucose content and pH values >6.0, and perhaps approaching 7.0, has important consequences for spoilage development.

STORAGE IN AIR

Compounds supporting bacterial growth

From the results of experiments in which sterile pieces and deproteinized buffer extracts of lamb meat were inoculated with pure cultures of fluorescent and non-fluorescent *Pseudomonas* spp., *Brochothrix thermosphacta* and an *Enterobacter* sp., Gill and co-workers (Gill, 1976; Gill & Newton, 1977) were able to conclude that glucose is used exclusively to support the initial phase of growth of those types of bacteria which typically dominate the spoilage flora of meat stored in air (see Dainty *et al.*, 1983). This phenomenon which has also been shown to apply in the case of growth on sheep liver surfaces (Gill & DeLacy, 1982), is clearly a result of the well estalished phenomena of catabolite repression and/or inhibition. The only exception found by Gill's group was an *Acinetobacter* sp. which metabolized amino and lactic acids in the presence of glucose during growth in lamb extract medium. Interestingly, despite these findings, surface spoilage of the sheep livers referred to above, which was associated with the growth of high numbers of *Acinetobacter* sp. as well as pseudomonads, manifested itself as visible growth rather than the off-odour development typical of amino acid metabolism (see below).

Only upon glucose depletion becoming apparent in the surface layers of the meat samples, which occurred as numbers reached *ca.* $10^8/cm^2$, did the metabolism of other compounds become detectable. In the case of the pseudomonads this included simultaneous utilization of several amino acids and lactic acid; for the *Enterobacter* sp., glucose-6-phosphate and then amino and lactic acids; and for *B. thermosphacta*, glutamic acid. This latter finding is most unusual because all other data in the literature shows *B. thermosphacta* to be strictly saccharolytic (e.g. Grau, 1979) in terms of energy production from single carbon sources. Evidence that isoleucine, leucine, valine and alanine are metabolized during its growth on meat is available (Dainty & Hibbard, 1983) and small amounts of the resulting end-products (see below) can be detected prior to glucose depletion (Dainty & Hofman, 1983). What function this metabolism serves is unknown, but some minor role in energy production cannot be ruled out.

A more recent study suggests that the metabolism of two other acids, namely gluconic and 2-oxogluconic acids, would also have been occurring during the second metabolic phase of the pseudomonads. Farber and Idziak (1982) showed that these two compounds accumulated in beef muscle inoculated with *P. fluorescens*, *P. putida* or a non-fluorescent pseudomonad as a result of partial glucose oxidation. In accord with classical findings

with laboratory strains (Lynch et al., 1975), the concentrations of these compounds in the meat subsequently fell, presumably as a result of transport into the bacterial cells followed by oxidation to carbon dioxide.

That metabolism of some of the other small molecular weight components of muscle tissue might be occurring upon glucose depletion appears likely, particularly in the case of the pseudomonads, which are known to metabolize creatine and some non-phosphorylated breakdown products of ATP in laboratory media (Shaw & Latty, 1982). Spectrophotometric evidence for nucleotide breakdown in beef inoculated with pseudomonads has been presented by Jay & Kontou (1967).

With regard to protein breakdown, the overwhelming majority of evidence indicates that this does not become a significant process until maximum population densities have been attained and spoilage is at an advanced stage as judged by sensory criteria (for detailed review see Dainty et al., 1983). Evidence has been sought using a variety of techniques including: electron microscopy of myofibril ultrastructure; staining intensity of individual myofibrillar and sarcoplasmic proteins separated by gel electrophoresis; measurement of specific microbial proteinase activity in meat tissues during storage; immunology of fresh and stored meat proteins; determination of amino acid concentrations; light microscopy to follow penetration of bacteria from surface to deeper layers. When the process does become evident, degradation of both sarcoplasmic and myofibrillar proteins can be expected and sometimes that of connective tissue proteins as well.

It is also doubtful whether bacterial lipolysis is a significant process prior to overt spoilage. In fact, growth of a non-lipolytic strain of P. fluorescens on lipid surfaces of lamb has been shown to be supported by the small concentrations of the same major growth supporting substrates as in lean i.e. glucose, lactic acid and amino acids (Gill & Newton, 1980). Of course many meat isolates are lipolytic, including P. fragi (Bala et al., 1977), and there can be little doubt from the identity of some of the volatile compounds produced during storage (see below), that lipolysis does occur. Lipolysis and the associated oxidative changes leading to the development of rancidity, which can also be catalysed by bacteria (Alford et al., 1971), may be of most concern in the case of minced meats (Branen, 1978). Attributing any of the changes unequivocally to bacterial activity in any meat sample is not easy in view of autoxidation and perhaps enzymatic processes. Furthermore attempts to demonstrate lipid metabolism could fail because of the ability of certain microbes to metabolize further some of the products of the process (Alford et al., 1971) and the possibility of interaction of the breakdown products of lipids and amino acids (Branen, 1978). More research is needed to clarify the situation which may become even more of a problem (Newton et al., 1977) as increased use is made of modified atmospheres enriched in oxygen to maintain colour during storage and prolong shelf-life.

In the vast majority of studies described above pure culture inoculation techniques were used. Although there is no reason to suppose that the findings of an initial, exclusively glucolytic phase followed by utilization of mixtures of low molecular weight substrates and ultimately of protein and perhaps lipid, cannot be extrapolated to the mixed flora contamination typical of naturally contaminated meats, a certain amount of caution is necessary in doing so. It is customary to ensure even contamination of the surface(s) of inoculated samples, a condition which rarely, if ever, applies in natural contamination. Glucolytic and mixed substrate phases of metabolism probably, therefore, coexist in close proximity. Sampling then becomes all important, particularly when use of chemical change as an index of acceptability/spoilage is proposed. There may also be conditions to which this order of substrate utilization does not apply even given uniform contamination. The most obvious one is the DFD condition in which glucose is severely limited, if present at all. Essentially there is only the mixed second phase of metabolism and, because metabolism of amino acids is the most commonly accepted mechanism of off-odour formation, earlier onset of spoilage can be expected. Gill's group showed that beef and lamb muscles of high pH exhibited off-odours as numbers of an inoculated pseudomonad reached $10^6/cm^2$ rather than at the $10^8/cm^2$ level associated with normal pH beef (Newton & Gill, 1978a). By manipulating the glucose content and pH value of high pH beef they also showed that the earlier belief 'that high pH increased growth rate and thus reduced time to spoilage' could be discounted. An interesting point, worthy of further investigation, is the identity of the substrates supporting growth of B. thermosphacta on DFD meat in view of its undoubted preference, if not complete reliance upon, carbohydrate as a source of energy. It has certainly been found to grow to high numbers on such meat (Dainty & Hibbard, 1980; Egan & Grau, 1981) and ribose from nucleotide or glycerol were suggested as possible substrates by the first authors.

Newton & Rigg (1979) suggested that the initial glucolytic phase would not be exhibited under conditions of oxygen limitation imposed by certain kinds of packaging films, because, at the resulting submaximal growth rates, catabolite repression phenomena are removed. Finally, the whole idea of an initial, exclusively glucolytic phase has been questioned by Molin (1985) on the basis of pure culture growth of P. fragi in laboratory media under batch and continuous culture conditions. While the author claims glucose, lactate, citrate (added as buffer) and aspartate-glutamate were metabolized simultaneously, the significance of the findings is difficult to judge because of the author's own lack of consistency of interpretation and because, in the present author's opinion, some of the data do not justify the stated conclusion. More research is clearly needed, particularly of the mixed, natural flora situation, to clarify these doubts.

End-products of bacterial growth

A large number of compounds have been detected in aerobically stored meats supporting bacterial growth. Not all of them are consistently present in higher concentrations than in fresh samples of meat and some of those that are, may be of non microbial origin. Nevertheless bacterial sources for a significant proportion of them have now been found.

The transient accumulation of gluconic and 2-oxogluconic acids during the initial glucolytic phase of growth of certain pseudomonads on meat as described above (Farber & Idziak, 1982), has been shown by my own group to be a property common to all the presently recognized clusters of pseudomonads found on meat, and to the so-called Moraxella-like strains. These Gram negative, oxidase positive, non-motile saccharolytic bacteria are frequently recorded contaminants of meat stored in air. Our own unpublished findings also indicate that measurable quantities of gluconic acid remain at relatively advanced stages of spoilage despite the ability of the bacteria listed, and possibly other elements of the flora including B. thermosphacta and

Enterobacteriaceae strains, to use it as an energy source.

Neither of these sugar acids are produced by the other common contaminants of meat stored in air. B. thermosphacta has its own incomplete oxidative pathway of glucose metabolism, resulting in the accumulation of acetoin (3-hydroxy-2-butanone), diacetyl (2,3-butanone), acetic acid and possibly, if the glucose concentration is high enough, 2,3-butanediol (Dainty & Hibbard, 1980; Dainty & Hibbard, 1983; Dainty & Hofman, 1983; Stanley et al., 1981). End products of glucose metabolism by the Enterobacteriaceae have not been studied during growth in meat though they presumably include carbon dioxide, which is also produced by the pseudomonads and B. thermosphacta, and possibly short chain fatty acids.

During the subsequent mixed substrate phase of growth, a wide variety of end-products is produced by the pseudomonads, the large majority of which are volatile and amongst which are the compounds responsible for spoilage odours. That not all pseudomonads have an equal capacity in this regard, is clearly illustrated in a study (Dainty et al., 1984) of five Pseudomonas strains selected on the basis of sensory properties and the need to have a representative of each of the four clusters defined in the original study of Shaw & Latty (1982). The two cluster 2 strains each produced a number of ethyl, and to a lesser extent methyl, esters of short chain fatty acids which were clearly the source of the 'fruity' off-odours which developed. For one of the two strains this 'fruity' odour was all but masked by a powerful 'sulphury', 'garlic' component of the odours which was attributed to the production of isopropyl mercaptan and the corresponding thio-acetate and sulphides. The cluster 3 strain produced a very characteristic 'cabbage-like' odour, the source of which appeared to be methyl mercaptan and its corresponding thioacetate and sulphides. Growth of the cluster 1 and 4 strains resulted in similar 'creamy', 'cheesey', 'rancid' odours which, while quite characteristic and distinct from the stale odours of stored, sterile meat, were not as unpleasant as the odours produced by the cluster 2 and 3 strains. Identification of the compounds responsible for the 'creamy' etc. odours is not certain, but the elevated concentrations of certain ketones and alcohols suggest they may have played a role. In keeping with earlier findings (McMeekin et al., 1978), none of the pseudomonads consistently produced detectable amounts of hydrogen sulphide under these growth conditions although they each produced at least one sulphur-containing compound. However, production of the short chain esters was restricted to the cluster 2 strains which identify with type strains of P. fragi (Shaw & Latty, 1982). It is worth noting that this cluster was consistently found to dominate the flora of meat obtained from a variety of sources and stored at a range of temperatures (Shaw & Latty, 1984). With a few exceptions, further work in progress in my own laboratory, indicates that these sensory and chemical properties are indeed reproducible and characteristic of the clusters. Interestingly not all type strains of P. fragi produced esters during growth on meat. In other studies with pure cultures (Stutz, 1978; Gibbs et al., 1979) the same classes of compounds, but with some differences in detail, have been detected. Stutz's study also showed that the patterns of volatile compound production did not vary at oxygen tensions varying between 2 and 20% (v/v) or at temperatures between 5° and 20°C. Two nitriles, and the O-methyl ethers of the corresponding aldoximes, were reported to be produced by a Moraxella-like strain by Gibbs et al. (1979) and we now have strong evidence for production of one of the aldoxime derivatives by a cluster 1 pseudomonad.

Another common volatile product of pseudomonad growth on meat is ammonia (Gill, 1976), which is presumed to be the cause of the increase in pH frequently observed during the post-glucose phase of growth. However, it appears not to have been recorded as a major sensory component in the studies cited until a very advanced stage of spoilage was reached. Nor have the volatile amines, although trimethylamine was recorded as a product of fluorescent and non-fluorescent pseudomonads growing on beef (Stutz, 1978). Finally, another amine, but in this case a relatively involatile one, putrescine, has been detected within the surface layers of meat inoculated with representative strains of all the common pseudomonads from meat (Slemr, 1981; Dainty et al., in press). The concentrations are sufficiently high for the suggestion to have been made that it could serve as an indicator of shelf-life/spoilage (see final section), but there is no indication that it contributes to spoilage odours.

Compounds characteristic of the growth of B. thermosphacta on meat include, in addition to those derived from glucose (see above), others derived from the branched chain amino acids. Isobutyric, isovaleric and 2-methylbutyric acids are the most common (Dainty & Hibbard, 1980; 1983) but combinations of the corresponding alcohols and aldehydes can also be expected (Dainty & Hofman, 1983). The relative proportions of these compounds will be different in normal and DFD meat because of the effects of glucose content and pH on their formation (Dainty & Hibbard, 1980; Dainty & Hofman, 1983).

End-products of the other common contaminants of meat stored in air, the cold tolerant Enterobacteriaceae, have not been studied in detail although sensory descriptions (Patterson & Gibbs, 1977) suggest that they include sulphur-containing compounds and amines. Although probably not a cause of any off-odours, one amine produced in significant amounts by Serratia and Klebsiella strains is cadaverine (Slemr, 1981). Like putrescine, and possibly in combination with it, cadaverine has been suggested as an indicator of spoilage.

From the proceeding paragraphs it is clear that the mixture of end-products associated with naturally contaminated meats could be very complex. The only detailed study of the volatile compounds is that of Dainty et al. (1985) in which the major compounds detected were:

ethyl acetate	acetoin	1-pentanol
ethyl propionate	diacetyl	1-octen-3-ol
ethyl n-butanoate	2-methylpropanol	
ethyl isovalerate	3-methyl-1-butanol	
ethyl n-hexanoate	1-undecene	
methanethiol	1,4-undecadiene	
dimethylsulphide	dimethylbenzene	
dimethyldisulphide	toluene	

These findings are entirely consistent with the pure culture results presented above and with the fact that Pseudomonas strains and B. thermosphacta dominated the microbial flora. The presence of ethyl esters and methane thiol and derivatives, further indicated that cluster 2 and 3 pseudomonads were the most numerous

types of pseudomonad (cf Shaw & Latty, 1984). A definite sequence of appearance of the various classes of volatile compounds, which was reflected in the odour descriptions, was also evident, namely:

1. acetoin, diacetyl, 3-methyl-1-butanol, 2-methyl propanol
2. ethyl esters
3. sulphur compounds.

On the basis of our present knowledge of the role of specific organisms in volatile compound formation, B. thermosphacta appeared to have played a more significant role in spoilage development than is normally attributed to it. More data are clearly needed before assigning any general validity to these findings but they certainly bring into question the notion propounded by Gill (1976) that spoilage onset necessarily coincides with that of amino acid metabolism. In situations where B. thermosphacta may dominate the flora, such as storage in modified gas atmospheres comprising elevated oxygen tensions and sufficient carbon dioxide to inhibit the pseudomonads (e.g. Newton et al., 1977), this patently will not be the case. However, in situations where pseudomonads are clearly dominant and B. thermosphacta fails to reach significant numbers, as in some storage trials recently completed by my group, Gill's theory is borne out by the nature of the volatiles being identified e.g. esters of branched chain fatty acids and sulphur compounds. In these experiments a slightly modified trapping procedure for volatile compounds is being used together with better capillary columns for gas chromatographic analysis. As a result more complex mixtures of compounds are being detected and evidence obtained of interactions between volatile compounds produced by different elements of the microbial flora to form compounds not detected in pure culture studies.

Data on non-volatile compounds formed during growth of natural, mixed floras is restricted, in the main, to the group of mono-, di- and poly-amines sometimes known as the biogenic amines. Elevated concentrations of both putrescine and cadaverine, believed to be formed from arginine via ornithine and lysine respectively, have been reported in stored, intact cuts of pork (Lakritz et al., 1975; Edwards et al., 1983) beef and lamb (Edwards et al, 1983); in minced pork (Nakamura et al., 1979), beef (Edwards et al., 1983); Sayem-El-Daher & Simard, 1985) and mixtures of the two meats (Wortberg & Woller, 1982). In general, increases in putrescine concentration were greater than those of cadaverine in accord with findings from pure cultures that pseudomonads are major producers of putrescine, Enterobacteriaceae of cadaverine. Elevated storage temperatures e.g. 10° or 20°C rather than 4°C increased cadaverine formation (Nakamura et al., 1979; Sayem-El-Daher & Simard, 1985) in line with their stimulation of growth of Enterobacteriaceae. Because of the method of analysis used in some of these studies, increased concentrations of other amines were recorded during storage including tyramine (Wortberg & Woller, 1982; Sayem-El-Daher & Simard, 1985), spermidine (Nakamura et al., 1979; Sayem-El-Daher & Simard, 1985), diaminopropane (Sayem-El-Daher & Simard, 1985) and agmatine (Wortberg & Woller, 1982). Pure culture studies have given no clue as to specific microbial sources of these compounds.

STORAGE IN VACUUM PACKS

Evidence presently available suggests that the chemical changes associated with bacterial growth under these conditions are less complex than those already discussed for storage in air.

Compounds supporting bacterial growth

In accord with classical findings for lactic acid bacteria, the type of organisms which typically dominate the spoilage flora of normal pH meat stored in this manner (see Dainty et al., 1983), glucose was shown to be the primary source of energy of a Lactobacillus strain inoculated onto sterile lamb muscle (Gill, 1976). Although arginine was shown to be metabolized when glucose became depleted in the surface layers, it did not result in any further increase in cell numbers, presumably because of a combination of the low energy yield from this fermentation and the relatively low concentration of the substrate. This explains why microbial numbers typically reach a maximum and plateau out at about 10^7-10^8 org/cm^2 rather than continue to increase to the climax populations found on meat stored in air i.e. 10^9-10^{10}/cm^2. Organisms growing under the latter conditions have the benefit of greater energy yield from oxidative reactions linked to electron transport processes and/or the ability to metabolize a much greater range of compounds, amino acids and lactic acid in particular.

During growth in a meat juice medium (Newton & Gill, 1978b), an Enterobacter sp. and B. thermosphacta representing the other common types of bacteria sometimes found on vacuum packed meat, also used glucose as the primary, and in the case of B. thermosphacta as the sole, source of energy. Growth of B. thermosphacta on the lean tissues of vacuum packaged meat is critically dependent upon the pH of the tissues and the permeability of the packaging. At pH values below 5.7, use of the highly impermeable films available today will greatly reduce, and possibly prevent its growth, lactic acid being the inhibitory agent under these conditions (Grau, 1980; Egan & Grau, 1981). The Enterobacter sp. was able to metabolize glucose-6-phosphate, but presumably, although it was not stated by the authors, only upon glucose depletion.

This was certainly shown to be the case for Enterobacter (Serratia) liquefaciens growing in a meat juice medium incubated anaerobically at the surprisingly high temperature of 30°C (Gill & Newton, 1979). Although the experiment was part of a study of growth on vacuum packaged DFD meat, the glucose concentrations were more typical of normal pH meat and no pH value of the medium was given. Bearing these points in mind, E. liquefaciens was shown to metabolize serine at the same time as glucose and glucose-6-phosphate. A strain of Alteromonas putrefaciens, an organism only found on DFD meat because of an inability to grow at pH values much below 6.0, also metabolized serine and glucose simultaneously and even showed some preference for the amino acid. Both organisms were able to metabolize lysine, arginine and threonine upon glucose depletion. The identity of the growth substrate(s) for lactic acid bacteria and B. thermosphacta on DFD meat, which is able to support high numbers of both organisms (Egan & Grau, 1981; Egan & Shay, 1984), are not known. Arginine seems an unlikely sole source of energy for the lactic acid bacteria in view of the arguments presented above regarding its low energy yield and low availability. Furthermore, not all the lactic acid bacteria isolated

from meat are able to metabolize arginine, for example the leuconostocs cannot (Shaw & Harding, 1984), and there is no evidence that growth of strains which can, is favoured on DFD meat. In the case of B. thermosphacta, possible candidates again appear to be ribose from nucleotides and/or glycerol (Dainty & Hibbard, 1983).

End-products of bacterial growth

Accumulation of acid end-products of carbohydrate metabolism under the fermentative (low oxygen tension) conditions of vacuum packs is often given as the explanation for the development of the 'sour'/'acid'/'cheesy' off-odours and off-flavours which characterize normal pH meat stored in this manner (Sutherland et al., 1976; Egan, 1984), but there is no definite proof of this.

In pure culture inoculation experiments, growth of a lactic acid bacterium, Serratia liquefaciens and B. thermosphacta on normal and high pH beef resulted in greater concentrations of acetic acid than in stored, sterile controls (Dainty, 1981). Taking into account the increases in the sterile controls, S. liquefaciens produced ca. 4-5 times the amount of the other two organisms. Growth of A. putrefaciens and an Aeromonas sp. on the high pH meat led to similar increases to those of S. liquefaciens. Only growth of B. thermosphacta on the high pH meat produced elevated concentrations of other fatty acids namely, isobutyric and isovaleric acids. These results provide adequate explanations of the higher concentrations of acetic, isobutyric and isovaleric acids detected in high pH samples of naturally contaminated beef compared to their normal pH counterparts (Dainty et al., 1979). The sources of the high levels of n-butyric and n-valeric acids detected in these high pH samples are unknown but non-microbial sources (Shank et al., 1962) cannot be ruled out. Acetic acid, with smaller amounts of propionic and isobutyric acids, was also the major component of the fatty acids detected by Sutherland et al. (1976) in their study of naturally contaminated beef. In addition to glucose, alanine could contribute to acetic acid formation in normal pH, and be a major precursor in high pH samples, while the branched chain acids are presumably formed from the corresponding amino acids.

Two of the three types of lactic acid bacteria commonly found on vacuum packed meats are homofermentative streptobacteria (Shaw & Harding, 1985) and formation of lactic acid seems highly probable during storage. Using a chemical assay Sutherland et al. (1976) were unable to detect increases in total lactic acid concentration in joints of beef stored in excess of 8 weeks. Nor were we able to detect increases in recently completed studies of chemical changes in normal and DFD pork and normal pH beef. Increases in total lactic acid content have been observed during storage of ground beef in packs of low oxygen permeability (Nassos et al., 1983; 1985). The increases were substantial, up to 30-40% of the original lactic acid content of the meat. One possible explanation of this increase is that, with the bacteria being distributed throughout the whole mass of meat rather than restricted to the surface, glucose availability would not be limited to the same extent and end-product formed per unit mass would be greater.

Compounds other than acids detected during storage of normal pH beef in vacuum packs include the diamines putrescine and cadaverine (Edwards et al., 1983). In contrast to the findings reported earlier for aerobic storage, cadaverine was present in higher concentrations than putrescine. A subsequent pure culture inoculation study has shown the Enterobacteriaceae, and Hafnia alvei and Serratia liquefaciens in particular, to be major sources of cadaverine while a combination of arginine dihydrolase positive streptobacteria and either H. alvei or S. liquefaciens was needed for significant putrescine formation (Dainty et al., in press). It also became apparent during the course of these studies that tyramine can be expected to accumulate during vacuum packaged storage and that the cluster 1 streptobacteria of Shaw & Harding (1985) are the causative organisms (Dainty et al., in preparation). The unidentified amines observed by Sutherland et al. (1976) in their study of beef could be some combination of these and the alkylamines reported by Dainty et al. (1979). In the latter study of commercial packs of beef, trimethylamine was detected in much higher concentrations in the DFD than in the normal pH samples examined.

Analysis of highly volatile compounds, other than alkylamines, has rarely been carried out in vacuum packs. In a recent examination of headspace volatiles associated with naturally contaminated samples of normal and high pH pork a number of sulphur-containing compounds have been detected (Edwards & Dainty, in press). These include hydrogen sulphide, methane thiol, dimethylsulphide, dimethyltrisulphide, methyl thioacetate, methyl thiopropionate, bis (methylthio) methane and methyl (1-methylthio) ethyl disulphide. Of these only the first five were detected in the normal pH samples and at much lower concentrations (20-500x) than in the high pH samples. Interestingly a number of ethyl esters of short chain fatty acids similar to those detected during storage in air were associated with the high pH samples as well. The sulphur compounds were clearly major contributors to the objectionable odours associated with the high pH meat and possible sources are under investigation.

CHEMICAL CHANGES AS INDICATORS OF SPOILAGE

The possibility of using microbially induced chemical change(s) as alternative or additional means of assessing spoilage/shelf-life/acceptability has been, and continues to be, a strong motivating force for studies such as those described in the previous sections. Such assessments are presently made from sensory data and the enumeration of total, and possibly specific types of, bacteria. Sensory assessments are subjective making the setting of criteria differentiating acceptable from non acceptable, spoiled from non spoiled almost impossible. Furthermore, while bacterial counts (total and/or specific) must bear some relationship to acceptability, there are some problems in their use. Not all bacteria, as illustrated earlier for the pseudomonads, have the same spoilage potential. In the case of vacuum packs, bacterial numbers can remain at maximum levels for significant periods of time without obvious sensory change. And, of course, the spoilage of DFD meat is now known to be rapid, not because of increased growth rate at higher pH values as once believed, but because the absence of an initial glucolytic phase means that objectionable compound formation from amino acid degradation occurs at lower cell densities.

Perhaps a more appropriate index would be some measure of the chemical changes involved directly or indirectly in spoilage development and sufficient data is now available to make a tentative appraisal of this possibility. Unfortunately, this has to be done in many cases in an indirect manner, by correlating chemical

types of pseudomonad (cf Shaw & Latty, 1984). A definite sequence of appearance of the various classes of volatile compounds, which was reflected in the odour descriptions, was also evident, namely:

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Evidence presently available suggests that the chemical changes associated with bacterial growth under these conditions are less complex than those already discussed for storage in air.

Compounds supporting bacterial growth

In accord with classical findings for lactic acid bacteria, the type of organisms which typically dominate the spoilage flora of normal pH meat stored in this manner (see Dainty et al., 1983), glucose was shown to be the primary source of energy of a Lactobacillus strain inoculated onto sterile lamb muscle (Gill, 1976). Although arginine was shown to be metabolized when glucose became depleted in the surface layers, it did not result in any further increase in cell numbers, presumably because of a combination of the low energy yield from this fermentation and the relatively low concentration of the substrate. This explains why microbial numbers typically reach a maximum and plateau out at about 10^7-10^8 org/cm^2 rather than continue to increase to the climax populations found on meat stored in air i.e. 10^9-10^{10}/cm^2. Organisms growing under the latter conditions have the benefit of greater energy yield from oxidative reactions linked to electron transport processes and/or the ability to metabolize a much greater range of compounds, amino acids and lactic acid in particular.

During growth in a meat juice medium (Newton & Gill, 1978b), an Enterobacter sp. and B. thermosphacta representing the other common types of bacteria sometimes found on vacuum packed meat, also used glucose as the primary, and in the case of B. thermosphacta as the sole, source of energy. Growth of B. thermosphacta on the lean tissues of vacuum packaged meat is critically dependent upon the pH of the tissues and the permeability of the packaging. At pH values below 5.7, use of the highly impermeable films available today will greatly reduce, and possibly prevent its growth, lactic acid being the inhibitory agent under these conditions (Grau, 1980; Egan & Grau, 1981). The Enterobacter sp. was able to metabolize glucose-6-phosphate, but presumably, although it was not stated by the authors, only upon glucose depletion.

This was certainly shown to be the case for Enterobacter (Serratia) liquefaciens growing in a meat juice medium incubated anaerobically at the surprisingly high temperature of 30°C (Gill & Newton, 1979). Although the experiment was part of a study of growth on vacuum packaged DFD meat, the glucose concentrations were more typical of normal pH meat and no pH value of the medium was given. Bearing these points in mind, E. liquefaciens was shown to metabolize serine at the same time as glucose and glucose-6-phosphate. A strain of Alteromonas putrefaciens, an organism only found on DFD meat because of an inability to grow at pH values much below 6.0, also metabolized serine and glucose simultaneously and even showed some preference for the amino acid. Both organisms were able to metabolize lysine, arginine and threonine upon glucose depletion. The identity of the growth substrate(s) for lactic acid bacteria and B. thermosphacta on DFD meat, which is able to support high numbers of both organisms (Egan & Grau, 1981; Egan & Shay, 1984), are not known. Arginine seems an unlikely sole source of energy for the lactic acid bacteria in view of the arguments presented above regarding its low energy yield and low availability. Furthermore, not all the lactic acid bacteria isolated

from meat are able to metabolize arginine, for example the leuconostocs cannot (Shaw & Harding, 1984), and there is no evidence that growth of strains which can, is favoured on DFD meat. In the case of B. thermosphacta, possible candidates again appear to be ribose from nucleotides and/or glycerol (Dainty & Hibbard, 1983).

End-products of bacterial growth

Accumulation of acid end-products of carbohydrate metabolism under the fermentative (low oxygen tension) conditions of vacuum packs is often given as the explanation for the development of the 'sour'/'acid'/'cheesy' off-odours and off-flavours which characterize normal pH meat stored in this manner (Sutherland et al., 1976; Egan, 1984), but there is no definite proof of this.

In pure culture inoculation experiments, growth of a lactic acid bacterium, Serratia liquefaciens and B. thermosphacta on normal and high pH beef resulted in greater concentrations of acetic acid than in stored, sterile controls (Dainty, 1981). Taking into account the increases in the sterile controls, S. liquefaciens produced ca. 4-5 times the amount of the other two organisms. Growth of A. putrefaciens and an Aeromonas sp. on the high pH meat led to similar increases to those of S. liquefaciens. Only growth of B. thermosphacta on the high pH meat produced elevated concentrations of other fatty acids namely, isobutyric and isovaleric acids. These results provide adequate explanations of the higher concentrations of acetic, isobutyric and isovaleric acids detected in high pH samples of naturally contaminated beef compared to their normal pH counterparts (Dainty et al., 1979). The sources of the high levels of n-butyric and n-valeric acids detected in these high pH samples are unknown but non-microbial sources (Shank et al., 1962) cannot be ruled out. Acetic acid, with smaller amounts of propionic and isobutyric acids, was also the major component of the fatty acids detected by Sutherland et al. (1976) in their study of naturally contaminated beef. In addition to glucose, alanine could contribute to acetic acid formation in normal pH, and be a major precursor in high pH samples, while the branched chain acids are presumably formed from the corresponding amino acids.

Two of the three types of lactic acid bacteria commonly found on vacuum packed meats are homofermentative streptobacteria (Shaw & Harding, 1985) and formation of lactic acid seems highly probable during storage. Using a chemical assay Sutherland et al. (1976) were unable to detect increases in total lactic acid concentration in joints of beef stored in excess of 8 weeks. Nor were we able to detect increases in recently completed studies of chemical changes in normal and DFD pork and normal pH beef. Increases in total lactic acid content have been observed during storage of ground beef in packs of low oxygen permeability (Nassos et al., 1983; 1985). The increases were substantial, up to 30-40% of the original lactic acid content of the meat. One possible explanation of this increase is that, with the bacteria being distributed throughout the whole mass of meat rather than restricted to the surface, glucose availability would not be limited to the same extent and end-product formed per unit mass would be greater.

Compounds other than acids detected during storage of normal pH beef in vacuum packs include the diamines putrescine and cadaverine (Edwards et al., 1983). In contrast to the findings reported earlier for aerobic storage, cadaverine was present in higher concentrations than putrescine. A subsequent pure culture inoculation study has shown the Enterobacteriaceae, and Hafnia alvei and Serratia liquefaciens in particular, to be major sources of cadaverine while a combination of arginine dihydrolase positive streptobacteria and either H. alvei or S. liquefaciens was needed for significant putrescine formation (Dainty et al., in press). It also became apparent during the course of these studies that tyramine can be expected to accumulate during vacuum packaged storage and that the cluster 1 streptobacteria of Shaw & Harding (1985) are the causative organisms (Dainty et al., in preparation). The unidentified amines observed by Sutherland et al. (1976) in their study of beef could be some combination of these and the alkylamines reported by Dainty et al. (1979). In the latter study of commercial packs of beef, trimethylamine was detected in much higher concentrations in the DFD than in the normal pH samples examined.

Analysis of highly volatile compounds, other than alkylamines, has rarely been carried out in vacuum packs. In a recent examination of headspace volatiles associated with naturally contaminated samples of normal and high pH pork a number of sulphur-containing compounds have been detected (Edwards & Dainty, in press). These include hydrogen sulphide, methane thiol, dimethylsulphide, dimethyltrisulphide, methyl thioacetate, methyl thiopropionate, bis (methylthio) methane and methyl (1-methylthio) ethyl disulphide. Of these only the first five were detected in the normal pH samples and at much lower concentrations (20-500x) than in the high pH samples. Interestingly a number of ethyl esters of short chain fatty acids similar to those detected during storage in air were associated with the high pH samples as well. The sulphur compounds were clearly major contributors to the objectionable odours associated with the high pH meat and possible sources are under investigation.

CHEMICAL CHANGES AS INDICATORS OF SPOILAGE

The possibility of using microbially induced chemical change(s) as alternative or additional means of assessing spoilage/shelf-life/acceptability has been, and continues to be, a strong motivating force for studies such as those described in the previous sections. Such assessments are presently made from sensory data and the enumeration of total, and possibly specific types of, bacteria. Sensory assessments are subjective making the setting of criteria differentiating acceptable from non acceptable, spoiled from non spoiled almost impossible. Furthermore, while bacterial counts (total and/or specific) must bear some relationship to acceptability, there are some problems in their use. Not all bacteria, as illustrated earlier for the pseudomonads, have the same spoilage potential. In the case of vacuum packs, bacterial numbers can remain at maximum levels for significant periods of time without obvious sensory change. And, of course, the spoilage of DFD meat is now known to be rapid, not because of increased growth rate at higher pH values as once believed, but because the absence of an initial glucolytic phase means that objectionable compound formation from amino acid degradation occurs at lower cell densities.

Perhaps a more appropriate index would be some measure of the chemical changes involved directly or indirectly in spoilage development and sufficient data is now available to make a tentative appraisal of this possibility. Unfortunately, this has to be done in many cases in an indirect manner, by correlating chemical

change with bacterial numbers and types and then making use of the rather ill-defined relationships between spoilage and bacterial numbers. Clearly this is one of the shortcomings to be rectified in future work.

From the data presented it is obvious that chemical changes associated with the initial glucolytic phase of growth should be amongst the earliest indicators of the extent of microbial growth, spoilage development, etc. And, because all of the commonly occurring meat psychrotrophs on meat stored in air and in vacuum packs metabolize glucose, its estimation in both circumstances should relate to a total count. A simple determination of glucose in the surface layers alone, or separate determinations in a number of consecutively deeper layers to measure the developing gradient, are possible. The latter, in particular, is quite time consuming if conventional enzymatic analysis of the extracted compound is envisaged. It might be possible, however, to measure glucose content *in situ* using miniature glucose electrodes, which have been described in the literature, but designed to penetrate to defined depths from the surface. Extrapolation of the limited data available suggests microbial numbers in the region of $10^6/cm^2$ should be detectable by these methods. In theory, because production of unit mass of bacteria per mole of substrate is far less under fermentative conditions, the method should be more sensitive for vacuum packaged growth.

A second possibility linked to glucose metabolism is the measurement of gluconic and/or 2-oxogluconic acid accumulation and particularly the former which can readily be assayed enzymatically. Such a method would be relevant to storage in air, but not in vacuum packs or necessarily in modified atmospheres. Farber & Idziak's (1982) results for pure cultures showed that gluconic acid was readily detectable at cell numbers typically associated with the onset of spoilage i.e. $10^8/cm^2$. No quantitative data for the oxo-acid were given. Although the decline in concentration observed on further growth posed a potential problem, a single result from a naturally contaminated sample of lamb showed gluconic acid to be still detectable at $10^9/cm^2$. Our own unpublished results confirm these results and indicate that gluconic acid can first be detected, using a straightforward nicotinamide nucleotide coenzyme dependent assay, at cell numbers in the region of $10^8/cm^2$. Of these methods, glucose gradient determination would be the least affected by the natural variation in glucose content of animal muscles of similar pH (Newton & Gill, 1978) but they would all become less reliable with increasing pH because of the inverse relationship between pH and glucose content. Of course the methods would be entirely inappropriate for obvious DFD muscles.

A further possibility linked to glucose metabolism is the estimation of lactic acid in vacuum packaged material. Nassos *et al.* (1973) reported that the total lactic acid content of ground beef correlated positively with bacterial numbers and negatively with panel assessments of odour acceptability and calculated concentrations corresponding to 50% panel acceptability with confidence limits. The relevance of such findings in the more general context of non-ground meat samples is lessened, however, in view of the findings of other authors reported earlier (Sutherland *et al.*, 1976). The method would be subject, of course, to the limitations described above for glucose etc.

The possibility of using compounds not derived from glucose has been considered from time to time in the form of increases in ammonia concentration or pH during aerobic storage. More recently Slemr (1981) reported a 10-fold increase in the combined putrescine/cadaverine content of pork stored at 5°C before obvious signs of organoleptic spoilage and at microbial counts of *ca.* $10^6/cm^2$. A 100-fold increase was evident at spoilage when numbers were 10^7-$10^8/cm^2$. In our own study of beef, pork and lamb cuts and minced beef stored at 5°C, detectable increases in these amines were only evident as numbers exceeded $10^7/cm^2$ and spoilage was imminent and the method seems to be of value as an objective confirmation of incipient spoilage rather than as a useful predictor. Similar conclusions can be drawn from the studies of Nakamura *et al.* (1979) and of Sayem-El-Daher & Simard (1985) but there were indications in the latter authors' study of increases in both putrescine and 2,3-diaminopropane at lower cell numbers when the meat was stored at 10°C rather than at 4°C.

Accumulation of diamines has also been shown in vacuum packaged beef (Edwards *et al.*, 1985), with first detection before off odour development when cell numbers were $6x10^6/cm^2$, a population density below the maximum. The apparent promise of these results has been lessened in the light of pure culture experiments showing that *Enterobacteriaceae* strains play a crucial role in their formation (Dainty *et al.*, in press), and such organisms are not always present on stored meats. However, organisms that are, the cluster 1 streptobacteria (Shaw & Latty, 1985) appear to produce tyramine during growth in vacuum packs of meat and the relationships between its detection, bacterial numbers and sensory criteria are being investigated.

Finally the possibility of using some of the highly volatile compounds in the headspaces above stored meat must be considered. The non invasive nature of the technique reduces the sampling problems inherent in all the other techniques as a result of non-uniform contamination. Furthermore it actually measures components of the off-odours which cause spoilage. A major drawback is, of course, the fact that many of the compounds are only produced in the post glucose phase of growth when spoilage is imminent. Despite this fact, in my own group's study of the time course of volatile compound formation for meat stored in air (Dainty *et al.*, 1985), some compounds were readily detectable at a stage when the odours, although different from those of the fresh material, were not objectionable. Amongst these compounds were acetoin and diacetyl, believed to be derived from glucose, and 3-methyl-1-butanol which was almost certainly formed from isoleucine. The possible use of such techniques cannot therefore be ruled out.

What all these methods lack in their attempts to measure directly the microbial quality or degree of spoilage of a product, is sufficient sensitivity. There is, of course, no reason why an incubation step at controlled, elevated temperatures could not be introduced to overcome this problem and give the methods more predictive value than they presently have. As in the case of impedance/conductance measurements, the time to detection of the chosen parameter would then be used to obtain the "desired" information about the non incubated sample from previously constructed calibration curves.

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