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Sulphite preservation of British fresh sausage.

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SULPHITE PRESERVATION OF BRITISH FRESH SAUSAGE

Submitted by Jeffrey Gordon Banks

for the degree of Ph.D.

of the University of Bath

1983

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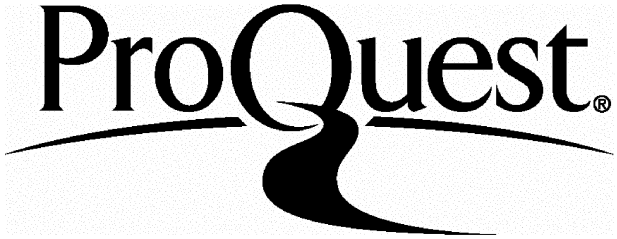

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SUMMARY

The microbial associations that developed in commercially produced sulphited and unsulphited sausages during storage for up to 8 days at a variety of temperatures were identified. Such associations were comprised of dominant, e.g. *Brochothrix thermosphacta*, yeasts and lactic acid bacteria, or minor, e.g. Pseudomonadaceae and Enterobacteriaceae, groups. A detailed analysis of numerous isolates of the previously-ignored Gram-negative microflora using computer-assisted numerical techniques revealed that sulphite concentration influenced the composition of the Enterobacteriaceae but not the pseudomonads.

A method was developed for the assay of free and bound sulphur IV oxospecies in culture media or meat-based samples. It was superior to existing techniques and demonstrated limited irretrievable - by oxidation - and extensive reversible - by binding - loss of sulphite preservative from freshly manufactured and stored sausage.

Selected micro-organisms isolated from sausage were tested for their tolerance of free and bound sulphur IV oxospecies in batch and "turbidometer" culture under various conditions. In general this technique reflected the elective/selective role of sulphite in sausage and allowed the microbial association to be defined more clearly in terms of tolerance of the active preservative.

The incidence and level of contamination of sausage and its ingredients with *Salmonella* was established using a 'most-probable number' method. Despite a high incidence of contamination of most of the meat ingredients and finished product with these organisms, the level of infection - with the exception of mechanically recovered meat - was low. Thus the role of sulphur IV oxospecies in determining the fate of these food poisoning organisms in sausage or culture media was assessed by deliberate infection with rifampicin-resistant *Salmonella* mutants.

With a view to extending the shelf life of fresh sausage, a limited survey of adjuncts or alternative "preservatives" to sulphite using pilot-scale batches of sausage meat was done.

INTRODUCTION

As British fresh sausages are made from diced or minced meat, fat and rind from various sources, there are ample opportunities for contamination with both food spoilage and food poisoning micro-organisms. Extensive comminution of the ingredients in a bowl chopper ensures not only that the contaminants are distributed randomly, but that they are suspended in an environment notable for its chemical activity as well as its potential to support extensive microbial growth. Thus three amylases - one present in rusk, another in porcine muscle and a third in the salivary gland - and a maltase of meat origin ensure that substrate levels of glucose, maltose, maltotriose and maltotetrose are available to contaminants of pork sausages stored at 4^{o} or $22^{o}C$ (Abbiss, 1978). The accumulation of valerate and changes in the lipid pattern, as indexed by g.l.c. analysis of stored sausages, have led also to the tentative conclusio n that enzyme-mediated changes of fats and proteins occur in sausages (Leads, 1979). As yet, however, the relative importance of enzymes of microbial and meat origin have not been established. There is evidence also that a small amount of the legally-permitted preservative of sausages, sulphite or metabisulphite, is irretrievably lost in stored sausages and a large amount is reversibly bound such that its antimicrobial properties are negated (Brown, 1977). Determinations of pH changes provide yet another index of chemical changes, there being an acid drift of *ca*. 1 pH in sulphited and *ca*. 2.8 units in unsulphited sausage (Brown, 1977). Although sulphite is regarded as a preservative, the physiological basis of its mode of action is unknown and the studies of the

microbiology of sausages permit only a general conclusion, namely that it selects a Gram-positive flora (Dyett and Shelley, 1962, 1966; Dowdell and Board, 1967, 1968, 1971). Brown (1977) was probably the first to note that *Pseudomonas* spp. grew in sulphited sausages and he demonstrated, also, that enterobacteria flourished in unsulphited ones stored at 22°C. Thus he supported indirectly the notion (Dyett and Shelley, 1962) that sulphite plays an important role in preventing the growth of *Salmonella* in sausages. As these studies (Dowdell and Board, 1968, 1971) which defined the microbial association - lactic acid bacteria, yeasts, micrococci and *Microbacterium thermosphactum* - of stored sausages did not analyse the concentration of free sulphite in the products examined, the general definition of this association may well mask an important feature, namely that its members differ markedly in their tolerance of free sulphite. A chemical method was developed in the present study so that the concentration of free and bound sulphite in sausages examined microbiologically could be established. Many of the isolates from these experiments were identified by computer analysis and their tolerance of free sulphite at different incubation temperatures established. A novel test system was developed to establish the sulphite tolerance of pure cultures at different incubation temperatures. These studies, which are discussed on pp. 147 -157 , have resulted in a better definition of the microbial association found in British fresh sausage.

As noted above, Dyett and Shelley (1962, 1966) surmised that sulphite was responsible for the good public health record of

British fresh sausage in spite of the high incidence of contamination with *Salmonella* (Roberts *et al.*, 1975). Although large numbers of samples were included in the surveys of Roberts *et al.* (1975) and those of Turnbull and Rose (1982) and Barrell (1982), no attempts were made to establish the level of contamination or to identify the ingredients responsible for such infection. In the present study, quantitative methods were used to establish the level of *Salmonella* contamination of British fresh sausages and ingredients taken in the course of routine production from a factory. This phase of the study was extended to define the behaviour of *Salmonella* in stored sulphited or unsulphited sausage. Previous studies of the microbiology of sausages have shown that batches made in experimental kitchens rarely contain a microflora comparable to that produced in a factory. It needs to be stressed therefore that the major work discussed in this thesis was done with material taken from a factory.

LITERATURE REVIEW

Manufacture of pork and pork and beef sausage

Recipes for the pork sausages and the pork and beef sausages included in this study are given in the Appendix (p.329). As the method of manufacture differs slightly between firms, a brief account of that used in this study will be given.

Large (*ca*. 50 x 20 x 20 cm) blocks of lean pork, and/or beef and pork backfat, minced semi-lean pork (head and belly meat) and cooked rinds were added to a bowl chopper (Laska, Austria) - capacity, 500 ℓ. The mincing of meat is associated with a rise in the glucose concentration due to the action of intrinsic α-amylases, maltases and amylo-1-6-glycosidases (Newbold and Scopes, 1971) and accounts in part for the increased chemical activity of the sausage meat. Seasonings, ice, water and poly-phosphates were added to the meat and a slurry produced by rapid chopping. The seasonings contained: (a) spices, some of which may act as bacteriostatic/bacteriocidal agents (Chipault *et al*., 1952) or antioxidants (Chipault *et al*., 1952, 1956); (b) a pigment ("Red 2G"); (c) preservative, (sodium sulphite or metabisulphite) to give a final concentration just above the legally-permitted maximum of *ca*. 600 μg SO_2/g freshly-made sausage, and (d) sodium chloride. Rusk was the last ingredient to be chopped into the meat slurry, its principal functions being to retain water (Haq *et al*., 1973) thereby ensuring successful extrusion of the slurry into casings and to contribute to flavour (Leads, 1979) . Of the two types of casing in common use, reconstituted collagen or synthetic cellulose,

the former was used.

Probable changes during preparation

Little work has been done on physical changes occurring during the manufacture of British fresh sausage. This section relies therefore on observations made in studies of related products, frankfurters, Thuringer and Bologna-type products.

"Bowl chopping" destroys the structure of the fat and meat. The cell contents dissolve in the iced water and it is surmised that very rapid comminution coats fat particles with a protein film (Borchert *et al.*, 1967). The presence of ice during processing probably favours the formation of a pseudo-emulsion simply because the solubility of protein in dilute brine is increased by chill temperatures (Karmas, 1977). Moreover, the addition of sodium chloride at an early stage of the preparation of a slurry has been shown to optimize the extraction of the salt-soluble proteins, actomysin and myosin, and provide an ionic environment (Karmas, 1977) which increases the rate of formation of the "pseudo-emulsion" (Theno *et al.*, 1978). The addition of polyphosphate salts - "Calgon" (sodium hexametaphosphate) or fibrisol V10 - to the mix in the early stages of bowl chopping, aid the retention of water by the meat fibres and protein. This is due in part to the stabilization of the pH at or near neutrality; as meat approaches its isoelectric point (pH 5.5) water and water-soluble materials are released from the meat matrix (Lawrie, 1978). The number of fat pockets are reduced also, thus favouring even "emulsification"

(Gerrard, 1976). The protein film is considered to be a major barrier to the coalescence of small fat globules formed by vigorous chopping (Acton and Saffle, 1972). It could be inferred from this discussion that British fresh sausage was basically a fat-in-water emulsion. Indeed this term is commonly used in the trade, even though the commodity rarely if ever has either the structure or the properties of a conventional oil-in-water emulsion.

Microbiology*

Sources of micro-organisms

As the initial size and composition of the microflora present in freshly made sausage can influence the development of the microbial association during storage (Dowdell and Board, 1971; Brown, 1977), it is pertinent to consider factors that contribute to the contamination of the meat ingredients, the principal depots of infection (Dowdell and Board, 1968, 1971). There are two major sources of the micro-organisms on meat, namely those of gut or mucous-membrane origin that infect the meat during slaughter and those acquired through contact of meat with equipment etc. Ingram (1949) was perhaps the first to distinguish between these two sources of infection when he alluded to organisms of

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* The names of micro-organisms used in this thesis are those given in the cited reports.

"intrinsic" and "extrinsic" origin respectively. He stated that the intrinsic micro-organisms in the tissues of healthy animals need not be pathogenic; the latter would be expected to occur in the carcasses of animals which had been suffering from overt or covert infections. The high temperature storage of carcass meat has been considered (Ingram and Dainty, 1971) to be the main reason for growth of intrinsic bacteria, such as *Clostridium* spp., which cause "bone taint" for example. The occurrence of organisms in meat suggests that some may have penetrated the wall of the gut or mucous membranes and been disseminated *via* the lymphatic and vascular systems (Arnold, 1928; Nickel and Gisske, 1941; Bogadi and Sewell, 1974; Gill *et al.*, 1976). Although many workers have reported the isolation of micro-organisms from within meat (e.g. Ayres, 1955; Lechowich, 1971; Lawrie, 1974) a critical review of the literature (Gill, 1979) drew attention to technical difficulties, particularly with respect to asepsis, that need to be controlled before valid conclusions can be made. Gill (1979) cites the observations of Haines and Scott (1940) and Sharp (1963) who showed that flaming of dissection instruments was at best only 70% successful in achieving sterilisation. It is evident from the literature (Table 1), that a greater incidence and level of contamination of deep muscle tissue was found by workers before 1970, whereas the consensus of opinion after this date favours the view that such tissues in healthy animals are usually sterile (e.g. Hasegawa *et al.*, 1970; Buckley *et al.*, 1976 and Gill *et al.*, 1978).

Indeed, Roberts (1980) concluded that the presence of small

Table 1. The bacteriological status of deep muscle tissue of slaughtered animals

Animal	Reference	Animals positive (%)	Micro-organisms/ g	Identity of micro-organisms
Pigs	Reith (1929)	100	"low numbers"	Staphylococci, G +ve rods
	Jensen and Hess (1941)	66	not reported	*Clostridium, Achromobacter* spp.
	Vanderzant and Nickelson (1969)	42	10^2	Staphylococci
	Lepovetsky *et al.*(1953)	7	10^2 - 10^4	*Streptococcus, Pseudomonas, Escherichia coli, Enterobacter* spp.
Cattle	Narayan (1966)	61	not reported	*Clostridium* spp.
	Vanderzant and Nickelson (1969)	27	10^3	Staphylococci, coryneforms
Sheep	Zender *et al.* (1958)	0	0	NG
	Radouco-Thomas *et al.* (1959)	0	0	NG
	Vanderzant and Nickelson (1969)	36	10^2	Staphylococci
Pigs	Hasegawa *et al.*(1970)	0	0	NG
Cattle	Zagaevskii (1973)	5	1	*Clostridium* spp.
	Buckley *et al.* (1976)	0	0	NG
Sheep	Gill *et al.* (1978)	0	0	NG

NG, Not given

numbers of viable micro-organisms in carcasses of healthy animals was of very limited commercial importance, and he suggested that the process of slaughter introduced micro-organisms into tissues. Thus in an investigation of bone marrow of hogs, Jensen and Hess (1941) isolated pigmented bacteria which had been used to contaminate the "sticking knife". Similar results were obtained when genetically-marked micro-organisms were used in this type of experiment (Mackey and Derrick, 1979). Moreover, the surmise (Wilson and Miles, 1964) that bacteria may reach the tissues by penetration of the intestinal epithelium was supported by the demonstration (Mackey and Derrick, 1979) that organisms administered orally to pigs were isolated from the lungs and spleen *post-mortem*. Although there is evidence of translocation (e.g. Arnold, 1928; Nickel and Gisske, 1941), Roberts (1980) was of the opinion that it makes a negligible contribution to the contamination of market meat.

Extrinsic micro-organisms

Micro-organisms of the general environment are the major contaminants of carcass meat. They infect initially cut surfaces but may penetrate into meat during storage (Gill and Penney, 1977). The following review deals with the two important aspects of contamination in factories in which slaughter, butchery and manufacture are done (Fig. 1), firstly the sources of contamination, and secondly the factors influencing the level of contamination.

Sources of contamination

The skin of the animal, dust, soil, water and air in lairages and in abattoirs can all harbour substantial numbers of micro-organisms (Empey and Scott, 1939; Stolle, 1981) and climatic conditions, particularly rainfall and temperature, may affect the size and composition of the populations (Newton *et al.*,1978).

Scalding, singeing and "black scraping" are processes that could be expected to have an important influence on the level of contamination of a carcass. Scalding may cause a reduction of 0.9 - 2.5 log cycles in the total viable (Dockerty *et al.*, 1970; Snijders, 1976; Snijders and Gerats, 1976) and of 3 log cycles in the Enterobacteriaceae count (Gerats *et al.*, 1981). High temperatures and alkaline water favour the reduction in bacterial numbers (Dockerty *et al.*, 1970). The attachment of micro-organisms to porcine skin appears to offer protection (Butler *et al.*, 1979). Thus the numbers of *Lactobacillus* and *Pseudomonas putrefaciens* inoculated intentionally onto pigs' skin were reduced by upwards of 3 - 4 log cycles during scalding (Butler *et al.*, 1980). Singeing kills only those micro-organisms on porcine skin with which the flame makes direct contact. Indeed some workers, (e.g. Dockerty *et al.*, 1970 ; Butler *et al.*, 1980) report small reductions only in the number of micro-organisms during this process whereas others (e.g. Snijders, 1976; Snijders and Gerats, 1976; Rasch *et al.*, 1978) infer that this process is much more effective. Thus the former group of workers noted a 0.4 - 1 and the latter a 2.5 - 3 log cycle reduction in the total viable count. Scraping by hand does not

reduce the number of micro-organisms on the carcass (Dockerty *et al.*, 1970); indeed, mechanical scraping may even contribute to contamination of the carcass (Gerats *et al.*, 1981).

Throughout all these processes, the stick wound is the principal avenue for microbial penetration of deep tissue; subsequently the cut along the mid-line of the belly - the initial step in evisceration - results in contamination of internal tissues and surfaces. Micro-organisms derived from workers, equipment or the animals' skin together with excessive handling of the carcass results in an increase in the number of contaminants (Dockerty *et al.*, 1970). Gardner (1980) and Roberts *et al.* (1980) demonstrated that the standards of hygiene at the evisceration stage determined the ultimate level of contamination of the carcass. Rupture of the intestines influences the size of the microbial load and the probability of contamination of the carcass with food poisoning organisms of gut origin (Gerats *et al.*, 1981). The low temperature and high humidity of the chill rooms favours the colonization of the carcass with Gram-negative, aerobic psychrotrophic micro-organisms (Gill, 1980) and subsequent hand butchering, cleaning of bones by machine to produce mechanically recovered meat (MRM), and mincing are processes which have the potential to increase further the level of contamination of meat. Thus there is a discord in the literature dealing with the microbiological status of deep tissues of freshly slaughtered animals, reports that micro-organisms can be isolated readily (e.g. Narayan, 1966; Narayan and Takacs, 1966; Pusztai, 1970), contrasting with

those which state that large amounts of sterile muscle can be removed from carcasses (e.g. Radouco-Thomas *et al.*, 1959; Gardner and Carson, 1967; and Ockerman *et al.*, 1969). The observations of the latter list of workers is supported by Jensen and Hess (1941) who failed to isolate micro-organisms in a biopsy of porcine muscle and yet noted an incidence of contamination of 66% in samples taken immediately *post-mortem*.

Microbial invasion of the tissues

Micro-organisms lacking the determinants of virulence will presumably fail to combat the antimicrobial systems should they invade an unstressed host. This presumably accounts for the germ-free state of the tissue fluids of healthy animals (Gill, 1979). With debilitated animals, however, opportunist micro-organisms may be the cause of short-lived, persistent or recurrent infections (von Graevenitz, 1977). From his critical review of the literature Gill (1979) concluded that *ante-mortem* invasion leads to "intrinsic contamination".

The microbial associations of fresh sausage

Many of the early reports on sausage were concerned with the contamination of the product with micro-organisms of public health significance, the so-called "sanitary quality" of sausage (Cary, 1916). Indeed Sulzbacher and McLean (1951), who studied American pork sausages, stated that little was known about the types of micro-organisms present in fresh sausage despite

several reports dealing with their numbers (e.g. van der Slooten, 1907; Savage, 1908; Cary, 1916). An examination of 316 isolates of pork sausage by Sulzbacher and McLean (1951), who based identification of isolates on descriptions in the 6th edition of Bergey's Manual (Breed *et al.*, 1948), revealed that *ca.* 74% could be assigned to six genera (Table 2). In the light of the present study, several features of this report need comment.

The large proportion of *Proteus* spp. is misleading because these organisms were isolated from *Salmonella-Shigella* agar after enrichment of sausage in tetrathionate broth. The majority (70%) of pseudomonads were active producers of lipases and proteases and, although they were isolated frequently from freshly-made sausage, they could not be recovered in large numbers from the stored product (Table 3). *Microbacterium* sp., which formed large populations in the chilled product, were implicated in the production of acidic flavours. All 47 isolates of *Microbacterium* comprised one species that differed from any of those given in Bergeys Manual (Breed *et al.*, 1948). These non-motile, Gram-positive asporogenous bacilli were unable to reduce nitrates to nitrites, were catalase-positive, gelatin-negative and produced lactate and carbon dioxide from carbohydrates. A later report (McLean and Sulzbacher, 1953) assigned these organisms to a new species, *Microbacterium thermosphactum*. The original report (Sulzbacher and McLean, 1951) did not state whether or not sulphite was included in the sausages.

Table 2. The microorganisms isolated from American pork sausage*

Genus	Number of isolates	% of total
Bacterium	65	20.6
Microbacterium	47	14.9
Achromobacter	40	12.7
Pseudomonas	34	10.8
Bacillus	28	8.9
Proteus	21	6.7

* Adapted from Sulzbacher and McLean (1951)

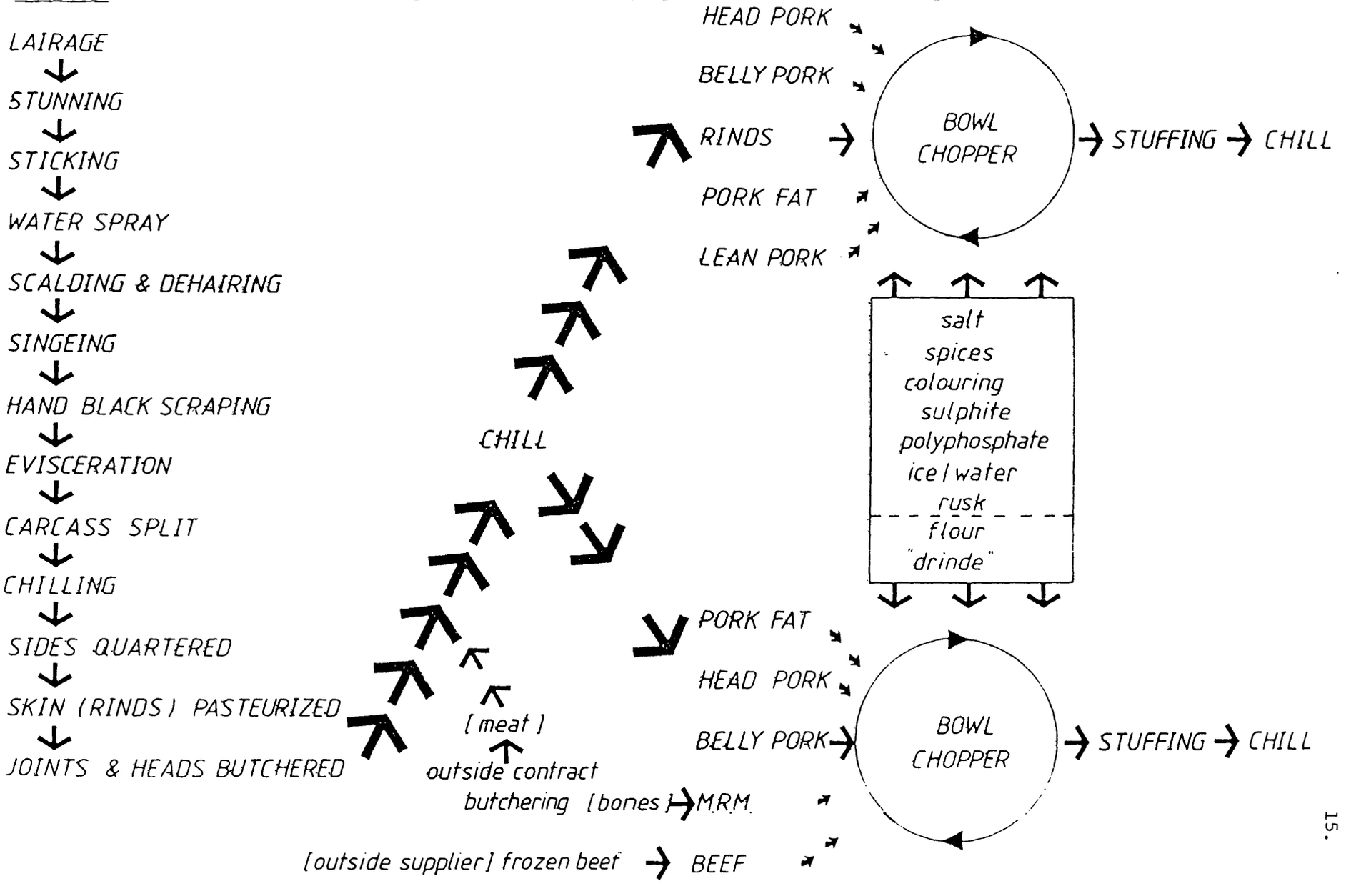
Table 3. Types and distribution of micro-organisms in sausage and on equipment†

Genus	Fresh sausage	stored sausage	Spices	Equipment
Pseudomonas	+++	-	-	+
*Microbacterium**	++	++++	-	-
Alcaligenes	+	++	-	-
Achromobacter	++	++	-	+
Bacterium	+++	++	-	+
Bacillus	+	+	++	+

*Now identified with *Brochothrix thermosphacta* (Sneath and Jones, 1976)

†Adapted from Sulzbacher and McLean (1951).

Figure 1. Flow diagram of pork and pork and beef sausage production in the factory studied.



British fresh sausage

The meat ingredients infect fresh sausage with those organisms (Table 4) which occur in large numbers both in the stored product and in the abattoir environment (Ayres, 1955). Dyett and Shelley (1962, 1966) were the first to examine the effect of sulphite on the keeping qualities of British fresh pork and beef sausages. They identified the majority of their isolates from sulphited sausage with *Bacillus, Micrococcus* or *Streptococcus;* the absence of *Microbacterium thermosphactum* was probably due to the incubation of the isolation medium (Plate Count agar) at 30°C. The first evidence that a particular microbial association was a common feature of British fresh sausages was provided by Dowdell and Board (1967) who established also that an "active fermentation" began in sausages which had been stored for > 24 h at 4° or 22°C. During the first 24 h of storage at 4°C, the numbers of the Gram negative, aerobic bacilli-the numerically-dominant group at time of manufacture - diminished, and rapid multiplication of *Microbacterium thermosphactum*, yeasts and lactic acid bacteria ensured that the characteristic Gram positive/yeast association was formed within 48 h. Further surveys showed that (1) pork sausage was similar to beef sausage in terms of the level and type of contamination, (2), there was an extensive range of contamination (3), the counts in particular brands of sausage fell within a part of the overall range and (4), the scatter of counts of a particular brand increased with an increased total viable count (Dowdell and Board, 1968). The initial microbial load was an important determinant in the selection of the microbial association. Using this 4th

Table 4. Arbitrary classification of contaminants comprising the microbial association of freshly made and stored British fresh sausage*

Category	Contaminant	Initial level of infection (c.f.u./g)	Significant growth at 4°C	Significant growth at 22°C
Dominant	*Microbacterium thermosphactum*	10^5_4	+	+
	Micrococcus spp.	10^4	-	+
	Lactobacillus spp.	$1\text{-}100 \times 10^2_3$	+	+
	Yeasts	$1\text{-}100 \times 10^3$	+	+
Major	*Streptococcus faecalis* *Streptococcus faecium*	$1\text{-}100 \times 10^2$	±	±
	Coliforms	$1\text{-}100 \times 10^2$	±	±
Minor	*Kurthi zopfii*	$< 10^5_2$†		
	Pediococcus spp.	$< 10^2_2$		
	Leuconostoc spp.	$< 10^2_2$	-	-
	Bacillus spp.	$< 10^2_1$		
	Clostridium spp.	$< 10^1$		
	Pseudomonas spp. *Acinetobacter* spp.	$1\text{-}10 \times 10^5$§		

* Adapted from Dowdell and Board (1971)

† Found in 4 samples only

§ Found at this level in heavily contaminated samples only

criterion, Dowdell and Board (1968) separated 40 samples of pork and beef sausages into the following categories: Type A (20% of samples) had an initial microbial load of 5 - 10 x 10^5 micro-organisms/g and was dominated numerically by yeasts; Type B (15%) contained initially 1 - 10 x 10^6 organisms/g and was dominated by unidentified Gram positive bacilli; *Microbacterium thermosphactum* and micrococci comprised a small fraction only of the microflora; Type C, the most commonly occurring (60% of samples) microbial association, was formed primarily of *Microbacterium thermosphactum,* although small numbers of other Gram-positive bacilli, yeasts and Gram negative bacilli were present also. Sausages of the last category, which had an initial level of contamination of 1 - 100 x 10^6 micro-organisms/g, appeared to be produced consistently by large manufacturers. Local butchers, on the other hand, produced another category of sausage (type D), having a different type of microbial association. The very high initial level of infection (1 - 10 x 10^7 micro-organisms/g) consisted mainly of aerobic Gram negative bacilli, identified with the '*Pseudomonas - Achromobacter* complex'.

Dowdell and Board (1971) noted also that on storage at refrigeration or room temperature the majority of pork or beef sausages favoured the growth of one or other of two microbial associations. Thus with storage at 4°C, *Microbacterium thermosphactum* and yeasts became dominant whereas at 22°C *Microbacterium thermosphactum*, yeasts, lactic acid bacteria and micrococci grew. The microbial contaminants were assigned to one of three

categories, dominant, major or minor. These distinctions were made not only with regard to the size of the initial infection but to the fate of the organisms in the stored product also (Table 4). Although it has been demonstrated unequivocally that microbial associations exist in commercial sausage (Dowdell and Board, 1967, 1968, 1971), other workers have failed on occasions to verify this finding (e.g. Ashworth *et al.*, 1974). Perhaps the main reason for such differences was that the latter studied sausages produced on a very small scale in a test kitchen. It has been established that the latter product is markedly different, particularly from a microbiological standpoint, from the typical commercial sausage (Brown, 1977). Further support for the existence of microbial associations was provided by Brown (1977), Abbiss (1978) and Leads (1979). They confirmed that *Microbacterium thermosphactum* dominated the microflora of the majority of commercially-made pork sausages, and that growth of lactic acid bacteria was stimulated by a high initial microbial load. Furthermore, they demonstrated that the characteristic 'yeast-dominated' microflora, which was associated with a low initial bacterial count, was a factory-specific phenomenon. Although the last mentioned group of workers agreed with the contentions of Dowdell and Board (1967, 1968, 1971) that microbial growth was faster and greater at the surface than at the centre of sausages, they did not give evidence in support of the conclusion that lactic acid bacteria grew more extensively at the last mentioned site (Dowdell and Board, 1971).

Thus it is evident from the literature that a particular group of micro-organisms tends to dominate the microbial flora of stored British fresh sausage and, although Brown (1977) provided evidence that the situation was caused by strong selective pressures, these still await definition. The role of sulphite in the selection of the microbial association was therefore studied in detail (pp.125 - 151).

Enterobacteriaceae

The family Enterobacteriaceae may be defined as a group of Gram-negative bacilli that are either motile - peritrichous flagella - or non-motile, which grow aerobically or anaerobically on simple media and MacConkey's bile-salt-lactose medium. They are oxidase-negative and, with one exception, catalase positive, capable of reducing nitrates to nitrites. They ferment glucose in peptone water with the production of either acid or acid and gas, and degrade glucose and other carbohydrates both fermentatively and oxidatively.

In 1937 Rahn proposed the family name Enterobacteriaceae to include organisms which had been assigned to the genera *Escherichia, Salmonella, Aerobacter, Klebsiella, Proteus, Erwinia, Eberthella* and *Shigella*. The new family included also strains of *Serratia, Pseudomonas, Flavobacterium* and *Achromobacter* which fermented glucose with the production of gas. All these "taxa" were placed in a single genus, *Entero-bacter*. With the adoption of the first Bacteriological Code

(Buchanan *et al.*, 1948) both *Enterobacter* Rahn, 1937 and Enterobacteriaceae Rahn 1937 became illegitimate because they did not conform to the rules which were made retroactive to bacterial names (Anon, 1951). Although the Judicial Commission (Anon, 1958) chose to conserve the name Enterobacteriaceae Rahn 1937 as Enterobacteriaceae Rahn 1937, *nom. fam. cons.* (Opin. 15, Jud. Comm., 1958) it was not included, however, in the Approved List of Bacterial Names (Skerman *et al.*, 1980) and was considered (Lapage, 1979) to have been formed in contravention of Rules 9 and 21a of the Code (Anon, 1951). The proposal (Lapage, 1979) of Enterobacteraceae as an alternative to Enterobacteriaceae has been opposed (Farmer *et al.*, 1980) because the former has not been validly published and thus has no standing. Furthermore, proposals to replace Enterobacteriaceae Rahn, 1937, *nom. fam. cons.* (Opin. 15, Jud. Comm., 1958) with Enterobacteriaceae *fam. nov. nom. rev.* (Ewing *et al.*, 1980) or with Escherichiaceae (Goodfellow and Trüper, 1982) have been made. The genera currently assigned to the family Enterobacteriaceae are given in Table 5. In view of the confusion surrounding the choice of family name noted above, the term Enterobacteriaceae will be used in this thesis, because of its common use in food microbiology.

Sources of Enterobacteriaceae

From the standpoint of contamination of pork with Enterobacteriaceae (Table 6), five main depots of infection can be considered:

Table 5. Members of the family Enterobacteriaceae*

(Buchanan and Gibbons, 1974)	Genera not included in Bergeys Manual (Buchanan and Gibbons,1974 but proposed recently
Citrobacter	*Cedecea*
Enterobacter	*Rahnella*
Escherichia	*Buttiauxella*
Hafnia	*Levinea*
Klebsiella	*Obesumbacterium*
Proteus	*Tatumella*
Salmonella	*Kluyvera*
Serratia	*Xenorhabdus*
Shigella	*Providencia*
Edwardsella	*Morganella*
Erwinia	*Branhamella*
Yersinia	*Ewingella*

* General discussions of the taxonomy of this family have been presented by Johnson *et al.* (1975); Sakazaki *et al.* (1976) and Sackin and Jones (1976).

Table 6. Occurrence of Enterobacteriaceae in meat and meat products

	Reference																
	1 a	1 b	2	3 a	3 b	3 c	4 a	4 b	5	6 a	6 b	6 c	7	8	9	10	11
Aerobacter sp.									22								
Citrobacter sp.									12								
amalonica																	
freundii																	
Enterobacter																	
aerogenes							1/-										
agglomerans							8/18										
cloacae				-/2/-													
liquefaciens													3				
Erwinia carotovora																	
Escherichia sp.									61								
coli							-/31										
Hafnia sp.										+/+/+							
alvei							1/-										
Klebsiella sp.									6	-/+/-							
ozaenae																	
pneumoniae																	
Paracolobactrum sp.																	
Proteus sp.														+	+	+	
rettgeri				1/-/-													
Serratia sp.															+		
liquefaciens				-/-/16			37/-										
marcescens																	
rubidaea							0/1										
Yersinia sp.			+														
enterocolitica	1/4						3/0										+

	Reference																
	12 a	12 b	13	14	15	16 a	16 b	16 c	16 d	17							
Aerobacter sp.										+							
Citrobacter sp.																	
amalonica			2														
freundii	1/-		5	+	+	+/-	/-	/-									
Enterobacter																	
aerogenes	2/-		51	+		+/-	/-	/-									
agglomerans	11/48			+	+	+/-	/+	/+									
cloacae				+	+	+/-	/-	/-									
liquefaciens	-/58																
Erwinia carotovora	-/16																
Escherichia sp.										+							
coli																	
Hafnia sp.																	
alvei	2/-		9	+	+	+/-	/-	/-									
Klebsiella sp.																	
ozaenae	2/1					+/-	/-	/-									
pneumoniae			11	+	+	+/-	/-	/-									
Paracolobactrum sp.										+							
Proteus sp.										+							
rettgeri			3														
Serratia sp.																	
liquefaciens	51/20			+	+	+/+	/+	/+									
marcescens			2			+/-	/-	/-									
rubidaea																	
Yersinia sp.																	
enterocolitica		+	14			+/-	/-	/-									

+ organism isolated Numeric symbols refer to number or percentage of strains isolated

Table 6. Reference

1 Asakawa *et al.* (1979) a, pork, b, sliced ham

2 Aulisio *et al.* (1980) pork

3 Blikstad *et al.* (1981) pork loin; a, fresh, b, air-stored, c, CO_2-stored

4 Cox *et al.*(1979) a, ground beef, b, pork sausage

5 Dowdell and Board (1968) British fresh sausage

6 Eddy and Kitchell (1959) a, beef, b, whale, c, eviscerated poultry

7 Enfors *et al.* (1979) pork loin

8 Haines (1933) pork loin

9 Jensen and Hess (1941) soured hams

10 Kirsch *et al.*(1952) putrid hamburger

11 Lee *et al.*(1980) beef and pork

12 Mercuri and Cox (1979) a, ground beef, b, pork sausage

13 Newton (1979) meat works and vacuum-packed meat

14 Ng and Stiles (1978) ground beef

15 Stiles and Ng (1981a) various meats

16 Stiles and Ng (1981 *b*) a, meat works, b, vacuum-packed beef, c, ground beef, d, frozen pork sausage

17 Sulzbacher and McLean (1951) fresh sausage

1. Animal feeds and ingredients are frequently contaminated with Enterobacteriaceae (Patterson, 1969; Edel *et al.*, 1973; Stott *et al.*, 1975). Levels of contamination of upwards of 1.3×10^5 Enterobacteriaceae/g have been implicated in the spread of *Serratia, Klebsiella, Citrobacter, Proteus* and *Escherichia* in a piggery although pelletting of feeds can lead to a $10^3 - 10^5$ reduction in the numbers of the organisms (Mossel *et al.*, 1967).
2. Most systems of pig husbandry offer little impediment to transfer of micro-organisms between animals. The skin of pigs may harbour a significant number of Enterobacteriaceae and the faeces upwards of 10^8/g (Willingale and Briggs, 1955). In addition to direct contact between pigs, infection *via* faeces, dirt, air and drinking water has been demonstrated (Willingale and Briggs, 1955).
3. Workers and
4. Equipment in the slaughterhouse and processing plant will be involved in the dissemination of Enterobacteriaceae. Chill rooms may contain appreciable populations of psychrotrophic Enterobacteriaceae (Newton *et al.*, 1978).
5. As the gut harbours a large number of Enterobacteriaceae, slaughtering techniques play an important role in the transfer of organisms from a depot to the butchered meat. For example, heavy contamination of the latter can occur when the gut is punctured during evisceration and excision of the rectum can lead also to heavy contamination (Gerats *et al.*, 1981).

The inferences drawn from analyses which showed heavy contamination of uncooked meats with Enterobacteriaceae have tended to be influenced by two assumptions: (1) that heavy contamination is likely to be associated with the presence of *Salmonella* spp., and (2) that heavy contamination is basically an indicator of poor hygiene during slaughter and butchering.

The well founded precept that lactose-fermenting Gram-negative rods - coliforms - and particularly *Escherichia coli* in water is indicative of recent faecal contamination and therefore of the probable occurrence of *Salmonella* and *Shigella* has been accepted uncritically by some food microbiologists (Anon, 1976). Although the MPN coliform index is widely used by them, certain features of the technique - the statistics, interpretation of results and range of organisms isolated - have not been sufficiently stressed. For example, the occurrence of false positive results in water examined for coliforms have been noted repeatedly (Meyer, 1918; Sears and Putnam, 1923; Leitch, 1925; Thompson, 1927; Koser and Shinn, 1927; Greer and Nyhan, 1928; Andrews and Presnell, 1972; Dutka, 1973; Bissonette *et al.*, 1975 and Dutka and Kwan, 1978). Undue emphasis has been given to the isolation of commensal or saprophytic micro-organisms which may or may not be members of the Enterobacteriaceae. Thus Wilson *et al.* (1935) isolated genera of Enterobacteriaceae which are associated principally with vegetation and Hussong *et al.* (1981) identified several aberrant, intermediate groups ("unclassified new species") of Enterobacteriaceae as well as members of the genera *Pseudomonas, Bacillus, Aeromonas* and

Staphylococcus. Although the plasmid borne character (Sackin and Jones, 1976) of lactose fermentation has been a key feature of the indicator organisms used in food or water microbiology, only a few food-borne pathogens of faecal origin have this property e.g. some *Salmonella* serotypes of sub genus I (Threlfell *et al.*, 1983) and III - the "Arizona" group. Thus some food microbiologists (Mossel, 1957; Mossel and Ratto, 1970; Newton, 1979) recommend an examination for all rather than merely the lactose fermenting members of the Enterobacteriaceae. This is achieved by using glucose instead of lactose in isolation and enumeration media.

Psychrotrophic Enterobacteriaceae (e.g. *Enterobacter*, *Hafnia* and *Serratia* spp.), occur and probably grow on unpreserved meat and meat products stored at chill temperatures.

However Newton and Gill (1978) reported that large numbers of lactobacilli on anaerobically stored meat inhibited the growth of a species of *Enterobacter*. Furthermore, Sutherland *et al*. (1975 *a,b*) noted that Gram-negative, fermentative bacilli comprised a small fraction (*ca*. 7%) only of the microflora of vacuum packed meat. The pH of the substrate also influences the rate of multiplication of psychrotrophic strains of Enterobacteriaceae (Newton and Gill, 1980) and Grau (1980) was of the opinion that the type of anion causing a reduction in the pH was an important determinant of the rate of growth of strains of *Enterobacter cloacae*, *Serratia liquefaciens* and *Yersinia enterocolitica*. In comparison with strains of *Pseudomonas*,

the relatively slow rate of growth of the individual contaminants at chill temperatures is the principal factor which selects against the establishment of large populations of Enterobacteriaceae on aerobically stored meat (Gill and Newton, 1977). Thus at temperatures below 20°C, pseudomonads - especially *Pseudomonas fragi* - tend to dominate the microbial blooms on meat (Shaw and Latty, 1982) and in meat products (Molin and Ternström, 1982).

The observations by Brown (1977) that presumptive Enterobacteriaceae grew in unsulphited sausages but to a limited extent only in sulphited ones led to a detailed study of the actual changes in the numbers as well as the types of these organisms in sausages or media having known concentrations of sulphite.

In addition, the search for glucose rather than lactose fermenting organisms in the study concerned with *Salmonella* contamination of sausages and ingredients was done with the objective of assessing the utility of the Enterobacteriaceae count as an index of contamination of the product with these food poisoning organisms.

Brochothrix thermosphacta

As little is known about the natural niche of *Brochothrix thermosphacta (Microbacterium thermosphactum)* and, until recently, its taxonomic relationships were confused, this section gives particular emphasis to the classification of this organism and the factors which appear to favour its growth in meat and meat products such as British fresh sausage.

Nomenclature and taxonomy

The genus *Microbacterium* was proposed for heat resistant, Gram-positive, catalase positive, asporogenous bacilli which had been isolated from dairy sources, especially pasteurized milk and milk products (Orla-Jensen, 1919). Four species were defined: *Microbacterium lacticum, flavum, mesentericum* and *liquefaciens*. In the 5th edition of Bergey's Manual (Bergey *et al.*, 1939) the genus was assigned to the family Bacteriaceae but was transferred to the tribe Lactobacillaae within the Lactobacteriaceae in the 6th edition (Breed *et al.*, 1948). *Microbacterium mesentericum* was renamed *Nocardia mesenterica* and *Microbacterium liquefaciens* was removed to the appendix (Breed *et al.*, 1948). The 7th edition (Breed *et al.*, 1957) excluded *Microbacterium liquefaciens* and established the genus *Microbacterium,* comprising two species, *Microbacterium flavum* and *Microbacterium lacticum,* within the Corynebacteriaceae. The genus was extended to accommodate *Microbacterium thermosphactum* (Buchanan and Gibbons, 1974) despite the fact that neither *Microbacterium lacticum* nor *Microbacterium flavum* were related closely to this new species (Table 7). On the basis of its biological properties (cell morphology, staining reactions, cellular inclusions, GC content of DNA, degree of DNA homology with *Corynebacterium diphtheriae* PW8, and pattern of enzymes) and chemical structure (peptidoglycan type, cell wall polysaccharides, phospholipids, glycolipids and fatty acids), *Microbacterium flavum* Orla-Jensen (1919) has been renamed *Corynebacterium flavescens* (Barksdale *et al.*, 1979) and is recognised by the latter epithet in the Approved

Table 7. Differentiating characteristics of microbacteria*

Character	*MICROBACTERIUM* *thermosphactum*	*flavum/lacticum*	Reference
Pleomorphic growth cycle	+	-	Davidson *et al.* (1968)
Psychrotrophic	+	-	" "
Heat-resistant	-	+†	McLean & Sulzbacher (1953)
Benzidine test	-	+	Deibel and Evans (1960)
Cytochromes	a,b_1,a_3	a,b,c	Davidson and Hartree (1968)
DNA % GC	36	58-64	Collins-Thompson *et al.* (1972)
Operational TCA cycle	-‡	+	" "

* Adapted from Gardner (1981)

† *Microbacterium flavum* not heat resistant (Barksdale *et al.*, 1979)

‡ Disputed by D. Kelly and R.H. Dainty (1983) 'Biosynthesis of glutamate *via* enzymes of the TCA cycle in *Brochothrix thermosphacta*', J. gen. Microbiol., in press.

List of Bacterial Names (Skerman *et al.*, 1980). *Microbacterium lacticum* is considered by some to be misplaced in the genus *Microbacterium* and a proposal to rename it as *Aureobacter liquefaciens* nom. nov. within the family Corynebacteriaceae has been made (M.D. Collins, pers. comm.). The name *Microbacterium thermosphactum* was coined by McLean and Sulzbacher (1953) for a heat sensitive contaminant of fresh pork sausage. This organism was not included in the 7th edition of Bergey's Manual (Breed *et al.*, 1957) but was assigned to the coryneform group in the 8th edition (Buchanan and Gibbons, 1974). Its taxonomic position remains uncertain, it being considered as a species *incertae sedis* in the current Approved List of Bacterial Names (Skerman *et al.*, 1980). Some workers have suggested that *Microbacterium thermosphactum* is probably related to the lactic acid bacteria (Diebel and Evans, 1960; Barlow and Kitchell, 1966) whereas others (Davidson, 1970) have supported its inclusion within the Corynebacteriaceae. The absence of unsaturated and cyclopropane fatty acids, both of which are commonly found in lactobacilli, from the lipids of *Microbacterium thermosphactum* (Shaw and Stead, 1970) and the presence of a functional cytochrome system (Davidson and Hartree, 1968; Kelly and Dainty, unpublished observations) would lend support to the views of the latter; The exacting nutritional requirements (London, 1976) in the absence of ammonium ions and cell wall type (Schleifer, 1970; Schleifer and Kandler, 1972) recognized the unsatisfactory to the former. Sneath and Jones (1976) classification of this organism by McLean and Sulzbacher (1953) and assigned it to a new genus, *Brochothrix*, containing one species. They proposed also that the new genus be assigned to

the Lactobacillaceae. These proposals were supported by Wilkinson and Jones (1977) who, in a taxonomic study of *Listeria* and related organisms, demonstrated that, although *Microbacterium thermosphactum* was related to the genus *Lactobacillus*, it formed a distinct phenon worthy of generic rank. The supposition that *Microbacterium thermosphactum* was related to *Kurthia* (Buchanan and Gibbons, 1974) was not supported by others (Davies *et al.*, 1969; Jones, 1975; Wilkinson and Jones, 1977; Shaw and Keddie, 1983).

Niche

Despite the numerous reports of the occurrence of *Brochothrix thermosphacta* in many types of meat product, its precise ecological niche is not known (Gardner, 1981). *Brochothrix thermosphacta* has been isolated from lairage slurry, cattle hair, rumen contents, slaughter hall soil, hands of workers, carcasses and chill room air (Patterson and Gibbs, 1978). The common occurrence of this organism on the hands of workers, equipment and carcasses during "boning-out" operations (Newton *et al.*, 1978; Patterson and Gibbs, 1978) indicate that ample opportunity exists for the transfer of this organism from these sites to the meat or products made therefrom.

Muscle *post rigour* is likely to contain sufficient L-lactate to prevent anaerobic growth of *Brochothrix thermosphacta* on chilled carcasses but the ability of the organism to multiply in the presence of this acid under aerobic conditions is well known (Grau, 1980). Despite the absence of esterases capable

of degrading fats containing fatty acids > C_{12} (Collins-Thompson *et al.*, 1972), glycerol is readily metabolised under aerobic conditions. Lipolytic action of meat or other microbial systems would thus appear to be a prerequisite, however, for the growth of *Brochothrix thermosphacta* on this substrate. Carbohydrates would appear to be the preferred metabolites; high glucose concentrations and an acidic pH favouring acetoin and acetic acid production, low glucose concentrations, causing the accumulation of *iso*butyrate and *iso*valerate from the breakdown of valine and leucine (Dainty and Hibbard, 1980). Neither the pH nor the temperature of carcass meat is likely to curtail the growth of *Brochothrix thermosphacta*. Although the optimum pH for growth is 7.0, it will tolerate a range of pH (5.0 - 9.0; Brownlie, 1966) and proliferate on carcass meat of pH 5.4 - 5.5 (Patterson and Gibbs, 1977). *Brochothrix thermosphacta* will grow at $0^{o}C$ (Brownlie, 1966) and has a high energetic efficiency over a wide range of temperatures (Rogers *et al.*, 1980).

The advent of wrapping films having a range of gas permeability (Barlow & Kitchell, 1966; Gardner *et al.*, 1967; Shay *et al.*, 1978) and their use in modified-atmosphere packaging (Weidemann, 1965; Davidson, 1970) led to the recognition of *Brochothrix thermosphacta* in the spoilage of meat and meat products. Some early reports noted the presence of "coryneform bacteria" or Gram positive bacilli on meat (Haines, 1937). Such organisms have been found on a variety of meats: beef (Rogers and McCleskey, 1957; Wolin *et al.*, 1957; Ayres, 1960; Weidemann, 1965), poultry (Thornley, 1957; Barnes and Shrimpton, 1968; Barnes

et al., 1979), lamb (Barlow and Kitchell, 1966; Newton *et al.*, 1978) and pork (Gardner *et al.*, 1967; Gardner and Patton, 1969). Comminution of meat, as in the production of sausage, appears to result in an increased level of contamination with *Brochothrix thermosphacta* and large populations have been reported in American pork sausage (Sulzbacher and McLean, 1951; Miller, 1964), irradiated frankfurter (Drake *et al.*, 1958) and British fresh sausage (Gardner, 1966; Leaton, 1968; Dowdell and Board, 1967, 1968, 1971). Cured sausage rarely contains *B. thermosphacta*, a situation which is probably attributable to the sensitivity of the organism to the NO_2^- ion (Gardner, 1981).

In the present study, *Brochothrix thermosphacta* was included in the studies concerned with sulphite tolerance of members of the association of British fresh sausage with the objective of assessing the contribution which this organism's tolerance to the preservative makes to its success as a colonizer of British fresh sausage.

Yeasts

The literature on the sources, types and behaviour of yeasts in meat and meat products is scant (Walker and Ayres, 1970). Their metabolic activity and biomass (Rose, 1976) would be expected to cause perceptible changes in foods and on occasions to favour growth of and spoilage by other micro-organisms. In practice, several factors are known to influence the ability of yeasts to compete with or aid other micro-organisms in food

e.g. numbers and types of yeasts, availability of nutrients, pH, redox potential, temperature, water activity (a_w) and presence of preservatives (Ingram, 1958).

Yeasts are ubiquitous (Lodder, 1970) but seldom cause spoilage of fresh red meats because of their slow rates of growth at refrigeration temperatures (Ayres, 1960), and their low level of initial contamination (Table 8). If bacterial growth is restricted, by irradiation or by antibiotics, yeasts may attain large populations on carcass or processed meat (Barnes *et al.*, 1979). Low a_w is another well-known elective factor (Scott, 1936, 1957) which operates in products such as salami, pepperoni, cervelat or Thuringer (Cesari, 1919; Cesari and Guilliermond, 1920). Some asporogenous, lipolytic yeasts do grow on meat, especially the fatty tissue, at low temperatures (Lea, 1931 *a,b;* Vickery, 1936) and they can attain populations comparable numerically to those of bacteria. Indeed Ingram (1962) was of the opinion that the importance of these lipolytic yeasts in the spoilage of meat had been generally underestimated by food microbiologists.

Reports of yeast contamination of sausages generally refer to products which have been heated, dried, smoked or fermented. Thus, Oglivy and Ayres (1953) reported a yeast-dominated microflora in packed frankfurter (a cooked, smoked product). Large populations of yeasts, especially on the outer surface, were associated with the spoilage of skinless pork sausage - a partly cooked version of British fresh sausage (Hockley, 1980; Legan, 1981). Yeast production of slime on sausages is well documented *viz.*

Table 8. Occurrence of yeasts in meat and meat products

Organisms identified with:	Reference												
	1	2	3	4	5	6	7	8	9	10	11	12	13
Aureobasidium pullulans								+					
Bullera alba											+		
Candida sp.	+		+	+	+			+		+	+	+	+
albicans											+		
curvata										+			
caninulata													+
humicola			+									+	
krusei								+					
lipolytica				+							+		+
mesenterica												+	
rubra			+										
rugosa											+		
saké										+			
tropicalis			+										
utilis			+										
valida											+	+	
vini												+	
zeylanoides					+						+	+	+
Cryptococcus sp.										+	+	+	
albidus										+	+		
gastricus											+	+	
hungaricus												+	

Table 8 continued

Organisms identified with:	Reference												
	1	2	3	4	5	6	7	8	9	10	11	12	13
Cryptococcus (contd.)													
infirmominiatus												+	
laurentii												+	
macerans												+	
melibiosum											+		
Debaryomyces kloeckeri													+
nicotianae													+
sub-globosus													+
Hansenula sp.			+										
anomola			+										
polymora											+		
subpelliculosa			+										
Kluyveromyces sp.			+										
Leucosporidium sp.			+								+	+	
scottii											+		
Rhodotorula sp.	+		+	+		+	+	+	+	+	+	+	
glutinis			+										
graminis										+			
rubra			+								+	+	
Saccharomyces sp.			+										
cerevisiae			+										
Sporobolomyces sp.			+										
Torulopsis sp.	+	+						+			+	+	+
gropengiesseri													+

Table 8 continued

Organisms identified with:	1	2	3	4	5	6	7	8	9	10	11	12	13
Torulopsis cont.													
famata											+		+
candida								+			+	+	+
versatalis											+		
Trichosporon sp.			+		+			+		+	+	+	+
beigelii										+			
cutaneum			+										
pullulans			+		+			+					+
Pichia etchellsii											+		

References:

1 Ayres (1960) chilled meat

2 Brooks and Hansford (1923) fresh beef

3 Davenport (1980) frozen meat and meat products

4 Ingram (1958) meat, ham treated with chlortetracyclines

5 Lodder (1970) chilled beef

6 Njouku-Obi *et al.* (1957) chilled beef, poultry

7 Walker and Ayres (1959) chilled meat

8 Walker and Ayres (1970) red meat, fish and poultry

9 Wells and Stadelmann (1958)

10 Dijkmann *et al.* (1980) minced meat

11 H. Dalton unpublished observations, sulphited and unsulphited sausages

12 H. Dalton unpublished observations, beef mince

13 Drake *et al.* (1958, 1959) frankfurters

saucisson (Cesari, 1919; Cesari and Guilliermond, 1920), weiner (Mrak and Bonar,1938; Mrak and Phaff, 1948) and British fresh sausage (Dowdell and Board, 1971).

The resistance of yeasts to sulphur dioxide or sulphite (Rehm and Wittman, 1962; Dennis, 1978; Dennis and Harris, 1979) has led to the surmise that their growth in British fresh sausage may be favoured (Brown, 1977) but Dyett and Shelley (1966) noted that, in most instances, the climax populations of yeasts in sulphited sausage were 1 - 2 log cycles lower than those in the unsulphited product. Their conclusion that sulphite at 450 ppm inhibited the growth of yeasts was not in accord with the many observations of others (e.g. Dowdell and Board, 1967, 1968; Ashworth *et al.*, 1974; Brown, 1977). Brown (1977) was of the opinion that yeasts were mainly responsible for producing sulphite-binding agents and that the growth of some of the bacterial contaminants may be dependent upon the consequent reduction in the concentration of free sulphite. This observation, together with the discordant views on the influence of the preservative on yeast growth *per se* led to the present study of a number of yeasts for sulphite-tolerance and potential to produce sulphite-binding compounds.

Sulphite preservation

Although the salts of sulphurous acid have been used as preservatives in a wide variety of foodstuffs (Schroeter, 1966; Roberts and McWeeny, 1972), they are of particular value in products having an acidic pH reaction, e.g. cider (Burroughs and

Sparks, 1964), orange juice (Ingram and Vas, 1950 *a,b*) fruit pulp (Robson, 1968), jams (Dennis and Bugahiar, 1980) and wines (Faparusi, 1969; Okafor, 1975; King *et al.*, 1981). This situation obtains because SO_2, the most antimicrobial moiety formed by dissociation of sulphurous acid is at a maximum concentration (Hammond and Carr, 1976). As SO_2 is a particularly reactive molecule (Joslyn and Braverman, 1954), having the potential to combine with many compounds in aqueous solution (e.g. aldehydes, ketones, olefins, sugars, organic acids, thiol groups, enzymes, cofactors, vitamins, nucleic acids, amino acids and lipids, Baird-Parker, 1980) , it is used also as an antioxidant (e.g. in potatoes, Lund, 1968) and an inhibitor of enzymic and non-enzymic (Burton *et al.*, 1963), browning in pickles and confectionary (Sullivan, 1971), and vegetables (Moussa, 1973). It needs to be stressed that the vast literature on the mechanism of SO_2-preservation in acidic foodstuffs has tended to direct attention away from the situation which obtains in sulphited products poised at, or near, a neutral pH. In the light of the present study on sausage preservation only the literature pertaining to the use of sulphite in "neutral pH" meat products will be considered in detail.

Sulphite preservation of meat products

Metabisulphite or sulphite is included in fresh sausage primarily to delay microbial spoilage. The content of preservative in the product has been determined traditionally by collection of the sulphur dioxide (SO_2) released from a sample suspended in boiling

acid (Monier-Williams, 1927). Indeed this analytical procedure has led to the general assumption that SO_2 *per se* is the only antimicrobial moiety. In practice, the pH of sausage (6.8 - 6.2) would not be expected to cause significant SO_2 formation from metabisulphite and sulphite (Table 9, Ingram, 1948; King *et al.*, 1981) and it is probable that bisulphite (HSO_3^-) and sulphite (SO_3^{2-}) are the active agents (Hammond and Carr, 1976). Vas and Ingram (1949) were probably the first food microbiologists to establish that the pH of a food product was an important determinant of the efficacy of SO_2 and they published a set of curves showing the proportions of the various molecular species based on the value of 5×10^{-6} for K_2 (2nd dissociation constant). Although these curves have been reproduced widely in the literature - e.g. Rehm and Wittman, (1962) - King *et al.* (1981) were of the opinion that they are in error by two full pH units (7.2 *vs.* 5.3) at the pK_2 point. But even with their calculated percentage (Table 9) of each molecular species of undissociated sulphurous acid (H_2SO_3) in the range of pH, 7.5 - 5.0, based on a K_1 of 1.7×10^{-2} and a K_2 of 6.31×10^{-8} (pK_1 = pH 1.77 and pK_2 = pH 7.20), SO_2 forms a negligible proportion. Although it is well known that the absolute content of SO_3^{2-}/HSO_3^- in sausage diminishes with time due to oxidation, little is known about the extent of binding of HSO_3^- and SO_3^{2-}. Oxidation of HSO_3^- and SO_3^{2-} to SO_4^{2-} is well documented (Abel, 1913; Anderton and Locke, 1956; Fridovitch and Handler, 1961; Hibbert, 1970) and may account for the irretrevable loss of preservative from stored sausage studied by Brown (1977). It has been established also that reversible combination of SO_3^{2-} with pyruvate (Burroughs and

Table 9. Percentage distribution of molecular species of sulphurous acid as a function of pH*

pH	% H_2SO_3	% HSO_3^-	% SO_3^{2-}
5.0	0.0580	99.31	0.63
5.5	0.0180	98.05	1.93
6.0	0.0060	94.15	5.84
6.5	0.0015	83.55	16.45
7.0	0.0002	61.30	38.70
7.5	0.0000	33.70	66.30

* Adapted from King *et al.* (1981)

Sparks, 1964), acetaldehyde and ketones (Joslyn and Braverman, 1954) to form hydroxysulphonates, glucose and maltose (Ingram and Vas, 1950 *a*,*b*) to form addition complexes or amines (Joslyn and Braverman, 1954) to produce amine-bisulphites can occur. As such compounds show little antimicrobial activity (Neuberg, 1929; Rehm, 1964), the present study was concerned with the development of an analytical procedure which would estimate the concentration of unbound (free) HSO_3^-/SO_3^{2-} referred to as 'free sulphite' for convenience.

Lafontaine and his co-workers (1955) were perhaps the first to note that the addition of sulphite to minced meat resulted in a failure of the resident microflora to grow. This situation was dependent not only on the concentration of sulphite but also on the temperature of storage and the initial concentration of micro-organisms. Thus a concentration of 300 µg/g total sulphite (as SO_2) caused bacteriostasis at 4°C but not at 20°C whereas higher concentrations (1000 and 3000 µg/ml) were effective at both temperatures. It is notable that these workers observed that the addition of sulphite favoured the growth of anaerobic micro-organisms and that the efficacy of preservation was inversely related to the initial size of the microbial population. Furthermore broth studies using selected isolates from minced meat corroborated the above observations. Krol and Moerman (1959/60) confirmed the findings of Lafontaine *et al.* (1955) and reported also that microbial growth, as indicated by a total viable count, was restricted for up to 6 days at refrigeration temperatures in sulphited (300 µg/g) minced meat balls and a

concentration of 900 µg/g resulted in the death of micro-organisms. Even at the former concentration of sulphite, the Enterobacteriaceae were particularly sensitive with upwards of a 90% kill during refrigerated storage for 6 days. Fournaud *et al.* (1971) also observed that sulphite was a most effective preservative against the Enterobacteriaceae and microbial contaminants in general in the minced pork ingredients of saucissons. Dyett and Shelley (1962) commented on the inhibition of growth of the Enterobacteriaceae - *Salmonella* spp. in particular - in sulphited pork and beef (Dyett and Shelley, 1966) sausages. Three observations in the latter report are of especial importance: firstly that with incubation at temperatures below 22°C, sulphite was most potent against the 'coli-aerogenes' group and other Gram-negative bacteria in general; secondly, that addition of sulphite resulted initially in a kill of the numerically dominant micro-organisms, and thirdly that it retarded the growth rate of the microbial association thereafter. According to Christian (1963) sulphite, at a concentration of 3.5 grains/lb was able to extend the shelf life of minced beef 2 - 3 fold with storage at 41°F. In accord with the above observations of Dyett and Shelley (1962, 1966) and Krol and Moerman (1959/60), Christian noted that addition of sulphite increased the "lag" period and suppressed the rate of growth of the dominant micro-organisms at 34° and 41°F. Furthermore there was some evidence that the preservative exerted a selective action on the microbial association: with storage of unsulphited minced beef at low temperatures, Gram-negative rods predominated whereas in the presence of sulphite short Gram-positive rods were numerically dominant. In the former case a

"putrid" spoilage was evident when microbial numbers had reached *ca.* 100 x 10^6 c.f.u./g whereas "souring" - a feature of sulphited mince - could not be demonstrated until a viable cell concentration > 500 x 10^6 c.f.u./g had been achieved. Gardner (1968) could not reproduce all of the findings of Christian (1963) with respect to sulphite preservation of vacuum-packed baconburgers. The former worker found that the composition of the normal microflora was not influenced by sulphite concentration. It should be recognised that before the addition of sulphite the product contained nitrite and nitrate which were likely to affect the composition and behaviour of the microbial association as well as negating the antimicrobial effect of sulphite (Tompkin *et al.*, 1980). With storage at either 5°, 10° or 22°C, however, sulphite exerted initially a lethal effect on the dominant micro-organisms and retarded the rate of their growth thereafter (Gardner, 1968). Thus with addition of sulphite, extensions to the shelf life of baconburgers were of the order of 2d, 10 d and 28 d at 22°C, 10°C and 5°C respectively. In the light of the present study it is noteworthy also that, regardless of the temperature of storage, sulphite prevented an acid drift in the baconburgers over the period of storage.

An investigation of the influence of sodium sulphite on *Escherichia coli, Salmonella typhimurium* and micro-organisms indigenous to raw minced meat (Moerman *et al.*, 1966) revealed that, with incubation at 15°C, a concentration of 0.03% sulphite caused bacteriostasis of these organisms but it was less effective against the normal microflora. With incubation at 20° or 25°C the addition of the preservative had a negligible effect on the rate

or extent of growth of any of the micro-organisms sought. Moerman and his co-workers (1966) were able to demonstrate a significant decrease in the concentrations of total sulphite over the storage period. Thus with incubation for 20 h at 37°C, only 110 µg/g of the original 300 µg/g sulphite could be recovered whereas after 41 h at 25°C, 170 µg/g remained. The utility of sulphite in the preservation of refrigerated raw mince was confirmed also by Mulder (1969) who showed that with storage at 1°C, addition of sulphite prevented microbial growth for upwards of 7 days.

Following an extensive survey (Dowdell and Board,1968) of British fresh sausages offered for sale at retail outlets, Dowdell and Board (1971) were able to define the composition of the "microbial association" (pp. 16 - 19) of the product. They observed also that the yeasts and *Microbacterium thermosphacta (Brochothrix thermosphactum)* - dominant members of this microbial association - were capable of profuse growth in sulphited culture media whereas pseudomonads and coliforms isolated from fresh sausage were not. The elective nature of sulphite for a particular type of microbial association in fresh sausage meat was first proposed by Hurst (1972) and confirmed by Brown (1977). A comparison of various systems of preservation for British fresh sausage manufactured on a small scale (Ashworth *et al.*, 1974) confirmed the observations of Dowdell and Board (1968, 1971) that *Microbacterium thermosphactum (Brochothrix thermosphacta)*, lactobacilli, micrococci and yeasts were the dominant microbial groups in the sulphited product. Ashworth and his co-workers (1974) demonstrated that although sulphite curtailed the proliferation of

coliforms, *Microbacterium thermosphactum* and pseudomonads, the lactobacilli, micrococci and yeasts were unaffected.

Furthermore they noted that a combination of polyphosphate and sulphite acted synergistically to reduce the rate of growth of the sensitive microbial groups. Indeed the addition of polyphosphate enhanced the antimicrobial action of sulphite against lactobacilli, pseudomonads and yeasts in the sausage meat studied by Tyson (1976). She concluded that sulphite alone delayed the onset of spoilage of fresh sausage principally by retarding the rate of growth of the lactobacilli. The preservative enhanced the rate and extent of growth of the yeasts, whilst only permitting growth of the pseudomonads when the concentration of the active (free sulphite) moiety had fallen below a critical level.

The role of sulphite in sausage preservation was studied in more detail by Brown (1977) who demonstrated that sulphite inhibited the growth of coliforms at chill temperatures only. He was of the opinion that sulphite "steered" the microbial association towards a "fermentation" dominated by *Microbacterium thermosphactum* and lactobacilli. He proposed also that free sulphite was bound by unidentified compounds produced by the developing microflora as well as by meat and rusk components.

The above review of the limited literature concerned with the nature of sulphite-preservation of neutral pH meat products has revealed several features. It has shown firstly, that the

preservative is effective against micro-organisms only when present in the free (unbound) state. Secondly, that sulphite is most efficient at low temperatures and its potential to inhibit microbial growth decreases with time. Thirdly, that sulphite appears to elect for a Gram-positive/yeast-dominated microflora, which invariably results in spoilage due to "souring", perhaps through selectively inhibiting Gram-negative micro-organisms - particularly the coliforms and Enterobacteriaceae. It is against this background of meat microbiology that the wider scope of literature concerned with the *modus operandi* of the salts of sulphurous acid against micro-organisms is now set.

Factors influencing the antimicrobial action of SO_2 and sulphite

Above all else it is evident that the efficacy of the salts of sulphurous acid depends on the degree of ionization of the molecule (Douglas, 1966; Hammond and Carr, 1976), and the literature tends to support the view that the antimicrobial efficacy decreases in the order: SO_2 (H_2SO_3) > HSO_3^- > SO_3^{2-} (Rehm and Wittman, 1962, 1963). Thus before conclusions regarding the sensitivity of particular micro-organisms to sulphite can be made, it is important that the pH of the test system is known. For example, the inhibition by sulphite of photosynthesis and respiration in unicellular green and nitrogen fixation in blue-green algae (Babich and Stotzky, 1974) has been shown to be strongly pH dependent. This phenomenon was also a feature of sulphite toxicity to fungi and coliphage (Babich and Stotzky, 1978) although broth studies indicated that a concentration of

5×10^{-2} M SO_2 (as SO_3^{2-} or HSO_3^-) at pH 7.0 would nevertheless still inhibit *Escherichia coli, Pseudomonas aeruginosa, Bacillus cereus* and *Serratia marcescens*. Vas and Ingram (1949) reported that the rate of multiplication of yeasts was directly proportional to the concentration of SO_2 (range, 0 - 560 mg/ℓ) at pH 3.4. The rate was retarded at pH 2.88. Rehm and Wittman (1962) quoted minimum inhibitory concentrations of SO_2 over the pH range 2.5 - 5.0 for other micro-organisms, (µg/ml in parentheses): *Saccharomyces cerevisiae* (80-160), *Saccharomyces ellipsoides* (20-80), *Hansenula anomola* (240), *Mucor* spp. (30-60) and *Penicillium* spp., (20-400).

Like most undissociated antiseptic acid molecules, SO_2 probably penetrates the microbial cell more rapidly than ionic species (Ingram *et al.*, 1956; Rahn and Conn, 1944; Oka, 1964). Some workers (e.g. Rahn and Conn, 1944; Macris and Markakis, 1974; King *et al.*, 1981) claim that undissociated H_2SO_3 is the only antimycotic moiety and others (Faparusi, 1969; Okafor, 1975; Hammond and Carr, 1976) state that at commercial concentrations even SO_2 is unsatisfactory for the preservation of products (e.g. palm wine) which have a pH reaction > 4.0.

The efficacy of sulphite is also influenced drastically by the presence and activity of binding agents (Hammond and Carr, 1976). Binding of sulphite has been demonstrated by many workers (e.g. Farnsteiner, 1904; Prater *et al.*, 1944; Ponting and Johnson, 1945; Ingram, 1948; Gehman and Osman, 1954; Richardson, 1970) and a list of compounds in sausage which are likely to bind $SO_2/HSO_3^-/SO_3^{2-}$ has been compiled by Brown (1977). Of primary importance

are carbonyls e.g. aldehydes, ketones and sugars (Lück, 1977); aldehydes forming hydroxysulphonates, amines forming amine-bisulphites (Joslyn and Braverman, 1954) and sugars forming addition compounds (Ingram and Vas, 1950 *a,b*). An acidic pH (3 - 5) favours the formation of such complexes (Rehm and Wittman, 1962, 1963) although temperature and concentration of reactants also influence the rate and extent of binding (Burroughs and Sparks, 1964). Binding compounds may be present in food (Ingram and Vas, 1950 *a,b*), formed during storage (Burroughs and Sparks, 1964) or produced by moulds and bacteria (Burroughs and Sparks, 1964; Weeks, 1969; Rankine and Pocock, 1969).

It is generally accepted that only free (unbound) sulphite is antimicrobial (Neuberg, 1929; Ingram, 1948; Rehm, 1964). Thus the claim (Fornachon, 1963 ; Lafonlafourcade and Peynaud, 1974), that bound sulphite killed micro-organisms is probably illfounded and due to dissociation of the complexed sulphite producing small concentrations of free preservative.

The salts of sulphurous acid are reported to be more effective against moulds than yeasts (Robson, 1968; Roberts and McWeeny, 1972; Baird-Parker, 1980). Of the yeasts, those with an oxidative metabolism (e.g. *Pichia membranaefaciens, Debaryomyces* spp. and *Rhodotorula* spp.) are considered (Rehm and Wittman, 1962) to be less resistant than those capable of fermentation (e.g. *Hansenula* spp., *Saccharomyces* spp.) and Reed and Peppler (1973) noted that *Pichia membranefaciens* and *Kloekera apiculata* were 'SO_2-sensitive' whereas *Saccharomycodes lugwidii, Saccharomyces baileii* and

Brettanomyces spp. would resist up to 500 µg/ml SO_2. It has been suggested that differential uptake of, and affinity for SO_2 is the reason for the apparent resistance of some yeasts, (Hammond and Carr, 1976) although uptake is now believed to be by passive diffusion (Stratford, 1983) and not by active transport (Macris and Markakis, 1974). Adaptation of yeasts to SO_2 tolerance has been reported also (Schanderl, 1959; Robson, 1968).

Bacteria are among the most sensitive micro-organisms to sulphite. Rehm and Wittman (1962) noted the following minimum inhibitory concentrations at pH 6.0 (µg/ml SO_2 in parentheses): *Pseudomonas fluorescens* (50), *Escherichia coli* (100-200), *Staphylococcus aureus* (80), *Bacillus* spp. (50) and *Lactobacillus casei* (100). It has been suggested also (Roberts and McWeeny, 1972) that Gram-negative are in general, more sensitive than Gram-positive bacteria.

Mechanism of action of sulphite

Several potential targets of microbial metabolism may be perturbed by sulphite. Most workers (e.g. Freese *et al.*, 1973; Hammond and Carr, 1976; Lück, 1977)are of the opinion that sulphite inhibition results from multisite disruption of metabolism.

It has been concluded by some (Macris, 1972; Macris and Markakis, 1974) but not all (Stratford, 1983) workers that SO_2 is actively transported into the yeast cell. The former workers

were able to demonstrate saturation kinetics with increasing SO_2 concentration whereas Stratford (1983) contends that transport is by diffusion only. Indeed he associated the low rate of diffusion of SO_2 into the cells of *Saccharomycodes ludwigii* with the organism's tolerance of 10 times the concentration of SO_2 which would inhibit *Saccharomyces cerevisiae* for example. Low (*ca.* 1 µg/g) concentrations of molecular SO_2 can inhibit, and higher concentrations, kill yeasts (Schimz, 1980), and stationary-phase cells are more sensitive than those in active growth (Schimz, 1980). Following exposure to sulphite, most micro-organisms exhibit a lag period before cell viability decreases rapidly. Sulphite damage in yeasts is usually irreversible after *ca.* 60 min exposure (Schimz and Holzer, 1979) and A.T.P. is released into the medium. A possible reason for the efflux of A.T.P. from yeast has been suggested by Warth (pers. comm.). He claims that sulphite lowers the internal pH of the cell. Thus when SO_3^{2-} enters the cell as an uncharged molecule, it becomes ionized due to the higher pH of the interior of the yeast cell and releases protons which lower the pH. As this would result in the disappearance of the membrane proton gradient, the yeast would expel A.T.P. in an attempt to release protons and re-establish the gradient.

It has long been known that the addition of sulphite to growing cells of *Saccharomyces cerevisiae* caused an increase in the concentration of glycerol in the medium (Neuberg and Reinfurth, 1918, 1919); small doses of sulphite added at intervals prevent cytotoxicity and merely "steer " the fermentation (Freeman and

Donald, 1957). There has been speculation that the glycerol formed is from glyceraldehyde-3-phosphate as a result of blockage of glycolysis (Gancedo *et al.*, 1968). Indeed, Stratford (1983) found that in aerobically-grown cells of *Saccharomyces cerevisiae* the rate of decarboxylation by pyruvate decarboxylase was maintained in the presence of sulphite despite binding of SO_3^{2-} to acetaldehyde. It was probable therefore, that NAD^+ was regenerated using oxygen as the terminal electron acceptor. Sulphite may then prevent A.T.P. generation from glycolysis or increase the rate of A.T.P. utilization. In the first instance, sulphite would act as an uncoupler of glycolysis thus facilitating intermediate flow to be maintained. whilst preventing concomitant ATP generation. In anaerobically-grown cells of this organism, Stratford (1983) noted that SO_3^{2-} bound intracellular acetaldehyde thereby preventing the regeneration of oxidised NAD^+ by ethanol dehydrogenase. Shortage of NAD^+ would prevent activity of glyceraldehyde-3-phosphate dehydrogenase and therefore prevent flow of intermediates *via* glycolysis and further decarboxylation of pyruvate. Accumulation of glyceraldehyde-3-phosphate would promote the formation of glycerol and regenerate some NAD^+. No net energy (as ATP) however, could be formed by this means.

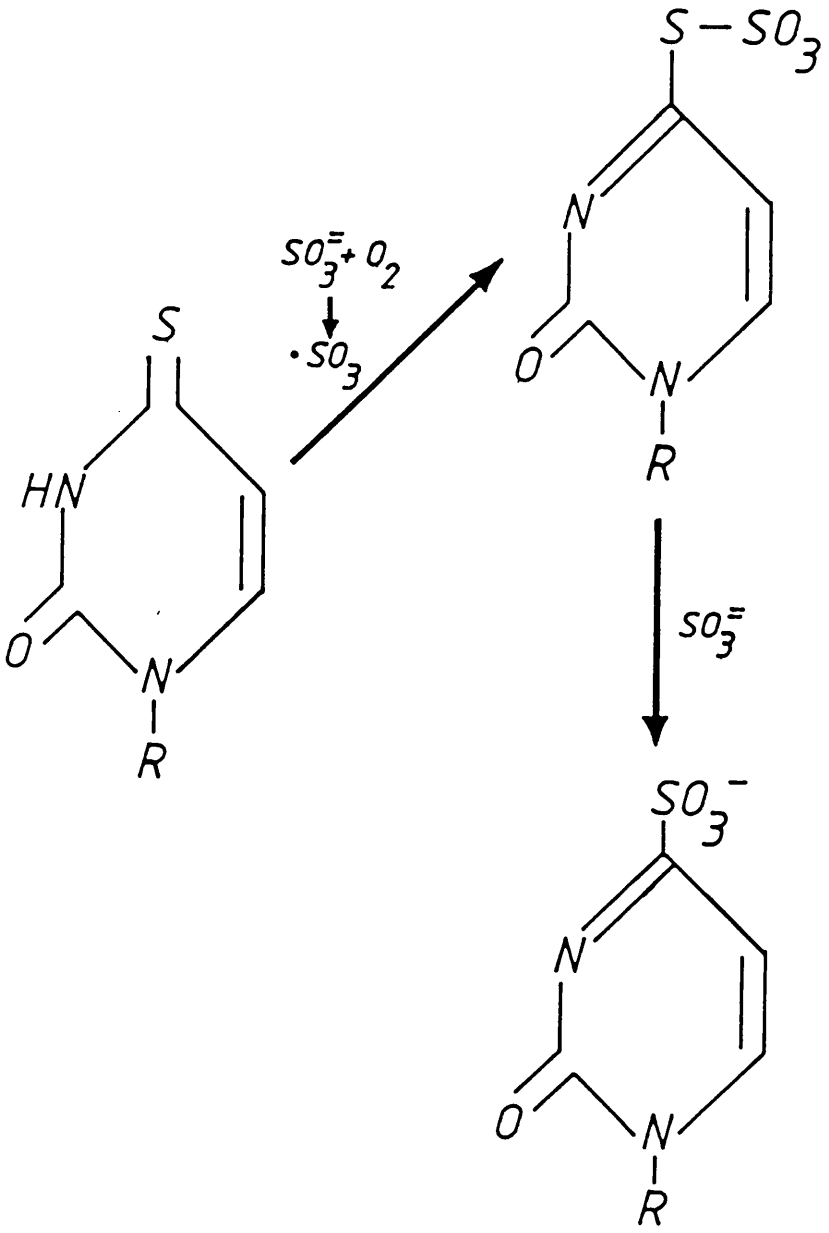
Such observations are in accord with those of others (e.g. Pfleiderer *et al.*, 1956) who showed that sulphite was active against -SH groups and was a powerful inhibitor of NAD-dependent reactions. Indeed, SO_2 can react directly with nicotinamide adenine dinucleotide (NAD) thus preventing the occurrence of energy-yielding oxidative reactions (Meyerhof *et al.*, 1938; Dupuy, 1959). Rehm (1964)

indicated that at least three steps in glycolysis in *Escherichia coli* and *Saccharomyces cerevisiae* were strongly inhibited by sulphite. The blocking of the glyceraldehyde-3-phosphate → 1,3-diphosphoglycerate step is probably the most crucial in yeasts whereas the NAD-dependent formation of oxaloacetate from malate is more important in *Escherichia coli* (Wallnöfer and Rehm, 1965).

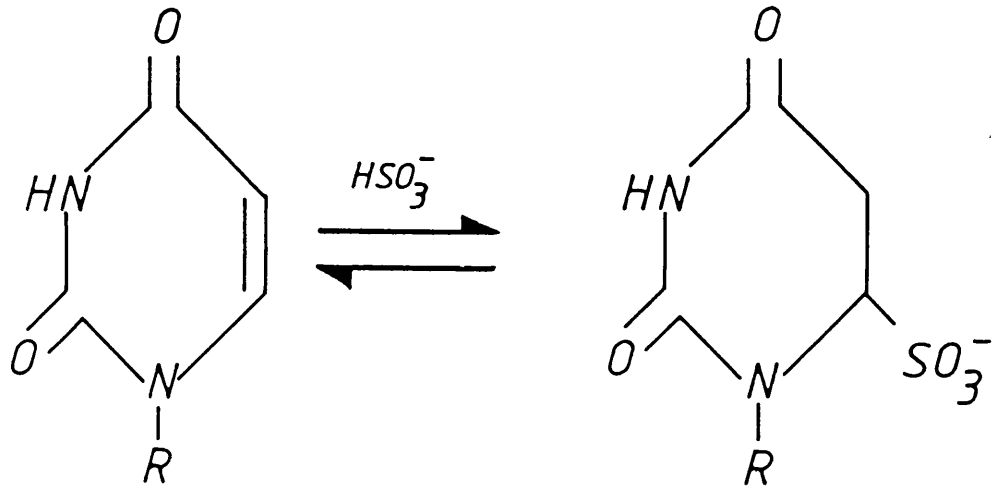
Several other reactions involving sulphite have been mentioned in the literature. For instance sulphite may inhibit growth by reacting with products of intermediate metabolism thus shifting the reaction equilibria to thermodynamically-inefficient states (Rehm and Wittman, 1962), or reversibly inhibiting β-galactosidase induction, RNA and protein synthesis in *Escherichia coli*. (Robakis, 1980). Sulphite may also break disulphide bridges by replacing the sulphur group from the bond of cystine or cystine peptides (Clarke, 1932) and as sulphonation of disulphide bridges in enzymes can lead to loss of enzyme function it is a likely site of sulphite toxicity. Some of the reactions of bisulphite and sulphite with important cell constituents are outlined below:

Reaction of HSO_3^- with 4-thiouridine

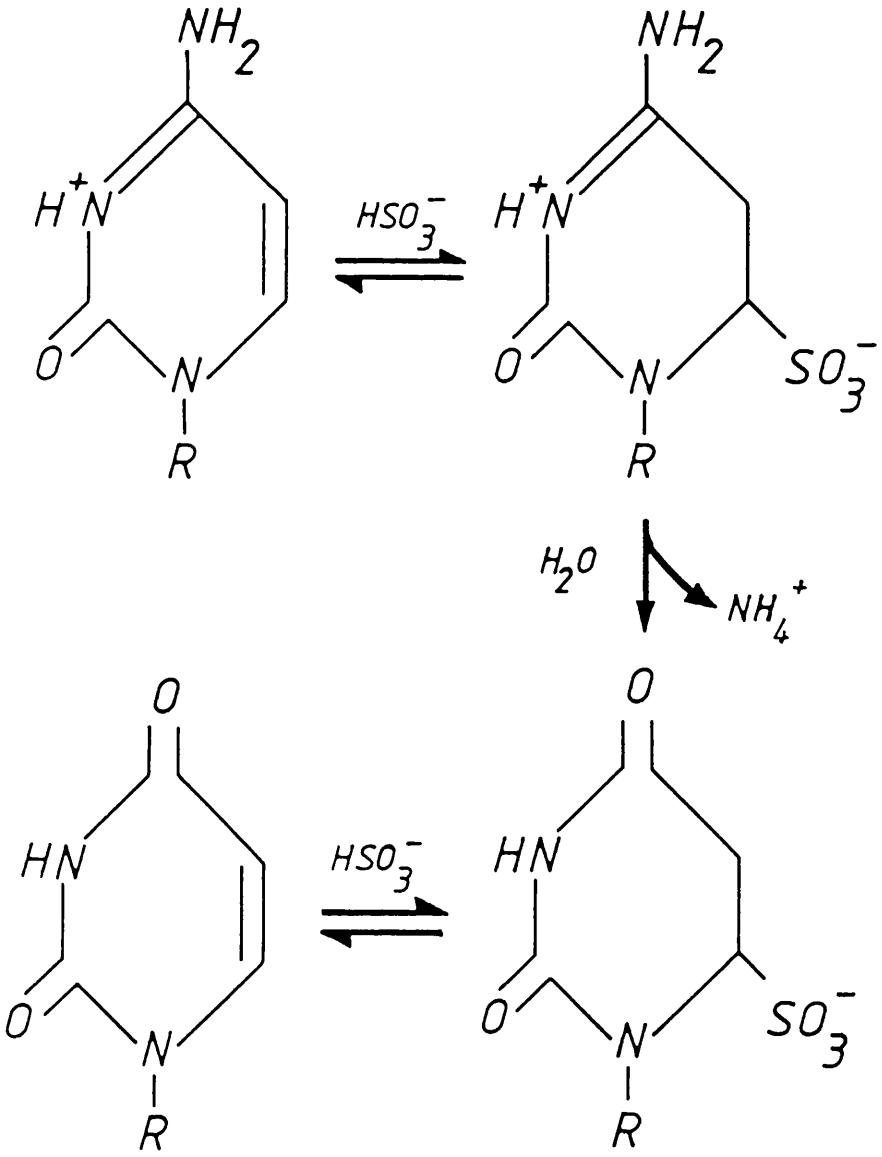
4-Thiouridine occurs in many bacterial tRNA's between the dihydrouridine stem and the CCA stem (Nishimura, 1972) and HSO_3^- reacts *via* an O_2-mediated free radical mechanism:


S
HN
O
N
R
$SO_3^= + O_2$
$\cdot SO_3$
S – SO_3
N
O
N
R
$SO_3^=$
SO_3^-
N
O
N
R

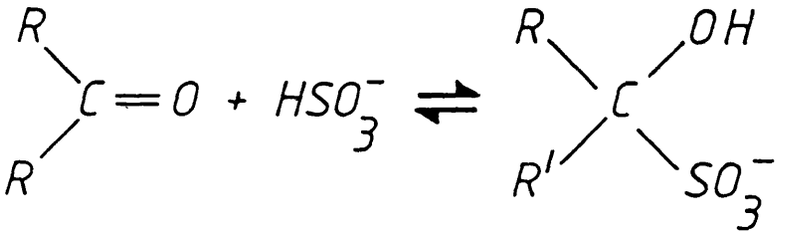
Addition of HSO_3^- to uracil



Deamination of cytosine by HSO_3^-



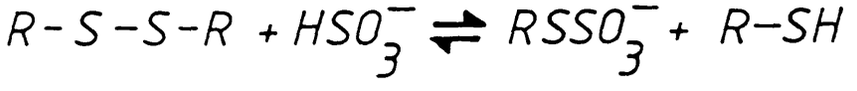
Reaction with carbonyl compounds



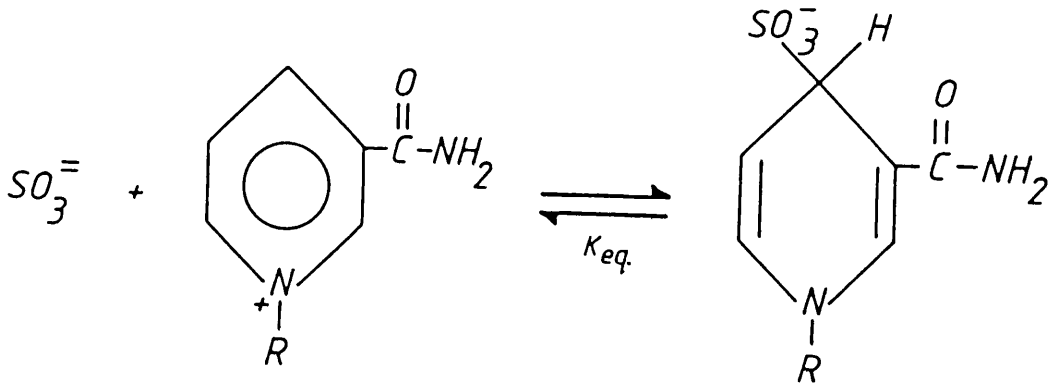
Finally it has been demonstrated also (Tanaka and Luker, 1978) that killing of bacteria and yeasts by SO_3^{2-} (up to 24 mM) was more effective under aerobic than anaerobic conditions. They found that when *Escherichia coli* was exposed to SO_3^{2-}, leakage of β-methyl-glucoside occurred, followed by rapid loss of ATP. Furthermore they noted that SO_3^{2-} (15.6 mM at pH 6.0) killed *Salmonella typhimurium* and *Bacillus subtilis* by breaking DNA as indicated by a more rapid kill of DNA-repair mutants than their prototrophs.

In summary, it is evident that SO_2/HSO_3^- or SO_3^{2-} can act in several ways to inhibit or kill micro-organisms.

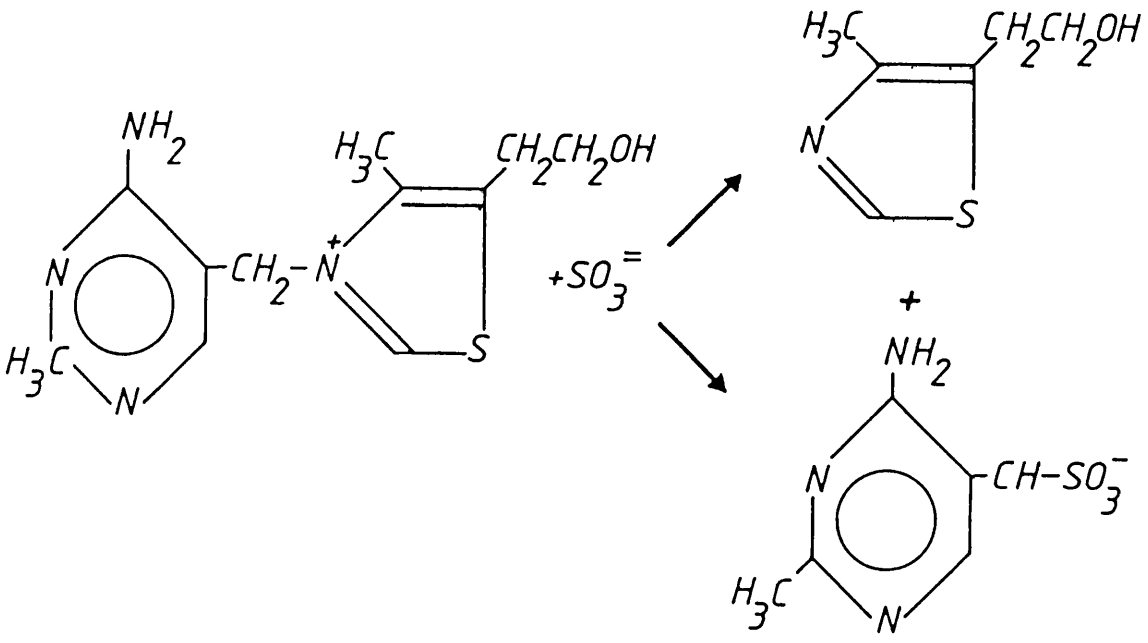
Reaction with disulphide bonds (sulphitolysis)



Reaction with NAD



Reaction with thiamine



As the reaction of the preservative is influenced greatly by the nature of the environment, e.g. pO_2, pCO_2, a_w, pH, nutrient status, availability of binding compounds, temperature and types,numbers and physiological status of the test micro-organisms, the action of sulphite in such a complex system as the British fresh sausage will be difficult to resolve. It was for this reason that the present study concentrated on the action of sulphite on microbial growth, measured by changes in opacity or colony-forming units.

MATERIALS AND METHODS

Sausage manufacture

Commercial scale

Sausage ingredients were weighed into a bowl chopper (Laska, Austria) in the proportions of a commercial sausage recipe (Appendix, p.329). The total capacity of the mix was 500 ℓ. When appropriate, the first batch was made without preservative, the second included preservative (sodium metabisulphite) in the seasoning (Rank-Hovis-McDougall 599,England), at the "commercial level" which was predicted to give a final concentration of *ca.* 600 µg SO_2 g^{-1} sausage. The meat/rusk slurry was extruded mechanically into reconstituted collagen casings (Devro, England), linked by hand, packed in eights and wrapped in food grade cellophane film (25 µm thickness). The oxygen permeability of the latter at 25°C was 400 cc/m^2/h/0.025 mm; water vapour permeability at 25°C and 75% relative humidity was 5 g/m^2/24 h/ 0.025 mm.

Laboratory scale

Sausage ingredients obtained from material awaiting use in sausage manufacture in a factory of a large-scale manufacturer were minced (Kenwood A901), and weighed into a clean stainless steel bowl in the proportions of a commercial sausage recipe (Appendix p. 329). The total capacity of the mix was 2 Kg. The ingredients were chopped vigcrously (Kenwood A901). Additives: sulphited or unsulphited seasonings at the commercial level,

potassium sorbate (2,000 µg/g) or solid carbon dioxide pellets (20% w/v) were chopped into the slurry in various combinations (Appendix, p. 309). The sausage meat was extruded through a sausage stuffer (Kenwood) into reconstituted collagen casings (Devro, England), linked by hand, packed in eights and wrapped in film (*vide supra*).

Seeding of sausage meat

Rifampicin-resistant *Salmonella* were inoculated into Brain Heart Infusion broth (BHIB, pH 7.0; Difco) and incubated at 37°C for 18 h. Freshly manufactured sulphited or unsulphited sausage meat obtained from the factory was inoculated with *Salmonella* at a level calculated to give a final concentration of *ca.* 1×10^3 *Salmonella* g^{-1} sausage. The inoculated meat slurries were mixed thoroughly (Kenwood A901) and samples (100 g) put into square Petri dishes (Sterilin). Uninoculated samples were prepared also, sterile distilled water being added to the sausage meat instead of a suspension of *Salmonella*.

Storage of sausages and sausage meat in Petri dishes

As with factory practice, all sausages and sausage meat were stored aerobically at 4°C for 24 h. Thereafter packs of sausages were kept at 4°, 10°, 15° or 22°C for up to 14 d. Petri dishes containing seeded sausage meat were kept at -20°, 4°, 9°, 15°, 20° or 25°C for up to 8 d.

Sampling of sausages and sausage ingredients

Samples of pork sausage, pork and beef sausage and all the ingredients used in their manufacture were obtained from a large factory during normal production in the period 1.10.79 to 31.9.82. One Kg of pork meats were taken at random from material awaiting processing. The meat came from pigs slaughtered at the factory; butchered meat had been stored at 4°C overnight. Cattle were neither slaughtered nor butchered on site and 1 kg samples were taken from blocks of frozen, deboned beef. Samples of rusks, seasoning, polyphosphate (Fibrisol V10), pasteurized and dried rinds, mechanically recovered meat and linked sausages were examined also. All samples were stored at 4°C and examined within 3 h. To minimise sampling errors (Elliott, 1977), all meats were comminuted in a sterile mincer (Kenwood). Ten sub-samples (each of ca. 2 g) were taken at random from a minced ingredient, bulked, and homogenised in 180 ml of ¼ strength Ringers diluent (Oxoid BR52) with a Colworth stomacher 400 (Seward, London) for 60 s. Further serial dilutions were done in Ringers diluent (9 ml). With linked sausage four sub-samples were taken from either the surface (depth up to 0.25 cm) or "core" sites (innermost 1 cm diameter cylinder) or from a transverse section, and, as with meat ingredients, a 20 g sample was used.

Determination of sulphur dioxide (SO_2) in sausage

Monier-Williams (1927) method, modified by Shipton (1959)

Specimen preparation. A sample (20 g) of sausage was taken from a

standard pack (454 g) using the sampling scheme described on p.62.

Total SO_2 determination. Analysis was done according to the modification of Shipton (1959). The specimen was acidified with orthophosphoric acid (2M, 50 ml) and distilled for 60 min. Hydrogen peroxide was used to collect the SO_2 (Burroughs and Sparks, 1963).

Electrometric ion-selective probe method (Bailey and Riley, 1975; Brown, 1977).

Specimen preparation. A sample (5 g) of sausage was taken from a standard pack, added to distilled water (45 ml) and homogenised in a Colworth Stomacher 400 (Seward, London) for 60 s. The homogenate was centrifuged (MSE Super Speed 18) at 35,000 *g* for 10 min. When the set speed was achieved, the compressor was regulated to cool at 2°C. Chilled centrifugation separated a fatty layer from the aqueous layer and a solid base, comprising of starch, meat and rind particles (Brown, 1977). The aqueous layer was decanted and stored in a stoppered volumetric flask (50 ml) in an ice bath to reduce SO_2 loss before analysis.

Total SO_2 determination . One part of sodium hydroxide (1M) was added to one part of specimen solution and stored at 25°C for 30 min to dissociate bisulphite-addition compounds. A sample of alkalised specimen (25 ml) was placed in an Erlenmeyer flask (100 ml) containing a magnetic stirring bar and an equal volume

of sulphuric acid (1 M) added. The flask was put in an ice bath on a stirrer bed (Chem Lab SS3H) and the probe (EIL, Chertsey) immersed to a depth of 1.5 cm. The peak deflection (mV) after 2 min as registered on an EIL 7030 pH meter (Monovalent cation setting) or as indicated on a flat-bed pen recorder (Servoscribe) noted.

Free SO_2 determination. Analysis was done immediately after specimen preparation *vide supra*. No alkalisation procedure was used and acidification with sulphuric acid released the free SO_2 from the specimen which was then estimated as for total SO_2 determination *vide supra*.

Spectrophotometric method

Specimen preparation. A sample (5 g) of sausage was taken from a standard pack, added to chilled distilled water (25 ml) in a screw-capped bottle (100 ml) which contained glass beads, and shaken 60 times through an arc of 0.5 m for 30 s. The homogenate was stored in an ice bath before analysis.

The specimen was placed in a three-necked round-bottom flask (Q&Q FR 250/35/22A). One side neck was connected to a supply of nitrogen (Air Products, Bristol) via a modified sparger (Q&Q MF 27/3/13). A vertical condenser (Q&Q C1/13) was connected to the centre neck and two Drechsel bottle heads (Q&Q 27/3/13) were attached in parallel to the condenser outlet by means of a "y"

junction adaptor (Q&Q MF 10 2B) with taps. The dip tubes of the Drechsel bottle heads were placed in boiling tubes (Q&Q MF 24/3) below the surface of solutions of 5,5'-dithiobis 2-nitrobenzoic acid (DTNB) (50 ml). DTNB reagent (final concentration 2.5 x 10^{-3} M; pH 8.0) was dissolved in phosphate buffer (1.6 x 10^{-2} M Na_2HPO_4 + 1.8 x 10^{-3} M KH_2PO_4) containing ethanol (10% v/v). Nitrogen was then passed through the distillation flask (20 $cm^3 s^{-1}$). Hydrochloric acid (2 M, 50 ml) was added via the remaining side neck and the flask was heated. On the completion of distillation (15 min) DTNB was added to the phosphate buffer (50 ml) and the absorbance measured at 412 nm on a Pye Unicam SP6-550 UV/VIS spectrophotometer (Wedzicha and Bindra, 1980).

Free SO_2 determination. Analysis was done immediately after specimen preparation by transfer of the specimen to a distillation flask (Q&Q 250/35/22A) containing a magnetic stirring bar. A condenser was not used and the tubing adaptor (Q&Q MF 10 2B) was attached directly to the flask. No heat was applied and the reaction was allowed to continue for 15 min after addition of hydrochloric acid (2M, 50 ml). Nitrogen was passed through the distillation flask (20 $cm^3 s^{-1}$) as above. Analysis of DTNB was done as for total SO_2 determination.

Standard sulphite solutions

Sulphite solutions were made up from potassium metabisulphite and contained ethylene-diamine-tetraacetic acid (EDTA, 1 x 10^{-4} M) to prevent oxidation. The solutions were standardised iodimetrically

by volumetric analysis.

Standard curves of sulphite concentration

Electrometric ion-selective probe analysis. Standard solutions of sulphite were treated in the same way as specimen solutions to give a standard curve (Fig. 2).

Spectrophotometric analysis. Standard solutions of sulphite were added directly to buffered DTNB reagent and analysed spectrophotometrically in the same way as specimen solutions to give a standard curve (Fig. 3). A freshly prepared reagent blank was used for each set of readings.

Determination of sulphite concentration of sausage seasonings

1. A sample (0.01 g) of seasonings was added to DTNB (25 ml), diluted with buffer to a final volume of 50 ml and analysed spectrophotometrically.
2. A sample (0.01 g) of seasonings was used. The concentration of free and total SO_2 was determined by the method described.
3. Iodimetric method (used by one large manufacturer of sausage). A sample (1 g) of seasonings was added to distilled water (25 ml) in an Erlenmeyer flask (250 ml). Two ml of sulphuric acid (10% w/v) and 3 drops of soluble starch were added and mixed thoroughly. This solution was titrated with iodine (0.1 N). The percentage SO_2 = titration value (ml) x 0.32%.

Determination of sulphite-binding capacity of sausage ingredients

Sterile lean pork was excised from large (300 x 200 mm) blocks of meat, minced aseptically (Kenwood A901) and samples (25 g) added to sterile Erlenmeyer flasks (250 ml). Samples (25 g) of minced lean, head and belly pork, rinds, fat or mechanically recovered meat (MRM) were added to flasks, as was rusk also. Solutions of filter-sterilised potassium metabisulphite (100 ml, *ca.* 600 µg SO_2/ml) were added aseptically to the flasks which were stoppered with rubber bungs. The flasks were incubated at 25°C in a shaking (88 reciprocal excursions over 5 cm) water bath for up to 6 h. Uninoculated sulphite solutions were used as controls. Concentrations of free and total sulphite of the slurries were determined by the method described on pp. 64 - 66.

Sulphite-binding agents

Preparation of detection solution. An aqueous solution (pH 8.0) of basic fuchsin (0.02 w/v) and sodium metabisulphite (0.125% w/v) was made in deoxygenated distilled water and stored under a blanket of scrubbed (Appendix, p.335) dinitrogen at *ca.* 4°C.

Preparation of standard curve. Samples (1 ml) of various concentrations of acetaldehyde were added to the detection solution (10 ml), sparged with scrubbed dinitrogen and the increase in absorbance (λ = 538 nm) measured immediately against a blank control (Pye Unicam SP UV-VIS spectrophotometer). The presence of sulphite-binding agents in unsulphited culture fluid was determined as above, and the concentrations, estimated by reference to the

standard curve (Fig. 4). In examination of sulphited culture fluid, correction was made for the presence of "endogenous" sulphite.

Sulphate (SO_4^{2-}) determination

A modified method based on that of Bertolacini and Barney (1957) was used. Deionised water was used. Micro-organisms were removed from culture fluid by centrifugation (7,000 *g*; 15 min) and a sample (1 ml) of the supernatant was added to 1 ml of 0.12 M potassium hydrogen phthalate ($COCH.C_6H_4.COOK$) in ethanol (50% v/v). Five mg of solid barium chloranilate ($BaC_6Cl_2O_4$) was added, the mixture "whirlimixed", centrifuged (7,000 *g*; 15 min) and the absorbance of the supernatant (chloranilic acid) measured (λ = 332 nm) on a Pye Unicam SP 6 UV-VIS spectrophotometer. A standard curve, using various concentrations of sodium sulphate ($Na_2SO_4.12H_2O$) was made using the same method (Fig. 5). Where there was a possibility of interference from cations or components which would absorb near 332 nm, a sample (30 ml) of culture supernatant was passed down a column of Dowex 50X8 resin (10 ml bed volume in the H^+ form) at *ca*. 5 ml/min. The effluent was brought to pH 4 with ammonium hydroxide (NH_4OH), adjusted to volume and a sample (4 ml), mixed with 0.05 M potassium hydrogen phthalate (1 ml). After vigorous agitation and centrifugation (7,000 *g*, 15 min) the absorbance of the supernatant was measured at 530 nm against a deionised water blank.

Concentrated solutions of freshly-prepared sulphite (2.5 - 12.5 mM) also gave some colour in this assay so a calibration curve

was prepared (Fig. 6) with sodium sulphite ($Na_2SO_3.7H_2O$). From this curve, absorbance corrections at 530 nm due to sulphite (determined independently with DTNB reagent) could be made in samples containing both SO_3^{2-} and SO_4^{2-} moieties.

Removal of thiol compounds from samples

Interference from thiol compounds was removed in two ways (1) formaldehyde (0.01 ml; 50 mM) was added to the sample (1 ml) which contained both sulphite and a thiol. The sulphite was complexed as formaldehyde sulphoxylate so that it did not react with the DTNB reagent. Residual absorbance at 412 nm was due to thiols and the appropriate correction made (Fig. 7). (2). The sample (1 ml) was diluted 10 fold and allowed to stand in air at 25°C for 20 min. This completely oxidised thiols to the corresponding disulphides after which gave no colour with DTNB (Fig. 8). Residual colour was due to sulphite.

pH

A sample (20 g) of sausage or an ingredient of sausage was homogenised in distilled water (pH 7.0; 180 ml) in a Colworth Stomacher 400 (Seward, London) for 60 s. The pH of the homogenate was measured with a glass pH electrode (Russell, England).

Isolation, enumeration and maintenance of micro-organisms from meat and sausage

Total viable count (TVC): From appropriate dilutions, samples (20 µl) were pipetted onto the dried surface (37°C, 60 min) of PCA. Using the technique of Miles and Misra (1938), 32 replicate drops were accommodated in one Petri dish (diameter, 9 cm). Incubation was at 20°C for 3 d, and colonies picked at random were purified by streaking on PCA. Pure cultures were stored on PCA slopes at 4°C with sub-culture at 4 week intervals.

Pseudomonas

Samples (0.2 ml) of an appropriate dilution were spread over the dried (37°C, 60 min) surface of Cetrimide-Fusidic acid-Cephalodrine (CFC, Mead and Adams, 1977) medium. Incubation was at 15°C for 48 h and 3 replicates at each dilution level were done. Colonies picked at random from the lowest countable dilution of CFC agar were purified by streaking on PCA and maintained thereafter on PCA slopes at 4°C with subculture every 4 weeks.

Lactobacillus

A sample (1 ml) of an appropriate dilution was added to 10 ml of MRS (de Man *et al.*, 1960) medium at 50°C. When set, an overlay (10 ml) of MRS was added. Three replicates were done at each dilution level and incubation was at 25°C for 5 d under anaerobic conditions. Colonies picked at random from the lowest countable

dilution of MRS agar were purified by streaking on Heart Infusion (HIA; Difco B44) agar, and maintained in Robertson's cooked meat medium (Appendix p. 333) at 4°C. The isolates were subcultured every 4 weeks.

Group D streptococci

Kanamycin aesculin azide agar (KAA; Kanamycin aesculin azide broth + 1.5% w/v agar) was sterilised by autoclaving at 121°C for 15 min. A sample (1 ml) of an appropriate dilution was mixed with KAA (20 ml) at 50°C. Three replicates were done at each dilution level and incubation was at 37°C for 24 h. Colonies picked at random from the lowest countable dilution of KAA were purified by streaking onto Todd Hewitt agar (TDA, Appendix p. 333), and maintained in Robertson's cooked meat medium (Appendix, p. 333) at 4°C with subculture every 4 weeks.

Brochothrix thermosphacta

A sample (0.1 ml) of an appropriate dilution was spread over the dried surface (37°C, 60 min) of streptomycin and thallous acetate actidione (STAA, Gardner, 1966) medium. Three replicates were done at each dilution level and incubation was at 20°C for 3 d. Colonies picked at random from the lowest countable dilution of STAA were purified by streaking on PCA and maintained on PCA slopes at 4°C with subculture every 6 weeks.

Yeasts

A sample (0. 1 ml) of an appropriate dilution was spread over

the dried (37°C, 60 min) surface of Rose Bengal chloramphenicol (RBC, Oxoid CM 549) medium. Three replicates were done at each dilution level and incubation was at 25°C for 3 d in the dark. Colonies picked at random from the lowest countable dilution on RBC were purified by streaking onto malt extract (MA, Appendix p. 333) agar. Isolates were maintained on MA slopes at 4°C and subcultured every 6 weeks.

Enterobacteriaceae

Lactose fermenting Enterobacteriaceae. A sample (1 ml) of an appropriate dilution was mixed with 10 ml of violet red bile (VRB, Oxoid CM 107) agar which had been cooled to 46°C. When set, the surface of the agar was overlaid with VRB (10 ml). Incubation was at 37°C for 24 h.

Glucose fermenting Enterobacteriaceae. A sample (1 ml) of an appropriate dilution was mixed with 10 ml of violet red bile glucose (VRBG Oxoid CM 485) agar cooled to 46°C. When set, the surface of the agar was overlaid with VRBG (10 ml). Incubation was at 30°C for 24 h. Six replicates at each dilution level were done and colonies selected at random were picked from VRB or VRBG and purified by streaking on PCA. Isolates were maintained on slopes of PCA at 4°C with subculture every 4 weeks.

Rifampicin-resistant *Salmonella*

Salmonella$^{rif\ R}$ were recovered on Desoxycholate citrate (DCA,

Difco B274) agar supplemented with rifampicin (Sigma) at a concentration of 50 μg ml^{-1} medium. DCA was boiled, cooled to 50°C and a filter-sterilised solution of rifampicin in methanol (BDH) added and the medium (DCAR) mixed. Petri dishes were poured immediately and the surface of the medium dried (37°C, 60 min) before use. A sample (0.1 ml) of an appropriate dilution was spread over the surface of the medium. Six replicates were done at each dilution level and incubation was at 37°C for 48 h. Isolates were maintained on PCA slopes at 4°C with subculture every 2 weeks.

Selective enumeration of drug-resistant *Salmonella*

Rifampicin and Nalidixin resistant *Salmonella*

Drug-resistant *Salmonella* , *Hafnia alvei* (E 58), *Escherichia coli* (E16), *Enterobacter agglomerans* (E85) and *Citrobacter freundii* (E91) were tested for growth on solid and in liquid media.

Test organisms were incubated at 30°C for 16 h in TSB after which time cell concentration was *ca.* 10^7 - 10^8 c.f.u. ml^{-1} broth in all cases. Samples (1 μl) were multipointed (Denley) onto the dried (37°C, 60 min) surface of DCAR, PCA + rifampicin (50 μg/ml) PCA and MacConkey agars and into TSB or TSB + nalidixic acid (30 μg/ml) contained in Petri dishes (Sterilin).

Influence of recovery medium on selective enumeration of *Salmonella*$^{rif\ R}$ mutants

Rifampicin-resistant strains of *Salmonella goerlitz, virchow, kedougou, hadar, anatum,* an unidentified group E4 *Salmonella* and strains of Enterobacteriaceae isolated from British fresh sausage (E 58, E 16, E 85 and E 91 *vide supra*) were incubated at 30°C for 16 h in TSB (10 ml). Decimal dilutions were made in sterile distilled water (9 ml). Five replicate samples (0.1 ml) of appropriate dilutions of single strains or mixed (in the ratio 1:1) strains were plated onto TSA, TSA + rifampicin(50 µg/ml) or DCAR.

Yeasts

The yeasts were characterised by the methods given below in order to assess whether or not sulphite was having a selective role in sausages.

Morphology

Gross. Colony appearance, pigment and texture on malt extract agar (Malt, 6% w/v) after incubation at 25°C for 5 d was noted.

Microscopic. The size and shape of cells and occurrence of capsules, arthrospores, pseudo- or true hyphae was noted.

Production of urease. The method of Christensen (1946) was used.

Assimilation of nitrate

The method of Wickerham (1946) was used. Difco carbon base (B391; 11.7% w/v) was used as the basal medium.

Fermentation of glucose

The method of Beech *et al.* (1968) with filter-sterilised glucose (6% w/v) was used.

Assimilation of organic substrates

The basal medium (Difco yeast nitrogen base, B392; 6.7% w/v) was filter-sterilised, dispensed into sterile universal bottles in 25 ml volumes and maintained at 55°C. Organic substrates were dissolved (4% w/v) in distilled water, filter-sterilised, dispensed into sterile universal bottles in 5 ml volumes and kept at 55°C . Purified agar (Oxoid L28; 4% w/v) was dispensed into 100 ml bottles, sterilized by autoclaving at 115°C for 10 min and cooled to 55°C, The basal medium and organic supplement was added aseptically to the agar base, mixed and kept at 55°C. A sample (250 µl) of the complete medium was dispensed into wells of sterile Microtitre plates (Sterilin) and allowed to set.

Test strains were incubated in 10 ml of yeast nitrogen base (6.7% w/v) containing D-glucose (0.1% w/v) at 25°C for 2 d. The suspensions were centrifuged (7,000 *g*, 15 min) and the pellets were resuspended finally in sterile distilled water (2 ml). Samples (200 µl) were pipetted into the wells of a sterile Microtitre plate (Sterilin).

Inoculation

Ninety-six map pins (Rexel Co. Ltd.) were glued (Araldite) into the wells of a Microtitre plate. This inoculation plate was soaked in ethanol (70% w/v) for 2 h, dried (45°C, 6 h) and used to inoculate suspensions of washed yeast strains into the wells in Microtitre plates containing single organic substrates. Incubation was at 25°C for 14 d in a moist air environment.

Identification was achieved by reference to traditional works (e.g. Lodder, 1970) or to a numerical data base (API 20C Auxanogram).

Lactic acid bacteria

For the preliminary characterization of organisms isolated on MRS agar (presumptive lactobacilli) or KAA (presumptive streptococci), the following properties were used:
Growth on modified (Dowdell and Board, 1968) Keddies (1951) medium (Appendix, p. 331) was tested. Incubation was at 25°C for up to 14 d. The Gram reaction according to the method of Kopeloff and Beerman (1922) was assessed. Production of catalase was tested. Hydrogen peroxide (10 vols.) was applied to a nutrient agar slope culture (72 - 96 h) of the organism. Growth at 15°, 37° or 45°C in MRS broth (9 ml) was assessed using 0.1 ml of an overnight culture grown in MRS at 30°C as inoculum. Incubation was for up to 14 d. The homo- or heterofermentative action on glucose in the medium of Gibson and Abd-el-Malek (1945) was determined; incubation was at 25°C for up to 14 d. Evidence of

oxidative or fermentative breakdown of glucose was tested with the OF medium (Appendix p.332) of Whittenbury (1963); incubation was at 25°C for up to 14 d. Further characterisation of the lactobacilli involved the monitoring of the following properties: The type of isomer of lactate which was formed from glucose breakdown was determined using the methods of Barker and Sommerson (1941) and Garvie (1967). The production of carbon dioxide from the breakdown of gluconate and the hydrolysis of aesculin were tested according to the methods of Collins and Lyne (1976).

The formation of ammonia from arginine was tested (Appendix p. 332). The API 50 CHL scheme (Paule, 1971) was used to study the fermentation of 49 carbohydrates (see below). The methods used were those recommended by the manufacturer (API Systems, Basingstoke); incubation was at 25°C for up to 72 h.

Organic substrates used in fermentation studies

0 Control	10 Galactose
1 Glycerol	11 Glucose
2 Erythritol	12 Fructose
3 D Arabinose	13 Mannose
4 L Arabinose	14 Sorbose
5 Ribose	15 Rhamnose
6 D Xylose	16 Dulcitol
7 L xylose	17 Inositol
8 Adonitol	18 Mannitol
9 β Methyl xyloside	19 Sorbitol

20 α Methyl mannoside

21 α Methyl glucoside

22 N acetyl glucosamine

23 Amygdalin

24 Arbutin

25 Esculin

26 Salicin

27 Cellobiose

28 Maltose

29 Lactose

30 Melibiose

31 Sucrose

32 Trehalose

33 Inulin

34 Melezitose

35 Raffinose

36 Starch

37 Glycogen

38 Xylitol

39 Gentiobiose

40 D Turanose

41 D Lyxose

42 D Tagatose

43 D Fucose

44 L Fucose

45 D Arabitol

46 L Arabitol

47 Gluconate

48 2 Keto gluconate

49 5 Keto gluconate

In addition to the preliminary rests used for the identification of the lactic acid bacteria *vide supra*,the following tests were used for classification of the streptococci: The ability to reduce tetrazolium and to grow in the presence of 0.4% potassium tellurite were tested using the methods of Facklam (1972). The tests described below were done under aerobic or anaerobic conditions in Repli-dishes (Sterilin): Tolerance of 40% bile, ability to grow at pH 9.6, 10^{o}C or in the presence of 6.5% NaCl in MRS broth; production of haemolysis on a modified Kneteman's (1947) medium (Appendix p. 332); oxidation or fermentation of carbohydrates (see below).

Carbohydrate (final concentration, 1% w/v) in basal medium of Whittenbury (1963; Appendix, p. 332)

Azelate; Arbutin; Citrate; Raffinose; Maltose; Salicin; D+trehalose; Mucate; Adonitol; D+Melezitose; D+Melibiose; Sorbitol; Inulin; Arabinose; Cellobiose; Sarcosine: Glycerol; Mannitol; Pimelate; Inositol; D- ribose.

Preparation of inoculum

Strains of Streptococci were incubated at 30°C for 24 h in 10 ml of *Streptococcus* medium (SM, Appendix, p.332). Cultures were centifuged (7,000 *g*; 5 min) and the pellet resuspended in sterile Ringers (10 ml). The washing procedure was repeated and *ca*. 1 µl placed in the liquid or, on the surface of solid media contained in Repli-dishes (Sterilin) by multipoint inoculation. For carbohydrate utilization studies, each well of the Repli-dish contained *ca*. 5 ml of Whittenbury's (1963) medium and provided aerobic and anaerobic conditions. This was indicated by the site of growth and by the reduction of methylene blue below the surface layer (2 cm) of uninoculated medium. Reducing conditions existed throughout the incubation period of 7 d at 30°C. Inoculated Repli-dishes were incubated in moist chambers or in anaerobic jars.

Serology of *Streptococcus* antigens

The counterimmunoelectrophoresis (CEIP) method was used.

Extract preparation

Pure cultures of the test strains were inoculated into 20 ml of sterile Todd-Hewitt broth (Oxoid DM 189) in plastic centrifuge tubes (capacity 25 ml). Incubation was at 37°C for *ca*. 18 h after which time the tubes were centrifuged at 7,000 *g* for 15 min. The pellets were resuspended in 1 ml amounts of 0.05 N HCl in saline (0.85%), and the pH was adjusted to 2.3. The suspensions were heated to 100°C for 10 min in a water bath, cooled to 25°C and brought to a neutral pH with NaOH (1 M). The suspensions were centrifuged (7,000 *g*; 15 min) and the supernatants were used as antigens in the CEIP procedure.

Gel and buffer preparation

Hydrochloric acid (0.2 N; 38.2 ml) was added to barbitone buffer (sodium methyl-barbiturate, 8.25 g; glass distilled water, 1000 ml) to give a final pH of 8.6. Agarose (1 g, Sigma) was dissolved in this barbitone-HCl buffer (100 ml) by heating in a water bath, maintained at 70°C and poured onto preheated (50°C) glass slides (300 mm x 200 mm) to a gel thickness of *ca*. 2 mm. When the gel had set holes (5 mm diameter) were punched 10 mm apart along the electrophoretic axis. Sixty-eight wells could be accommodated on a slide. *Streptococcus* antisera (30 µl; ZJ series, Wellcome, Beckenham) were pipetted into anodal wells; antigens (30 µl) into cathodal wells.

Electrophoresis

The gel was placed in an electrophoresis tank which was filled

with barbitone-HCl buffer. The current was adjusted to 2.5 mA cm^{-1} width; voltage to *ca.* 300 V and the gel was "run for 2h".

Fixing and staining

The gel was fixed in an aqueous solution of perchloric acid (8%, v/v) for 2 min, rinsed in distilled water (1000 ml) and stained with Comassie brilliant blue (0.04%, w/v) in perchloric acid (3.5% w/v) for 1 h at 37°C.

Enterobacteriaceae

The following tests were used to characterise the organisms isolated on Violet Red Bile and Violet Red Bile Glucose agar, and to determine whether or not sulphite preservative influenced the composition of the Enterobacteriaceae: The Gram stain was determined by the method of Kopeloff and Beerman (1922). The mode of breakdown of glucose under aerobic or anaerobic conditions was determined using the method of Hugh and Leifson (1953). The presence of catalase was tested by the application of hydrogen peroxide (6%, v/v) to a PCA slope culture (18 - 24 h at 37°C) of the test organism. The production of oxidase was tested using the method of Kovács (1956). The ability to grow on MacConkeys medium (containing crystal violet and bile salts) and Eosin methylene blue (EMB, Oxoid CM 69) agar was noted; incubation was at 30°C for 48 h.

The motility of strains incubated in TSB for 18 h at 30°C was tested by the hanging drop method (Collins and Lyne, 1976).

The API 20 E scheme (API, Basingstoke) was used to assess the following characteristics:

The ability of a strain to produce β-galactosidase, arginine dihydrolase, lysine and ornithine decarboxylases, urease, tryptamine deaminase, indole from tryptophan, hydrogen sulphide from sodium thiosulphate, acetoin from sodium pyruvate, nitrites and/or dinitrogen from nitrates and gelatinase. Incubation was at 30°C for up to 48 h.

The ability to grow using citrate as a sole carbon source; incubation at 30°C for up to 48 h.

The ability to ferment mannitol, sorbitol, rhamnose, sucrose, inositol, melibiose, amygdalin and L(+)arabinose. Incubation was at 30°C for up to 48 h.

The API 50 CHE scheme (API, Basingstoke) was used to determine the ability of a strain to ferment the following carbohydrates:

1	Glycerol	13	Mannose	25	Esculin
2	Erythritol	14	Sorbose	26	Salicin
3	D Arabinose	15	Rhamnose	27	Cellobiose
4	L Arabinose	16	Dulcitol	28	Maltose
5	Ribose	17	Inositol	29	Lactose
6	D Xylose	18	Mannitol	30	Melibiose
7	L Xylose	19	Sorbitol	31	Sucrose
8	Adonitol	20	α Methyl Mannoside	32	Trehalose
9	β Methyl Xyloside	21	α Methyl glucoside	33	Inulin
10	Galactose	22	N Acetyl Glucosamine	34	Melezitose
11	Glucose	23	Amygdalin	35	Raffinose
12	Fructose	24	Arbutin	36	Starch

37	Glycogen	42	D Tagatose	46	L Arabitol
38	Xylitol	43	D Fucose	47	Gluconate
39	Gentiobiose	44	L Fucose	48	2 Keto Gluconate
40	D Turanose	45	D Arabitol	49	5 Keto Gluconate
41	D Lyxose				

Incubation was at 30°C for up to 96 h.

Data from all the tests mentioned above were converted into discrete, binary data and analysed by the Clustan numercial analysis package (Wishart, 1978). The data were sorted by the similarity coefficients of Jaccard (S_J), Gower (S_G), and Simple Matching (S_{SM}); clustering was by single linkage (nearest neighbour), complete linkage (furthest neighbour) and average linkage (unweighed pair group - UPGMA) methods. Identification was done by reference to culture collection strains or to a data base (API Floppy Disk).

Culture studies

All media used are listed in the Appendix (pp. 334 - 335).

Turbidometer culture

The test organism was inoculated into the test medium (90 ml) in an Erlenmeyer flask (250 ml) and incubated until a cell concentration of *ca*. 1×10^6 bacterial cells/ml medium or *ca*. 1×10^5 yeast cells/ml medium was attained. Samples (1 ml) were transferred to sterile medium (500 ml) in modified flat-bottomed Quickfit flasks (2 ℓ). The flasks were maintained at the desired temperature

in a through-flow water bath and were stirred with magnetic stirring bars. For aerobic studies, filter-sterilised air (Air Products, Bristol) was used to sparge the cultures (20 $cm^3 s^{-1}$) via Dreschel bottle heads (Q&Q 27/3/13) fitted to the necks of the flasks. Flasks were fitted also with modified fermentation locks containing 'Lysol' (20% v/v) to prevent aerosol production (Fig. 9). The optical density (λ = 600 nm for bacteria, 540 nm for yeasts) of the cultures was monitored continuously using a spectrophotometer (Pye-Unicam, UV-VIS) and recorded on a flat-bed pen recorder (Servoscribe). Culture fluids were drawn through sterilised 'through-flow' glass cuvettes by a peristaltic pump and returned to the culture flasks via side-arm attachments covered by rubber seals (Subaseal). Narrow bore P.T.F.E. tubing was used to draw the culture fluids out of the flasks and silicon rubber tubing was fitted around the peristaltic drive shaft. Samples (1 ml) of culture fluid were withdrawn aseptically from the culture flasks. With yeast cultures, the 10^{-1} dilution in ¼ Ringers was sonicated for 15 s (Pringle and Mor, 1975) at an amplitude of 6 - 7 microns (peak to peak) in order to disrupt clumps of cells (P. Thompson, pers. comm.). Serial dilutions were made in ¼ Ringers (9 ml) and from appropriate dilutions, samples (20 µl) were pipetted onto the dried surface (37°C, 1 h) of PCA. Using the technique of Miles and Misra (1938) 32 replicate drops were accommodated on a 9 cm diameter Petri dish (Sterilin).

Batch culture

The test organism was grown in the test medium to a cell concentration of *ca.* 10^6 ml^{-1} medium (bacteria) or *ca.* 10^5 ml^{-1}

medium (yeasts). Samples (0.1 ml) were transferred to sterile medium (90 ml) in Erlenmeyer flasks (250 ml). Flasks were maintained at the desired temperature in a water bath and left static, or stirred with magnetic stirring bars or shaken (88 reciprocal movements/min through a distance of 5 cm). For aerobic studies, loose-fitting cotton wool bungs covered with cotton gauze were inserted into the flasks whereas for anaerobic studies, 250 ml Erlenmeyer flasks (Q&Q FE 250/4) were sparged (20 $cm^3 s^{-1}$) with filter-sterilised, scrubbed (Appendix, p. 335), white spot dinitrogen (Air Products, Bristol) *via* modified Dreschel bottle heads (Q&Q 27/3/13). A sample (0.5 ml) of culture broth was withdrawn aseptically from the culture flasks and the optical density (λ = 600 nm, bacteria; 540 nm, yeasts) read in microcuvettes (Sterilin) on a spectrophotometer (Pye-Unicam, SP 600 UV-VIS). Cell viability was estimated as in Turbidometric culture (*vide supra*).

Preparation of sulphite solutions

Stock solutions of potassium metabisulphite were prepared in oxygen-free distilled water, filter-sterilised and kept at $4^{o}C$ under a blanket of filter-sterilised dinitrogen. The concentrations of total sulphite were determined iodimetrically and by addition of stock solutions to Ellman's reagent - 5,5'-dithiobis-2-nitrobenzoic acid (DTNB) using the spectrophotometric method described previously (p. 64 - 66). Sulphite solutions were added aseptically to cultures in mid-exponential phase, 2 fold dilutions into batch culture flasks or incrementally until stasis was observed on the flat bed recorder in turbidometer culture flasks. Thereafter, samples (1 ml) of culture fluid were withdrawn

aseptically from flasks, free and total SO_2 concentrations determined as before (see p. 66), or after filtration or centrifugation (13,000 *g*; 2 min) of the broth to remove micro-organisms.

Hydrogen sulphide (H_2S) production

Filter paper strips (Whatman) were soaked in a saturated solution of lead acetate, dried at 70°C, and clipped onto the outlets of modified fermentation locks or filters of culture flasks.

Binding or neutralising agents

Solutions of acetaldehyde (BDH, Poole) or hydrogen peroxide (Sigma, Poole) were made up in deoxygenated, distilled water, filter-sterilised, and kept in tightly-stoppered bottles at 4°C. In order to bind completely the free sulphite in culture flasks, acetaldehyde was added in excess whereas for neutralisation, hydrogen peroxide was added in an equimolar concentration to sulphite.

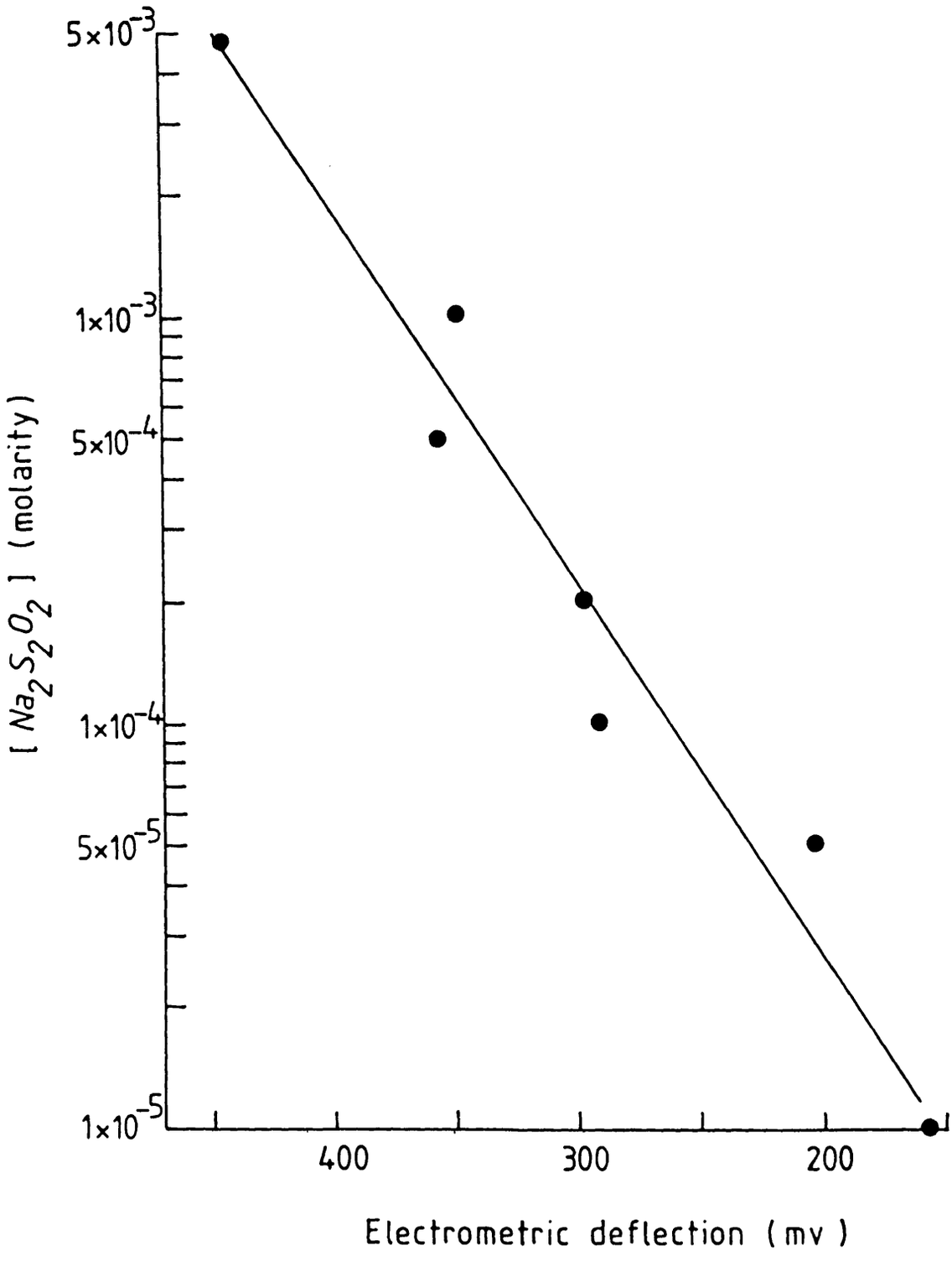


Figure 2. Influence of sodium metabisulphite concentration on electrometric deflection as measured by the SO_2 probe.

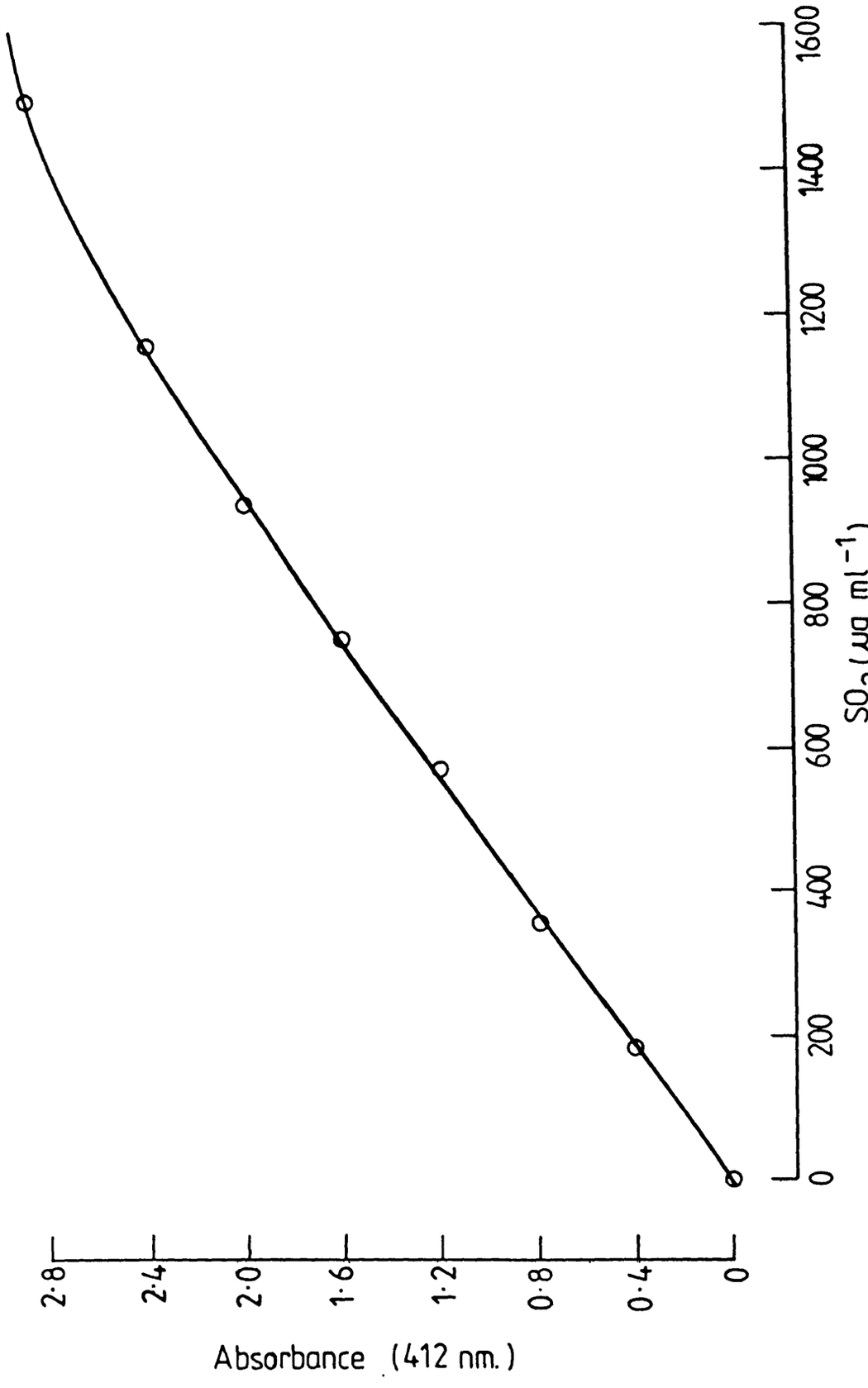


Figure 3. Standard curve of SO_2 concentration as measured by the DTNB-modified spectrophotometric assay.

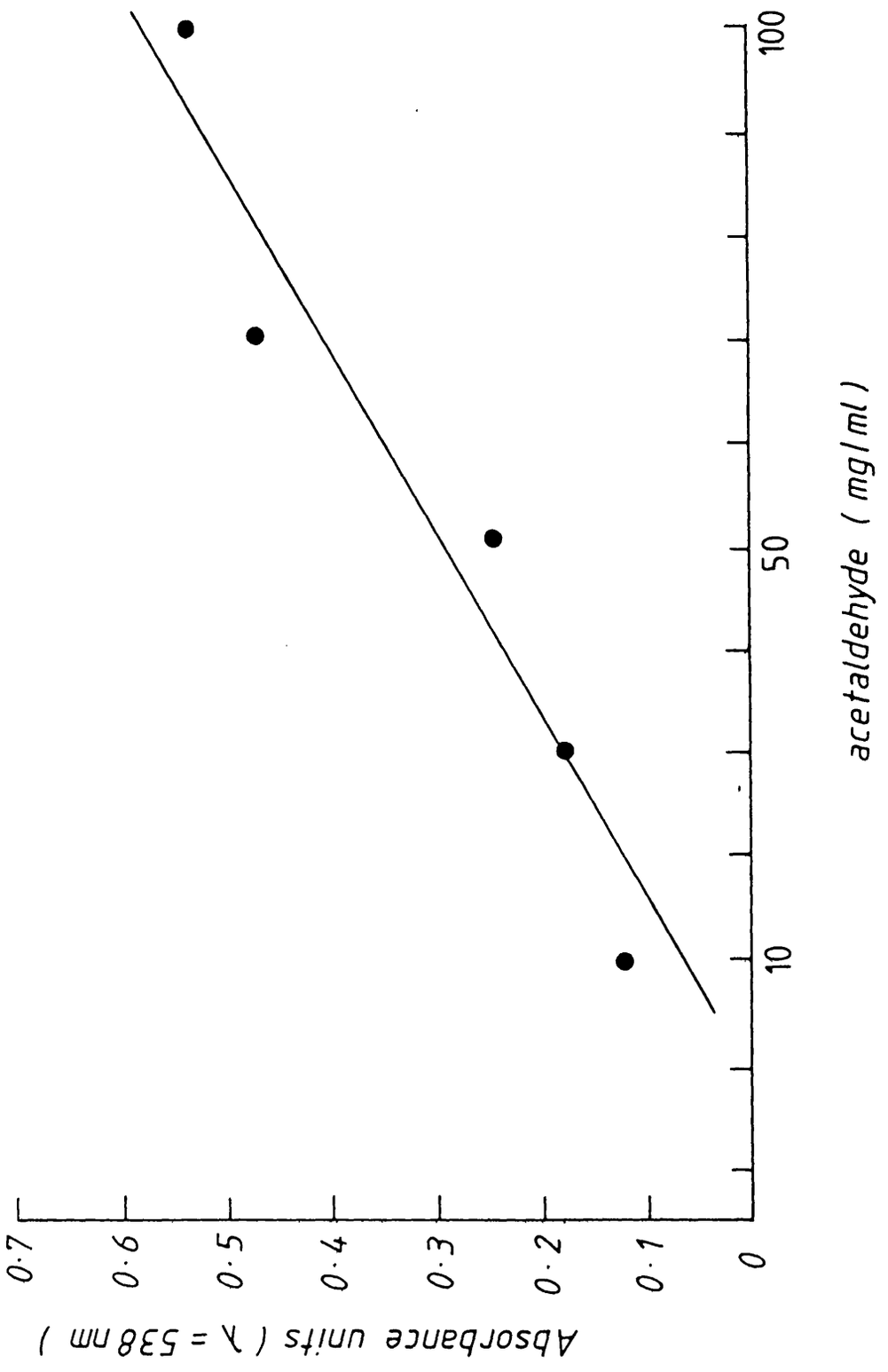


Figure 4. Standard curve of acetaldehyde concentration as measured by the sulphite-basic fuchsin assay.

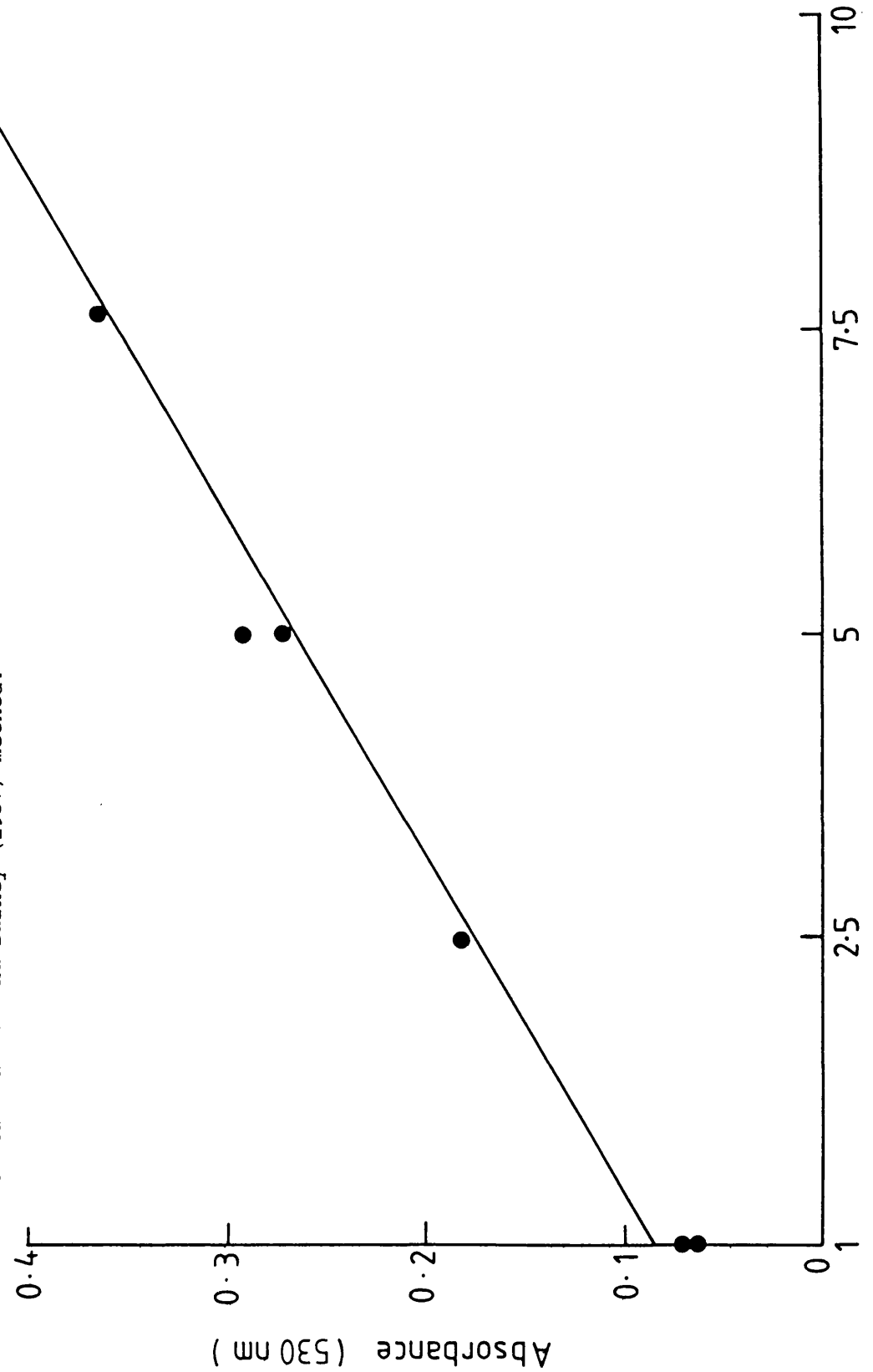


Figure 5. Standard curve of sulphate concentration as measured by a modification of the Bertolacini and Barney (1957) method.

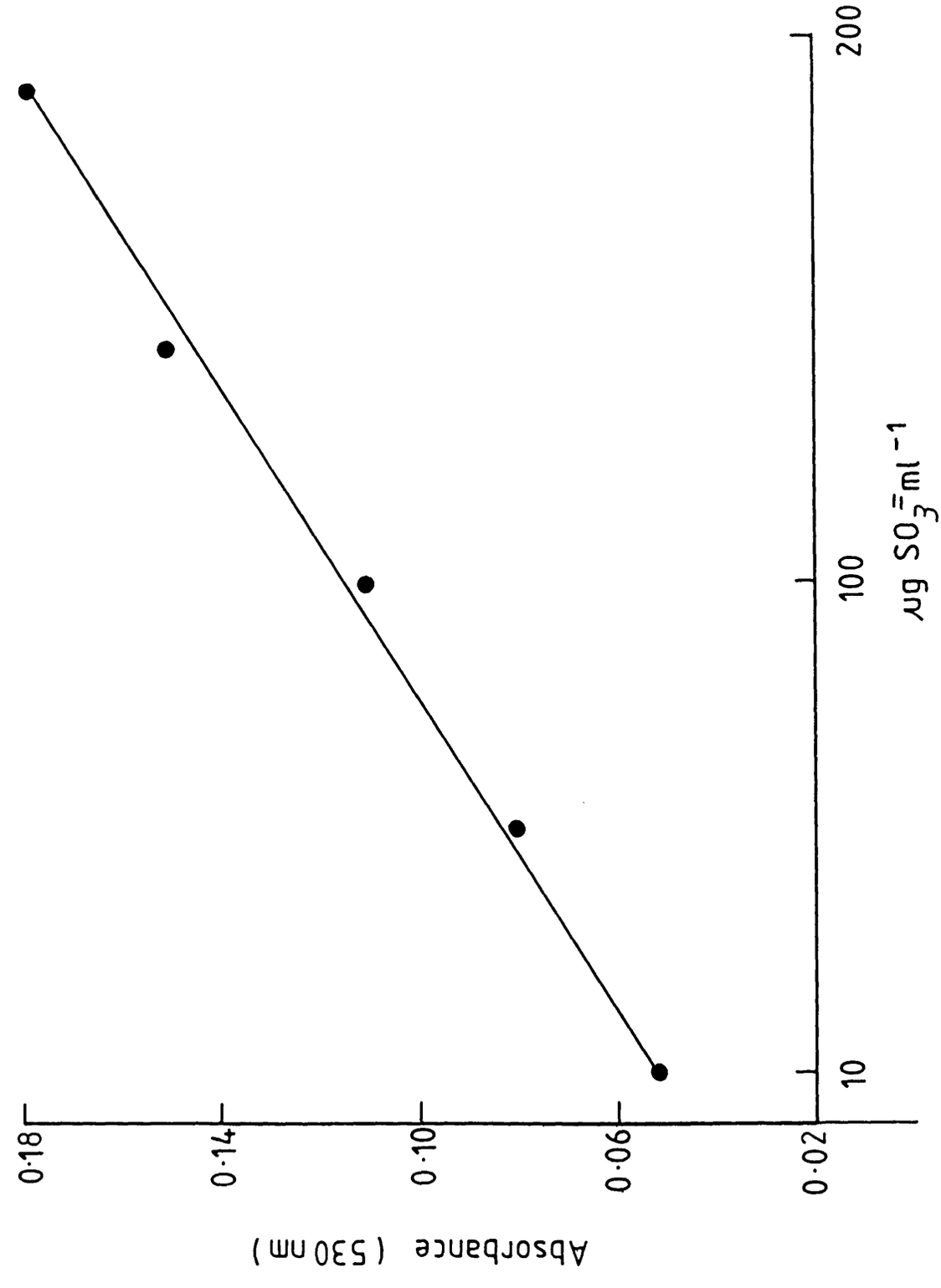
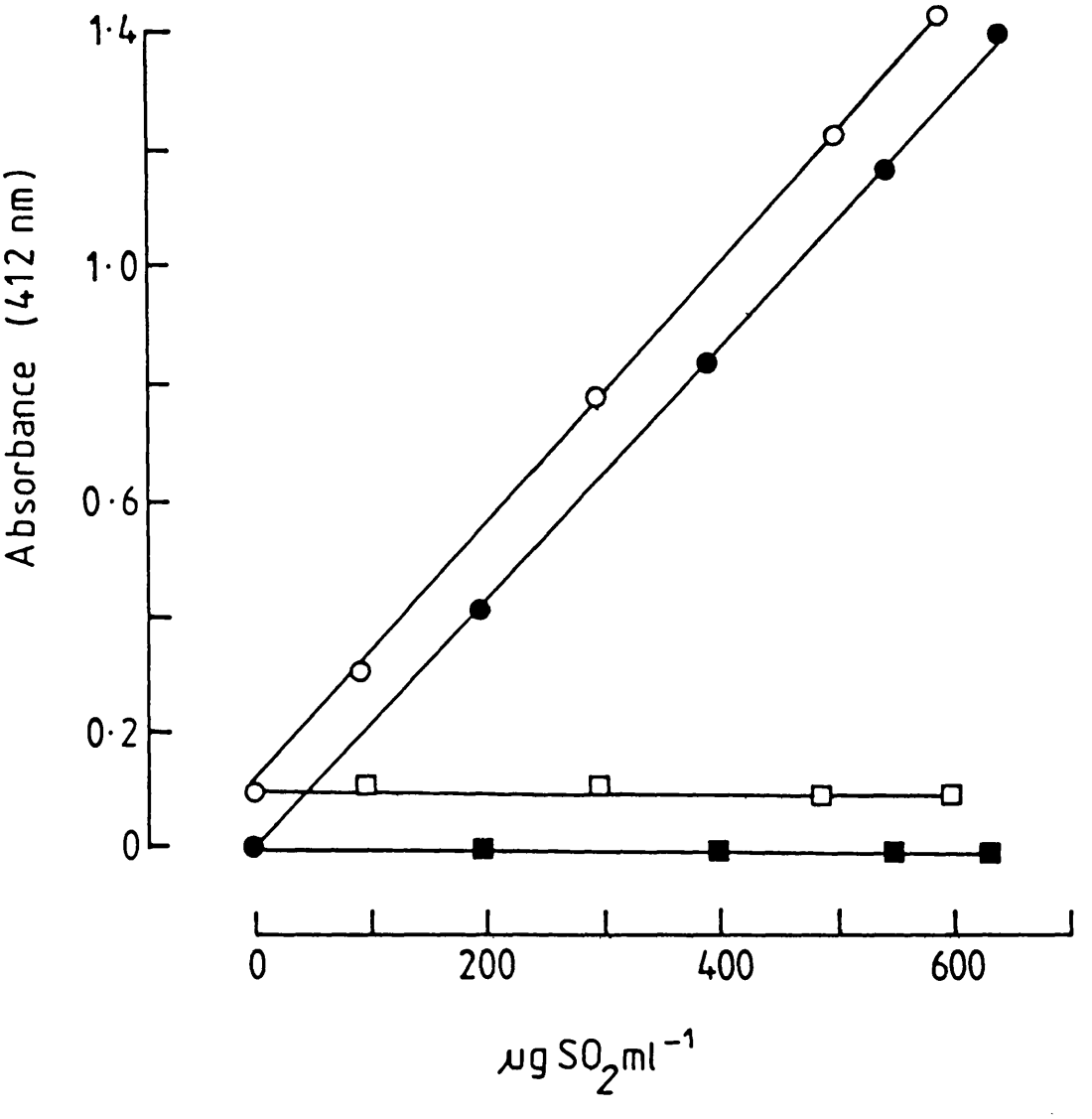


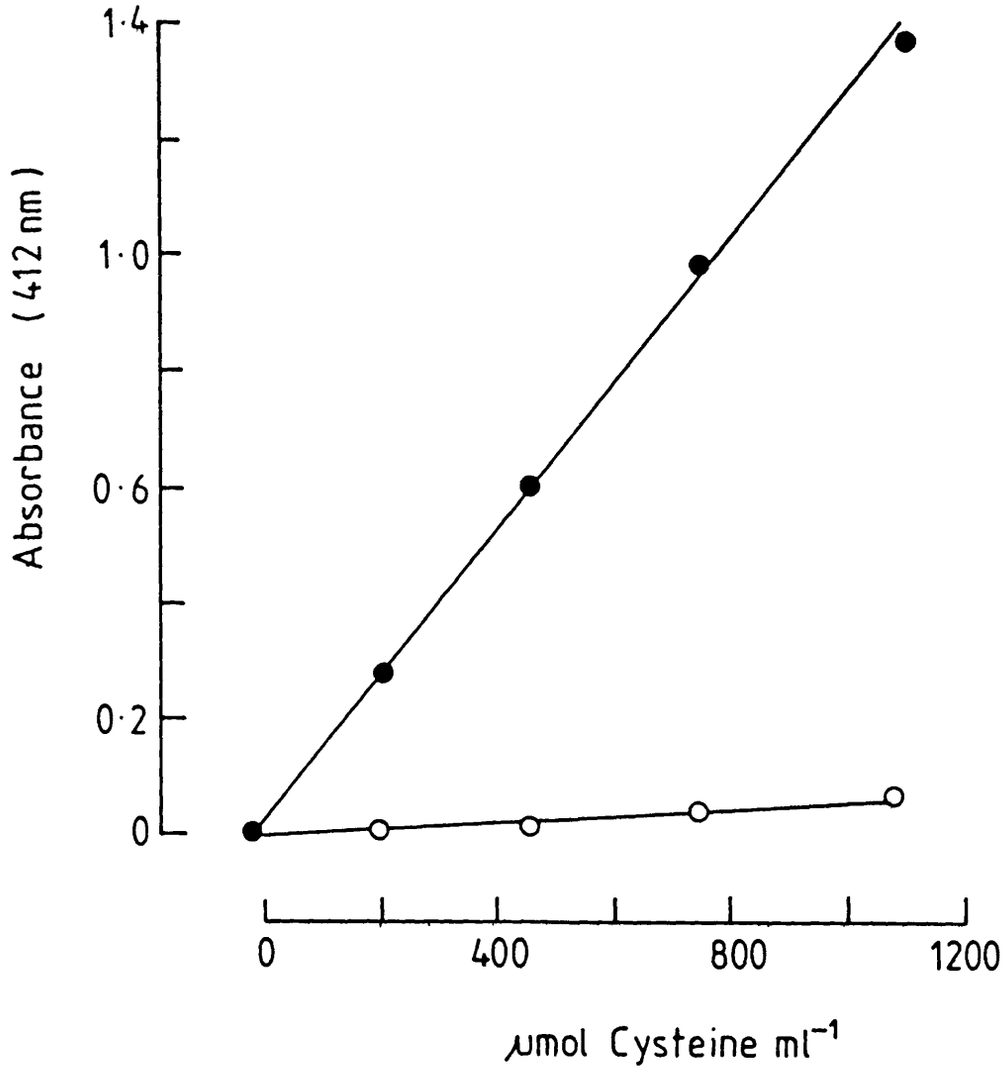
Figure 6. Interference by sulphite concentration in the measurement of sulphate concentration.

Figure 7. Interference by thiols in the measurement of sulphite concentration. Detection by means of formaldehyde treatment.



Key: ○ sulphite and thiol; ● thiol;
□ sulphite and thiol + formaldehyde (50 mM);
■ sulphite + formaldehyde (50 mM)

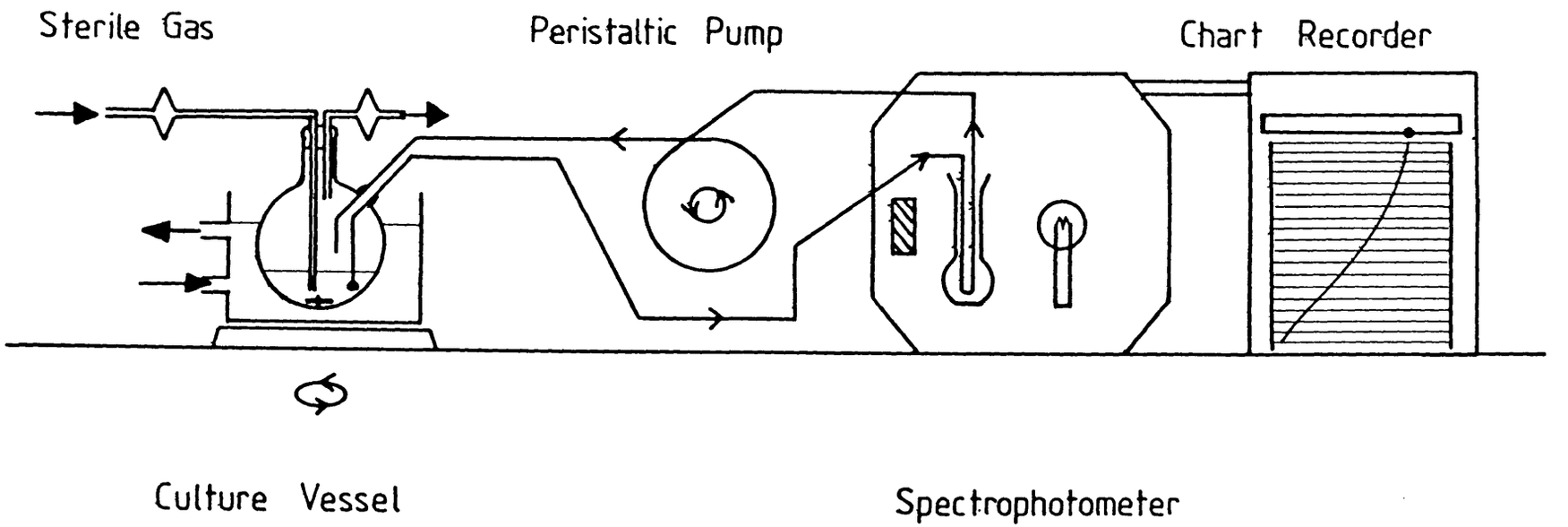
Figure 8. Interference by cysteine in the measurement of sulphite concentration. Quenching of cysteine by oxidation.



Key: ● Freshly-prepared solutions of cysteine

○ Solutions of cysteine stored aerobically at 25 °C for 20 min.

Figure 9. Flow diagram of turbidometer apparatus used in sulphite-sensitivity culture studies.



RESULTS

Spectrophotometric method - theory and practice

The sulphite ion reacts with many organic disulphides to displace a thiol anion thus forming the organic thiosulphate, commonly referred to as a "Bunte" salt.

(1) $RSSR + SO_3^{2-} \rightleftharpoons RSSO_3^- + RS^-$ (see Fig. 10)

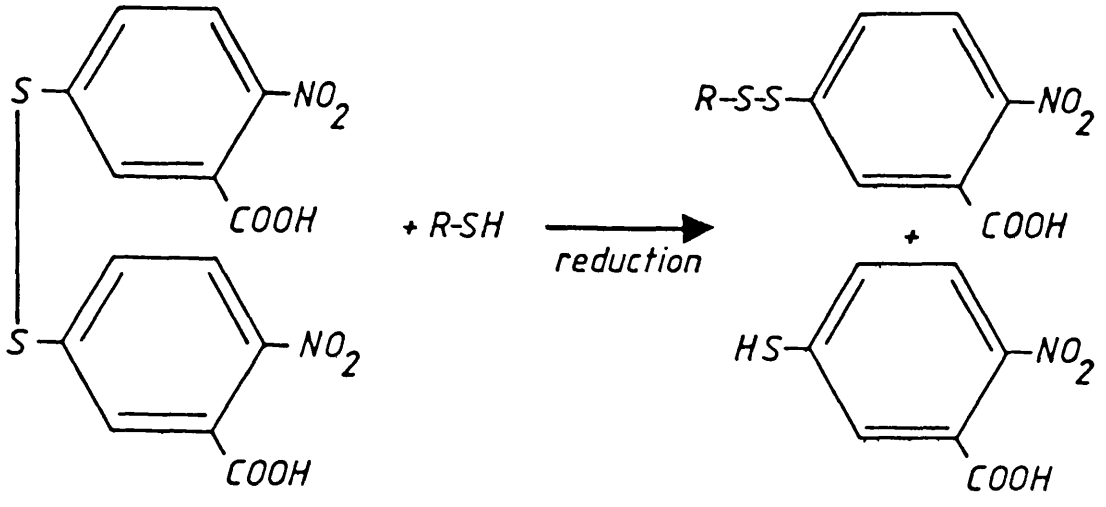
A large excess of sulphite ion is normally needed to estimate quantitatively the disulphide and the thiol anion is usually titrated with silver nitrate (Cecil and McPhee, 1954).

Some disulphides e.g. 5,5'-dithiobis(2-nitrobenzoic acid), "Ellman's reagent", and 4,4'-dithiodipyridine (Grassetti and Murray, 1967) are used also for the determination of thiol groups. The disulphide is reduced to the corresponding thiol which absorbs at a different wavelength from the disulphide. As the disulphide is in excess, a mixed disulphide is probably formed:

(2) $R'SSR' + RSH \rightleftharpoons R'SSR + R'SH$

The disulphide absorbs maximally at 324 nm and the 5-mercapto-2-nitrobenzoic acid at 412 nm (Fig. 10). When the concentration of the disulphide was *ca.* 5×10^{-5} M, a mole ratio plot (Fig. 11) of SO_3^{2-} indicated a stoichiometric reaction, 1 mole of SO_3^{2-} reacting with 1 mole of DTNB. The accentuated inflection seen in this plot suggests that there are no intermediates formed and

that the 5-mercapto-2-nitrobenzoate moiety is produced as one of the products in each reaction.



5,5 - dithiobis (2-nitrobenzoic acid)

Pale yellow

5-mercapto-2-nitrobenzoate

Dark yellow

Concentration range for SO_2 determination

At a wavelength of 412 nm and a light path of 1 cm, 0.04 µg SO_2/ml was equivalent to 0.001 absorbance units (SP6-550 UV-VIS spectrophotometer; Pye-Unicam). A straight-line plot (Fig. 12) was obtained up to an absorbance of 2.04 units, this being equivalent to a concentration of 950 µg SO_2/ml. To avoid saturation of DTNB and loss of SO_2 from the system, the flow of SO_2 in the carrier gas was diverted by means of a 'Y' junction adaptor to duplicate receiving vessels containing the reagent.

Stability of sulphite and formation of thiol

Sulphite solutions containing EDTA (1×10^{-4} M) maintained under a blanket of dinitrogen at *ca.* 4°C were stable for at least 60 min. The coloured complex was formed within 2 min of the addition of SO_2 to DTNB and, although there was no appreciable loss of colour for 60 min (Table 10), the absorbance decreased significantly with prolonged (18 h) aerobic storage due to oxidation of the thiol anion (Humphrey *et al.*, 1970).

Rate and efficiency of SO_2 uptake by DTNB

For free and total SO_2 determinations in samples (5 g) of sausage meat, a 900 s reaction time gave a 95% and 100% recovery of SO_2 respectively (Table 11). Several receiving vessels containing DTNB connected in series to the primary vessel did not recover any measurable SO_2. A carrier gas flow rate of 20 $cm^3 s^{-1}$ (equivalent to *ca.* 100 bubbles min^{-1} in the receiving vessel containing 50 ml of DTNB) allowed complete harvesting of the SO_2 coming from the reaction vessel.

Stability of DTNB

The observation that DTNB *per se* showed no tendency to form the highly absorbing thiol over a storage period of 28 d (pH 8.0; 25°C) is in accord with that of others e.g. Wedzicha and Johnson (1979); Wedzicha and Bindra (1980). The sulphite ion reacts quantitatively with DTNB over the range of pH 6 - 9 (Humphrey *et al.*, 1970); above pH 8.0, however, there is

Table 10. Influence of incubation time on absorbance of DTNB + SO_2 stored aerobically at *ca*. 25°C

	Minutes					
	0	1	2	5	60	960
Blank control	0.000*	NT	NT	NT	0.000	0.000
Standard A	0.099	0.102	0.101	0.099	1.000	0.072
B	0.111	0.117	0.121	0.123	0.119	0.077
C	0.351	0.362	0.361	0.361	0.361	0.264
D	0.473	0.492	0.497	0.496	0.500	0.327
E	0.521	0.544	0.545	NT	0.545	0.430
F	1.344	1.367	1.375	1.375	1.372	1.122
G	2.106	2.234	2.245	2.246	2.237	1.734

* Absorbance at 412 nm

NT not tested

Table 11. Rate of SO_2 harvesting by DTNB (5 g sausage)

Time (s)	Free SO_2 (%)	Total SO_2 (%)
0	0	0
150	32	47
300	90	96
600	94	99
900	95	100
1800	100	100

some evidence of breaking of S-S linkages, probably due to attack by OH^- radicals, leading to the formation of the highly absorbing thiol. As it was considered that a DTNB solution buffered at pH 8.0 would be consistent with efficient trapping of SO_2 from the gas phase, it was adopted in the present work.

Influence of sample alkalization on total SO_2 determination

Bound sulphite compounds are dissociated very slowly in acid solutions at room temperature, Vas (1949); they are released rapidly under alkaline (pH 9 - 11) conditions (Ponting and Johnson, 1945; Brown, 1977). Although extended (60 min) distillation would be expected to dissociate bound SO_2 moieties (Shipton, 1959) it has been suggested that short (15 min) distillation periods may result in a reduced yield (Wedzicha and Bindra, 1980). Alkalization of sausage homogenate for 30 min before acidification and distillation did not result (Table 12) however in a significantly greater ($p = 0.05$, paired t-test) yield of total SO_2 with subsequent measurement by the method developed in this study.

Interference with DTNB

Unsulphited sausage meat

Using the spectrophotometric method developed in this study, no interference from components of freshly produced or stored unsulphited sausage was detected with sparging (free SO_2 determination) or distillation (total SO_2 determination). If sausage (0.1 g) was added directly to DTNB, an inflated blank value resulted; the

Table 12. Effect of alkalization on recovery of total SO_2 in the spectrophotometric method

Sample	Spectrophotometric method	Addition of NaOH (1 M; 25 ml) to sample 30 min before acidification and distillation
	A	B
1	292 *	275
2	316	322
3	347	354
4	349	329
5	382	376
6	456	441
7	471	484
8	489	462
9	492	501
10	530	522

* µg SO_2 g^{-1} sausage

interference by these unknown components of meat or microbial origin increased during storage at 4°C(Table 13)

Thiols

Cysteine reacts with DTNB to produce a complex. There is a straight line plot up to at least 1050 µg freshly prepared cysteine/ml (Fig. 8). Such interference was rarely encountered, however, in routine estimation of SO_2 concentration in sausage or broth cultures. Most thiols will oxidise in air after *ca*. 20 min at 25°C and thus fail to form a chromogen with DTNB (Fig. 8). The addition of 0.01 ml of formaldehyde (50 mM) to a sample containing sulphite and a thiol leads to the formation of formaldehyde sulphoxylate which does not react with DTNB (Fig. 7).

Electrometric ion-selective probe method

The theory and use of gas-sensing membrane probes for the determination of SO_2 in solution has been discussed by Bailey and Riley (1975). Such probes incorporate an SO_2 permeable membrane so that a hydrogen ion-sensing electrode is separated from the test solution by a buffered aqueous solution of hydrogen sulphite. In this study, the concentration of SO_2 was usually maximised by poising the test solution below pH 1.0 before analysis. The equilibria associated with the hydrolysis of SO_2 in aqueous solution can be summarised (King *et al.*, 1981):

(3) $$SO_2 + H_2O \rightleftharpoons H_2O.SO_2$$

Table 13. Interference with DTNB from components of stored ($4^{o}C$) unsulphited sausage

Days	Apparent SO_2 concentration (µg/g sausage)	
0	8*	0†
1	6	0
2	0	0
3	21	0
4	14	0
8	41	0

* Direct addition of sausage (0.1 g) to DTNB reagent

† Method for total SO_2 determination

Table 14. Interference in total SO_2 determination of unsulphited sausage*

Storage at $4^{o}C$ (days)	Apparent SO_2 (µg SO_2 g^{-1} sausage)
0	12
1	4
2	0
3	26
4	17
5	34
6	0
7	12
8	15

* Shiptons (1959) modification of Monier-Williams's (1927) method

(4) $H_2O.SO_2 + H_2O \rightleftharpoons HSO_3^- + H_3O^+$

(5) $HSO_3^- + H_2O \rightleftharpoons SO_3^{2-} + H_3O^+$

The use of the probe in this study was based largely on the methods of Brown (1977). Two technical points need to be stressed. Firstly chilled centrifugation of homogenised samples was needed because pork fat tended to coat the membrane of the probe. This resulted in (a) a loss of sensitivity and progressively lower estimations of SO_2 concentration and (b) a short membrane life. The poor recovery of both free and total SO_2 in samples can be explained in part because the SO_2 level of sausage serum only was measured without regard to SO_2 trapped in the fat or solid phases of centrifuged sausage homogenate. Secondly, a deflection (mv) was registered on an EIL pH meter (monovalent cation setting) following immersion of the probe in the acidified specimen. At very low concentrations (< 50 µg/ml) of SO_2, the peak deflection after 2 min was often difficult to measure because of (a) a slow response period and (b) interference by unidentified factors. At high concentrations (> 400 µg/ml) of SO_2, the peak deflection after 2 min was easily measured but the recovery period (dialysis against distilled water) required to remove excess SO_2 from the internal buffer solution of the probe was seldom less than 10 min.

Concentration range for SO_2 determination

The probe could detect as little as 5 µg SO_2/ml and a plot of the logarithm of preservative concentration against the electrometric deflection (mv) was linear up to at least 5×10^{-3} M $Na_2S_2O_3$ (Fig. 2).

Steam distillation method

The modification (Shipton, 1959) of Monier-Williams's (1927) method was used for reference purposes in this study.

Interference

The steam distillation method (Table 14) was prone to interference. This resulted in an inflated blank value of *ca.* 15 µg SO_2 g^{-1} sample from unidentified compounds in unsulphited sausage.

Influence of sample size

Although analysis of ten replicates of sausage meat gave identical mean recoveries (412.6 µg SO_2/g) for 10 g and 20 g samples, the variation in the former (standard deviation = 38.18) was greater than in the latter (standard deviation = 22.84). The better reproducibility obtained with the 20 g sample led to its adoption for all assays based on steam distillation.

Comparison of methods

Total SO_2 determination

In direct comparisons, the spectrophotometric method proved to be less variable than the steam distillation method (Table 15) There was no significant difference (Students t-test $p = 0.01$) in the level of recovery of SO_2 and an excellent correlation between the two methods (Spearman's rank correlation coefficient ($r_s = 0.99$). A regression line (least squares method) fitted to the data had a coefficient of determination (r^2) of 0.96

Table 15. Analysis of variance between the steam distillation, spectrophotometric and probe methods for estimation of total SO_2 in sausage

Replicate	Spectrophotometric (5 g)*	Steam distillation (20 g)*	Probe (5 g)*
1	393+	425	370
2	400	400	351
3	415	439	392
4	381	440	385
5	401	396	349
6	412	410	393
7	380	440	360
8	390	405	345
9	392	390	362
10	387	382	380
$\bar{x}$	395.1	412.6	368.7
σn-1	11.9	22.8	18.0

+ µg total SO_2 g^{-1} sausage

* Sample size

(intercept (y) at 0.08, slope (m) of 0.98). A more detailed comparison between twenty duplicate samples confirmed that there was no significant difference (Paired t-test $p = 0.01$) between total SO_2 recovery and a regression line fitted to the data was described: $r^2 = 0.99$, $y = 6.03$ and $m = 1.00$ (Fig. 13). Comparison of the steam distillation with the probe method showed that the latter was less variable. The differences in SO_2 recovery between the methods were significant ($p = 0.05$) when analysed by the t-test and correlation between the methods was poor, $r_s = 0.67$.

Comparison of methods for total SO_2 estimation after freezing of the sample

A limited study showed that freezing ($-20^{o}C$) of samples of sausage meat for up to 48 h had no appreciable effect on recovery of total SO_2 by the steam distillation or the spectrophotometric methods. Although the latter recovered on average more SO_2 ($\bar{x}$ = 401.66 μ g SO_2/g) before sample freezing, this level was not significantly higher ($p = 0.05$) than those achieved by either method after freezing (Table 16). The error, as indicated by the standard deviation ($\sigma n-1$), was very small ($\sigma n-1 = 2.89$) for the assay of fresh sausage but relatively large ($\sigma n-1 = 12.65$) for the assay of stored freezer material.

Free SO_2 determination

The spectrophotometric method recovered significantly more free SO_2 than the probe (t-test, $p = 0.05$) and was less variable

Table 16. Influence of sample freezing (-20°C; 48 h) on recovery of total SO_2

Replicate	Spectrophotometric		Steam distillation
	Before freezing	After freezing	After freezing
1	401.2*	393.2*	403.2*
2	399.4	400.2	388.8
3	406.7	415.8	396.0
4	400.3	381.2	408.0
5	400.7	401.8	349.6
$\bar{x}$	401.66	398.40	389.10
σn-1	2.89	12.65	23.26

* µg SO_2 g^{-1} sausage

(Table 17). Correlation between the methods was very good (r_s = 0.99) according to Spearman's rank correlation test and a regression line fitted to these data had an r^2 value of 0.92, (y = 0.44, m = 0.88). A more detailed study of 20 duplicate samples confirmed that the spectrophotometric method recovered significantly (p = 0.05, paired t-test) more free SO_2 than the probe method. A regression line (Fig. 14) described these data (r^2 = 0.98, y = 4.41 and m = 0.88).

Estimation of SO_2 concentration in sausage seasonings

Analysis of sausage seasonings (RHM 599) used commercially in the preparation of sulphited or unsulphited batches of sausage is summarised in Table 18. The iodimetric method, used by the large-scale manufacturers for determination of SO_2 was prone to error due to unidentified moieties in the unsulphited seasoning. On average this error accounted for *ca.* 14% of the apparent SO_2 concentration and explains the consistently higher values obtained using the iodimetric, rather than the spectrophotometric, method.

Sulphite binding

Sterile solutions of sulphite maintained under a blanket of oxygen-free dinitrogen did not bind significant amounts of free, or cause the loss by oxidation, of total SO_2 (Table 19). Aerobically stored solutions of sulphite led to an initial binding of 15% of the free SO_2, a level which was maintained during the period of incubation. All meat ingredients of sausage bound significantly (p = 0.05) greater amounts of free SO_2 and catalysed also the loss

Table 17. Comparison of methods for recovery of free SO_2 in 10 replicate samples of sausage

Replicate	µg SO_2 g^{-1} sausage	
	Spectrophotometric (5 g)*	Probe (5 g)*
1	160	145
2	144	120
3	139	110
4	135	130
5	152	97
6	150	121
7	165	147
8	150	99
9	151	105
10	145	102
$\bar{x}$	149.10	117.6
n	10	10
$\sigma n-1$	8.99	18.3

* sample size

Table 18. SO_2 concentration of sausage seasonings

RHM 599 Seasoning		Iodimetric method	Spectrophotometric: Direct addition to DTNB	Spectrophotometric: Method used on pp.
1	+ SO_2	35,760† (608)*	32,450 (552)	31,940 (543)
	- SO_2	5,760	0	0
2	+ SO_2	31,150 (530)	29,260 (497)	28,750 (489)
	- SO_2	3,820	0	0
3	+ SO_2	34,250 (582)	31,660 (538)	30,980 (527)
	- SO_2	4,660	0	0

* Figures in parentheses : µg SO_2 g^{-1} sausage mix assuming seasoning added at 1.7% w/w

† µg SO_2 g^{-1} seasoning

No interference was detected with either of the spectrophotometric methods

Table 19. Sulphite-binding capacity of sausage ingredients

	Time (h) 0			3			6			
	Total	Free	% bound	Total	Free	% bound	Total	Free	% bound	% Total SO_2 lost
										t_o-t_6
sterile lean pork	670*	502	25	431	146	66	206	65	69	69
lean pork	645	483	25	402	167	59	234	82	65	64
head meat	532	371	30	370	202	45	181	104	43	66
belly meat	571	492	14	422	198	53	369	121	67	35
rinds	603	504	16	524	364	31	440	253	43	27
fat	643	263	59	278	92	67	76	21	72	88
MRM†	584	147	75	163	83	49	102	45	56	83
rusk	612	470	23	525	322	39	370	270	27	40
aerobic control	602	514	15	556	461	17	491	367	25	18
anaerobic control	623	537	14	604	549	9	587	520	11	6

† MRM = mechanically-recovered-meat

* µg SO_2 ml^{-1} medium determined by spectrophotometry

of greater (p = 0.05) amounts of total SO_2 (Table 19). With the exception of mechanically-recovered-meat, binding and irretreviable loss of SO_2 increased with storage time in the presence of meat ingredients. On average, rusk bound only 30% of the free SO_2; after 6 h, however, the absolute level of SO_2 had fallen by 40%.

SO_2 loss from stored British fresh sausage

Influence of site

There was no significant difference (p = 0.05) between the levels of total SO_2 recovered from the centre and from the surface layer (0.25 cm) of sausage at the storage temperatures used in this study (Fig. 15 a,b,c). The level of free SO_2 recovered from the surface was significantly lower (p = 0.05) than from the centre at all three storage temperatures, 4, 10 or 22°C (Figs. 15 a, b and c respectively).

Influence of temperature of sausage

Total SO_2

On average 24% of total SO_2 added to the ingredients in the bowl chopper was lost irretreviably; loss of total SO_2 occurred at a rapid rate from samples taken immediately following manufacture to the 3rd day of storage but at a much reduced rate thereafter (Fig. 16). Although the initial level of SO_2 in the freshly manufactured sausage did not influence the rate of loss it did dictate the final level on day 8. Loss was related directly to temperature. Thus after 8 d at 4°, 10° or 22°C, 82%, 79% and 76% of the total SO_2 level (at t_o) remained.

Free SO_2

On average 27% of the total SO_2 added to sausage ingredients in the bowl chopper was bound reversibly; thereafter the loss of free SO_2 from day 0 to day 5 at all storage temperatures conformed to the equation:

(6) $$\log_{10} SO_2 = mx + b$$

where x = storage time in days, m = slope/gradient and b = $\log_{10} SO_2$ at t_o. After day 5, the rate of loss diminished (Fig. 16). The rate of loss was significantly different ($p = 0.05$) at the 3 storage temperatures ($22^\circ > 10^\circ > 4^\circ C$). After 8 d at 4°, 10° or $22^\circ C$, 27%, 18% and 11% respectively of the initial level of free SO_2 remained.

Binding of free SO_2

The rate and extent of binding was related directly to the storage temperature. Thus only storage at $4^\circ C$ prevented substantial loss of free SO_2 during the period day 0 - day 5.

Loss of SO_2 from sausage meat stored in Petri dishes

Although in these experiments 600 µg SO_2 were added per gram of sausage ingredients in the bowl chopper, 150 µg were lost irretrievably The absolute level of total SO_2 in sausage meat (100 g) stored in sealed square Petri dishes at 4°, 9°, 15°, 20° or $25^\circ C$ diminished rapidly from day 0 to day 4; thereafter the rate of loss decreased (Fig. 17). The initial rate of loss was related

Figure 10. Formation of 5-mercapto-2-nitrobenzoic acid from reaction of SO_2 with 5,5'-dithiobis-2-nitrobenzoic acid Key: ○ DTNB ● DTNB + SO_2

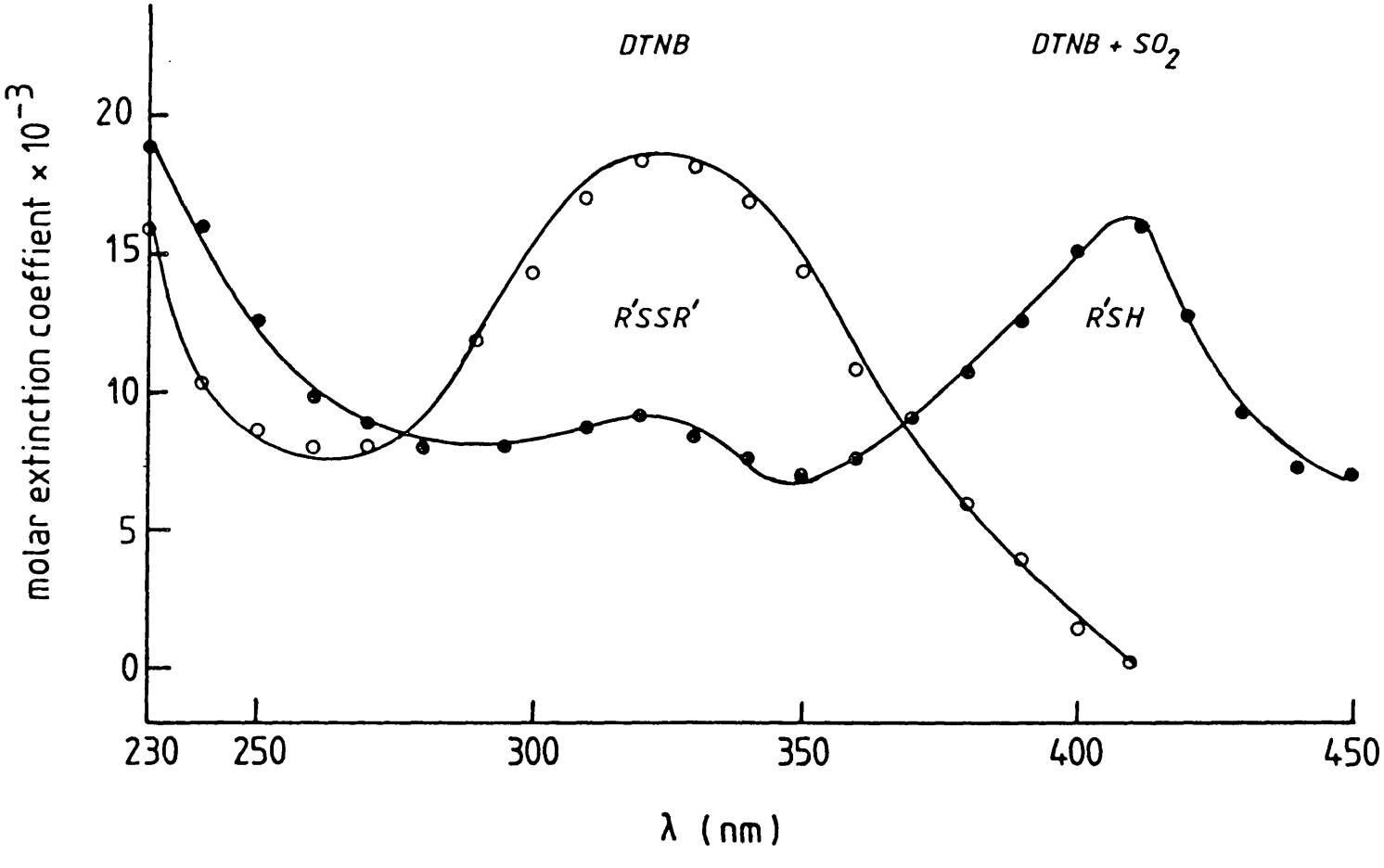
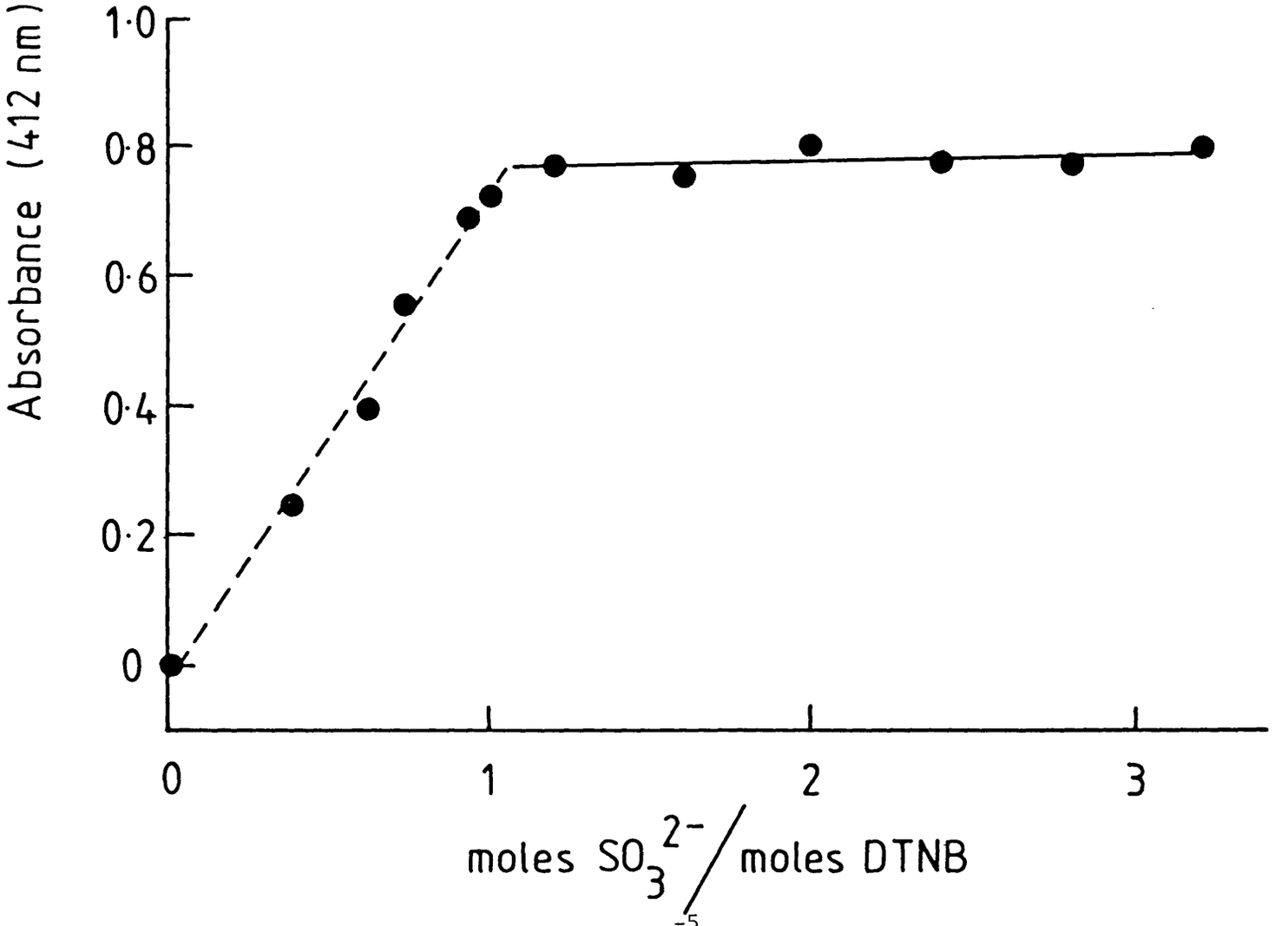


Figure 11. Mole ratio plot of sulphite and DTNB*



* Concentration of disulphide = *ca.* 5 x 10^{-5} M)

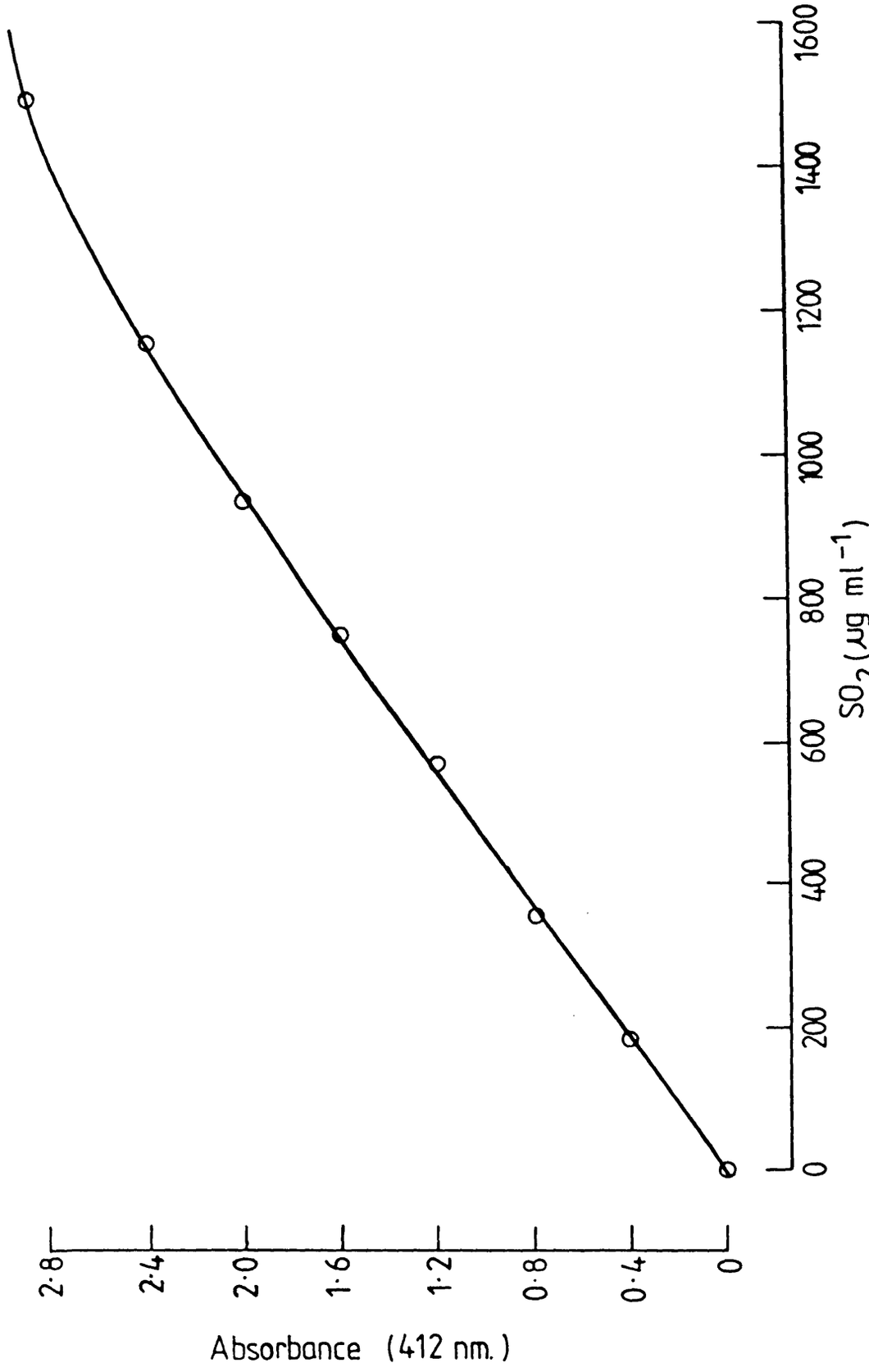


Figure 12. Standard curve of SO_2 concentration as measured by the spectrophotometric assay.

Figure 13. Comparison of methods for the determination of total SO_2 concentration in fresh sausage.

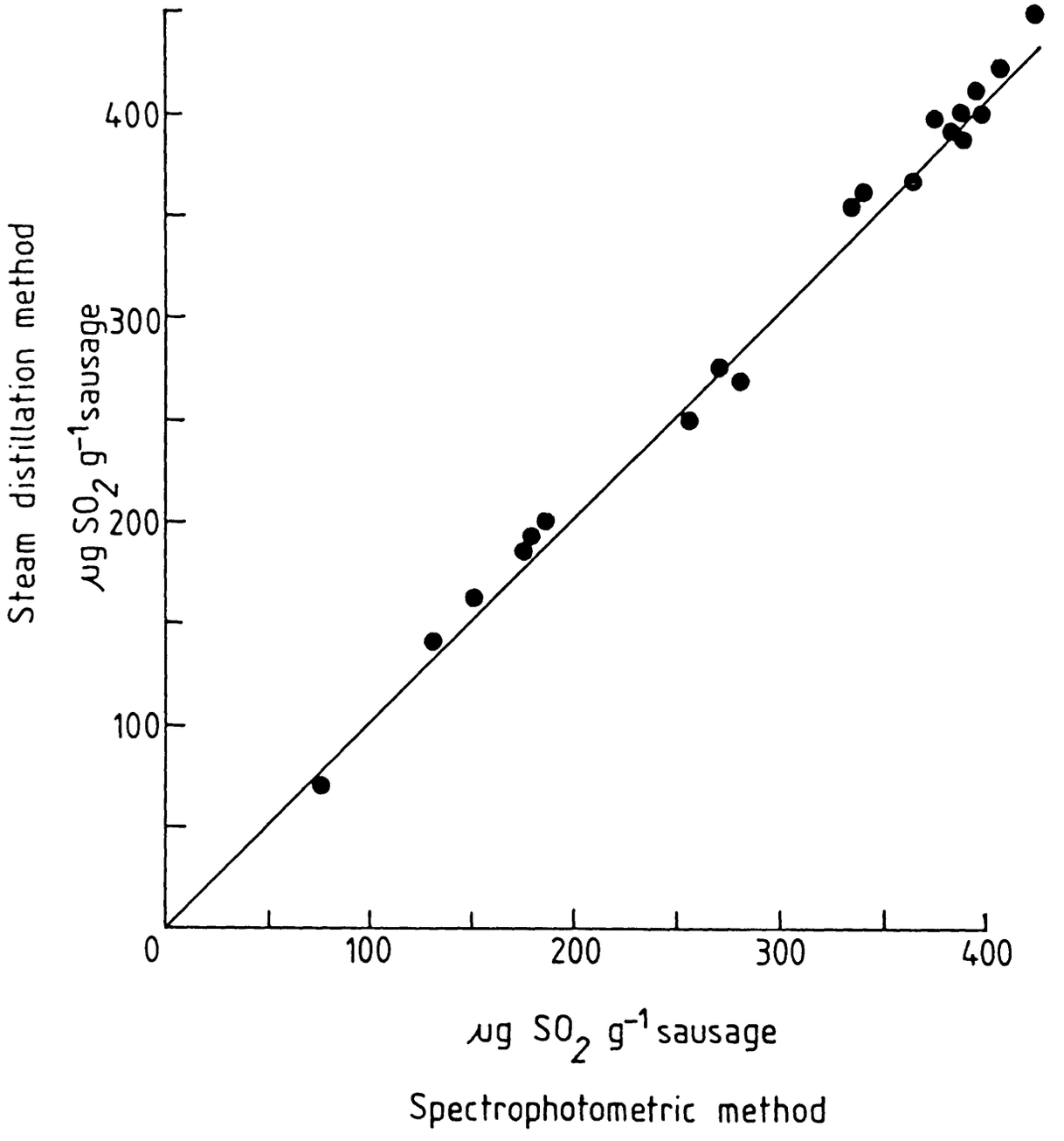


Figure 14. Comparison of methods for the determination of free SO_2 concentration in fresh sausage.

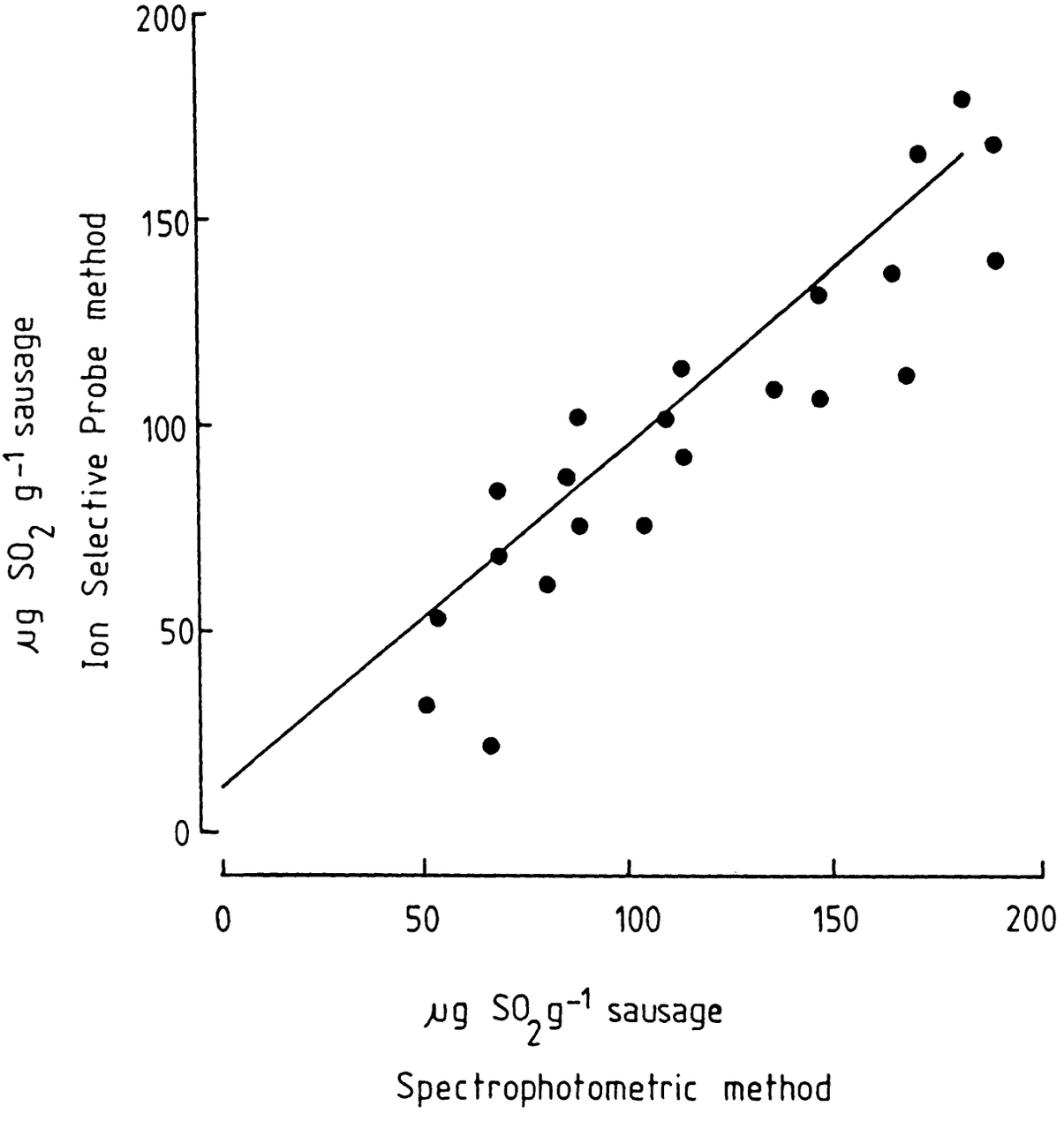


Figure 15. Influence of temperature and location on the loss of total and free sulphite from stored fresh sausage.

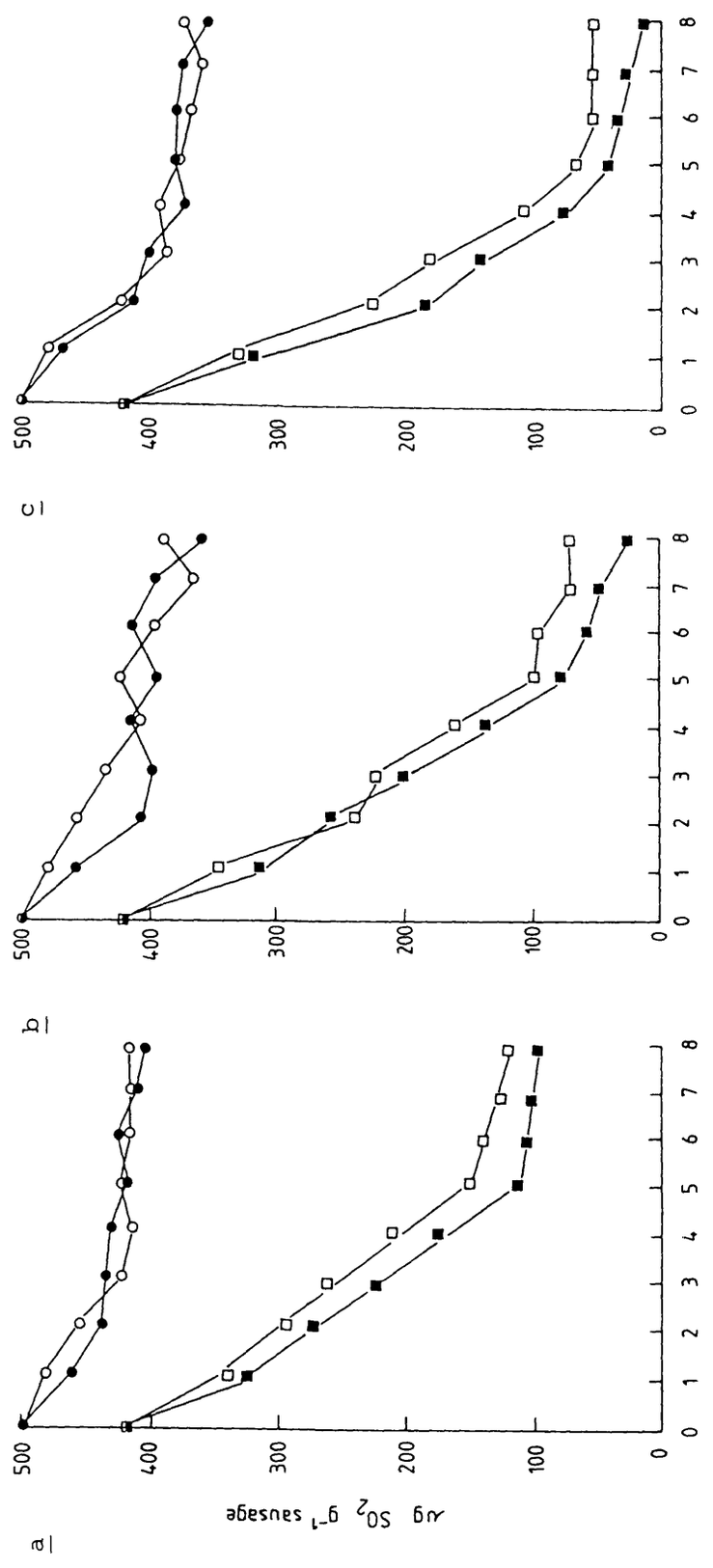


Figure 16. Influence of temperature on the loss of free and total sulphite from stored fresh sausage

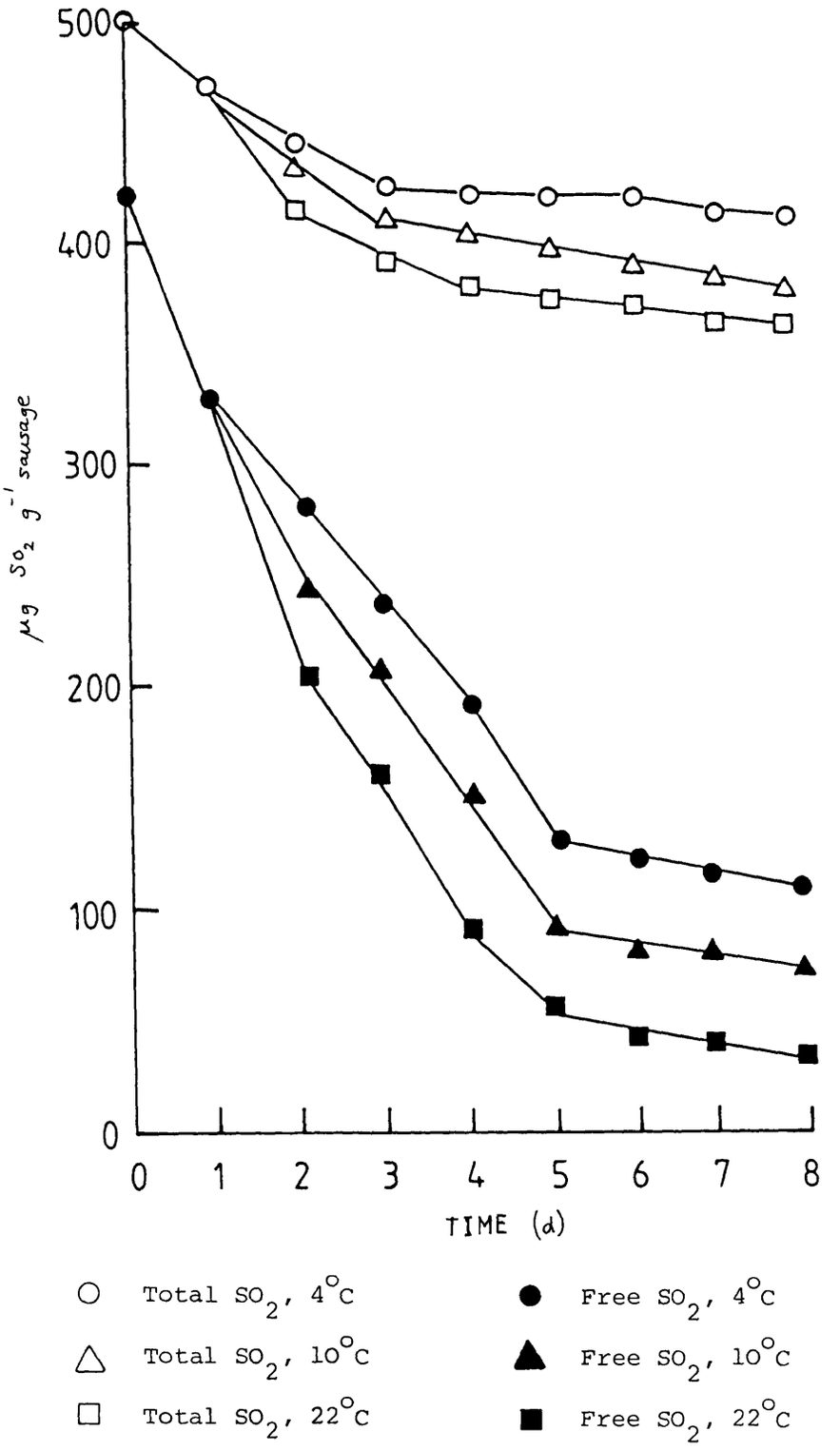


Figure 17. Influence of temperature on the loss of free and total sulphite from sausage meat stored in Petri-dishes

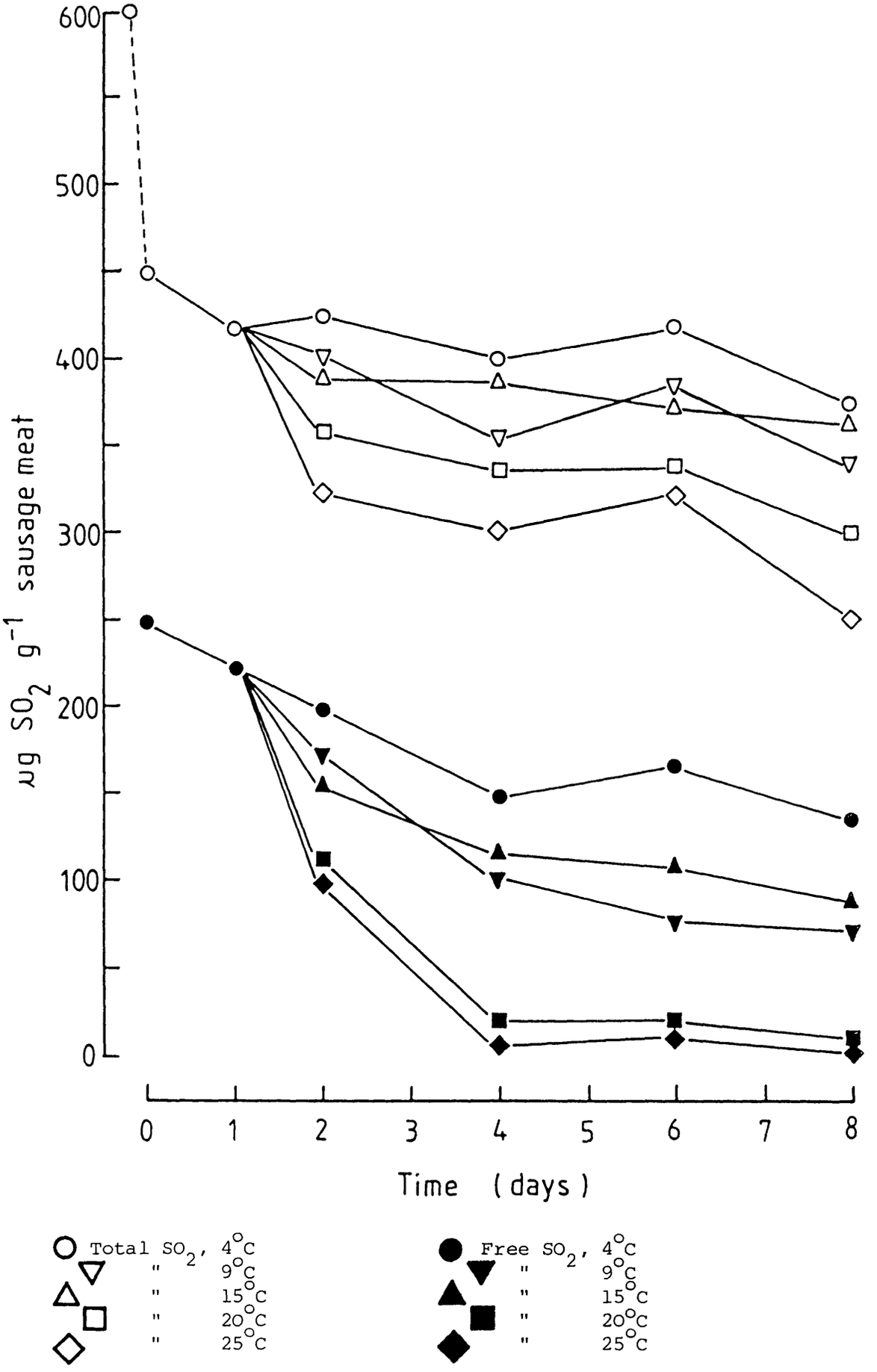


Figure 18. Exponential regression line describing the influence of storage temperature on the rate of loss of total SO_2 from sausage meat

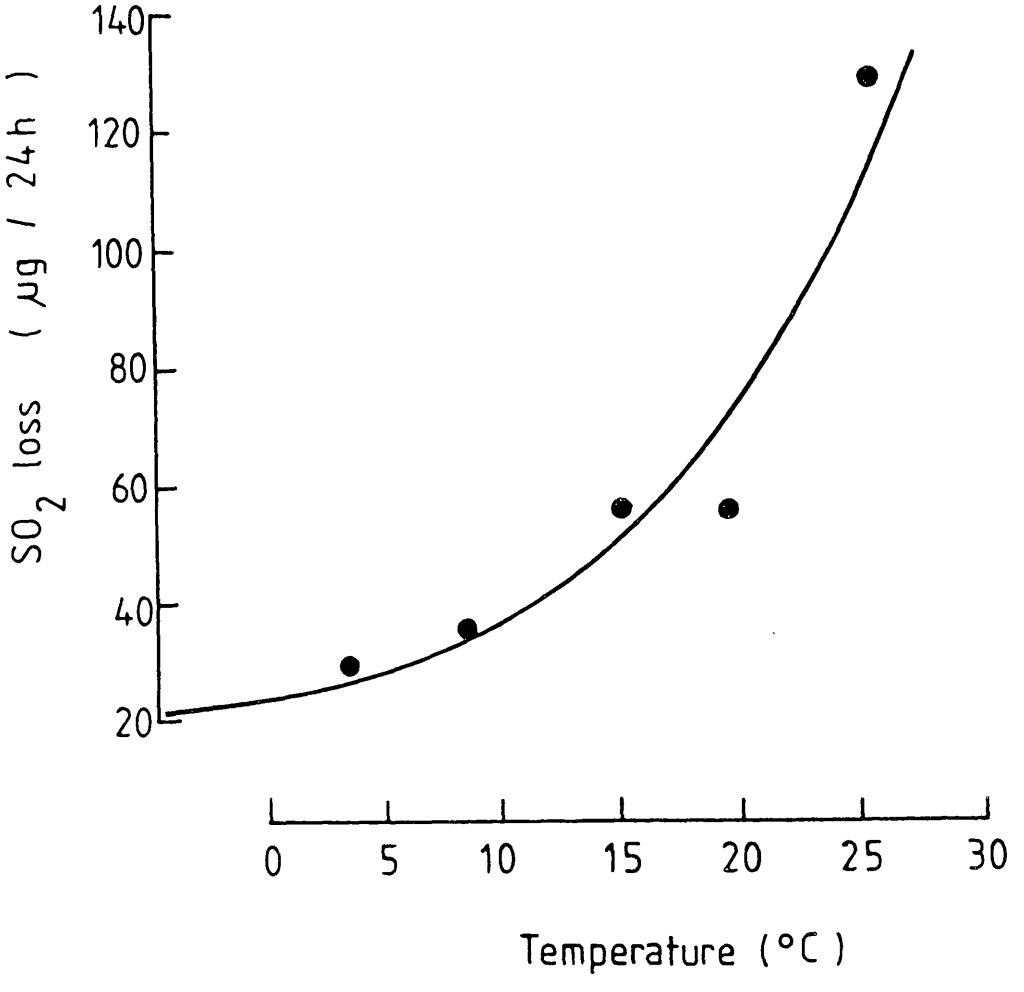
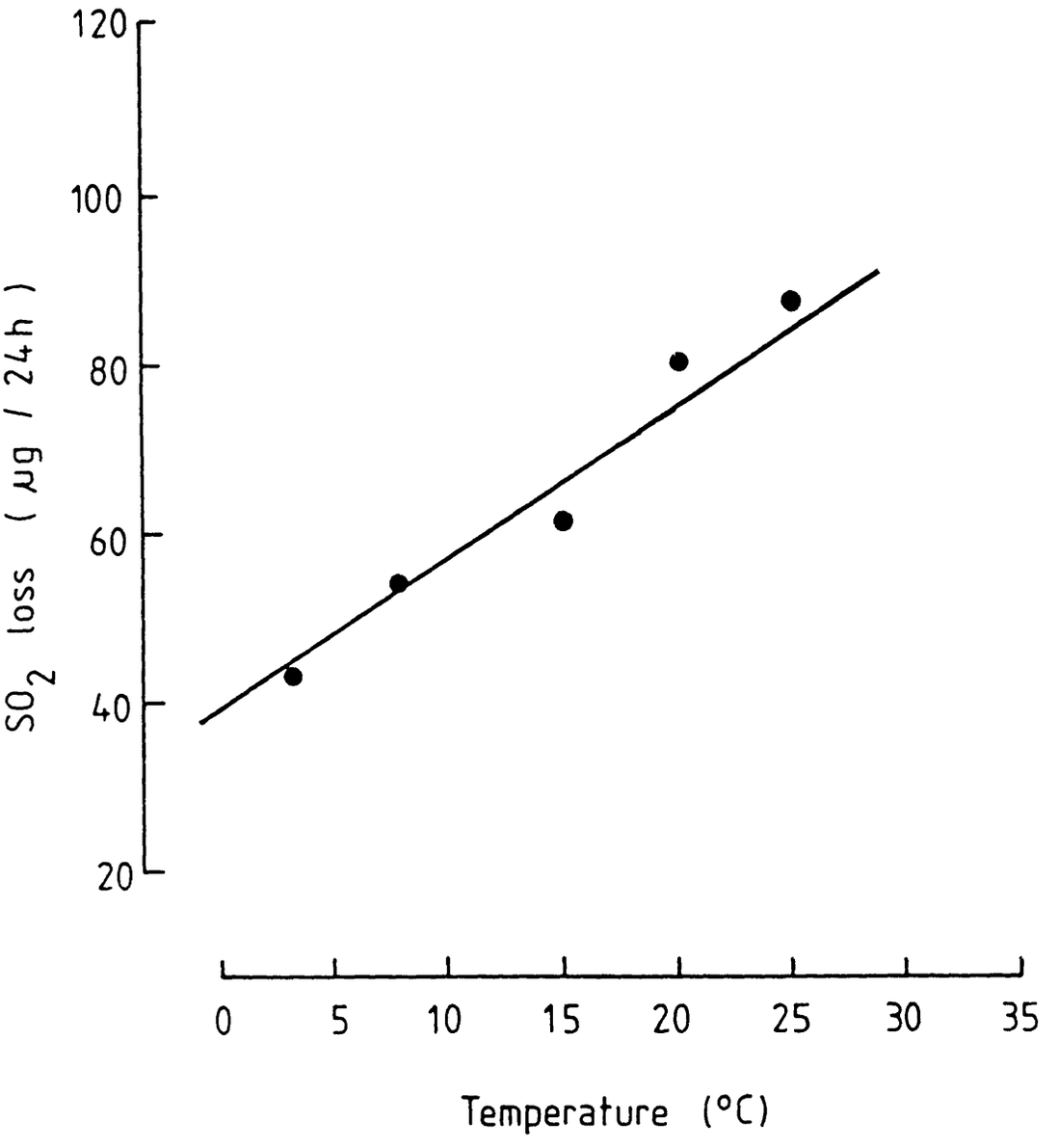


Figure 19. Linear regression line describing the influence of storage temperature on the rate of loss of free SO_2 from sausage meat.



directly to storage temperature, a linear regression line (Fig. 18) fitted to the data had a coefficient of determination (r^2) = 0.98, intercept (y) 2 34.5 and slope (m) = 1.92. Free sulphite levels declined rapidly (Fig. 17) and loss was related exponentially (Fig. 19) to temperature, r^2 = 0.87, y = 21.76 and m = 0.06.

Sources and extent of contamination of British fresh sausage of factory origin

Analysis of 35 batches of freshly made sulphited pork sausage over the period 1.10.79 - 31.9.82 showed that *Brochothrix thermosphacta*, lactobacilli, Enterobacteriaceae and yeasts were always present at levels greater than 10^2/g; streptococci and pseudomonads were present in low numbers (10^3/g or less) only (Table 20). In all cases the sausage microflora was dominated numerically by *Brochothrix thermosphacta* (isolated on STAA medium), substantial contamination with lactobacilli or pseudomonads was of infrequent occurrence. Of the organisms noted above only the pseudomonads and Enterobacteriaceae were present in significantly greater numbers in the freshly made unsulphited product. With the exception of the streptococci, most raw meat ingredients awaiting use in the factory harboured large (> 10^3/g) populations of all of the members of the microbial association (Table 20). The minced ingredients, belly and head meat, were usually more heavily contaminated with all types of micro-organisms than were whole pieces of lean pork. Back fat contained relatively low numbers of contaminants, especially Enterobacteriaceae, whereas rinds, which had been cooked, minced and chilled, were always heavily infected (Table 20).

Table 20. Sources and extent of microbial contamination of British fresh sausage of factory origin

	Sulphited sausage	Unsulphited sausage	Lean pork	Belly pork	Head pork	Cooked rinds	Back fat
	35*	8	20	20	20	20	20
Yeasts	4.22†	3.88	3.73	3.42	4.17	3.90	3.07
	3.21-4.92‡	2.51-4.65	2.14-6.17	2.64-4.98	3.92-5.55	2.92-6.75	2.1-5.44
Br. thermosphacta	5.86	5.93	4.46	5.67	5.92	6.22	5.20
	4.3-7.92	4.7-8.24	< 2-6.73	4.4-8.4	5.9-7.67	4.73-8.44	3.84-6.87
Lactobacillus spp.	3.62	3.71	3.45	3.15	3.84	4.02	3.77
	2.2-5.37	2.34-6.41	2.2-6.89	< 2-4.21	2.6-6.48	< 2-6.64	< 2-5.80
Streptococcus spp.	2.94	2.60	1.54	1.07	1.94	2.89	1.04
	< 1-4.60	< 1-4.32	< 1-2.2	< 1-2.5	1.78-2.62	< 1-4.48	< 1-2.0
Pseudomonas spp.	3.35	4.42	3.11	3.47	4.25	4.77	3.18
	< 2-6.13	2.98-5.89	<2-5.89	< 2-6.47	< 2-5.73	2.92-8.49	< 2-6.07
Enterobacteriaceae	3.59	4.42	3.78	4.64	4.85	5.83	2.86
	2.4-4.42	3.46-4.92	< 1-5.14	2.4-5.94	2.6-6.37	2.81-6.48	1.40-5.29

* Number of samples tested

† Mean count ($\log_{10}$ c.f.u. g^{-1} sample)

‡ Range of counts ($\log_{10}$ c.f.u. g^{-1} sample) for all samples

Influence of day of manufacture

Although the composition of the microbial association of the sausages produced at the factory was always dominated initially by *Brochothrix thermosphacta* and, with storage at a variety of temperatures, by *Brochothrix thermosphacta*, lactobacilli and yeasts, the chilled storage of carcass meat over the weekend influenced the extent of the initial contamination (Fig. 20). Thus sausages produced on Mondays or Tuesdays with meat which had been stored for upwards of 96 h at *ca*. 4°C, contained significantly ($p = 0.05$) more micro-organisms ($\bar{x}$ $\log_{10}$ 7.27; $\sigma = \log_{10}$ 0.62) than those produced on Wednesdays or Thursdays with meat which had been stored for 30 h ($\bar{x} = \log_{10}$ 5.85; $\sigma = \log_{10}$ 0.62).

Influence of initial population size on extent of growth of micro-organisms during storage

The initial level of contamination with micro-organisms was related inversely to the extent of microbial growth during storage at various temperatures (Fig. 21), a regression line fitted to the data had a coefficient of determination, $r^2 = 0.91$ and $y = 3.25$, $m = -0.28$. The data of Brown (1977) and Dowdell and Board (1968, 1971) were analysed also; those of the former were described by a linear regression line $r^2 = 0.84$, $y = 3.44$, $m = -0.31$ and the latter by $r^2 = 0.63$; $y = 1.97$, $m = -0.11$.

Influence of sulphite and temperature on the size and composition of the microbial association

Loss of sulphite

Analysis of the seasonings used in five experiments (3 commercial, 2 pilot scale), indicated that of the 580 µg or so sulphite added per gramme of ingredients in the bowl chopper, *ca.* 26% was lost irretrievably and 23% bound reversibly during mixing. Likewise comparable rates and extents of loss of free and total sulphite from sausages stored at 4°, 10°, 15°, 20° or 22°C were noted in all the other four batches examined. For purposes of clarity, the fate of free and total sulphite in one batch of sausage only is shown in Fig. 22.

Development of the microbial association

As measured by the total viable count (Fig. 23), the presence of sulphite reduced significantly both the rate and extent of growth of the principal bacterial contaminants at all storage temperatures (Fig. 30 a). The size of the climax populations of yeasts was not affected significantly by sulphite (Fig. 30b). Its presence enhanced however the rate of multiplication of these organisms especially with storage at 4° and 10°C, (Fig. 24). The preservative influenced the dominant bacterial contaminant, *Brochothrix thermosphacta*, in as much as larger populations were formed in unsulphited sausages as compared to sulphited ones (Fig. 31a). The time taken to attain a climax population of the organisms was influenced by both temperature and sulphite; the most rapid growth occurred in unsulphited sausage at 22°C, (Fig. 25).

The growth of *Lactobacillus* spp. in sulphited sausages at 22°C was similar to that of *Brochothrix thermosphacta;* at 10°C or less, the rate and extent of growth of the former, especially in preserved sausages, was markedly reduced (Figs. 26, 31b). The rate and extent of growth of members of the genus *Streptococcus* were only affected by sulphite with storage at 10°C or less (Figs. 27, 32a). Judging from changes in the size of the populations of pseudomonads and Enterobacteriaceae, sulphite was more effective as a preservative, especially at low storage temperatures, against the Gram-negative fraction of the initial contaminants of sausages. Thus with the pseudomonads, no demonstrable growth occurred in sulphited sausage stored at 4°C, whereas growth occurred at 10° or 22°C. The rate of increase and eventual size of the populations in sulphited sausages were always less than in unsulphited ones (Fig. 32b). Moreover, the temperature of storage did not influence appreciably either the rate or extent of growth of these organisms in the unpreserved product (Fig. 28). Members of the Enterobacteriaceae appeared to be the most sensitive indicators of the preservative role of sulphite. These organisms formed large populations in unsulphited sausages stored at 10° or 22°C but remained quiescent at other storage temperatures, other than 22°C, or when free sulphite was present at concentrations > 25 μg SO_2 g^{-1} sausage (Figs. 29, 33).

Influence of temperature and sulphite concentration on pH drift

There was little change in the pH of sulphited sausages at 4°, or 10°C (Fig. 34) during storage for 7 d, but a marked acid

drift with storage at 22°C (Fig. 34).The marked changes in the pH correlated with the formation of large populations of lactic acid bacteria. In other experiments (see pp.157 - 164) the rate of pH drift in sulphited sausage meat was associated exponentially with the storage temperature, (coefficient of determination, $r^2 = 0.79$) thus supporting the notion that sulphite was effective in preventing acid formation only up to a certain temperature threshold. Changes in pH were invariably greater with unsulphited sausage meat and were significantly larger (Students paired t-test) than those in sulphited ones at 4° ($p = 0.05$); 9° ($p = 0.05$); 15° ($p = 0.01$) and 20°C ($p = 0.01$). At 25°C, however, there was no significant difference between the pH of the preserved or un-preserved product over the storage period of 2 - 8 days.

Influence of a high concentration of sulphite and temperature

During the course of examination of sulphited and unsulphited batches of sausage produced under commercial conditions in a factory, a particular batch was exceptional in having an extremely high concentration of sulphite. Analysis of this batch gave some interesting trends. Of the 660 µg total sulphite g^{-1} sausage present initially, 270 µg were bound reversibly during manufacture and, as with other batches tested, the absolute content of sulphite decreased slowly with storage (Fig. 35). The rate of decline of free sulphite was slower than in sausages containing normal levels of the preservative (Fig. 35). Indeed, on day 7, the concentrations of active preservative were 2 - 3 fold higher than in commercially-made sausages containing legally-permitted levels

of sulphite. These differences in the rate of loss of the active form of the preservative were reflected in the results obtained from a microbiological analysis, especially with the populations of the sulphite tolerant organisms, the yeasts. In contrast to sausages containing legally permitted levels of sulphite, the rate but not the extent of growth of the yeasts was suppressed by the presence of abnormally high concentrations of the preservative (Fig. 36a). The temperature of storage also influenced the doubling times and size of the populations attained by day 4 or 7 (Fig. 39a). The rate and extent of growth of *Brochothrix thermosphacta* were severely curtailed by the high level of sulphite (Fig. 36b); in the absence of the preservative, the doubling times and size of the climax populations (Fig. 39b) were of the same order as those noted in other batches of unsulphited sausage. As noted previously (p. 129), the temperature of storage influenced the rate and extent of growth of the lactobacilli and streptococci. The former group was not affected, however, by the high concentrations of preservative (Fig. 36c). Indeed, the climax population formed at $4^{o}C$ was significantly greater than in sausage containing the normal levels of sulphite (Fig. 40a). The streptococci likewise formed substantially larger populations in the highly sulphited product stored at refrigeration temperatures when compared with those developing in normal batches of sausage (Figs 37a, 40 b). The response of the pseudomonads was similar to those in sausage containing the legally permitted levels in that their growth in the sulphited product was not evident until the 5th day of storage at $15^{o}C$, at which time the level of free sulphite had diminished to *ca.* 110 µg/g sausage (Figs. 37b, 41a). The marked

sensitivity of the Enterobacteriaceae to sulphite, which was noted above (p. 129), was reflected by the significantly greater doubling times and smaller climax populations (Fig. 41b) in the highly sulphited product stored at 4^{o} or $15^{o}C$ (Fig. 37c).

Composition of the microbial associations

Influence of sulphite concentration

The identification schemes used in previous studies of the microbial associations of British fresh sausage (Brown, 1977; Abbiss, 1978; Leads, 1979) were such that subtle changes, if any, in the composition of microbial groups would have been undetected. As the results presented above suggested that the Gram-negative bacteria were the most sulphite-sensitive members of the microbial association, it was surmised that the changing concentrations of free sulphite may exert a subtle selection such that the composition of the populations of these minor members of the association would vary, particularly between sausages made with or without the preservative. This hypothesis was tested by detailed analysis of the composition of the Enterobacteriaceae and *Pseudomonas* isolates.

Enterobacteriaceae

Thirty seven attributes were used for the initial characterization (Table 21) which assigned the isolates to twelve distinct groups. Sixty-four representative strains from these groups were characterized further using the API 50 CHE scheme. The Clustan

Table 21. Characteristics of Enterobacteriaceae isolated from meat and sausage

Cluster:	1	2	3	4	5	6	7	8	9	10	11	12
Strains in cluster:	8	13	34	37	32	14	42	49	52	24	5	4
Colony type on VRBG: (30°C 24 h)												
>2 mm diameter	0*	23	88	81	100	50	67	100	100	100	0	100
<2 mm diameter	100	77	12	19	0	50	33	0	0	0	100	0
Halo of bile salts	0	54	100	100	100	100	83	100	100	100	40	25
Gram reaction												
Positive	0	0	0	0	0	0	0	0	0	0	0	0
Negative	100	100	100	100	100	100	100	100	100	100	100	100
Presence of endospores	0	0	0	0	0	0	0	0	0	0	0	0
Motility	0	0	53	95	86	100	80	86	100	83	0	0
Growth on:												
MacConkeys medium	100	100	100	100	100	100	100	100	100	100	100	100
Acid formed on Mac. medium	25	0	85	100	100	43	86	94	88	75	100	100
Metallic sheen formed on EMB	0	8	91	0	0	0	0	0	0	0	20	25

Table 21 (continued)

Cluster:	1	2	3	4	5	6	7	8	9	10	11	12
Strains in cluster:	8	13	34	37	32	14	42	49	52	24	5	4
Catalase production	100	100	100	100	100	100	100	100	100	100	100	100
Oxidase production	0	0	0	0	0	0	0	0	0	0	0	0
Action on Glucose:												
Oxidation	100	100	100	100	100	100	100	100	100	100	100	100
Fermentation	100	100	100	100	100	100	100	100	100	100	100	100
Production of:												
β galactosidase	100	92	100	100	91	50	90	100	100	0	100	100
arginine dihydrolase	0	0	0	27	0	0	100	0	0	0	0	0
lysine decarboxylase	0	100	94	0	82	100	0	0	100	100	100	0
ornithine decarboxylase	100	100	32	67	100	100	100	0	100	100	0	0
urease	25	0	100	0	0	0	0	0	0	0	60	0
tryptophane deaminase	0	0	0	0	0	0	0	0	0	0	0	0
indole from tryptophane	25	0	100	0	0	0	0	0	0	0	60	0
H_2S from thiosulphate	0	0	0	7	0	0	0	0	0	0	0	0
Acetoin from sodium pyruvate	0	0	0	0	0	0	0	0	0	12	0	0
Gelatinase	0	0	0	0	73	100	10	0	0	0	0	0

Table 21 (continued)

Cluster:	1	2	3	4	5	6	7	8	9	10	11	12
Strains in cluster:	8	13	34	37	32	14	42	49	52	24	5	4
$NO_3^- \rightarrow NO_2/N_2$	100	85	100	100	100	100	100	100	100	100	100	100
Growth on citrate as sole C source	0	0	0	7	9	0	70	0	0	31	80	0
Acid from: (aerobic)												
Mannitol	100	39	100	100	100	100	100	100	100	100	100	0
Inositol	75	0	0	0	100	67	0	0	0	0	100	0
Sorbitol	100	0	81	73	100	83	50	2	0	5	100	0
Rhamnose	0	0	97	93	0	0	100	96	100	5	100	0
Sucrose	100	0	47	53	100	100	70	98	13	0	100	0
Melibiose	25	0	97	73	91	33	100	92	0	5	100	0
Amygdalin	63	8	31	93	100	67	100	98	0	83	100	0
L(+) Arabinose	38	0	97	93	100	33	100	98	93	100	100	0
Glucose	100	100	100	100	100	100	100	100	100	100	100	100
Strains tested:	2	2	8	8	8	2	8	8	8	6	2	2
Acid form: (anaerobic)												
Glycerol	+	+	+	+	+	+	+	+	+	+	+	+
Erythritol												

Cluster:	1	2	3	4	5	6	7	8	9	10	11	12
Strains tested:	2	2	8	8	8	2	8	8	8	6	2	2
D-Arabinose				+			V		V	+	+	
L-Arabinose	+	+	+	+	+		+	+	+	+	+	+
Ribose	+	+	+	+	+	+	+	+	+	+	+	+
D-Xylose	+	+	+	+	+		+	+	+	+	+	+
L-Xylose												
Adonitol			V			+					+	
β methyl-xyloside												
Galactose	+	+	+	+	+	+	+	+	+	+	+	+
D-Glucose	+	+	+	+	+	+	+	+	+	+	+	+
D-Fructose	+	+	+	+	+	+	+	+	+	+	+	+
D-Mannose	+	+	+	+	+	+	+	+	+	+	+	+
L-Sorbose	V			+							+	
Rhamnose		+	V	+			+	+	+	+	+	+
Dulcitol				V				+			+	+
Inositol	+			V	+	+	+				+	
Mannitol	+	+	+	+	+	+	+	+	+	+	+	+
Sorbitol	+		+	+	+	+	+	+			+	+

Table 21 (continued)

Cluster:	1	2	3	4	5	6	7	8	9	10	11	12
α Methyl D Mannoside												
α methyl D Glucoside				V			+				+	
N-acetyl Glucosamine	+	+	+	+	+	+	+	+	+	+	+	+
Amygdalin												
Arbutin	+		V		+	+	+	+	V		+	+
Aesculin	+				+	+		+	V		+	+
Salicin	+				+	+	+	+	V		+	+
Cellobiose	V			+	V		+	V			+	+
Maltose	+	+	+	+	+	+	+	+	+	+	+	+
Acid from: (Anaerobic)												
Lactose	V		+	+	+		+	+	+		+	+
Melibiose	V		+	V	+		+	+			+	+
Saccharose	+			V	+	+	+	+			+	+
Trehalose	+	+	+	+	+	+	+	+	+	+	+	+
Inulin												
D-Raffinose	V		V		+		+	+			+	+
Melezitose	V				+						+	

Table 21 (continued)

Cluster:	1	2	3	4	5	6	7	8	9	10	11	12
Starch						+					+	
Glycogen												
Xylitol						+						
β Gentiobiose	V	+		+			+	+	+	+	+	+
D Turanose	V				+						+	
D Lyxose						+						
D-Tagatose											+	
D-Fucose								V				
L-Fucose	V	+	+				V	V	+	+	+	
D-Arabitol	V		V	+							+	
L-Arabitol						V					+	
Gluconate	+	+	+	+	+	+	+	V	+	+	+	+
2 Keto gluconate	V	+	V	+	+	+	+	V	+		+	
5 Keto gluconate	+			+	+	+		V			+	+

*% strains positive + All strains positive V Variable All strains negative

numerical analysis package (Wishart, 1978) was used to sort the binary data. Sorting was by the Jaccard (S_J); Gower (S_G); or simple matching (S_{SM}) similarity coefficients, clustering was by single complete or average, (UPGMA) linkage. The dendrogram (Fig. 42) supported the initial conclusion that 12 distinct clusters occurred, these joined at a level of similarity of 69.5% (69.5%S).

Three hundred pure cultures of presumptive Enterobacteriaceae were assembled from Violet Red Bile Glucose agar (VRBG) inoculated with dilutions of sulphited or unsulphited sausages or ingredients used in their manufacture (Table 22). The following seven stock cultures were added to this collection: *Citrobacter freundii*, ATCC 11102; *Citrobacter intermedius*, NCIB 4143; *Enterobacter cloacae*, ATCC 13047; *Escherichia coli*, ATCC 25922; *Hafnia alvei*, ATCC 25927; *Serratia liquefaciens*, NCIB 9321; *Serratia marcescens* ATCC 13880. As a result of initial characterization by traditional methods, (Edwards and Ewing, 1972) as well as with the API 20 E and 50 CHE schemes, 16 strains could not be assigned to a cluster nor a genus. The remainder, which fell into 12 clusters, were identified with *Hafnia alvei* (76 strains); *Enterobacter agglomerans* (49 strains); *Enterobacter cloacae* (42 strains); *Citrobacter freundii* (37 strains); *Escherichia coli* (34 strains); *Serratia liquefaciens* (32 strains); *Serratia marcescens*, (14 strains); *Yersinia* spp. (21 strains) or *Klebsiella* spp. (5 strains).

Analysis of the results indicated appreciable differences between the types of Enterobacteriaceae which occurred in the sulphited or unsulphited product (Table 22). Thus, *Escherichia*

Table 22. Sources and identification of O.T.U.s in dendrogram (Fig. 42)

Cluster	Number of strains	Identified with:	SOURCE									
			Sausage				Sausage ingredients					Stock cultures
			$+SO_2$	$+SO_2/CO_2$	$-SO_2$	$-SO_2/+CO_2$	MRM	Lean	Belly	Head	Rinds	
1	8	*Yersinia* spp.	4	2	0	1	0	1	0	0	0	-
2	13	*Yersinia* spp.	0	4	0	2	3	0	1	2	1	-
3	34	*Escherichia coli*	27	5	0	1	0	0	0	0	0	ATCC 25922
4	37	*Citrobacter freundii*	15	4	16	0	0	0	0	0	0	ATCC 11102 and NCIB 4143
5	32	*Serratia liquefaciens*	20	1	10	0	0	0	0	0	0	NCIB 9321
6	14	*Serratia marcescens*	0	3	5	2	1	0	0	2	0	ATCC 13880
7	42	*Enterobacter cloacae*	29	7	0	5	0	0	0	0	0	ATCC 13047
8	49	*Enterobacter agglomerans*	0	4	39	6	0	0	0	0	0	-
9	52	*Hafnia alvei*	17	5	17	4	2	2	1	3	0	ATCC 25927
10	24	*Hafnia alvei*	0	0	11	5	0	1	1	2	4	-
11	5	*Klebsiella* spp.	0	0	0	1	2	0	0	2	0	-
12	4	Unassigned	0	0	0	0	0	2	2	0	0	-
			112	35	98	27	8	6	5	11	5	7

coli, *Enterobacter cloacae* and *Yersinia* spp. were isolated frequently from the former, whereas *Enterobacter agglomerans* and one biotype of *Hafnia alvei* from the latter type. These findings contrast with those obtained in a detailed analysis of the pseudomonad flora which indicated that no such selection occurred. (pp. 258 - 293).

Lactobacillus

Of the 92 cultures isolated from sulphited or unsulphited sausage on MRS agar, all grew on a modified (Dowdell and Board, 1968) Keddie's medium (1951), were Gram-positive catalase-negative and occurred typically as bacilli in pairs or chains. Furthermore all strains produced acid in Whittenbury's (1963) medium although strains L64 and L65 grew poorly. On the basis of these and other tests (Table 23), 90 strains were assigned to the homofermentative and 2 strains (L64 and L65) to the hetero-fermentative group (Sharpe, 1979). Of the homofermentative group, 10 (L10, 37, 46, 55, 72, 73, 79-82) were assigned to the thermobacterium sub-group 1A, and 80 to the streptobacterium sub-group 1B (Table 24). The four major fermentation profiles which were typical of 81 of the strains are summarized in Fig. 43. The majority of the homofermentative streptobacteria were identified with *Lactobacillus casei* var. *casei* (43 strains Fig. 43a; 28 strains, Fig. 43b), 4 minor profiles described the remaining 9 strains (L2, 14 ,22, 28, 33, 47, 69, 78, 101) which were left unassigned to species. Two distinct profiles were typical of the 10 strains of homofermentative thermobacteria, 6 strains (L72, 73, 79-82) were assigned to *Lactobacillus salivarius* (Fig. 43c) and 4 strains (L10, 37, 46,

Table 23. Physiological attributes of lactobacilli isolated from sausage

Test	Number of isolates	Culture Collection Code (L1 - L92)
Ability to grow at:		
15°C	50	
37°C	92	
45°C	64	
Homofermentative metabolism	90	
Heterofermentative metabolism	2	L64, 65
Isomer of lactic acid formed:		
L(+)	80	
D(-)	0	
DL	12	L10, 37, 46, 55, 64, 65, 72, 73, 79-82
Carbon dioxide formed from gluconate		
+	82	
-	10	L10, 37, 46, 55, 72, 73, 79-82
Hydrolysis of aesculin		
+	81	
-	8	L64, 65, 72, 73, 79-82
+/- (weak)	3	L10, 46, 55

Table 23 (continued)

Test	Number of isolates	Culture Collection Code (L1 -L92)
Ammonia formed from arginine		
+	2	L64, 65
-	88	
+/- (weak)	2	L80, 81

Table 24. Sources and identification of lactobacilli isolated from fresh sausage

Number of strains	Isolated from sausage		Fermentation profile	Identified with:		Assigned to *Lactobacillus*
	+ SO_2	- SO_2		Group	Sub group	
43	24	19	Fig. 43a	Homo*	Streptobacteria	*casei* var. *casei*
28	12	16	Fig. 43b	Homo	Streptobacteria	*casei* var. *casei*
6	5	1	Fig. 43c	Homo	Thermobacteria	*salivarius*
4	1	3	Fig. 43d	Homo	Thermobacteria	*helveticus*
9	4	5	4 minor profiles (data not shown)	Homo	Streptobacteria	Unassigned
2	2	0	1 minor profile (data not shown)	Hetero+	-	Unassigned

* Homofermentative

\+ Heterofermentative

55) to *Lactobacillus helveticus* (Fig. 43d). One carbohydrate fermentation profile was typical of the two heterofermentative lactobacilli (L64 and L65).

The presence of sulphite preservative did not influence significantly the range of *Lactobacillus* spp. isolated from sausage (Table 24).

Streptococcus

All of the 48 isolates from KAA agar - 24 strains from sulphited sausages (S1 - S24) and 24 from unsulphited sausages (S25 - S48) - produced visible growth on modified (Dowdell and Board, 1968) Keddie's (1951) medium, gave a positive reaction in the Gram stain test, a negative catalase reaction and were typically arranged as single, paired or chains of cocci. All strains grew at 10°, 15°, 37° or 45°C and were homofermentative. Furthermore, all initiated growth at pH 9.6 and in 6.5% NaCl and were tolerant of 40% bile. On the basis of these and other tests (Table 25) they were assigned to the "faecal" group (Sharpe, 1979) of streptococci. Thirty-four strains were identified tentatively with *Streptococcus faecalis* and 14 with *Streptococcus faecium* . Results from a screen of oxidative and fermentative breakdown of sugars (Figs 44 , 45) suggested however, that there were 3 groups. Thus 25 isolates could be identified with *Streptococcus faecalis,* 10 with *Streptococcus faecium* and 13 with an 'intermediate biotype' which was left unassigned (Figs 44, 45). The presence of sulphite preservative did not appear to influence

Table 25. Physiological attributes of Streptococci isolated from sausage

Test	Number of strains	Culture collection code (S1 - 48)
Reduction of tetrazolium		
+	36	
-	12	S2,5,6,15,16,17,23,25,29,38,39,45
Growth in 0.4% potassium tellurite		
+	31	
-	17	S2,5,6,15,16,17,23,25,29,38,39,41, 42,43,44,45,46
Type of haemolysis on blood agar:		
none	36	
α only	7	S6,23,40,41,44,47,48
β only	3	S32,34,37
α and β	2	S30, 31
Serological group (Lancefield)		
C.E.I.S. method:		
Group D	48	
Cross reaction with other antisera	0	

significantly the composition of the *Streptococcus* microflora and isolates identified with the different species or biotypes noted above originated from both sulphited and unsulphited sausage (Table 26).

Yeasts

Of the eighty-nine yeasts isolated from ingredients of pork sausage and from the unsulphited or sulphited sausage which had been stored for upwards of 7 d at 4°, 10° or $22^{\circ}C$, 67 were non-pigmented (white, cream or buff) and 13 pigmented strains (pink or orange). With subculture on malt extract agar, 48 were mucoid, 35 were smooth and 6 were rough in texture (Table 27). All strains showed multipolar budding from circular or oval cells, a minority possessed arthrospores, a pseudo- or true mycelium and none produced chlamydospores (Table 27). On the basis of morphological and biochemical tests the strains were assigned to 8 groups. The majority (53%) were assigned to the genus *Candida* or *Torulopsis*, 25% to the genus *Debaryomyces*; 15% to the genus *Rhodotorula* and 7% to the genus *Trichosporon* (Table 28). This survey of a limited number of yeasts suggested that sulphite did not influence significantly the composition of the yeast flora in sausages (Table 27).

Culture studies

The observations discussed on pp. 125 - 132 identified the bacteriostatic role of free sulphite in sausages. This led me to the hypothesis that the contribution or otherwise of a particular

Table 26. Identification of Streptococci from sausage

Group*	Identified with	Number of strains	Origin of strains		Culture collection code
			+ SO_2 sausage	- SO_2 sausage	
1	*Streptococcus faecalis*	25	14	11	S1-48 inclusive except:
2	Unassigned	13	5	8	S1,6,9,11,23,34,35,37, 41-44, 46
3	*Streptococcus faecium*	10	5	5	S2,5,15,16,17,25,29,38, 39, 45

* See Figs. 44, 45

Table 27. Sources and morphological attributes of yeast strains from sausage ingredients and sausage

Group	Number of strains	Ingredients	Sausage		Colony*		Cell shape†	Microscopic morphology			
			$+SO_2$	$-SO_2$	Type	Colour		Chlamydospores	Arthrospores	Pseudo-mycelium	True mycelium
1	7	0	5	2	M/R	W	Cl/OV	0	7	0	7
2	10	1	6	3	M	W	Cl	0	0	0	0
3	22	7	6	9	M/S	W/C/B	Cl	0	0	0	0
4	29	5	10	14	S	W	Cl/OV	0	0	27	0
5	5	1	3	1	M	W	Cl/OV	0	0	5	0
6	3	0	2	1	M	W	OV	0	0	3	0
7	12	3	5	4	M/S	P/O	Cl	0	0	12	0
8	1	1	0	0	S	0	OV	0	0	1	0
	89	18	37	34							

Footnote: * M Mucoid
R Rough
S Smooth

W White
C Cream
B Buff
P Pink
O Orange

† Cl Circular
OV Oval

Table 28. Physiological attributes of yeasts isolated from sausage and sausage ingredients

* + = all strains positive, † number of strains positive, NT not tested

Group	1	2	3	4	5	6	7	8
Number of strains	7	10	22	29	5	3	12	1
Assimilation:								
Control	-	-	-	-	-	-	-	-
Glucose	+*	+	+	+	+	+	+	+
Glycerol	+	+	+	+	+	-	7†	+
2-keto-D-gluconate	+	+	+	-	+	+	-	+
L-arabinose	+	+	+	-	-	-	11	+
Xylose	+	+	+	+	-	-	11	+
Adonitol	-	+	-	-	-	-	8	-
Xylitol	-	+	-	+	-	-	4	+
Galactose	+	+	+	+	-	-	9	+
Inositol	-	-	-	-	-	-	-	-
Sorbitol	+	+	+	+	+	-	10	+
Methyl-D-Glucoside	+	+	+	-	-	-	2	-
N acetyl D Glucosamine	+	+	+	+	+	-	-	+
Cellobiose	+	+	+	-	-	-	-	-
Lactose	+	+	+	-	-	+	-	-
Maltose	+	+	+	-	-	-	10	+
Saccharose	+	+	+	-	-	-	+	+
Trehalose	-	+	-	-	+	-	+	+
Melezitose	+	+	+	-	-	-	+	+
Raffinose	+	+	+	-	-	-	+	+
Glucose fermentation	0	0	0	0	0	3	0	0
Nitrate assimilation	7	0	0	0	0	3	12	0
Urease production	NT	0	NT	0	0	1	12	1
Group assigned to:	*Trichosporon cutaneum*	*Torulopsis candida*	*Debaryomyces hansenii*	*Candida rugosa*	*Candida zeylanoides*	*Candida*	*Rhodotorula glutinis* var. *glutinis*	*Rhodotorula rubra*

group of organisms to the microbial association was perhaps associated with their relative tolerance of sulphite. This hypothesis was tested with isolates from sulphited and unsulphited sausages.

Batch culture

Preliminary studies were done with actively-growing static or agitated cultures in 90 ml of a chemically-defined medium (pH 7.0) in 250 ml Erlenmeyer flasks to which sulphite was added. The concentration of sulphite which inhibited growth was monitored by viable counts. Thus, for example, a concentration of 250 µg free sulphite/ml MGGM caused growth stasis of *Hafnia alvei* strain E 34 at pH 7.0 and 30 °C whereas 125 µg free sulphite/ml reduced the rate and extent of growth; 500 µg free sulphite/ml resulted in death (Fig. 46).

The rate of growth of the six strains of yeasts and 8 out of the 9 strains of *Brochothrix thermosphacta* was not influenced by *ca*. 500 µg free sulphite/ml at 20°C (Fig. 47). The growth rate of the exceptional strain of *Brochothrix thermosphacta* was retarded by 470 µg/ml. A lower range of sulphite concentration (255 - 415 µg sulphite/ml) inhibited
the growth of Gram-negative bacteria and in some instances the concentration causing bacteriostasis was very close to that causing death. Thus with 9 strains of Pseudomonads and 24 strains of Enterobacteriaceae the minimum inhibitory concentrations (MIC's) ranged from 160 - 330 and 15 - 270 µg free sulphite/ml respectively (Fig. 47), within the latter family some members - *Enterobacter*

agglomerans (MIC 50 - 100 µg/ml), *Yersinia enterocolitica* (MIC 50 - 100 µg/ml) and *Salmonella* spp. (MIC 25 - 125 µg/ml) were particularly susceptible, whereas others - *Hafnia alvei* (MIC 210 - 265 µg/ml), *Serratia liquefaciens* (MIC 200 - 250 µg/ml) - were less susceptible to free sulphite.

Pure cultures of dominant members of the microbial association of sulphited sausage were substantially more tolerant of the preservative than were those of organisms (e.g. *Pseudomonas* spp., Enterobacteriaceae) which did not contribute significantly to the association. As such experiments were done with "physiologically-fit" micro-organisms at temperatures which allowed good growth, the situation is not analogous to that in a freshly prepared, chilled sausage. Thus these preliminary experiments with batch cultures were extended to studies of the influence of high initial concentrations of free or bound sulphite at temperatures of 4^{o} and $12^{o}C$ on *Salmonella*, these organisms of public health significance being chosen because of their apparent sensitivity to low concentrations of free sulphite.

The 6 isolates of *Salmonella* did not grow in modified glucose glutamate medium (MGGM) at $4^{o}C$ but did so at $12^{o}C$. As the response of all the 6 strains to sulphite was similar at both temperatures (Fig. 48), only the results obtained with *Salmonella virchow* rif^{R} - the serotype used in sausage-seeding experiments (pp. 157 - 164) - are described.

When 900 - 1000 µg sulphite was added per ml of sterile MGGM,

ca. 200 - 300 µg were bound reversibly within minutes; inoculation of the medium with *Salmonella* did not cause appreciable binding of the residual free sulphite (Fig. 49). The latter was easily bound by the addition of an excess of acetaldehyde without influencing appreciably the absolute level of total sulphite (Fig. 49). The loss of free and total sulphite from uninoculated MGGM was faster and occurred to a greater extent at 12°C (Fig. 50) than at 4°C (Fig. 49). Moreover, the transfer after 7 days of flasks of sulphited medium from 4°C to 12°C resulted in a rate of loss of free and total sulphite that was greater than that occurring in flasks stored only at 4°C (Fig. 51). Actively growing or quiescent *Salmonella* did not influence substantially the rate or extent of loss of free or total sulphite (Figs. 49 - 51).

Sulphite protected to a large extent all the strains of *Salmonella* inoculated in MGGM and stored at 4°C from death. There was a gradual decline only - *ca.* 10^6 c.f.u./ml to *ca.* 10^5 c.f.u./ml in 20 days - in the size of their populations (Fig. 48). In the absence of sulphite, the populations of all the strains of *Salmonella* declined at a significantly faster rate, those of *Salmonella anatum, goerlitz* and *kedougou* from *ca.* 10^6 c.f.u./ml to *ca.* 10^3 c.f.u./ml in 20 days and those of *Salmonella hadar, typhimurium* nal.^{R} and *virchow* (Fig. 52) from *ca.* 10^6 c.f.u./ml to *ca.* 10^3 c.f.u./ml in 3 - 6 days (Fig. 48).

The addition of sulphite to slowly growing cultures of *Salmonella* at 12°C also caused rapid decline in the number of viable organisms from *ca.* 10^3 - 10^4 c.f.u./ml to numbers below

the limit for detection (*ca.* 10^1 c.f.u./ml) by days 4 - 6 (Fig. 53). Sulphite bound by acetaldehyde reduced both the rate and extent of growth as compared with the unsulphited cultures. It was notable, however, that large populations (*ca.* 10^7 - 10^8 c.f.u./ml) were attained after 7 - 13 days (Fig. 53).

When cultures of *Salmonella* stored at 4°C for 7 days were transferred to 12°C, the following observations were made: (1) *Salmonella* in sulphite-free MGGM initiated growth; (2) the protective effect of sulphite was negated and rapid death ensued, and (3) the sulphite-acetaldehyde complex did not prevent multiplication of *Salmonella*, (Fig. 54).

The observations made with batch cultures supported the hypothesis that the sulphite tolerance of members of the microbial association was such that this property can be considered to be a major cause of the selection of the association described on pages 125 - 151. Moreover, the evidence supports the conclusion that sulphite , in the free but not bound form, acts mainly as a bacteriostatic agent at neutral pH. The results obtained with *Salmonella* are of particular interest because they suggest a protective role for sulphite with susceptible organisms stored at temperatures preventing growth.

Turbidometer culture

As the batch culture system relied on determination of viable counts to index the action of sulphite there was no opportunity for

critical assay of sulphite tolerance particularly as the range of tolerance was extensive. Thus a novel assay system was developed (Fig. 9). The preservative was added incrementally to a continuously monitored culture in the exponential phase of growth. When the trace of optical density plotted on the chart recorder indicated that growth had been arrested, the concentration of free sulphite in the medium was determined immediately. Moreover, the method allowed constant adjustment to the concentration of free sulphite, in the culture medium thereby compensating for irretrievable - by oxidation - or reversible - by binding - loss of active sulphite. This dynamic approach to the assay of sulphite sensitivity confirmed the preliminary results obtained with batch culture experiments.

Thus stasis was not induced in any of the 8 strains of yeast or 2 strains of *Brochothrix thermosphacta* by a concentration of 500 µg free sulphite/ml whereas 125 - 270 µg free sulphite/ml inhibited the growth of 2 strains of *Pseudomonas fragi* and 1 strain of *Pseudomonas fluorescens* (Fig. 55). With the exception of members of the genus *Salmonella*, growth of representative Enterobacteriaceae was suppressed by 50 - 270 µg free sulphite/ml. A lower range (15 - 109 µg free sulphite/ml) inhibited *Salmonella* obtained from culture collections (e.g. *Salmonella virchow* rif.^{R}; Fig. 56) or from sulphited sausage (e.g. *Salmonella derby*; Fig 57). It needs to be stressed that growth stasis as indicated by turbidometry was confirmed by viable counts. Sulphite-induced stasis of the *Salmonella* cultures persisted for *ca*. 20 h at 30^{o}C after which time the organisms died (Figs. 56, 57). The addition

of a binding (acetaldehyde) or neutralizing (hydrogen peroxide) agent at this time neither released *Salmonella* from growth inhibition nor prevented their death (Figs. 56, 57). With the notable exception of *Enterobacter agglomerans*, an organism which responded to sulphite in the manner described above, other members of the Enterobacteriaceae were inhibited but not killed by sulphite in the course of the experiments. Indeed the addition of a sulphite-binding or neutralizing agent led to the growth of organisms which had been inhibited by sulphite for 20 h at 30°C (Fig. 58).

The results obtained from batch and turbidometer culture permitted a comparison of the sulphite-sensitivity of common contaminants of commercial sausage (Fig. 55) tested under aerobic conditions in buffered, defined media at pH 7.0 and at near optimum temperatures for growth. Further studies showed that the actual range of sulphite concentration causing growth inhibition but not the rank order of the various ranges discussed above was influenced by incubation temperature and redox potential. Thus an organisms'tolerance of free sulphite diminished as the incubation temperature moved away from the optimum for growth. Free sulphite was generally more effective under anaerobic conditions although 4 strains of *Salmonella* - *anatum, derby, kedougou* and *virchow* - were notably tolerant. Indeed, in the absence of oxygen, high concentrations (> 250 µg free sulphite/ml) were required to inhibit growth at pH 7.0 and 30°C.

There was no demonstrable difference noted in trials which compared the sulphite sensitivity of isolates grown in chemically-

defined or complex media, even though the latter had a much larger potential for binding sulphite.

Fate of a selected pathogen in sausage

Because of the particular importance of *Salmonella* in food-borne disease and the good public health record of British fresh sausages with respect to this group of organisms as well as their apparent marked sensitivity to free sulphite, a series of experiments were done to establish their fate in sausage. The low and unpredictable levels of natural contamination of sausage with *Salmonella* (pp. 230 - 257) meant that samples taken from a factory could not be used in studies of the fate of these organisms during storage. Thus sausage meat was inoculated with low numbers (*ca.* 10^3/g) of *Salmonella* and other Enterobacteriaceae which had been isolated from sausage, grew on PCA, MacConkey agar or in TSB whereas, with the exception of *Salmonella typhimurium* 340 nal^R, only the former grew (Table 29) on PCA or DCA supplemented with rifampicin (50 µg/ml). In contrast strains of *Salmonella typhimurium* 340 nal^R, *Enterobacter agglomerans* and *Citrobacter freundii* only grew in TSB supplemented with 30 µg/ml nalidixic acid (Table 29). Moreover, of the *Salmonella* serotypes studied, *goerlitz* was the only one affected adversely by the rifampicin-supplemented DCA medium (Table 30a.) Preliminary experiments showed also that yeasts in sausage grew on rifampicin-supplemented TSA but not on rifampicin supplemented DCA. Thus the latter medium was selected for use in this study.

Table 29. Influence of recovery medium on isolation of Enterobacteriaceae

Organisms	Growth on recovery medium: DCAR	PCAR	PCA	TSBN	TSB	MaC
*Hafnia alvei**	-	-	+	-	+	+
*Escherichia coli**	-	-	+	-	+	+
*Enterobacter agglomerans**	-	-	+	+	+	+
*Citrobacter freundii**	-	-	+	±	+	+
Salmonella typhimurium †	-	-	+	+	+	+
goerlitz§	+	+	+	-	+	+
virchow§	+	+	+	-	+	+
kedougou§	+	+	+	-	+	+
"E4" §	+	+	+	-	+	+
hadar §	+	+	+	-	+	+
anatum§	+	+	+	-	+	+

* Isolated from sulphited/unsulphited British fresh sausage
† nalidixin resistant strain
§ rifampicin resistant strain
DCAR Desoxycholate citrate agar supplemented with rifampicin (50 µg/ml)
PCAR Plate count agar supplemented with rifampicin (50 µg/ml)
TSBN Tryptone soya broth supplemented with nalidixic acid (30 µg/ml)
MaC MacConkey agar

\- No growth
\+ growth

Table 30a Influence of recovery medium on quantitative isolation of *Salmonella*

Salmonella:	Isolation medium		
	TSAR	TSA	DCAR
goerlitz	431*	472	216
virchow	230	228	232
kedougou	379	411	360
"E4"	301	337	275
hadar	459	471	460
anatum	226	234	206

* Arithmetic mean of 5 replicate counts at a dilution level of 10^{-5} in TSB

TSAR Tryptone soya agar supplemented with rifampicin (50 µg/ml)

DCAR Desoxycholate citrate agar supplemented with rifampicin (50 µg/ml)

When diluted mixtures of cultures of *Salmonella* and other Enterobacteriaceae were incubated on rifampicin-supplemented DCA, significant ($p = 0.05$) diminution in the numbers of *Salmonella anatum* in the presence of *Escherichia coli* or *Enterobacter agglomerans*, and a group E 4 *Salmonella* sp. in the presence of *Escherichia coli* was noted (Tables 30b 31). Conversely, significantly greater ($p = 0.05$) recovery of *Salmonella hadar* in the presence of either *Enterobacter agglomerans , Citrobacter freundii* or *Hafnia alvei* on rifampicin-supplemented DCA, was observed (Table 30b 31). As *Salmonella virchow* was not influenced significantly by the type of recovery medium or competing organism, it was chosen for further studies.

Influence of sulphite concentration and temperature of storage

As similar trends were evident in all the three major experiments on this topic, for purpose of brevity the results of one particular batch only are described below. It should be stressed also that although the unsulphited and sulphited sausage meat was obtained from a large factory in the course of routine production, inoculation of meat slurries with rifampicin-resistant *Salmonella* was done in the laboratory.

With the exception of storage at -20°C, (Fig. 60) Enterobacteriaceae grew at all temperatures in unpreserved sausage meat, the rate of growth was related to temperature (Figs. 61 - 65). The numbers of these organisms did not change in sulphited sausage meat stored for up to 7 days at -20°, 4° (Fig. 61), 9° (Fig. 62) or 15°C (Fig. 63). After 4 days at 20°C (Fig. 64) however, the

Table 30b Influence of competing micro-organisms on quantitative isolation of *Salmonella* on TSA

	Organism Code									
	1	2	3	4	5	6	7	8	9	10
1	180*/-†									
2		230/-								
3			216/-							
4				215/-						
5	250/326	274/351	285/344	NT/344	472/-					
6	185/204	211/229	238/222	219/222		228/-				
7	253/296	258/321	302/314	248/313			411/-			
8	NT/259	257/284	274/277	232/276				337/-		
9	350/326	356/351	385/344	300/343					471/-	
10	162/207	244/232	264/225	224/225						234/-

* Arithmetic mean of 5 replicate counts at a dilution level of 10^{-5} (observed mean recovery)

† Expected mean recovery

1 *Citrobacter freundii*
2 *Enterobacter agglomerans*
3 *Escherichia coli*
4 *Hafnia alvei*
5 *Salmonella goerlitz*
6 *Salmonella virchow*
7 *Salmonella kedougou*
8 *Salmonella* "E4"
9 *Salmonella hadar*
10 *Salmonella anatum*

Table 31. Influence of competing micro-organisms on quantitative isolation of *Salmonella* on DCAR

	1	2	3	4	5	6	7	8	9	10
1	0*/180†									
2		0/230								
3			0/216							
4				0/215						
5	126/125	140/137	180/142	139/-	216/472					
6	118/93	116/105	134/119	91/108		232/228				
7	133/127	112/129	154/151	170/124			360/411			
8	116/-	109/128	77/137[b]	117/116				275/377		
9	240/175[a]	259/178[a]	174/192	285/150[a]					460/471	
10	107/81	64/122[b]	71/132[b]	79/112						206/234

* Arithmetic mean of 5 replicate counts at a dilution level of 10^{-5} (observed mean recovery)

† Expected mean recovery

Organism code - as in footnote to Table 30*b*

a significantly greater recovery than expected (p = 0.05) b significantly poorer recovery than expected (p = 0.05)

decline in the concentration of free sulphite to non-inhibitory levels (*ca.* 50 - 60 µg free sulphite/g) was associated with a small increase in their numbers (Fig. 66). *Salmonella virchow* rif.^{R} present in unsulphited sausage meat stored at -20°, 4° or 9°C did not grow, it did so to a limited extent at 15°C and extensively at 20° or 25°C (Fig. 65). Growth of this serotype in sulphited sausage meat at 25°C or 20°C was not observed until after incubation for 2.5 and 4 days respectively, by which times the concentrations of free sulphite were 20 and 45 µg/g sausage meat (Fig. 66). It was noteworthy that these values were of the same order as those noted in the experiments with pure cultures in broth even though there was a progressive acid drift in the sausage meat and hence a possible accentuation of sulphite toxicity (Fig. 67). There was a pronounced acid drift in the pH of unsulphited but not sulphited sausage meat (Fig. 68). With the former the data were described by a linear regression line (coefficient of determination, $r^2 = 0.73$) whereas with the latter by an exponential regression line ($r^2 = 0.79$), see (Fig. 69).

Influence of a high initial level of microbial contamination

It was notable also that rifampicin-resistant *Salmonella virchow* failed to grow at 4°, 15° or 20°C within 8 days in sulphited or unsulphited sausage meat which had been made from meat stored for upwards of 96 h in the factory (Fig. 70c). As would be expected, the freshly made sausage had a very high microbial population, dominated by *Brochothrix thermosphacta* and other Gram-positive bacilli, as determined by microscopical examination of

Gram stained films made from colonies on Plate Count agar (Fig. 70a). The extent of growth of the dominant microbial group as well as that of the Enterobacteriaceae was curtailed (Fig. 70b). The last mentioned group, although present initially in high numbers did not attain the large populations in unsulphited sausage meat which would have been predicted from the analysis of sausage containing normal levels of contaminants (pp. 125 - 130). Thus the extent of the initial contamination as well as the free sulphite content influences not only the growth of the Enterobacteriaceae, but also salmonellae in sausage meat.

Figure 20. Influence of day of manufacture on the initial level of microbial contamination of fresh sausage.

Key: Batches of sausage manufactured on Wednesdays, Thursdays or Fridays

Batches of sausage manufactured on Mondays or Tuesdays; $\bar{x}$ Mean $\log_{10}$ count of initial infection with micro-organisms

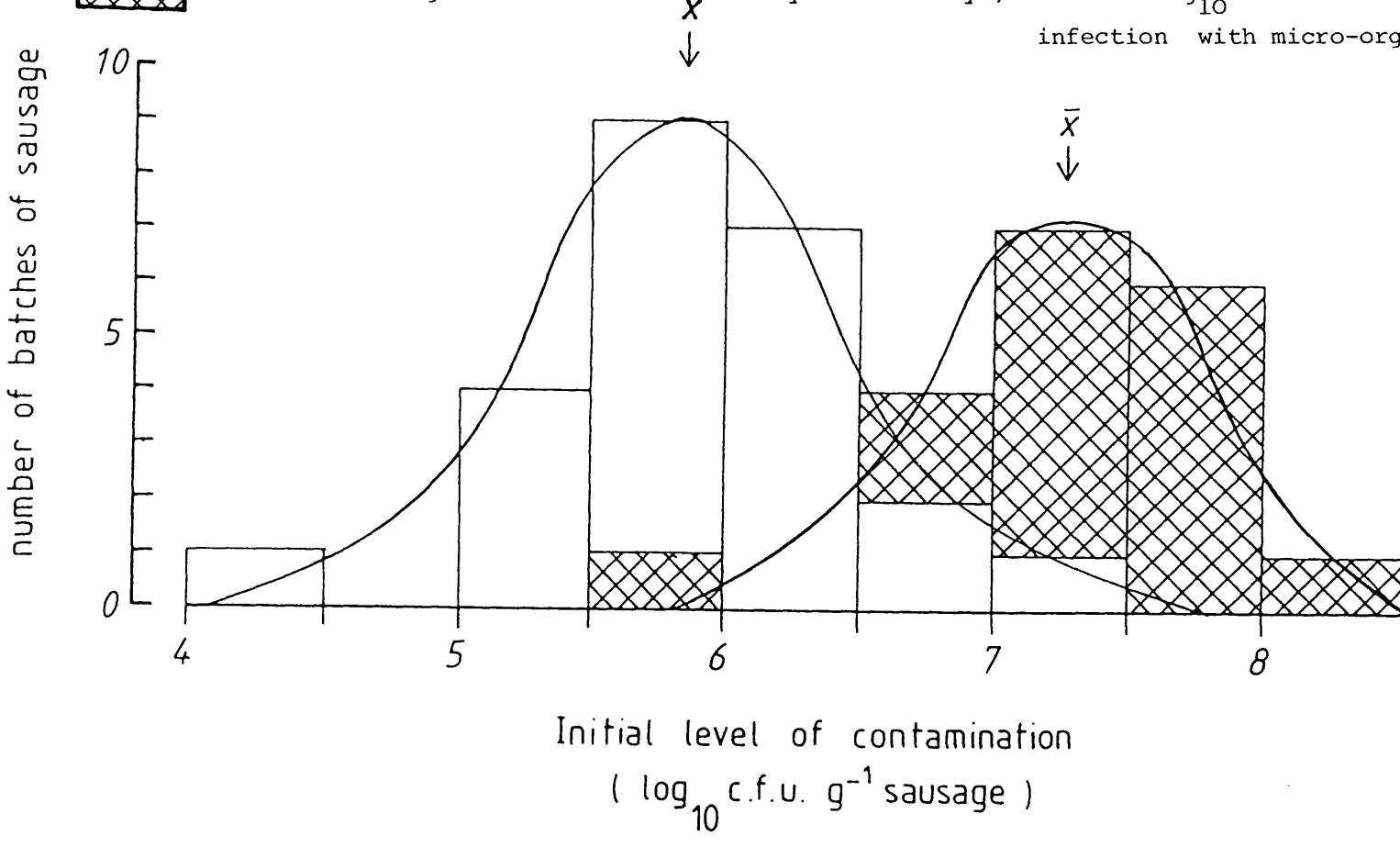


Figure 21. Regression lines describing the influence of the initial level of microbial contamination on the final climax population size in fresh sausage.

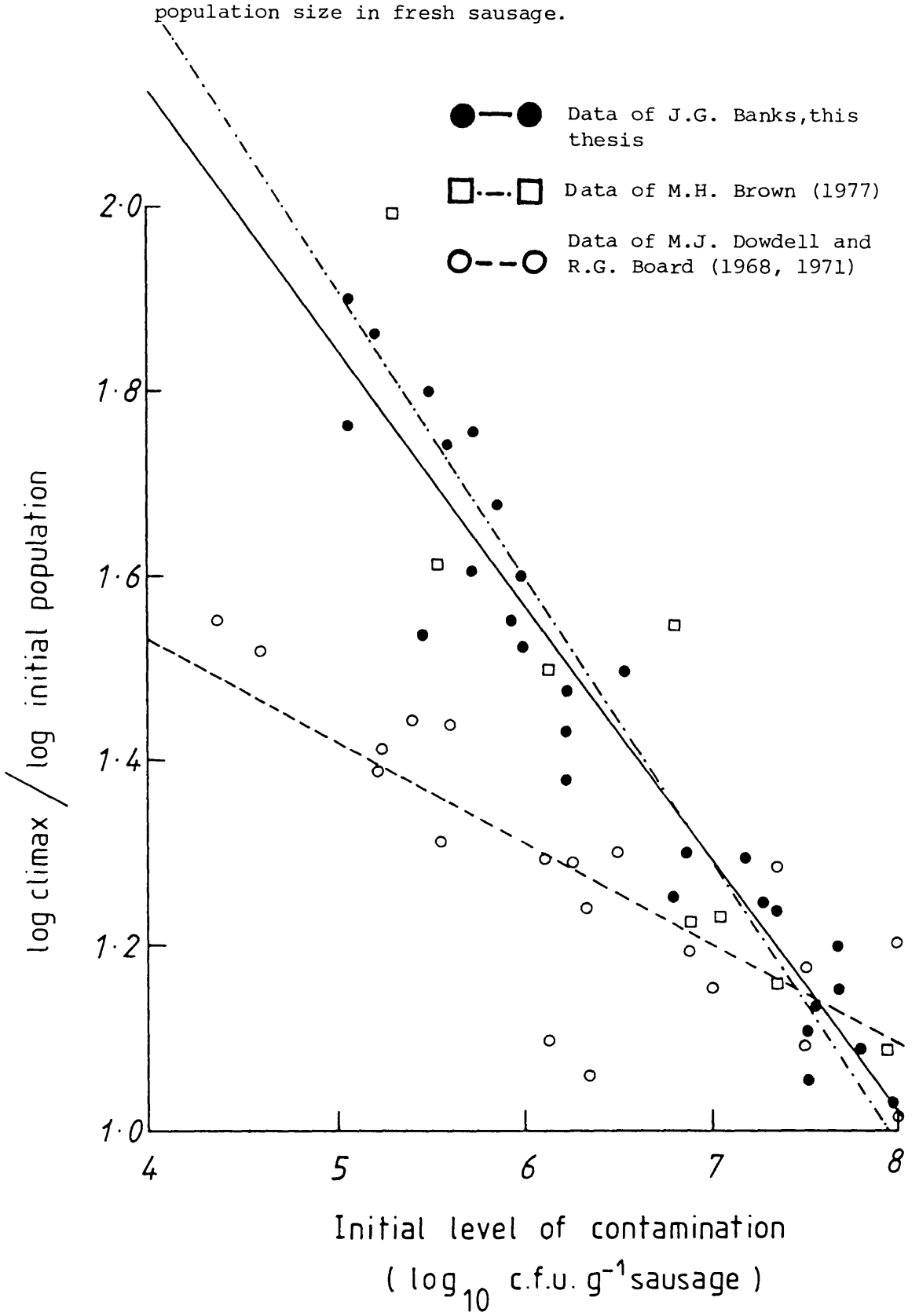
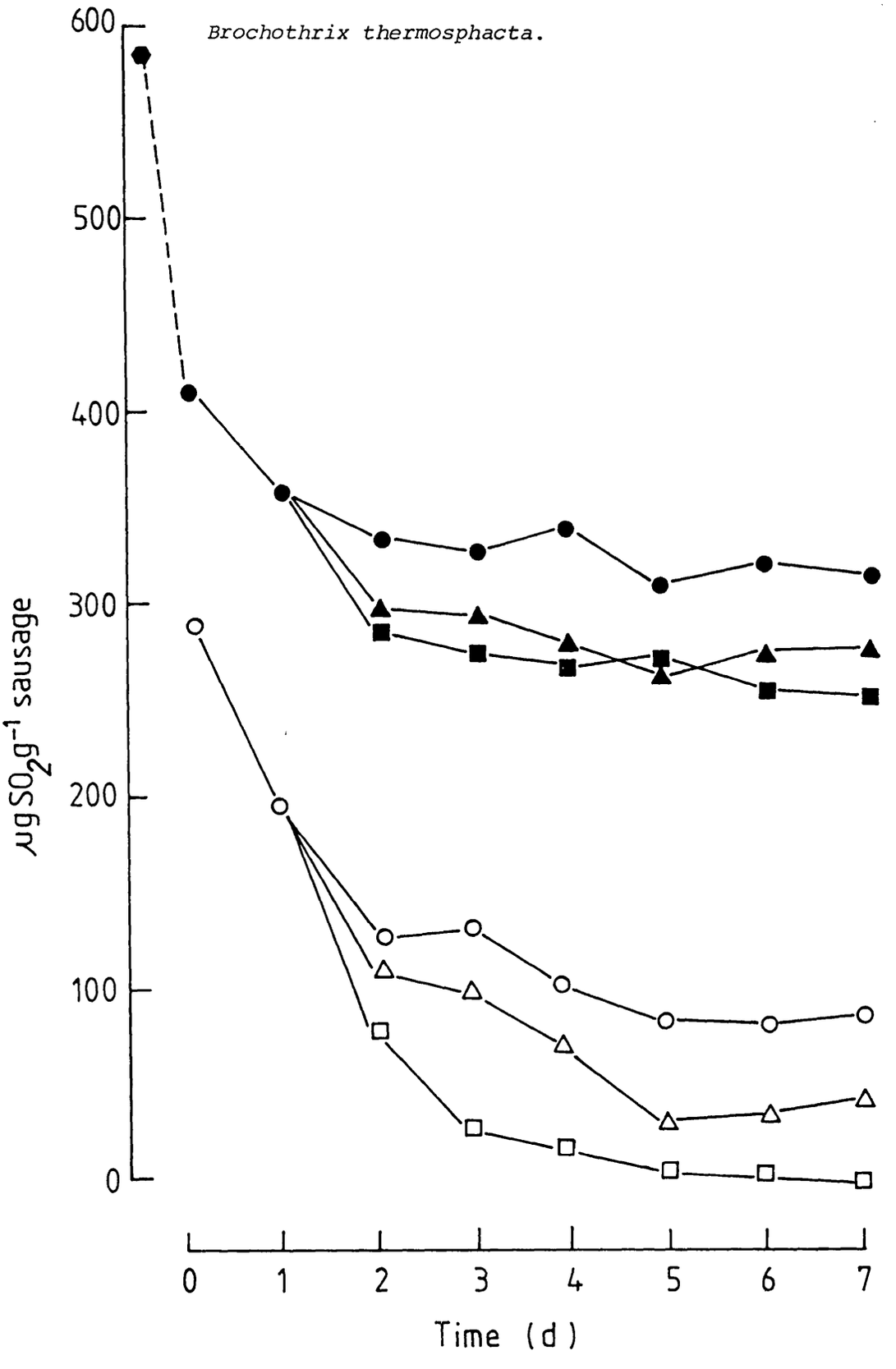


Figure 22. Influence of temperature of storage on the loss of free and total sulphite from a batch of sausage dominated by *Brochothrix thermosphacta*.



Key: Predicted contribution of total SO_2 to sausage by retrospective analysis of the seasonings.

Total SO_2, 4°C
" 10°C
" 22°C

Free SO_2, 4°C
" 10°C
" 22°C

Figure 23. Influence of temperature of storage and sulphite concentration on the total viable count in sausage dominated by *Brochothrix thermosphacta*.

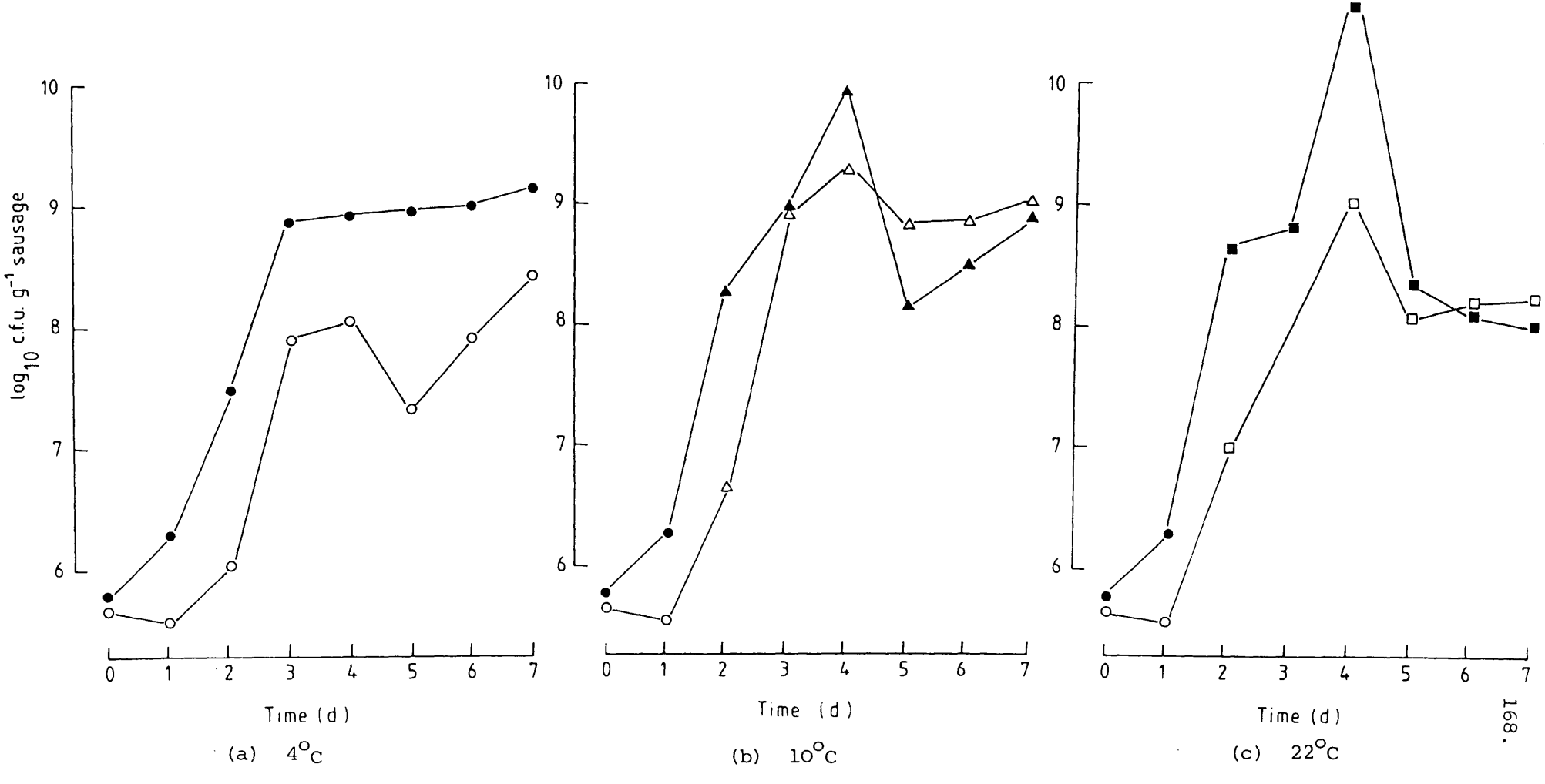


Figure 24. Influence of temperature of storage and sulphite concentration on the growth of the yeasts in sausage dominated by *Brochothrix thermosphacta*.

Open symbols + sulphite

Closed symbols, - sulphite

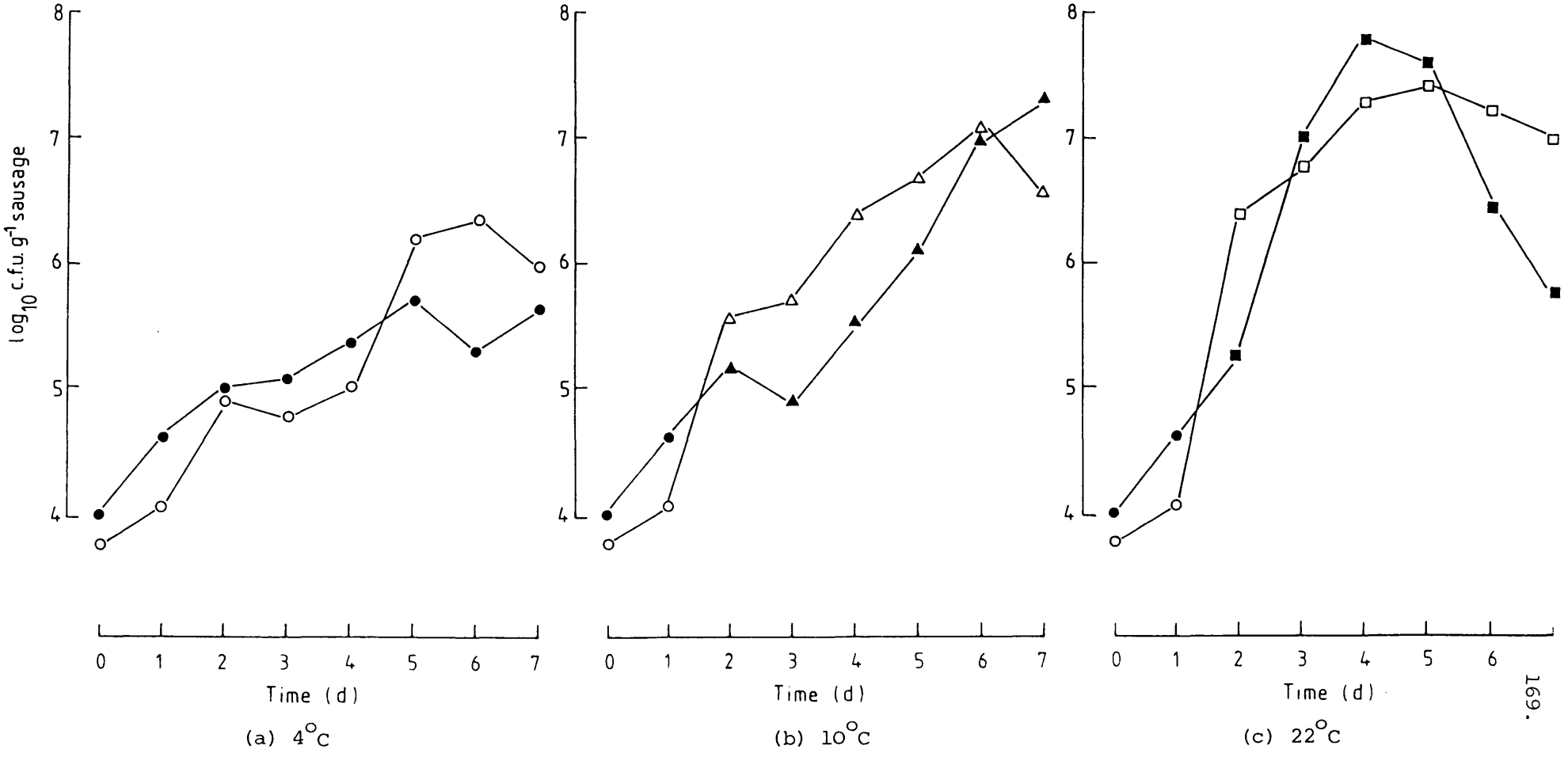


Figure 25. Influence of temperature of storage and sulphite concentration on the growth of *Brochothrix thermosphacta* in sausage dominated by this organism.

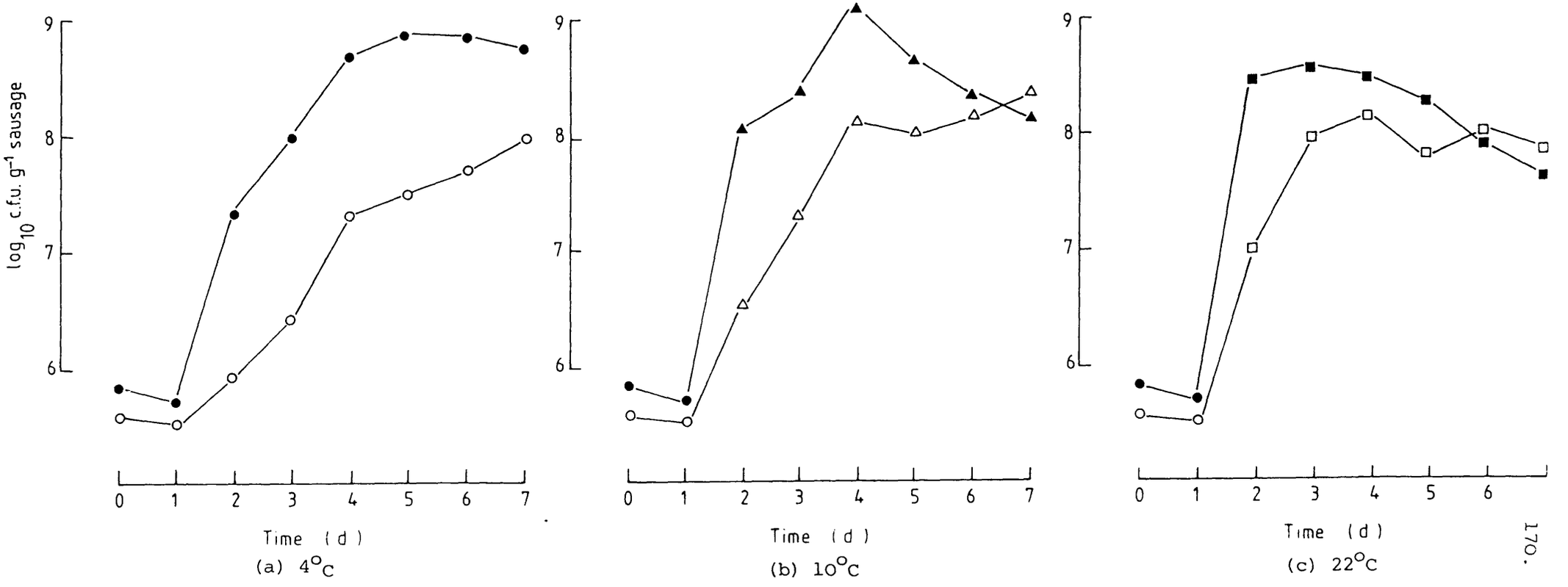


Figure 26. Influence of temperature of storage and sulphite concentration on the growth of *Lactobacillus* spp. in sausage dominated by *Brochothrix thermosphacta*.

Open symbols, + sulphite

Closed symbols - sulphite

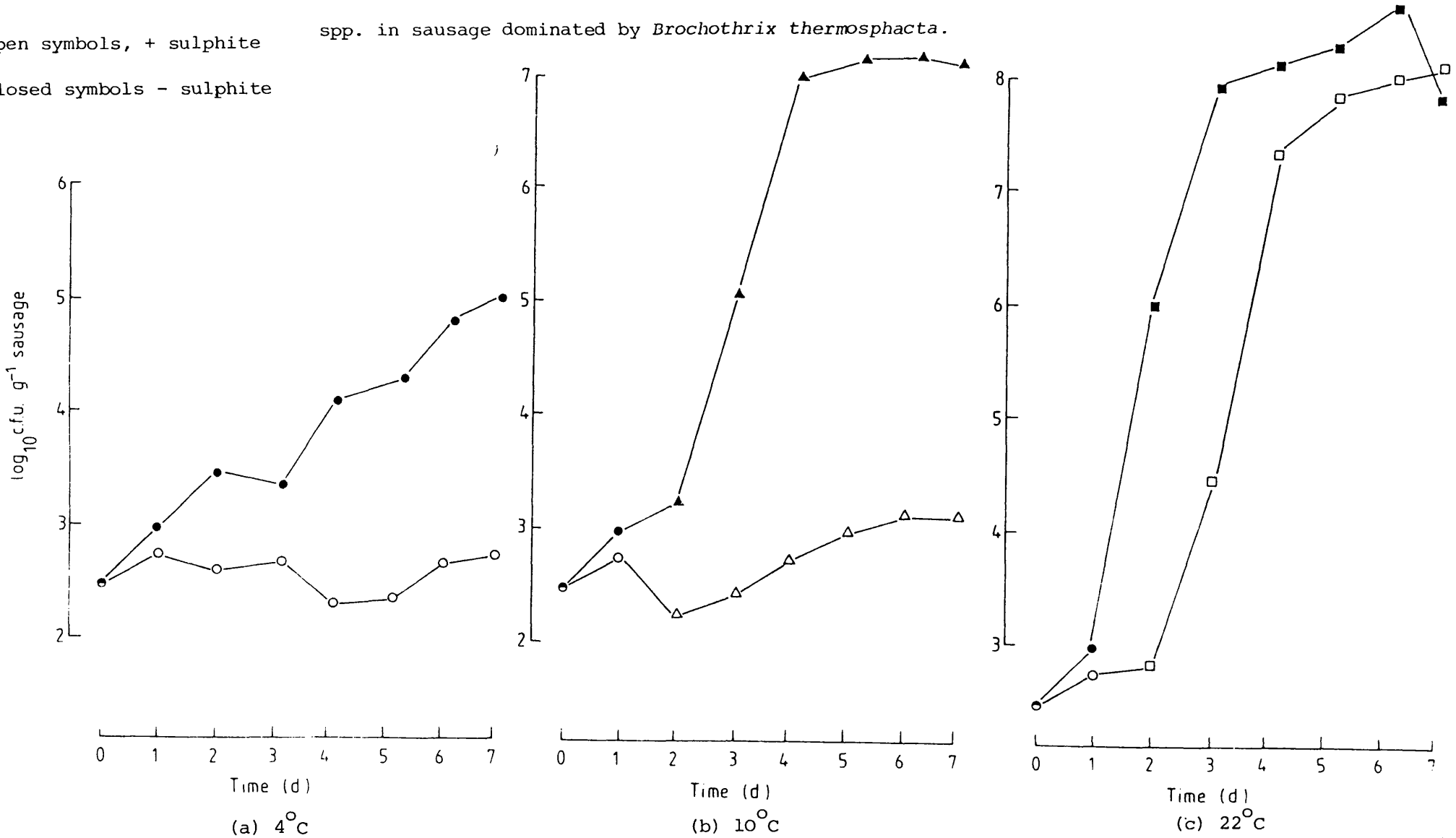


Figure 27. Influence of temperature of storage and sulphite concentration on the growth of *Streptococcus* spp. in sausage dominated by *Brochothrix thermosphacta*.

Open symbols, + sulphite

Closed symbols, - sulphite

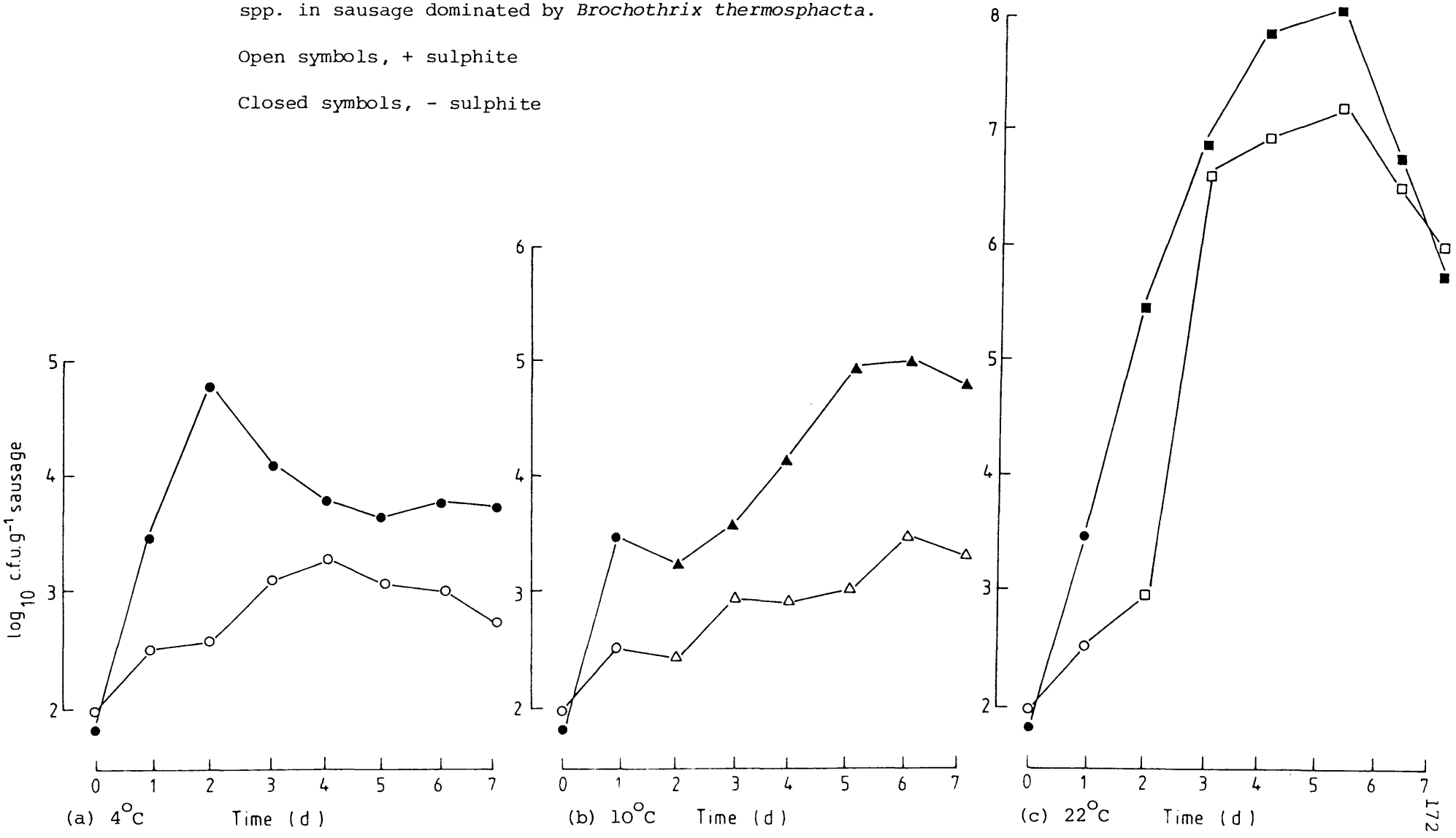


Figure 28. Influence of temperature of storage and sulphite concentration on the growth of *Pseudomonas* spp. in sausage dominated by *Brochothrix thermosphacta*.

Open symbols, + sulphite

Closed symbols, - sulphite

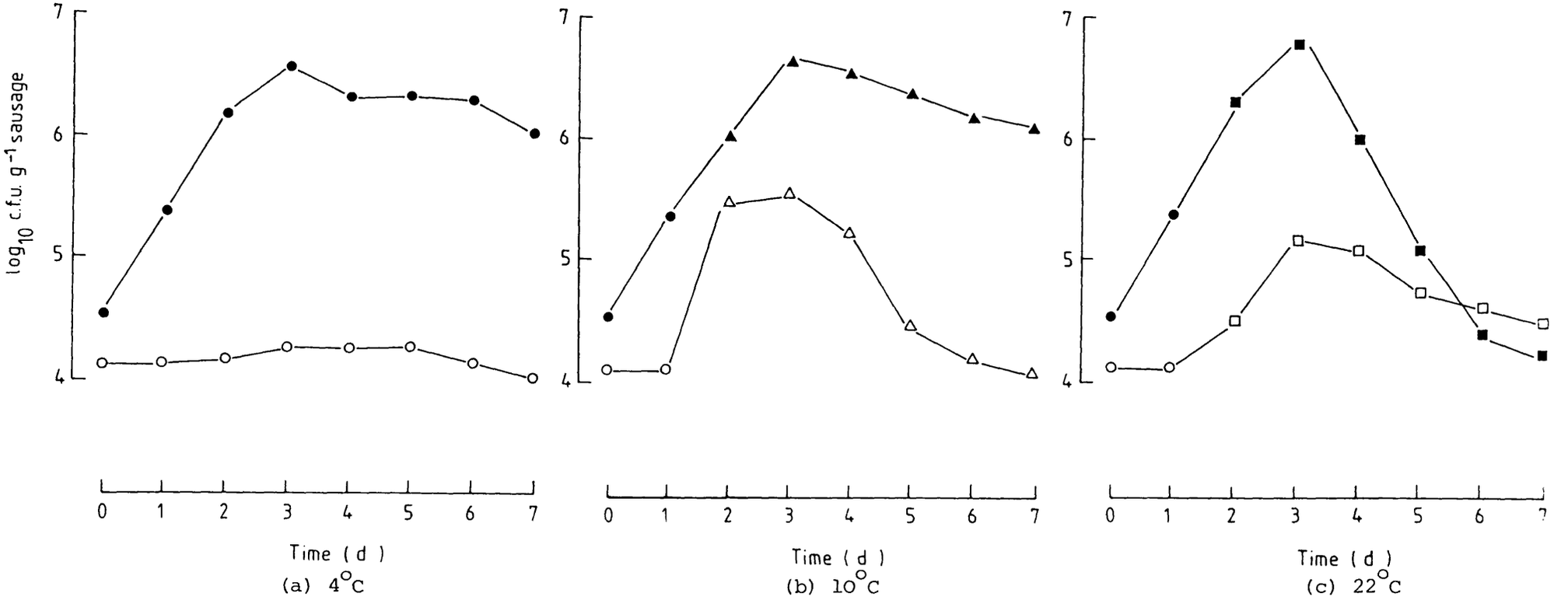


Figure 29. Influence of temperature of storage and sulphite concentration on the growth of Enterobacteriaceae in sausage dominated by *Brochothrix thermosphacta*. Open symbols, + sulphite; Closed symbols, - sulphite

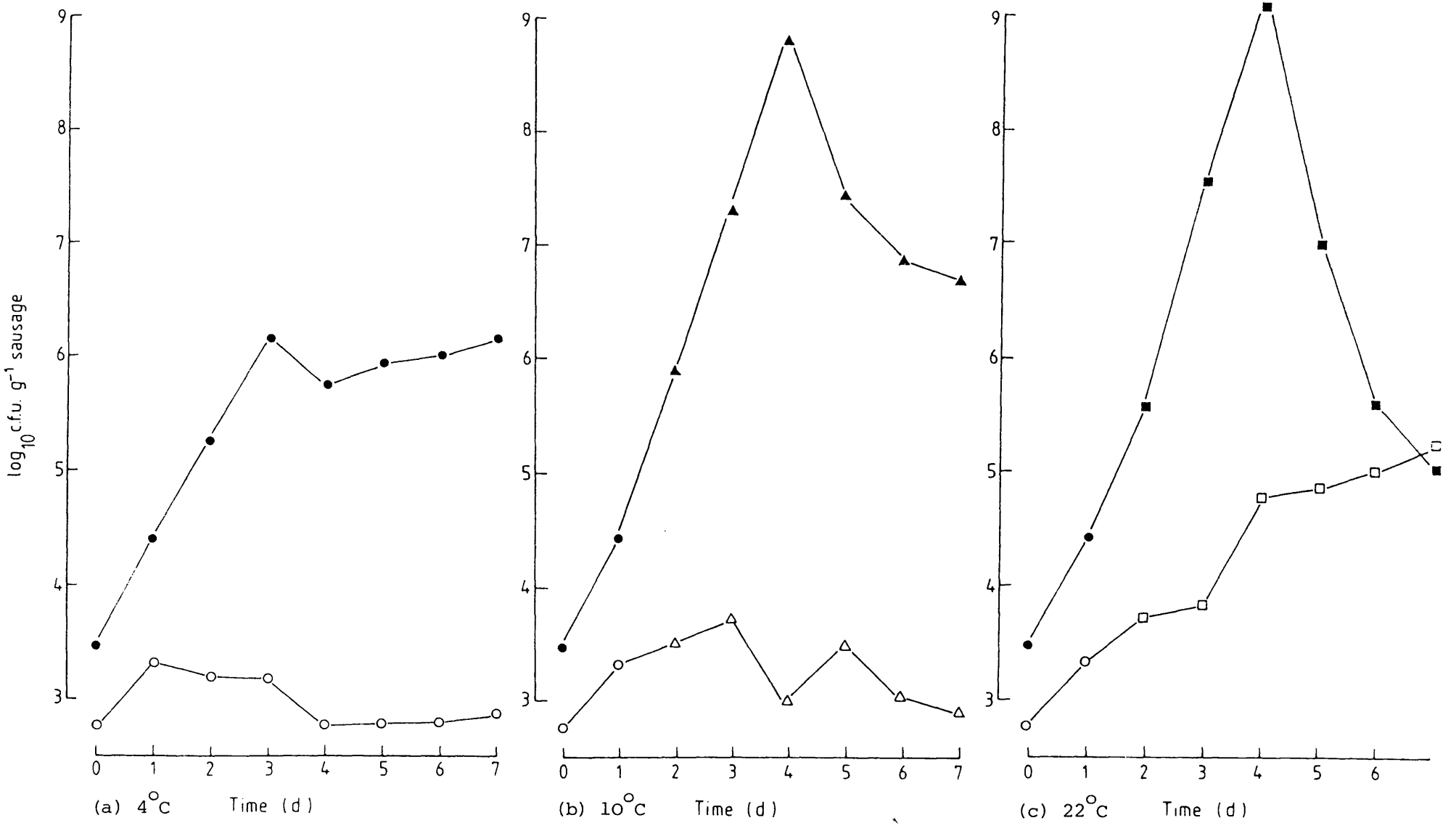
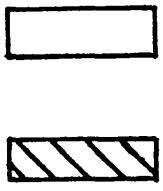


Figure 30. Influence of temperature of storage and sulphite concentration on the rate and extent of growth of a, the total viable count, and b, the yeasts in sausage dominated by *Brochothrix thermosphacta*.



Mean doubling time up to climax population in sulphited sausage

Mean doubling time up to climax population in unsulphited sausage

Climax population in sulphited sausage

Climax population in unsulphited sausage

a; Total viable count

b; Yeasts

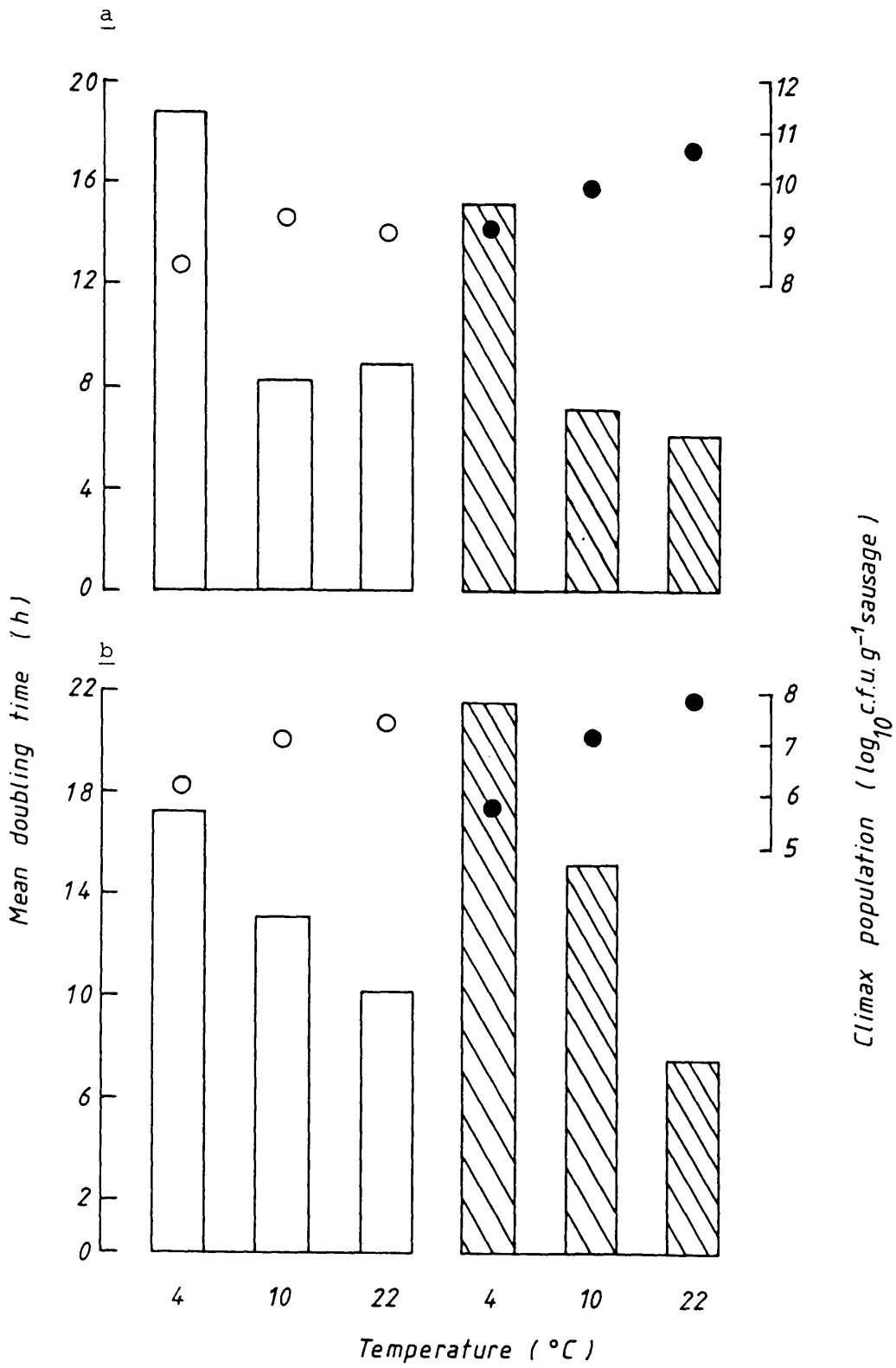
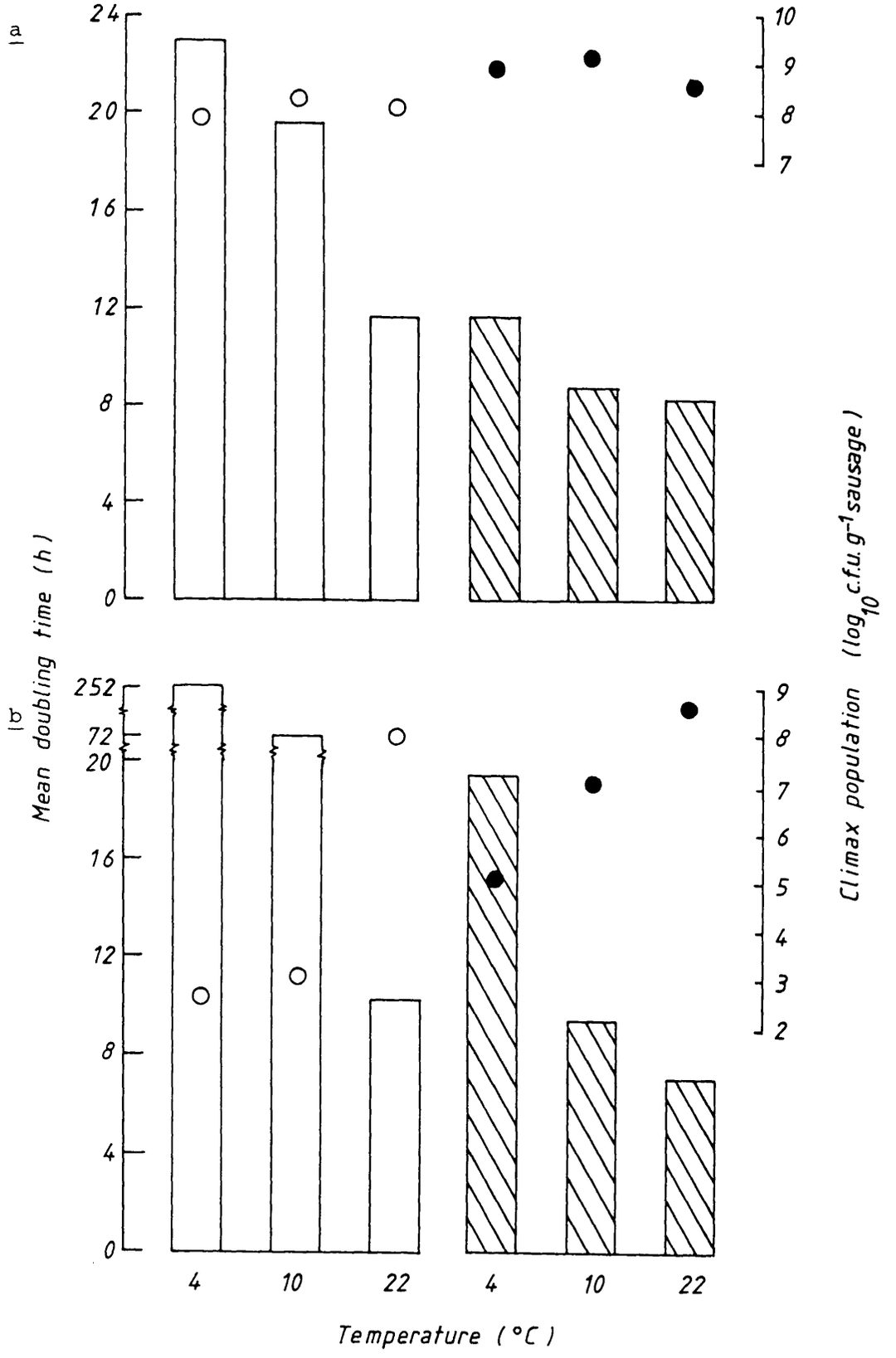

a
b
20
16
12
8
4
0
22
18
14
10
6
2
0
12
11
10
9
8
8
7
6
5
4
10
22
4
10
22
Mean doubling time (h)
Climax population (log10 c.f.u. g-1 sausage)
Temperature (°C)

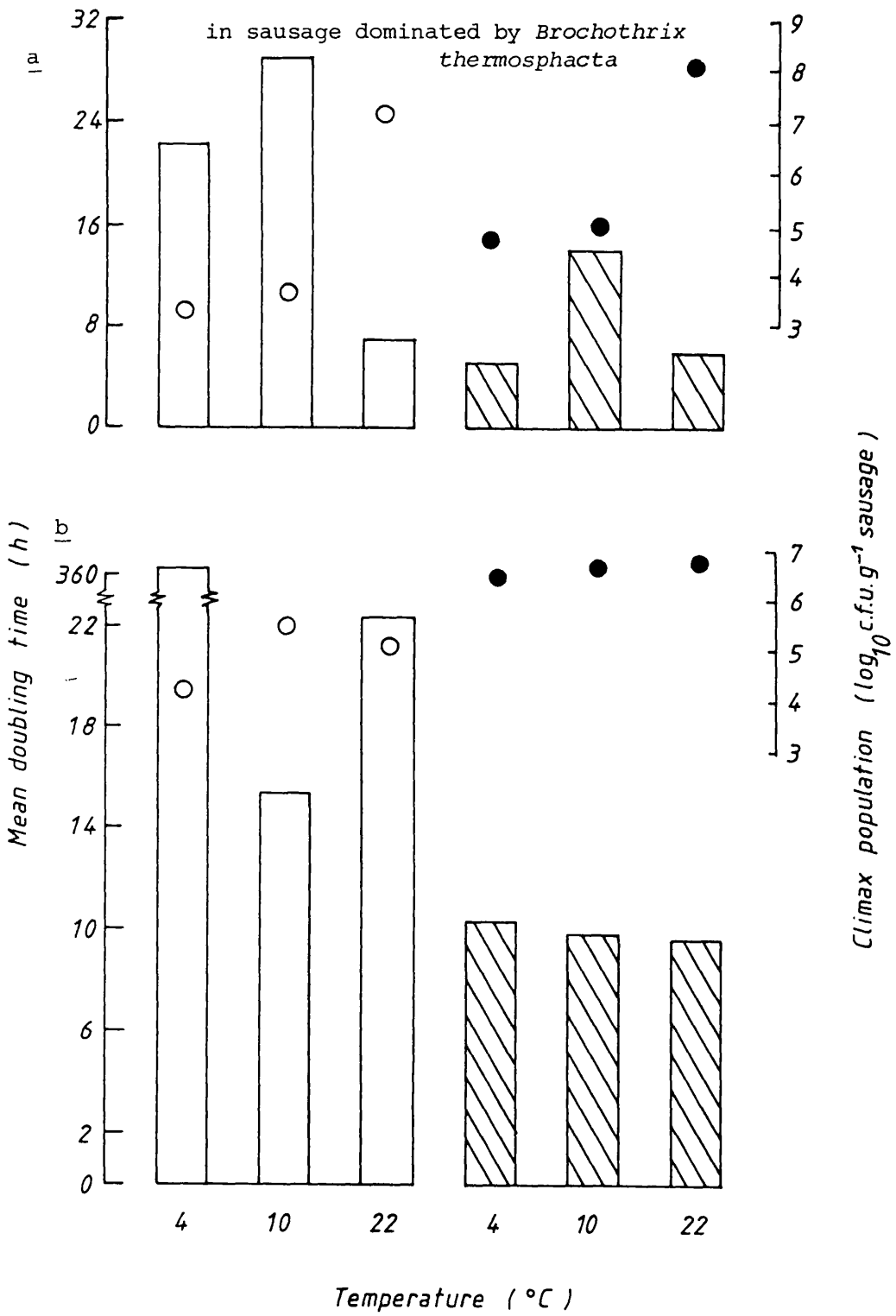
Figure 31. Influence of temperature of storage and sulphite concentration on the rate and extent of growth of a, *Brochothrix thermosphacta* and b, *Lactobacillus* spp. in sausage dominated by *Brochothrix thermosphacta*.



Key: As Figure 30.

a; *Brochothrix thermosphacta*
b; *Lactobacillus* spp.

Figure 32. Influence of temperature of storage and sulphite concentration on the rate and extent of growth of a, *Streptococcus* spp. and b, *Pseudomonas* spp. in sausage dominated by *Brochothrix thermosphacta*

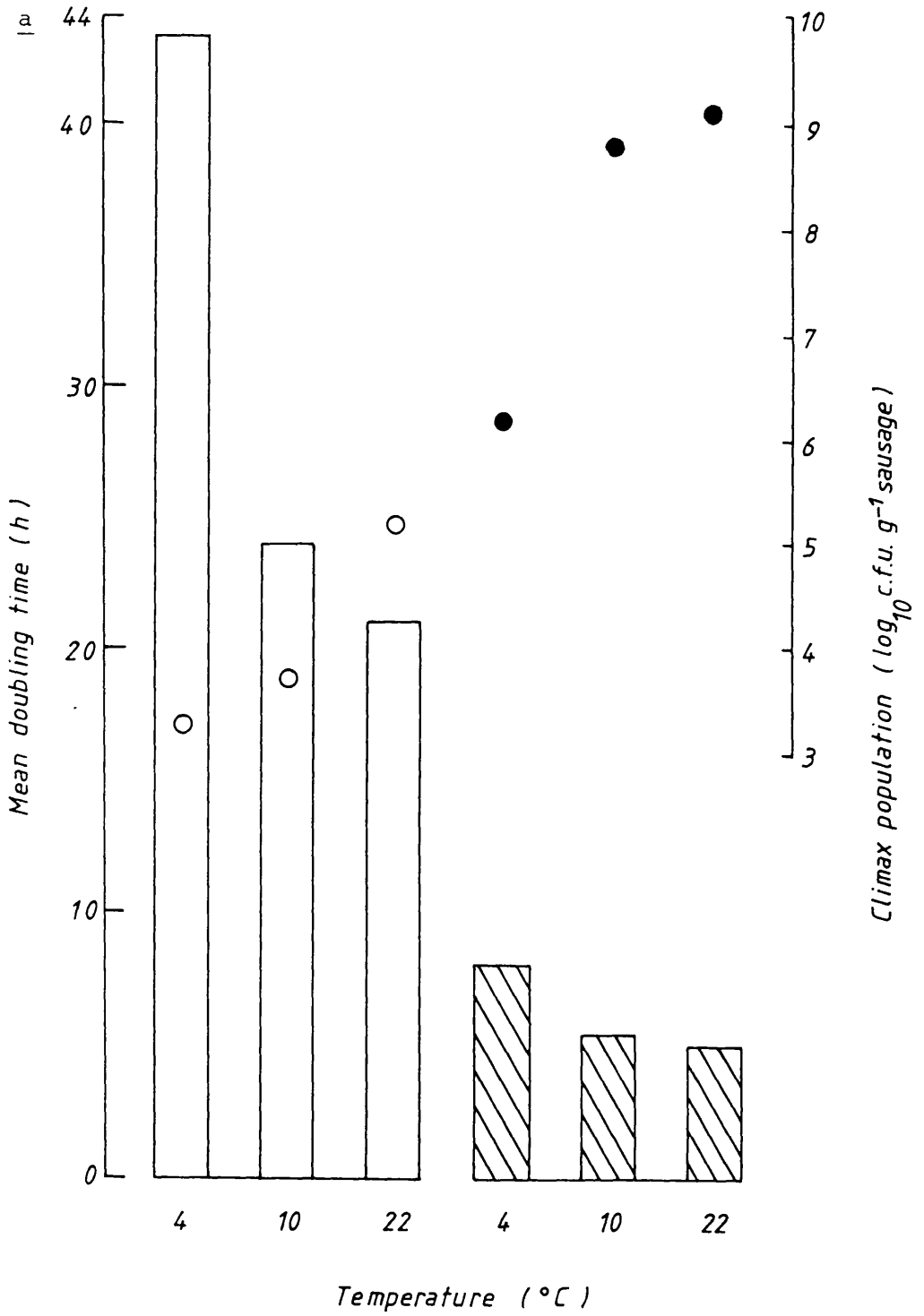


a, *Streptococcus* spp.

b, *Pseudomonas* spp.

Key: As Fig. 30.

Figure 33. Influence of temperature of storage and sulphite concentration on the rate and extent of growth of Enterobacteriaceae in sausage dominated by *Brochothrix thermosphacta*.



a Enterobacteriaceae

Key: As Fig. 30.

Figure 34. Influence of temperature of storage and sulphite concentration on the pH drift in sausage dominated by *Brochothrix thermosphacta*.

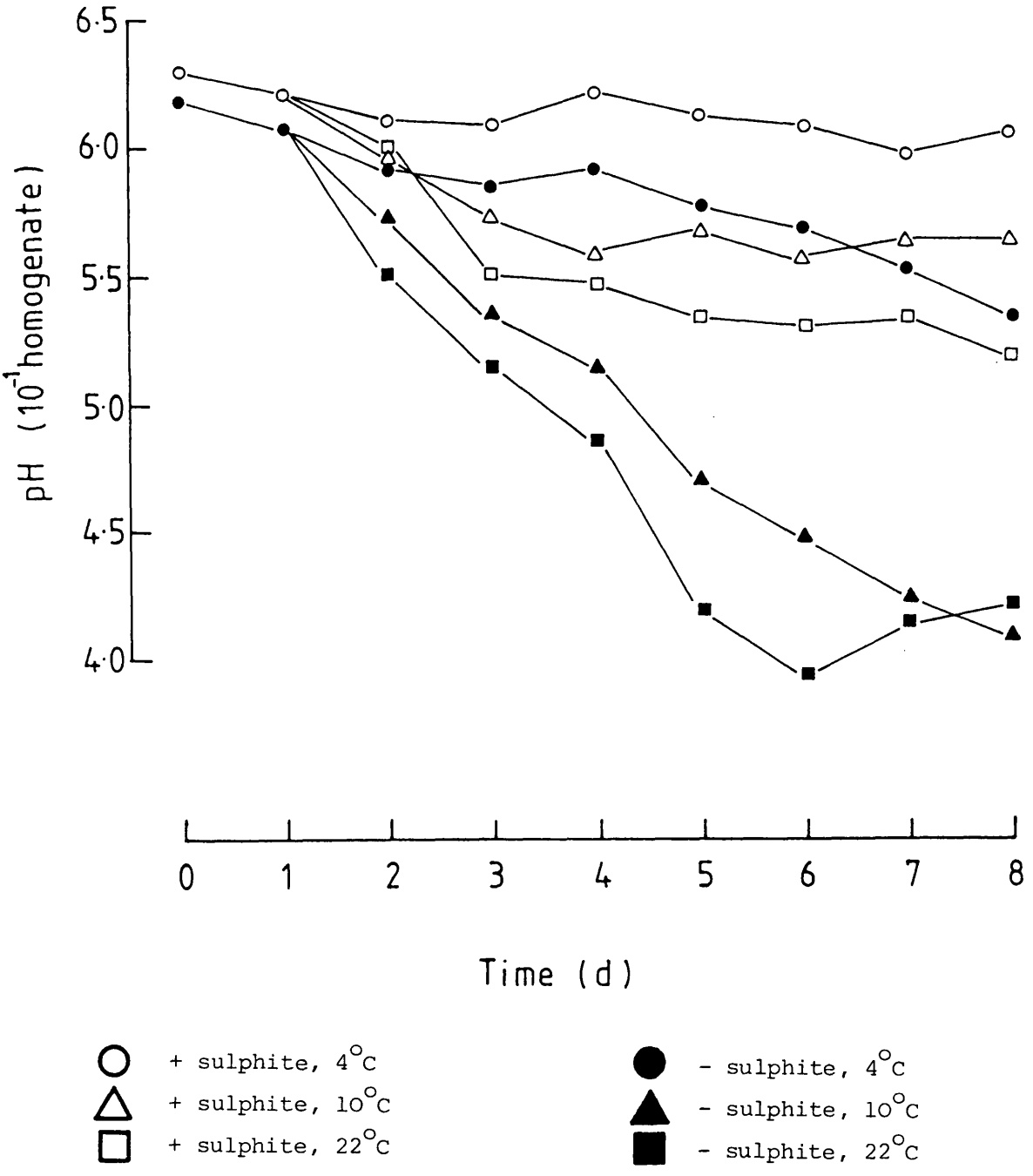


Figure 35. Influence of temperature on the loss of free and total sulphite from sausage dominated by yeasts

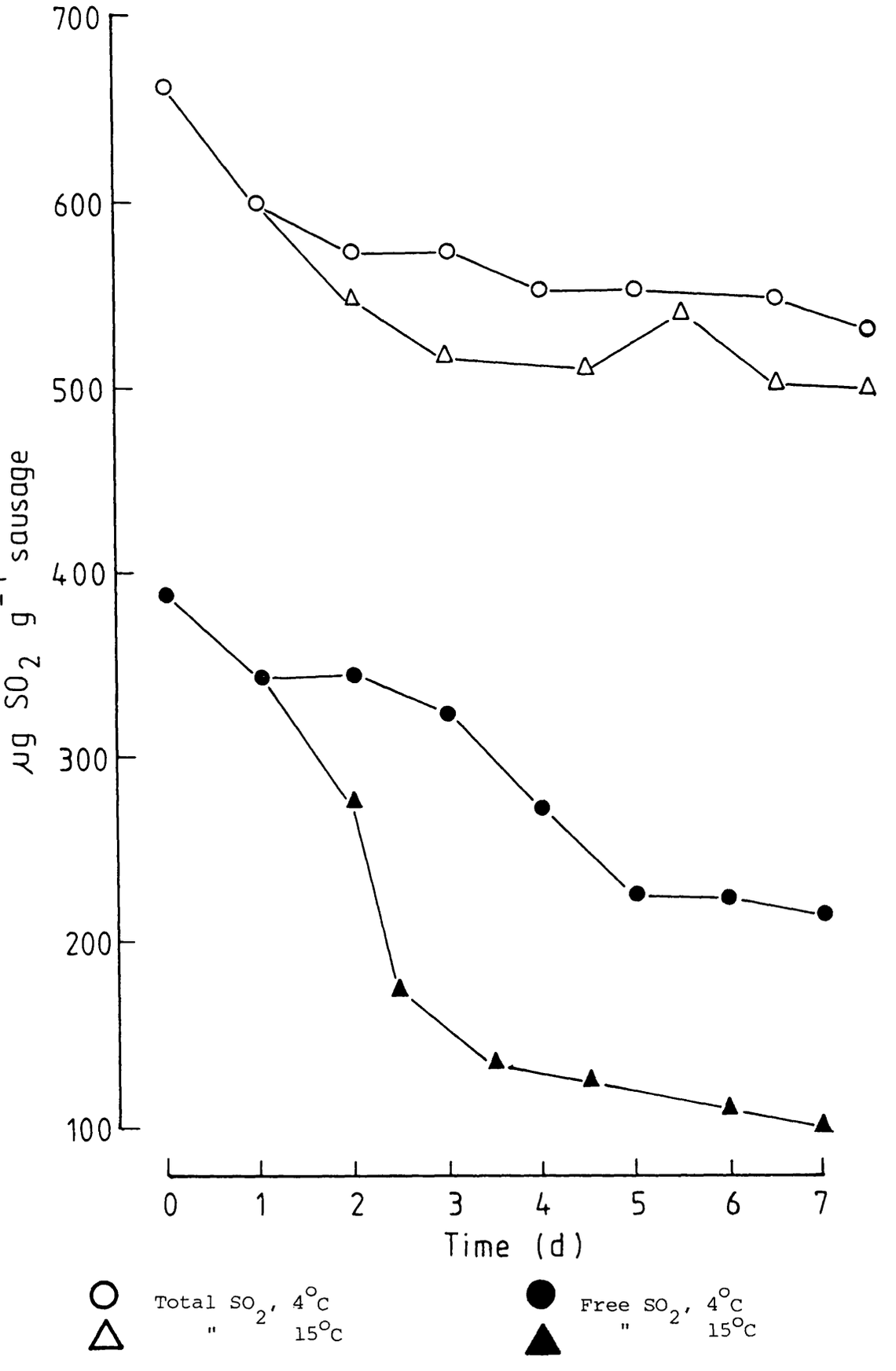


Figure 36. Influence of temperature and sulphite concentration on the growth of a, the yeasts, b, *Brochothrix thermosphacta* and c, *Lactobacillus* spp. in sausage dominated by yeasts

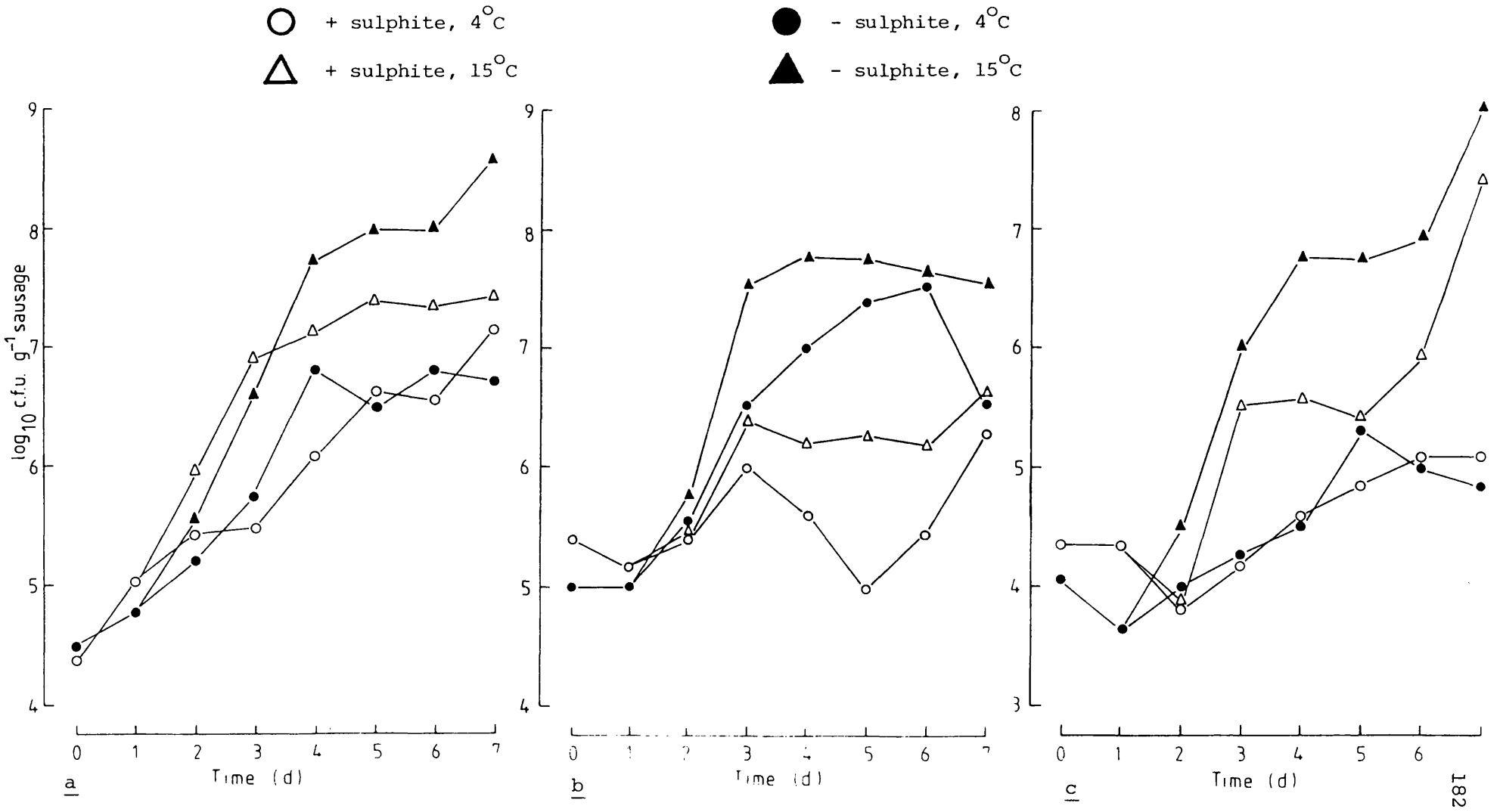


Figure 37. Influence of temperature and sulphite concentration on the growth of a, *Streptococcus* spp. b, *Pseudomonas* spp. and c, Enterobacteriaceae in sausage dominated by yeasts.

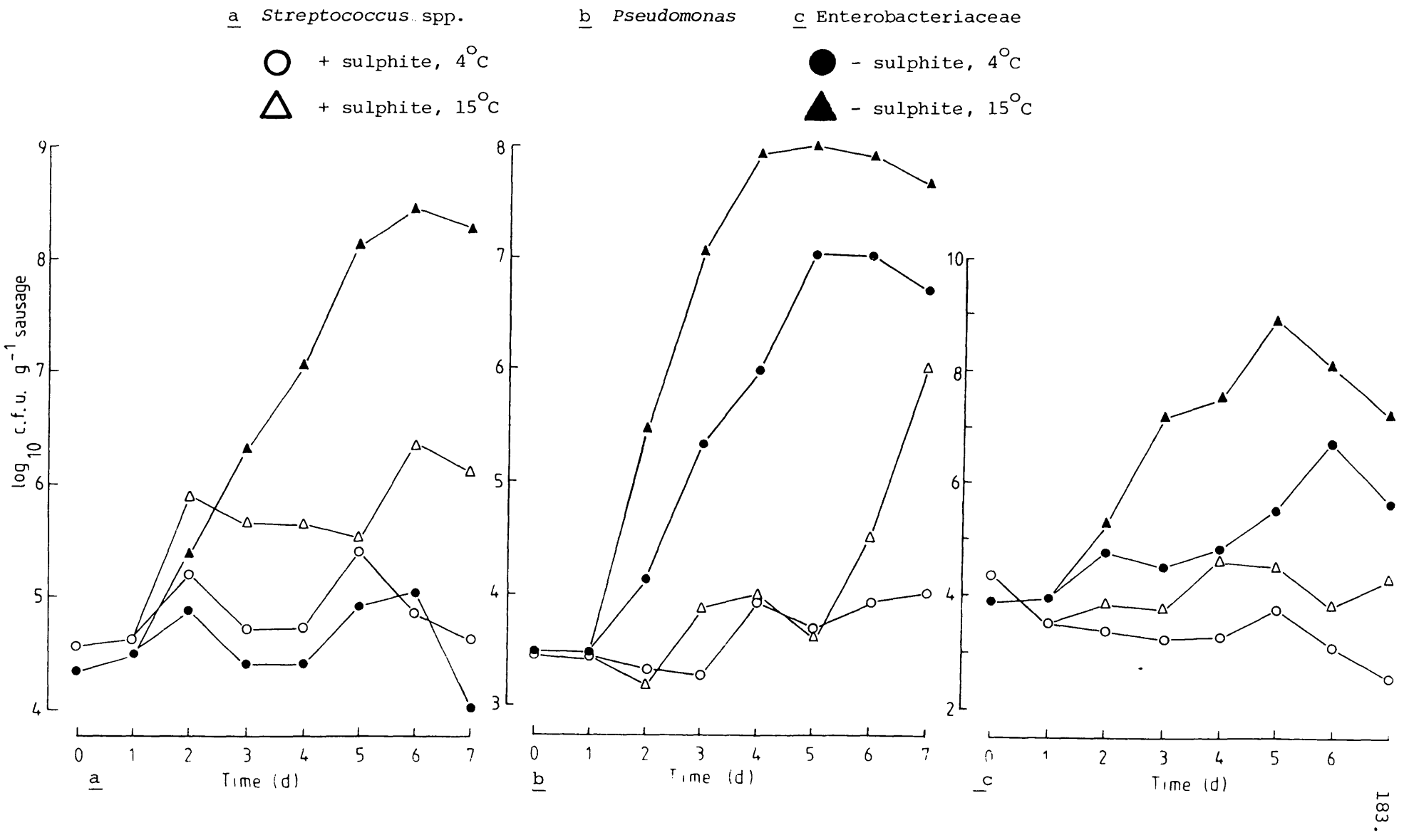


Figure 38. Influence of temperature of storage and sulphite concentration on the pH drift in sausage dominated by yeasts

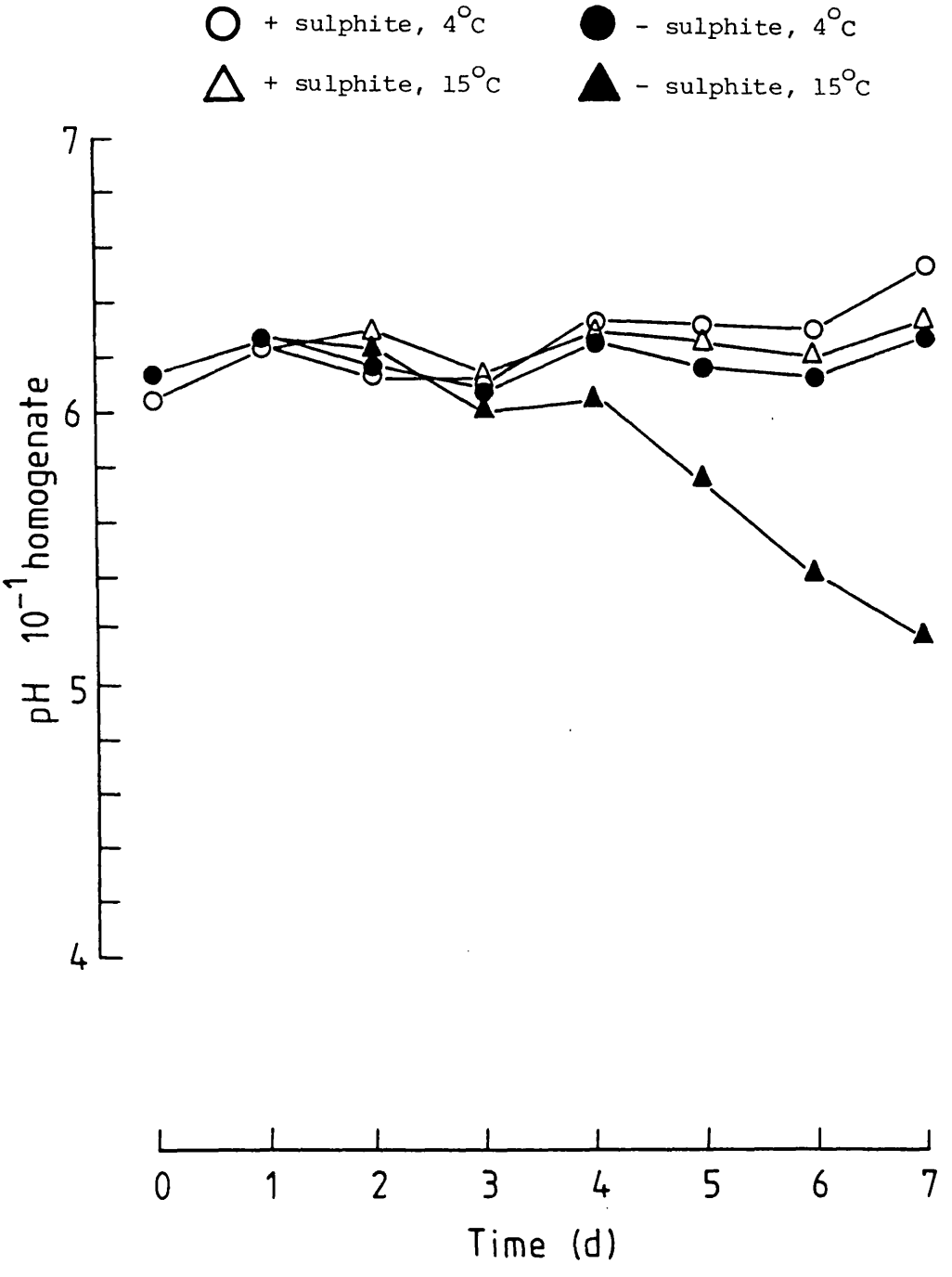
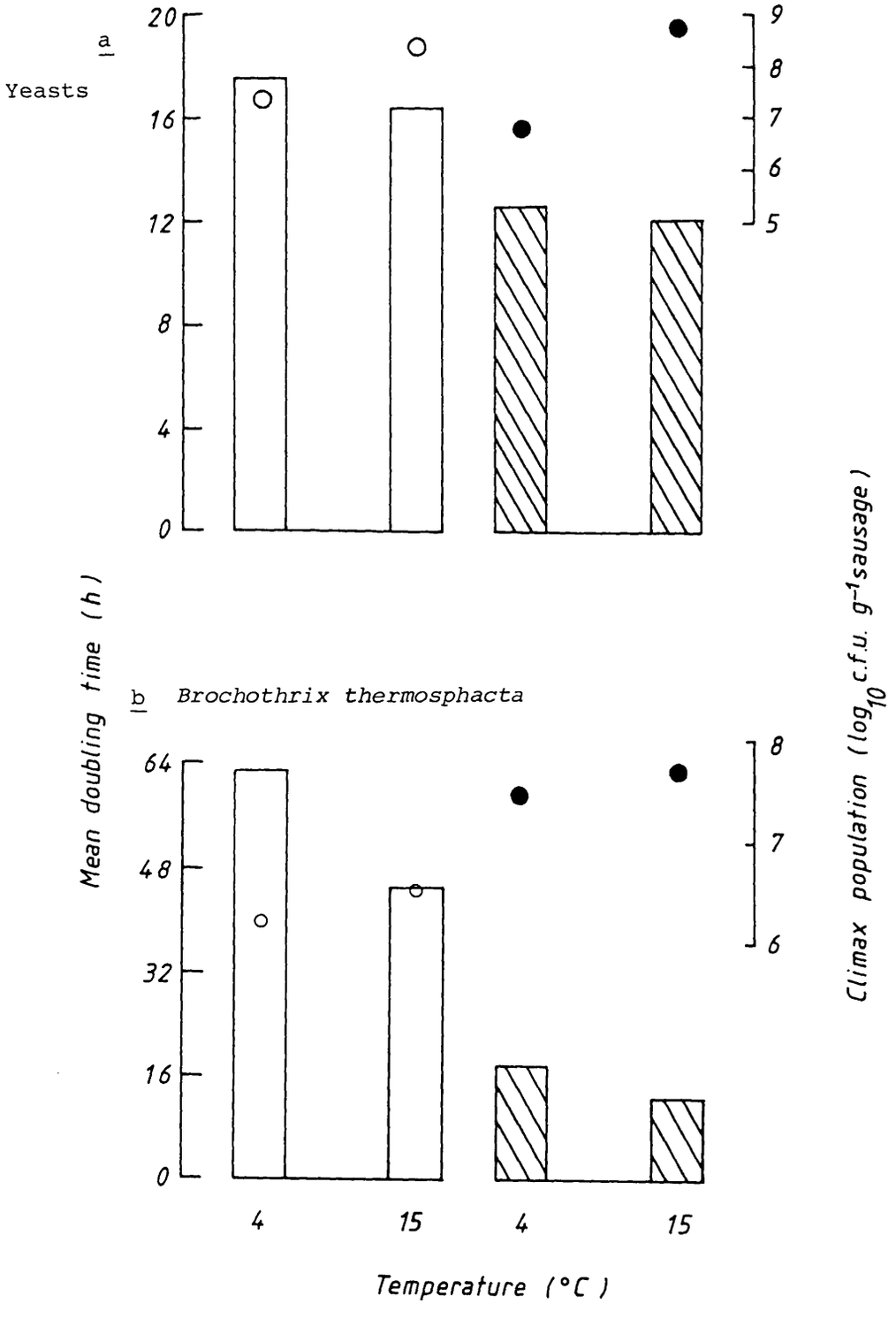


Figure 39. Influence of temperature of storage and sulphite concentration on the rate and extent of growth of a, the yeasts and b, *Brochothrix thermosphacta* in sausage dominated by yeasts.



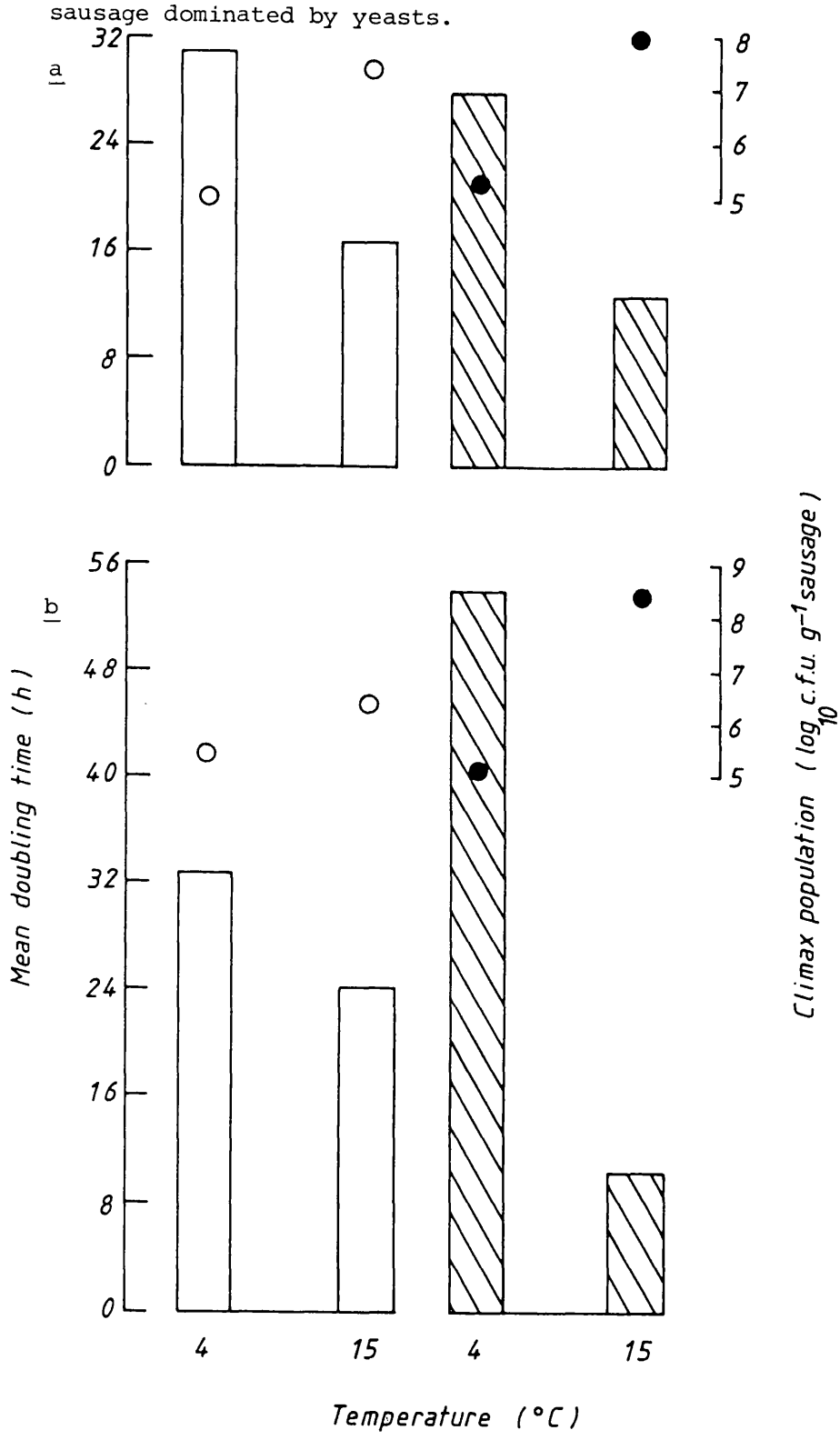
Mean doubling time up to climax population in sulphited sausage

Mean doubling time up to climax population in unsulphited sausage

Climax population in sulphited sausage

Climax population in unsulphited sausage

Figure 40. Influence of temperature of storage and sulphite concentration on the rate and extent of growth of a, *Lactobacillus* spp. and *Streptococcus* spp. in sausage dominated by yeasts.

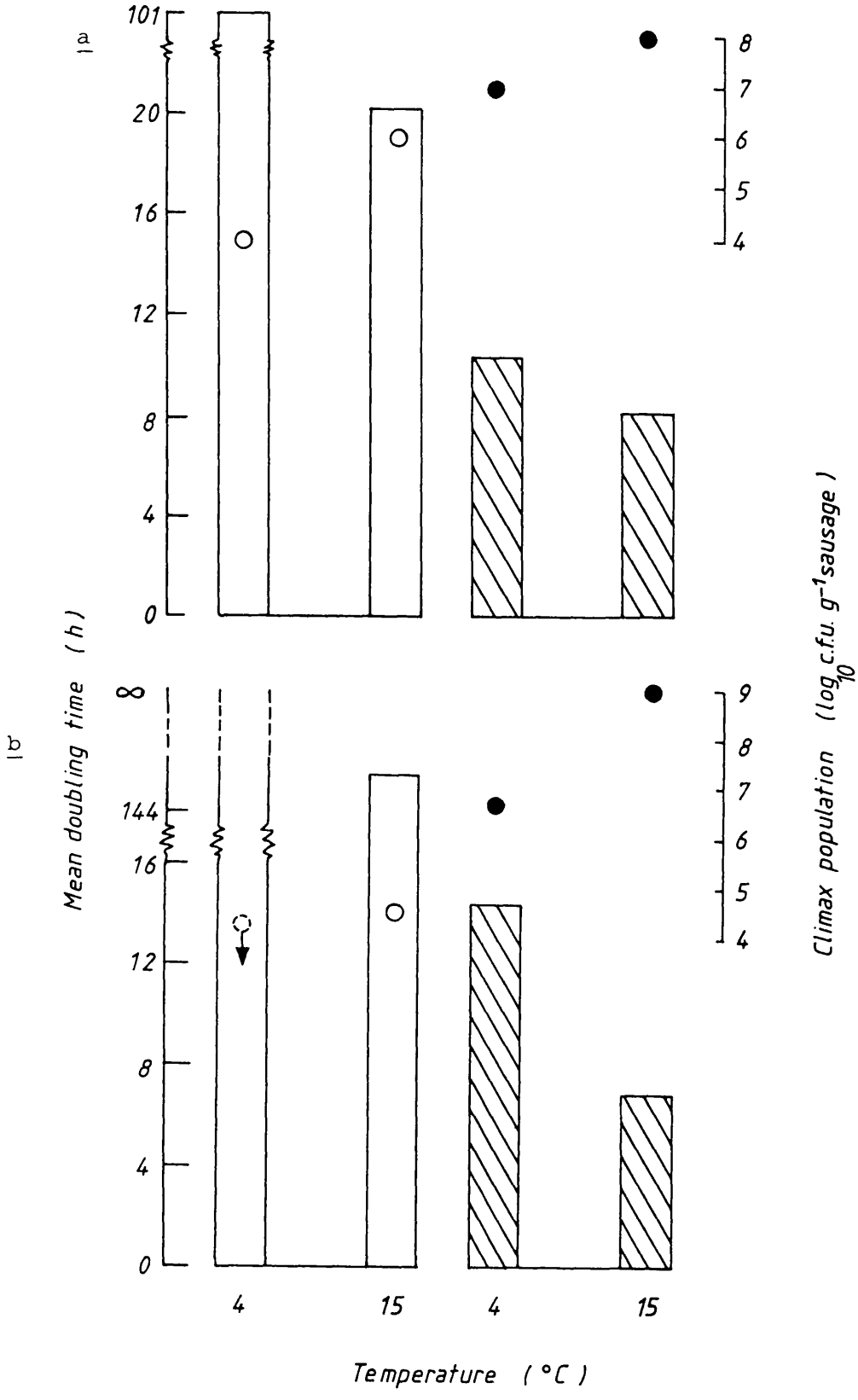


a *Lactobacillus* spp.

b *Streptococcus* spp.

Key: As in Fig. 39.

Figure 41. Influence of temperature of storage and sulphite concentration on the rate and extent of growth of a, *Pseudomonas* spp. and b, Enterobacteriaceae in sausage dominated by yeasts.



a *Pseudomonas* spp.

b Enterobacteriaceae

Key: As in Fig. 39.

Figure 42. Simplified dendrogram of isolates of Enterobacteriaceae from sausage and sausage ingredients

Footnote: % similarity calculated with the S_{SM} coefficient, clustering by UPGMA

Cluster:

1 *Yersinia* spp.

2 *Yersinia* spp.

3 *Escherichia coli*

4 *Citrobacter freundii*

5 *Serratia liquefaciens*

6 *Serratia marcescens*

7 *Enterobacter cloacae*

8 *Enterobacter agglomerans*

9 *Hafnia alvei*

10 *Hafnia alvei*

11 *Klebsiella* spp.

12 Unassigned

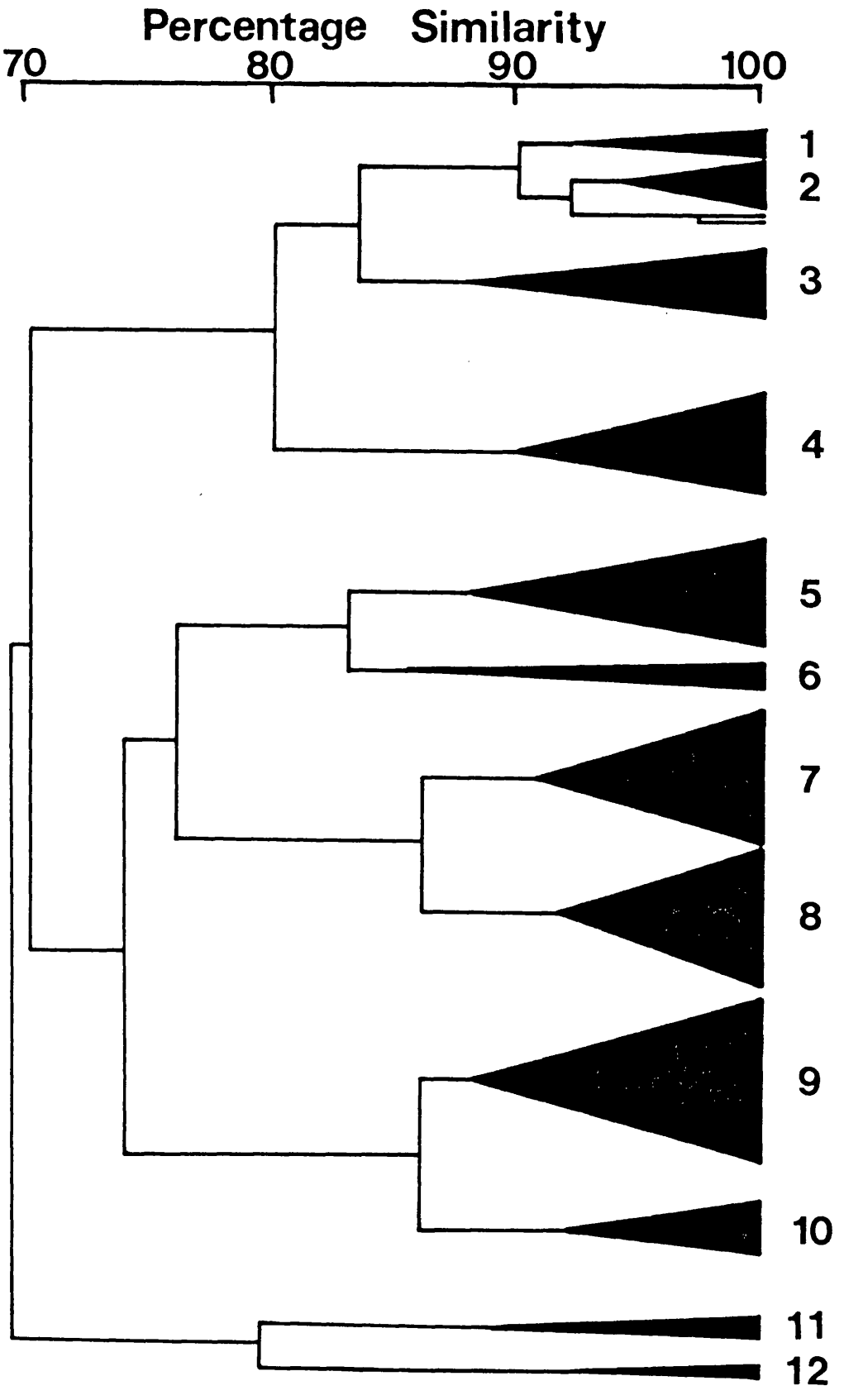

Percentage Similarity
70
80
90
100
1
2
3
4
5
6
7
8
9
10
11
12

Figure 43. Fermentation characteristics of *Lactobacillus* spp. isolated from sausage.

Footnote: a Homofermentative streptobacteria group 1B (Sharpe, 1979). Isolates (43) identified with *Lactobacillus casei* var. *casei*

b Homofermentative streptobacteria group 1B (Sharpe, 1979). Isolates (28) identified with *Lactobacillus casei* var. *casei*

c Homofermentative thermobacteria group 1A (Sharpe, 1979). Isolates (6) identified with *Lactobacillus salivarius* (L72, 73, 79-82).

d Homofermentative thermobacteria group 1A (Sharpe, 1979). Isolates (4) identified with *Lactobacillus helveticus* (L10, 37, 46, 55)

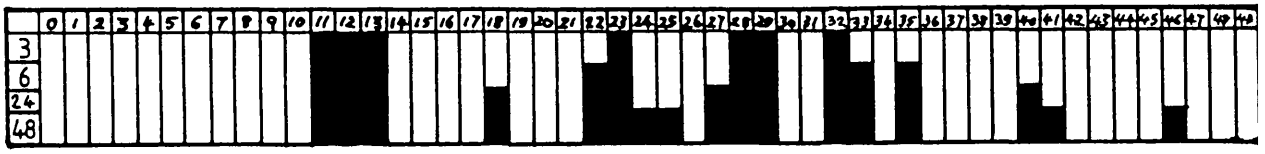
No fermentation

Fermentation of organic compound after 3, 6, 24 or 48 h incubation

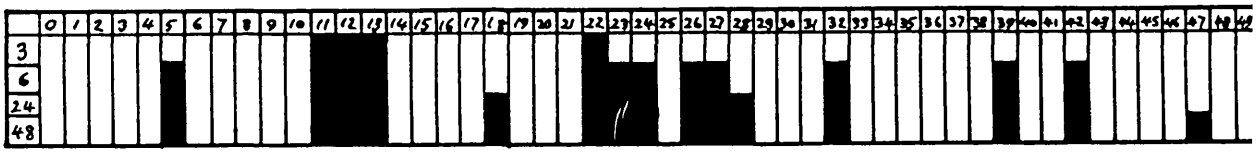
Substrates

0	Control	21	α methyl-D-glucoside		
1	Glycerol	22	N-acetyl glucosamine		
2	Erythritol	23	Amygdalin		
3	D-arabinose	24	Arbutin		
4	L-arabinose	25	Aesculin		
5	Ribose	26	Salicin		
6	D-xylose	27	Cellobiose		
7	L-xylose	28	Maltose		
8	Adonitol	29	Lactose		
9	βmethyl-xyloside	30	Melibiose		
10	Galactose	31	Saccharose	41	D-lyxose
11	D-glucose	32	Trehalose	42	D-tagatose
12	D-fructose	33	Inulin	43	D-fucose
13	D-mannose	34	Melezitose	44	L-fucose
14	L-sorbose	35	D-raffinose	45	D-arabitol
15	Rhamnose	36	Starch	46	L-arabitol
16	Dulcitol	37	Glycogen	47	Gluconate
17	Inositol	38	Xylitol	48	2 Keto-gluconate
18	Mannitol	39	β gentibiose	49	5 Keto-gluconate
19	Sorbitol	40	D-turanose		
20	α methyl-D-mannoside				

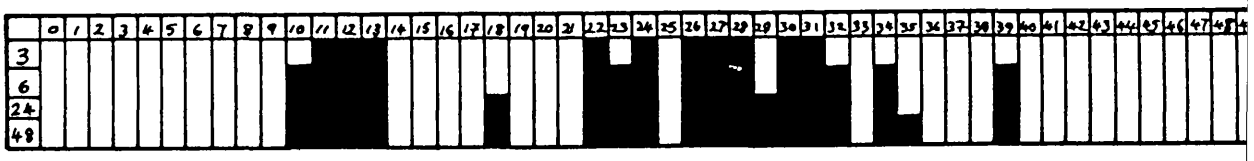
a


0 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24 25 26 27 28 29 30 31 32 33 34 35 36 37 38 39 40 41 42 43 44 45 46 47 48 49
3
6
24
48

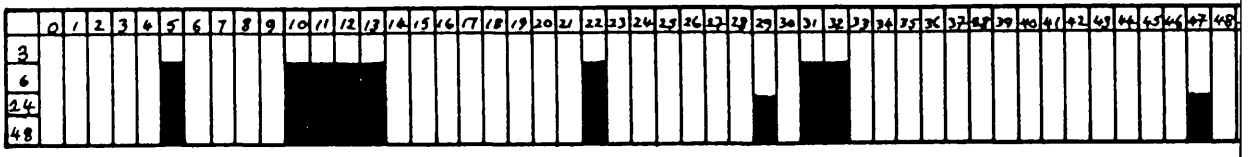
b


0 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24 25 26 27 28 29 30 31 32 33 34 35 36 37 38 39 40 41 42 43 44 45 46 47 48 49
3
6
24
48

c


0 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24 25 26 27 28 29 30 31 32 33 34 35 36 37 38 39 40 41 42 43 44 45 46 47 48
3
6
24
48

d


0 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24 25 26 27 28 29 30 31 32 33 34 35 36 37 38 39 40 41 42 43 44 45 46 47 48
3
6
24
48

Footnote to Fig. 44.

a Aerobic incubation

b Anaerobic incubation

● Reaction positive in all strains

◐ Reaction variable

Reaction negative in all strains

i. *Streptococcus faecalis*

ii *Streptococcus faecium*

iii *Streptococcus* Group I

iv *Streptococcus* Group II

v *Streptococcus* Group III

Substrates:

0 Basal medium
1 Salicin
2 Sorbitol
3 Glycerol
4 Azelate*
5 D(+) trehalose
6 Inulin
7 Mannitol
8 Arbutin
9 Mucate*
10 Arabinose
11 Pimelate
12 Citrate*
13 Adonitol
14 Cellobiose
15 Inositol
16 Raffinose
17 D(+) Melizitose
18 Sarcosine
19 D-ribose
20 Maltose
21 D(+) melibiose

* No reaction due to endogenous acid in organic supplement

Figure 44. Aerobic and anaerobic metabolism of organic substrates by *Streptococcus* spp. isolated from sulphited sausage

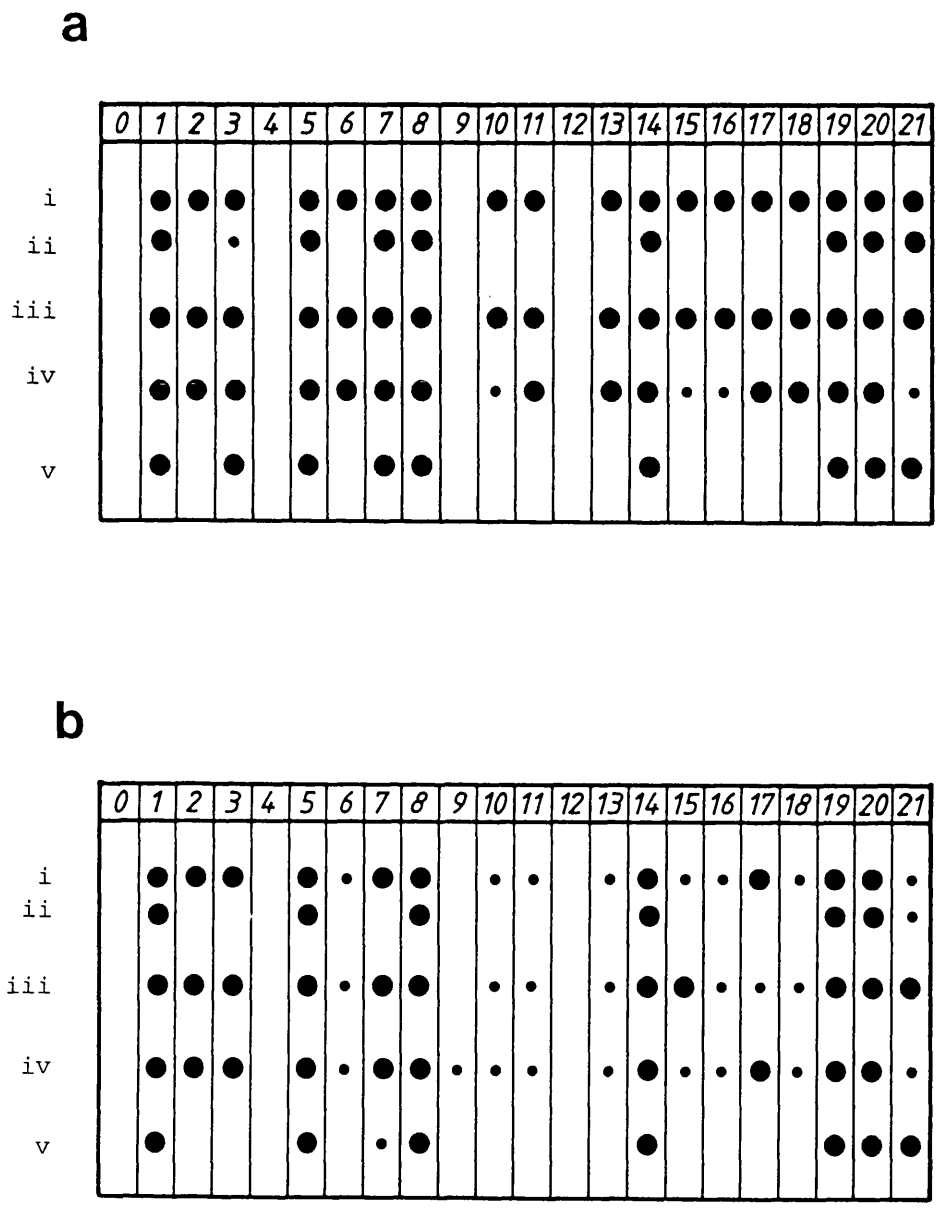
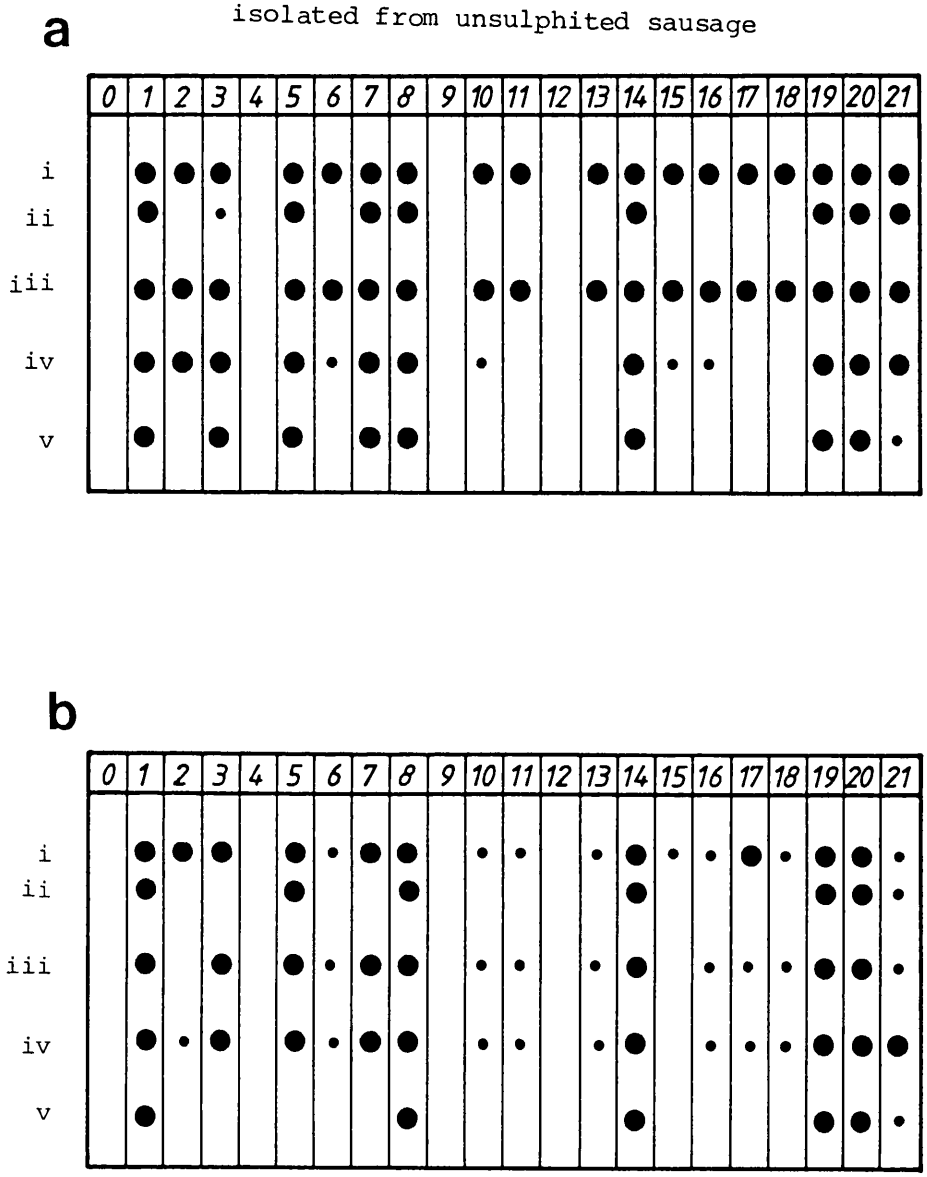


Figure 45. Aerobic and anaerobic metabolism of organic substrates by *Streptococcus* spp. isolated from unsulphited sausage



Key: As in Fig. 44.

Figure 46. Influence of sulphite on *Hafnia alvei* in culture medium

● Modified glucose glutamate medium (MGGM) pH 7.0, 30°C

△ MGGM + *ca*.125 µg free SO_2/ml

○ MGGM + *ca*.250 µg free SO_2/ml

□ MGGM + *ca*.500 µg free SO_2/ml

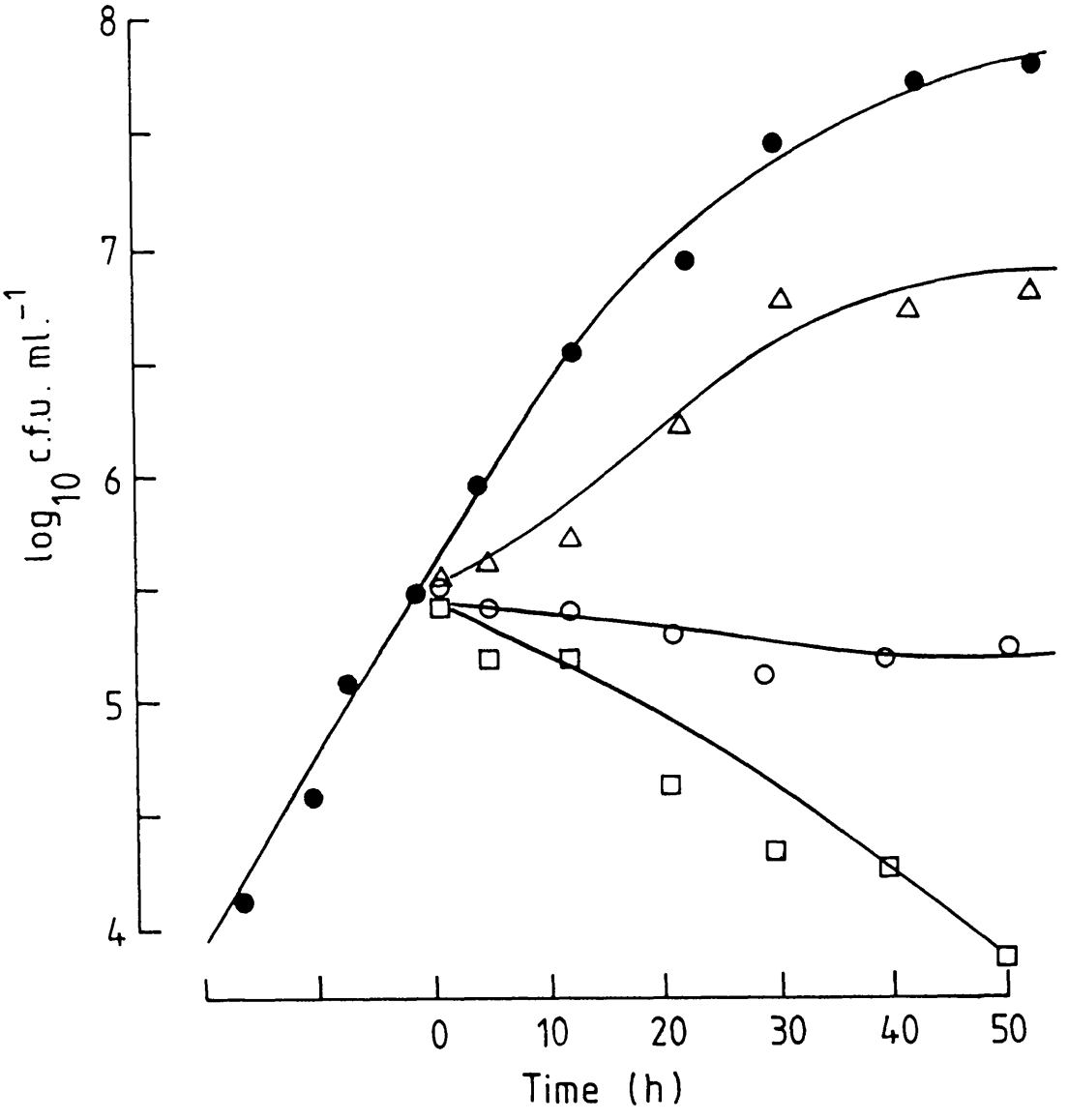


Figure 47. Range of sensitivity of representative microbial groups to free sulphite in batch culture medium

Footnote:  Range of inhibition

Microbial Group	Strains tested	Medium used	Temperature of incubation
Yeasts	6	Yeast nitrogen base-glucose broth	30°C
Streptococcus spp.	8	Buffered Whittenbury's broth	"
Lactobacillus spp.	10	" " "	"
Pseudomonas spp.	9	Palleroni and Doudoroff's (1972) broth	"
Salmonella spp.	12	Modified glucose glutamate broth	"
Enterobacteriaceae	24	" " " "	"
Brochothrix thermosphacta	9	Grau's (1980) broth	20°C

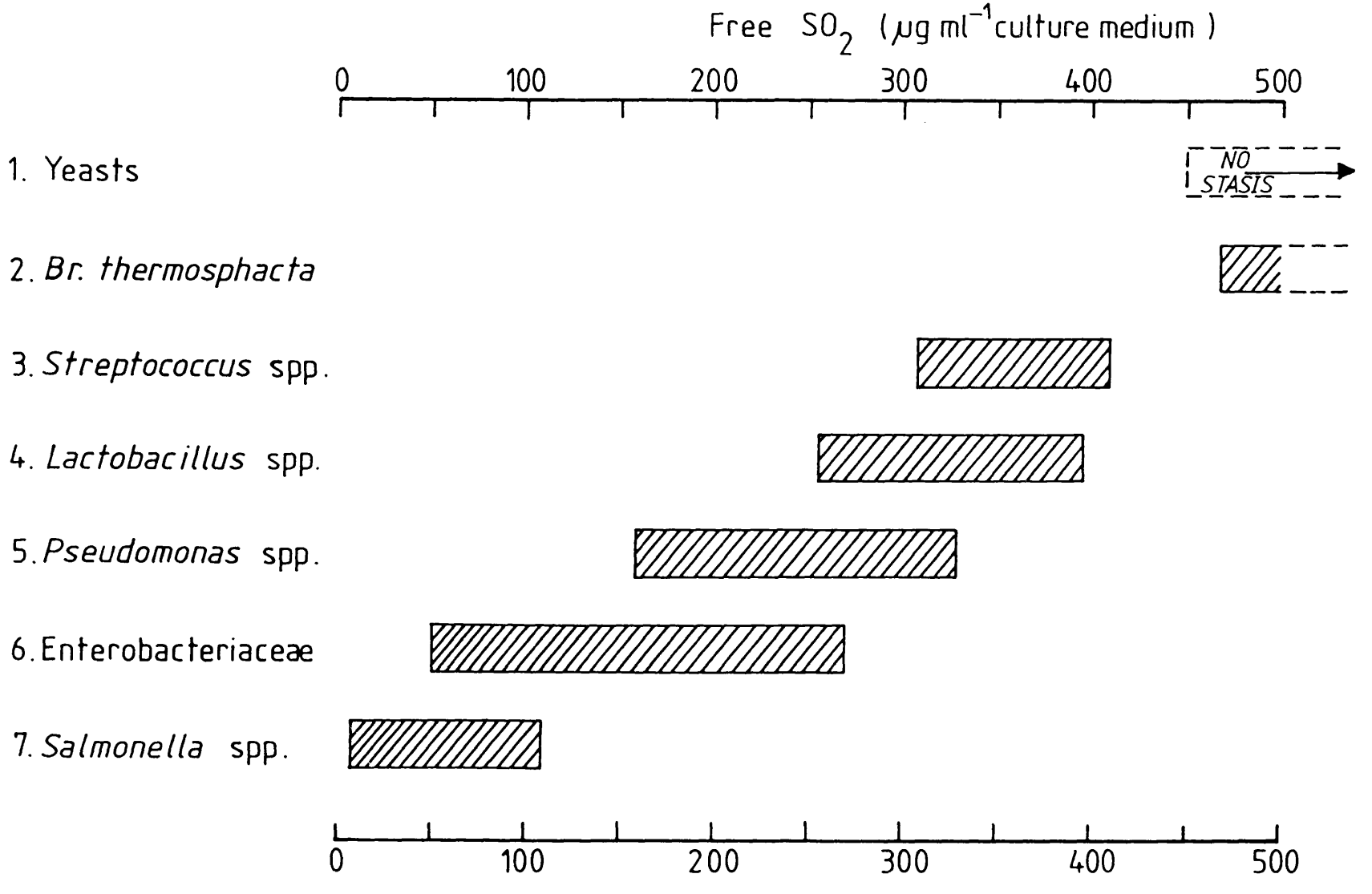
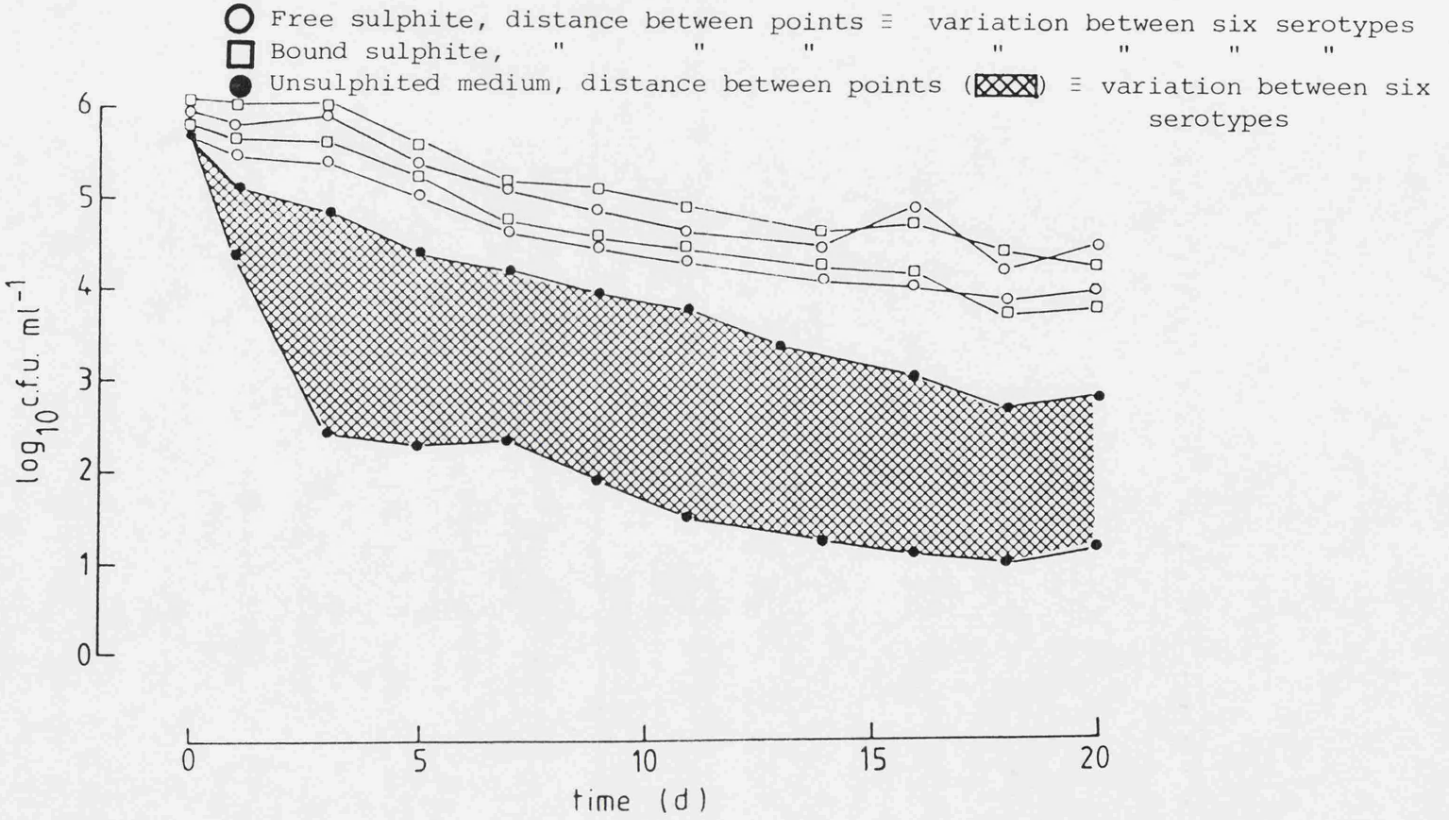

Free SO_2 ($\mu g\ ml^{-1}$ culture medium)
0
100
200
300
400
500
1. Yeasts
NO STASIS
2. Br. thermosphacta
3. Streptococcus spp.
4. Lactobacillus spp.
5. Pseudomonas spp.
6. Enterobacteriaceae
7. Salmonella spp.
0
100
200
300
400
500

Figure 48. Influence of high concentration of free and bound sulphite on six serotypes of *Salmonella* in batch culture at 4°C.

Footnote: Sulphite concentration = *ca.* 900-1000 µg total SO_2/ml MGGM

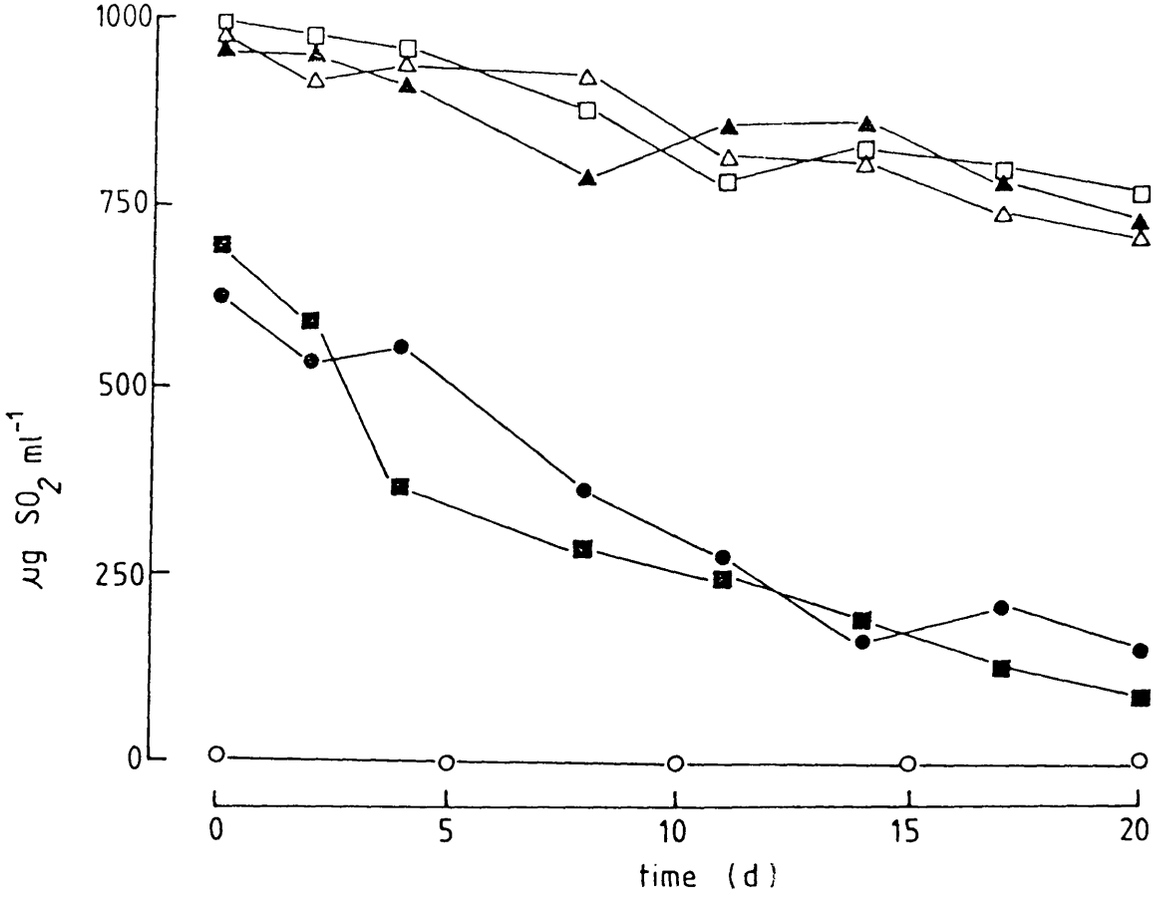


Salmonella serotypes: *anatum, goerlitz, kedougou, hadar, typhimurium* and *virchow*

Figure 49. Loss of free and total sulphite from MGGM at 4°C.

Footnote:

▲ Total SO_2, uninoculated MGGM
□ Total SO_2, MGGM inoculated with *ca.* 10^6 cells of *Salmonella virchow* rif^R
△ Total SO_2, MGGM + acetaldehyde inoculated with *Salmonella virchow* $rif.^R$
● Free SO_2, uninoculated MGGM
■ Free SO_2, MGGM inoculated with *Salmonella virchow* $rif.^R$
○ Free SO_2, MGGM + acetaldehyde inoculated with *Salmonella virchow* $rif.^R$



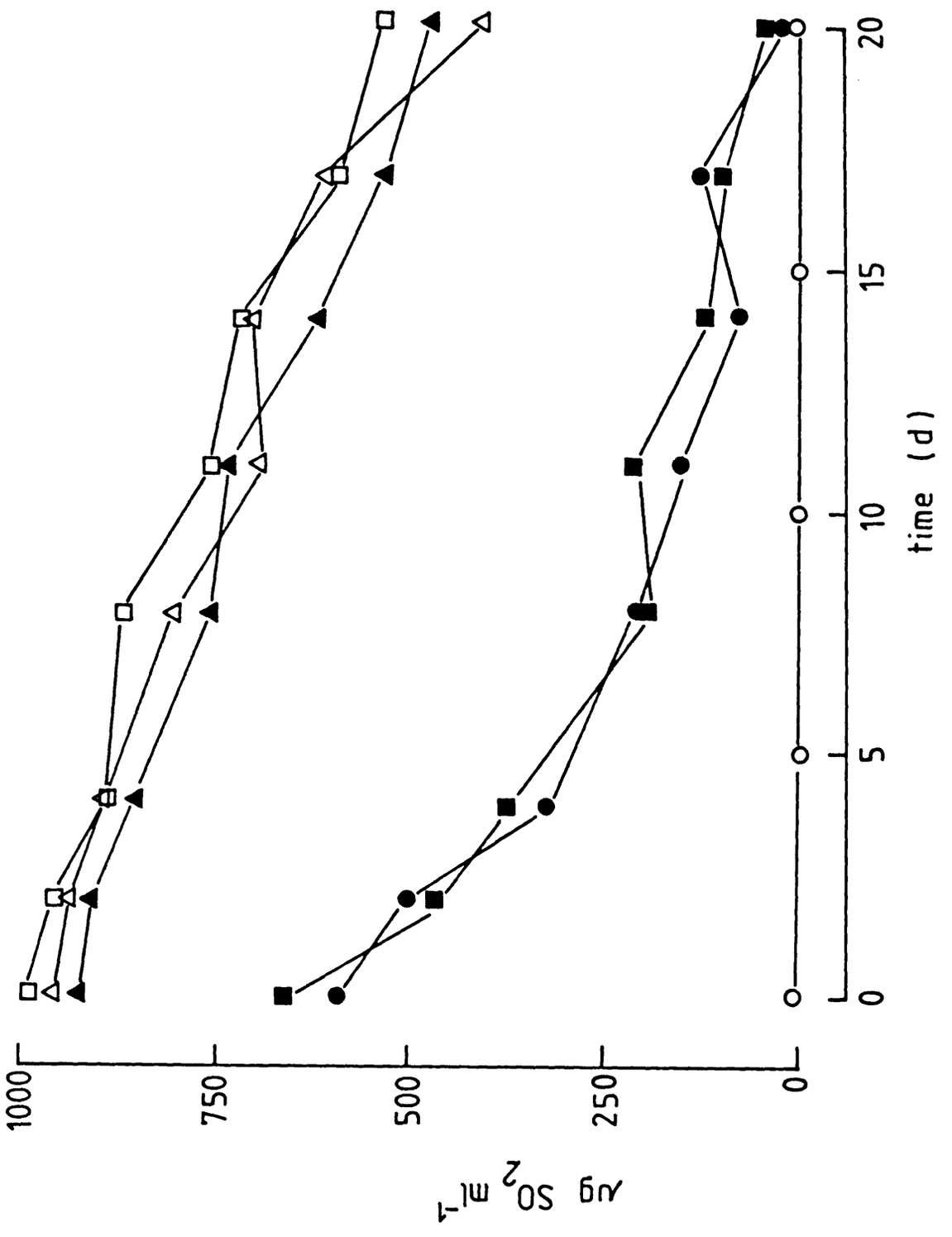


Figure 50. Loss of free and total sulphite from MGGM at 12°C.

Key: As in Fig. 49.

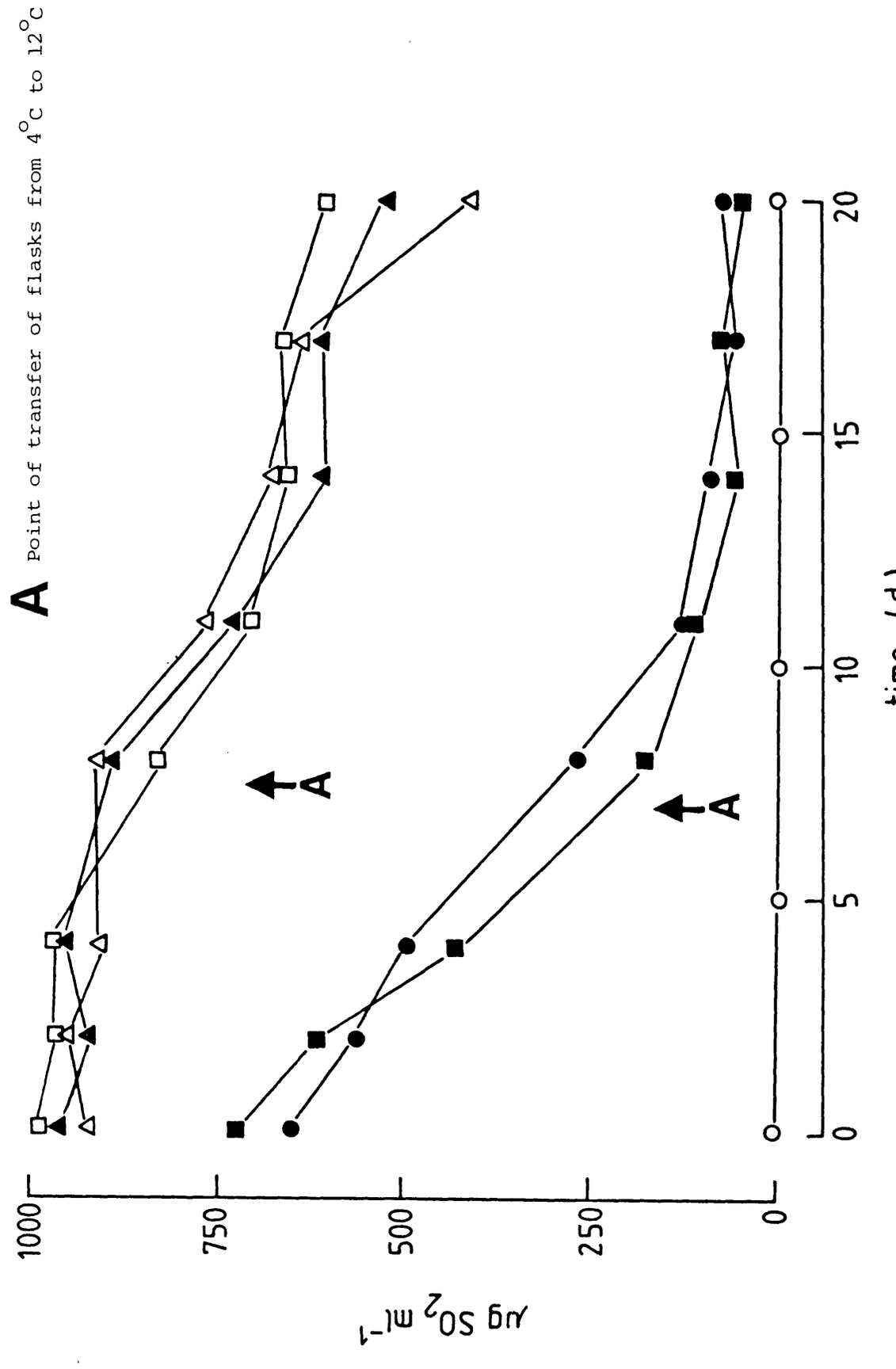


Figure 51. Loss of free and total sulphite from MGGM stored for 7 days at 4°C, then at 12°C for 18 days

Key: As in Fig. 49.

A Point of transfer of flasks from 4°C to 12°C

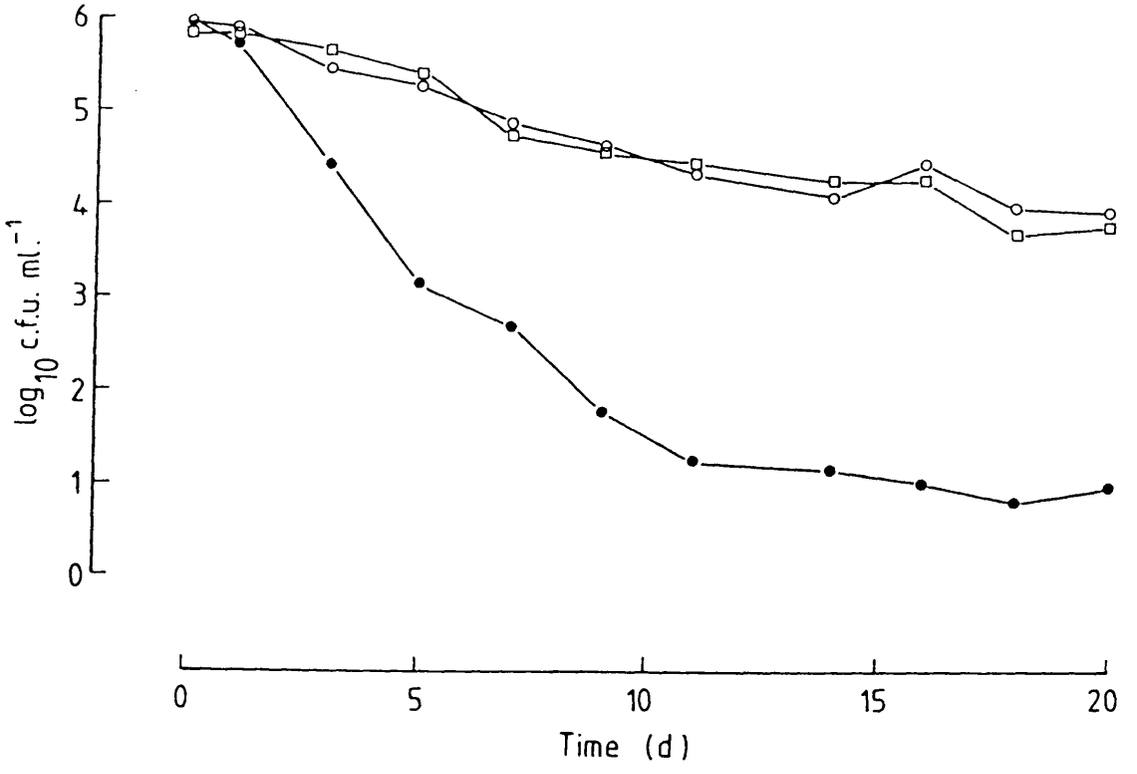
Figure 52. Influence of free and bound sulphite on *Salmonella virchow* rif.^{R} at 4^{o}C

Footnote:

● Unsulphited MGGM inoculated with *ca*.10^{6} cells of *Salmonella virchow* rif.^{R}

○ Sulphited MGGM inoculated with *Salmonella virchow* rif.^{R}

□ MGGM + acetaldehyde + sulphite inoculated with *Salmonella virchow* rif.^{R}



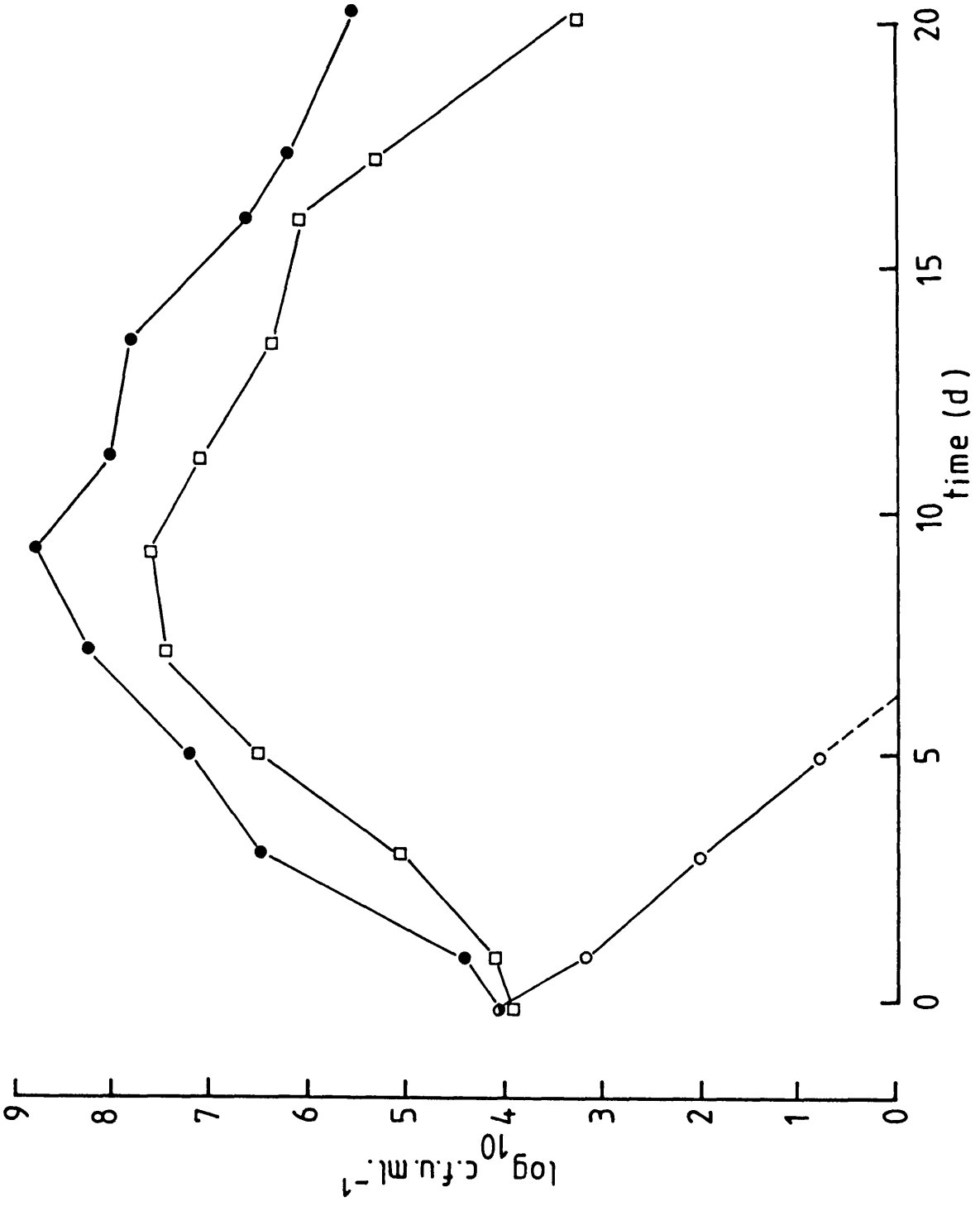


Figure 53. Influence of free and bound sulphite on *Salmonella virchow* rif.^{R} at 12^{o}C.

Footnote: As in Fig. 52.

Figure 54. Influence of free and bound sulphite on *Salmonella virchow* rif.^{R} stored for 7 days at 4°C, then at 12°C for 18 days.

Footnote: As in Fig. 52, also:

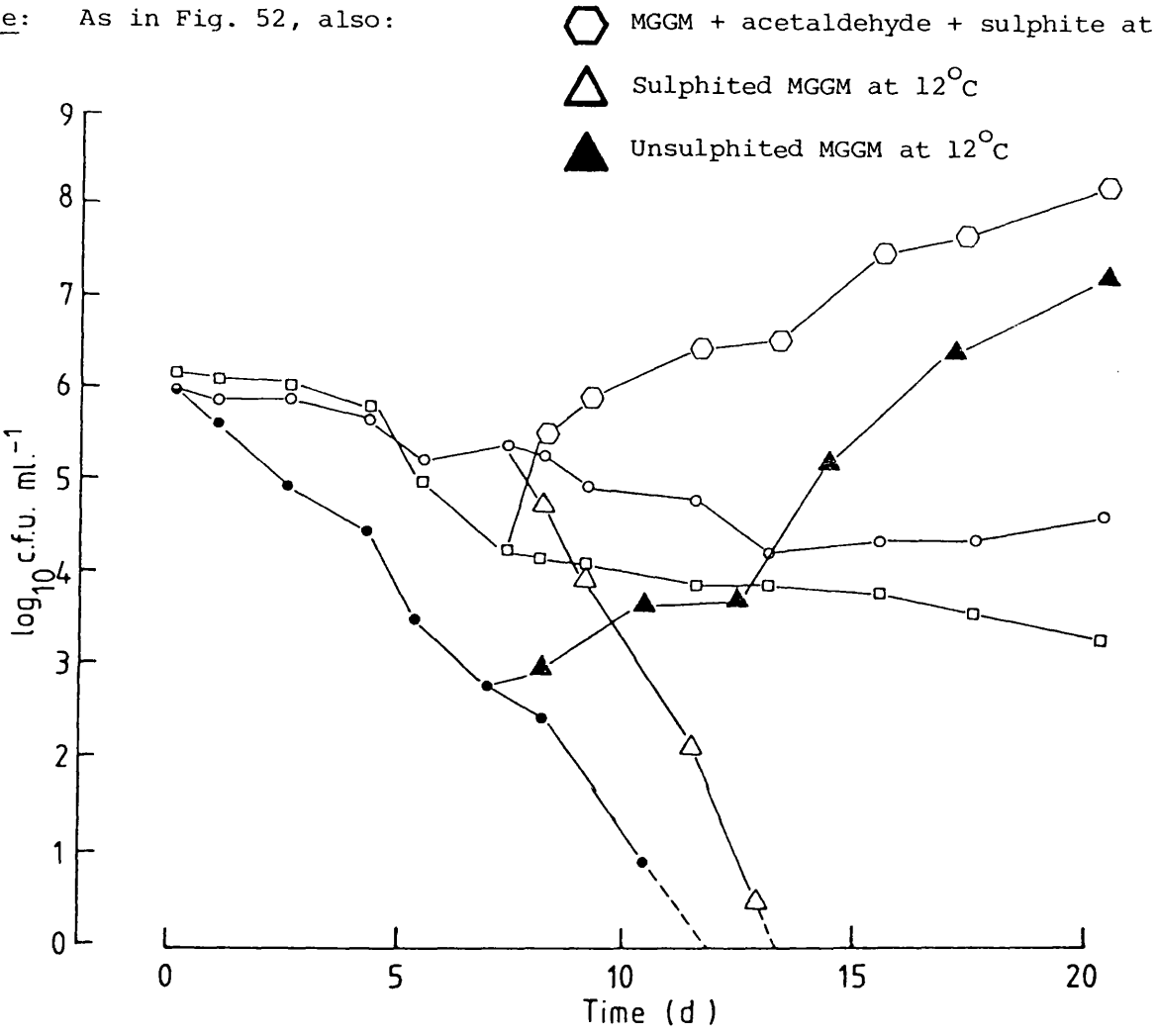
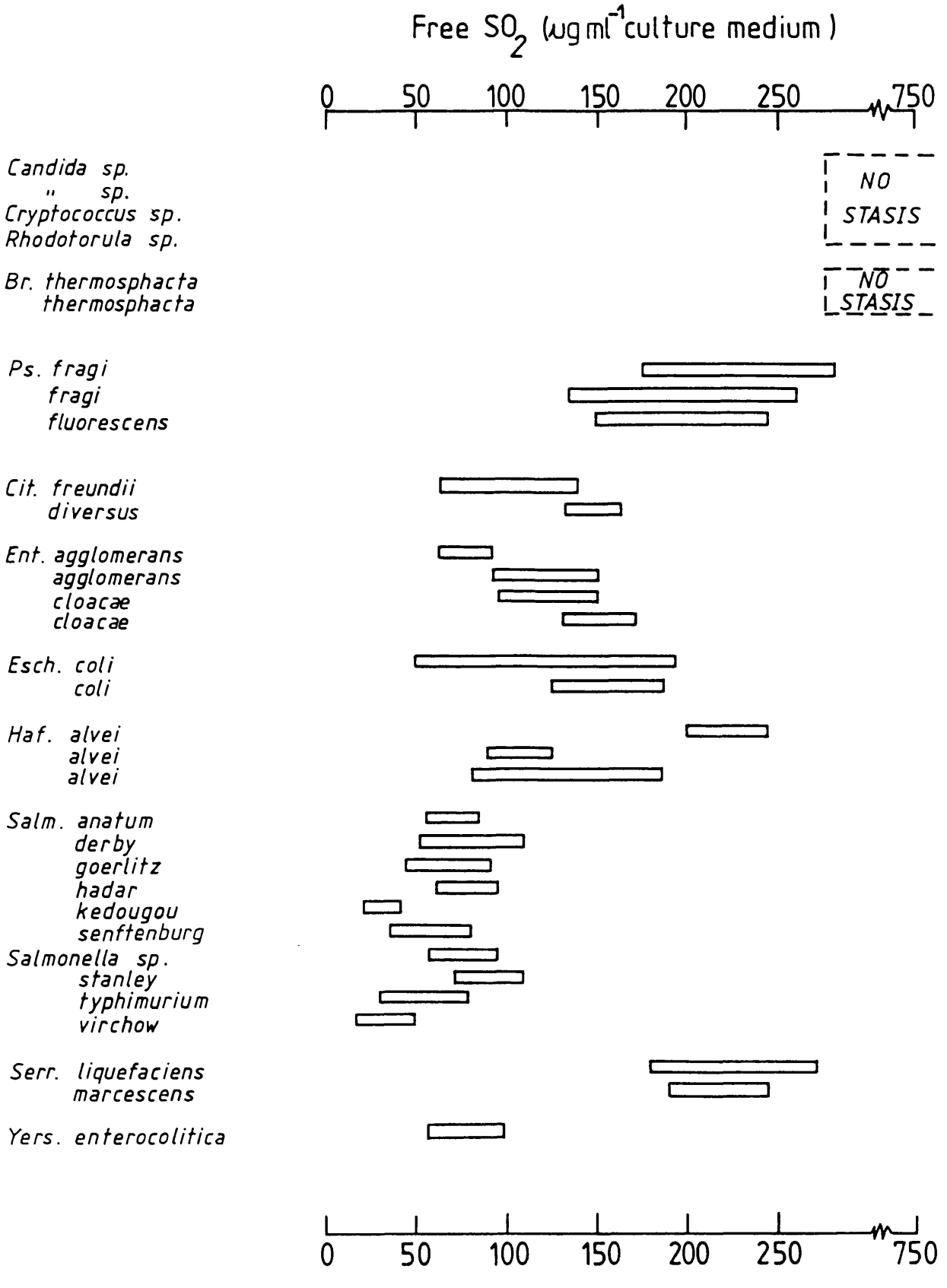


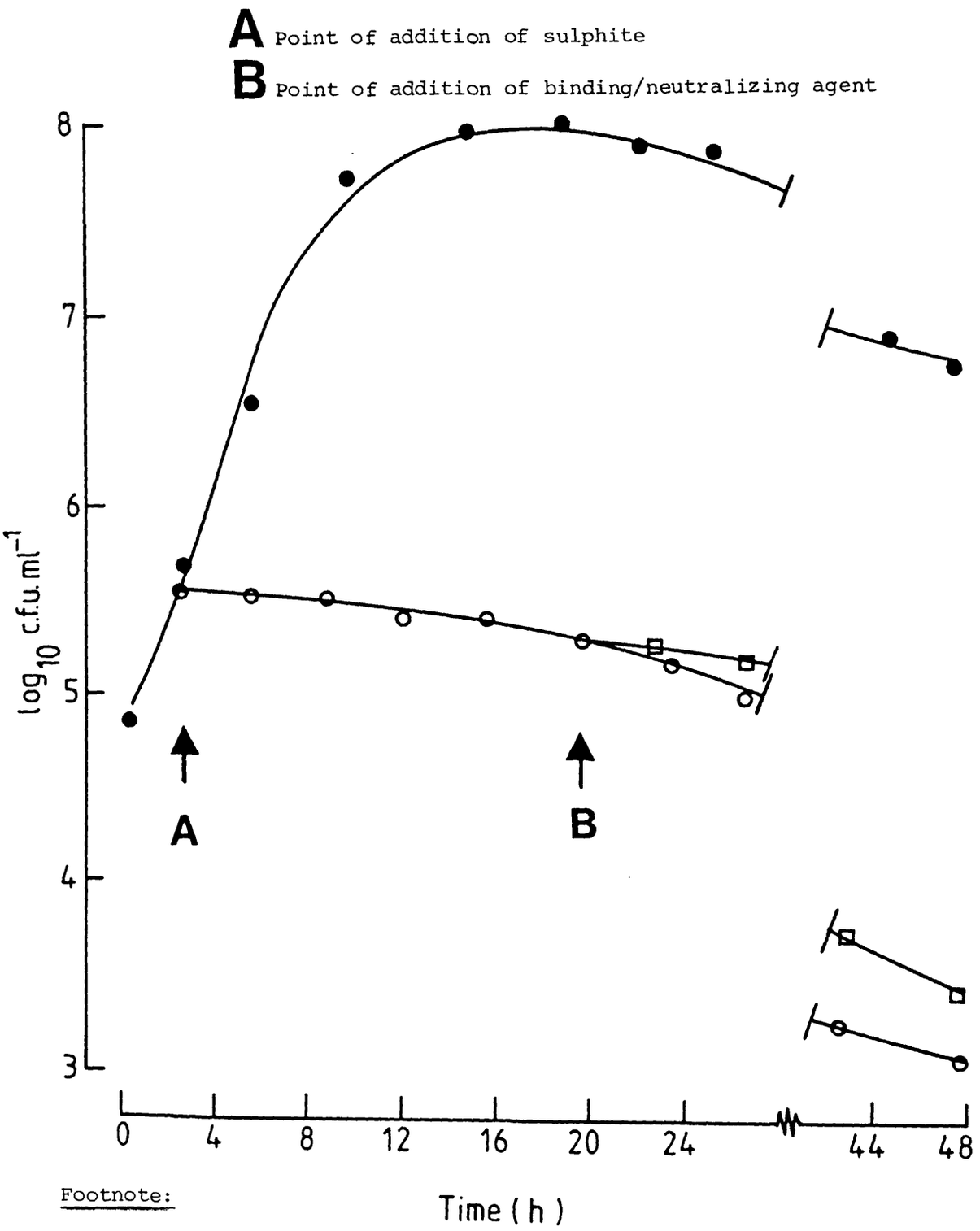
Figure 55. Range of sensitivity of representative micro organisms to free sulphite in turbidometer culture medium.



Footnote: ▭ Range of inhibition

Media and temperatures of incubation used as in Fig. 47.

Figure 56. Influence of free and bound sulphite on *Salmonella virchow* rif.^{R} in turbidometer culture medium at 30°C



Footnote:

● Unsulphited MGGM

○ Sulphited MGGM

□ MGGM + acetaldehyde/hydrogen peroxide + sulphite

Figure 57. Influence of free and bound sulphite on *Salmonella derby* in turbidometer culture medium at 30°C.

Footnote: As in Fig. 56.

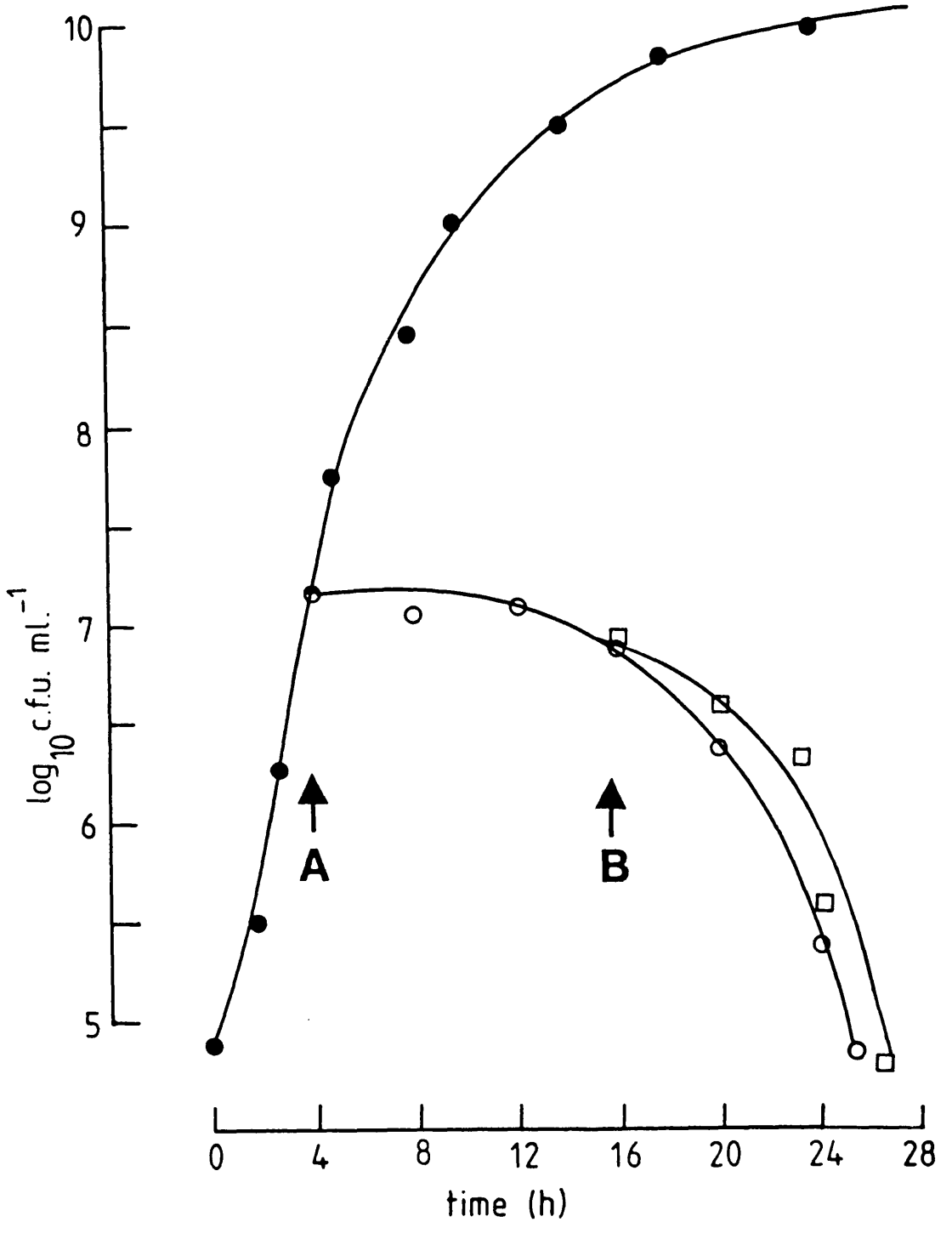


Figure 58. Influence of free and bound sulphite on *Hafnia alvei* in turbidometer culture medium at 30°C

Footnote: As in Fig. 56.

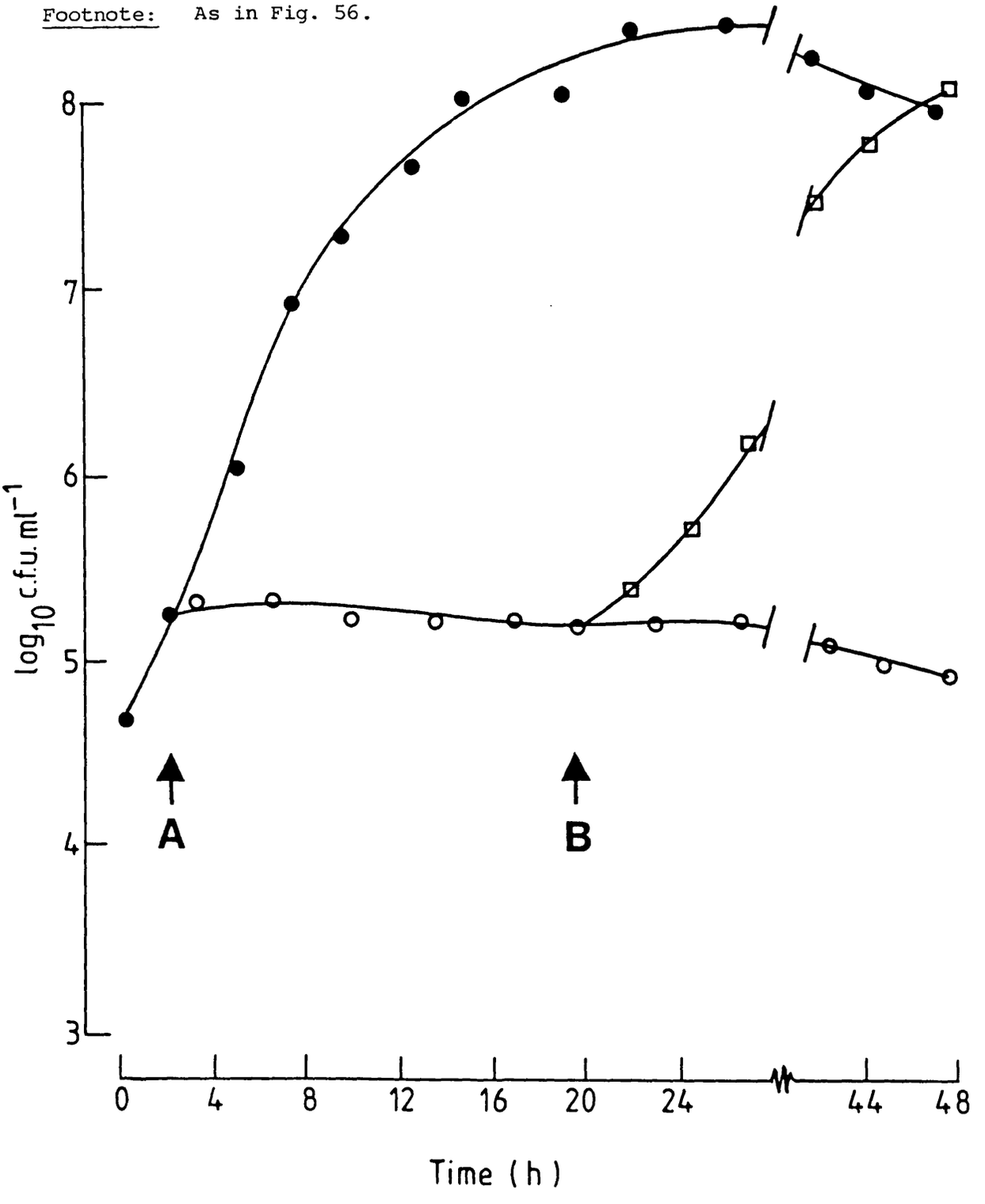


Figure 59. Influence of free sulphite concentration on the growth rate of, and production of binding agents by a yeast isolated from sulphited sausage

Footnote:

a ⬡ Unsulphited, glucose-supplemented yeast nitrogen base medium inoculated with yeast, 90

● Sulphited (650 g total SO_2/ml) medium inoculated with Y90

▲ " (450 " " ") " " " "

■ " (150 " " ") " " " "

b ○ Total sulphite, high preservative

△ " " , moderate preservative

□ " " , low preservative

☆ " " , medium not inoculated with yeast, Y90

● Free sulphite, high concentration of preservative

▲ " " , moderate concentration of preservative

■ " " , low concentration of preservative

✪ " " , medium not inoculated with yeast , Y90

c Symbols as in b, *vide supra*

d Symbols as in a, *vide supra*. Measurement of binding agent concentration using the basic fuchsin-sulphite assay.

Incubation was at 30°C under aerobic conditions.

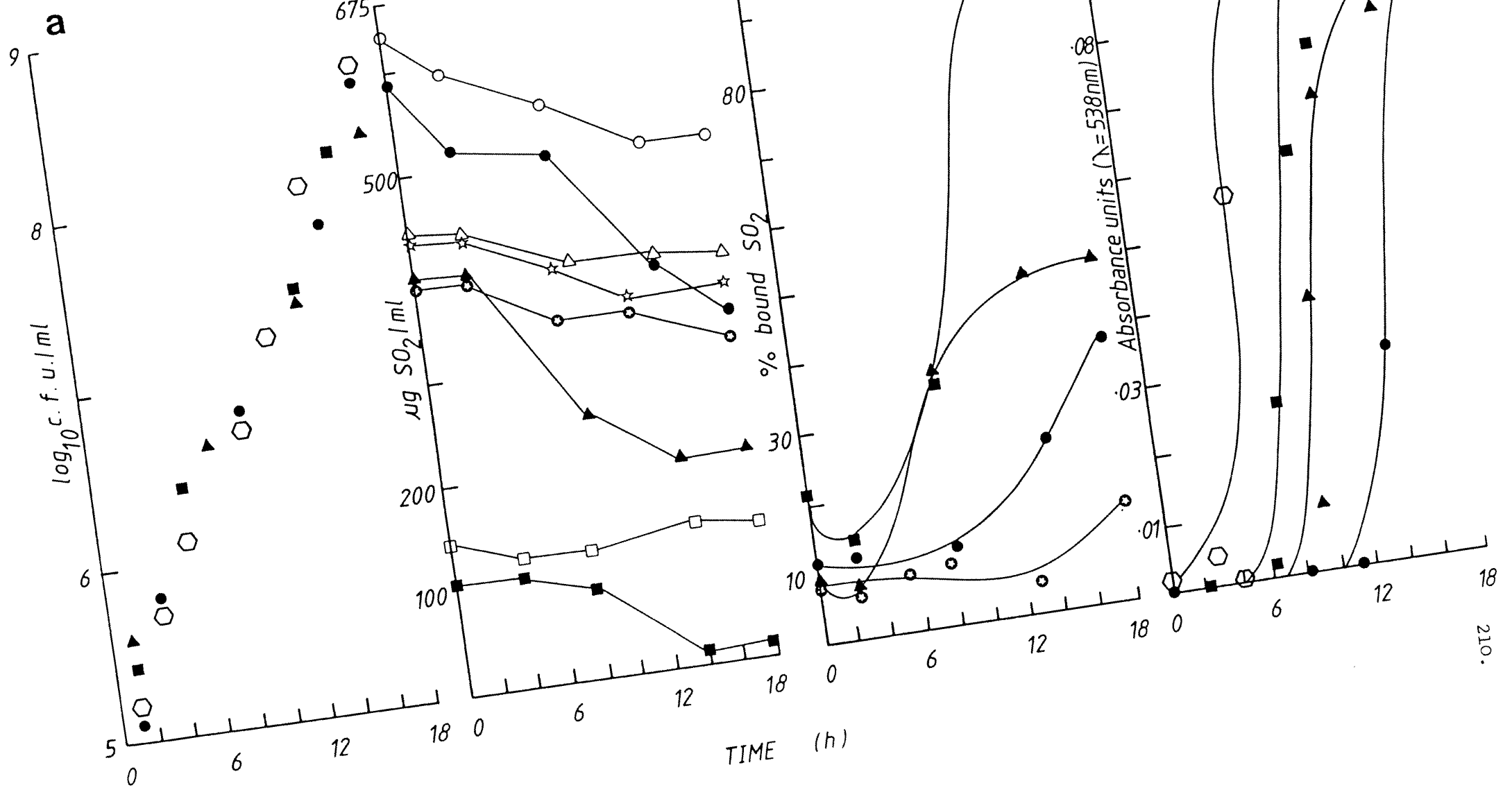

a
b
c
d
9
8
6
5
675
500
200
100
100
80
30
10
·10
·08
·03
·01
0
6
12
18
log_{10} c. f. u./ml
µg SO_2/ml
% bound SO_2
Absorbance units (λ=538nm)
TIME (h)

Figure 60. Influence of sulphite concentration on the growth of *Salmonella virchow* rif.^{R} and Enterobacteriaceae in sausage meat at -20°C

Footnote:

- ● *Salmonella virchow* rif.^{R} inoculated into unsulphited sausage meat
- ○ *Salmonella virchow* rif.^{R} inoculated into sulphited sausage meat
- ■ Enterobacteriaceae in unsulphited sausage
- □ Enterobacteriaceae in sulphited sausage.

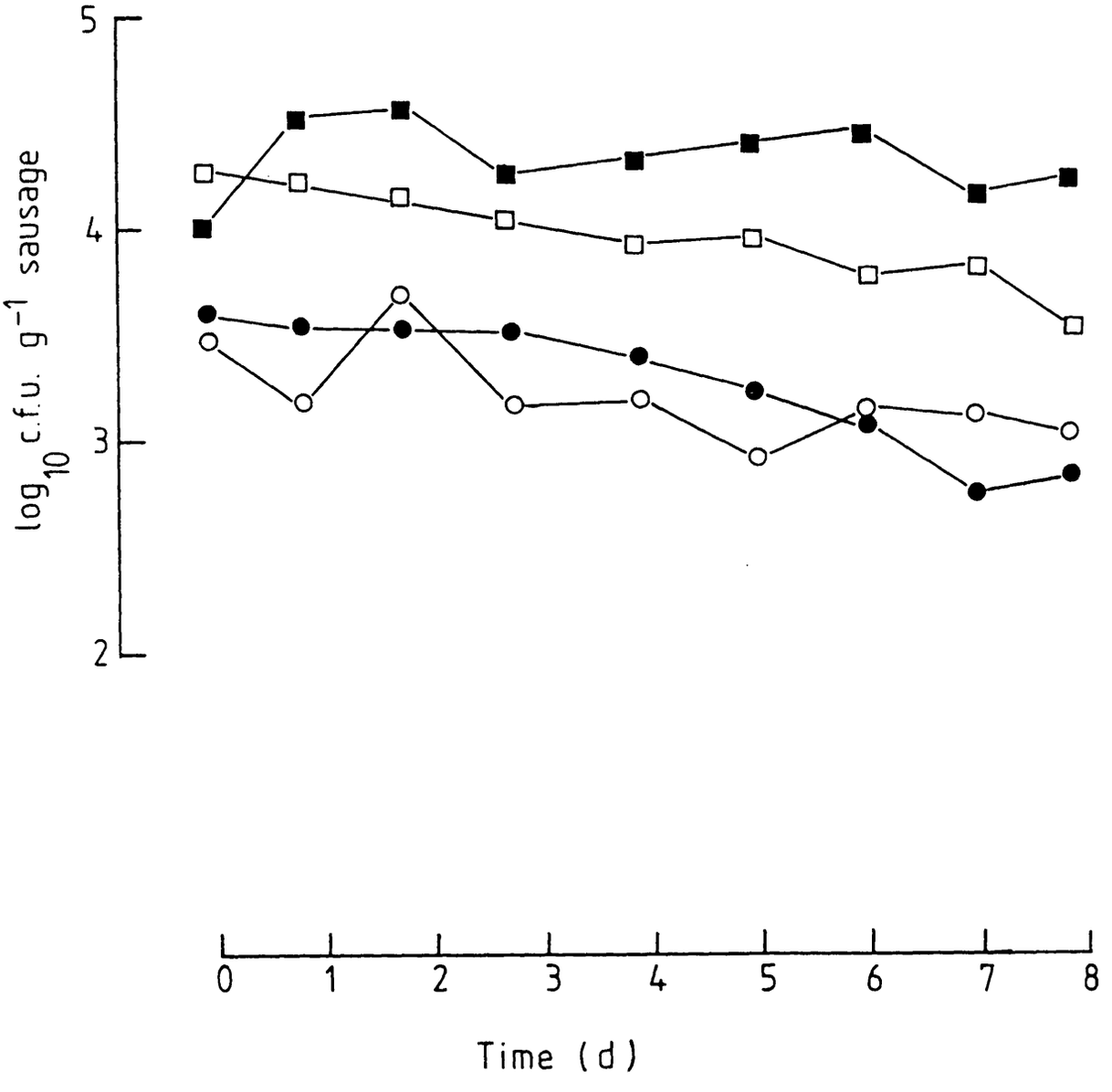


Figure 61. Influence of sulphite concentration on the growth of *Salmonella virchow* rif.^{R} and Enterobacteriaceae in sausage meat at 4°C.

Footnote: As in Fig. 60.

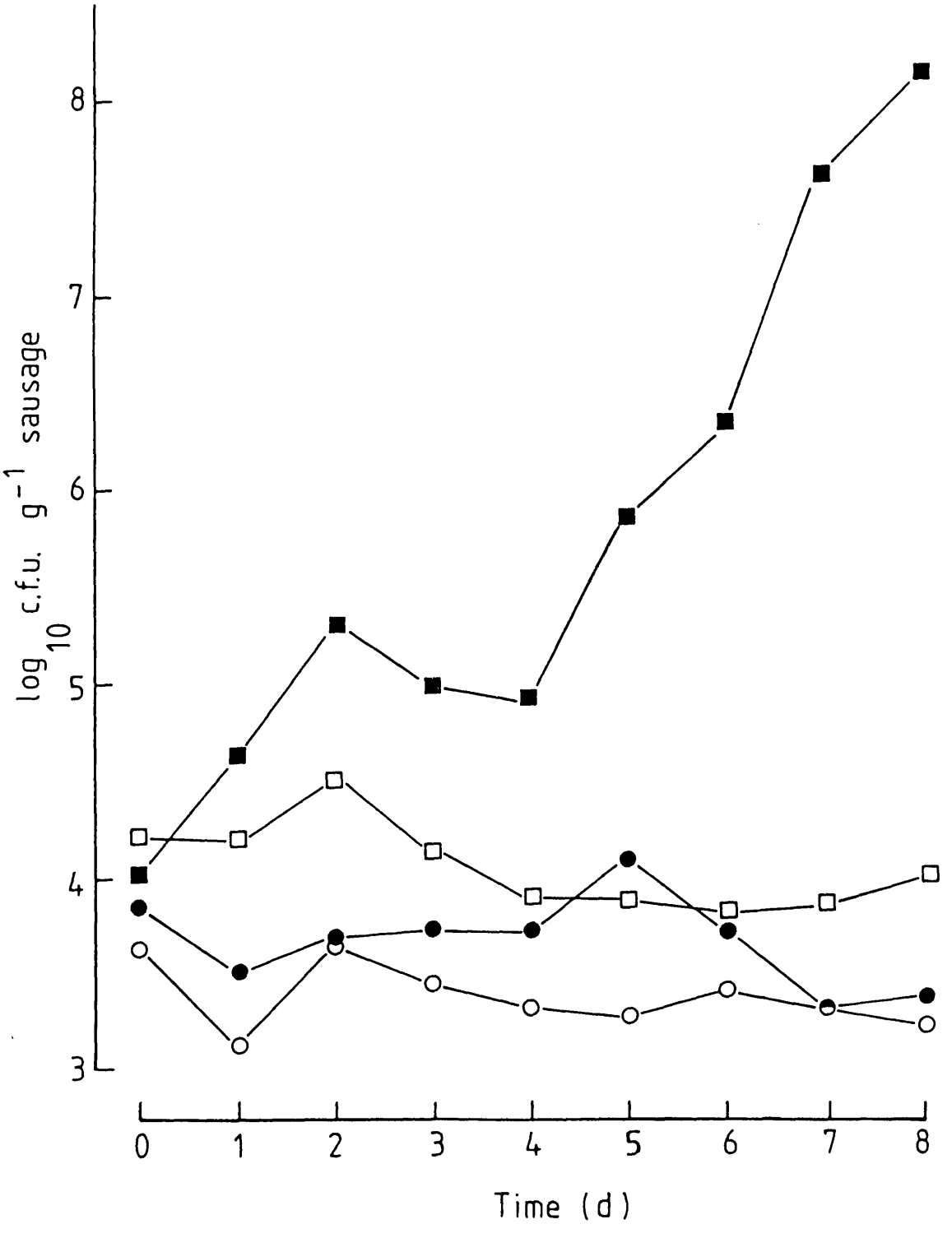
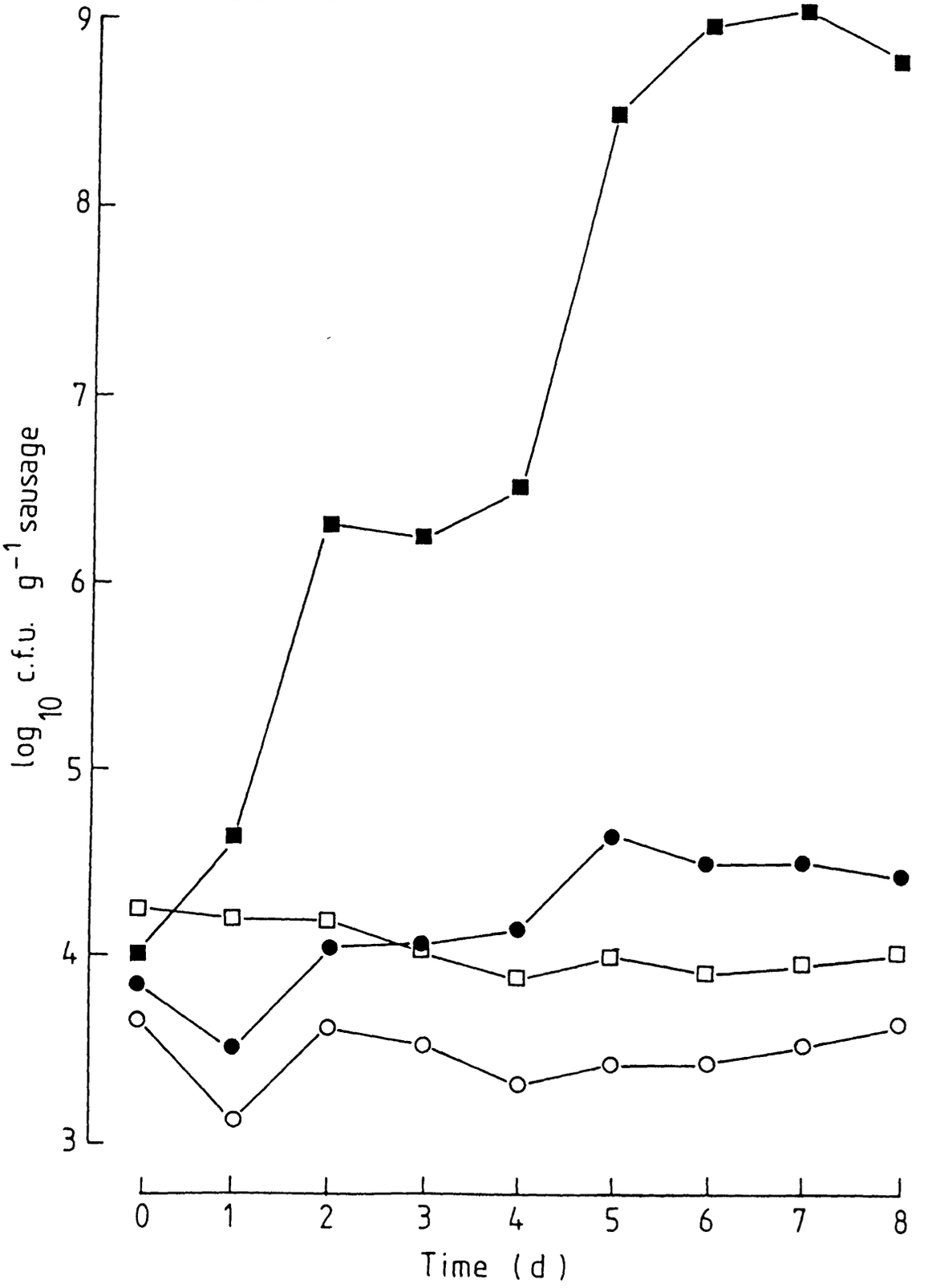


Figure 62. Influence of sulphite concentration on the growth of *Salmonella virchow* rif.^{R} and Enterobacteriaceae in sausage meat at 9°C.



Footnote: As in Fig. 60.

Figure 63. Influence of sulphite concentration on the growth of *Salmonella virchow* rif.^{R} and Enterobacteriaceae in sausage meat at 15°C.

Footnote: As in Fig. 60.

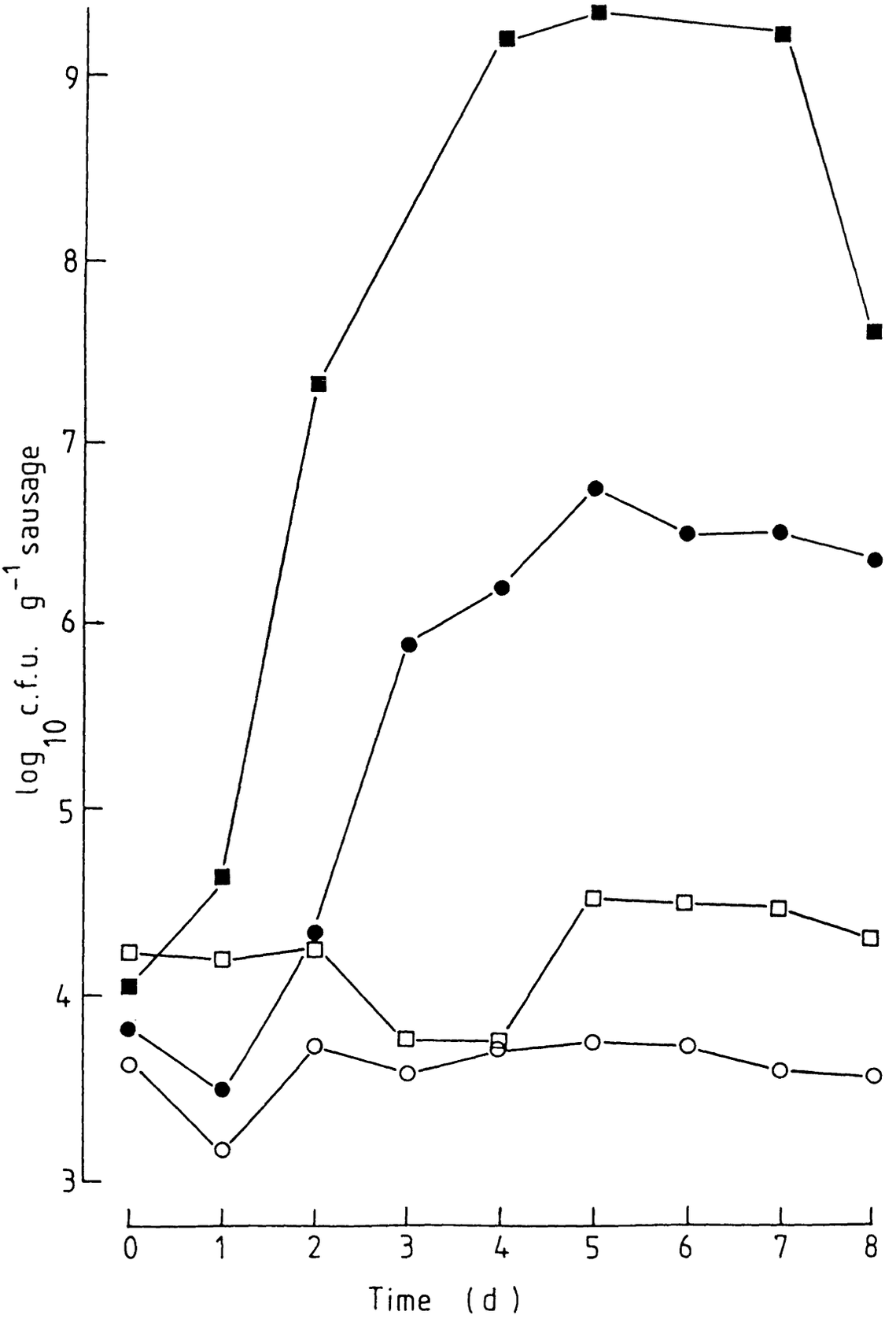
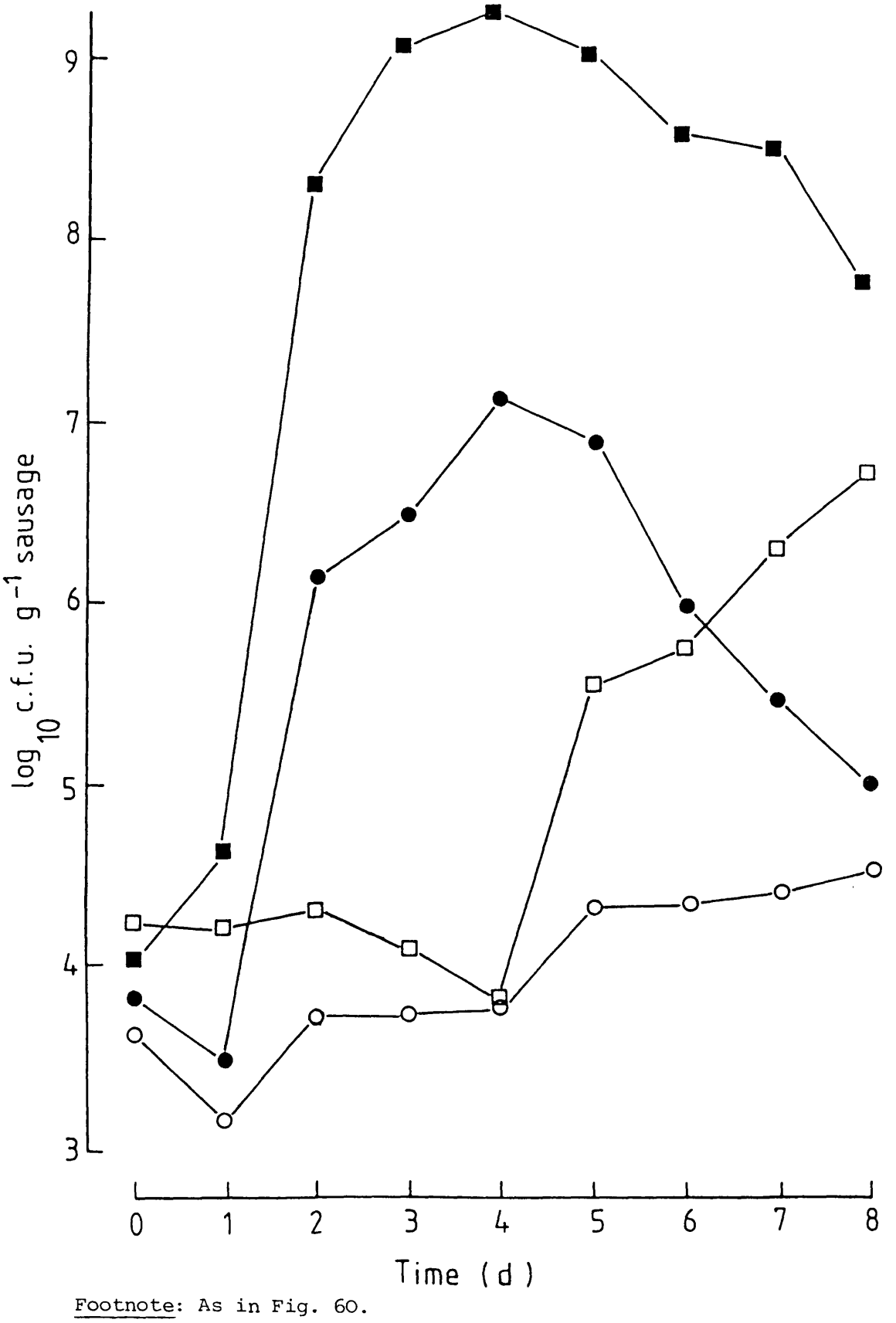
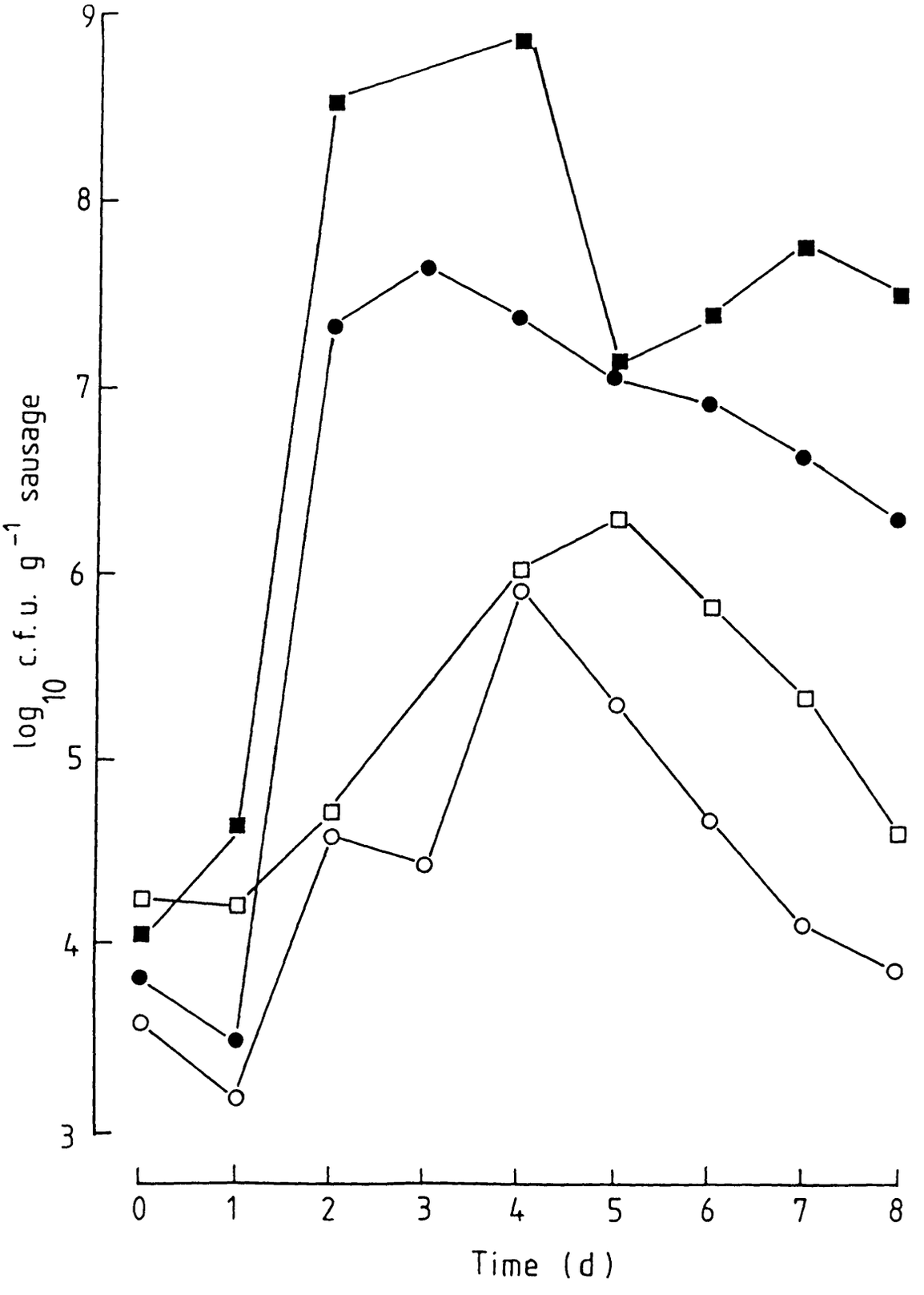


Figure 64. Influence of sulphite concentration on the growth of *Salmonella virchow* rif.^{R} and Enterobacteriaceae in sausage meat at 20°C.



Footnote: As in Fig. 60.

Figure 65. Influence of sulphite concentration on the growth of *Salmonella virchow* rif. and Enterobacteriaceae in sausage meat at 25°C.

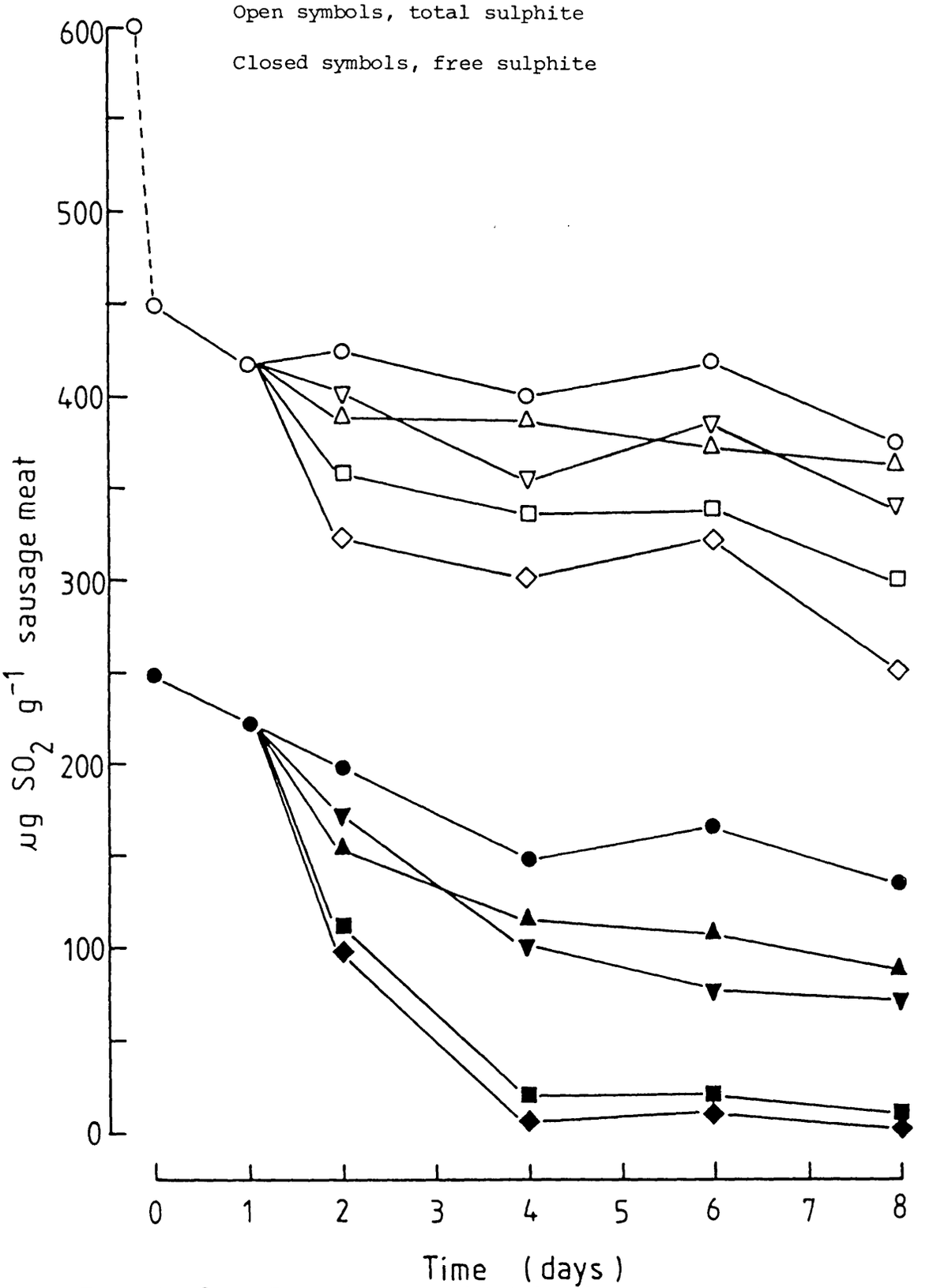


Footnote: As in Fig. 60.

Figure 66. Influence of temperature of storage on loss of free and total sulphite from sausage meat stored in Petri-dishes

Open symbols, total sulphite

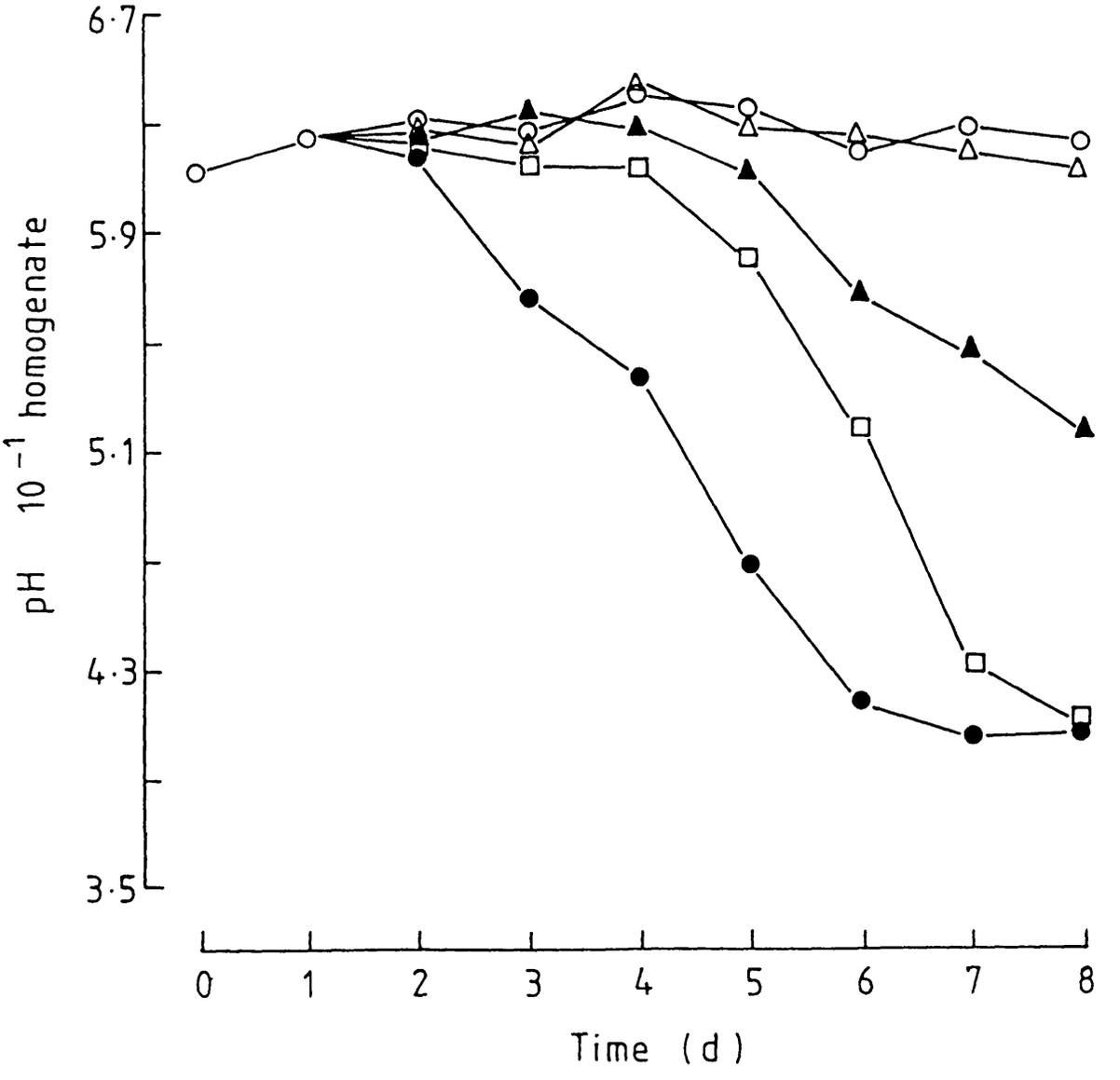
Closed symbols, free sulphite



Footnote: O---O Probable fate of total sulphite added from ingredients to bowl chopper

○ 4°C
▽ 9°C
△ 15°C
□ 20°C
◇ 25°C

Figure 67. Influence of temperature of storage on pH drift in sulphited sausage meat stored in Petri-dishes



Footnote:

○ 4°C
△ 9°C
▲ 15°C
□ 20°C
● 25°C

Figure 68. Influence of temperature of storage on pH drift in unsulphited sausage meat stored in Petri-dishes

Footnote: As in Fig. 67.

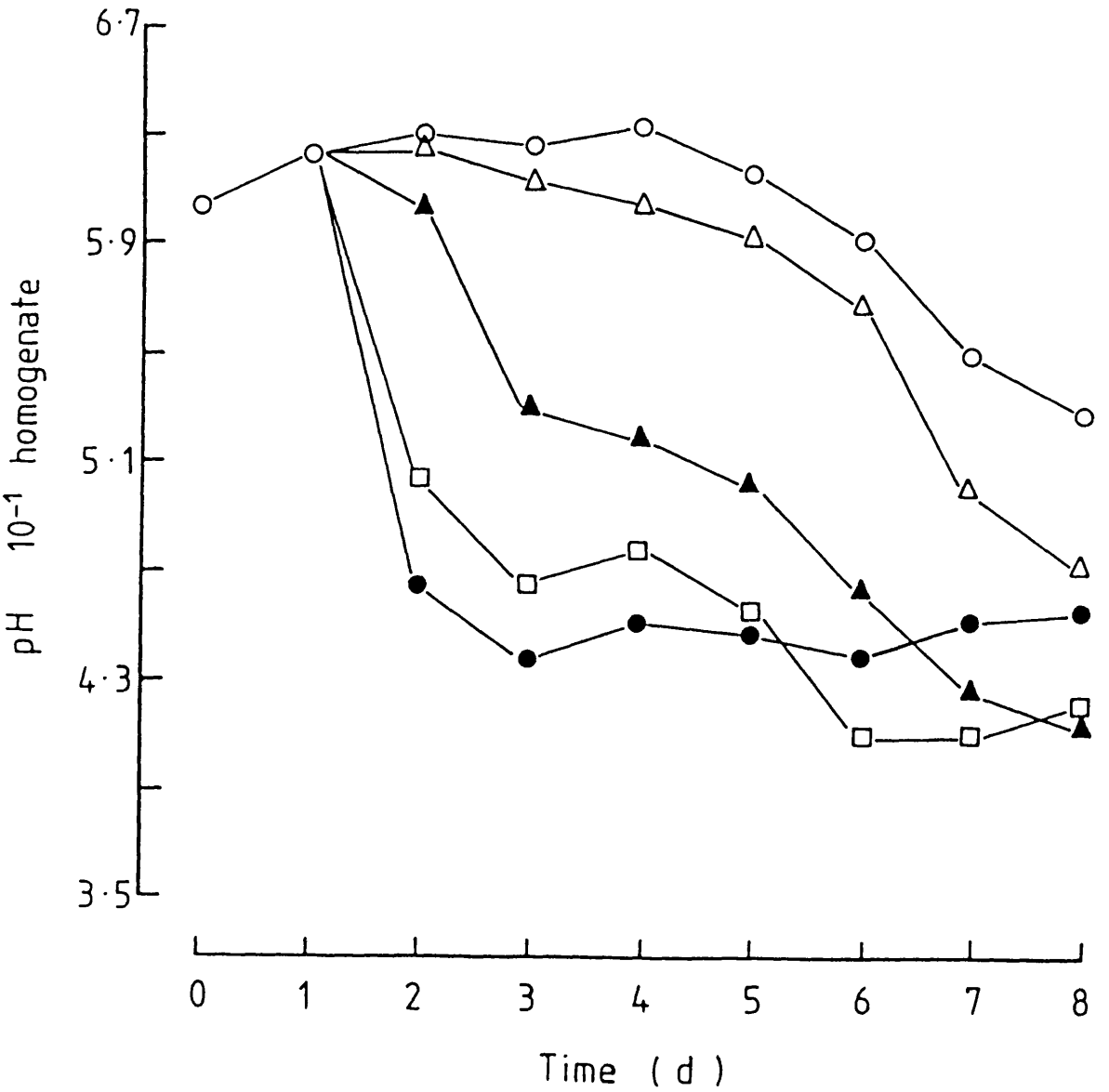
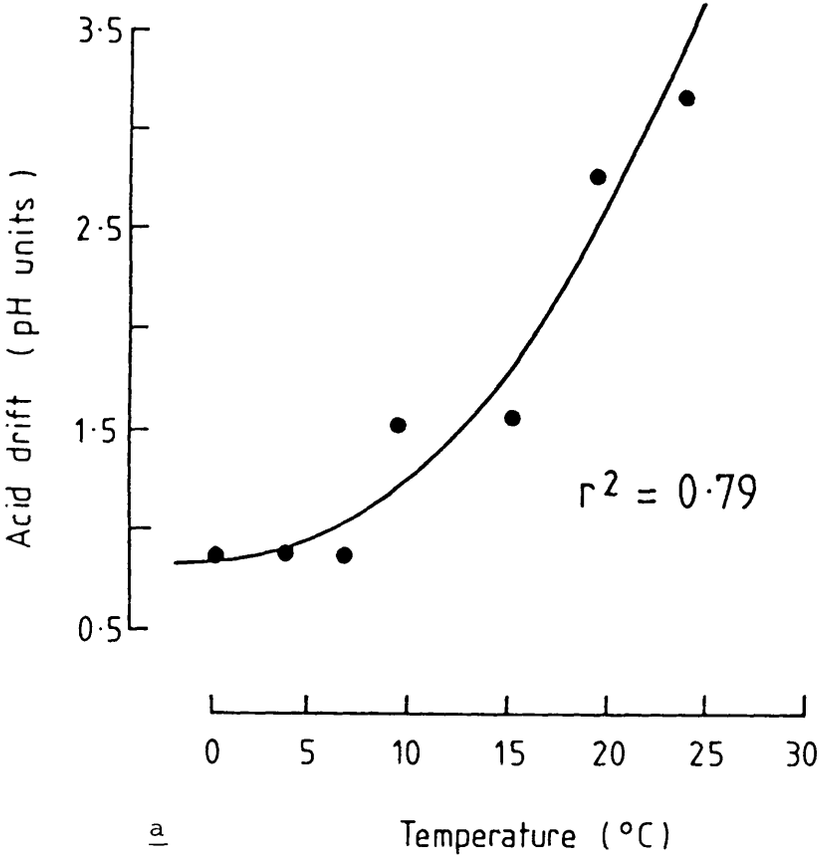


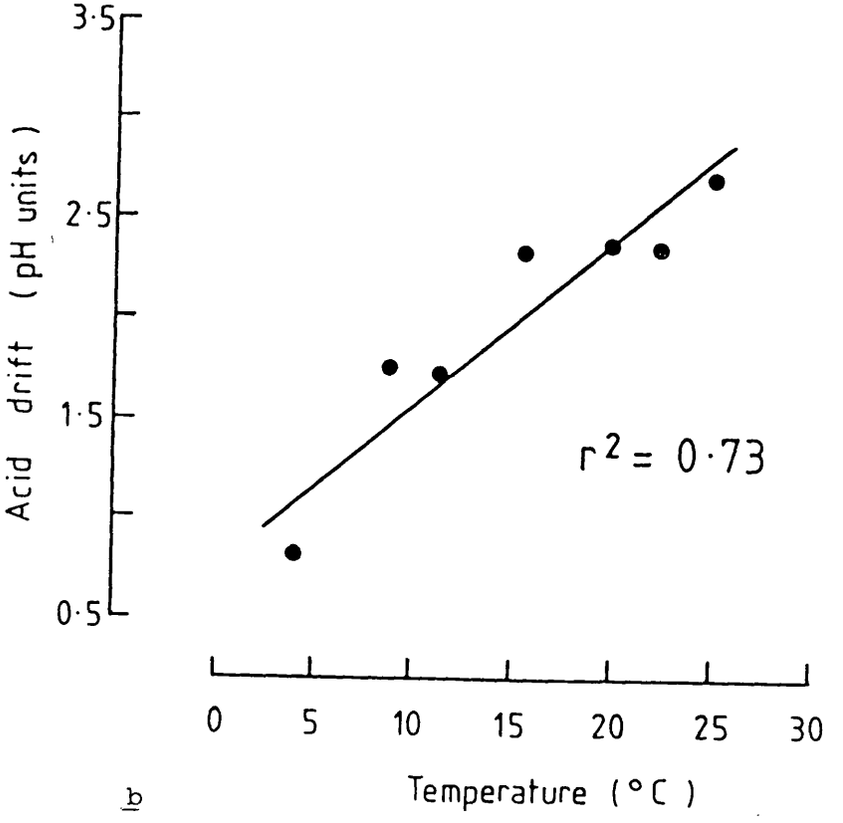
Figure 69. Influence of temperature of storage on the rate of pH drift in a, sulphited and b, unsulphited sausage meat stored in Petri-dishes

Footnote: a Exponential regression line describing the influence of temperature on the rate of pH drift in sulphited sausage meat. Coefficient of determination (r^2) = 0.79

b Linear regression line describing the influence of temperature on the rate of pH drift in unsulphited sausage meat. Coefficient of determination (r^2) = 0.73.



a



b

Figure 70. Influence of temperature of storage and sulphite concentration on growth of a, the total viable count, b, the Enterobacteriaceae and c, *Salmonella virchow* rif.^{R} in sausage meat having an unusually high initial microbial load.

Footnote: a Total viable count

: b Enterobacteriaceae

c *Salmonella virchow* rif.^{R}

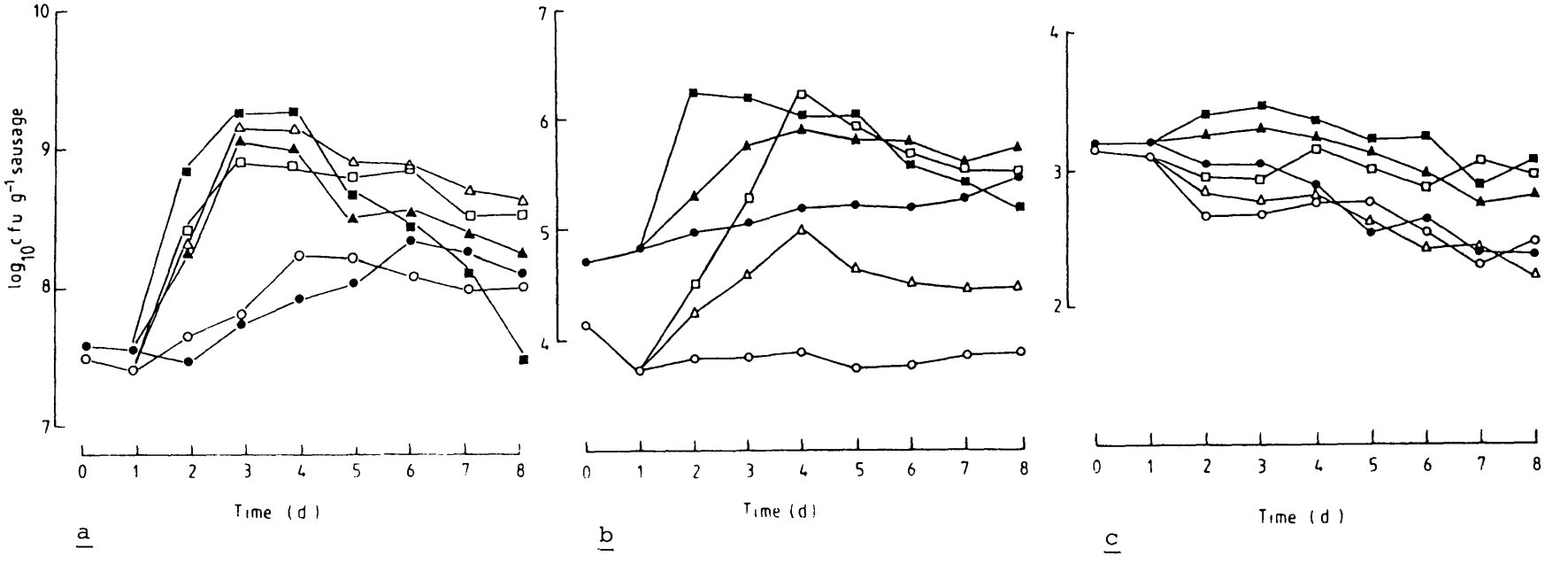
○ 4°C

△ 15°C

□ 20°C

Open symbols, sulphited sausage

Closed symbols, unsulphited sausage



SALMONELLA CONTAMINATION OF SAUSAGE

The incidence and level of contamination of British fresh sausage with *Salmonella*

Farm animals, especially pigs and poultry are the major depots of *Salmonella*, one of the commonest causes of food poisoning in developed countries (Bryan, 1980; Silliker, 1980). Indeed, there is abundant evidence that food products, for example sausages and sausage meat (Table 32), containing materials from such animals commonly have a high incidence of contamination with these organisms. Major surveys of the British fresh sausage for example, have demonstrated a wide variation in the incidence of these organisms. Public health reports, on the other hand, indicate that only occasionally have British fresh sausages been implicated in outbreaks of food poisoning (Table 33). Of the many reasons that can be put forward to account for this situation, the general practice of cooking sausages immediately before consumption may be the major one. Another might be the actual level rather than the incidence of contamination, the former can only be important in view of the observations (pp.157 - 164) that the bacteriostatic activity of sulphite is temporary in sausages subjected to temperature abuse. Indeed the recent observations (Gill *et al.*, 1983) that *ca.* 10^3 *Salmonella* cells can cause salmonellosis calls into question the long held view that at least 10^5 *Salmonella* are needed for an infective dose (Bryan, 1979). The work described in this section sought to establish the level of contamination of sausages made in a large factory, and to identify the major sources among the ingredients.

Table 32. Isolation of *Salmonella* from sausage and sausage meat

Source	% positive for *Salmonella*	Level of contamination (where given)	Major serotypes of *Salmonella* found (percentages in parentheses)	Reference
Sausage and s. meat	16	present in 25-30 g samples	*typhimurium* (17); *heidelberg* (6); *bredeney* (4)	Barrell (1982)
Pork sausage	25	" " 3-4 g "	"*Enteritidis*" group	Cary (1916)
Fresh sausage	2	" *ca.* 15 g "	*typhimurium* only	Cherry *et al.* (1942)
Pork sausage	14	" " 50 g "	*agona* (25); *infantis* (25); *typhimurium* (19)	Duitschaever and Buteau (1979)
Pork sausage	3	-	*give* *bredeney*	Dyett and Shelley (1965)
Various	13	-	various	Eenick (1963)
Fresh sausage	17	-	*cholerae-suis* (66); *typhimurium* (15); *derby* (5)	Felsenfeld *et al.* (1950)
Fresh sausage	23	present in 30 g sample	*derby* (36); *anatum* (31); *bredeney* (5)	Galton *et al.* (1954)
Sausage	6	-	*paratyphosus*	Hübener cited by Rommeler (1909)
Pork sausage	12	" 25 g "	*derby* (23); *anatum* (14); *agona* (11)	Johnston *et al.* (1982)
Pork sausage	5-18	-	*bredeney*	Jones *et al.* (1964)
Beef sausage	17	-		
Sausage	29	-	*paratyphosus*	Komma (1910)
British fresh pork saus.	43	4-6/100 g (10 tube MPN with 120 g sample)	"group D" (50); *agona* (25); *ljubljani* (25)	Pain (1981)
Pork sausage	5	<5-161/100 g (10 tube MPN with 110 g sample)	*typhimurium* only	Patterson (1969)
British fresh pork saus. and saus. meat	30	present in 10-15 g sample	*infantis* (28); *agona* (25); *derby* (12) (Factory B)	Roberts *et al.* (1975)
Visceral sausage	16	-	*parathyphosus*	Rommeler (1909)
Sausage and sausage meat	15	-	*typhimurium*	Spencer (1964)
Pork sausage	28	(84% +ve in 25 g sample (15% +ve in 1 g sample (0.6% +ve in 0.1 g sample	various	Surkiewicz *et al.* (1972)
Sausage and sausage meat	3	present in sample of varied size	various	Turnbull and Rose (1982)
Sausage and "made-up meat"	-	565 isolations	various	Vernon and Tillet (1974)
Pork sausage	38	present in 30 g sample	various	Weissman and Carpenter (1969)
Pork sausage	9	-	various	Wilson *et al.* (1961)

Table 33. Outbreaks of *Salmonella* food poisoning involving sausage or sausage meat

Serotype of *Salmonella* implicated	Food implicated	Probable source of contamination	Number of cases involved in outbreak	Reference
newport	various, including sausage	Infected pig carcase	450-500	Anon.(1950)
st. paul	sausage meat	Hospital kitchen and drains of food processing factory	6	Anon. (1964)
typhimurium	pork sausages	Butcher's shop drain	4	
newport	pork sausage meat and pie meat	Retail shop and food processing factory drain	65	
heidelberg	sausage meat	Drains from butchers' shops and abattoir	9	
brandenburg	pork and beef sausage mixes, sausages at retail outlet	Abattoir drains, factory drains, carcasses, butchered meat	214	Bevan-Jones *et al.*(1964)

Literature Review

Sources of *Salmonella* contamination of pigs

Many surveys (Table 34) have indicated a wide range of *Salmonella* contamination of pigs examined before or immediately after slaughter. In general, analysis of mesenteric lymph nodes or some other organ such as the spleen, rather than faeces, may provide the best index of contamination of pigs with these organisms because those in the blood and lymph are probably concentrated by filtration. Analysis of excrement, either directly or by rectal swab, may fail to recover low numbers of organisms particularly with animals that are not persistent excretors (McCall *et al.*, 1966; Haddock, 1970). Indeed some have advocated analysis of the previously noted organs because they contend that there is a good correlation between their contamination and that of the meat taken from the carcass (Cherry *et al.*, 1943; Floyd *et al.*, 1953; Newell *et al.*, 1959). Surveys such as those listed in Table 34 have directed attention at two topics, the sources of *Salmonella* to which farm animals are exposed and practices, both on the farm and in the factories, that influence the incidence of *Salmonella* contamination.

Contaminated feed has been identified as an important primary source of *Salmonella* (Adam, 1957; Walker, 1957). This view is supported by the many surveys (e.g. Anon, 1959; Pomeray and Grady, 1961; Schotts *et al.*, 1961), which have revealed a high incidence of *Salmonella* in feeds or in ingredients used in their production. In practice, feeds or ingredients appear to be subjected to sporadic rather than persistent contamination, contamination of some rather than all batches is a common finding in these surveys (e.g. Jacobs *et al.*, 1963; Clise and Swecker, 1965; Dawkins and

Table 34. Incidence of *Salmonella* contamination in pigs prior to, and after slaughter

Source and reference	% positive samples	Source and Reference	% positive samples
Mesenteric lymph nodes and spleens:		Caecum	
Anon (1947)	2.5	Craven and Hurst (1982)	70[1] 49[2] 41[3]
Anon. (1955)	0.4		
Anon. (1964)	1.9	Harvey *et al.* (1977)	10.8
Cherry *et al.* (1943)		Newell *et al.* (1959)	2
Edel and Kampelmacher (1970)	34.1*	Faeces	
Hormaeche and Salsamendi (1939)	48	Chau *et al.* (1977)	75†
Levine *et al* . (1945)	13	Galton *et al.*(1954)	7[4] 25[5] 51[6]
Ruben *et al.* (1942)	40	Harvey *et al.* (1977)	-
		Lo *et al.* (1967)	27
Hormaeche and Salsamendi(1936)	2.2	McKinley *et al.* (1980)	35[7]
		Newell *et al.* (1959)	9†;
		Tsai *et al.* (1971)	69

* Portal, mesenteric lymph nodes and faeces

† rectal swabs

1 1 day
2 2 days in lairage
3 3 days

4 farm
5 lairage
6 killing floor

Robertson, 1967; Harvey and Price, 1967). Thus from 7 - 34% of vegetable ingredients, bone and meat and bone meal were found to be contaminated in one survey (Anon, 1961) whereas *Salmonella* was not detected in any of the samples of fish, cereal or vegetable protein meals, and in 7% only of samples of meat and bone meal in another (Patterson, 1969). The variety in the range of serotypes isolated in these surveys is additional evidence of the sporadic nature of the contamination. Single batches of feed have been implicated in the introduction of *Salmonella heidelberg* and *Salmonella st. paul*, for example, into swine herds (Newell *et al.*, 1959; Galbraith *et al.*, 1961), and the *Salmonella* excretion by symptomless carriers can be important in the subsequent dissemination of the organisms (Sojka *et al.*, 1977). The use of contaminated feed has been linked without dubiety to outbreaks of food poisoning. The evidence supports therefore the notion that the use of *Salmonella*-free feed could play an important role in protecting humans from *Salmonella* of animal origin. Indeed Kampelmacher *et al.* (1965) did not isolate *Salmonella* from slaughtered pigs which had been fed *Salmonella*-free feed.

Of the many methods that have been used to achieve feed of this status, the use of heat during pelleting has attracted most attention. This process can cause a 10^2 - 10^5 (Mossel *et al.*, 1967) reduction in the Enterobacteriaceae count and the carrier rate for *Salmonella* in pigs. Thus only 0.4% of pigs fed with pelleted feed material compared with 4.7% receiving meal were carriers (Edel *et al.*, 1973).

Stress associated with the transport of pigs from farms to

markets or abattoirs can result in an increase in the number of animals excreting *Salmonella* as indicated by the results of some (e.g. Galton *et al.*, 1954; Kampelmacher *et al.*, 1963; Williams and Newell, 1970) but not all surveys (e.g. Tsai *et al.*, 1971; McKinley *et al.*, 1980). Similarly,residence times in the lairage has been shown by some (e.g. McDonagh and Smith, 1958; Anderson *et al.*, 1961; Bevan Jones *et al.*, 1964) but not all workers (e.g. Craven and Hurst, 1982) to increase the incidence of pigs excreting *Salmonella*.

Once pigs enter an abattoir, faeces (Childers *et al.*, 1973; Stolle and Reuter, 1978), the skin and hair of the animals (Norval, 1961; Patterson and Gibbs, 1978) and equipment (Lehmann, 1964; Watson, 1975) can become important vehicles in the dissemination of *Salmonella* and operations such as scalding at 60 - 62^{o}C or dehairing do not always diminish the incidence of contamination of carcasses with these organisms (Kampelmacher *et al.*, 1961; Chau *et al.*, 1977). Indeed such practices (Fig. 1) may contribute to *Salmonella* contamination (Galton *et al.*, 1954) because the organisms are afforded protection by hair follicles or faeces. This protection probably accounts for the frequent occurrence of these organisms in the dregs in the scald tank (Kampelmacher *et al.*, 1961) and effluent drains (Chau *et al.*, 1977). *Salmonella* contamination of porcine carcasses can be reduced by exposure to naked flames, but the process that is used to remove the singed appearance, "black scraping", can cause recontamination, (Kampelmacher *et al.*, 1961). The potential of the abattoir environment to be an important depot of infection was

shown in a survey (Anon.) published in 1964. It reported a 1.9% incidence of *Salmonella* contamination of pig tissue immediately after slaughter but recovery rates of 74%, 11% and 0.5% from swabs taken from drains in the abattoirs, meat factories and butcher's shops handling the meat included in the survey. The frequent occurrence of identical serotypes and phage types in this particular survey provided further evidence in support of the generally accepted view (McDonagh and Smith, 1958; Lo *et al.*, 1967; Chau *et al.*, 1977) that a chain of infection can, under unsatisfactory conditions, link the farmyard to the kitchen of the home or canteen.

In attempts to rectify problems associated with traditional slaughtering systems, attention has been directed at methods that reduce the number of spoilage organisms as well as *Salmonella* on carcasses that are about to enter chill rooms. Spraying the carcasses with water containing acetate, stannous chloride, hydrogen peroxide or steam (Biemuller *et al.*, 1973) as well as chlorine, antibiotics, β-propiolactone, citrate and succinate have been shown to be effective, at least under experimental conditions. The purpose of this study was to establish the actual numbers of *Salmonella* in sausages produced in a factory in which traditional methods of slaughter and butchery ensured that meat used in the product was liable to exposure to the several depots of infection noted above.

Salmonella in sausages

Few attempts have been made to establish the level of contamination of sausages with *Salmonella* (Surkiewicz *et al.*, 1972; Patterson, 1969; Pain, 1981). Indeed the major survey (3309 samples) by Roberts *et al.* (1975) as well as the minor ones (Turnbull and Rose, 1982; Barrell, 1982) of British fresh sausages could be criticized because of the emphasis given to the incidence rather than the level of *Salmonella* contamination. As it is generally accepted that, with the exception of chocolate products (Gill *et al.*, 1983), at least 10^5 *Salmonella* are required to produce overt symptoms of disease in undebilitated humans (Bryan, 1980), the level as well as the incidence of contamination of a food should be considered to be of equal importance. It was for these reasons that a quantitative method for the recovery of *Salmonella* was adopted in this study.

Inadequate knowledge about the distribution of micro-organisms within food and the inaccuracies inherent in the available methods can give misleading information concerning the incidence and level of contamination of food products with *Salmonella*. For example, the assumption that a log-normal distribution of micro-organisms exists in samples taken from a batch of food may fail to take account of the skew in the distribution (Kilsby and Pugh, 1981). At least 20 - 30 replicates/sample must be examined in order to define this skew. If the variance of the log counts obtained from such preliminary studies is reduced , however, the increase in precision will allow a reduction in the number of replicates. There is evidence also which suggests that micro-organisms in

general, and low numbers of *Salmonella* in particular, are distributed contagiously on whole pieces of meat (Kilsby and Pugh, 1981). Thus thorough comminution is needed in order to achieve a random distribution of the contaminants. Moreover as the variance of a series of counts decreases, the apparent mean (average) of those counts increases. In practice therefore examination of a sample of whole meat may not yield isolates of *Salmonella* whereas they are isolated from a comminuted sample. Similarly it has to be recognised that a random but modest scheme of sampling of the carcass may fail to detect one pig carcass which may have been infected at slaughter with thousands of *Salmonella*. As butchering and especially bowl chopping of sausage ingredients tend to randomize the distribution of *Salmonella*, the probability of detection of these organisms in samples of the finished product is increased (Kilsby and Pugh, 1981). In the case of sausages, problems associated with the sampling of ingredients such as meat can be overcome by the use of multiple sub-sampling (Brown, 1977). If the sample of finished product examined is considered in terms of the contribution by individual ingredients, then it is evident that very small samples of ingredients contribute to the bulk sample (Table 35). This situation would be expected to favour the isolation of *Salmonella* from (1) minced ingredients and (2) ingredients which comprise a small proportion of the total mix (e.g. rinds). Thus, fewer sub samples of these ingredients were chosen from meats awaiting use in the factory and whole pieces of meat (e.g. lean, fat) were minced (Kenwood A901) in the laboratory to achieve a particle size similar to that in factory-processed ingredients. (e.g. head, belly, rinds).

Table 35. Influence of sample size and composition of sausage mix on proportion of ingredient examined by MPN method

Ingredient	% mix	kg*	% ingredient examined by MPN scheme	
			10 tube (120 g)	5 tube (60 g)
Lean pork (W)	26.4	79.2	0.15	0.08
Fat (W)	22.0	66.0	0.18	0.09
Belly pork (M)	7.0	21.0	0.57	0.29
Head pork (M)	7.0	21.0	0.57	0.29
Rinds (M)	4.0	12.0	1.00	0.50

* Assuming average batch mix of 300 kg; (W) whole pieces of meat 30 x 20 x 20 cm;

(M) Minced at factory

Even with all these precautions, experience showed that the level of contamination was too low to permit enumeration by direct plating and the MPN system had to be used.

Most Probable Number method

The most probable number (MPN) technique, a dilution method, was developed to estimate the density of micro-organisms in a liquid. Sub-samples of varying sizes of the liquid with several replicates of each are analysed for the presence or absence of particular micro-organisms using an appropriate medium for the organisms sought. Three assumptions have to be made: (1) that the micro-organisms being sought are distributed randomly throughout the liquid, (2) that they do not attract or repel each other, and (3) that growth in the medium inoculated with a sub-sample of the bulk liquid will be initiated by one or more micro-organisms. The probability of a negative result (no growth) in a particular subsample replicate is related to the density of micro-organisms in the original liquid thus: where a subsample (v ml) is taken from the sample (V ml) in which there are x organisms, the probability of any organism being in the sample = v/V and of it not being in the sample = (1 - v/V). The probability (p) that none of the organisms are in the sample is $p = (1 - v/V)^x$ and when v/V is small, it is approximately the same as $p = e^{-vx/V}$ as x/V is the density (ρ) of organisms per ml, $p = e^{-v\rho}$. If n samples, each of volume v are taken, and if s of these are found to be negative, the proportion s/n of samples is an estimate of ρ.

Therefore an estimate of the density (ρ) can be obtained by $s/n = e^{-vp}$ which gives:

$$d = (-1/v)\ \ell n\ (s/n) = -(2.303/v)\ \log_{10}(s/n)$$

where d = the "most probable number" or organisms/ml.,
It follows that the precision of the MPN method is extremely poor when all or none of the samples are negative since when all are positive, the estimated density is infinite, and when all are negative, it is zero, according to the confines or threshold limits of the particular experimental design. So v must be chosen so that some of the sub-samples are positive and some negative. In practice, several levels of dilution are chosen to achieve this end. Cochran (1950) showed that if a fixed number of sub-samples is used, then the average precision obtained is nearly the same for any dilution ratio between 2 and 10 although a low dilution ratio gives a more constant precision over the range of densities being covered. Thus for a fixed total number of samples, the choice of dilution ratio is a compromise between the extra work involved with using several levels of low dilution ratio and the fluctuation in precision associated with a higher dilution ratio.

The number of replicates at each dilution level is important also (Cochran, 1950). The standard error attached to an estimated density (d) is usually computed from $\log_{10}d$ as this is closer to a normal distribution than 'd' which is skewed. For a dilution ratio of 10, the standard error of $\log_{10}d$ is:

$$S.E.\ (\log_{10} d) = 0.58 / \sqrt{n}$$

where n is the number of replicate samples per dilution. The precision of the MPN method can be estimated from plotting the number of replicates/dilution level against the standard error ($\log_{10} d$).

The estimate of the MPN was obtained by reference to tables (McCrady, 1915; de Man, 1977; 1983; Parnow, 1972) or computer program. A program was written by Dr. A. Robinson (School of Mathematics, University of Bath) for the present study such that the MPN/g original material for any combination of replicates or dilution levels could be calculated. At 3 levels of dilution and a dilution ratio of 10,5 replicates/dilution were done. Thus the detection level is theoretically 2 *Salmonella*/g. Twenty complete batches were analysed.

Materials and Methods

Sampling and isolation of *Salmonella* (5 tube MPN scheme)

Samples of pork sausage, pork and beef sausage and all the ingredients used in their manufacture were obtained from a large factory during normal production in the period 2.3.80 to 5.4.81. One kg of pork meat was taken from material awaiting processing. The meat came from pigs slaughtered at the factory; butchered meat was stored at 4°C overnight. Cattle were neither slaughtered nor butchered on the site and 1 kg samples were taken from blocks of frozen,de-boned beef. Samples of rusks, seasoning, polyphosphate (Fibrisol V10), rinds and linked sausages were

examined also. All samples were stored at 4°C and examined within 3 h of collection. To minimize sampling errors, all the meats were comminuted with a sterile mincer (Kenwood A901). Sixty g of samples and 540 ml of pre-enrichment broth (Appendix p. 330), usually buffered peptone water (BPW) - were blended in a Colworth Stomacher 400 (Seward, London) for 60 s. Five samples (each of 100 ml) of the homogenate were distributed in bottles. Five samples (each of 10 ml) were added to 90 ml of sterile pre-enrichment broth in bottles and shaken for 10 s, and 5 samples (each of 1 ml) of homogenate were added to 99 ml of pre-enrichment broth and shaken for 10 s.

Incubation was at 37°C for 24 h and after vigorous shaking of the bottles, 1 ml samples were transferred to enrichment broth (Appendix p.330)- usually tetrathionate (Difco) broth - (9 ml amounts), and these subcultures incubated at 43°C for 48 h. At 24 and 48 h, loopfuls of enrichment cultures were streaked out on dried surfaces of Brilliant Green agar (BGA; Oxoid CM 329), bismuth sulphite agar (BSA; Oxoid) and Desoxycholate citrate agar (DCA; Difco) according to the methods of Edel and Kampelmacher (1969). Inoculated selective agar in Petri dishes was incubated at 37°C for 48 h; Colonies of presumptive *Salmonella* spp. were transferred to Plate Count agar (PCA; Oxoid) after 24 and 48 h.

Characterization of *Salmonella*

Pure cultures on PCA were inoculated into Kohns I and II media (Oxoid) and the API 20 E scheme (API, Basingstoke, England).

Isolates presumptively identified with *Salmonella* were characterized further by the biochemical tests of Edwards and Ewing (1972) and analysis of somatic and flagellar antigens using slide and tube agglutinations with antisera from Burroughs Wellcome (Beckenham, Kent). The MPN of *Salmonella*/g sample was established with McCrady's (1915) tables or by reference to the computer program of Dr . A. Robinson only after complete confirmation of isolates.

Determination of antibiotic resistance of *Salmonella*

Strains of *Salmonella* were incubated in Tryptone Soya broth (TSB; Oxoid) at 37°C for 18 h and a sample (0.2 ml) spread over the dried (37°C, 1 h) surface of Diagnostic sensitivity test agar (Oxoid CM 261). Antibiotic-seeded "multodisks" (Oxoid) were placed aseptically on the surface of the agar. Incubation was at 37°C for 18 h.

Isolation of Enterobacteriaceae

Twenty g samples of minced meat, other ingredients and sausages were homogenized (Colworth Stomacher) in 180 ml of quarter-strength Ringers solution and decimal dilutions made in this solution. One ml of appropriate dilutions was mixed with 15 ml of Violet Red Bile Agar (VRB; Oxoid) cooled to 45°C. When set, the surface of the agar was overlaid with 10 ml of VRB, incubated at 37°C for 24 h and colonies of coliform organisms counted. One ml samples were added also to 15 ml of Violet Red Bile Glucose Agar (VRBG; Oxoid) cooled to 45°C. When the agar had set, its surface was overlaid

with 10 ml of VRBG, incubated at 30°C for 24 h, and the colonies of Enterobacteriaceae counted. All counts were done in triplicate. Randomly selected colonies from Petri dishes containing the lowest, countable dilution were purified by plating on PCA. Gram-negative, oxidase-negative, fermentative bacilli were tentatively identified with Enterobacteriaceae. They were characterized further by the methods of Edwards and Ewing (1972) and the API 20E and API 50CHE systems (API, Basingstoke).

Statistical analysis

A Hewlett-Packard 97 calculator and appropriate programes were used to do simple linear regressions and Spearman's rank correlation.

Results

The MPN scheme outlined in the Materials and Methods section was adopted following extensive preliminary work in which comparison was made with 0.1% (w/v) peptone water, lactose broth and buffered peptone water for pre-enrichment (Table 36); selenite-cystine, lysine iron cystine neutral red, and tetrathionate broths for enrichment with incubation at 37° and 43°C (Table 37). A combination of buffered peptone water, tetrathionate broth and desoxycholate citrate or Brilliant green agar allowed best recovery of *Salmonella* from samples of British fresh sausage (Tables 36, 37). More isolations were made after 48 h compared with 24 h enrichment and from enrichment at 43°C compared with

Table 36. Effect of pre-enrichment, enrichment and selective plating media on recovery of *Salmonella* from British fresh sausage‡

Pre-enrichment at 37°C	Enrichment and plating media: Selenite			Tetrathionate			LICNR		
	BG	BS	DC	BG	BS	DC	BG	BS	DC
Peptone water	3*	0	2	6	5	4		NT	
Lactose broth	3	0	2	3	4	2		NT	
Buffered peptone water	4	0	4	6	5	6	3	0	2

* Number of samples containing *Salmonella* (after 24 or 48 h incubation of enrichment/plating media)

‡ 10 samples tested

NT Not tested

Table 37. Effect of duration and temperature of incubation of enrichment broths on recovery of *Salmonella* from British fresh sausage‡

	Enrichment media							
	Selenite				Tetrathionate			
	24 h		48 h		24 h		48 h	
Plating medium	37°	43°	37°	43°	37°	43°	37°	43°
BG	8*	8	12	14	12	18	17	16
BS	6	4	0	2	9	8	14	7
DC	11	7	9	10	14	12	16	19

* Number of samples containing *Salmonella*

‡ 32 samples tested

37°C (Table 37).

Although *Salmonella* spp. were isolated frequently (Table 38) from all the meat ingredients other than back-fact of pork sausages as well as the finished product, the actual levels of contamination were low (3 - 40 salmonellae/g ingredient). Thirty percent of the cooked and comminuted rinds contained *Salmonella* spp. but they were not isolated from the non-meat ingredients (rusk, spices, polyphosphates) of pork sausage. *Salmonella* spp. were isolated from all the meat ingredients other than pork back-fat of pork and beef sausages (Table 39) but in low numbers only, viz 8 - 40/g of beef flank and pigs' head meat. In contrast, 95% of the mechanically recovered meat (MRM) contained 60 - 378 salmonellae/g. One sample of dried rinds but no samples of the non-meat ingredients of pork and beef sausages yielded *Salmonella* spp.

Of the 8 serotypes of *Salmonella* isolated from ingredients and sausages, *derby* and *dublin* (Tables 40 and 41) occurred most frequently. *Salmonella agona* was isolated on 4 occasions only and *S. infantis* was the only serotype recovered from the cooked rinds. The incidence of serotypes (7 out of the 8 isolated) was highest with the mechanically recovered meat.

On no occasion was a serotype isolated from sausages without it being recovered also from one or more of the ingredients used to produce that particular batch. On several occasions, however, serotypes were isolated from ingredients but not from sausages

Table 38 . Contamination of ingredients and pork sausage with *Salmonella*

	Sausage	Lean pork	Belly meat	Head meat	Semi-lean meat	Rinds
Samples:						
number tested	20	15	20	20	20	20
number positive	13	6	7	2	7	6
% positive	65	40	35	10	35	30
mean MPN/g	20	24	21	7	20	11
MPN range for positive samples	7-40	11-40	7-23	3-11	11-37	6-24

Table 39. Contamination of ingredients and pork and beef sausage with *Salmonella*

	Sausage	Beef flank	MRM*	Head meat	Rinds†
Samples:					
number tested	20	20	20	20	10
number positive	11	4	19	6	1
% positive	55	20	95	30	10
mean MPN/g	17	11	265	23	0.8
MPN range for positive samples	8-24	8-17	60-378	8-40	-

* Mechanically-recovered meat

† Heat-processed, dried rinds

Table 40. Separate isolations of *Salmonella* from ingredients and pork sausage

	Lean pork	Belly meat	Head meat	Semi-lean meat	Rinds	Sausage
Salmonella serotype:						
derby	4	3	1	4	-	12
dublin	4	3	1	2	-	9
newport	2	2	-	1	1	5
heidelberg	3	-	-	-	2	5
stanley	2	2	-	1	-	5
infantis	-	-	-	-	4	4
typhi-murium	2	-	-	-	-	2
agona	-	1	1	-	-	2
	17	11	3	8	7	44

Table 41. Separate isolations of *Salmonella* from ingredients and pork and beef sausage

	MRM*	Head meat	Beef flank	Rinds†	Sausage
Salmonella serotype:					
derby	8	-	1	-	9
newport	5	1	1	-	7
stanley	4	2	1	-	6
dublin	5	2	-	-	6
typhi-murium	3	-	1	1	4
heidelberg	3	1	-	-	4
infantis	2	-	-	-	2
	30	6	4	1	38

* Mechanically recovered meat

† Heat-processed, dried rinds

containing the ingredients. In such instances, the level of contamination was low or the ingredient was used in small amounts only.

As the level of *Salmonella*-contamination of ingredients and the latter's contribution to the sausage were known, it was possible to compare the predicted and the observed levels of contamination of pork sausages providing there were at least 2 of these organisms/g of ingredient. With this type of sausage, the predicted contribution by all ingredients and the observed level of contamination were in accord (Fig. 71a) a linear regression line described the data with a coefficient of determination, $r^2 = 0.96$. In addition there was a good correlation ($r^2 = 0.92$) of the predicted contribution by lean pork alone and the observed levels of *Salmonella*-contamination of sausages. As all the other meat ingredients gave low r^2 values (footnote to Fig. 71) it was concluded that their contribution to *Salmonella*-contamination of pork sausages were subordinate to that of lean pork, the principal meat ingredient. An acceptable correlation ($r^2 = 0.79$) of the predicted and observed contamination of ingredients and pork and beef sausages respectively was also noted (Fig. 71b). In this instance, the threshold level of contamination of an ingredient was 5 salmonellae/g (*cf.* 2/g for pork sausages).

The observations summarized in Fig. 71 direct attention at the role of dilution in diminishing the level of contamination of a product by an ingredient that is relatively heavily contaminated. This was particularly notable with MRM, the ingredient of pork and beef sausages with the highest level of *Salmonella*-contamination (Table 39)and the greatest variety of serotypes (Table 41). In

practice, the extent of dilution of this material was such that there was a poor correlation (r^2 = 0.49) of the predicted and observed levels of contamination of a product.

Enterobacteriaceae and coliform organisms were present in all the samples of meat and sausages but none of the non-meat ingredients of pork sausages (Table 42). There was little variation in the levels of contamination of sausages with these organisms but a pronounced variation with ingredients, especially whole pieces of meat (Fig. 72). It was concluded that the former reflected random contamination resulting from the vigorous mixing of ingredients in sausage production and the latter contagious contamination resulting from contact of meat with dirty equipment etc. In addition, dilution of contaminants was again noted. Thus MRM, the ingredient containing the largest number of coliform organisms, was used only in pork and beef sausages which contained fewer of these organisms than pork sausages. Only one sample of a dry ingredient, rinds, of pork and beef sausages contained coliforms and this was the sample from which *S. typhimurium* was isolated (Table 43).

In general, the Enterobacteriaceae count (VRBG medium) appeared to be a more reliable index of possible *Salmonella*-contamination of sausages and ingredients than was the coliform count (VRB medium). This is evident in the results obtained with the lean, semi-lean pork and belly meat used in pork sausages (Fig. 72b). There was no apparent association between the level of contamination of head meat, cooked rinds and pork and beef sausages with

Table 42. Extent of contamination of ingredients and pork sausage with Enterobacteriaceae and coliforms

	Number of samples tested	Enterobacteria $\bar{x}$	($\log_{10}$ c.f.u./g) σ	Coliforms $\bar{x}$	($\log_{10}$ c.f.u./g) σ
Sample:					
Sausage	20	3.26	0.10	2.92	0.10
Lean pork	15	4.01	0.51	2.96	0.33
Semi-lean meat	20	3.82	0.36	3.16	0.29
Head meat	20	3.64	0.37	3.31	0.32
Belly meat	20	3.42	0.27	2.81	0.36
Rinds	20	3.17	0.45	2.49	0.42
Fat	20	2.58	0.38	1.94	0.37

$\bar{x}$ = mean of three replicates

σ = standard deviation of three replicates

Table 43. Extent of contamination of ingredients and pork and beef sausage with Enterobacteria and coliforms

	Number of samples tested	Enterobacteria $\bar{x}$	($\log_{10}$ c.f.u./g) σ	Coliforms $\bar{x}$	($\log_{10}$ c.f.u./g) σ
Sample:					
Sausage	20	1.73	0.24	1.44	0.12
Mechanically recovered meat	20	3.89	0.19	3.35	0.25
Head meat	20	3.80	0.25	3.26	0.35
Beef flank	20	3.27	0.52	2.81	0.59
Fat	20	1.99	0.48	1.69	0.41
Rinds	10	0.60*	-	0.48*	-

* One sample only positive

$\bar{x}$ = mean of three replicates

σ = standard deviation of three replicates

Enterobacteriaceae (Fig. 72b). As would be expected, Enterobacteriaceae in a heat-processed, dry material such as the rinds used in pork and beef sausages provides evidence not only of post-processing contamination but, judging from these observations (Table 43), a warning that food poisoning organisms such as *Salmonella* may have gained access to the product. *Salmonella* spp. and members of the 'Arizona' group isolated from sausages and mechanically recovered meat during the preliminary and main experiments were screened for their sensitivity to a variety of antibiotics. All of the strains tested were resistant to at least one of the antibiotics used in the challenge test (Table 44), many were multiply-resistant and one strain of *Salmonella dublin* grew in the presence of 5 antibiotics. The majority of strains (88%) were resistant to benzylpenicillin (Penicillin G) and chloramphenicol (60%) and appreciable proportions (43% and 33%) to oxytetracycline and sulphafurazole respectively. All strains were sensitive to erythromycin, streptomycin, furozolidine, nalidixin and cycloheximide. Although insufficient numbers of organisms were tested to indicate clear patterns of resistance, some trends were evident; Arizona organisms were all described by the profile CH^R, P^R, OT^R and *Salmonella infantis*, P^R.

In order to achieve an overall perspective of *Salmonella* contamination of a commodity such as British fresh sausage, the results of surveys of the finished product must be considered together with observations on the precision of quantitative methods, variations due to season and causes of contamination of ingredients. Finally, of course, the behaviour of the food poisoning organism

Table 44. Antibiotic resistance of *Salmonella* isolated from sausage ingredients and sausage

Salmonella	Culture Code	Antibiotic Code: CH	E	SF	P	S	T	FR	N	OT	C
agona	S46 (1)				+					+	
derby	S62 (1,1)	+		+							
	SO5 (1,1)	+		+	+						
	S41 (1,1)				+						
	S42 (1,2)	+		+							
	S69				+						
dublin	S33 (1,1)	+		+	+		+			+	
	S25 (1,1)	+		+	+						
	S26 (1,1)	+		+	+						
	S10 (1,2)	+									
	S62 (2,2)	+			+		+			+	
	S59 (2)	+			+						
newport	SO4 (2,2)			+	+						
	SO6 (2,2)	+			+						
	S12 (2)				+						
stanley	S27 (1,1)	+		+						+	
	S28 (1,1)	+		+	+						
	SO1 (1,1)				+					+	
	SO7 (1,1)									+	
	SO2 (1,2)	+		+	+						
	SO3 (2)	+		+	+						

Table 44 continued

Salmonella	Culture Code	CH	E	SF	P	S	T	FR	N	OT	C
typhimurium	SO4[1]				+		+				
	S2O[1]			+	+		+				
	S43[2]	+			+					+	
	S47[2]	+			+						
heidelberg	S34[1]	+			+						
	SO8[1]			+	+						
infantis	S29[1]				+						
	S35[1]				+						
"Arizona"	SF[3]	+			+					+	
	SG2[3]	+			+					+	
	SG3[3]	+			+					+	
Untyped:											
Group B	SA[1]	+			+					+	
B	SB[1]	±			+					+	
B	SC[1]				+					+	
B	SD[1]				+					+	
C[1]	SE[1]				+					+	
C[1]	SG1[1]	+			+					+	
D[1]	SH[3]	+			+					+	
D	SJ[3]				+						
% of strains resistant		60	0	33	88	0	10	0	0	43	0

Key: Isolated from:

1 Pork sausage
2 pork and beef sausage
3 MRM

CH chloramphenicol
E erythromycin
SF sulphafurazole
P penicillin G
T tetracycline
FR furazolidine
N nalidixin
OT oxytetracycline
C cycloheximide
S streptomycin

in a product post-manufacture has to be considered also. As the MPN method had to be used to enumerate *Salmonella*, the number of replicates per dilution and the precision of the methods have to be considered in the context of the work involved. Indeed the number of samples that had to be examined dictated the use of 5 tubes per dilution in the work discussed. As the standard error of the logarithm of the MPN is inversely proportional to the square root of the number of tubes at a single dilution (Cochran, 1950), it might be anticipated that a higher incidence, and maybe a higher level, of *Salmonella* contamination of sausages would be demonstrated by doubling the number of tubes per dilution. In practice, there was no appreciable difference in the results presented above and those obtained in a limited survey (Pain, 1981) using 10 tubes/dilution. Indeed, as the latter survey was done in the late autumn/winter, it showed a diminishing incidence of contamination of ingredients and sausages with *Salmonella* and thus confirmed the many observations (e.g. Roberts *et al.*, 1975) that ambient temperature influences *Salmonella* contamination of meat and meat products. Additional confidence in the MPN method was given by the observations that a serotype recovered from sausages was invariably isolated also from one or more of the ingredients. Apart from dissemination of *Salmonella* spp. of gut origin through a pigs tissues at slaughter (Kampelmacher *et al.*, 1961), puncturing of the gut at evisceration can be an important cause of contamination of a particular carcass (Kampelmacher *et al.*, 1961) and a source for cross contamination in subsequent processing (Kampelmacher *et al.*, 1961; Chau *et al.*, 1977). Indeed, it has been shown that a 'comet tail' type of spread of *Salmonella* follows

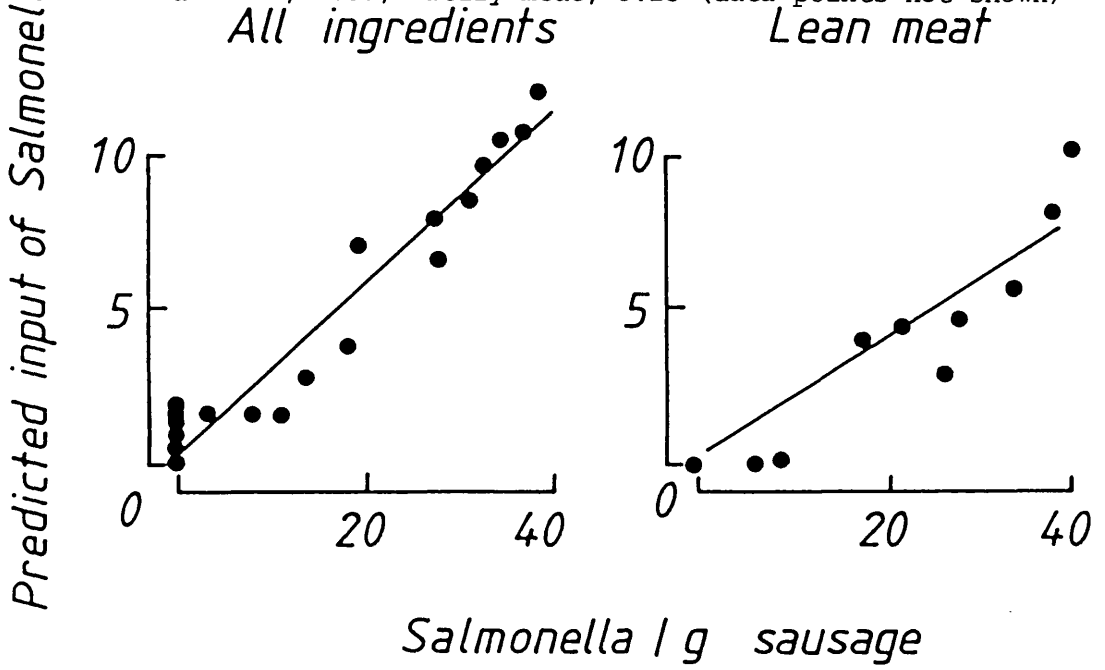
the processing of a contaminated carcass until the organisms are eventually diluted out from the system. In this instance, contagious (Elliott, 1977) rather than random distribution will occur. In other instances, a process, such as the washing and de-hairing of pigs' carcasses or the mincing of material obtained therefrom, can provide not only a longer term source of infection but also opportunities for the contaminants to be randomly rather than contagiously distributed in or on meat or meat products (Elliott, 1977). The influence of mincing or some other form of comminution on the distribution of contaminants has been demonstrated by Kilsby and Pugh (1981). Moreover, the variation in the viable counts of organisms, be they *Salmonella* or coliforms, decreases as the contaminants become more randomly distributed. Conversely the apparent mean of the counts will increase with a more random spread of organisms. These influences were noted in this work also and account for the differences between the observed levels of contamination of the major sources of *Salmonella* and the predicted level in the final product (Fig. 71). The present work has shown also that the influence of dilution must be taken into account when considering the relative importance of ingredients as causes of contamination of a product. Thus although MRM contained the largest numbers and greatest variety of *Salmonella* spp., the use of small amounts (e.g. 1.7% of sausages) meant that its contribution to the contamination of the product was negligible when compared with belly meat (cf. Table 39). Indeed, the latter would appear to be the ingredient that ought to be monitored routinely in sausage manufacture when the objective was to minimize contamination of the product with *Salmonella*. The viable

counts of Enterobacteriaceae appeared to be poor indices of *Salmonella* contamination of the majority of uncooked ingredients included in this study (head meat and backfat in pork sausage and MRM, head meat, beef flank and backfat in pork and beef sausage). From the viewpoint of management in a factory, they are of particular value as would be expected in the routine examination of processed ingredients. Thus there was a good correlation of Enterobacteriaceae contamination of rinds and dried rinds with the recovery of *Salmonella*. Moreover, the isolation of *Salmonella infantis* from one ingredient only, the cooked rinds, may indicate that a human carrier rather than pigs was the source of this serotype.

Although the survey of Roberts *et al*. (1975) included both sulphited and unsulphited sausages, the small number of samples (312) of the latter did not permit an assessment of the possible influence of the preservative on *Salmonella* in the commodity mainly because the survey was qualitative in nature. Our observations that the actual was invariably greater than the predicted level of contamination of sausages with *Salmonella* can be taken in part as evidence that sulphite had little if any bactericidal action on these organisms during the manufacturing stage. Moreover, the low and unpredictable levels of contamination of sausages with *Salmonella* means that samples taken from a factory cannot be used in studies of the fate of these organisms during storage.

Figure 71. Linear regression of predicted number of *Salmonella* from ingredients with number of *Salmonella* in a, pork sausage and b, pork and beef sausage.

a Regression coefficients (r^2) in pork sausage. All ingredients, 0.96; Lean meat, 0.92; Semi-lean meat, 0.44; Rinds, 0.07; Head meat, 0.03; Belly meat, 0.15 (data points not shown)



b Regression coefficients (r^2) in pork and beef sausage. All ingredients, 0.79; M.R.M. 0.49; Beef flank, 0.20; Head meat, 0.42 (data points not shown)

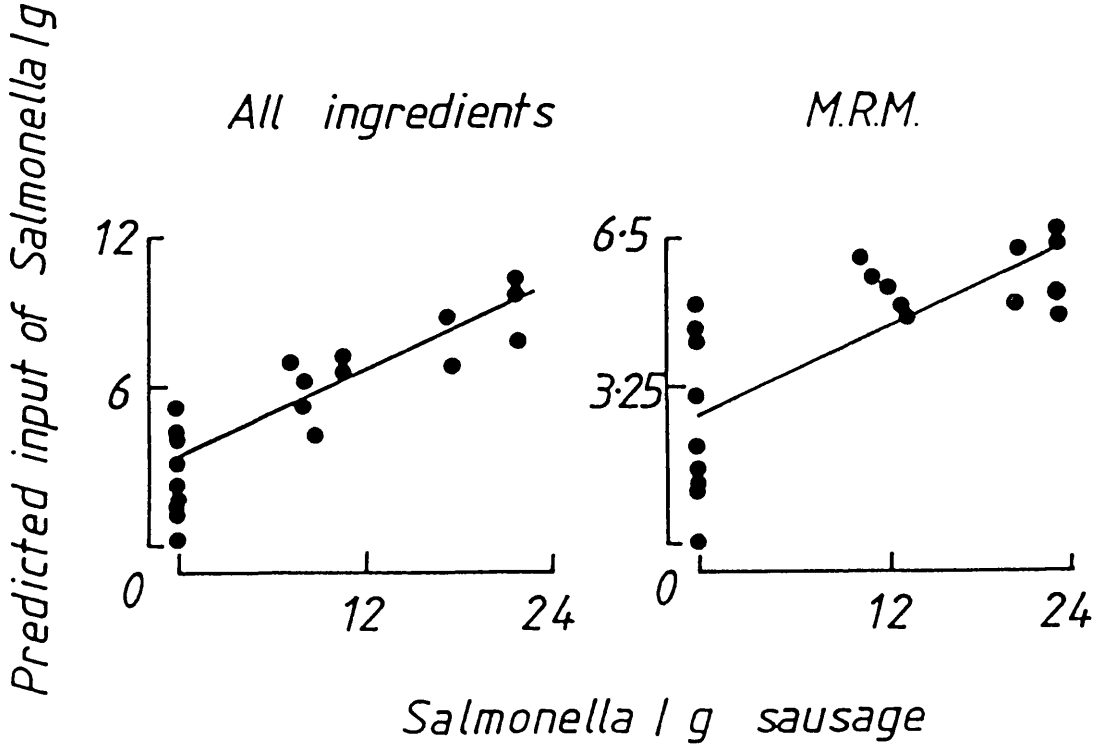
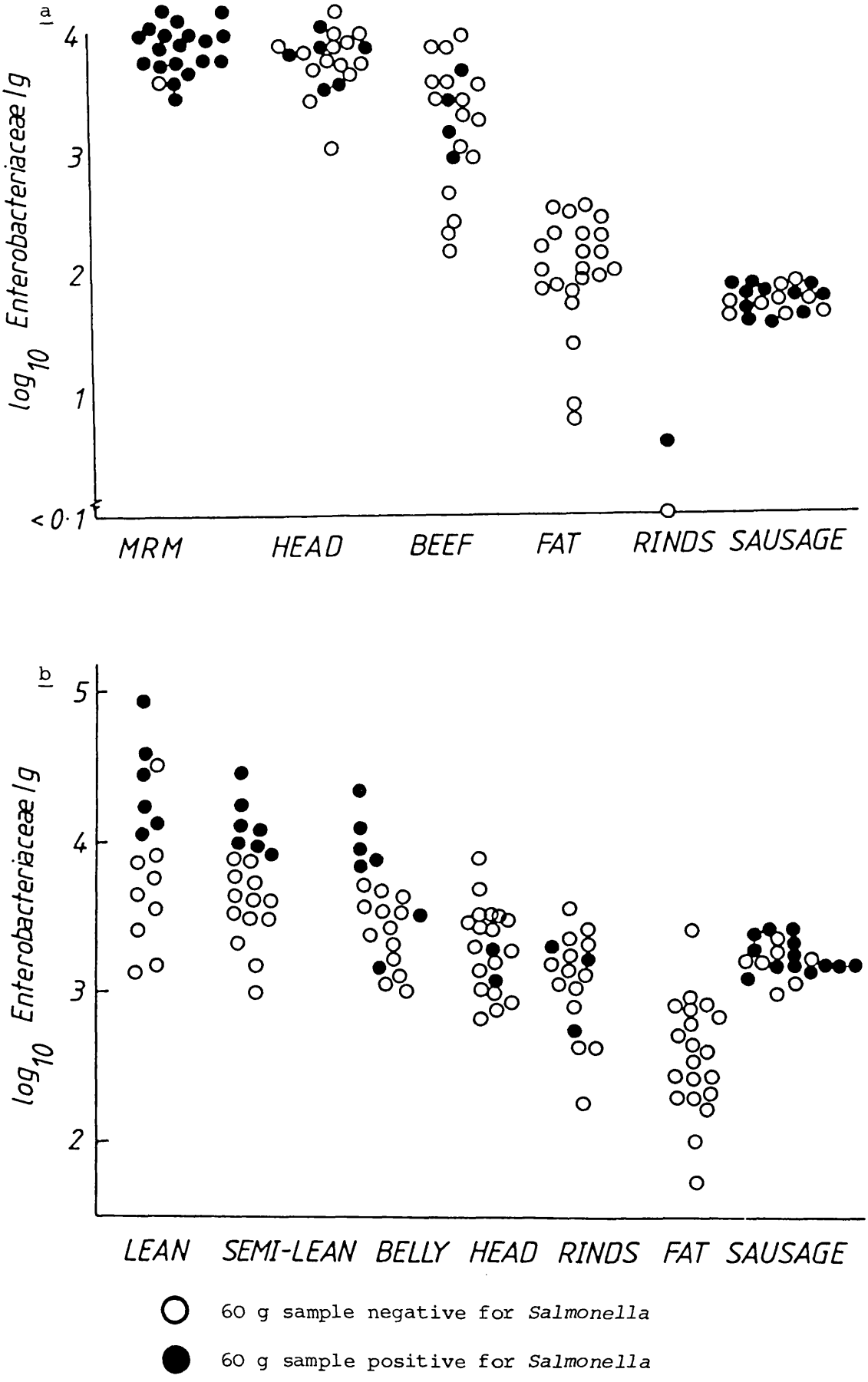


Figure 72. Relationship between numbers of Enterobacteriaceae and presence of *Salmonella* in ingredients of a,pork and beef and b, pork sausage



PSEUDOMONAS

The importance of members of the genus *Pseudomonas* in the spoilage of proteinaceous foods stored at chill temperatures was recognised slowly, a situation due in part to the inadequate descriptions of the genus (e.g. Bergey *et al.*, 1939; Breed *et al.*, 1948). The major taxonomic study of the aerobic pseudomonads by Stanier *et al.* (1966) marked the beginning of a new approach to the classification of these organisms even though initially the wealth of data produced by such studies were difficult to analyse routinely. Indeed food microbiologists continued to rely on simple keys (e.g. Shewan *et al.*, 1960*a*) to identify their strains. Computer-aided numerical taxonomy has negated the difficulties associated with the analysis of large amounts of data, affirmed the significant contribution made by the pseudomonads to the microbial populations on meat and in meat products and enabled the identification of the principal species.

Historical perspective

The first description of the genus *Pseudomonas* (Migula, 1894) gave emphasis to the presence and location of flagella as well as to general morphology. As the former criteria were not accepted generally by bacteriologists, misidentification of micro-organisms led to considerable confusion and contradiction in the literature relating to foods.

Early studies with meat isolates

Glage (1901) was perhaps the first person to note that motile aerobic bacilli capable of liquefying gelatin occurred in the slime developing on the surface of chilled meat. In the 1st edition of Bergey's Manual the Gram-negative saprophytes were sub-divided on the basis of pigment production without any regard to the presence or location of flagella. Thus,organisms producing yellow pigments were assigned to flavobacteria, green pigments to pseudomonads and no pigments to achromobacters. These traits were reflected in subsequent studies of the aerobic organisms recovered from meat. Thus,Haines (1933), who studied the bacterial flora which developed on chilled lean meat, identified the majority of isolates with *Achromobacter* because they failed to produce pigment. Haines and Smith (1933) referred to the principal component of the "microbial association" on spoiled meat as the *Pseudomonas/Achromobacter* complex, a term that was still in use in 1957 (Rogers and McCleskey). The implicit weakness of basing classification on pigmentation alone has been highlighted repeatedly. Thus Ingram (1934) reported that all his isolates from pork mince were pseudomonads whereas Empey and Vickery (1933) and Empey and Scott (1939) identified the majority of their meat isolates with *Achromobacter.* Kluyver and van Neil (1936) can be considered to have been instrumental in causing a fundamental change in the classification of the Pseudomonadaceae; they emphasised the taxonomic importance of polar flagella (Bergey *et al.*, 1939). Even so, reorganization of the 5th edition of the Manual included 19 such organisms in the genus *Achromobacter.* These organisms were later transferred to the genus *Pseudomonas* (Breed *et al.*, 1948).

The failure of food microbiologists to accord taxonomic significance to the presence and location of flagella led to persistent misidentification. Thus, although Brown and Weidemann (1958) examined the isolates of Empey and his collaborators, and re-identified the majority with *Pseudomonas*, the view that *Achromobacter* spp. were important spoilage organisms persisted. For example, despite the implication of members of the genus *Pseudomonas* in the production of off odours and slime on flesh meat (Jensen, 1944), some contemporary reports (Jepsen, 1947) suggested that *Achromobacter* spp. were the predominant organisms in the slime.

Although the 6th edition of the Manual added to the number of species in the Pseudomonadaceae, it provided only vague clues for the identification of pseudomonads from food. Thus, Sulzbacher (1950) was unable to identify organisms from frozen lamb/pork with the species definitions in this edition of the Manual. Indeed, of the 541 isolates of *Pseudomonas* from refrigerated beefburger, Kirsch and his co-workers (1952) could identify a minority only, 147 with *Pseudomonas geniculata*, 88 with *Ps. fragi*, 62 with *Ps. rugosa* and 9 with *Ps. aeruginosa*. The lack of suitable criteria in the Manual for the identification of the unpigmented species of *Pseudomonas* also resulted in a tendency to over-estimate the importance of the pigmented types. For example, Ingram and Hobbs (1954) identified all the pseudomonads from cured and uncured hams with *Ps. fluorescens* and Kitchell and Ingram (1956) the dominant species on slimy pork with *Ps. multistriata*. Likewise, Wolin and his collaborators (1957) identified 95% of these isolates with *Pseudomonas geniculata*. The need for further

systematic study of species within the Pseudomonadaceae was stressed by Ayres (1960*a*) who, from a review of the literature in which identification had been based on definitions given in the 6th edition of the Bergey Manual (Breed *et al.*, 1948), noted that at least seven species (*Ps. fluorescens, fragi, putida, ambigua, convexa, taetrolens* and *incognita*) had been associated with the spoilage of proteinaceous foods. The need for a simple, utilitarian scheme for the identification of pseudomonads led Shewan and his co-workers (1960 a) to devise a key which proved to have most value in the classification of aerobic Gram-negative micro-organisms isolated from fish (Shewan *et al.*, 1960*b*). The inadequacies of the descriptions of *Pseudomonas* in Bergey's Manual can be considered as the major cause of the dubious identifications noted above as well as the reason for many microbiologists adopting routinely the scheme of Shewan and his collaborators (1960 *a*).

A major, but in some ways, a traditional study of the taxonomy of *Pseudomonas* was done by Stanier and his co-workers (1966). They based their definitions of species and biotypes on the ability of an organism to use a specific pattern of compounds as carbon and energy sources, an approach inspired by the work of den Dooren de Jong (1926). It was notable, however, that Stanier did not favour the use of computer analysis; subsequent studies with this method (Sneath *et al.*, 1981) were in accord with the major trends proposed by the original workers. Davidson and his co-workers (1973), who adopted the methods of Stanier *et al.* (1966) to study 231 pseudomonads isolated from meat, reported a very low incidence of identification of their isolates - 16 were identified with *Ps.*

fluorescens, 19 with *Ps. putida* - with the descriptions given by Stanier *et al.* (1966). The majority of isolates were non-fluorescent and non-pigmented - the Group II of Shewan *et al.* (1960*a*). Similar difficulties were encountered in studies of isolates from milk (Juffs, 1973) and fish (Gillespie and Macrae, 1975; Gray and Stewart, 1980; Gillespie, 1981). It is notable,however, that Gyllenberg *et al.* (1963), who applied computer analysis to isolates characterized by traditional methods, recognized *Pseudomonas fragi* as an important spoilage organism of milk. This organism, first described by Eichholz (1902) and Grüber (1902), had long been regarded as an important dairy organism (Hussong *et al.*, 1937) and is now recognised as the dominant pseudomonad on chilled meats. Thus it accounted for 67% of the isolates which Molin (1981) studied, an observation which has been confirmed by others (Martin and Patterson, 1982; Shaw and Latty, 1982; Molin and Ternström, 1982).

Sources of Pseudomonas

Early reports suggested that hides and wash water were the most important sources of carcass contamination with psychrotrophic micro-organisms (Haines, 1933; Empey and Scott, 1939), and the isolation of *Pseudomonas* from dressed carcasses has led to suggestions that the hide is important in the transfer of organisms to meat (Nottingham *et al.*, 1974). As psychrotrophic strains of *Pseudomonas* are common in soil, water and on vegetation, many opportunities exist also for their transfer to meat *via* the factory workers, their cutting knives or machinery. The walls and floors

in chill rooms and cutting boards in boning rooms also harbour substantial populations of pseudomonads (West *et al.*, 1972; Newton *et al.*, 1978). Although structural and work surfaces might be expected to spread organisms onto meat, the actual level of psychrotroph contamination on meat has been shown to decrease during the butchering stage (Newton *et al.*, 1978). The seasonal fluctuations in the level of psychrotroph contamination of meat have been attributed mainly to changes in ambient temperature (Empey and Scott, 1939), and rainfall (Newton *et al.*, 1978). The low temperature and high relative humidity in chill rooms are factors which are considered to favour pseudomonad growth and the ratio of the psychrotroph count to that of the total count on porcine carcasses increases with chilled storage (Scholfield *et al.*, 1981). The present study has associated high levels of contamination of sausages with prolonged chill-room storage of meat (p. 165). Although it is generally accepted that *Pseudomonas* spp. are the dominant micro-organisms on whole pieces of chilled carcass meat, the behaviour of this group in meat products has been seldom reported. As the pseudomonads generally comprise a fraction only of the microbial associations which develop in meat products, a major cause of the failure to monitor the fate of this group of micro-organisms has been the lack of a suitable selective medium. Indeed, the cetrimide-fusidic acid-cephalodrine medium of Mead and Adams (1977) appears to be the only one that has been recommended (Gardner, 1980) for use in meat microbiology. Thus the early reports by Dyett and Shelley (1962, 1966) and Dowdell and Board (1967, 1968) did not comment on the behaviour of pseudomonads in British fresh sausage although the last mentioned workers demonstrated that this group of organisms formed

a significant proportion of the microbial association in heavily contaminated sausages made by local butchers (Dowdell and Board, 1971). They noted also that pseudomonads multiplied in sulphited sausage which had been stored at 4°C or room temperature. Plate count agar, as used by Dowdell and Board (1971), was supplemented with hexadecyltriammonium bromide by Ashworth *et al.* (1974). They found that pseudomonads grew well in unsulphited but not sulphited sausage at refrigeration temperatures. Brown (1977), who used the medium of Masurovsky *et al.* (1963), confirmed these findings, and noted also that the pseudomonads in sulphited sausage did not die during storage at 22° or 4°C.

This section of the thesis is concerned with a detailed characterization of *Pseudomonas* spp.; the behaviour of these organisms in sausages is considered on pp. 125 - 132 and their tolerance of sulphite on pp. 151 *et seq*.

The classification of pseudomonads and other aerobic Gram-negative bacteria from sausage

The above review has shown that the classification of *Pseudomonas* spp. isolated from chilled proteinaceous foods has tended to lag behind developments in the taxonomy of the genus. There has been a trend also for food microbiologists to isolate from the "blooms" which develop during the storage of such foods. As this can be considered to be a form of enrichment culture, the study of Shaw and Latty (1982), for example, may have been inadvertently biased by the dominance of enriched organisms. This possibility was

avoided in the present study simply by using isolates taken from meat stored in a factory at chill temperatures for a short while only, or from stored sausage.

Materials and Methods

Sampling and Isolation

Samples (1 kg) of pork were obtained from meats awaiting use in the factory - the pigs were slaughtered on site and the butchered meat stored at *ca.* 4°C for 24 h. Samples of sulphited and un-sulphited British fresh sausage were taken also. All samples were stored at *ca.* 4°C and examined within 3 h. To achieve random rather than contagious distribution of the micro-organisms (Kilsby and Pugh, 1981), whole pieces of meat were minced (Kenwood A901, England). Samples (20 g) of minced meat or sausage were added to ¼ strength Ringers diluent (180 ml) and homogenized in a Colworth Stomacher 400 (Seward, London) for 60 s. Serial dilutions were made in ¼ strength Ringers (90 ml) and 0.2 ml of an appropriate dilution spread over the dried surface of Cetrimide Fusidin-Cephalodrine (CFC; Mead and Adams, 1977) medium. Incubation was at 15°C for 48 h.

One hundred and sixty-five strains of Gram-negative aerobic bacteria were isolated from 20 samples of sausage ingredients and the corresponding freshly-prepared sausage and those stored at 4°C, 10°, 15° or 20°C for up to 8 days. Colonies were picked at random from the lowest countable dilution on CFC medium, purified by restreaking on Plate Count agar (PCA) and maintained on slopes

of PCA at *ca.* 4°C with sub-culture every 4 weeks.

Preliminary characterization

Isolates on CFC agar were examined for colony form and pigmentation; their Gram reaction, (Kopeloff and Beerman, 1922), cell morphology, flagella arrangement (Mayfield and Inniss, 1977) and oxidase reaction, (Kovács, 1956) were determined also. Production of acid from glucose was tested in the O-F medium (incubation, 15°C for 10 days) of Hugh and Leifson (1953).

Culture characteristics

Pigment production: Cultures were streaked onto the medium of King *et al.* (1954) and incubated at 15°C for 7 days.

Accumulation of poly β hydroxybutyrate: The method of Stanier *et al.* (1966) was used.

Growth temperatures: Ability to grow at 41°C in 48 h, 30°C in 7 d, 15°C in 10 d and at 4°C in 14 d was tested in the YE medium of Stanier *et al.* (1966); 0.05 ml of culture grown at 15°C for 48 h in YE medium was used as inoculum.

Growth factor requirement: The ability to grow on the YEA basal medium of Stanier *et al.* (1966) containing acetate (0.1% w/v) was

tested at 30°C for 7 d. Failure to grow was assumed to be due to a deficiency in growth factors.

Denitrification: The method of Stanier *et al.* (1966) was used.

Biochemical tests

Catalase: Hydrogen peroxide (20 vol.) was added to cultures grown on Nutrient agar containing glucose (1% w/v) at 15°C for 48 h.

Tween 80 hydrolysis: The method of Sierra (1957) was used (incubation, 30°C for 7 days).

Starch hydrolysis: The method of Stanier *et al.* (1966) was used (incubation, 30°C for 7 days).

Gelatin hydrolysis: The method of Frazier (1926) was used (incubation, 30°C for 7 days).

Ornithine and lysine decarboxylation: The method of Møller (1955) was used (incubation, 15°C for 7 d).

Egg yolk reaction: The method described by Anon. (1957) was used.

Carbon sources

The ability to grow on 126 compounds as the sole source of

carbon was tested. The methods and media of Stanier *et al.* (1966) were used with the exceptions that (1) all compounds were filter-sterilised, and (2) isolates were suspended in sterile ¼ strength Ringers and *ca.* 1 µl was placed on the surface of dried solid medium containing a specific substrate by multipoint inoculation (Denley, England). Incubation was at 30°C and growth was scored (at 1, 3, 5 and 7 days), as strongly positive (2+) or weakly positive (1+) if growth was visibly greater than on the control medium. If growth was equal to or less than that on the control medium, it was scored as negative (0-). All the tests were repeated on 10% of the isolates and the average probability of error (p) was calculated (Sneath and Johnston, 1972).

Computer analysis

Multistate characters were analysed using the Taxpak program (M.J. Sackin, Department of Microbiology, University of Leicester, England). The Gower (S_G), Simple Matching (S_{SM}) and Jaccard (S_J) coefficients together with unweighted pair-group average (UPGMA), and single linkage clustering were calculated on the ICL 1900 computer at the University of Nottingham, England. Data were analysed by the Ovclust program also (Sneath, 1979). A program, DICHAR (Sneath, 1980) was used to list the most diagnostic properties of the phenons and these results form the basis of the key to the identification of clusters (Fig. 74).

Results

The majority of the isolates were Gram-negative, non-fermentative,

oxidase-positive, motile bacilli with polar flagella and were identified with *Pseudomonas*. A small number of Gram-negative, oxidase-positive, non-motile bacteria were identified with *Moraxella*. Both groups of organisms were included in the computer analysis as were type strains of *Pseudomonas*, pseudomonads implicated in the spoilage of pork and organisms from the genera *Alteromonas*, *Acinetobacter* and *Aeromonas* (Table 45). All the isolates were tested for their ability to use a range of substrates as carbon sources. Ten percent of the isolates were re-tested and the reproducibility calculated (Sneath and Johnston, 1972). As the average probability of error (p) was *ca*. 3%, no appreciable distortion in the computer analysis of the results was to be expected. The S_{SM} coefficient (matrix not shown) and clustering by UPGMA linkage sorted 138 operational taxonomic units (OTU's) into six clusters, A-F (Fig. 73 at the 77% level of similarity (77% S). Ten of the 13 stock cultures were loosely clustered; one (*Ps. fluorescens* NCIB 9046) clustered with A, another (*Ps. fragi* NCIB 8542) with C, and the third (*Alteromonas putrefaciens* NCIB 10471) with group II (Fig. 73). The remaining OTU's (25) were either scattered among the stock cultures or contained in groups I (OTU's 15-18) or III (16 isolates).

Cophenetic discrepancies, the contributions made by each strain to the distortion between (1) the similarities in the similarity matrix and (2) the corresponding values implied by the phenogram (dendrogram), were measured by:

$$\Delta_j = \left[\sum_{k=1}^{t} (S_{jK} - C_{jK})^2 \right]^{1/2}$$

Table 45. Origin of isolates of *Pseudomonas*

Organism	Number of strains	Strain/culture collection no.	Source of Isolate
Pseudomonas spp. from sausage ingredients	9	R 1-9	Pork rinds
	2	R 1-2	Pork fat
	12	R 1-12	Lean pork
Pseudomonas spp. from sausage	86	S+ 1-86	Sulphited sausage
	56	S- 1-56	Unsulphited sausage
Pseudomonas spp. from Culture Collections:			
Pseudomonas			
aeruginosa	2	NCIB 8295/ATCC 10145, NCTC 5083	
alcaligenes	1	NCIB 9945	
cepacia	2	NCIB 9089 *FD 2001	Unknown
fluorescens	2	NCIB 9046/ATCC 13525, NCTC 10038	
fragi	1	NCIB 8542/ATCC 4973	
maltophilia	1	NCIB 9023/ATCC 13637	
putida	1	NCIB 9887/CCEB 520	
stutzeri	1	NCIB 10783	
testosteroni	1	NCIB 8893	

Table 45 continued

Organism	Number of strains	Strain/culture collection no.	Source of Isolate
Pseudomonas spp. associated in food spoilage:			
fluorescens	1	*N904A	Pork
Pseudomonas sp.	1	*FD1984	Pork
Pseudomonas sp.	2	*N902C	Pork
		*N902D	Pork
aeruginosa	1	*N903	Pork
Other reference strains:			
Alteromonas putrefaciens	1	NCIB 10471	
Acinetobacter calcoaceticus	1	NCIB 8250	
Aeromonas liquefaciens	1	*N212B	Pork

* Author's isolates

where S_{jK} and C_{jK} are the similarity between OTU's j and K in the similarity matrix and in the phenogram respectively (Sneath and Sokal, 1973; Sneath *et al.*, 1981).

The cophenetic correlation coefficient, r = 0.8899, (Sneath, 1978) demonstrated that the results given in Fig. 73 did not differ markedly from the data contained in the similarity matrix. The mean inter- and intra-group similarities with associated values for the variance and standard deviation (Table 46) provided further evidence of the validity of the separation of the organisms into clusters A-F and groups I-III.

The Ovclust program of Sneath (1979) demonstrated, however, that although clusters A, B and F were distinct entities, C, D and E were not (p = 10%). Indeed the considerable overlap between clusters C, D and E suggests that they ought to be considered as one cluster (Table 47). Groups II and III were separated significantly from all the other clusters but group I could not be distinguished readily from clusters A-E (Fig. 73). These findings are supported by other results (*vide infra*).

Distinctness of clusters/groups and their relationships in hyperspace

The overlap statistics of phenons (clusters of groups) were calculated by a program which tests for the significance of overlap of 2 clusters in Euclidean space. This program used the number of operational taxonomic units (OTU's) in each sub-cluster/group and the inter- and intra-centroid distances. d[based on the

Table 46. Combined matrix of mean inter- and intra-group similarities for clusters formed by S_{SM} and UPGMA

Cluster/Group									
A	.879 *								
	.002 **								
	.041 ***								
B	.826	.959							
	.001	.000							
	.029	.021							
C	.816	.790	.883						
	.001	.001	.002						
	.028	.024	.038						
D	.785	.768	.833	.904					
	.001	.000	.001	.001					
	.028	.019	.032	.037					
E	.753	.741	.819	.773	.888				
	.001	.001	.001	.001	.002				
	.027	.027	.038	.025	.047				
F	.747	.699	.783	.744	.760	.855			
	.001	.001	.001	.001	.002	.003			
	.031	.024	.034	.028	.039	.059			
I	.675	.650	.693	.659	.720	.647	.736		
	.005	.004	.003	.002	.004	.001	.005		
	.073	.064	.052	.050	.060	.035	.074		
II	.632	.603	.684	.631	.729	.745	.627	.854	
	.003	.002	.001	.002	.001	.002	.003	.005	
	.051	.047	.037	.043	.032	.041	.056	.067	
III	.557	.578	.502	.540	.475	.412	.484	.304	.816
	.007	.005	.007	.004	.004	.007	.006	.008	.008
	.084	.071	.081	.065	.060	.081	.074	.087	.088
	A	B	C	D	E	F	I	II	III

* mean group similarity
** variance of similarity
*** standard deviation of similarity

Table 47. The distinctness of clusters as measured by their overlap*

Cluster/Group									
A	-	>99**	>99	>90	>99	>99	<90	>99	>99
B	-	-	>99	>99	>99	>99	<90	>99	>99
C	-	-	-	<90	<90	<90	<90	>99	>95
D	-	-	-	-	>99	>99	<90	>99	>99
E	-	-	-	-	-	>95	<90	>99	>99
F	-	-	-	-	-	-	>95	>99	>99
I	-	-	-	-	-	-	-	> 90	>90
II	-	-	-	-	-	-	-	-	>99
III	-	-	-	-	-	-	-	-	-
	A	B	C	D	E	F	I	II	III

* Ovclust output estimated with the V(O) = 0.025 overlap setting (Sneath, 1977, 1979)

** Probability that overlap is less than 2.5%

relationship, $d = (1 - S_{SM})^{\frac{1}{2}}$] to calculate an index of overlap (V_G). The level of overlap expected for a rectangular distribution (Sneath, 1977) was used as a critical level of overlap to compare all pairs of subgroups/clusters.

Thus from Table 47 the distinctness of each cluster/group from every other cluster/group shown on the dendrogram (Fig. 73) is measured by the "w" statistic (Sneath, 1977; 1979; 1980).

Cluster A (16 OTU's; 85% S). This group included 11 isolates from sausage and 2 from ingredients. More than 80% of cluster A strains used 48 out of 126 of the carbon sources tested (Table 48), and all produced fluorescent pigments. Organisms of this cluster were identified with *Pseudomonas fluorescens* biotype A (Stanier *et al.*, 1966).

Cluster B (8 OTU's; 92% S). This group contained 7 isolates from sausage and 1 from an ingredient. More than 80% of these closely-related isolates used sixty out of the 126 carbon sources tested (Table 48) and 63% produced fluorescent pigments and lipases although one strain only gave a typical egg-yolk reaction. Organisms of this cluster were identified with *Pseudomonas fluorescens* biotype C, Stanier *et al.* (1966).

Cluster C (88 OTU's; 85% S). This group contained 70 isolates from sausage and 17 from ingredients. All the organisms of this major cluster (54% of pseudomonad isolates), which contained *Pseudomonas fragi* NCIB 8542 also, were non-fluorescent and did not produce extracellular lipases or proteases. More than 80 %

Table 48. Reaction of cluster/group isolates to tests used in numerical analysis of organisms from fresh and processed meat

Cluster/Group	A	B	C	D	E	F	I	II	III
No of Strains	15	8	88	4	10	12	4	6	16
Colony form on CFC agar:									
Smooth	87*	88	47	100	60	75	0	100	75
Rough	13	12	53	0	40	25	100	0	25
Pigmented	47	88	9	0	0	0	25	100	31
Unpigmented	53	12	91	100	100	100	75	0	69
Gram reaction	0	0	0	0	0	0	0	0	0
Flagella arrangement:									
Monotrichous	100	100	100	100	100	0	100	100	100
Multitrichous	0	0	0	0	0	0	0	0	0
Pigments:									
Fluorescent	100	88	0	0	0	0	0	0	25
Phenazine	0	0	0	0	0	0	0	0	0
Pink/Brown	7	0	0	0	0	0	0	100	0
Growth at:									
4^{o}	100	100	100	100	100	100	100	100	100
15^{o}	100	100	100	100	100	100	100	100	100
30^{o}	100	100	100	100	100	100	100	100	100
41^{o}	0	0	0	0	0	0	0	0	0
Acid from D-Glucose									
Aerobic	100	100	100	100	100	0	100	50	100
Anaerobic	0	0	0	0	0	0	0	0	0
Catalase	100	100	100	100	100	100	100	100	100
DNAse	0	0	0	0	0	0	0	100	0

Table 48 continued

Cluster/Group	A	B	C	D	E	F	I	II	III
No of strains	15	8	88	4	10	12	4	6	16
Acid from D-Glucose									
Egg yolk reaction	73	12	0	0	0	0	25	17	56
H_2S	0	0	0	0	0	0	0	100	0
Lysine decarboxylase	0	0	0	0	0	0	0	0	0
Ornithine decarboxylase	0	0	0	0	0	0	0	100	0
$NO_3^- \rightarrow N_2$	13	100	0	0	0	0	0	50	0
Oxidase	100	100	100	100	100	100	100	100	100
Urease	100	88	91	100	100	50	75	50	100
Hydrolysis of:									
Arginine	100	100	100	100	100	25	100	0	100
Gelatin	60	25	0	0	0	0	25	100	75
Starch	0	0	0	0	0	0	0	0	6
Tween 80	0	75	0	0	0	0	0	50	63
Growth of sole carbon source:									
Carbohydrates:									
D-Arabinose	0	0	94	100	100	75	75	0	81
L-Arabinose	100	0	75	25	60	92	25	17	100
Cellobiose	0	0	2	0	0	0	0	0	94
D-Fructose	100	100	99	100	100	0	100	33	100
D-Galactose	100	13	21	0	20	92	25	0	94
Gluconate	100	100	100	100	100	83	25	40	100
D-Glucose	100	100	100	100	100	0	100	67	100
Inulin	0	0	1	0	0	0	0	0	94
2-keto-D-Gluconate	100	100	69	0	80	0	100	0	100
Lactose	0	0	0	0	0	0	0	0	94
Maltose	0	0	1	0	0	0	75	60	86
D-Mannose	100	100	68	100	60	42	100	0	100
Mucate	100	0	10	25	0	17	100	0	100
L-Rhamnose	0	88	3	0	60	20	0	0	92
D-ribose	100	100	97	100	100	92	100	33	100
Salicin	6	0	0	0	0	0	0	0	100
Starch	0	0	0	0	0	0	0	0	94

Table 48 continued

Cluster/Group	A	B	C	D	E	F	I	II	III
Sucrose	31	0	83	75	100	8	100	0	100
Trehalose	71	100	81	75	80	0	100	0	100
D-Xylose	0	0	97	100	0	0	100	0	100
Fatty acids:									
Acetate	100	100	99	100	100	67	50	33	100
Butyrate	0	0	0	0	0	0	0	0	0
Iso-Butyrate	0	0	0	0	0	0	0	0	0
Caprate	100	100	100	100	100	92	100	0	94
Caprylate	100	0	100	0	90	50	33	0	93
Heptanoate	81	100	80	50	0	0	25	50	38
Propionate	0	0	0	0	0	0	75	0	0
Valerate	94	88	88	50	20	25	25	50	100
Iso-Valerate	0	0	7	50	0	8	25	0	13
Dicarboxylic acids:									
Azelate	6	100	1	0	0	0	0	0	88
Eicosanedioate	0	0	0	0	0	0	0	0	38
Fumarate	100	100	100	100	100	92	25	50	94
Glutarate	100	100	100	100	100	83	100	0	94
Maleate	0	0	1	0	0	0	75	0	88
Malonate	83	100	1	0	0	0	0	67	94
Oxalate	0	0	0	0	0	0	75	0	88
Pimelate	0	88	0	0	0	0	75	0	88
Sebacate	6	100	0	0	0	0	0	0	88
Suberate	14	100	0	0	0	8	0	0	75
Succinate	100	100	100	100	100	92	50	83	94
Hydroxyacids:									
Glycollate	6	0	0	0	0	0	0	0	94
DL-Glycerate	93	100	24	0	0	0	0	40	100
DL-β-Hydroxybutyrate	100	100	100	100	100	10	75	0	92
DL-Lactate	100	100	100	100	100	8	50	50	100
D-Malate	25	50	67	75	50	0	25	33	88

Table 48 continued

Cluster/Group	A	B	C	D	E	F	I	II	III
L-Malate	100	100	85	100	0	88	50	80	71
D-(-)Tartrate	20	0	6	0	0	0	50	0	50
L-(+)Tartrate	0	0	32	75	20	0	50	0	94
meso-Tartrate	21	0	67	50	60	17	75	40	38
Miscellaneous organic acids:									
Aconitate	69	0	6	0	10	0	25	0	100
Citraconate	0	25	0	0	0	0	0	0	94
Citrate	100	100	98	100	100	91	100	0	100
Itaconate	69	13	86	100	90	17	25	0	19
α-Keto glutarate	100	100	98	100	100	100	100	33	100
Pyruvate	100	100	100	100	100	88	75	50	100
Polyalcohols and glycols:									
Adonitol	50	100	0	0	0	50	0	0	0
2,3 Butyleneglycol	19	0	2	0	10	0	0	0	100
Ethyleneglycol	0	0	0	0	0	0	75	0	94
Erythritol	56	100	0	25	10	0	100	0	100
Glycerol	100	100	100	100	100	91	50	0	100
meso-Inositol	50	100	13	0	10	67	25	0	100
Mannitol	100	100	53	50	40	67	25	0	100
Propyleneglycol	0	0	5	25	10	8	0	0	94
Sorbitol	56	0	0	0	0	25	100	0	100
Alcohols:									
iso-Butanol	6	0	0	0	0	0	25	0	94
n-Butanol	25	25	17	0	0	0	75	33	94
Ethanol	0	0	0	0	0	0	0	0	94
Geraniol	33	0	8	0	0	0	0	0	21
Methanol	0	0	0	0	0	0	0	0	94
iso-Propanol	81	100	57	100	100	75	50	0	94
n-Propanol	0	0	11	0	0	0	0	0	94

Table 48 continued

Cluster/Group	A	B	C	D	E	F	I	II	III
Non-nitrogenous aromatic and other cyclic compounds:									
Benzoylformate	0	0	0	0	0	0	0	0	94
δ-Hydroxybenzoate	0	0	0	0	0	0	0	0	0
m-Hydroxybenzoate	0	25	0	0	0	17	0	0	94
p-Hydroxybenzoate	100	100	91	100	50	83	25	33	88
D-Mandelate	0	0	0	0	0	0	0	0	94
L-Mandelate	0	0	0	0	0	0	0	0	94
Phenol	0	0	0	0	0	0	0	0	81
Phenylacetate	13	13	16	75	0	8	0	17	93
Phenylethanediol	0	0	1	0	0	0	0	0	81
Phthalate	0	0	0	0	0	0	75	0	94
iso-Phthalate	0	0	0	0	0	0	0	0	94
Quinate	75	100	92	0	0	0	0	0	100
Testosterone	0	0	24	75	40	80	100	0	92
Aliphatic amino acids:									
D-α-Alanine	100	0	100	0	0	50	100	0	50
β-Alanine	100	100	100	100	100	50	100	0	100
DL-α-Aminobutyrate	0	0	1	0	0	0	0	0	0
γ-Aminobutyrate	100	100	100	100	10	92	0	0	69
DL-α-Aminovalerate	25	0	0	0	0	0	50	0	13
δ-Aminovalerate	69	100	73	100	60	33	0	0	94
DL-Arginine	94	100	100	100	0	92	50	0	64
L-Aspartate	100	100	100	100	100	100	100	50	100
DL-Citrulline	25	100	6	100	10	8	50	0	100
L-Glutamate	100	100	100	100	10	83	50	67	69
Glycine	0	0	43	100	30	0	0	33	94
L-Leucine	100	100	100	100	100	42	100	67	100
L-Isoleucine	100	100	90	100	100	42	100	17	88
L-Lysine	100	100	94	100	0	83	50	17	69
DL-Norleucine	13	0	0	0	0	0	50	0	13
DL-Ornithine	75	100	92	50	0	58	50	0	69

Table 48 continued

Cluster/Group	A	B	C	D	E	F	I	II	III
L-Serine	100	100	53	100	50	0	75	67	100
L-Threonine	56	100	80	75	80	0	75	50	100
L-Valine	100	100	24	100	30	25	25	33	50
Amino acids and related compounds:									
p-Aminobenzoate	0	0	0	0	0	0	0	0	44
L-Histidine	100	100	96	100	100	25	100	33	100
Kynurenate	31	13	3	0	10	0	33	0	94
L-Kynurenine	22	100	14	75	0	70	0	50	100
L-Phenylalanine	93	100	95	100	10	83	50	40	63
L-Proline	100	100	100	100	10	92	50	17	69
D-Tryptophan	0	0	1	0	0	0	25	0	69
L-Tryptophan	69	100	7	0	0	0	75	33	100
L-Tyrosine	100	100	100	100	10	83	50	33	69
Amines:									
α-Amylamine	0	13	0	0	0	0	0	0	94
Benzylamine	19	0	0	100	0	17	0	0	88
Butylamine	0	0	0	0	0	0	0	0	94
Ethanolamine	86	100	15	0	20	0	25	0	100
Histamine	46	0	3	100	0	20	0	0	100
Methylamine	0	0	1	0	0	0	0	0	94
Tryptamine	31	13	0	100	0	25	0	17	85
Miscellaneous nitrogenous compounds:									
Acetamide	6	0	0	0	0	0	0	0	88
Betaine	94	100	91	100	80	0	50	0	100
Creatine	13	0	57	50	30	8	50	0	94
Hippurate	31	75	77	100	80	25	100	0	94
Nicotinate	19	0	6	75	0	25	0	0	94
Pantothenate	0	100	0	0	0	0	50	0	100
Sarcosine	100	100	82	75	50	0	75	0	100

*Percentage of strains giving a positive reaction

of the strains utilized 45 of the 126 carbon substrates tested (Table 48). Organisms of this cluster were identified with *Pseuomonas fragi*.

Cluster D (4 OTU's; 86.5% S) contained 4 isolates from sausage and was related closely (83.5% S) to cluster C. The isolates of this cluster did not produce fluorescent pigments, proteases or lipases. Forty-five out of the 126 carbon sources tested were used by at least 80% of strains (Table 48). Isolates of cluster D, which were separated from those of cluster C on the pattern of substrate utilization (Fig. 74) were identified with *Pseudomonas fragi*.

Cluster E (10 OTU's; 83% S) comprised 10 isolates from sausage. Organisms of this cluster used 28 out of the 126 substrates tested (Table 48) and were identified with *Pseudomonas fragi*. All the strains in this cluster failed to grow on quinate, benzylamine, histamine, tryptamine and D-xylose for example (Fig. 74).

Cluster F (12 OTU's; 82.5%S) included 11 isolates from sausages and comprised Gram-negative, non-motile bacilli capable of utilizing 23 out of the 126 carbon sources tested (Table 48). Members of this cluster, which were similar to the group A isolates from meat (Davidson *et al.*, 1973), were identified with *Moraxella*.

Group I (4 OTU's; 73% S) comprised 3 isolates from sausage and 1 from an ingredient. These strains, which used 23 out of the

126 substrates tested (Table 48) have not been assigned to a species. The pattern of substrate utilization suggests that 3 OTU's resemble *Pseudomonas fluorescens* whereas one resembles *Pseudomonas fragi*, (Table 48).

Group II (6 OTU's; 77% S) included 4 isolates from sausage. These strains produced pink/brown pigments on the medium A of King *et al.* (1954), H_2S and DNAase. Few carbon sources were utilized (Table 48). Members of this group were identified with *Alteromonas*. They did not have an obligate requirement for salt (their tolerance of salt was not tested).

Group III (16 OTU's; 80% S) comprised 16 isolates from sausage. These strains used 98 out of the 126 substrates tested (more than 80% of strains, positive) and resembled *Pseudomonas fluorescens* biotype G of Stanier *et al.* (1966), who stated "Biotype G is frankly a provisional group, which lacks the uniformity characteristic of the other six biotypes". It is noteworthy that 3 of Stanier's strains (1, 99, 124), which clustered together, in the numerical analysis of the original data (Sneath *et al.*, 1981), shared many properties with organisms of group III of this study.

Discussion

The results presented above have confirmed the observations of several workers (e.g. Davidson *et al.*,1973; Shaw and Latty, 1982; Molin and Ternström, 1982) that the majority of pseudomonads isolated

from meat differed from those studied by Stanier *et al.* (1966). Indeed only *ca.* 19% of our strains of *Pseudomonas* from British fresh sausage and ingredients could be identified with the species described by Stanier *et al.* (1966).

Despite some claims that fluorescent pseudomonads predominate on chilled meat (e.g. Kirsch *et al.*, 1952; Wolin *et al.*, 1957, and Ayres, 1960), this report supports the findings of other workers (e.g. Davidson *et al.*, 1973; Shaw and Latty, 1982, and Molin and Ternström, 1982) that non-fluorescent types dominate. This study indicated also why the over-simplistic scheme of Shewan *et al.* (1960 *a*) has been used extensively by food microbiologists and has reinforced the findings of others (e.g. Gardner, 1965; Shewan, 1974; Barnes, 1976; Law *et al.*, 1979; Gillespie, 1981) that the dominant pseudomonads of chilled proteinaceous foods form a distinct group (Group II of Shewan *et al.*, 1960 *a*). The pseudomonads included in this study were not isolated from"blooms" and therefore the conclusion that *Pseudomonas fragi* is the dominant organism both of fresh, spoiled and processed meats appears to be justified (e.g. Blickstad *et al.*, 1981; Shaw and Latty, 1982; Molin and Ternström, 1982; Martin and Patterson, 1982).

In the past, unsatisfactory media have been used for the isolation and enumeration of *Pseudomonas* from meat and meat products in which these organisms were not numerically dominant. The contention of Gardner (1980) that CFC agar (Mead and Adams, 1977) is a useful medium for the recovery of *Pseudomonas* is supported by the results presented above. Although *Pseudomonas* spp. are

considered to be minor contaminants of sausage (Dowdell and Board, 1971), and are not implicated in the spoilage of the product, the biochemical changes brought about by these metabolically versatile organisms may be out of all proportion to the size of their climax populations in sausage and their potential to contribute to spoilage of the product should be considered in future studies.

The presence of sulphite did not appear to exert any selective pressure on the species of *Pseudomonas* which were recovered from meat and sausage. Indeed isolates from sulphited and unsulphited samples were represented in all the major clusters or groups shown in Fig. 73

Addendum

Numerical taxonomy of *Pseudomonas* spp. isolated in previous studies of sausages and meat

The data upon which Davidson,Dowdell and Board (1973) based their discussions of the properties of Gram-negative aerobes isolated from meats, including British fresh sausage, was available for numerical analysis. The Clustan package of Wishart (1978) was used with the objectives of (1) establishing whether or not *Pseudomonas fragi* was a common isolate in their work and (2) assessing whether or not the Gram-negative aerobic flora of sausage had changed over a 15 year period.

Of the 231 polar-flagellate Gram-negative rods studied by

Davidson *et al.* (1973), the properties of 218 isolates were available for study, 104 strains had been isolated from beef (Davidson, 1970) and 114 from British fresh sausage.

The majority (206 strains) of isolates were contained in 6 major clusters which fused at 65.8%S (Fig. 75). Twelve unclustered strains and those of clusters 1, 2 and 3 (161 OTU's) were closely related (82.3%S). There was little relationship between clusters 4 (20 OTU's), 5 (4 OTU's) and 6 (21 OTU's), however, with fusion at 69.65%S and 65.8% S respectively (Fig. 75). A separate analysis of the 104 beef strains assigned 93 to 8 clusters. Although the majority (55 OTU's) were contained in cluster 1, the extent of overlap between the 78 OTU's in clusters 1 - 5, which were separated from the remaining 15 OTU's of clusters 6 - 8 at 72.5%S (Fig. 76), suggests that they comprise one group only.

One hundred and four of the 114 strains from British fresh sausage were contained in 5 clusters (Fig. 77); the majority (62 OTU's) of strains being in cluster 1 (87.2%S). This cluster was closely related to cluster 2 (84 S) and 3 (78.4 S). Cluster 4 and 5 (15 OTU's) formed minor fractions of the isolates and were not closely related to the cluster 1 - 3 complex, linking at 75.9%S and 62.6%S respectively (Fig. 77).

The conclusion that the majority (79%) of the 218 strains depicted in Fig. 75 were *Pseudomonas fragi* (clusters 1, 2, 3 and 12 unclustered strains) is supported by a detailed analysis of utilization of specific substrates and other features (data not presented). It was notable that these organisms were easily distinguished from

the fluorescent ones contained in clusters 4 - 6 (Fig. 75). The proportion (75%) of isolates from beef which were identified with *Pseudomonas fragi* was of the same order as that (83%) obtained with isolates from British fresh sausage (Fig. 77) and in the study (73%) described on p. 258 - 285 . Thus it was concluded that changes in composition due to manufacturing methods had not influenced appreciably the pseudomonad flora of sausages over a 15 year period.

Figure 73. Simplified dendrogram of pseudomonads and other obligately aerobic Gram-negative bacteria isolated from sausage ingredients and fresh pork sausage.

Footnote: Percentage similarity calculated with the S_{SM} coefficient; clustering by UPGMA

A *Pseudomonas fluorescens* biotype A (Stanier *et al.*, 1966)

B *Pseudomonas fluorescens* biotype C (" " ")

C *Pseudomonas fragi*

D *Pseudomonas fragi*

E *Pseudomonas fragi*

F *Moraxella* spp.

I Unassigned

II *Alteromonas putrefaciens*

III *Pseudomonas fluorescens* biotype G (Stanier *et al.*, 1966)

1. *Pseudomonas cepacia* NCIB 9089 + FD 2001

2 *Ps. aeruginosa* NCIB 8295

3 *Pseudomonas* ex. sausage

4 *Ps. maltophilia* NCIB 9203

5 *Pseudomonas* sp. N902C

6 *Ps. stutzeri* NCIB 10783

8 *Acinetobacter calcoaceticus* NCIB 8250

9 *Ps. testosteroni* NCIB 8893

10 *Ps. alcaligenes* NCIB 9945

11 *Ps. aeruginosa* NCTC 5083

12 *Pseudomonas* sp. N902D

13 *Ps. aeruginosa* N903

14 *Ps. putida* NCIB 9887

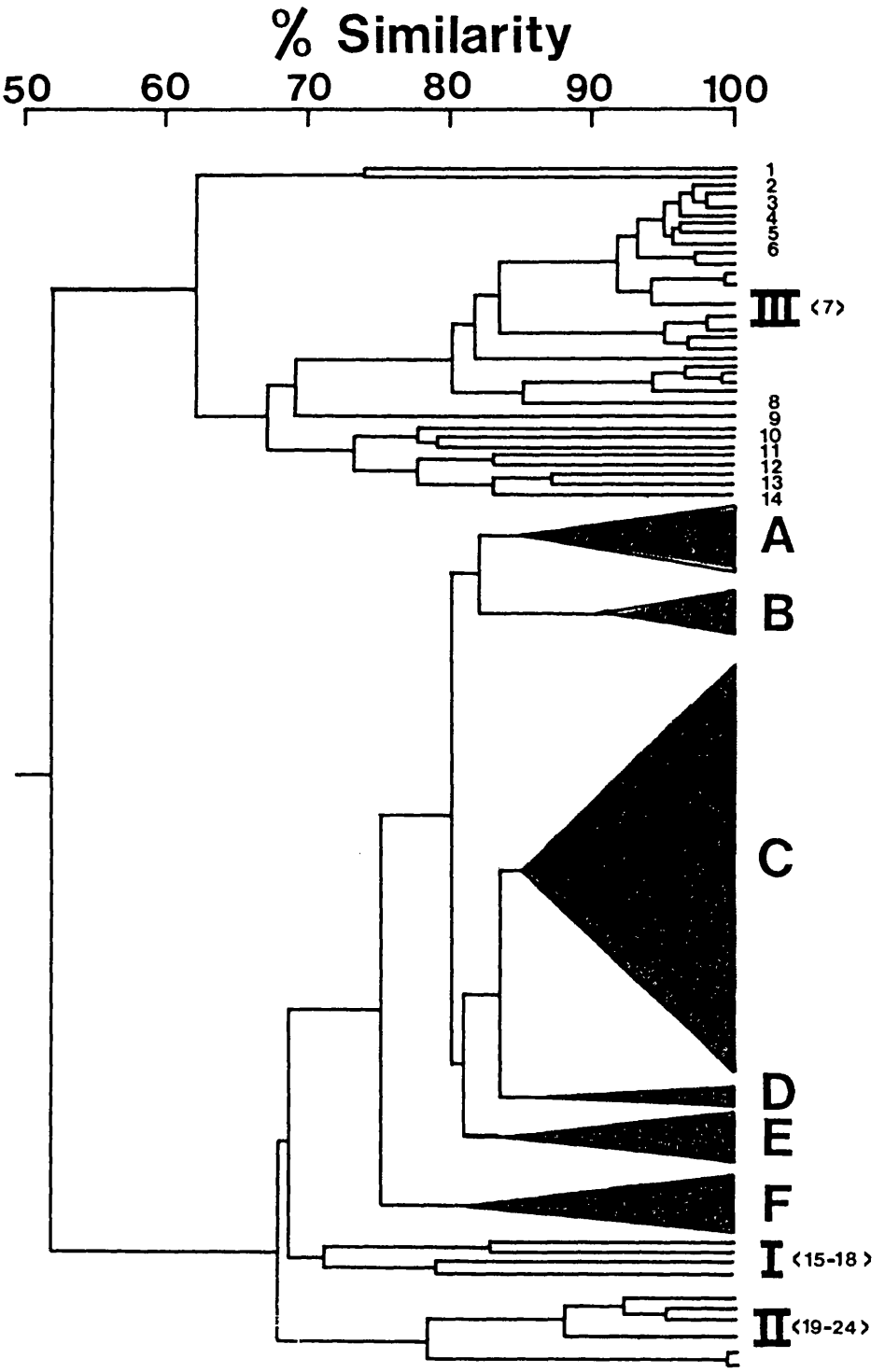
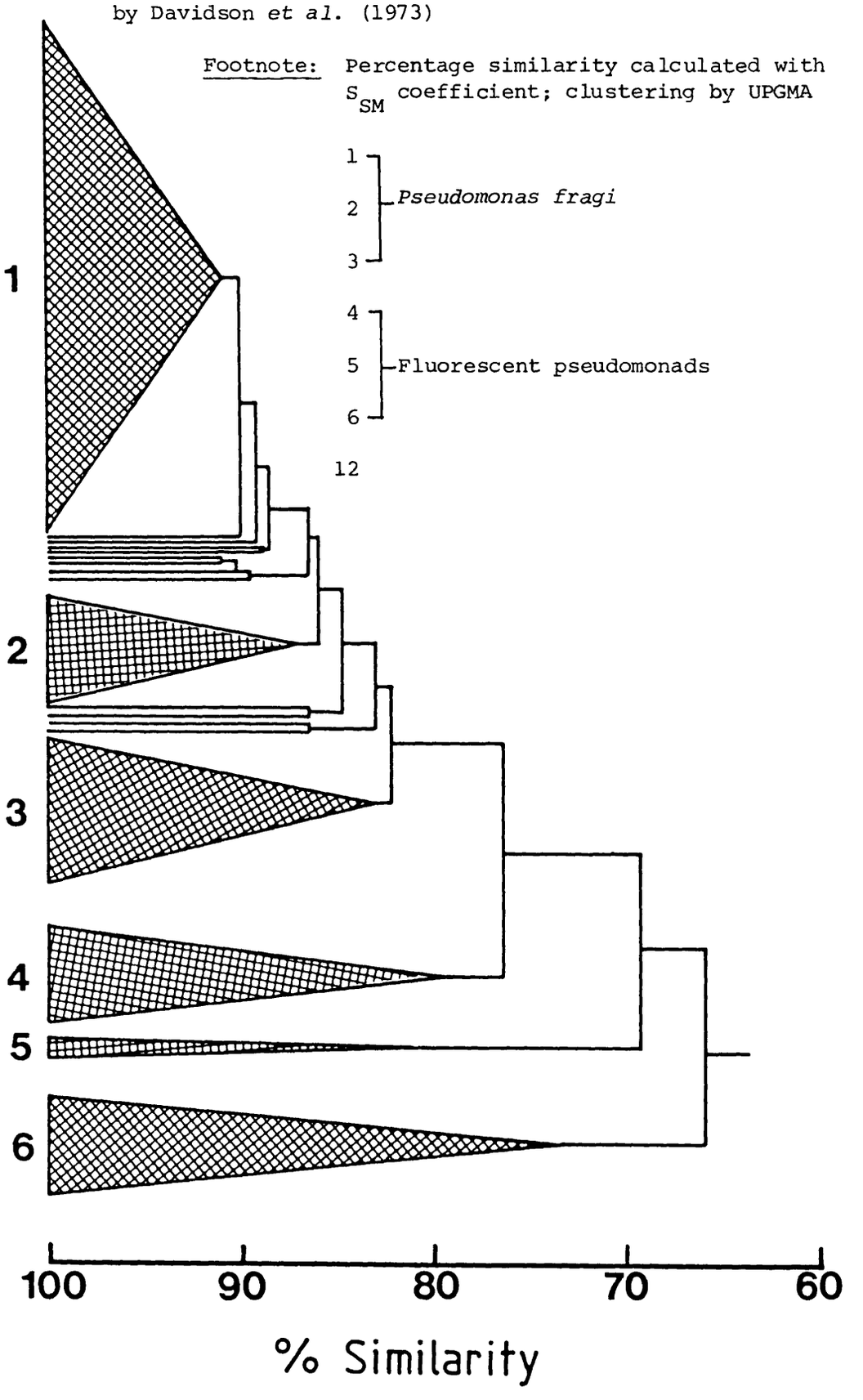

% Similarity
50
60
70
80
90
100
1
2
3
4
5
6
III (7)
8
9
10
11
12
13
14
A
B
C
D
E
F
I (15-18)
II (19-24)

Figure 75. Simplified dendrogam of 218 strains of *Pseudomonas* spp. isolated from fresh beef and fresh pork sausage by Davidson *et al.* (1973)



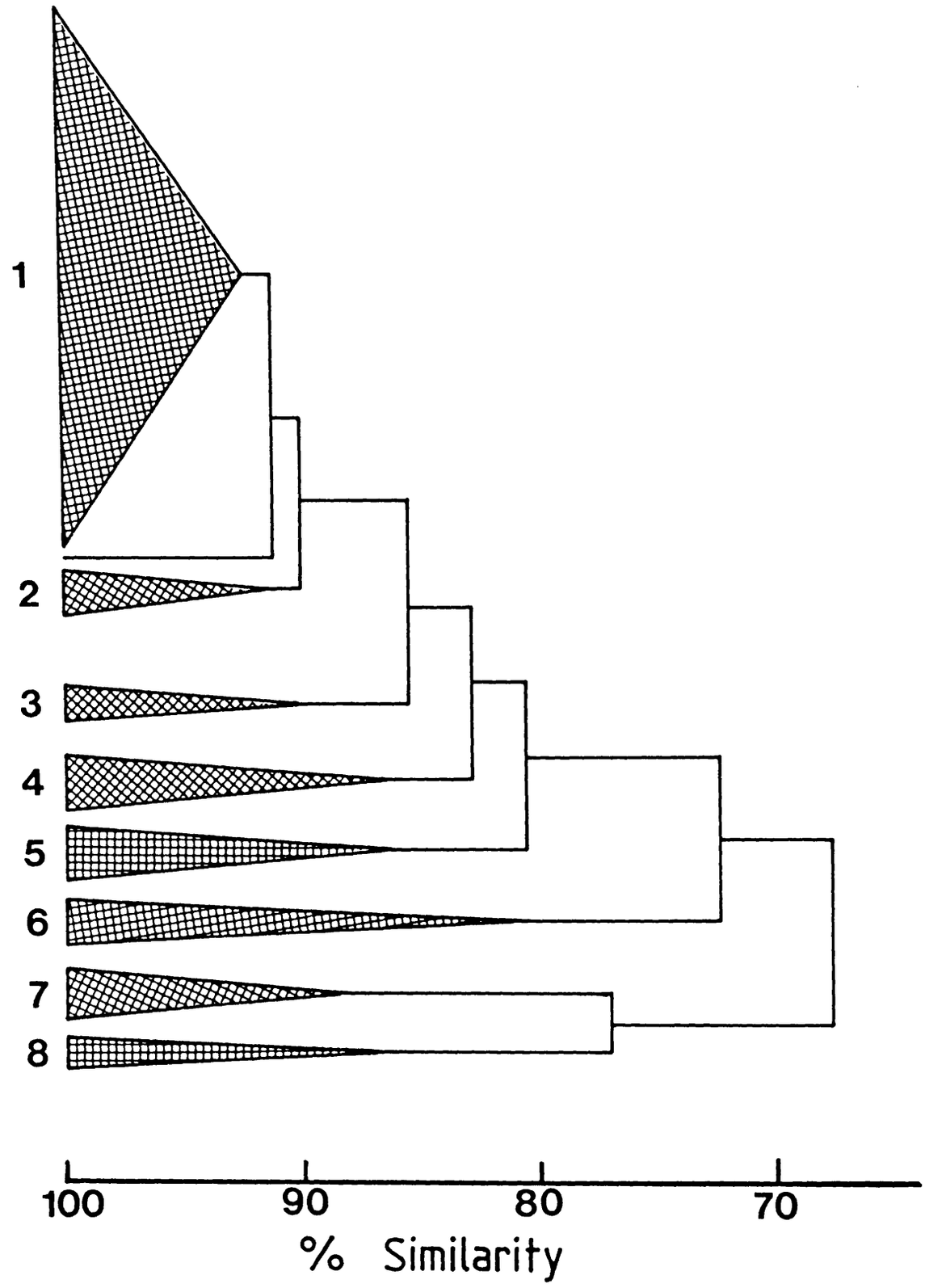
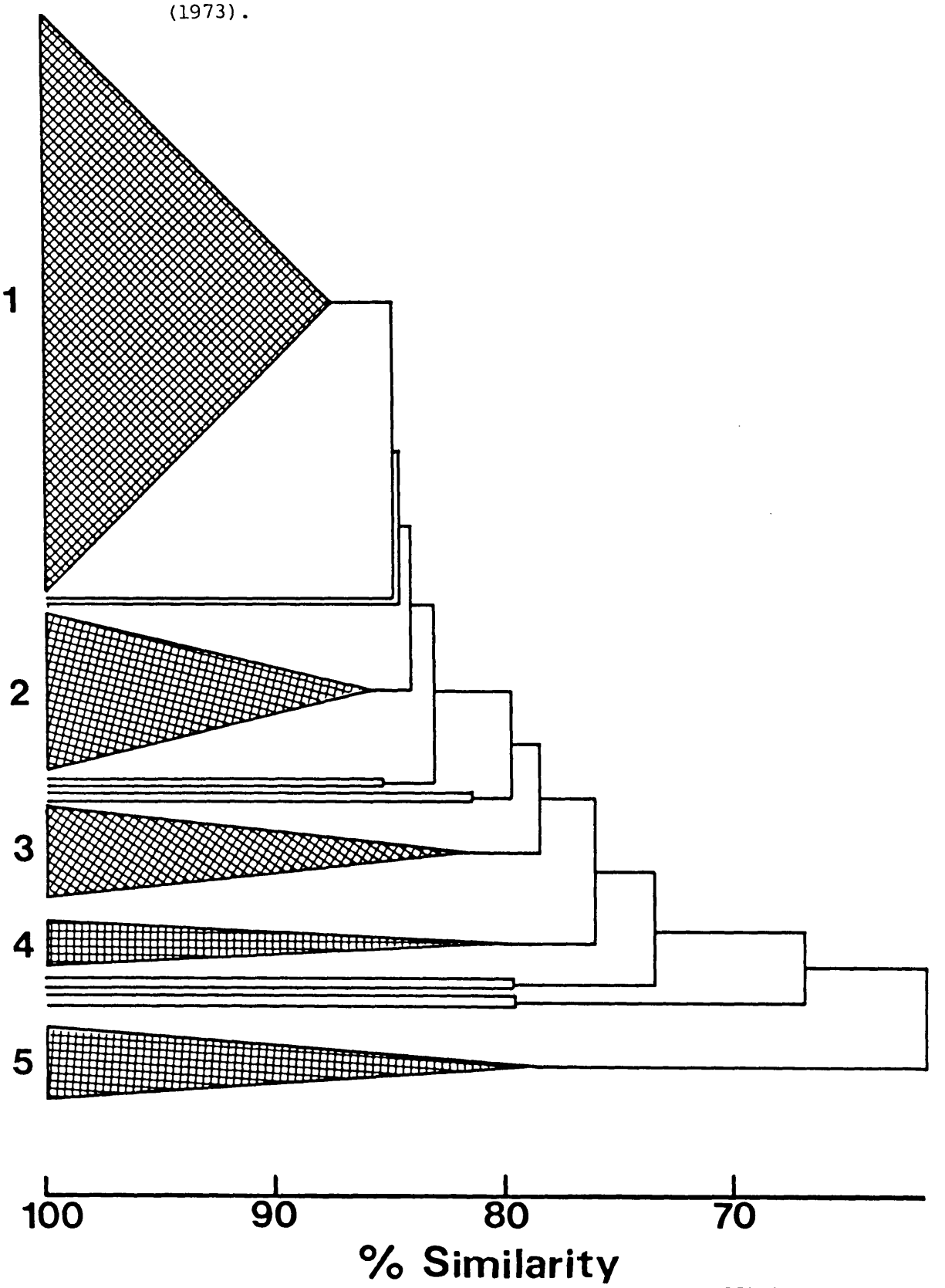


Figure 76. Simplified dendrogram of 104 strains of *Pseudomonas* spp. isolated from fresh beef by Davidson *et al.* (1973)

Footnote: Percentage similarity calculated with S_{SM} coefficient, clustering by UPGMA.

Clusters 1 - 8 unassigned.

Figure 77. Simplified dendrogram of 114 strains of *Pseudomonas* spp. isolated from fresh pork sausage by Davidson *et al.* (1973).



Footnote: Percentage similarity calculated with S_{SM} coefficient; clustering by UPGMA.

Clusters 1 - 5 unassigned.

DISCUSSION

When this project began there was circumstantial evidence (Brown, 1977; Abbiss, 1978) that sausages made on a small scale in a test kitchen or laboratory rarely mimiced the commercial product especially with respect to the size and composition of the micro-flora. As a consequence the majority of the present study was done with material taken at the point of bowl-chopping in the factory of a large sausage manufacturer. Thereafter the material was treated in such a manner that it simulated commercial production. During studies of changes in the growth and composition of the microflora, particular attention was given to the previously ignored Gram-negative organisms, *Pseudomonas* spp. and Entero-bacteriaceae. The development of an analytical technique that distinguished between bound and free sulphite was an essential stage in this study also. Indeed the technique proved to be particularly valuable in studies of sulphite-inhibition of *Salmonella*.

Analysis of the ingredients of fresh sausage showed that all the major members of the microbial association were present, the size of the populations being influenced by the ingredient and methods of preparation. Thus mincing of ingredients and then storage at chill temperatures was associated with a moderate increase in the numbers of micro-organisms. This was attributed in part to the re-distribution of micro-organisms on the meat but mainly to the growth of psychrotrophs. As mincing of meat releases enzymes which cause a rise in the concentration of glucose (Newbold and Scopes, 1971), microbial growth was probably aided

also by a fresh supply of nutrients. Indeed Ayres (1955) contended that comminuted meat can support a 10-fold greater population than jointed meat because of the increased surface area and an enhanced supply of nutrients. Backfat harboured fewer organisms than minced meat. This could be due to the low nutrient status of such material as well as the limited contamination to which fat is exposed because of the protection afforded by the skin until butchering. Oxygen-catalysed toxic substances e g. hydroxyls (OH^-), singlet oxygen ($^1O_2$), peroxides (O_2^-) and hydrogen peroxide (H_2O_2) may also be involved in restricting microbial growth on fat. The rinds invariably contained large populations of micro-organisms. As this was a cooked ingredient, post-cooking contamination before, during and after mincing,together with opportunity for microbial growth all contributed to the large populations. Thus it was the practice of the factory to cook the rinds and then allow them to cool slowly at ambient temperature before mincing and storage overnight at chill temperatures. Further evidence of factory practices increasing the level of contamination of ingredients came from studies of sausages made in the course of a working week. The chill storage of butchered meat over the weekend was shown to influence significantly the extent of the initial contamination of sausages (Fig.20). Thus on Mondays and Tuesdays sausage contained > 20 fold more micro-organisms than those produced on Wednesdays, Thursdays or Fridays. This situation is likely to be due to the growth of micro-organisms on the meat rather than to build up of contaminants over the weekend in or on the processing equipment which was thoroughly cleaned at the end of each shift.

It was notable that the size of the initial microbial population

in fresh sausage was related inversely to that of the final (climax) population (Fig. 21). This was noted by Dowdell and Board (1971) and Brown (1977). It is possible that in a sausage having a high initial load (usually lactobacilli) the ratio of one of the co-dominant groups of micro-organisms (e.g. yeasts or *Brochothrix thermosphacta*) to another may influence the capability of the microbial association as a whole to achieve high climax numbers. Although the reasons for this have not been elucidated in the present work, it is possible that preferential utilization of a particular carbon or energy source or production of a selective growth-stimulating/inhibiting compound may be involved. Of practical significance, however, is the fact that the presence of a low initial microbial load, as compared with a high initial load, enables the microbial association to develop at a faster rate. Thus caution should be exercised by the "trade" should they aim for a product having a low initial contamination. By doing so, the selection of a microbial association capable of faster growth might accelerate spoilage.

The hypothesis that the contribution of a particular group of micro-organisms to the microbial association in sausage might be determined by the concentration of sulphite which inhibited their growth was tested in broth culture with strains isolated from sausage (pp. 151 - 157). In general the strains of yeasts and *Brochothrix thermosphacta* tested were tolerant of concentrations of free sulphite in excess of those which would occur in fresh sausage. The lactobacilli and streptococci were inhibited by intermediate concentrations, especially with incubation at chill temperatures, of free preservative (Fig. 47) whereas the Gram-negative bacteria, pseudomonads and Enterobacteriaceae, were prevented from growth by

a lower range of sulphite (Fig. 55). Thus the microbial association can be defined in terms of sulphite-tolerance. There is evidence (p.168 - 187) that the efficacy of sulphite is enhanced by chill temperatures. The more rapid binding and oxidative loss of active sulphite at higher temperatures are factors which are likely to diminish the effectiveness of the preservative. Indeed the evidence presented on pp. 160 - 163 (Figs. 64 - 66) and pp. 155- 157. (Fig. 58) suggests that the decline in the concentration of free sulphite in sausage or broth is associated with the subsequent growth of Pseudomonadaceae and Enterobacteriaceae.

In an exceptional case, a batch of sausage was found to contain unusually high concentrations of sulphite. Although the rate of growth of the yeasts was suppressed slightly, those of *Brochothrix thermosphacta,* pseudomonads and Enterobacteriaceae were drastically affected (Figs. 39 b, 41 a, b). Thus yeasts dominated during storage. The substantial growth of lactobacilli and streptococci may have been associated with an increased availability of growth factors of yeast origin. It is notable that Brown (1977) suggested that a'yeast-dominated' sausage was a factory-specific phenomenon associated with a concomitant low initial bacterial (i.e. *Brochothrix thermosphacta*) count. Although he did not speculate upon the reasons, analysis of his data reveals that the sulphite content of such sausages was significantly higher than in those dominated by *Brochothrix thermosphacta*. He also noted that the loss of free sulphite was faster in the yeast-dominated sausages and attributed this to increased binding by agents of yeast origin. Although sulphite-binding compounds were producedby pure cultures of yeast in broth

systems (Fig. 59), the evidence obtained in the present study did not link unequivocally the growth of yeasts in sausages with free sulphite depletion.

As the results presented above suggested that the Gram-negative portion of the microbial association was the most sensitive to sulphite, the composition of their populations in unsulphited and sulphited sausage was assessed with the view to detecting whether or not subtle selection of the organisms among the initial contaminants occurred during storage.

There was no such selection in the Pseudomonadaceae, pp. 258-285. Isolates from the ingredients, sulphited or unsulphited sausage were identified with the major clusters shown in Fig. 73. Analysis of the composition of the populations of Enterobacteriaceae, on the other hand, highlighted differences between the species dominant in preserved or unpreserved sausage (Table 22). Thus *Escherichia coli, Enterobacter cloacae, Serratia liquefaciens* and *Yersinia* spp. were isolated frequently from the former, whereas *Enterobacter agglomerans* and a biotype of *Hafnia alvei* from the latter. The hypothesis that this selection was due to sulphite-sensitivity was tested in batch broth culture. *Enterobacter agglomerans* (minimum inhibitory concentration, MIC = 50 - 100 µg free sulphite/ml) was particularly sensitive whereas some strains of *Hafnia alvei* (MIC = 210 - 264 µg/ml) and *Serratia liquefaciens* (MIC = 200 - 250 µg/ml) were relatively tolerant of the preservative. Although such results tend to support this hypothesis, the range of sensitivity of strains of *Escherichia coli* to sulphite was such that the relative abundance of this species in the sulphited, but

not the unsulphited product was not due to the concentration of sulphite alone. It should be stressed that sausage contains a wealth of carbon and energy sources in the form of starch (Abbiss, 1978). Thus rusk (*ca.* 840 mg insoluble carbohydrate/g) and lean pork (*ca.* 2.2 mg/g) are likely to contribute as substrates for microbial growth. Because of the occurrence of two α-amylases and a maltase of meat origin, glucose and maltose are available to the microbial association throughout storage of sausage (Abbiss, 1978). Although lipids are probably used also (*c.f.* accumulation of valerate, Abbiss, 1978) there is probably a "sparing effect" (Mossel and Ingram, 1955) on protein. Indeed it has been shown recently (Dainty and Hibbard, 1983) that *iso*-valeric, *iso*-butyric and 2-methylbutyric acids may be formed by *Brochothrix thermosphacta* from leucine, valine and *iso* leucine respectively and contribute to spoilage odours (Dainty and Hibbard, 1980; Stanley *et al.*, 1981). A β-amylase has been detected in rusk also and probably contributes to changes in the concentration of carbohydrate in sausage. As amylases contain relatively few disulphide bridges they are resistant to denaturation by sulphite, but it is possible that hydroxy-sulphonate derivatives are formed, removing sugars from sausage as a consequence. Abbiss (1978) concluded, however, that the concentrations of sugars (glucose, maltose, maltotriose and malto-tetrose) found in fresh sausage were substantially greater than those required theoretically to bind all the sulphite at point of manufacture. As the presence of the preservative is always reflected in the composition of the microbial association, by inference, free sulphite must always be present. Moreover as lactate and acetate accumulate in the unsulphited but diacetyl and acetoin predominate in the sulphited sausage (Abbiss, 1978), the preservative

appears, as suggested by Brown (1977) to influence the fermentation pathways of members of the association. It is notable, moreover that the addition of sulphite (*ca.* 450 µg total sulphite/g) to minced beef or pork results in selection akin to that observed in fresh sausage (Nychas, pers. comm), the growth of *Pseudomonas fragi* and Enterobacteriaceae being severely curtailed. Sulphite results also in a sparing effect on the low concentrations (*ca.* 0.1 - 0.2%) of glucose, and eventual spoilage is manifested by souring in sulphited mince *cf.* putrefaction in the control. The evidence presented in the above discussion suggests that sulphite elects for a particular type of microbial association in fresh sausage, selectively inhibiting certain groups, e.g. the Pseudomonadaceae and Enterobacteriaceae, whilst allowing others e.g. the yeasts and *Brochothrix thermosphacta* to grow. This situation directs attention to possible mechanisms of action of the preservative.

Gould and his co-workers (1983), for example, surmised that sulphite probably reacts with enzymes, particularly those associated with the tricarboxylic acid (TCA) cycle whereas others (e.g. Freese *et al.*, 1973; Warth, 1977) inferred a reaction as a "proton ionophore". The oxidative function of the TCA cycle, mediated by dehydrogenases, produces the reduced nucleotide, nicotinamide adenine dinucleotide (NADH), subsequent reoxidation to NAD^+ being coupled to ATP production. Thus NADH may be considered not only as an end-product, but as a repressor of citrate synthase (Fig. 85). Weitzmann (1966) noted this phenomenon in *Escherichia coli*. The citrate synthases of Gram-positive organisms, on the other hand, are rarely affected by NADH (Weitzmann and Jones, 1968; Table 49).

Table 49. Regulatory patterns among bacterial citrate synthases*

NADH inhibition	
AMP reactivation	No AMP reactivation
Acinetobacter spp.	*Arizona arizonae*
Moraxella spp.	*Aeromonas formicans*
Pseudomonas spp.	*Erwinia uredovora*
	Escherichia coli
	Hafnia alvei
	Klebsiella spp.
	Proteus spp.
	Salmonella spp.
	Serratia marcescens
No NADH inhibition	
Arthrobacter spp.	
Bacillus spp.	*Streptomyces* spp.
Clostridium spp.	*Microbacterium thermosphactum*
Micrococcus spp.	*Staphylococcus aureus*
Nocardia spp.	*Kurthia zopfii*

* Adapted from Weitzmann (1980)

The repressed enzymes of strictly-aerobic Gram-negative bacteria were reactivated by adenosine monophosphate (AMP); AMP had no demonstrable action on those of facultatively-anaerobic Gram-negative bacteria, (Table 49). It is noteworthy that these patterns and those of sulphite-sensitivity appear to be common. Thus those micro-organisms - the yeasts, *Brochothrix thermosphacta*, micrococci and *Kurthia zopfii* (Dowdell and Board, 1971) - which are not influenced by sulphite possess citrate synthases which are not inhibited by NADH. In contrast, the Enterobacteriaceae, markedly sulphite-sensitive organisms, have citrate synthases which are inhibited irreversibly (i.e. no AMP reactivation) by NADH. Those organisms in which NADH inhibition is relieved by AMP, e.g. *Moraxella* spp. and *Pseudomonas* spp. are ones which are inhibited only by moderate concentrations of sulphite in broth and which grow to a limited extent in sulphited sausage (Table 49).

Sulphite reaction with NAD^+ that prevents energy-yielding oxidative reactions is well known (Meyerhof *et al.*, 1938; Dupuy, 1959). Indeed this reaction is most marked (Dupuy, 1959) at the pH values found in sausages. Rehm (1964) and Wallnöfer and Rehm (1965) tentatively identified the NAD-dependent step from malate to oxaloacetate in *Escherichia coli* as being the most probable target for sulphite.

As well as citrate synthase, malate dehydrogenase and pyruvate dehydrogenase are also subject to NADH inhibition and are thus possible targets for sulphite (Fig. 85). As citrate synthase activity is inhibited by α-oxoglutarate (Gottschalk, 1979), it is

possible that feedback repression by high concentrations of α-oxoglutarate may occur with sulphite blocking of the NAD-dependent α-oxoglutarate → succinyl-CoA step in the TCA cycle (Fig. 85). Such a lesion could result from an indirect reaction of sulphite with NAD, or by direct inhibition of the α-oxoglutarate dehydrogenase complex (Fig. 85). It is notable that the above reaction occurs only in Enterobacteriaceae (Sanwal, 1970) - the most sulphite-sensitive micro-organisms. During anaerobic growth, which might be expected to occur in the centre of sausage, energy is generated by fermentation.

Thus in Enterobacteriaceae, α-oxoglutarate dehydrogenase is absent and the TCA cycle is modified (Fig. 86). Although the branched non-cyclic pathway satisfies the biosynthetic demands for both α-oxoglutarate and succinyl-CoA (Amarasingham and Davies, 1965), sulphite may impede further utilization of α-oxoglutarate, thereby inhibiting citrate synthase by feedback repression.

Although it is evident from the above discussion that sulphite may inhibit aerobic metabolism, the Enterobacteriaceae are capable also of anaerobic mixed acid and butanediol fermentations. Organisms comprising the genera *Escherichia, Salmonella* and *Shigella* ferment sugars to lactic, succinic and formic acids (Fig. 87) with the concomitant production of carbon dioxide, hydrogen and ethanol. Species of the genera *Enterobacter, Serratia* and *Erwinia* produce less acid but more carbon dioxide, ethanol and 2,3-butanediol (Fig. 88).

It is theoretically possible that sulphite will interfere with

dehydrogenase enzymes (Figs 87, 88) and may even combine with acetaldehyde and remove the latter from subsequent reactions, e.g. regeneration of oxidised NAD^+ by ethanol dehydrogenase. Shortage of NAD^+ would prevent activity of glyceraldehyde-3-phosphate dehydrogenase and therefore prevent flow of intermediates *via* glycolysis and further decarboxylation of pyruvate. In this respect it is noteworthy that sulphite prevents two features from occurring in fresh sausage, (1) the rapid and extensive proliferation of the Enterobacteriaceae and (2) a dramatic decrease in the pH (pp. 129 - 130, Figs. 29 and 34). It follows that sulphite- perturbation of the metabolism of the Enterobacteriaceae may account for the non-occurrence of both features noted above.

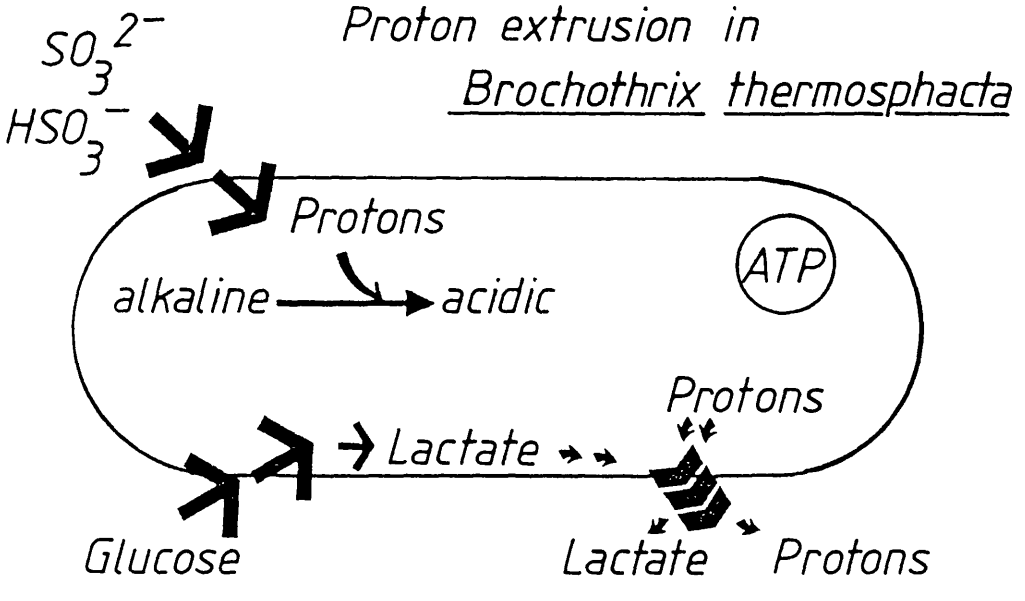
It needs to be stressed, however, that the above hypothesis and that discussed later (pp. 305 - 307) do not explain fully the anomolous results obtained with rifampicin-resistant *Salmonella* serotypes in sulphited broth stored at 4° and 12° C (pp. 152 - 154). Pseudomonadaceae use the Entner-Doudoroff pathway for sugar degradation. As in the pentose phosphate cycle, glucose-6-phosphate, is first dehydrogenated to yield 6-phosphogluconate (Fig. 89) which in turn is converted by a dehydratase and an aldolase to one molecule of 3-phosphoglyceraldehyde (3-PGA) and 1 molecule of pyruvate. The 3-PGA can then be oxidised to pyruvate by the enzymes of the Embden-Meyerhof pathway. Some pseudomonads, e.g. *Pseudomonas aeruginosa*, *Ps. fluorescens* and *Ps. putida*, contain a glucose dehydrogenase which converts glucose to gluconate. The latter can be alternatively phosphorylated to 6-phosphogluconate or further oxidised to 2-ketogluconate which is subsequently phosphorylated and reduced to yield 6-phosphogluconate (Fig. 90).

Thus the membrane-bound glucose and gluconate dehydrogenases facilitate the extracellular conversion of glucose into compounds which are less preferred substrates for competing micro-organisms. The pseudomonads are then capable of transporting and degrading glucose, gluconate and 2-ketogluconate. As sulphite inhibits the growth of pseudomonads in sausage and mince, whilst conserving the level of glucose in these products, it is feasible that it reversibly inhibits the dehydrogenase step from glucose-6-phosphate to 6-P-gluconate (Entner-Doudoroff pathway) as well as limiting the activities of glucose and gluconate dehydrogenases in extracellular glucose oxidation. One additional hypothesis which can be discussed in order to explain both the growth inhibition of the Enterobacteriaceae and the successful colonization by *Brochothrix thermosphacta* of sausage needs comment.

It has been suggested (p. 52) that sulphite may cause inhibition of growth by entering the cell, lowering the pH and releasing protons. In an attempt to expel protons and re-establish the membrane proton gradient, the cell might expel ATP. As internal acidification must be avoided, removal of protons against a steep concentration gradient, is mandatory. Such "proton export" is energy demanding and diminishes the amount available for cell synthesis. As the growth rate is reduced, the fraction of a cells' energy utilized for maintenance rises so that energy available for additional proton-extrusion becomes even less (Gould *et al.*, 1983).

Michels *et al.* (1979), on the other hand, have surmised that

lactate excretion causes proton efflux thereby generating an appreciable electrochemical proton gradient. Under conditions of homolactic fermentation of glucose this gradient, if coupled to energy transduction, would increase the theoretical ATP yield by *ca.* 30%. *Brochothrix thermosphacta* grows well at 4 - 9°C (Rogers *et al.*, 1980) and 25°C (Hitchener *et al.*, 1979) with L-lactate being the major product of glucose breakdown. Thus it is proposed that during metabolism of glucose to lactate, protons, derived from diffusion of sulphite into the cell, are continually expelled thus sparing ATP and permitting growth *viz.*:



Such symport of protons by homofermentative micro-organisms in the presence of high concentrations of sulphite would be of considerable advantage and may explain in part the significant contribution by *Brochothrix thermosphacta* - and perhaps of

homofermentative lactobacilli (pp. 141 - 145) also - to the microbial association of sausage.

From the above discussion it is evident that the inclusion of sulphite in sausage is largely ineffective against the spoilage association. Thus although future work may elucidate the *modus operandi* of the preservative, there remains little prospect of extending the shelf-life of the product. With this situation in mind an attempt (pp. 308 - 322) was made to identify systems which could be used as adjuncts to, or alternatives to sulphite.

COMMERCIAL IMPLICATIONS

It is evident from the results presented above that, in terms of its influence on the major members of the microbial association, sulphite ought not to be regarded as a preservative when used at the legally accepted levels. Even with the minor members, sulphite at these levels does not act only as a preservative but as an elective agent also. In the light of this evidence, attempts were made to identify, tentatively, systems that could be considered for commercial use with the objective of retarding microbial growth in sausages containing reduced amounts of sulphite.

The efficiency of two preservative systems were examined: sulphite:CO_2 and sulphite:potassium sorbate, these combinations being included in 6 batches of sausage produced on a pilot scale (Table 50). It needs to be stressed that such small scale production of sausage seldom mimics, particularly from a microbiological standpoint, the commercial product. In spite of this reservation, several important trends were evident in the results which may be of significance to "the trade".

Sources and extent of contamination of ingredients of pilot scale sausage

All of the meat ingredients were contaminated heavily with most of the members of the microbial association, although streptococci were present in low numbers only (Table 51). The relatively high initial level of infection of the final sausage mix (*ca.* 10^7 c.f.u./g)

Table 50. Composition of pilot scale batches of sausages (additions to basic recipe)

Batch number	Code	Additions to basic recipe			
		Unsulphited seasonings (RHM 599,1.7%w/w)	Sulphited seasonings (RHM 599,1.7%w/w)	Solid CO_2 (20% w/w)	Potassium sorbate (2,000 µg/g sausage)
1	$-SO_2$	+	-	-	-
2	$+SO_2$	-	+	-	-
3	$-SO_2/+CO_2$	+	-	+	-
4	$+SO_2/+CO_2$	-	+	+	-
5	$-SO_2$/+ sorbate	+	-	-	+
6	$+SO_2$/+ sorbate	-	+	-	+

Table 51. Extent of contamination of ingredients of pilot scale sausage

	Ingredients				
	Lean pork	Belly pork	Head pork	Cooked rinds	Back Fat
Total viable count	7.08*	5.08	5.47	8.22	7.14
Yeasts	3.91	3.76	4.29	4.87	4.51
Br. thermosphacta	ND	ND	ND	ND	ND
Lactobacillus spp.	5.34	3.84	3.84	6.60	5.83
Streptococcus spp.	1.00	1.00	1.77	<1.00	<1.00
Pseudomonas spp.	5.88	4.04	4.90	7.73	6.07
Enterobacteriaceae	4.66	2.44	2.57	5.32	3.97

* Mean count ($\log_{10}$ c.f.u./g sample)

ND Not done

probably reflected the substantial populations in the components of the product.

Loss of sulphite

The inclusion of CO_2 (Fig. 78 b) or sorbate (Fig. 78 c) reduced significantly both the rate and extent of loss of total sulphite. Both of these additives also reduced significantly the initial extent of binding of free sulphite, CO_2 being more effective than sorbate during storage for 7 days at 4° or $10^{\circ}C$ (Table 52). Thus with storage at $4^{\circ}C$ in the former case, the concentration of active preservative was maintained within 50 µg of the initial level, whereas in the latter case, *ca*. 150 µg had been bound by day 7 (Fig 78 b, c).

Influence of sulphite concentration

As in batches of sulphited or unsulphited sausage examined previously (pp. 125 - 132), the temperature of storage influenced directly and the inclusion of sulphite inversely, the rate and extent of growth of the total viable count (Fig. 79a). The yeasts grew in sulphited sausage at $10^{\circ}C$, but not at $4^{\circ}C$; they did not grow at either temperature in unsulphited sausage (Fig. 80a). Although sulphite delayed the onset of growth of *Lactobacillus* spp. at $4^{\circ}C$ for 24 h,the rate and extent of growth thereafter was not influenced by sulphite concentration and large populations were formed by day 7 (Fig. 81 a). The pseudomonads did not grow in either the preserved or unpreserved sausage during the course of the experiment although the populations in sulphited sausage

Table 52. Influence of CO_2 or sorbate on binding of free sulphite

Composition of Batch	Storage temperature (^{o}C)	Duration of Storage (d)	
		0	7
+ SO_2	4	74*	8†
	10	-	4
+ SO_2/+CO_2	4	74	61
	10	-	70
+ SO_2/+ Sorbate	4	80	38
	10	-	17

* Free sulphite as a percentage of total sulphite at day 0

† Free sulphite as a percentage of total sulphite at day 7

were invariably smaller than those in unsulphited ones (Fig. 82 a). Sulphite reduced significantly the rate and extent of multiplication of the Enterobacteriaceae, nevertheless a significant increase in the numbers of these organisms in preserved sausage stored at $4^{o}C$ or $10^{o}C$ occurred by the 7th day of storage, (Fig. 83 a). This situation may be due in part to the rapid loss of free sulphite from the product, (Fig. 78 a). Although temperature of storage influenced directly the rate and extent of acid formation, higher temperatures favouring a marked acid drift, sulphite prevented substantial production of acid even at $10^{o}C$, (Fig. 84 a).

Influence of CO_2 concentration

Addition of CO_2 to sulphited sausage delayed the onset of an increase in the total viable count for up to 48 h at 4^{o} or $10^{o}C$, thereafter the rate of growth at $4^{o}C$ was substantially reduced, (Fig. 79 b). Inclusion of CO_2 in unsulphited sausage did not influence the rate or extent of increase of the total viable count (Fig. 79 b). Although a combination of CO_2 and sulphite reduced significantly the size of the yeast populations at $4^{o}C$ during the 48 h post-manufacture, these organisms grew subsequently, albeit poorly (Fig. 80 b). The presence of CO_2 in the unsulphited product stored at $4^{o}C$ stimulated growth, a situation not evident at the higher temperature of storage (Fig. 80 b). Neither CO_2 in the unsulphited sausage stored at 4^{o} or $10^{o}C$ nor in the sulphited sausage stored at $10^{o}C$ was effective in preventing substantial proliferation of lactobacilli, although the combination of CO_2, sulphite and storage at $4^{o}C$ curtailed growth (Fig. 81 b). Carbon

dioxide alone retarded the growth rate of the pseudomonads such that their populations were lower in the unsulphited product at the 5th day of storage, (Fig. 82 b). As in sausage containing sulphite only, the addition of CO_2 prevented growth of pseudomonads for up to 7 days (Fig. 82 b). Carbon dioxide was effective in retarding the rate of growth of the Enterobacteriaceae in unsulphited sausages and prevented multiplication completely in sulphited ones (Fig. 83 b). The drifts in pH of preserved or unpreserved sausages containing CO_2 were not substantially different from those of 'uncarbonated' batches (Fig. 84 b).

Influence of sorbate

The rate and extent of growth of micro-organisms was drastically reduced in the presence of sorbate and, in unsulphited sausage stored at $4^{o}C$ for example, no significant increase in numbers was evident during the period of storage (Fig. 79 c). The combination of sulphite, sorbate and chilled storage reduced significantly the size of the yeast population for upwards of 48 h post-manufacture, thereafter they began to grow (Fig. 80 c). Sorbate alone, or in combination with sulphite, was not lethal at $4^{o}C$ and $10^{o}C$ or $10^{o}C$ respectively, but it did prevent the growth of the yeasts for up to 7 d (Fig. 80 c). The rate of multiplication of members of the genus *Lactobacillus* was retarded significantly by the addition of sorbate, especially at chill temperatures (Fig. 81 c). The numbers of pseudomonads were reduced to the greatest extent in sausages containing both sulphite and sorbate although after 4 days they resumed growth, albeit slowly, in all batches of sausage, (Fig. 82 c). As with the addition of CO_2, sorbate acted synergis-

tically with sulphite to maintain the Enterobacteriaceae in a quiescent state at 4° or 10°C for up to 7 days; sorbate alone was effective also in preventing growth, (Fig. 83 c). The pH of sausages containing sorbate did not change significantly at either 4° or 10°C over the period of storage, a situation which, in the absence of sulphite, was probably due to the suppression of growth of the acid-producing lactobacilli and Enterobacteriaceae, (Fig. 84 c).

Figure 78. Influence of temperature on loss of free and total sulphite in a, pilot-scale sausage, b, sausage supplemented with carbon dioxide (20% w/w) and c, sausage supplemented with potassium sorbate (2,000 µg/g).

Footnote: ○ 4°C Closed symbols, total sulphite

△ 15°C Open symbols, free sulphite

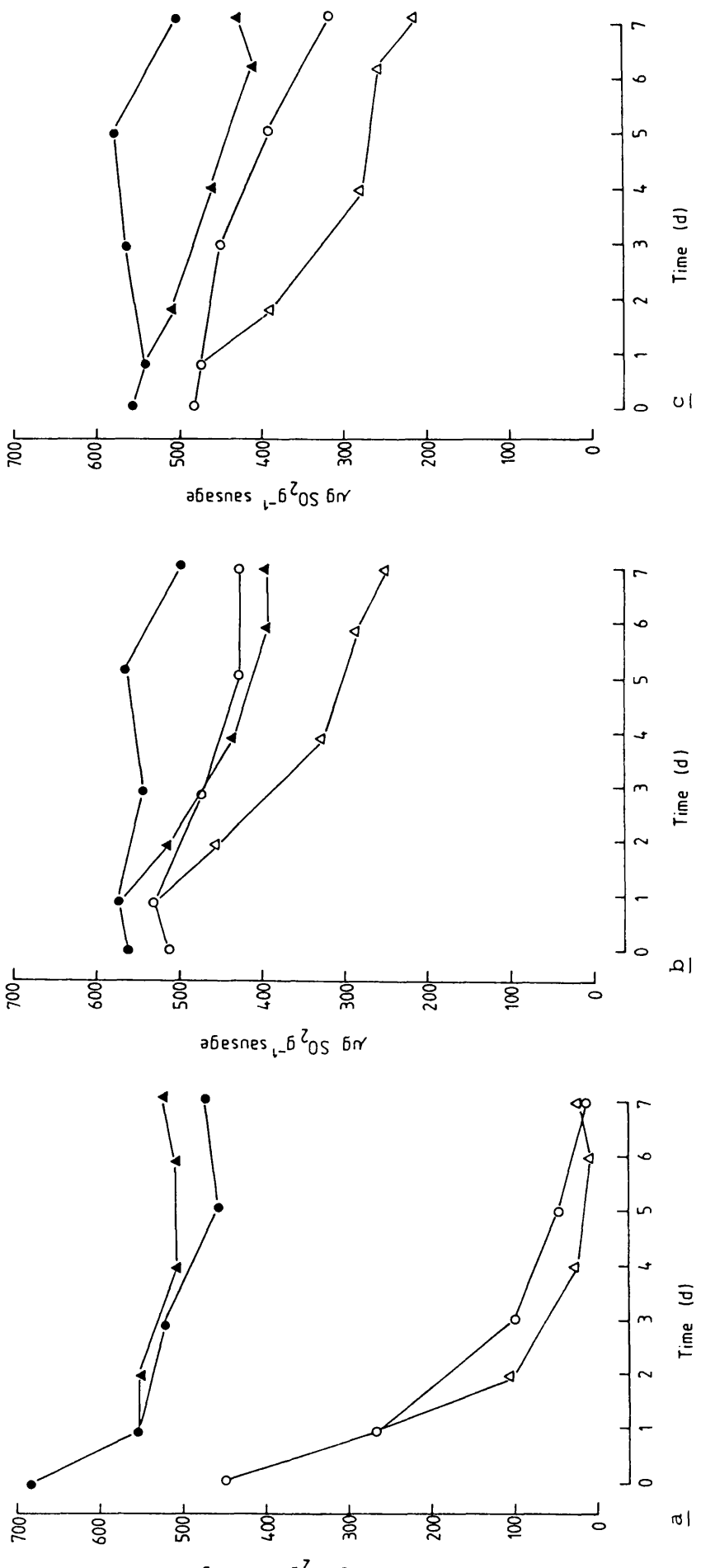


Figure 79. Influence of temperature of storage on growth of the total viable count in a, pilot-scale sausage, b, sausage supplemented with carbon dioxide (20% w/w) and c, sausage supplemented with potassium sorbate (2,000 µg/g).

Footnote:

○ 4°c — Open symbols, sulphited sausage

△ 15°C — Closed symbols, unsulphited sausage

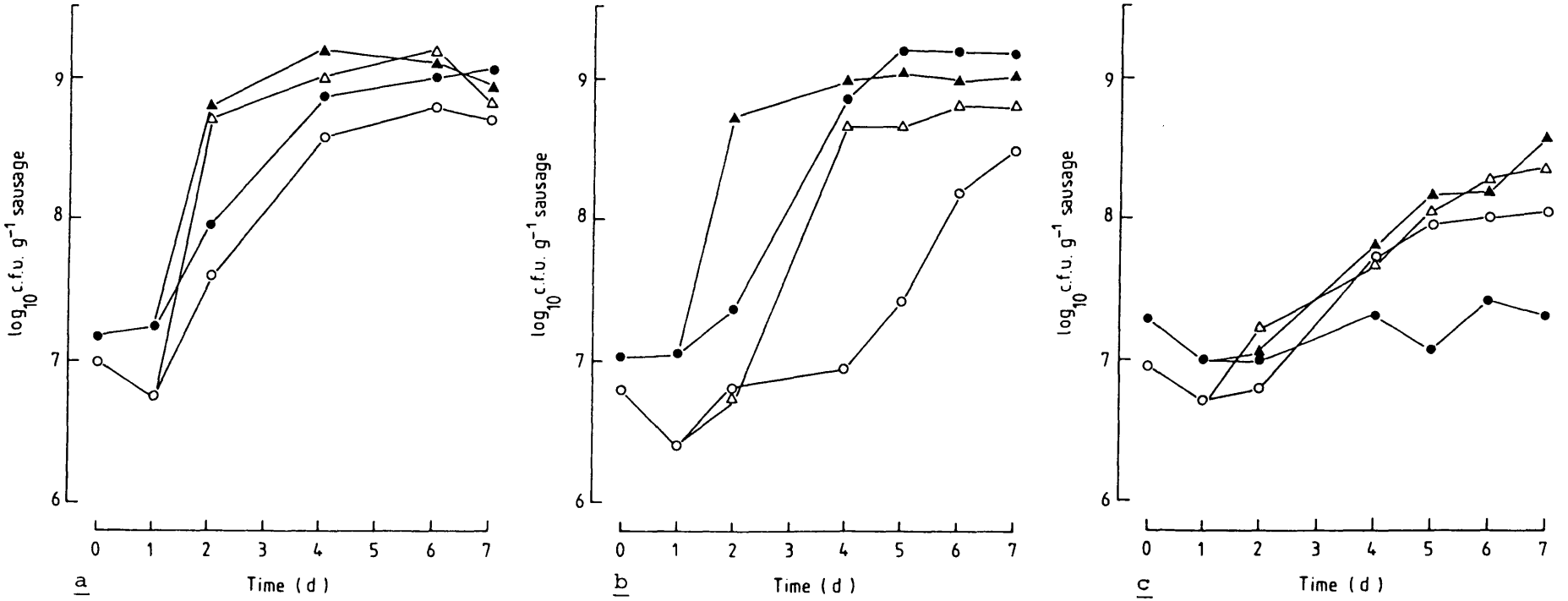


Figure 80. Influence of temperature of storage on growth of yeasts in a, pilot-scale sausage, b, sausage supplemented with carbon dioxide (20% w/w) and c, sausage supplemented with potassium sorbate (2,000 µg/g).

Footnote: As in Fig. 79.

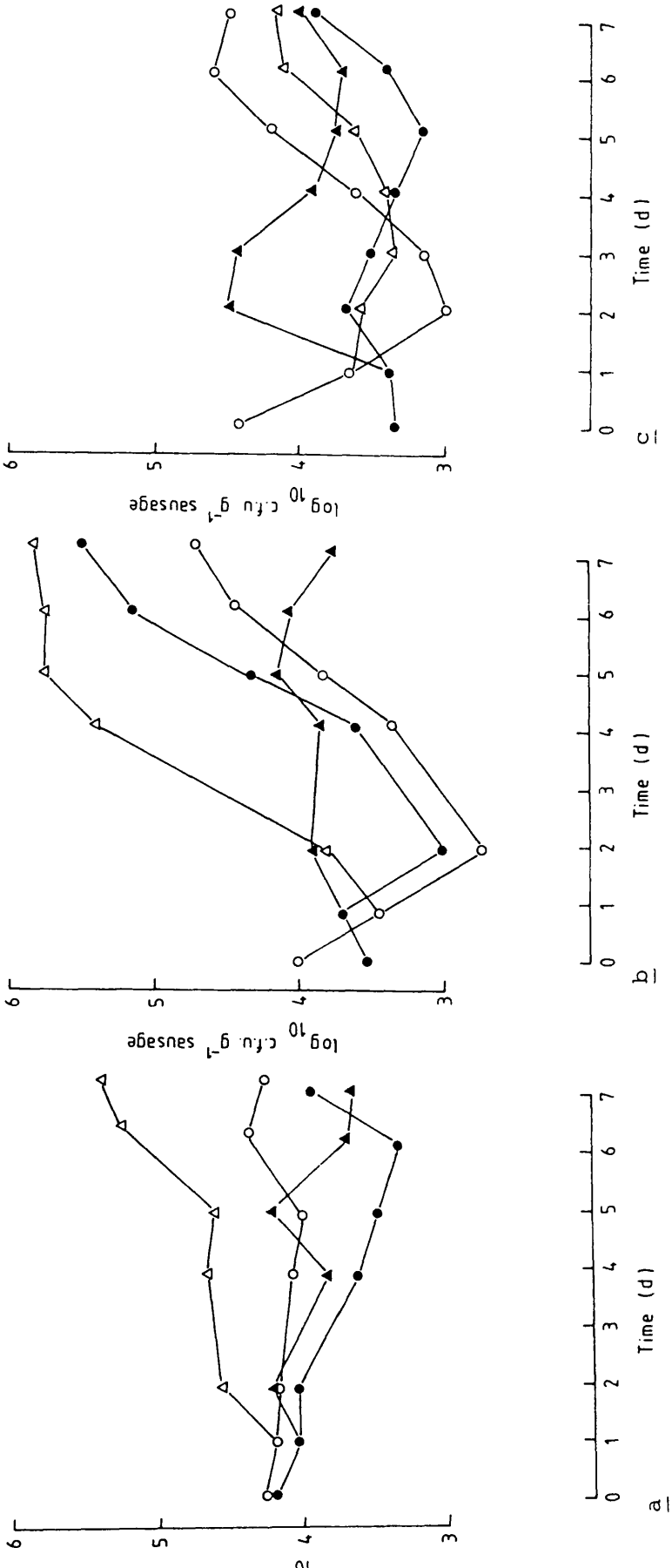


Figure 81. Influence of temperature of storage on growth of *Lactobacillus* spp. in a, pilot-scale sausage, b, sausage supplemented with carbon dioxide (20% w/w) and c, sausage supplemented with potassium sorbate (2,000 µg/g).

Footnote: As in Fig. 79.

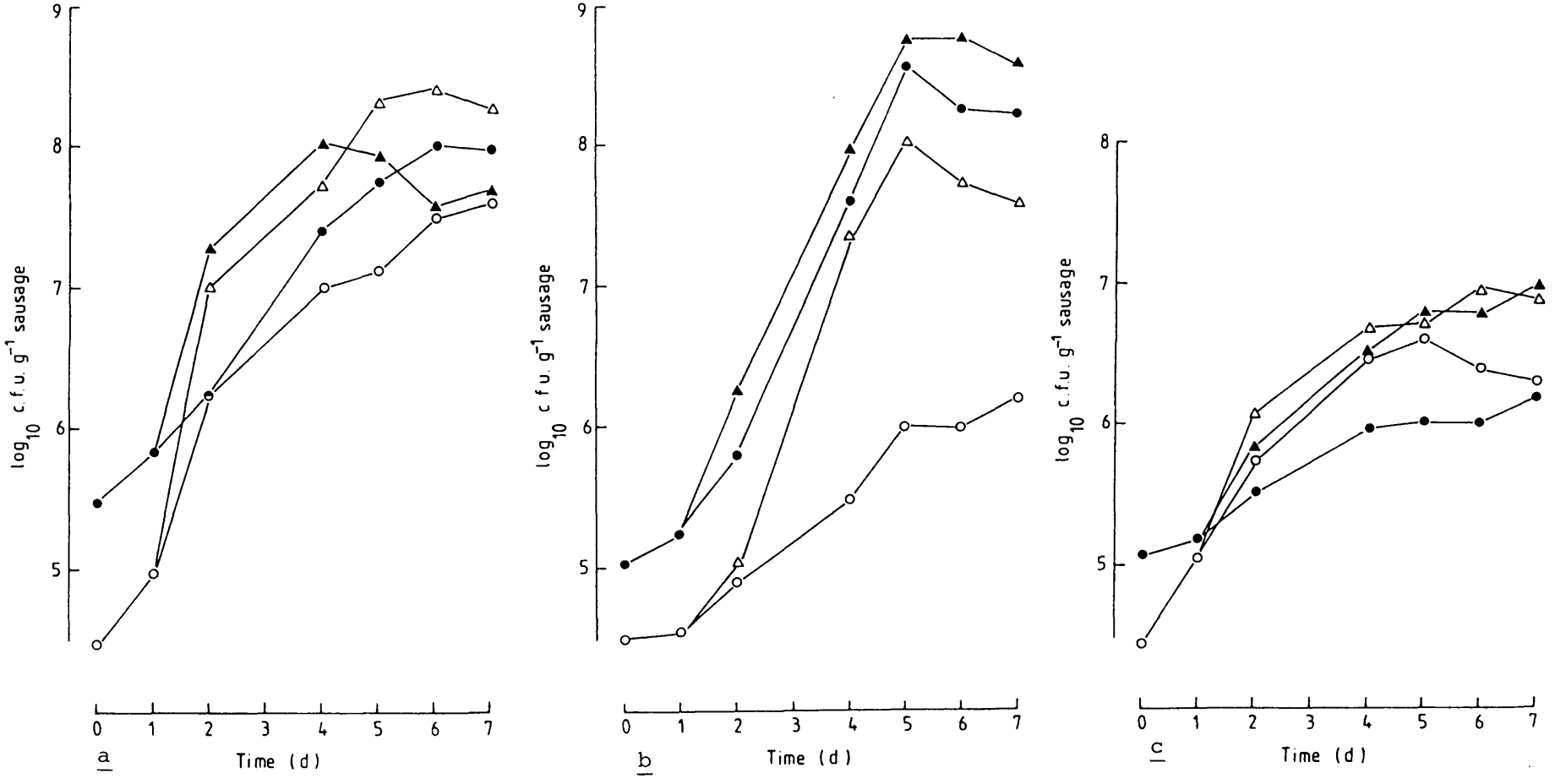
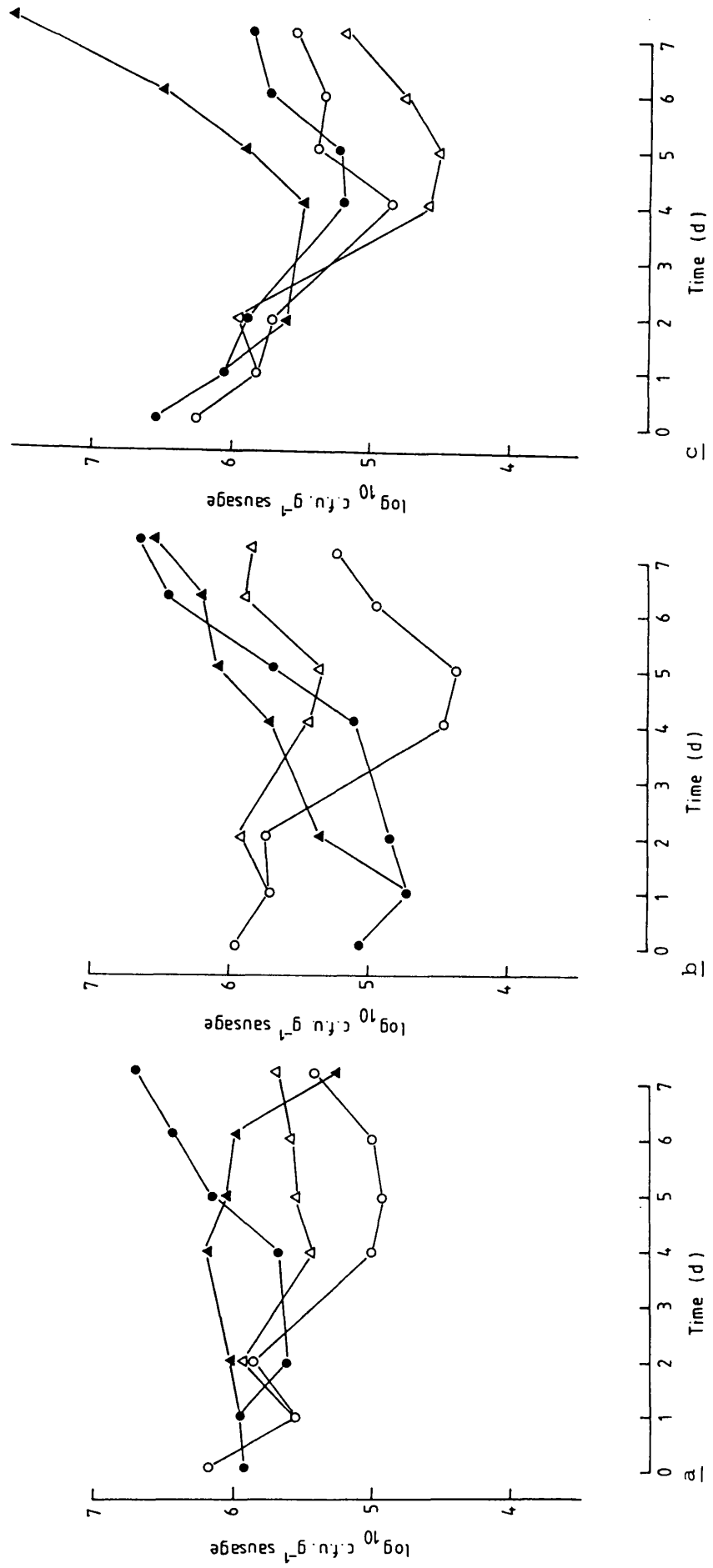


Figure 82. Influence of temperature of storage on growth of *Pseudomonas* spp. in a, pilot-scale sausage, b, sausage supplemented with carbon dioxide (20% w/w) and c, sausage supplemented with potassium sorbate (2,000 µg/g).

Footnote: As in Fig. 79.



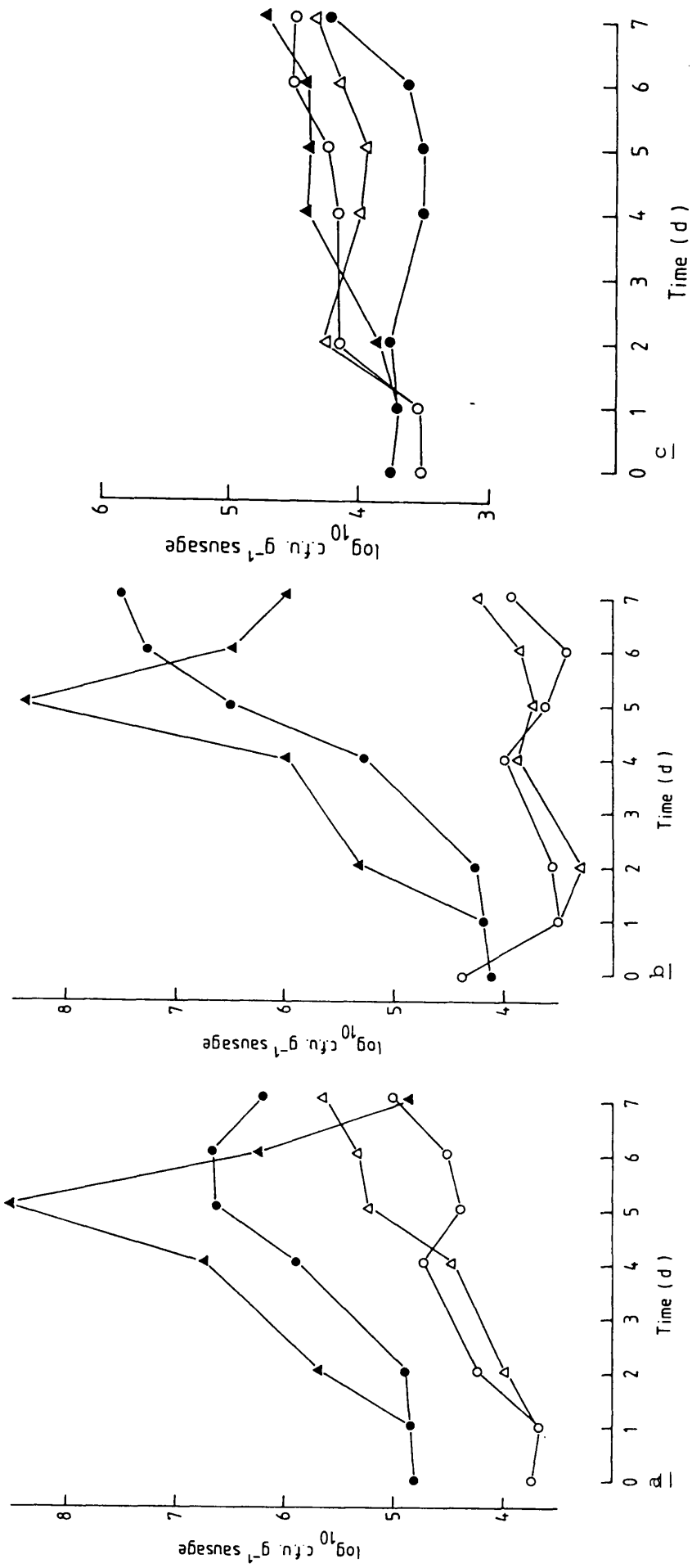


Figure 83. Influence of temperature of storage on growth of Enterobacteriaceae in a, pilot-scale sausage, b, sausage supplemented with carbon dioxide (20% w/w) and c, sausage supplemented with potassium sorbate (2,000 µg/g).

Footnote: As in Fig. 79.

Figure 84. Influence of temperature of storage on pH drift in a, pilot-scale sausage, b, sausage supplemented with carbon dioxide (20% w/w) and c, sausage supplemented with potassium sorbate (2,000 µg/g).

Footnote: As in Fig. 79.

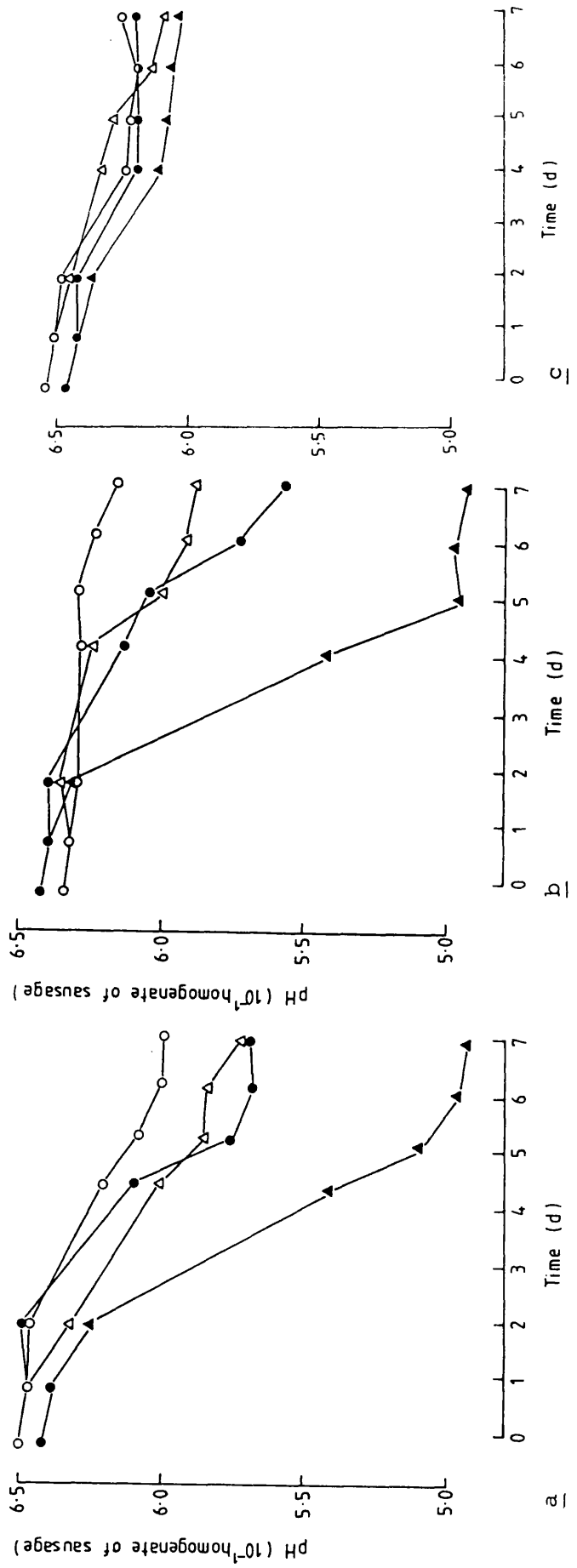
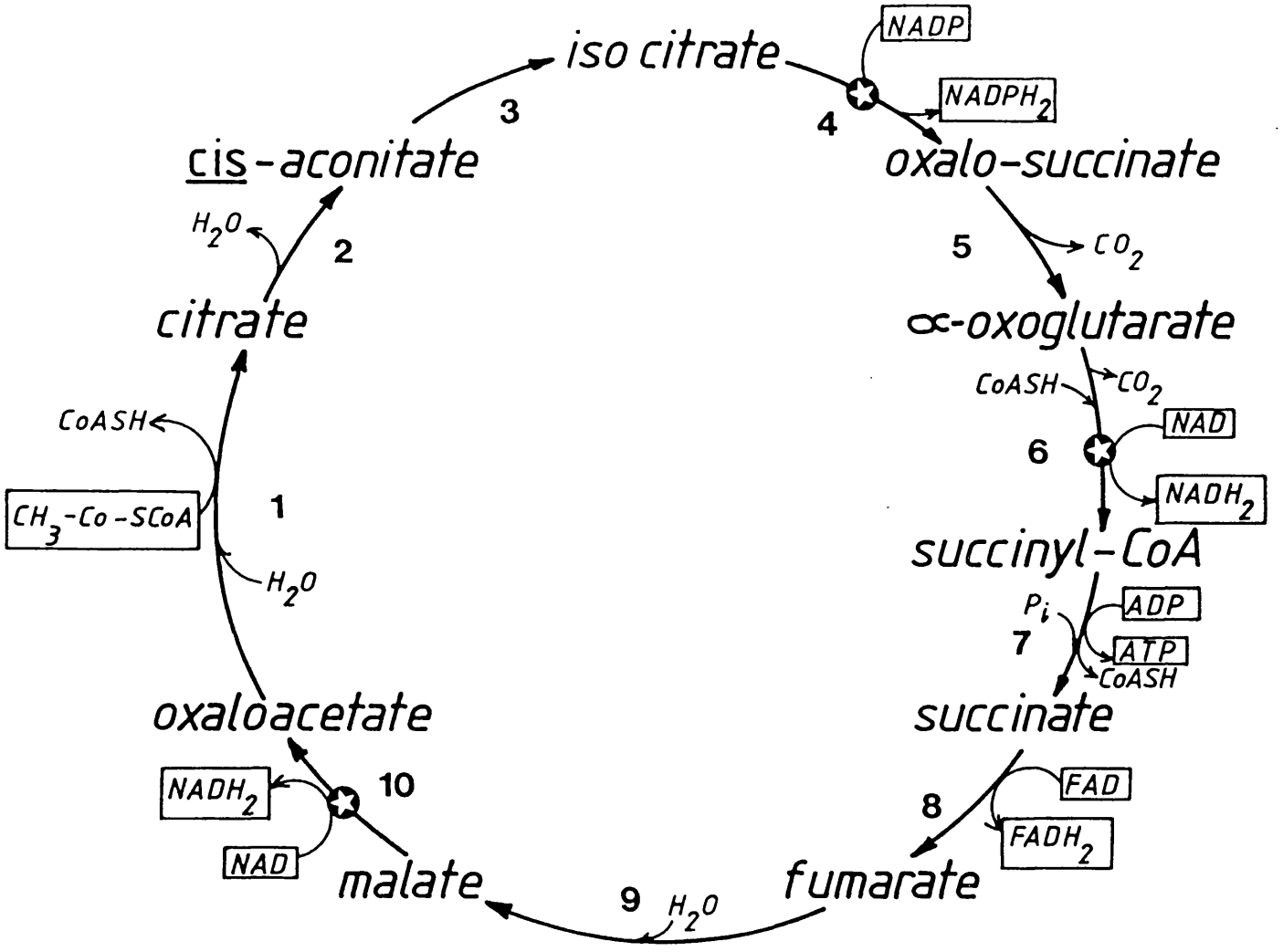


Figure 85. Oxidation of acetyl-CoA *via* the tricarboxylic acid cycle and probable sites of sulphite action*



Footnote: ✪ Probable sites of sulphite action

1. citrate synthase; 2,3 *cis*-aconitate hydratase; 4,5 isocitrate dehydrogenase; 6 α-oxoglutarate dehydrogenase complex; 7 succinate thiokinase; 8 succinate dehydrogenase; 9 fumarase; 10 malate dehydrogenase

* Adapted from Gottschalk (1979)

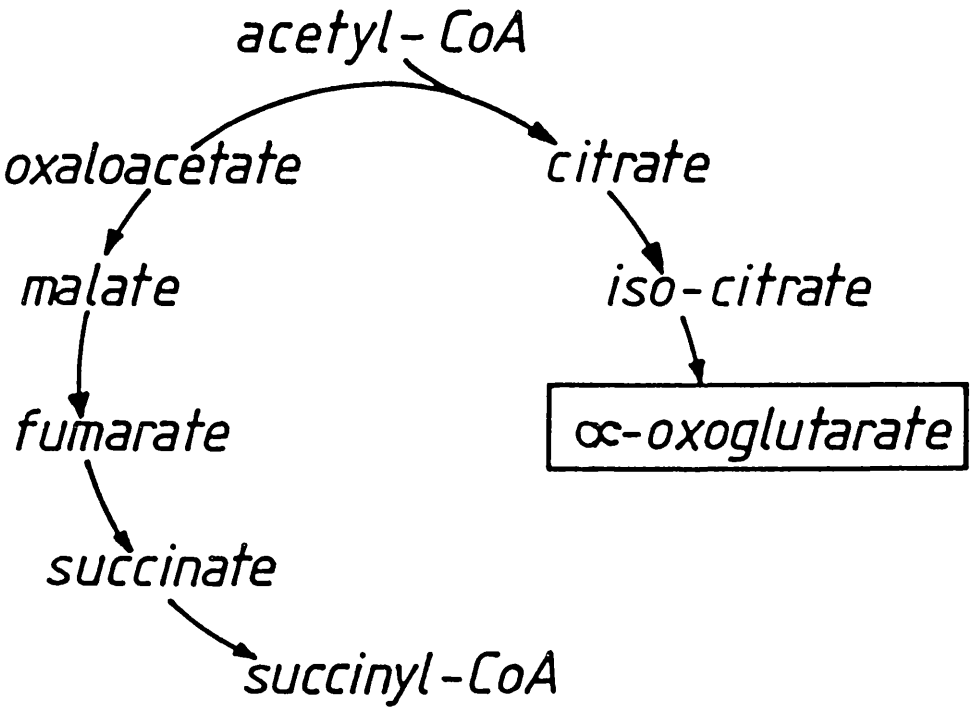
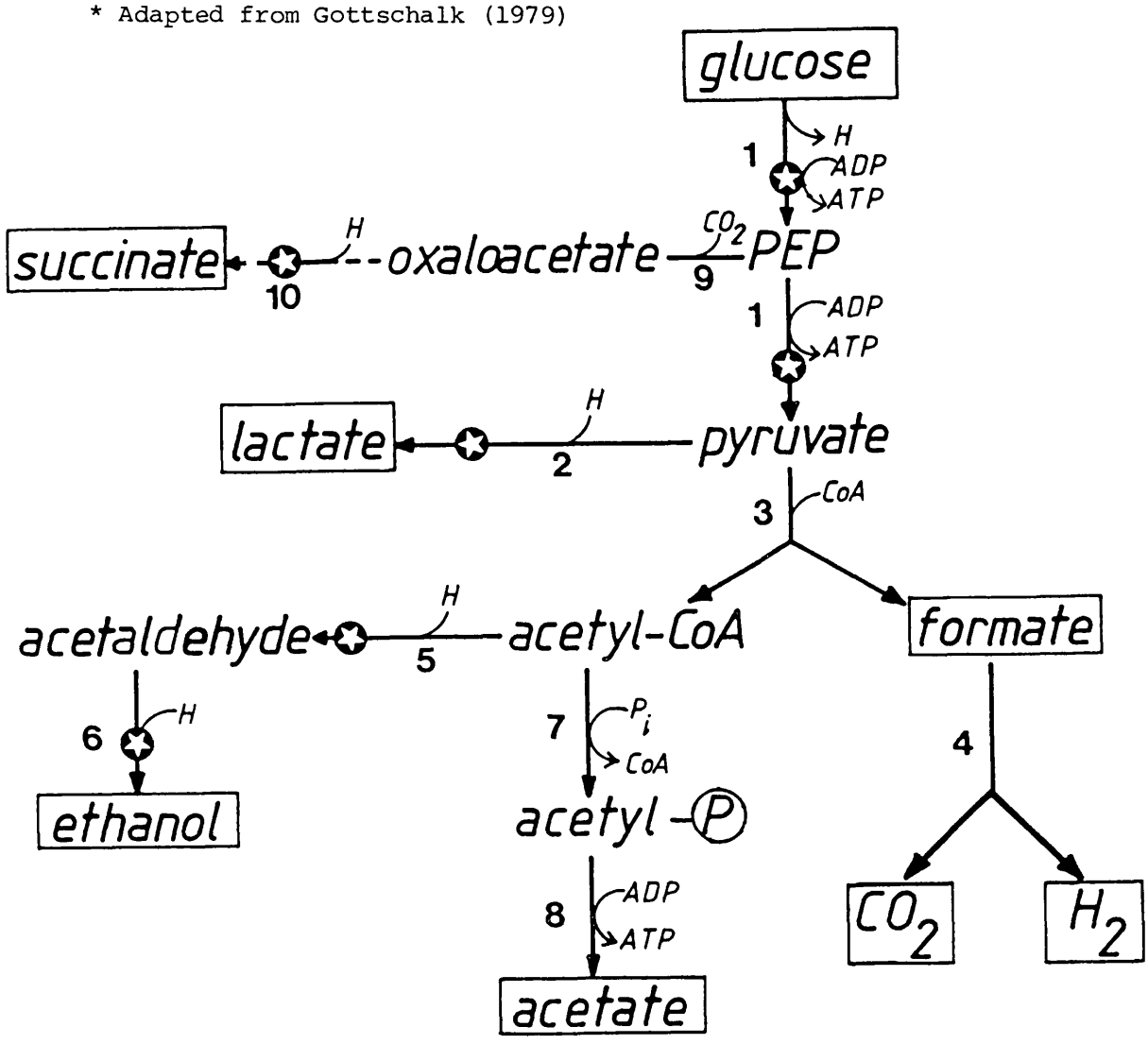


Figure 86. Modified TCA cycle operative in *Escherichia coli* growing anaerobically.

Figure 87. The mixed acid fermentation in Enterobacteriaceae and probable sites of sulphite action*

* Adapted from Gottschalk (1979)



Footnote: ✪ Probable sites of sulphite action

1 Embden-Meyerhof pathway enzymes

2 lactate dehydrogenase

3 pyruvate-formate lyase

4 formate-hydrogen lyase

5 acetaldehyde dehydrogenase

6 alcohol dehydrogenase

7 phosphotransacetylase

8 acetate kinase

9 PEP carboxylase

10 malate dehydrogenase , fumarase, fumarase reductase

Figure 88. The butanediol fermentation in Enterobacteriaceae and probable sites of sulphite action*

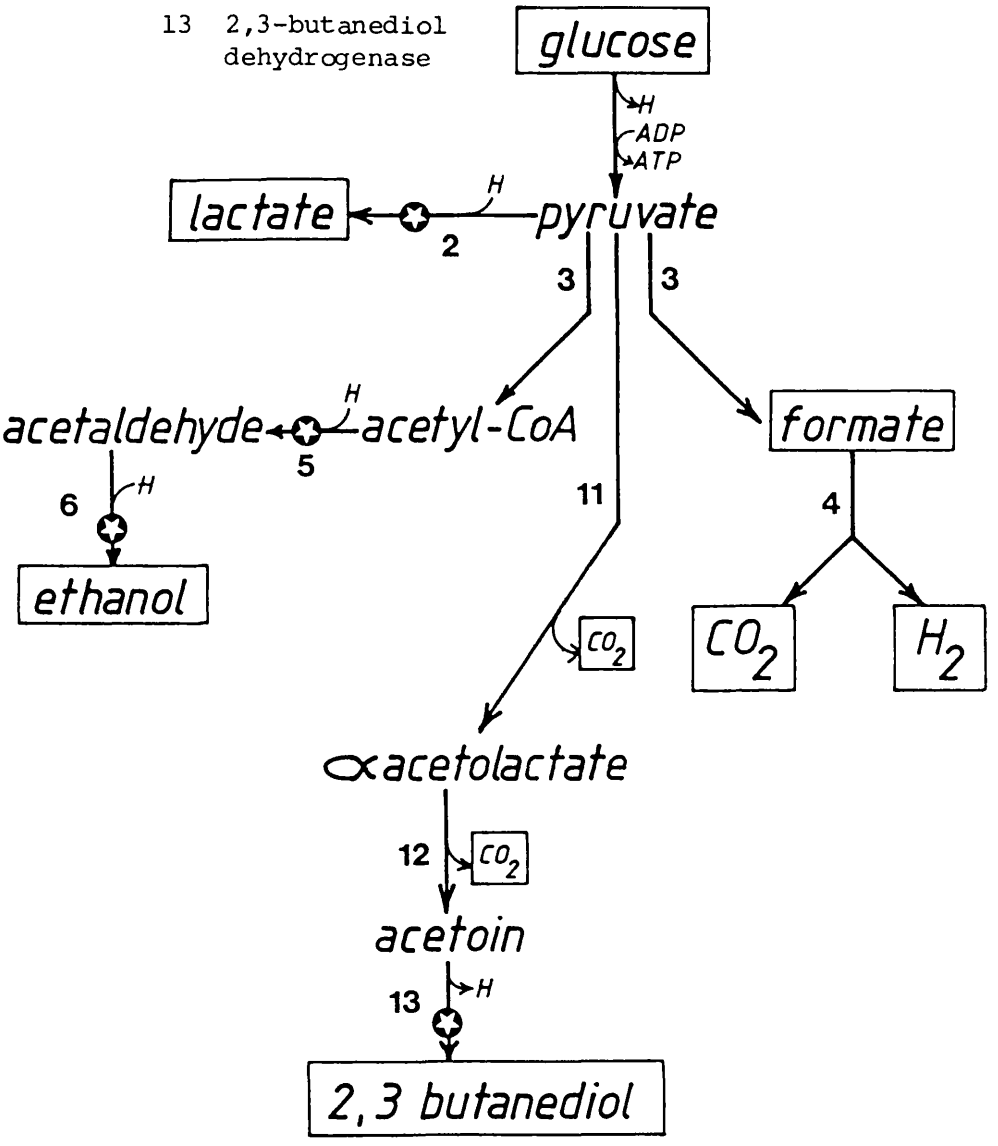
Footnote: ✪ Probable sites of sulphite action

1 - 10 as in Fig. 87

11 α -acetolactate synthase

12 α -acetolactate decarboxylase

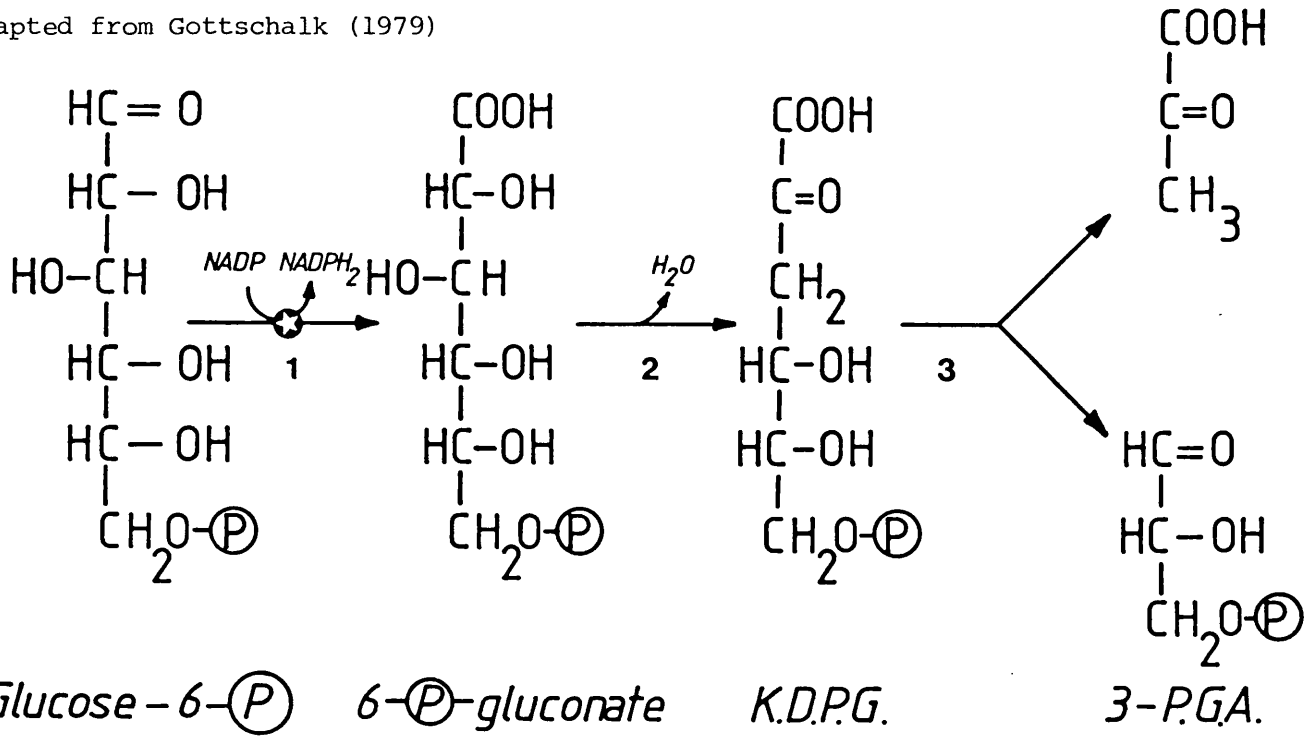
13 2,3-butanediol dehydrogenase



* Adapted from Gottschalk (1979)

Figure 89. The Entner-Doudoroff pathway for sugar breakdown in the *Pseudomonadaceae* and probable sites of sulphite action*

* Adapted from Gottschalk (1979)



Footnote:

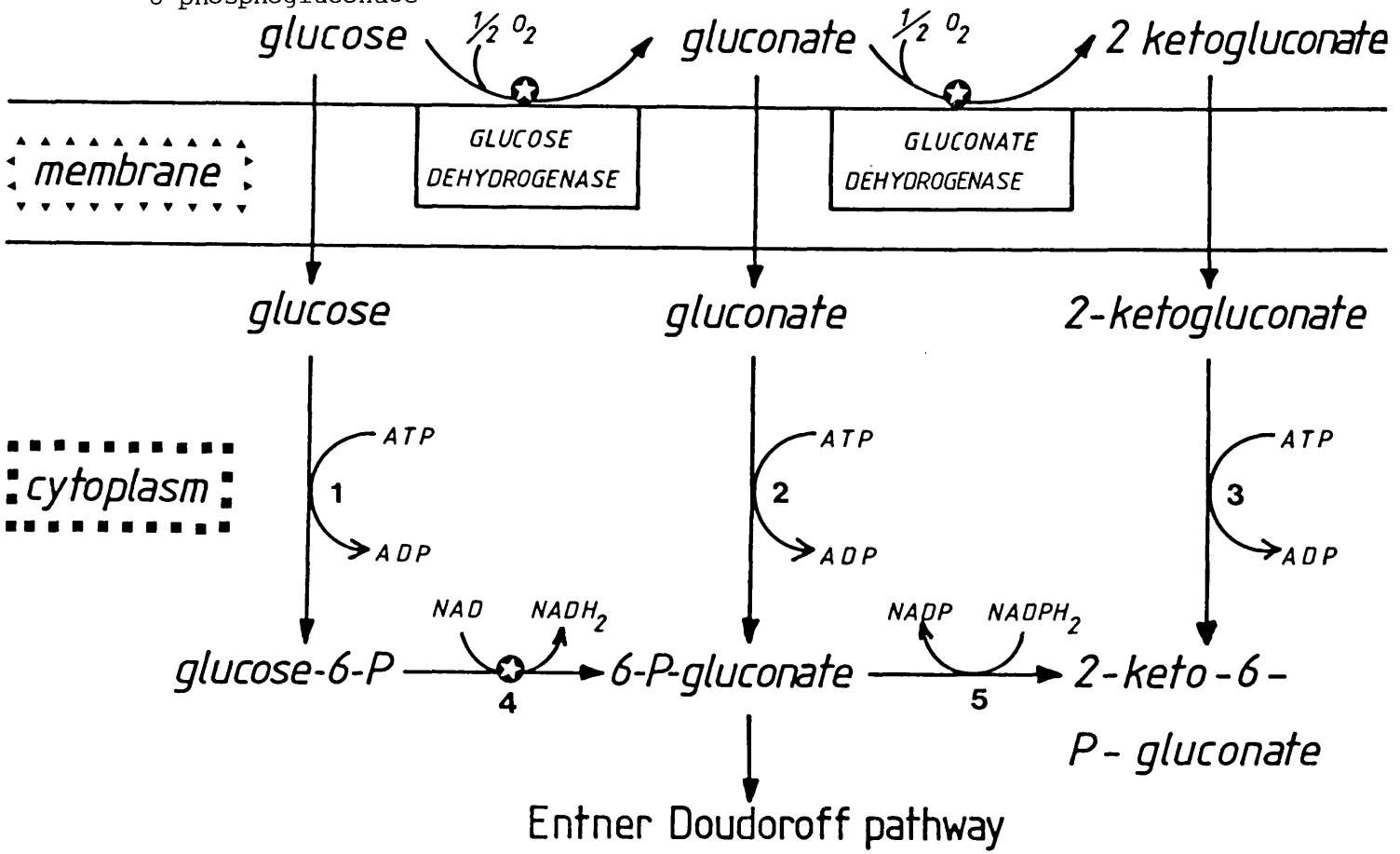
✪ Probable sites of sulphite action

1 Glucose-6-phosphate dehydrogenase

2 6-phosphogluconate dehydratase

3 2-Keto-3-deoxy-6-P-gluconate (KDPG) aldolase

Figure 90. The probable sites of sulphite action in the extracellular oxidation of glucose by pseudomonads and intracellular conversion of glucose, gluconate, and 2-ketogluconate into 6-phosphogluconate*



Footnote: ✪ Probable sites of sulphite action

1 hexokinase

2 gluconate kinase

3 2-ketogluconate kinase

4 glucose-6-phosphate dehydrogenase

5 2-keto-6-phosphogluconate reductase

* Adapted from Gottschalk (1979)

Appendix 1.

Standard specifications for sausage

Pork and Beef		Pork	
Ingredient	% w/w sausage	Ingredient	% w/w sausage
Beef flank	20.4	Lean pork	26.4
Pork back fat	11.7	Pork back fat	22.0
Pork head meat	9.6	Ice/water	19.3
Processed rinds ("drinde")	2.5	Rusk	12.3
M.R.M.	1.7	Pork belly meat	7.0
Flour		Pork head meat	7.0
Rusk		Pasteurized rinds	4.0
Water/ice	54.1	Seasoning	1.7
Seasoning		Polyphosphate	0.3
Polyphosphate			
	100.0		100.0

Appendix 2.

Isolation, identification and maintenance media

Salmonella

Buffered peptone water (BPW), g/ℓ: Peptone (Oxoid L37), 10; sodium chloride, 5; disodium hydrogen phosphate ($Na_2HPO_4.12H_2O$, 9; potassium dihydrogen phosphate, 1.5; distilled water to 1 ℓ. pH 7.0 at 25°C after sterilization at 121°C for 15 min.

Lactose broth, g/ℓ: 'Lab Lemco' beef extract, 5; peptone (Oxoid L37), 5; lactose, 5; distilled water to 1 ℓ. pH 6.7 at 25°C after sterilization at 121°C for 15 min.

Peptone water, g/ℓ : Peptone (Oxoid L37), 1; sodium chloride, 5; distilled water to 1 ℓ. pH 7.2 at 25°C after sterilization at 121°C for 15 min.

Tetrathionate broth, Tetrathionate broth base (Difco B104), 46 g; distilled water to 1 ℓ. Heated to 100°C, cooled to 25°C. Iodine solution (iodine, 6 g; potassium iodide, 5 g; distilled water to 20 ml), 20 ml added to broth base.

Selenite broth, g/ℓ: selenite broth (Oxoid CM 39), 23; distilled water to 1 ℓ. Heated to 100°C for 10 min.

Lysine iron cystine neutral red (LICNR) broth, g/ℓ ; L-lysine, 10; tryptone (Oxoid L42), 5; yeast extract (Oxoid L21), 3; mannitol, 5; D-glucose, 1; salicin, 1; ferric ammonium citrate, 0.5; sodium thiosulphate, 0.1; L-cystine, 0.1; neutral red, 0.025;

distilled water to 1 ℓ. pH adjusted to 6.2, medium heated to 100°C, cooled to 25°C.

Brilliant green agar (BGA), BGA (Oxoid CM 329), 52 g; distilled water to 1ℓ, heated to 100°C, cooled to 45°C and poured (20 ml) into Petri-dishes.

Bismuth sulphite agar (BSA), BSA (Oxoid CM 201), 52 g; distilled water to 1ℓ, heated to 100°C, cooled to 45°C, mixed and poured (25 ml) into Petri-dishes. Medium stored at 4-6°C for 3 d before use.

Desoxycholate citrate agar (DCA), DCA (Difco B274), 70 g; distilled water to 1ℓ, heated to 100°C, cooled to 45°C, and poured (20 ml) into Petri-dishes.

Kohns medium, No. I. (Oxoid CM 179); No. II, (Oxoid CM 181) made up and used according to manufacturers recommendations.

Lactic acid bacteria

Dowdell and Board's (1968) modification of Keddies (1951) medium (g/ℓ): Peptone (Difco B118), 5; Lab Lemco (Oxoid L29), 5; yeast extract (Oxoid L21), 5; D-glucose, 10; manganous sulphate ($MnSO_4.4H_2O$), 0.1; Tween 80, 0.5 ml; potassium citrate, 1; agar No. 2 (Lab M), 15; cycloheximide, 50 mg; distilled water to 1ℓ. pH adjusted to 5.4 before sterilization at 121°C for 15 min. Medium cooled to 50°C, added (9 vols.) to 2 M acetate buffer (pH 5.4; 1 vol.), mixed

and poured (20 ml) into Petri-dishes.

Whittenbury's (1963) medium. Lab Lemco (Oxoid L29), 5 g; peptone (Difco B 118), 5 g; yeast extract (Oxoid L21), 5g; Tween 80, 0.5 ml; D-glucose, 0.5% w/v; agar No. 2 (Lab M), 1.5% w/v; manganous sulphate ($MnSO_4.4H_2O$), 0.01% w/v; tap water to 1 ℓ. Bromocresol purple (14 ml., 1.6% w/v in ethanol) was added, the pH adjusted to 6.9 and sterilization was at 121°C for 15 min.

Whittenbury's medium (for carbohydrate utilization tests). Above medium excluding D-glucose. Filter-sterilized carbohydrate (1% w/v) added aseptically to sterilized basal medium at 55°C.

Streptococcus medium (SM), g/ℓ: Tryptone soya broth (Oxoid CM 129), 12; Lab Lemco (Oxoid L29), 2.5; yeast extract (Oxoid 21), 5; TRIS buffer, 3; hemin, 0.01; cystine, 0.4; distilled water to 1 ℓ. pH adjusted to 7.6, sterilization at 121°C for 15 min.

Kneteman's (1947) hydrogen peroxide medium. The medium of Whittenbury (1963) *vide supra* was overlaid to a depth of *ca*. 2 mm with the same containing manganese dioxide (4% w/v).

Arginine medium (g/ℓ): Tryptone (Oxoid L42), 5; yeast extract (Oxoid L21), 5; dipotassium hydrogen phosphate, 2; L-arginine dihydrochloride, 3; D-glucose, 0.5; agar No. 2 (Lab M), 15; distilled water to 1 ℓ. pH adjusted to 7.0 and sterilized at 115°C for 10 min.

Bile agar (g/ℓ): Tryptone (Oxoid L42), 5; yeast extract (Oxoid L21), 5; dipotassium hydrogen phosphate, 2; bile (40% w/v); D-glucose, 0.5; agar No. 2 (Lab M), 15; distilled water to 1ℓ. pH adjusted to 7.0 and sterilized at 115°C for 10 min.

Todd-Hewitt agar. Todd-Hewitt broth (Oxoid CM 189), 36.4 g; agar No. 3 (Oxoid L13), 15 g; distilled water to 1ℓ; sterilized at 115°C for 10 min.

Robertson's cooked meat medium. Fresh pork (500 g) was minced (Kenwood A901) and simmered (1 h) in distilled water (500 ml) containing 1N NaOH (2 ml). The slurry was filtered through cheese cloth and samples (*ca.* 5 g) of dried meat, distributed into 1 oz screw-capped bottles. Ten ml of Lab Lemco (Oxoid L29) broth was added to each bottle. Sterilization was at 115°C for 10 min.

Yeasts

Malt extract agar. Malt extract (Difco B 186), 30 g; agar No. 3 (Oxoid L13), 15 g; distilled water to 1ℓ. Sterilization at 121°C for 15 min.

Appendix 3.

Media used in sulphite tolerance work

All micro-organisms: Tryptone soya broth (Oxoid CM 129) or Nutrient broth (Oxoid CM 1).

Lactic acid bacteria: Buffered Whittenbury's broth. Lab Lemco (Oxoid L 29), 5 g; yeast extract (Oxoid L 21), 5 g; peptone (Oxoid L 37), 5 g; manganous sulphate ($MnSO_4.4H_2O$), 0.01% w/v; Tween 80, 0.5 ml; tri-sodium citrate, 2.5 g; sodium dihydrogen phosphate 195 ml; disodium hydrogen phosphate, 305 ml; distilled water to 1 ℓ. Sterilization was at 121°C for 15 min. Filter-sterilized D-glucose (1% w/v) was added to the basal medium at 25°C.

Yeasts: Yeast nitrogen base-glucose broth. Yeast nitrogen base (Difco B 392), 6.7 g; D-glucose, 10 g; sodium dihydrogen phosphate + disodium hydrogen phosphate buffer (10% v/v); distilled water to 1ℓ. pH adjusted to 7.0, medium filter-sterilized.

Enterobacteriaceae: Modified glucose-glutamate medium (MGGM). (g/ℓ): sodium glutamate, 12.7; sodium formate, 0.5; L-cystine, 0.04; L-aspartate, 0.048; L+ arginine, 0.04; thiamine, 0.002; nicotinic acid, 0.002; pantothenic acid, 0.002; magnesium sulphate, 0.2, ferric ammonium citrate, 0.02; calcium chloride, 0.02; ammonium chloride, 2.5; D-glucose, 20. pH buffered at 7.0 with sodium dihydrogen phosphate (0.2 M) and disodium hydrogen phosphate (0.2 M) at a final concentration of 20% (v/v). Medium was filter-sterilized.

Pseudomonas: Palleroni and Doudoroff's (1972) medium. Ammonium chloride, 0.1%; magnesium sulphate, 0.05%; ferric ammonium citrate 0.0005%; D-glucose, 1%; phosphate buffer (M/30) to pH 7.0. Medium was filter-sterilized.

Brochothrix thermosphacta. Grau's (1980) medium.

Appendix 4

Nilox scrubbers

In order to remove the last traces of oxygen from high purity white spot dinitrogen (Air Products, Bristol), 3 Dreschel bottles containing brown Nilox (2 bottles) and blue Nilox (1 bottle) scrubbers were arranged in series. Composition of brown Nilox (g): potassium hydroxide, 200; 1,2-naphthoquinone 4 sulphonic acid, (sodium salt), 10; lead wool, 250; distilled water, 900. Composition of blue Nilox (g): chromic sulphate, 300; sulphuric acid (10 M), 45; zinc granules, 250; distilled water, 900.

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