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The growth of salmonellas on cooked cured pork

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SUMMARY

With the use of streptomycin-resistant mutants to facilitate recovery, 5 strains of 4 species of *Salmonella* were shown to grow rapidly at 22° C. on low salt ham even from an inoculum of 10–20 organisms. *S. pullorum* did not grow well. All 6 strains of *Salmonella* survived but did not grow on 'high salt ham'. We conclude that cooked ham containing approximately 2·8 g. NaCl/100 g. H_2O once infected is more likely to give rise to food poisoning than is ham with the higher salt content traditionally used.

INTRODUCTION

Large doses of salmonellas are required to initiate food poisoning; doses that can arise only through massive contamination or through light contamination followed by the opportunity for growth before ingestion. Foods responsible for salmonella food poisoning are frequently not identified (e.g. the source of salmonellas was detected in only 20 % of the recorded general outbreaks and 2 % of the recorded family outbreaks in 1966 in England and Wales; Vernon, 1967) and so general vigilance is required. Rare outbreaks associated with cooked cured pork have been reported (Wildman, Nicol & Tee, 1951; Bailey *et al.* 1972), but the possibility of more ready growth of salmonellas on some such products arising from the recent tendency to use less sodium chloride than traditionally used appears not to have been adequately assessed. This paper reports a study of the growth of salmonellas on such a product and on a more traditional cured pork and assesses the effect of various factors. Previous studies have involved adding salmonellas to 1 % glucose in sterile jelly from ham (Koelensmid & van Rhee, 1964) or to ground pork containing the normal flora (Alford & Palumbo, 1969). However, in our opinion the ability of salmonellas to grow on meats should be assessed not by using artificial preparations but by using the meat in slices in the form in which it would be purchased, and the inoculum should be labelled in some way so that it is not confused with any salmonellas already on the meat. Therefore, to determine the ease with which salmonellas could grow on two different types of cooked cured pork we used streptomycin-resistant mutants inoculated on portions of the meat as purchased from shops.

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METHODS

Organisms

Salmonella enteritidis 4753/70, *S. typhimurium* 4718/70 and *S. newport* 3313/70, all isolated from raw meat and kindly provided by the Salmonella Reference Laboratory, Colindale Avenue, London, N.W.9 and *S. enteritidis* NCTC 5765, *S. choleraesuis* NCTC 5736 and *S. pullorum* NCTC 5776 were used. Slide tests showed that each strain was agglutinated by the appropriate group specific O antiserum (Wellcome Reagents, Beckenham, Kent). Other organisms were *Escherichia coli* NCTC 8984, 8986, 9434, 10487, 10537, all of which were stated to grow well at 22° C., *E. coli* 16A (from our departmental collection) and *Proteus vulgaris* NCTC 10015.

Media

Figures refer to % (w/v) in demineralized water. Nutrient broth was peptone (Evans), 0·5; Lab Lemco (Oxoid), 0·5; NaCl, 0·5; sterilized at 121° C. for 20 min.; pH 7·0. Nutrient agar was No. 3 agar (Oxoid), 1·2; other ingredients as for nutrient broth; sterilized at 121° C. for 20 min.; pH 7·0. MacConkey agar was CM7 (Oxoid). Glucose yeast Lemco agar (GYLA) was peptone (Evans), 1·0; Lab Lemco (Oxoid), 1·0; yeast extract (Difco), 0·25; Japanese shredded agar, 2·0; glucose, 0·5; sterilized at 121° C. for 15 min., pH 7·0. Minimal agar was minimal agar of Clowes & Hayes (1968) with 0·2 % lactose substituted for glucose. Ringer's solution ($\frac{1}{4}$ strength) was prepared by dissolving 1 BR 52 tablet (Oxoid) in 500 ml. glass distilled water and sterilizing at 121° C. for 15 min. Streptomycin sulphate B.P. (Glaxo, Greenford) was used, concentrations being expressed in terms of amount of streptomycin base.

Production of streptomycin-resistant mutants

Streptomycin-resistant mutants (str^r) were produced by the method of Meynell & Meynell (1970), by adding 100 ml. nutrient broth containing 2000 μg./ml. streptomycin to an overnight culture at 37° C. of each of the parental streptomycin-sensitive salmonellas (str^s) in 100 ml. nutrient broth and plating samples from the cultures after further incubation for 8 hr. and 24 hr. on nutrient agar containing 1000 μg./ml. streptomycin. After incubation of the plates for 24 hr. a single colony of each strain was used to establish stock cultures of str^r mutants, identity being confirmed by slide agglutination. Maintenance was on nutrient agar slopes from which streptomycin was omitted to stop development of streptomycin dependance, periodic counts on several media indicating that reversion to str^s was not a problem.

Cured meat samples for growth experiments

Over 6 months, samples of prepacked sliced cooked shoulder of cured pork were obtained in $3\frac{3}{4}$ oz. packs containing 4 slices from a refrigerated display cabinet at a supermarket. The manufacturing specification for this product was: NaCl, 1·8–2·2 % (w/w); KNO_2, 60 p.p.m.; no nitrate; total colony count less than 500/g. This material had a water content of *ca.* 70 % and was called by us 'low salt

ham'; analysis for Cl^- using an automated application of the mercuric thiocyanate and ferric alum reagents confirmed that the NaCl content was *ca.* 2 % (w/w) i.e. *ca.* 2·8 g./100 g. H_2O. For comparison samples of cooked shoulder of cured pork sliced and packed at the point of sale were obtained from a small store. This product contained *ca.* 4 % (w/w) NaCl i.e. *ca.* 6 g. NaCl/100 g. H_2O and was called by us 'high salt ham'.

Inoculation and incubation of meat

Overnight broth cultures at 37° C. in a shaking water bath contained *ca.* 1×10^9 salmonellas/ml. Cultures were diluted in aqueous NaCl (0·5 %), either alone or as mixtures, to give approximately the required number of organisms in 0·02 ml. for inoculation. Meat free from fat was aseptically cut with a No. 13 cork borer into pieces 20 mm. diam. which weighed about 0·5 g. and were about 2 mm. thick, and placed in Petri dishes. Pieces were inoculated with 1 drop (0·02 ml.) of cell suspension from a calibrated pipette. Petri dishes containing the portions were then incubated in polythene bags aerobically at 5, 10, 17 or 22° C. The number of organisms in the inoculum was determined by making colony counts on the suspension and on one of the pieces immediately after inoculation.

Counts of viable bacteria

A piece of meat was transferred to a 1 oz. universal bottle which contained 10 ml. Ringer's solution and blended at *ca.* 14,000 rev./min. for 3 min. using a top drive homogenizer (M.S.E., London S.W.1). Appropriate dilutions (0·1 ml.) of the supernatant fluid in Ringer's solution were inoculated on plates (2/dilution) that had been dried at 55° C. for 40 min. and spread. Counts of bacteria in liquid media were performed similarly. For counts of salmonellas, nutrient agar + 1000 μg. streptomycin/ml. incubated overnight at 37° C. was used; for *E. coli*, minimal agar incubated at 37° C. for 48 hr. was used; for *P. vulgaris* MacConkey agar incubated overnight at 37° C. was used – no swarming occurred; and for general colony count, GYLA incubated at 22° C. for 3 days was used. Counts were expressed as the number of organisms per piece of meat or per ml. of liquid medium.

Growth of organisms in liquid media

Nutrient broth (15 ml. in conical flask with attached test tube for nephelometry, or 50 ml. in 250 ml. conical flasks) with various additions was inoculated with 0·5 or 1·0 ml. of overnight shaken culture at 37° C. and was incubated at 22° C. Growth was estimated by colony counts or by relative turbidity using a nephelometer with filter OR2 (Evans Electroselenium Ltd., Halstead, Essex).

RESULTS

Suitability of streptomycin-resistant mutants for studying growth rates of salmonellas on cured meats

Comparisons by nephelometry of growth rates in shaken nutrient broth of str^r mutants and str^s parental strains of salmonellas indicated that there was little difference between the two, whether the broth was incubated at 22 or 37° C. or

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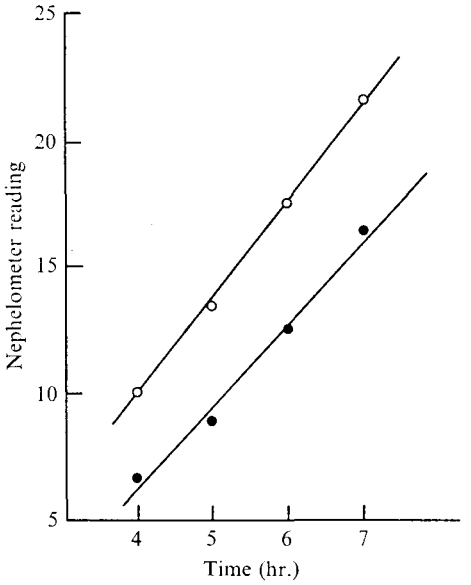

Fig. 1

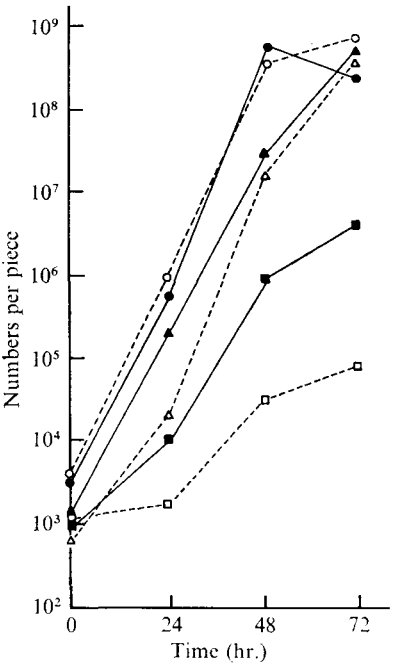

Fig. 2

Fig. 1. Comparative growth rates at 22° C. of *Salmonella enteritidis* NCTC 5765 str^s wild type and an str^r mutant in nutrient broth containing 3 % (w/v) NaCl. Each point is the average of nephelometer readings given by 2 flasks. ○, *S. enteritidis* str^s; ●, *S. enteritidis* str^r.

Fig. 2. Growth of str^r mutants of various salmonellas on 'low salt ham' at 22° C. ●, *S. enteritidis* 4753/70; △, *S. enteritidis* NCTC 5765; ○, *S. newport* 3314/70; ▲, *S. typhimurium* 4718/70; ■, *S. choleraesuis* NCTC 5736; □, *S. pullorum* NCTC 5776.

contained 0·5 % or 3 % NaCl (e.g. Fig. 1). No evidence was obtained of reversion of str^r to str^s and so we concluded that str^r mutants could be used to study the growth rate of salmonellas on cured meats.

Survival and growth of salmonellas on cured meat

When inoculated on to separate pieces of 'low salt ham' incubated at 22° C. numbers of *Salmonella enteritidis* NCTC 5765, *S. enteritidis* 4753/70, *S. choleraesuis* NCTC 5736, *S. newport* 3314/70 and *S. typhimurium* 4718/70 increased from 10- to more than 100-fold within 24 hr. The increases continued with further incubation. *S. pullorum* showed virtually no growth at 24 hr. and only slight growth thereafter. Typical results are shown in Fig. 2. Experiments with *S. enteriditis* NCTC 5765 indicated that the initial rate of growth was similar, irrespective of the number of organisms in the inoculum (Fig. 3), though with a smaller inoculum the number of salmonellas after 3 days was less than with a larger inoculum, presumably because other organisms already on the ham were able to grow to constitute a larger proportion of the flora before growth ceased for some reason. Even when the inoculum was only *ca.* 10–20 per piece the salmonellas increased more than 100-

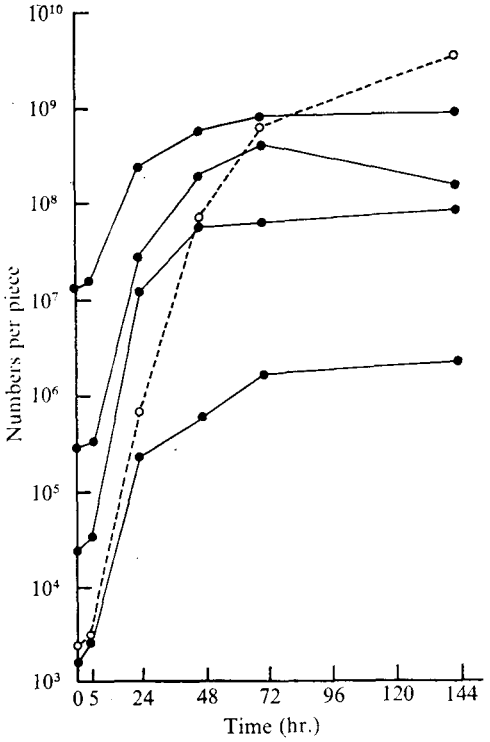

Fig. 3

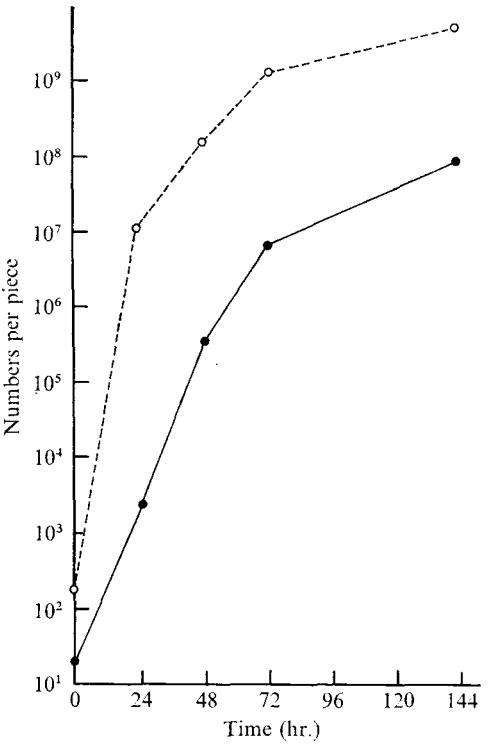

Fig. 4

Fig. 3. Growth of str^{r} mutants of *Salmonella enteritidis* NCTC 5765 from various sizes of inoculum on 'low salt ham' at 22° C. The colony count on GYLA of total flora on samples carrying the smallest inoculum of salmonellas is shown, but all samples taken after the same incubation period gave comparable counts on GYLA. ●, *S. enteritidis* grown from various inoculum sizes; ○, Colony count on GYLA.

Fig. 4. Growth of a str^{r} mutant of *Salmonella enteritidis* NCTC 5765 from an inoculum of *ca.* 20 organisms on 'low salt ham' at 22° C. ●, *S. enteritidis*; ○, Colony count on GYLA.

fold in 24 hr. at 22° C. and at 48 hr. the count was 10^{4} times more than the inoculum (Fig. 4). Incubation of meat portions inoculated with *S. enteritidis* NCTC 5765 at lower temperatures indicated that growth occurred at 17° C., although not until after 24 hr., but not at 5 or 10° C., where the number of salmonellas remained constant for at least 3 days (Fig. 5). When salmonellas were inoculated on to separate pieces of 'high salt ham' and incubated at 22° C. they grew slightly or not at all although the total flora increased considerably (Fig. 6).

Escherichia coli and *Proteus vulgaris*, two species that under natural conditions might be expected to be inoculated on to meat with salmonellas, were examined for their ability to affect growth of *S. enteritidis* NCTC 5765. Pieces of 'low salt ham' inoculated with 3×10^{6} *E. coli* 16A supported rapid growth of *S. enteritidis* (Fig. 7). Similar results were obtained with *E. coli* NCTC 8984, which was chosen after studies in nutrient broth had shown that in the presence of *S. enteritidis* it could grow faster than the other four *E. coli* strains from NCTC. *P. vulgaris* (1×10^{7}) inoculated together with *S. enteritidis* NCTC 8984 (5×10^{3}) into nutrient broth

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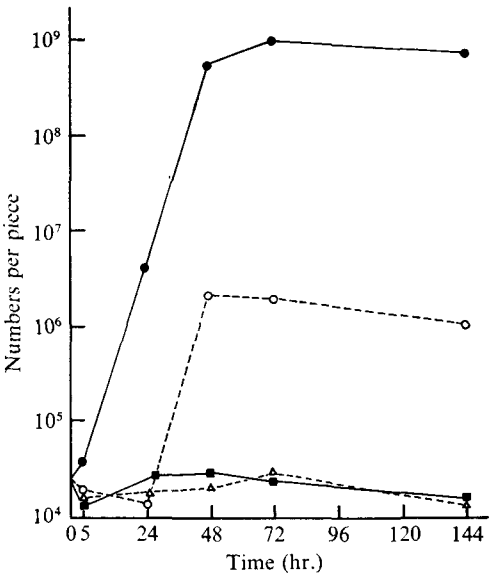

Fig. 5

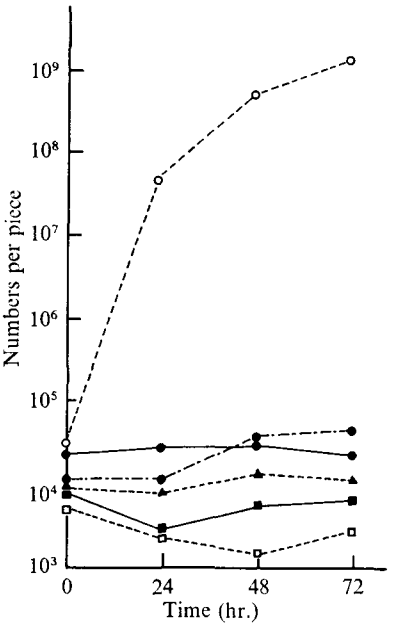

Fig. 6

Fig. 5. The effect of incubation temperature on growth of a str^r mutant of *Salmonella enteritidis* NCTC 5765 on 'low salt ham'. ●, 22° C.; ○, 17° C.; △, 10° C.; ■, 5° C.

Fig. 6. Growth of str^r mutants of various salmonellas on 'high salt ham' at 22° C. Colony counts on GYLA of meat samples inoculated with *S. enteritidis* NCTC 5765 are included. Meat samples inoculated with other salmonellas gave comparable results after the same incubation periods for colony counts on GYLA. ●——●, *S. enteritidis* NCTC 5765; ●—·—·—●, *S. typhimurium* 4718/70; ▲---▲, *S. newport* 3314/70; ■——■, *S. choleraesuis* NCTC 5736; □---□, *S. pullorum* NCTC 5776; ○---○, Colony counts on GYLA.

incubated at 22° C. also failed to restrict the growth of salmonellas, the counts being, after 24 hr.: *Salmonella*, 9×10^6; *Proteus*, $1{\cdot}5 \times 10^9$. In contrast *Staphylococcus aureus* is much inhibited by *E. coli* or *P. vulgaris* in broth, even when it outnumbers them by 100 to 1 in the inoculum (DiGiacinto & Frazier, 1966).

Effect of NaCl on growth of salmonellas

Static incubation at 22° C. of nutrient broth containing various concentrations of NaCl and inoculated with *ca.* 200/ml. *S. enteriditis* NCTC 8984 showed that 6% NaCl, the concentration of NaCl in the 'high salt ham', inhibited the growth of the salmonellas (Fig. 8). Experiments with all 6 strains of *Salmonella* showed by turbidimetry that good growth occurred in nutrient broth containing 0·5% or 3% NaCl and that virtually no growth occurred in nutrient broth containing 5·5% NaCl within 24 hr.

Effect of $NaNO_2$ on growth of salmonellas

Turbidimetric assessment of growth of *S. enteritidis* NCTC 8984 and *S. enteritidis* 4753/70 in nutrient broth incubated at 22° C. showed that nitrite at 100 p.p.m. added as $NaNO_2$ reduced by *ca.* 30% the growth of each strain at pH 5·0 in the

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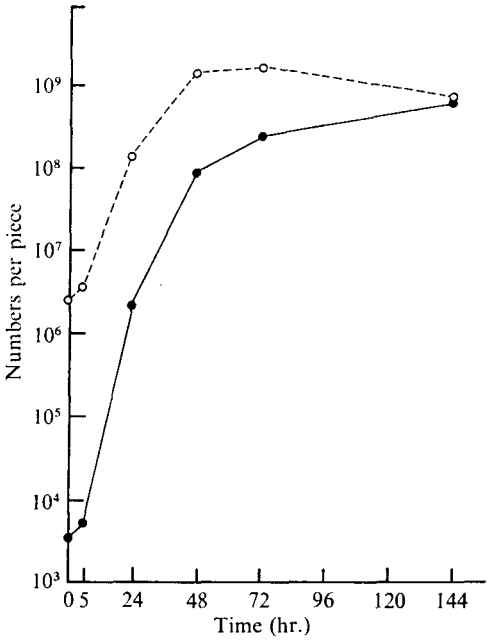

Fig. 7

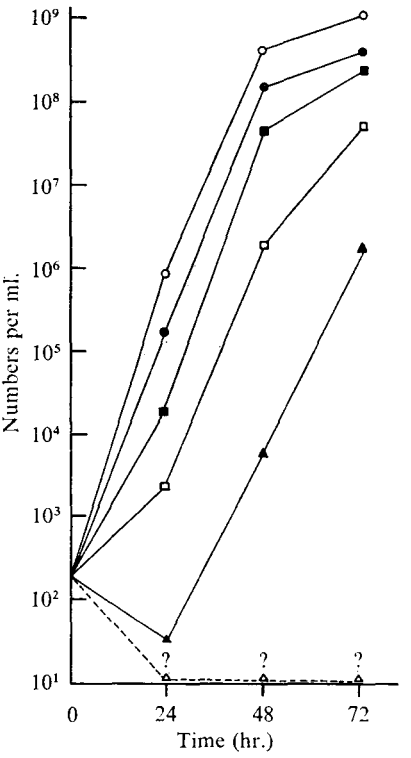

Fig. 8

Fig. 7. Growth at 22° C. of a str^r mutant of *Salmonella enteritidis* NCTC 5765 and *Escherichia coli* 16A on 'low salt ham' when the inoculum contained 1000 times more *E. coli* than *S. enteritidis*. ●, *S. enteritidis*; ○, *E. coli*.

Fig. 8. Growth at 22° C. of *Salmonella enteritidis* NCTC 5765 in nutrient broths containing various concentrations of NaCl. ○, 2 % NaCl; ●, 3 % NaCl; ■, 4 % NaCl; □, 4·5 % NaCl; ▲, 5 % NaCl; △, 6 % and 7 % NaCl.

presence of 5 % NaCl. At pH 5·0 in the presence of 3 % NaCl the effect was slight and, as was expected, at pH 7·0 in the presence of either 3 % or 5 % NaCl the nitrite had no detectable inhibitory effect.

DISCUSSION

The use of streptomycin-resistant mutants to determine the conditions under which salmonellas could grow on cured pork was convenient and avoided confusion between inoculated salmonellas and any organisms initially present. The method would seem to have general applicability and has been used by Greenberg (1969) for study of population changes in the gut flora of flies. Drug resistance transfer between organisms from the inoculum and contaminants already present on the meat was not expected, because usually only resistance to concentrations up to 25 μg. streptomycin/ml. is conferred by an R factor (Weisblum & Davies, 1968), and did not appear to occur; all colonies on the isolation medium containing streptomycin resembled the colonies of the original str^r mutants and those tested by slide agglutination reacted like the original strains.

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Choice of the method for inoculation of the meat samples was difficult because we wanted to deposit an inoculum of a known number of organisms without altering growth conditions from those normal on the product. We used 1 drop (0·02 ml.) prepared by diluting an overnight culture with 0·5 % (w/v) NaCl. This method seemed justifiable because under normal conditions contamination might occur via a drop of liquid (e.g. water dripping from thawing frozen meat; or blood dripping from meat) and because calculation indicated that the effect of the inoculum on the concentration of NaCl in the sample would be negligible. Thus, each sample of 'low salt ham' had a wet weight of 0·5 g., and contained *ca.* 0·35 g. H_2O. Addition of an inoculum in 0·02 ml. of 0·5 % (w/v) NaCl would reduce the content of NaCl by *ca.* 5 % e.g. from 2·87 g./100 g. H_2O to 2·73 g./100 g. H_2O.

Our results showed that 5 of the 6 salmonella strains examined grew rapidly at 22° C. on sliced cooked shoulder of pork with a NaCl content of *ca.* 2·8 g./100 g. H_2O, even when only a few organisms were inoculated and when other types of bacteria outnumbered the salmonellas, but that growth of salmonellas did not occur on a similar product with a NaCl content of *ca.* 6 g./100 g. H_2O. Experiments in nutrient broth with various concentrations of NaCl showed that little or no growth of salmonellas occurred in 3 days in the presence of more than 5 % NaCl. The results were comparable with those obtained by Vargues (1962; quoted by Jones, 1964) using *E. coli* and by Matches & Liston (1969) using *S. heidelberg*, *S. typhimurium* and *S. derby*, although these latter workers found greater tolerance to NaCl in nutrient broth at 37 than at 22° C. Using nutrient broth containing 1 % glucose and ham jelly containing 1 % glucose Koelensmid & van Rhee (1964) obtained good growth of salmonellas in 10 days at 20° C. but not at 5° C. with 6 % salt; with 8 % salt they obtained no growth at either temperature. Alford & Palumbo (1969) using nutrient broth apparently incubated for up to several weeks found that none of 23 salmonellas grew at 10° C. and pH 5·8 with 5 % NaCl but that at 20° C. all strains grew under these conditions and 6 strains grew with 8 % NaCl. At 30° C. 15 strains grew in 8 % NaCl. These workers also found that in ground pork at 10° C. and pH 5·75 no growth of salmonellas occurred with 5 % NaCl but some growth occurred with 3·5 % NaCl. They did not study growth on this material at higher temperatures. These various studies indicate that salmonellas are sensitive to the NaCl concentration present in the 'high salt ham' but that the sensitivity depends on temperature. Nitrite which is present in various amounts in cured meats is known to be bactericidal under certain conditions (Eddy & Ingram, 1956) but we did not investigate this in detail. Tests indicated that in nutrient broth nitrite was not very inhibitory at 100 p.p.m., while Koelensmid & van Rhee (1964) using ham jelly containing 500 p.p.m. $NaNO_2$ found growth of 3 of 4 salmonellas at 20° C., though this growth occurred only after several days during which time the nitrite concentration might have been reduced through reaction with amino groups.

The sizes of the populations of salmonellas reached on the 'low salt ham' after 24–48 hr., except in the case of *S. pullorum* which showed little growth, were comparable with $1{\cdot}5 \times 10^5$ organisms given by Wilson & Miles (1964) for minimum infective *S. newport* dose for adults. Nevertheless it must be appreciated that the

size of dose required to cause food poisoning may be much larger than this, depending on a variety of factors such as age of host and on characters of the strain of salmonella. We have made no attempt experimentally to determine infective doses of our strains or to assess either qualitatively or quantitatively the risk of contamination at the point of sale or in the home. However, salmonellas are common on raw meats (Hobbs, 1961; Prost & Riemann, 1967; Weissman & Carpenter, 1969) and salmonella food poisoning due to meat products constitutes a significant percentage of reported cases of food poisoning. We consider our examination indicates that cooked cured pork with a low NaCl content, once contaminated, is more likely to be a source of salmonella food poisoning than is a comparable product containing more NaCl.

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