

## Factors affecting attachment of *Salmonella typhimurium* to sausage casings

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*Effects of various substances on attachment of Salmonella typhimurium to artificial sausage casings were investigated. No inhibitory effects were observed when cells were incubated with glucose, galactose, hyaluronin, chondroitin sulphate, Tween 20, EDTA, gelatin, glycine and bovine albumin. There was a small decrease in attachment with increasing sodium chloride levels from 0–100 mg ml<sup>-1</sup>. Mannose did not inhibit attachment, implying that attachment was not mediated by type 1 fimbriae. Cells killed by irradiation (Cs-137) attached at a rate similar to non-irradiated cells, therefore viability and motility were not required for bacterial attachment. Bacterial attachment was inhibited by solubilized collagen, indicating that a specific interaction may be involved between a binding site on the collagen molecule and the micro-organism.*

### Introduction

Attachment of micro-organisms to meat surfaces is thought to involve multiple mechanisms, including hydrophilic and hydrophobic bonding (Dickson and Koohmaraie 1989) and specific interactions between the surface of the organism and meat (Old 1972, Sanderson et al. 1991). Once attached, under suitable conditions, bacteria can proliferate, which may lead to food spoilage or the risk of food poisoning.

Bacteria have been shown to attach to collagen fibers in meat and poultry tissues (McMeekin et al. 1984, Benedict et

al. 1991). Artificial sausage casings, composed principally of collagen, have been used as a model for studying attachment of salmonellae to meat (Walls et al. 1993). Advantages of sausage casings over meat are that they are sterile, they present a uniform surface, which reduces non-specific physical entrapment of cells, and they allow interactions with a single meat protein to be studied.

In previous studies standard plate counts (SPC) and counts made using scanning electron microscopy (SEM) were used to estimate numbers of bacteria attached to sausage casings (Walls et al. 1993). Good correlation was obtained between counts ( $r^2 = 0.89$ ). In the current study, SPC were used for estimating bacterial numbers except where bacterial growth would be affected by treatment with the substance under test, in which cases attachment was monitored using SEM.

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The objective of this work was to investigate the mechanisms responsible for attachment of salmonellae casings as a means of gaining insights into adherence of pathogenic bacteria to meat tissues.

## Materials and Methods

### *Micro-organism*

*Salmonella typhimurium* strain 14028 cells were grown in tryptose phosphate broth (TPB) (Difco, Detroit, MI, USA) at 37°C for 18 h, washed and resuspended in sterile isotonic saline. Standard plate counts (SPC) were used to determine inoculum levels, using a Spiral Plater, (Spiral Systems Instruments, Bethesda, MD, USA) with tryptose phosphate agar (TPA) (Difco) as the solid medium. Plates were incubated at 37°C for 24 h and colonies were counted using a laser bacteria colony counter with the computer assisted spiral bio-assay data processor (Model 500A Spiral Systems Instruments, Bethesda, MD, USA).

### *Sausage casings*

Artificial sausage casings (diameter = 40 mm) (DEVRO, Somerville, NJ, USA), formed from bovine hide corium, comprise 65% collagen, with minor components being cellulose, carboxymethylcellulose and mineral oil (Dever, pers. comm).

### *Preparation of potential inhibitors*

Solutions of 0.25 M D-mannose (Sigma, St Louis, USA), 0.25 M glucose (Mallinckrodt, Paris, K, USA), 0.25 M D-galactose (Eastman Organic Chemicals, Rochester, NY, USA), 1.0 mg ml<sup>-1</sup> chondroitin sulphate (Serva, GmbH & Co., Heidelberg, Germany) 1.0 mg ml<sup>-1</sup> glycine (Sigma), 1.0% (m/v) Tween 20 (Sigma), 250 mM EDTA (International Biotechnologies, New Haven, CT, USA) were prepared in distilled deionized water and sterilized by filtration through a 0.22 µm membrane (Nalgene, Rochester, NY, USA). Hyaluronin (1.0 mg ml<sup>-1</sup>; Sigma), was prepared in sterile distilled deionized water. Gelatin (Type A from Pork skin, 225 bloom; Knox, Englewood Heights, NJ, USA) was suspended in 70% ethanol to sterilize. The ethanol was decanted and the gelatin was air-dried in a biological safety hood. From this, a suspension of gelatin (1% m/v), was prepared in sterile isotonic saline. A sterile solution of purified

pepsin-solubilized bovine dermal collagen was used (Vitrogen 100, Celtrix Laboratories, Palo Alto, CA, USA). Before use, Vitrogen 100 was neutralized by mixing 8 ml of collagen solution with 1.0 ml of phosphate buffered saline solution, then adjusting to pH 7.4 with 0.1 M NaOH solution. All preparations were tested for sterility by streaking a loopful of each on to TPA and incubating at 37°C for 24 h.

### *Effect of potential inhibitors on attachment of Salmonella typhimurium to sausage casings*

*S. typhimurium* suspension (1.0 ml of approximately 10<sup>9</sup> cfu ml<sup>-1</sup>), was incubated with 9.0 ml of the inhibitor or control solution (sterile distilled deionized water or sterile isotonic saline) at 37°C for 30 min. A loopful of incubated *S. typhimurium* suspension was streaked on to TPA to demonstrate that the potential inhibitors to attachment had no inhibitory effect on the cells themselves, while 5.0 ml of suspension was added to sausage casings (0.5 g) in 45 ml sterile distilled deionized water. Casings were incubated at 37°C for 30 min. Casings were removed aseptically from the Petri dishes and washed twice by agitation for 30 s in approximately 50 ml of sterile isotonic saline. Casings were placed in stomacher bags with 9.9 ml of peptone water (0.1% m/v) and stomached for 2 min. Suspensions were diluted in 0.1% peptone water, then plated on to TPA using a Spiral Plater. Plates were incubated at 37°C for 24 h and counted. Counts were expressed as colony forming units per gram of casing.

### *Effect of sodium chloride on attachment of Salmonella typhimurium to sausage casings*

*S. typhimurium* were washed and resuspended in sterile deionized water. Cell suspensions of 10<sup>8</sup> cfu ml<sup>-1</sup> were incubated in 10.0 ml volumes of sterile NaCl solutions prepared in distilled deionized water at concentrations of 0, 25, 50, 75 and 100 mg ml<sup>-1</sup> at 37°C for 30 min. Sausage casings were cut aseptically into disks (diameter 12 mm) using a sterile cork borer. Disks were immersed in 1.0 ml of the incubated *S. typhimurium* suspensions and incubated at 37°C for 30 min. After incubation, disks were washed twice by agitation in sterile isotonic saline. For viewing under the scanning electron microscope,

disks were fixed by immersion in 1.0 ml of 1% glutaraldehyde/0.1 M sodium cacodylate buffer solution, (pH 7.4) for at least 24 h. Disks were washed in 0.1 M cacodylate buffer, post-fixed in 2% osmium tetroxide in 0.1 M cacodylate buffer, washed again in buffer, dehydrated in a graded series of ethanol solutions and critical point dried with carbon dioxide. Dried disks were mounted on aluminium stubs with colloidal silver adhesive, coated with a thin layer of gold by DC sputtering and viewed in the secondary electron imaging mode using a JEOL Model 840A scanning electron microscope. Sausage casing disks were viewed under 2000× magnification. Forty fields were chosen at random and all organisms in each field of view were counted. A mean count per field was obtained. The field area was measured and estimated to be 0.00168 mm<sup>2</sup> so counts could be expressed in terms of cells/mm<sup>2</sup> (Walls et al. 1993).

#### *Effect of irradiating Salmonella typhimurium cells on their attachment to sausage casings*

*S. typhimurium* cells were killed by irradiation, (Cs-137), under conditions to minimize cell surface alterations, (5.4 kGy, 5°C). Irradiated cells were streaked on to TPA to ascertain non-viability. Sausage casings were cut aseptically into disks (diameter 12 mm) using a sterile cork borer. Casing disks were dipped into approximately 20 ml of sterile isotonic saline to allow them to rehydrate. From the *Salmonella* suspension (approximately 10<sup>9</sup> cfu ml<sup>-1</sup>) 0.1 ml was placed on to the inner surface of the casing. Rate of attachment of both irradiated and non-irradiated cells was determined by exposing sausage casings to the bacterial suspension for 1, 15, 30, 45 or 60 min at 37°C. Sausage casing disks were washed twice by agitation for 30 s in approximately 20 ml of sterile isotonic saline to remove unattached cells. Disks were prepared for viewing using SEM as described above.

Surface structures of irradiated cells were compared with non-irradiated cells using TEM. Suspensions of *S. typhimurium* in 0.01 ml sterile isotonic saline solution were adsorbed to Formvar/carbon coated specimen grids for 1 min, washed with 5 drops of 0.1 M Tris buffer solution (pH 7.2) and negatively stained with 2% uranyl acetate solution. Images of whole single cells were recorded at 2000× magnification at 100 kV accelerating

voltage. Two grids were prepared for each type of cell and from each grid 100 isolated cells were chosen at random. Presence or absence of flagellae and fimbriae was noted. Fimbriated cells were subdivided into two groups, those with many (>5) fimbriae and those with few (1–5).

#### *Statistics*

Experiments were performed in duplicate and repeated two times. Bacterial attachment before and after treatment of cells with potential inhibitors was compared using Student's *t*-test.

## **Results**

### *Effect of potential inhibitors on bacterial attachment*

In the SPC studies, potential inhibitors to attachment had no effect on the growth of *S. typhimurium* cells. Carbohydrates, hyaluronin, chondroitin sulphate, gelatin, glycine, Tween 20, EDTA and bovine albumin had no inhibitory effect on attachment of *S. typhimurium* to sausage casings while solubilized collagen significantly ( $P < 0.05$ ) inhibited attachment (Table 1).

**Table 1. Effect of potential inhibitors on attachment of *Salmonella typhimurium* to sausage casings.**

Medium	Bacterial count of control (log <sub>10</sub> cfu g <sup>-1</sup> )	Bacterial count after treatment with potential inhibitor (log <sub>10</sub> cfu g <sup>-1</sup> )
Mannose	5.69	5.82
Glucose	5.59	5.63
Galactose	5.49	5.91
Hyaluronin	6.11	5.87
Bovine albumin	6.36	6.44
Chondroitin sulphate	6.08	6.39
Gelatin	5.77	5.70
Solubilized collagen	6.10	4.42 <sup>a</sup>
Glycine	6.38	6.56
Tween 20	5.87	5.96
EDTA	6.38	6.36

<sup>a</sup>Significantly different from control ( $P < 0.05$ ).

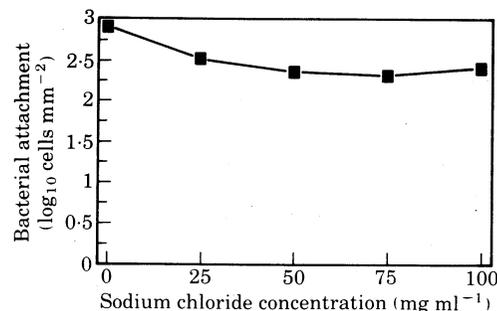
### Effect of sodium chloride on bacterial attachment

Sodium chloride, at concentrations ranging from 25–100 mg ml<sup>-1</sup> had little effect on bacterial attachment (Fig. 1). Increasing NaCl levels to >50 mg ml<sup>-1</sup> decreased bacterial numbers by about 0.5 log, but this was not statistically significant ( $P < 0.05$ ).

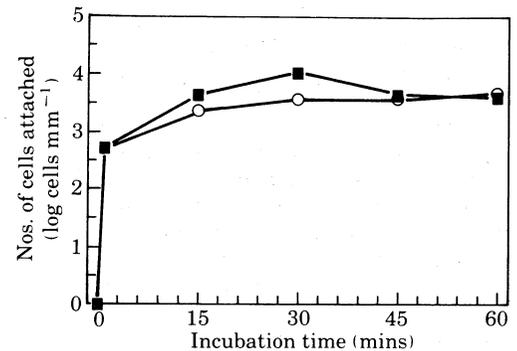
### Effect of irradiating *Salmonella typhimurium* cells on their attachment to sausage casings

**Rate of attachment.** Rate of attachment of irradiated cells was similar to non-irradiated cells, i.e. an initial rapid rate in the first minute of incubation, followed by a plateau (Fig. 2).

**Presence of surface structures.** Fimbriae were observed on both non-irradiated and irradiated *S. typhimurium* cells. Most non-irradiated cells, (61.5%) had detectable flagellae, 28.5% had both flagellae and fimbriae and 2.5% exhibited fimbriae only. This contrasts with irradiated cells; only 30% possessed flagellae and 8% possessed both flagellae and fimbriae. Eleven percent exhibited fimbriae only, a greater percentage than non-irradiated cells, although these may have been cells which previously had both flagellae and fimbriae but had lost their flagellae.



**Fig. 1.** Effect of sodium chloride on attachment of *Salmonella typhimurium* to sausage casings.



**Fig. 2.** Effect of irradiation on rate of attachment of *Salmonella typhimurium* to sausage casings. ○, irradiated cells; ■, non-irradiated cells.

### Discussion

Carbohydrates are thought to be involved in specific interactions with bacteria (Sharon et al. 1981). Mannose has been shown to inhibit binding, where binding is dependent on type I fimbriae (Old 1972). Since mannose did not inhibit binding of *S. typhimurium* to sausage casings, it may be inferred that type I fimbriae are not involved in this attachment process as reported previously by Lillard (1986). Benedict et al. (1991) observed that mannose inhibited attachment of *S. typhimurium* to meat tissues. However, Campbell et al. (1987) showed that *S. typhimurium* attached to chicken muscle equally well in the presence or absence of 1% mannose and suggested that fimbriae associated adhesions probably play little part in attachment to collagen. Also, Lillard (1989) demonstrated that attachment of *Salmonella* to chicken skin is independent of presence of fimbriae, which would agree with our findings. Glucose and galactose, present in insoluble collagen (Veis 1964), did not inhibit attachment of *S. typhimurium* to sausage casings. Glucose has been associated with attachment of *Pseudomonas*

*fluorescens* to mild steel, (Beech and Gaylarde 1989) while galactose has been shown to inhibit attachment of *Actinomyces* spp. to human erythrocytes, (Costello et al. 1979).

Solubilized collagen from bovine hide dermis inhibited attachment of *S. typhimurium* to sausage casings. This was not due to non-specific blocking of binding sites as bovine albumin did not inhibit attachment. This implies that there is a specific attachment receptor site to *S. typhimurium* present on the surface of sausage casings which is also present in solubilized collagen. In previous studies (Walls et al. 1993) cells appeared to be attaching to specific areas of the sausage casings rather than at random, implying that specific binding sites were present. Also, specific binding sites appear to be present on collagen molecules for an enterotoxigenic strain of *Escherichia coli* (Visai et al. 1990), streptococci (Speziale et al. 1987) and *Staphylococcus aureus* (Holderbaum et al. 1987). Gelatin did not inhibit attachment of *S. typhimurium* to sausage casings. This implies that denaturation of the collagen molecule removes its ability to specifically bind salmonellae. However, in previous studies, gelatin was observed to inhibit binding of lactobacilli and *Staphylococcus aureus* to collagen (Alejung et al. 1991, Switalski et al. 1989). In the current study, gelatin used was derived from acid treated pork skin, whereas sausage casings are produced by alkali treatment of calf skin. While solubilized collagen from calf skin may be sufficiently similar to collagen in sausage casings, acid treated pork skin may have undergone a transition which removes specific binding sites. This may explain the difference in results. Glycine, the principal amino acid residue on collagen, was not found to inhibit attachment, therefore this is probably not the attachment site.

Cells killed by irradiation attached at levels similar to non-irradiated cells. From this, it may be inferred that this adhesion required neither motility, energy production nor *de novo* gene expression by the organisms. Stanley (1983) reported that *Pseudomonas aeruginosa* cells made non-viable by heating or treatment with formaldehyde attached to stainless steel, although at a reduced rate. Notermans and Kampelmacher (1974) reported that non-motile bacteria rarely attach to chicken skin. However, in other studies (McMeekin and Thomas 1978, Lillard 1985, 1986, Campbell et al. 1987) presence of flagellae and motility were not involved in attachment.

Irradiated cells were observed to have approximately 50% less flagellae than non-irradiated cells. This appears to be the first study to investigate the effect of lethal irradiation doses on surface structures. Two studies indicated that a sub-lethal dose of irradiation does not prevent motility (Zelle and Hollaender 1955). These findings require further study to assess their significance.

Attachment of salmonellae to sausage casings showed a small, non-significant decrease with increasing concentrations of sodium chloride. Similar findings were observed by Thomas and McMeekin (1981, 1991) and Campbell et al. (1987) who reported that addition of physiological saline inhibited attachment of *Salmonella* to chicken muscle connective tissue. Lillard (1985) observed fewer bacterial cells on the surface of chicken muscle tissue in the presence of NaCl than in the absence of NaCl when investigated using SEM. However, when measured using plate counts, attachment of salmonellae to poultry skin was enhanced by about 1.5 log in the presence of 0.85% saline while no significant difference in attachment was observed with muscle tissue. Roller (1991) reported

an increase in attachment of *Pseudomonas fragi* to polystyrene after 4 h, from  $2.5 \times 10^3$  cfu/mm<sup>2</sup>/mg dry weight to  $7.0 \times 10^3$  cfu/mm<sup>2</sup>/mg dry weight with increasing saline levels from 0–35 g NaCl per liter. Our findings indicate that electrostatic bonding may not be the primary mechanism of attachment.

The connective tissue components hyaluronin and chondroitin sulphate did not inhibit attachment of *S. typhimurium* to sausage casings. Hyaluronin has been reported to inhibit attachment of *Salmonella* to chicken skin while chondroitin sulphate did not (Sanderson et al. 1991). This led to the hypothesis that there is a specific binding reaction between salmonellae and hyaluronin. It is likely that hyaluronin is not present in sausage casings, which would account for our results.

The surfactant Tween 20 did not inhibit bacterial attachment to sausage casings. This is similar to findings by Lillard (1988), who observed no effect of 1% Tween 80 on attachment of *S. typhimurium* to poultry skin. Triton-X-100 has been found to have no effect on

attachment of *Pseudomonas fragi* to stainless steel (Herald and Zottola 1989).

The divalent chelator EDTA did not inhibit attachment of *S. typhimurium* to sausage casings. This suggests that the presence of divalent cations is not required for attachment. Similar observations were made by Herald and Zottola (1989), who found that EDTA did not inhibit attachment of *Pseudomonas fragi* to stainless steel. However, presence of calcium and magnesium ions has been associated with the production of extracellular polymers, which occurs after the initial attachment process (Fletcher and Floodgate 1976, Lewis et al. 1989).

These studies indicate that attachment of *Salmonella typhimurium* to collagen may involve a specific binding mechanism but further studies are required to elucidate the adhesion involved.

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## References

- Alejung, P., Paulsson, M., Emody, L., Andersson, M., Naidu, A. S. and Wadstom, T. (1991) Collagen binding by Lactobacilli. *Curr. Microbiol.* **23**, 33–38.
- Beech, I. B. and Gaylarde, C. C. (1989) Adhesion of *Desulfovibrio desulfuricans* and *Pseudomonas fluorescens* to mild steel surfaces. *J. Appl. Bacteriol.* **67**, 201–207.
- Benedict, R. C., Schultz, F. J. and Jones, S. B. (1991) Attachment and removal of *Salmonella* spp. on meat and poultry tissues. *J. Food Safety* **11**, 135–148.
- Campbell, S., Duckworth, S., Thomas, C. J. and McMeekin, T. A. (1987) A note on adhesion of bacteria to chicken muscle connective tissue. *J. Appl. Bacteriol.* **63**, 67–71.
- Costello, A. H., Cisar, J. O., Kolenbrauder, P. E. and Gabriel, O. (1979) Neuraminidase-dependent hemagglutination of human erythrocytes by human strains of *Actinomyces viscosus* and *Actinomyces naeslundii*. *Infect. Immun.* **26**, 563–572.
- Dickson, J. S. and Koohmaraie, M. (1989) Cell surface charge characteristics and their relationship to bacterial attachment to meat surfaces. *Appl. Environ. Microbiol.* **55**, 832–836.
- Fletcher, M. and Floodgate, G. D. (1976) The adhesion of bacteria to solid surfaces. In *Microbial ultrastructure*. (Eds Fuller, R. and Lovelock, D. W.) pp. 101–107. New York, Academic Press.
- Herald, P. J. and Zottola, E. A. (1989) Effect of various agents upon the attachment of *Pseudomonas fragi* to stainless steel. *J. Food Sci.* **54**, 461–464.
- Holderbaum, D., Spech, T., Ehrhart, L. A., Keys, T. and Hall, G. S. (1987) Collagen binding in clinical isolates of *Staphylococcus aureus*. *J. Clin. Microbiol.* **25**, 2258–2261.

- Lewis, S. J., Gilmour, A. and Johnston, D. E. (1989) Factors affecting the detachment of a polymer-associated *Acinetobacter* sp. from stainless steel. *Int. J. Food Microbiol.* **8**, 155–164.
- Lillard, H. S. (1985) Bacterial cell characteristics and conditions influencing their adhesion to poultry skin. *J. Food Protect.* **48**, 803–807.
- Lillard, H. S. (1986) Role of fimbriae and flagella in the attachment of *Salmonella typhimurium* to poultry skin. *J. Food Protect.* **51**, 54–56, 65.
- Lillard, H. S. (1988) Effect of surfactant or changes in ionic strength on the attachment of *Salmonella typhimurium* to poultry skin and muscle. *J. Food Sci.* **53**, 727–730.
- Lillard, H. S. (1989) Factors affecting the persistence of *Salmonella* during the processing of poultry. *J. Food Protect.* **52**, 829–832.
- McMeekin, T. A. and Thomas, C. J. (1978) Retention of bacteria on chicken skin after immersion in bacterial suspensions. *J. Appl. Bacteriol.* **45**, 383–387.
- McMeekin, T. A. and Thomas, C. J. and Pennington, P. I. (1984) Contamination and decontamination of poultry carcass neck tissue. *J. Food Safety* **6**, 79–88.
- Notermans, S. and Kampelmacher, E. H. (1974) Attachment of some bacterial strains to the skin of broiler chickens. *Br. Poult. Sci.* **15**, 573–585.
- Old, D. C. (1972) Inhibition of the interaction between fimbrial hemagglutinins and erythrocytes by D-mannose and its derivatives. *J. Gen. Microbiol.* **71**, 149–157.
- Roller, S. D. (1991) Effect of sodium chloride on the adhesion of *Pseudomonas fragi* to polystyrene. *Biofouling* **5**, 57–63.
- Sanderson, K., Thomas, C. J. and McMeekin, T. A. (1991) Molecular basis of the adhesion of *Salmonella* serotypes to chicken muscle fascia. *Biofouling* **5**, 89–101.
- Sharon, N., Eshdat, Y., Silverblatt, F. J. and Ofek, I. (1981) Bacterial adherence to cells surface sugars. In *Adhesion and micro-organism pathogenicity* (Eds Elliot, K., O'Connor, M. and Whelan, J.) New York, Pitman Medical.
- Speziale, P., Raucci, G., Meloni, S., Meloni, M. L. and Wadstrom, T. (1987) Binding of collagen to group A, B, C, D, and G streptococci. *FEMS Microbiol. Lett.* **48**, 47–51.
- Stanley, P. M. (1983) Factors affecting the irreversible attachment of *Pseudomonas aeruginosa* to stainless steel. *Can. J. Microbiol.* **29**, 1493–1499.
- Switalski, L. M., Speziale, P. and Hook, M. (1989) Isolation and characterization of a putative collagen receptor from *Staphylococcus aureus* strain Cowan 1. *J. Biol. Chem.* **264**, 21080–21086.
- Thomas, C. J. and McMeekin, T. A. (1981) Attachment of *Salmonella* spp. to chicken muscle surfaces. *Appl. Environ. Microbiol.* **42**, 130–134.
- Thomas, C. J. and McMeekin, T. A. (1991) Factors which affect retention of *Salmonella* by chicken muscle fascia. *Biofouling* **5**, 75–87.
- Veis, A. (1964) *The macromolecular chemistry of gelatin*. New York, Academic Press.
- Visai, L., Speziale, P. and Bozzini, S. (1990) Binding of collagens to an enterotoxigenic strain of *Escherichia coli*. *Infect. Immun.* **58**, 449–455.
- Walls, I., Cooke, P. H., Benedict, R. C. and Buchanan, R. L. (1993) Sausage casings as a model for attachment of *Salmonella* to meats. *J. Food Protect.* **56**, 390–394.
- Zelle, M. R. and Hollaender, A. (1955) Effects of radiation on bacteria. In *Radiation biology*, Vol. II. (Ed. Hollaender, A.) New York, McGraw-Hill.